eman ta zabal zazu

Universidad del País Vasco
Euskal Herriko Unibertsitatea

MEDIKUNTZA ETA ERIZAINTZA FAKULTATEA
FACULTAD DE MEDICINA Y ENFERMERÍA

Departamento de Inmunología, Microbiología y Parasitología
Immunologia, Mikrobiologia eta Parasitologia Saila

Caenorhabditis elegans eta *Galleria mellonella* kandidiasi inbaditzailearen ereduak *Candida* espezie emergenteen birulentzia eta farmako antifungikoen eraginkortasuna ebaluatzeko

Modelos de candidiasis invasiva en *Caenorhabditis elegans* y *Galleria mellonella* para el estudio de la virulencia de especies emergentes de *Candida* y de la actividad de los fármacos antifúngicos

Ainara Hernando Ortiz

Doktorego-tesia

Leioa, 2021

AURKIBIDEA

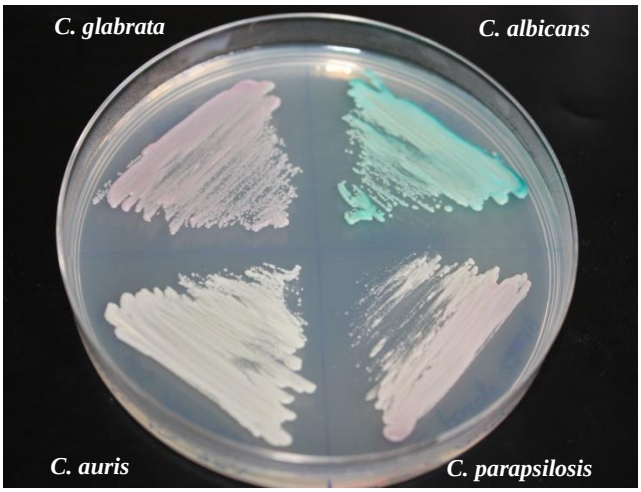
1. SARRERA

1. Sarrera

1.1. *Candida* generoa

Candida generoa onddo mota bat da, legamia alegia, eta oso zabalduta dago naturan. Giza aho-barrunbeko, heste-traktuko eta baginako mukosen mikrobiotan onddo zelulabakarreko genero horren espezieak aurki daitezke (Sardi et al., 2013). Normalean, *Candida* espezieak mukosak asintomatikoki kolonizatzen dituen komentsalea izaten da, baina patogeno oportunista da eta hainbat faktoreren arabera infekzio bat eragin dezake. Infekzio hori, kandidiasia izenekoa, batez ere immunoeskasiak dituzten pazienteetan gertatzen da edo baita prozedura kirurgiko inbaditzaileak eta espektro zabaleko antibiotikoak bezalako praktika medikoen erabileragatik. (Sardi et al., 2013; Pais et al., 2019). Izan ere, mikosi inbaditzailerik ohikoena kandidiasia da, mikosi inbaditzaile guztien % 70-90 sortuz (Cornistein et al., 2013). Gainera, heriotza-tasa %30etik gorakoa da eta AEBetan odol-uharraren bidez ospitalean hartutako infekzioen laugarren kausa ohikoena da (Pais et al., 2019).

Historian zehar 150 *Candida* espezie baino gehiago deskribatu dira, baina odoleko isolamenduen %95-97a hurrengo bost espeziek izaten dira: *Candida albicans*, *Candida glabrata*, *Candida parapsilosis*, *Candida tropicalis* eta *Candida krusei* (Pemán eta Salavert, 2012; Sardi et al., 2013; Quindós, 2018). *C. albicans* da eragile etiologiko nagusia, eta bere intzidentzia %27,8tik %69,8raino doa (Quindós et al., 2018). Intzidentzia handi honen arrazoia *C. albicans*ek hainbat birulentzia-faktore dituelako da; hala nola, hifen eraketa ehunak inbaditzeko eta fagozitoziari aurre egiteko, eta, biofilmen eta entzima hidrolitikoen eraketa (Pais et al., 2019). Hala ere, azken urteotan beste espezie batzuek eragindako kandidiasiak areagotu egin dira, besteak beste, *C. parapsilosis*, *C. glabrata* edo, orain dela gutxi deskribatutakoa, *Candida auris* (1. Irudia). Espezie horiek eragindako infekzioen gorakadaren faktore nagusietako bat da farmako antifungikoekiko sentsibilitate murriztua edo horiekiko erresistentzia garatzeko gaitasuna (Shadegui et al., 2018, Quindós et al., 2018, Fuller et al., 2019).



1. Irudia: *Candida* espezie desberdinen kolonien hazkuntza CHROMagar Candida agar kromogenoan.

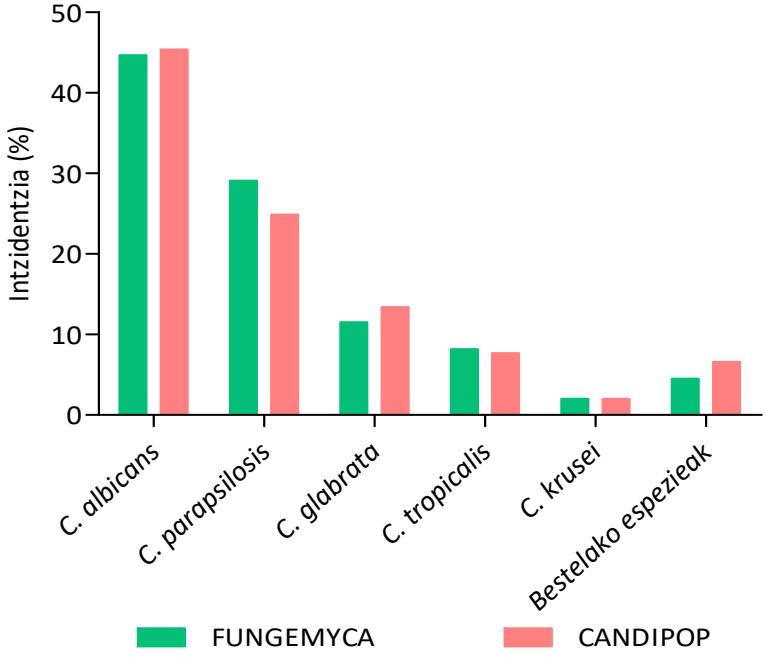
1.1.1. *Candida*ren epidemiologia

Epidemiologikoki FUNGEMYCA ikerketa multizentrikoa (2011) eta CANDIPOP populazio-azterketa (2014) Espainian egindako lan epidemiologiko berrienak dira, *Candida* espezie inbaditzaileen banaketa argia erakusten dutenak (2. Irudia) (Pemán et al., 2011; Puig-Asensio et al., 2014).

Bi azterlan horien emaitzak aztertzean ikusten da *Candida* espezie isolatu nagusien intzidentzian dagoen aldea ez dela gehiegi aldatzen. *C. albicans* da oraindik ere eragile etiologiko nagusia, azterlanen artean %0,7ko aldearekin. *C. parapsilosis* bigarren espezierik isolatuena da, eta %4,2ko aldea hauteman zen ikasketen artean. *C. glabrata* hirugarren espezie isolatuena izan zen, %1,9ko aldearekin. Azkenik, *C. tropicalis* laugarren espezierik isolatuena izan zen, %0,5 aldatuz, *C. krusei* bosgarren espezierik isolatuena izan zen bitartean, eta ez zen azterketen arteko alderik ikusi. FUNGEMYCA azterlanean, *C. orthopsilosis*en intzidentzia %2,4koa izan zen, *C. krusei*ren aurretik (Cantón et al., 2011).

*C. glabrata*ren intzidentziak gora egin du kandidiasi inbaditzaileen etiologia gisa, eta Espainian ez ezik, herrialde askok, hala nola, AEB, Australia, Belgika edo Danimarka

deskribatu dute espezie horren isolamendua areagotu egin dela odolean (Cleveland et al., 2015; Chapman et al., 2017; Trouvé et al., 2017; Astvad et al., 2018).



2. Irudia: Kandidemietan isolatutako espezien intzidentzia Espainian, FUNGEMYCA eta CANDIPOP azterlanen arabera.

C. parapsilosis Txinan, Japonian, Latinoamerikan, Afrikan, Asian eta Europako herrialde mediterraneoetan lehen edo bigarren espezierik isolatuena da (Quindós et al., 2018). Espezie horrek azala eta azazkalak koloniza ditzake, eta, batzuetan, giza mukosak ere. Nagusiki, minbizia duten pazienteengan eta ZIUn (Zainketa Intentsiboetako Unitatean) sartutako nerabeengan edo jaioberriengan isolatzen da, normalean nutrizio parenteralerako erabiltzen diren kateter benoso zentralen kolonizazioarekin lotuta, horien gainazalean atxikitzeko eta biofilmak garatzeko duten gaitasunagatik (Sardi et al., 2013; Quindós, 2014). *C. parapsilosis*arekin estuki lotutako bi espezie daude, *Candida orthopsilosis* eta *Candida metapsilosis*, eta elkarrekin *C. parapsilosis* konplexua osatzen dute. Bi espezie kriptiko horien intzidentzia *C. parapsilosis sensu stricto*rena baino txikiagoa bada ere, garrantzitsua da isolamendu klinikoak behar bezala identifikatzea,

espezie-espezifikoak diruditen farmako antifungikoekiko birulentzia eta sentsibilitate desberdinarekin lotutako akats terapeutikoak saihesteko (Lockhart et al., 2009; Miranda-Zapico et al., 2011; Pemán et al., 2012).

C. albicans ez diren *Candida* espezieen banaketa asko aldatzen da herrialdeen artean, ospitale batetik bestera eta paziente motaren arabera (Quindós, 2014) (3. Irudia).

3. Irudia: *C. albicans* ez diren *Candida* espezie ohikoenen banaketa (Quindós, 2014)

1.1.2. *Candida*ren birulentzia-faktoreak

*Candida*k eragindako infekzioak infekzio oportunistak dira, eta komentsala izanda patogeno oportunista bihurtzen duten mekanismoak oraindik ez daude ondo definituta. Kandidiasien patogenian inplikatutako faktore gisa honako hauek aipatzen dira: epitelioarekiko atxikidura, morfogenesia, entzima hidrolitikoen ekoizpena, *switching* edo aldaketa fenotipikoa eta biofilmen eraketa.

Birulentzia-faktoreak hainbat genek kontrolatzen dituzte, gene horiek kopuru eta une zehatz batean espresatzen dira, eta andui bakoitzaren fenotipoa eta birulentzia zehazten dituzte. Birulentzia-geneen espresioaren erregulazioa funtsezkoa da onddoaren patogenesian eta ostalariaren ehunetara egokitzerakoan. Erregulazio hori, kasu askotan, zelulen arteko komunikazio- edo seinaleztapen-mekanismo nagusiak gauzatzen du, *quorum sensing* bezala ezagutzen den mekanismoak, zelula-dentsitatearen menpe dagoena (Padder et al., 2018).

1.1.2.1. Entzima hidrolitikoen jariaketa

Candida hainbat entzima hidrolitiko ekoizten ditu, *Candida* espezieen birulentzian oinarrizko papera dituztenak. Entzima horiek funtsezkoak dira infekzio-prozesuan, ostalariaren ehunen inbasioan eta suntsipenean. Parte hartzen dute, hala nola atxikiduran, kolonizazioan, ehun edo zeluletan sartzean, ostalariaren immunitate-sistematik ihes egitean eta barreiatzean.

Ostalariaren mintz zelularraren osagai nagusiak fosfolipidoak eta proteinak dira. Beraz, hidrolizatzeko gai diren entzimak, fosfolipasak eta proteasak kasu, ostalariaren mintzaren desegonkortze-prozesuetan inplikatuta daude eta ehunen inbasioa errazten dute.

Fosfolipasak entzima multzo heterogeneoa dira, ostalariaren mintz zelularraren fosfolipidoen artean dauden ester-loturak hidrolizatzeko gai direnak. Fosfolipasak A, B, C eta D azpitaldeetan banatzen dira beren ekintza-moduaren eta diana-molekulen arabera. Entzima horien funtzioa mintz zelularra ezegonkortzea eta kolonizazioan laguntzea da. Jakinarazi da B fosfolipasak onddoen birulentzia-faktore garrantzitsua dela funtzio bikoitza duelako, hidrolasa eta lisofosfolipasa-transamilasa gisa (Ghannoum, 2000; Karkowska-Kuleta et al., 2009). Argitaratutako azterlan gehienak *C. albicans* espeziarekin egin dira, eta ikusi da entzima horien ekoizpena isolamenduaren eta bere jatorriaren araberakoa dela; odol-isolamenduak gernu-isolamenduak edo mukosa-kolonizatzaileak baino fosfolipasa-ekoizle handiagoak direla. Gainera, odol-isolamenduek entzima hidrolitikoak ekoiztu ditzakete ostalariaren ehunak inbaditu ahal izateko (Karkowska-Kuleta et al., 2009). *C. albicans* ez diren beste espezie batzuekin

egindako ikerketetan andui mendeko ekoizpena ere ikusi da, baina, oro har, andui horiek ez dute *C. albicans*en anduiek bezalako fosfolipasen kontzentrazio hain handia sortzen, hamar aldiz txikiagoa izan daiteke (Ghannoum, 2000).

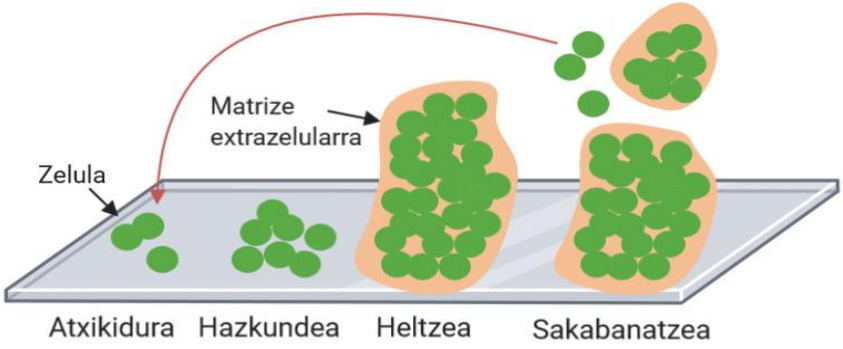
Proteasen funtzioak dira elikagaien xurgapena eta ostalariaren erantzun immuneko proteinen suntsiketa, hala nola, immunoglobulinak eta konplementuaren proteinak (Amani et al., 2018). Proteasa moten artean, aspartil proteasa edo proteasa aspartikoak (SAP) dira garrantzitsuenak euren jarduera proteolitikoagatik. Horiek *C. albicans* sortutako proteasarik ohikoenak dira, eta ostalariaren proteina gehienak degradatzeko gai dira, hala nola, kolagenoa, listu-laktoferrina, immunoglobulina gehienak, 1β-interleukina zitokina proinflamatorioa eta koagulazio-faktore batzuen aitzindariak. Gainera, ehun eta organo desberdinetako infekzioan parte hartzen dute, 2-7ko pH tartean aktiboa izan baitaiteke (Karkowska-Kuleta et al., 2009). Ikusi da *C. parapsilosis*ak *C. albicans* baino SAP gutxiago ekoizten dituela, eta espezie horretako bagina- eta larruazal-jatorriko isolamenduek SAP gehiago sortzen dituztela odol- jatorriko isolamenduek baino (Ramos et al., 2015). Beste *Candida* espezie batzuek beste proteasa motak sortzeko gai dira; adibidez, *C. glabrata*k yapsinak (YPS familiako proteinak) ekoizten ditu (Figueiredo-Carvalho et al., 2016).

Bestalde, hemolisinak polipeptido zitotoxiko multzo bat dira, zelulaz kanpo jariatzen direnak eta eritrozitoen lisia sustatzen dituztenak. Eritrozitoen mintz zelularra suntsitzen dute eta ateratzen den barruko hemoglobina eta burdina *Candida*k bere metabolismorako eta hazkunde zelularrerako erabiltzen ditu. Hemolisinek sortzen duten hemolisia hiru motatan sailkatzen da: alfa-hemolisia (hemolisi partziala), beta-hemolisia (hemolisi osoa) eta gamma-hemolisia (hemolisirik eza). Entzima horien ekoizpena *C. albicans* eta *Candida*ren beste espezie patogeno batzuetan detektatu da baina hemolisi mota (alfa edo beta) desberdinak deskribatu dira bai espezieen artean, bai espezie bereko anduien artean ere (Amani et al., 2018).

1.1.2.2. Biofilmen ekoizpena

Biofilmak gainazalei itsatsita agertzen diren mikroorganismo-komunitateak osatutako egiturak dira. Biofilm bat bere eraketaren azken etapetan dagoenean biofilm heldua deitzen zaio eta egitura horiek arkitektura konplexua erakusten dute. Mikroorganismoek beraiek jariatzen dituzten zelulaz kanpoko matrize polisakarido batez inguratuta daude eta matrize barruan aurki daitezkeen zelulak desberdinak izan daitezke. Zelula sesilak biofilma sortzen ari denean atxikitzen diren zelulak dira, zelula horiek biofilmaren eraketan eta zahartzapenean parte hartzen dute. Biofilmatik azkatzen diren zelulak, oro har, biofilma heldua denean gertatzen da, eta zelula planktoniko horiek sakabanatu edo barreiatu daitezke (4. Irudia).

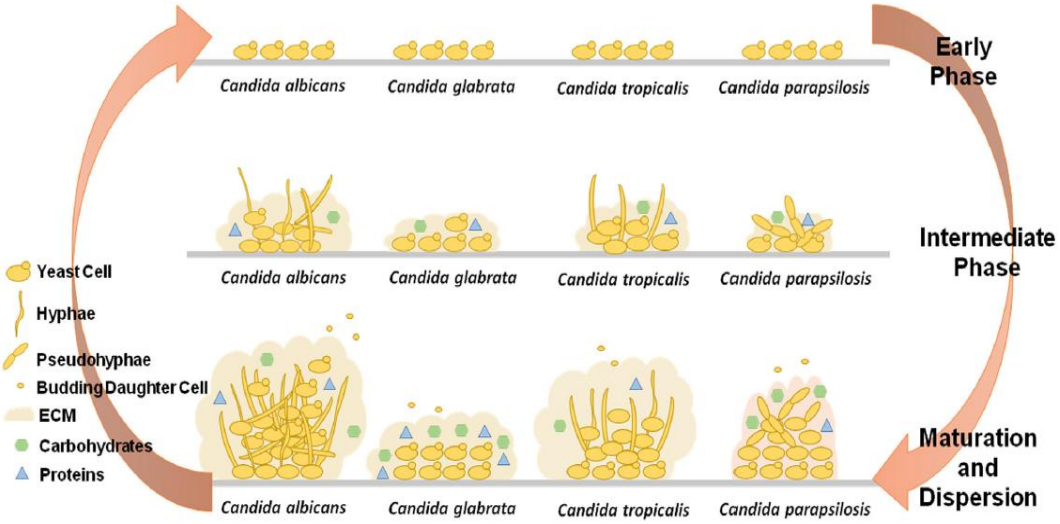
Biofilmak birulentzia-faktore garrantzitsua da infekzioa garatzeko, eta zelulen gainazaletan, hortzetan, gailu medikuetan, kateterrak eta balbulak kasu, garatu daitezke. Garrantzi medikoa duten *Candida*ren espezie gehienek biofilmak ekoizteko gai dira, hala nola *C. albicans*, *Candida dubliniensis*, *C. glabrata*, *C. krusei*, *C. tropicalis*, *C. parapsilosis* eta *C. auris* (Douglas, 2003; Muñoz et al., 2020).



4. Irudia: Biofilmak ekoizteko etapak

Candida espeziearen arabera sortzen den biofilmaren egituraren konposizioa aldatzen da. *C. albicans* espezieak sortzen duen biofilma zelulaz kanpoko matrizean murgildutako blastosporaz eta hifaz osatutako egitura heterogeneoa da. *C. glabrata*k sortutako biofilmaren kasuan, legamia formadun zelulez soilik osatuta dago, estu lotutako geruza anitzeko egitura batean. *C. tropicalis* espezieak berriz, legamia formadun zelulen, hifen

eta pseudohifen sare bat osatzen du, hifen hazkunde esanguratsu batekin. Bestalde, *C. parapsilosis* legamia formadun zelulez osatutako biofilma ekoizten du, zelulaz kanpoko matrize kantitatea oso txikia delarik (5. Irudia). Horregatik, espeziearen identifikazioa egitea eta biofilmaren berezitasunak ezagutzea bere biziko garrantzia du horien aurka zuzentasunez jarduteko (Cavalheiro eta Teixeira, 2018).



5. Irudia: *C. albicans*, *C. glabrata*, *C. tropicalis* eta *C. parapsilosis* espezieek sotutako biofilmen eskema konparatiboak. Bertan, zelulaz kanpoko matrizea (MEC) sortzeko gaitasun desberdinak, MECn dauden osagaiak eta morfologia zelular desberdinak erakusteko gaitasuna nabarmentzen dira (Cavalheiro eta Teixeira, 2018).

Kontutan hartu behar da in vitro ikerketetan biofilmak espezie bakarra osatutako matrizeak aztertzen direla. Baina normalean biofilmak *Candida* espezie desberdinez edo *Candida* eta beste mikroorganismoz, bakterioak askotan, osatutako komunitateak dira, horien arteko elkarrekintzetatik askotan onura metabolikoak lortzen dituztenak (Cavalheiro eta Teixeira, 2018).

Biofilmak farmako antimikrobianoen aurkako erresistentzia-mekanismo bat dira. Farmakoen ekintza oztopatzen dute eta mikroorganismoen erresistentzia sustatzen dute beste mekanismo batzuen bidez, adibidez, kanporatze- ponpa gehiago ekoiztuz (Perlin et al., 2017). Izan ere, ikusi da *C. albicans*, *C. glabrata*, *C. tropicalis*, *C. parapsislosis*, *C. orthopsilosis*, *C. metapsilosis* eta *C. auris* espezieek sortutako biofilmek B

anfoterizinarekiko, ekinokandinekiko eta azolekiko sentikortasun murriztua dutela edo erresistenteak direla (Pierce eta al., 2015; Cavalheiro eta Teixeira, 2018; Romera et al., 2019). Gainera, *Candida*k sortzen dituen biofilmek hesi fisiko gisa jarduten dute, giza immunitate-sistemako osagai eta zelulen aurka, eta makrofagoen aktibitatea murriztu dezakete (Alonso et al., 2017).Honetaz gain, biofilmei esker mikroorganismoek beste onura batzuk lortzen dituzte, hala nola ingurumen-baldintza kaltegarriekiko erresistentzia handiagoa (Cavalheiro eta Teixeira, 2018).

1.1.3. Farmako antifungikoak eta horien aurkako erresistentzia-mekanismoak

*Candida*k eragiten dituen infekzioak tratatzeko estrategia konplikatzen da infekzio horien larritasunaren arabera. Azaleko kandidiasien tratamenduak errazak diren bitartean, barreiatutako kandidiasiena (kandidiasi inbaditzaileena) konplexuagoak dira. (Quindós et al., 2019; Costa-de-Oliviera eta Rodrigues, 2020; Frías-de-León et al., 2020). Kandidiasi inbaditzaileen tratamenduak goiz egin behar dira, atzerapen bat hilkortasun handiagoarekin lotzen baita. Ezaguna da kandidemia duen paziente batek tratamendu antibiotiko enpiriko desegokia jasotzen badu eta tratamendu antifungiko egokia jasotzeko denbora gehiegi pasatzen bada, paziente horrek arrisku handia duela. Beraz, praktika horren atzerapenak, honek dakarren hilkortasun handia azal dezake.

Aktibitate antifungikoa duten zenbait farmako daude eta horiek ekintza-mekanismo desberdinak dituzte. Farmako horiek hainbat familiatan taldekatuta daude, hala nola polienoetan, azoletan eta ekinokandinetan, indikazio terapeutiko desberdinekin. Hala ere, gaur egun farmako horien eraginkortasuna murriztu egin da, batez ere, legamia erresistenteak sortu direlako, espezie patogeno berriak agertu direlako, farmako antifungikoak profilaxi gisa irrazionalki preskibitu direlako eta dosi terapeutikoak handitu direlako.

Farmako antifungikoekiko erresistentzia bi motatan sailka daiteke: lehen mailakoa edo intrintsekoa eta bigarren mailakoa. Erresistentzia primarioa edo intrintsekoa agertzen da onddoa aurrez farmako antifungikoaren eraginpean jarri ez denean, eta bigarren mailako erresistentzia onddoa farmakoaren eraginpean jarri ondoren garatzen denean. (Tam et al.,

2015). Bigarren mailako erresistentzia hori da, zalantzarik gabe, medizinaren esparruan kezkarik handiena sortzen duena kandidiasien tratamendu klinikoan eragiten duen arazoengatik (1. Taula).

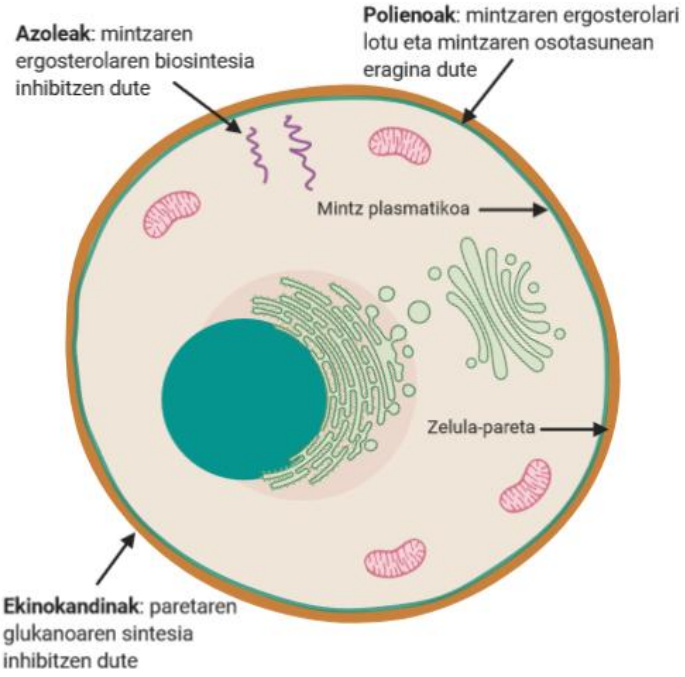
1.1.3.1. Polienoak

Polieno izeneko farmako antifungikoek onddoen heriotza zelularra eragiten dute ergosterolarekin interakzioan aritzean. Ergosterola arintasuna, simetria eta osotasuna ematen dizkio mintz zelularrari (6. Irudia; 1. Taula). Farmako talde hori da antifungikoen artean lehen agertu zena: B anfoterizina eta nistatina dira ezagunenak. Polienoek onddoaren mintzean poro handiak sortzea eragiten dute, iragazkortasuna eta metabolitoen garraioa aldatzen dituztenak. Ondorioz, ioiak eta zelularen beste osagai batzuk zelulatik ateratzen dira eta horrek zelularen heriotza eragiten du (Tam et al., 2015; Perlin et al., 2017).

B anfoterizina, 1957an onartu zenetik, kandidiasi inbaditzaileen tratamenduan gehien erabiltzen den polienoa da, nahiz eta duen toxikotasunak haren erabilera mugatu. Toxikotasun hori giza mintz zelularren kolesterolarekin dituen interakzioek eragiten dute, eta arazo hori sahiesteko asmoz, gaur egun, B anfoterizina liposomala bezalako formulazioak daude; efektu toxiko horiek murrizten dituztenak (Laniado-Laborín et al., 2009; Hamill, 2013; Adler-Moore et al., 2016; Bellmann eta Smuszkiewicz, 2017; Grazziotin et al., 2018).

*Candida*k polienoekiko duen erresistentzia oso ohikoa ez den arren, artikulu batzuetan deskribatzen dira B anfoterizinaren kontzentrazio minimo inhibitzaile (MIC) altuak , edo *C. albicans*, *C. krusei* eta *C. glabrata* espezieek eragindako kandidiasian tratamenduek huts egiten duten kasu klinikoak. (Krcmery eta Barnes, 2002; Krogh-Madsen et al., 2006; Borman et al., 2008; Khan et al., 2008; Kalantar et al., 2014). Gainera, hainbat eta hainbat lan argitaratu dira emergentea den *C. auris* espezie multierresistentearen isolamendu klinikoen aurkako B anfoterizinaren MIC altuak; kasu batzuetan, aztertutako isolamenduen %35 ere izan daitezkenak (Arendrup eta Patterson, 2017; Chowdhary et al., 2017; Lockhart et al., 2017; Chowdhary et al., 2018).

B anfoterizinarekiko erresistentzia mekanismoak oraindik ez daude argi. Ikertzaile batzuek, hala ere, mintz zelularraren ergosterol-kantitate murrizketarekin erlazionatzen dute, ergosterolaren biosintesian parte hartzen duten funtsezko proteinak inaktibatzen direlako, eta horren ordez, farmakoekiko afinitate txikiagoa duten beste esterol batzuk gehiago sintetizatzen direlako (Jensen et al., 2015).



6. Irudia: Ekinoandina, azol eta polieno farmako antifungikoen familien ekintza-dianak

1.1.3.2. Azolak

Azolak farmako antifungiko erabilienak dira *Candida*k eragiten dituen infekzioak tratatzeko. Talde horretan sartzen dira, besteak beste, triazol flukonazola , borikonazola , posakonazola, itrakonazola eta isabukonazola. Bere ekintza-diana onddoen mintz zelularraren ergosterolaren biosintesia da (6. irudia; 1. Taula). Zehazki, bere eragina 14-α-lanosterol-desmetilasa entzima inhibitzea da. Entzima hori P450 zitokromoaren menpekoa da, ergosterolaren biosintesirako nahitaezkoa. Beraz, entzima horren funtsioaren oztopaketak onddoaren mintz fungikoaren iragazkortasunean kalteak sortzen ditu (Zonios et al., 2008; Roberts et al., 2014; Tam et al., 2015).

Oro har, hobeto toleratzen diren farmakoak dira, eta gutxi dira polienoekin alderatuta sortzen dituzten albo-ondorioak (Maertens, 2004; Zonios et al., 2008; Roberts et al., 2014; Allen et al., 2015; Clark et al., 2015; Bellmann eta Smuszkiewicz, 2017; Ledoux et al., 2018). Horregatik, kontzentrazio handietan erabiltzea, flukonazolaren kasuan bezala, beste farmako antifungiko batzuk baino seguruagoa da.

Farmako antifungikoen talde horrekiko sentsibilitate txikiena duten *Candida* espezieak *C. glabrata*, *C. krusei* eta *C. auris* dira. Kandidiasi inbaditzaileen tratamenduan azolak erabiltzeak farmako horiekiko erresistenteak diren anduiak hautatzen lagundu du (Roberts et al., 2014).

Azolekiko erresistentziaren mekanismoak hiru dira nagusiki. Mekanismo bat da zelula-hormaren edo mintz plasmatikoaren konposizioan gertatzen diren aldaketek farmakoen sarrera eragozten dutela (Colombo et al., 2017; Perlin et al., 2017; Pristov et al., 2019). Beste bat *ERG11* genearekin dago erlazionatuta. *ERG11* genearen mutazioek aldaketak sortzen dute geneak kodetzen duen P-450 lanosterol-metilasa entziman, eta farmakoak entzimarekiko afinitatea galtzen du, beraz, ergosterolaren biosintesia ez da kaltetzen (Pristov et al., 2019).

Hirugarren erresistentzia-mekanismoa, berriz, kanporatze-ponpei esker da. Ponpa horiek farmako antifungikoak kanporatzen dituzte mintz zelularrean dauden garraio-proteinen bitartez. *ATP-Binding Cassette* (ABC) eta *Major Facilitator Superfamily* (MFS) garraiatzaileak daude implikatuta, farmakoak aktiboki zelulatik kanporatzen dituztenak ATP gastuaren bidez. ABC garraiatzaileek kodetzen dituzten *Candida Drug Resistance* (*CDR*) geneen gehiegizko espresioia azolen erresistentziarekin lotuta dago. MFS garraiatzaileek kodetzen dituzten *Multidrug Resistance* (*MDR*) geneen gehiegizko espresioia, berriz, flukonazolarekiko erresistentziarekin soilik erlazionatzen da (López-Ávila et al., 2016; Perlin et al., 2017; Pristov et al., 2019).

1.1.3.3. Ekinokandinak

Ekinoandinak farmako antifungiko berrienak dira. Farmako taldea anidulafungina, caspofungina eta micafungina izeneko lipopeptidoek osatzen dute. Farmako erdisintetikoak dira eta onddoen zelula-horman dute ekintza-diana (6. Irudia; 1. Taula) (Perlin et al., 2017). Kasu honetan, farmakoaren eragina 1,3-β-D-glukano sintasa entzimaren inhibizioan datza, eta, beraz, horma fungikoan dagoen 1,3-β-D-glukano polisakaridoaren sintesia blokeatzen da (Roberts et al., 2014; Perlin et al., 2017). Polisakarido hori onddoen hormako osagai nagusietako bat da eta zelularentzat bizitzeko beharrezkoa da.

Farmako horiek, azolek bezala, segurtasun-profil ona dute, eta, beraz, aukera terapeutiko ona dira kandidiasi inbaditzaileak tratatzeko (Alexander et al., 2013; Roberts et al., 2014; Bellmann eta Smuszkiewicz, 2017). Gainera, hainbat azterlanetan argitaratu da farmako horiekiko erresistentzia txikiagoa dela *Candida* espezie-sorta handi batean (Alexander et al., 2013; Roberts et al., 2014; Tam et al., 2015; Lockhart et al., 2017; Perlin et al., 2017).

Izan ere, *Candida*ren espezie gehienetan isolamenduen %1ek baino ez du sentsibilitate murriztua edo erresistentzia farmako horiekiko (Pfaller et al., 2013). Hala ere, *C. glabrata*ren kasuan, isolamenduen %4raino irits daiteke, eta hori arazo bat da espezie horrek azolei dien erresistentzia handiari gehituta (Pham et al., 2014; Perlin et al., 2017).

Ekinoandinen aurka garatutako erresistentzia-mekanismoak farmakoaren eta 1,3-β-D-glukano sintasa entzimaren arteko elkarrekintzan oinarritzen da. Entzimaren azpiunitateak hiru genek kodetzen dituzte: *FKS1*, *FKS2* eta *FKS3*. Lehenengo bi geneetan, *FKS1* eta *FKS2*, mutazioak gertatzen dira onddoa farmakoaren eraginpean jarri ondoren, hau da, bigarren erresistentzia-mekanismo motakoak dira. Mutazioak gene horien *hot spots* deitutako lekuetan gertatzen badira, sortzen diren aminoazidoen ordezkapenak handiagoak dira eta ekinokandinei erresistentzia ematen diete. Oro har, *FKS1* genean gertatzen diren mutazioak *Candida* espezie batzuetako ekinokandinen aurkako erresistentziarekin erlazionatzen da; baina *C. glabrata* espeziean zehazki, *FKS2* geneko mutazioak *FKS1* genean gertatutakoak baino garrantzitsuagoak dira ekinokandinen

aurkako erresistentzian (Katiyar et al., 2012; Colombo et al., 2017; Perlin et al., 2017; Pristov et al., 2019). *C. auris* espezie multierresistentearen kasuan, orain arte *FKS1* genean soilik detektatu dira ekinokandinekiko erresistentziarekin lotutako mutazioak (Chaabane et al., 2019).

1. Taula: Farmako antifungikoak eta horiekiko erresistentzia-mekanismoak

Familia	Ekintza	Erresistentzia-mekanismoak
Polienoak: B anfoterizina, nistatina	Mintz zalularraren ergosterolari lotzea	Mintz zelularrean ergosterol-edukia murriztea eta afinitate txikiagoko beste esterol batzuk gehitzea
Azolak: flukonazola, borikonazola, posakonazola, itrakonazola, isabukonazola	P450 zitokromoaren mendeko 14-α-lanosterol-desmetilasa entzimaren inhibizioa, ezinbestekoa mintz zelularrean dagoen ergosterolaren biosintesi-prozesuan	Farmakoaren sarrera murriztea, horma fungikoaren edo mintz plasmatikoaren konposizioan aldaketak eginez
		Azolen afinitate-galera P-450 lanosterol-metilasa entzimari, *ERG11* genearen mutazioen bidez
		Mintz zelularreko proteina garraiatzaileen bidez farmako antifungikoak kanporatzeko ponpak: *ATP-Binding cassette* (ABC) eta *Major Facilitator Superfamily* (MFS)
Ekinokandinak: anidulafungina, kaspofungina, mikafungina	Onddoen hormaren 1,3-β-D-glukanoren sintesia inhibitzea, 1,3-β-D-glukano sintasa entzimaren inhibizioaren bidez	1,3-β-D-glukano sintasa entzima kodifikatzen duten *FKS1* eta *FKS2* geneetan mutazioak

1.1.4. Farmako antifungikoekiko sentikortasuna in vitro aztertzeko metodoak

Farmako antifungikoekiko sentsibilitatea aztertzeko metodologia estandarizatuak garatzea beharrezkoa da azterketa epidemiologikoak behar bezala egin ahal izateko, erresistentzien garapenaren zaintza egokia egiteko edo farmako antifungikoen edo ahalmen antifungikoa duten molekula berrien in vitro efektua aztertzeko. Metodo horietako gehienak hazkuntza mikrobianoa murrizteko edo inhibitzeko behar den farmako antifungikoaren MICaren zehaztapenean oinarritzen dira. MIC hori hazkuntza fungikoaren % 50, 90 edo % 100 inhibitzen den arabera balora daiteke, aztertutako farmako antifungikoaren arabera.

Zenbait metodo estandarizatu daude legamiek farmako antifungikoekiko duten sentsibilitatea in vitro ebaluatzeko. Erabilienak *Clinical and Laboratory Standards Institute* (CLSI) eta *European Committee on Antimicrobial Susceptibility Testing* (EUCAST) sortutako gidak izan dira.

CLSIk garatutako M27-A3 eta M27-A3 S4 protokoloak farmako antifungikoen salda-mikrodiluzio bidezko in vitro sentsibilitatearen azterketan oinarritzen dira, diluzio bikoitz seriatuak eginez. Kasu honetan, plakak bisualki irakurtzen dira (CLSI, 2010 eta 2012).

EUCASTek garatutako E.DEF 7.3.2 protokoloa M27-A3n oinarritutako mikrodiluzio-metodoa da, baina zenbait aldaketa ditu, hala nola plaken irakurketa kasu honetan espektrofotometria bidez modu automatizatuan egiten da (Arendrup et al., 2020).

Bestalde, Xake-taularen metodologia (*Checkerboard*) erabiltzen da legamiek farmako antifungikoen aurrean duten sentsibilitatea aztertzeko, konbinatuak erabiltzen direnean. Hala ere, oraindik ez dago protokolo estandarizaturik farmako antifungikoen konbinaketa-saiakuntzak egiteko. Farmako antifungikoen konbinazioaren efektuaren analisia farmakoen MICa bakoitza bere aldetik zehaztean eta konposatu horien arteko interakzioetan oinarritzen da (Mukherjee et al., 2005). Horrela, monoterapia antifungikoaren efektua aztertu dezakegu eta baita bi farmako edo gehiagoren konbinazioarena ere.

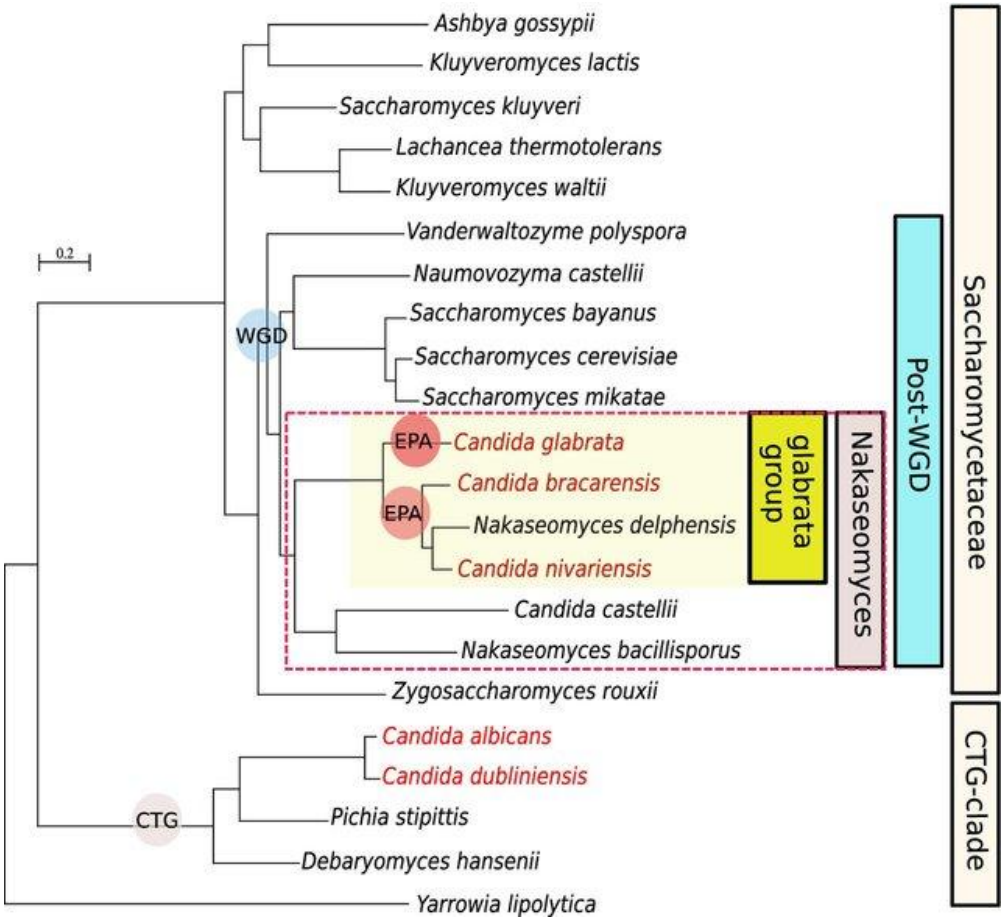
Farmakoen arteko interakzioak aztertzeko, gehien erabiltzen den metodologietako bat Loeweren gehigarritasunaren teorian oinarritzen dena da, zeinak kontzentrazio inhibitorio zatikatuaren indizea (FICI) kalkulatzea ahalbidetzen du (Te Dorsthorst et al., 2002; Mukherjee et al., 2005; Bellmann eta Smuszkiewicz, 2017). Literaturan aurkitu daitezke interakzioen sailkapen desberdinak, beraz, ikertzaile bakoitzak bere irizpideak har ditzake sailkapen horretarako erabilitako tarteak. Hala ere, autore batzuek uste dute Mukherjeek eta laguntzaileek 2005ean proposatutako sailkapena egokiena dela. Ikertzaile horien irizpideei jarraituz, interakzioak sinergikotzat hartzen dira FICIren balioa 0,5 edo txikiagoa denean (konposatuen batuketa-efektua), indiferenteak balioa 0,5 eta 4 artekoa denean (ez da inolako efekturik ikusten) edo antagonikoak balioa 4tik gorakoa denean (eragin negatiboa) (Mukherjee et al., 2005; Bellmann eta Smuszkiewicz, 2017). Beste autore batzuek bitarteko balio bat hartzen dute kontuan, FICI balioa 0,5 eta 1 artean dagoenean interakzio gehigarri gisa definitzen dute (efektu gehigarri bat ikusten da, sinergikoa izatera iritsi gabe) (Te Dorsthorst et al., 2002).

Akzio-diana desberdinak dituzten farmako antifungikoak konbinatzen direnean, hala nola, ekinokandinak eta B anfoterizina edo ekinokandinak eta azolak, konposatu horien aktibitatea handitu daiteke eta mintz zelularrerako sarbidea erraztu (Mukherjee et al., 2005; Bellmann eta Smuszkiewicz, 2017). Ekinoandinen eta beste farmako antifungiko batzuen artean deskribatutako konbinazio-azterlan gutxi daude argitaratuta, baina horietan in vitro eta in vivo interakzioetan emaitza positiboak ikusten dira (Mukherjee et al., 2005).

1.2. *Candida glabrata*, *Candida bracarensis* eta *Candida nivariensis*

Candida glabrata, *Candida bracarensis* eta *Candida nivariensis*, harreman filogenetiko estua duten hiru espezieak dira eta *Nakaseomyces* kladoren barruan dauden gizakiaren hiru patogeno bakarrak dira. Bestalde, filogenetikoki *Saccharomyces cerevisiae* ereduko legamiatik gertuago daude *C. albicans*etik baino (7. Irudia).

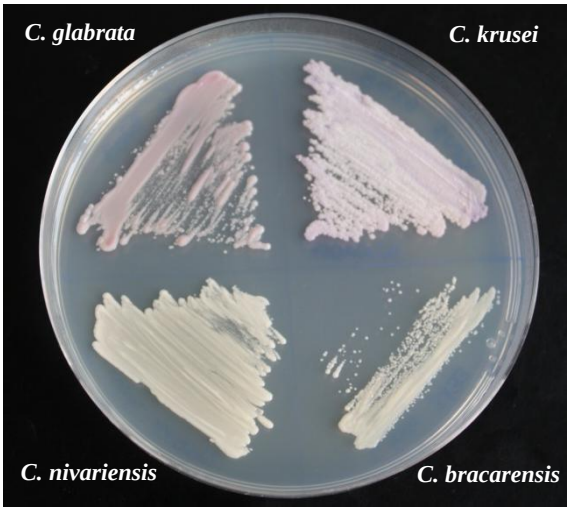
Beraz, gaur egundo espezie horien taxonomia hurrengo hau da: Erresuma *Fungi*, Suberresuma *Dikarya*, Dibisioa (Phylum) *Ascomycota*, Subdibisioa (Subphylum) *Saccharomycotina*, Klasea *Saccharomycetes*, Ordena *Saccharomycetales*, Familia *Saccharomycetaceae*, Generoa *Nakaseomyces*, Kladoa *Nakaseomyces*/*Candida* (NCBI:txid284593; NCBI:txid418086; NCBI:txid273131).



7. Irudia: *C. glabrata*, *Nakaseomyces* espezie sekuentziatuen eta beste *Saccharomycotina* espezie batzuen arteko harreman filogenetikoa. Espezie patogenoak gorriz adierazita daude. *CTG*= kode genetikoaren trantsizioa; *WGD*= genoma osoaren bikoizketa/hibridazio zaharra; *EPA*= EPA geneen bi hedapen independenteak gertatu ziren leinuak adierazten ditu (Gabaldón & Carreté, 2016).

Hiru espezie horiek ezaugarri morfologiko oso antzekoak dituzte eta ezin dira metodo fenotipiko konbentzionalen bidez bereizi. CHROMagar Candida agarrrean, *C. glabrata*

kolonien kolorea bioleta da, *C. nivariensis* eta *C. bracarensis* kolonien kolorea zuria den bitartean (8. Irudia). Hala ere, medio kromogeno horretan *C. glabrata*ren isolamendu klinikoak kolore zurixka erakutsi zuten kasuak deskribatu dira. Galeria biokimikoekin egindako identifikazio proben bidez ere ezin dira behar bezala bereizi, ikerketa batzuek *C. nivariensis* trehalosa hartzitu dezakeela deskribatzen baitute, *C. glabratak*, ordea, ez (Alcoba-Flórez et al., 2005; Gabaldón et al., 2013). Hala ere, bi espezieek karbohidrato hau hartzitzeko gai direla ere ikusi zen. Beraz, metodo molekularrak behar dira, hala nola PCR (*Polymerase Chain Reaction*) edo DNA erribosomalaren (rDNA) ITS (*Internal Transcribed Spacer*) zatiaren sekuentziazioa, behar bezala identifikatzeko (Alcoba-Flórez et al., 2005; Lockhart et al., 2009; Romeo et al., 2009; Swoboda-Kopec et al., 2014; Dudiuk et al., 2017).



8. Irudia: *Candida*ren hazkundea CHROMagar Candida agar kromogenoan.

C. nivariensis eta *C. bracarensis* espezieen intzidentzia *C. glabrata*rena baino askoz txikiagoa dela ikusi da. Izan ere, azterlan batzuek bi espezie horien intzidentzia %0,2 baino gutxiagoan kokatzen dute (Pemán et al., 2011). Hala ere, aurrez *C. glabrata* gisa identifikatutako isolamendu klinikoen atzera begirako azterlanetan ikusi zen *C. glabrata* bezala identifikatutako isolamenduen ehunekoa %93,5 eta % 100 bitartekoa zela. Beraz, *C. glabrata* gisa oker identifikatutako isolamenduen %6,5 *C. nivariensis* edo *C. bracarensis* ziren. Bi espezie horien intzidentzia txikia bada ere, garrantzitsua da

identifikazio egokia egitea akats terapeutikoak saihesteko. Izan ere, flukonazol eta ekinokandinekiko *C. glabrata* espeziea baino sentsibilitate baxuagoa duten *C. nivariensis* eta *C. bracarensis* espezieen isolamendu klinikoak deskribatu dira (Asadzadeh et al., 2019).

1.2.1. *Candida glabrata*

C. glabrata espezie haploide bat da eta bere tamaina zelularra 1-4 µm artekoa da, *C. albicans*ek tamaina zelular handiagoa duen bitartean (4-6 µm) (Fidel et al., 1999; Bialková eta Subil, 2006; Gabaldón et al., 2020). *C. albicans* ez bezala, ez da gai legamia fenotipotik hifara aldatzeko, legamia moduan soilik haziz (Timmermans et al., 2018). Izan ere, *C. glabrata* hasiera batean *Cryptococcus glabrata* deitzen zen (1917. urtean), eta geroago *Torulopsis glabrata* bezala sailkatu zen (1938. urtean), hifak edo pseudohifak sortzeko gai ez zelako eta *C. albicans* bezalako proteasarik jariatzen ez zuelako. Baina 1978an, *Torulopsis* eta *Candida* generoak *Candida* izenpean fusionatu ziren eta *C. glabrata* genetikoki *Saccharomyces cerevisiae* generotik gertuago egon arren, *Candida* generoan sartu zen patogenizitateagatik (Tam et al., 2015; Gabaldón et al., 2020).

*C. albicans*en aldaketa morfologikoa birulentzia-faktore garrantzitsuenetako bat den arren, *C. glabrata* gizakia infektatzeko ere egokitua dago. Birulentzia-faktore ohikoenak biofilmen eta adhesinen ekoizpena dira, batez ere Epa proteinak (*glicosilfosfatidilinositol-linked epitelial adhesins*). Zelula-hormako adhesina horiek azalera desberdinetara lotzen laguntzen diote, gailu mediko artifizialak zein zelularrak izanda (Gabaldón et al., 2013; Tam et al., 2015). *C. glabrata*ren biofilmek beste *Candida* espezie batzuek baino jarduera metaboliko txikiagoa erakusten dute, nahiz eta zelula kultibagarrien, proteina eta karbohidratoen kopurua handiagoa izan.

C. glabrata proteasa klasikoak sortzeko gai ez den arren, egile batzuek albuminaren degradazioa YPS familiako yapsinen ekoizpenaren ondorio dela uste dute. Proteina horiek kodetzen dituzten geneen espresioa lotuta dago giza makrofagoetan onddo honen biziraupen-ahalmena nabarmen handitzearekin (Figueiredo-Carvalho et al., 2016). Bestalde, *C. glabrata* gai da infekzio-prozesuan inplikatutako beste entzima hidrolitiko

batzuk, fosfolipasak adibidez, sortu eta sekretatzeko. Izan ere, Ghannoum eta laguntzaileek (2000) entzima horien jarduera espezie horrek sortutako kandidemien iraupenarekin lotzen dute. Hemolisinak ere sortzen ditu, ostalariaren odol-zelulak apurtzeko eta legamiaren prozesu metabolikoetarako beharrezkoa den burdina lortzeko gaitasuna ematen diotenak. Gainera, *C. glabrata*k fagozitosian estres oxidatiboari aurre egiteko erabiltzen dituen katalasak kodetzen dituzten geneak ditu (Tam et al., 2015).

*C. glabrata*ren patogeniaren beste faktore garrantzitsu bat da kontrako ingurumen-baldintzeekiko ez dela hain sentikorra, hala nola estres oxidatiboa, nutrienteen mugaketa edo beste mikroorganismo batzuekiko lehia, *C. albicans* ez bezala. *C. glabrata*k gainazal baten gainean, mantenugairik gabe, bost hilabetera arte iraun dezakeen bitartean, *C. albicans*aren iraupena ez da lau hilabetetik gorakoa (Kramer et al., 2006). Gainera, azterlan batzuetan frogatu da espezie horren ahalmen hidrofoboa, zelula-azalerari dagokionez, *C. albicans*ena baino askoz handiagoa dela (Tam et al., 2015). Alde nabarmenak daude *C. glabrata* eta *C. albicans* artean (2. Taula).

2. Taula: *C. glabrata* eta *C. albicans* espezien ezaugarri morfologikoen eta patogenesiaren arteko desberdintasunak (Kumar et al., 2019)

Ezaugarria	*Candida glabrata*	*Candida albicans*
Ploidia	Haploidea	Diploidea
Zelulen morfologia	Legamia	Legamia, pseudohifa eta hifa
Tamaina zelularra	1-4 µm	4-6 µm
Filogenia	Ez da CTG kladokoa	CTG kladokoa
Kolonien aldaketa fenotipikoa	Bai	Bai
Karbonoaren asimilazioa	Glukosa eta trealosa	Glukosa, trealosa, maltosa eta galaktosa
Auxotrofia	Niazina, tiamina, piridoxina	Ez
***Crabtree* (etanolaren ekoizpena)**	Positiboa	Negatiboa
DNA mitokondrialaren urritasuna (azolekiko erresistentziarekin lotuta)	Positiboa	Negatiboa
Gene ortologoen presentzia	Bai	Bai
Hemo errezeptorea	Ez	Bai
Hemoglobina eta trasferrinaren erabilpena	Ez	Bai
Berezko erresistentzia flukonazolarekiko	Bai	Ez
Aspartil proteasak	Ez	Bai
Bizimodua	Ziurrenik, komentsala eta patogenoa	Komentsala eta patogenoa
Ohiko infekzio-lekuak	Baginala, orala, barreiatu	Baginala, orala, barreiatua
Adhesina garrantzitsuenak	Lektinak (Epa)	Lektinak (Als eta Hwp)
Biofilmaren ekoizpena	Bai	Bai
Inbazioa	Ezezaguna	Endozitosi induzitua eta barneratze aktiboa
Hostalariaren zeluletan kaltea	Kalte ez-adierazgarria	Kalte adierazgarria

*C. glabrata*k infekzioan immunitate-sistemaren aurka erabiltzen duen biziraupen-estrategia garrantzitsua da bere birulentzian. *C. glabrata* ostalariaren zelulei aurre egiteko gai da, bereziki makrofagoei, eta zelula fagozitatuen kopuru bat gizaki zein murinoen makrofagoen barnean eusteko eta erreplikatzeko gai da (Timmermans et al., 2018). Proposatzen den estrategia da legamiak fagozitoen barrukoaldean erreplikazio masiboa egiten duela eta, ondorioz, horien lisia, karga fungiko handia dela eta (Kasper et al., 2015; Pais et al., 2019).

*C. glabrata*k eragindako infekzioak abdomeneko kirurgiak, tumore solidoak, adinekoak (> 65 urte), neutropenia duten jaioberriak, transplanteen hartzaileak edo aldez aurretik kortikoideekin tratatutako pazienteetan ikusten dira (Pemán et al., 2012; Quindós, 2014). *C. glabrata*ren intzidentziaren handipena izan daiteke, besteak beste, terapia immunogutxitzaileen erabilera areagotu delako, eta, ondorioz, immunoeskasiak dituzten pazienteen populazioa handitu egin delako, biztanleria zahartu egin delako, diagnostiko-ikuspegia hobetu delako edo infektatutako pazienteen tratamendu farmakologikoetan zailtasunak daudelako (Tam et al., 2015). Porrot terapeutikoa, batez ere, *C. glabrata*k gehien erabiltzen diren farmako antifungikoekiko duen erresistentzia-tasa altuarekin lotzen da, bereziki flukonazolarekiko (Kasper et al., 2015; Tam et al., 2015; Pfaller et al., 2019). Ekinoandinak, horregatik, *C. glabrata*k eragindako infekzioen aurkako tratamenduak dira (Katiyar et al., 2012; Pham et al., 2014; Colombo et al., 2017; Morales-Lopez et al., 2017).

C. glabrata da Espainian kandidiasia duten pazienteen artean hirugarren espezie isolatuena odolean (%11-15), *C. albicans* (%50) eta *C. parapsilosis* (%25-40) espezieen atzetik (Pemán et al., 2012; Puig-Asensio et al., 2014). Gainera, *C. glabrata*k eragindako kandidiasi inbaditzaileek gora egin dute ia herrialde guztietan (Quindós et al., 2018).

1.2.2. *Candida nivariensis*

C. nivariensis 2-6 µm-ko onddo zelulabakarra da, *C. glabrata* bezala hifak edo pseudohifak sortzeko gai ez dena. *C. nivariensis* espeziearen nitxo ekologikoa ezezaguna da, baina landareen gainazaletik isolatu da, eta, beraz, giza infekzioaren jatorria

ingurunekoa izan daiteke (Alcoba-Flórez et al., 2005). Espezie horrek infekzio desberdinak eragiten ditu, baina adierazpenik ohikoenak kandidemiak (%24), baginitisa (%22,7) eta kandiduriak (%8) dira. Espektro zabaleko terapia antibiotikoen erabilera eta kateterren erabilera dira *C. nivariensis*ek eragindako kandidiasiaren garapenean ohikoenak diren arrisku-faktoreak (Arastehfar et al., 2019).

C. nivariensis atzera begirako azterlanetan identifikatu da lau kontinentetan, non *C. glabrata* bezala identifikatuta zegoen (Alcoba-Flórez et al., 2005; Borman et al., 2008; Fujita et al., 2007; Lockhart et al., 2009; Chowdhary et al., 2010; Lopez-Soria et al., 2013; Li et al., 2014; Swoboda-Kopeć et al., 2014; Tay et al., 2014; Figueiredo-Carvalho et al., 2016). Lehen aldia 2005ean izan zen, Kanarietako ospitale bereko hiru pazientetan lortutako isolamenduak *C. glabrata* bezala oker identifikatuta zeuden, une horretan izan zen *C. nivariensis* espeziea deskribatu zenean (Alcoba-Flórez et al., 2005). Ondoren, Europan, Asian, Australian, Hego Amerikan eta Afrikan *C. nivariensis* eragindako kandidiasi kasuak erregistratu dira, Europan kasu gehienak izanda. Orain arte Ipar Amerikan ez da kasurik detektatu (Cuenca-Estrella et al., 2001; Asadzadeh et al., 2019; Malek et al., 2019). Iberiar penintsulan berriz, lehen kandidemia kasua *C. nivariensis* espezieagatik ez zen 2013ra arte argitaratu. Bai kasu kliniko horretan zein beste batzuetan, triazolen akats terapeutikoa ikusi da, eta ekinoandinak erabili behar izan dira *C. nivariensis* eragindako infekzioaren aurkako tratamendu gisa. B anfoterizina edo triazol, bereziki flukonazola, bezalako farmako antifungikoei deskribatutako sentsibilitate murriztua aldagarritasun geografikoari eta anduiari berari lotuta dago, fenomeno hori *C. glabrata*ren kasuan ere ikusi da (Lopez-Soria et al., 2013; Asadzadeh et al., 2019). Izan ere, zenbait azterlanetan, *C. nivariensis*aren farmako antifungikoekiko sentikortasun murriztua *C. glabrata*rena baino handiagoa izan daitekela deskribatu zen (Borman et al., 2008; Figueiredo-Carvalho et al., 2016).

Espezie horren birulentzia-faktoreen ezagutza mugatua da. Entzima hidrolitikoen eta biofilmen ekoizpena edo atxikitzeko gaitasuna gutxitan argitaratu dira (Figueiredo-Carvalho et al., 2016; Campos-García et al., 2019). *C. glabrata*rentzat deskribatu den bezala, *C. nivariensis* espeziean albuminaren degradazioa yapsinen ekoizpenari lotuta

dago, eta ez proteasa klasikoei. Figueiredo-Carvalho eta laguntzaileek (2016) ez zuten ikusi *C. nivariensis* fosfolipasak, hemolisinak eta esterasak ekoizten zituela, baina bai hauteman zuten isolamendu batzuk fitasa-ekoizle moderatuak zirela. Fitasa entzimak fitatoa degradatzen du, zelula fungikoentzat funtsezko mantenugaiak diren fosfato inorganikoa eta inositola askatuz. Bestalde, *C. nivariensis* espeziearen isolamendu kliniko batzuetan *C. glabrata*aren antzeko biofilma ekoizteko gaitasuna deskribatu den arren, *C. nivariensis* biofilmen ekoizpena mugatuagoa da *C. glabrata* isolamenduekin alderatuz (Figueiredo-Carvalho et al., 2016; Campos-García et al., 2019).

Infekzioa garatzeko kontutan hartu beharreko beste faktore bat patogenoa ostalariari atxikitzeko gaitasuna da. *C. nivariensis* espezieak substratu abiotikoekiko, itsaspen-ahalmena du, bai beirarekiko bai poliestirenoarekiko (Figueiredo-Carvalho et al., 2016).

1.2.3. *Candida bracarensis*

C. bracarensis ere hifak edo pseudohifak sortzeko gai ez den onddo zelulabakarra da, 30 °C-tan hobeto hazten dena eta 3-4,5 µm arteko tamaina zelularra duena (Malek et al., 2019). *C. bracarensis* 2006an deskribatu zen lehen aldiz Portugalen, okerki *C. glabrata* bezala identifikatu ondoren. Argitaratutako isolamendu kopurua *C. nivariensis* baino txikiagoa da. *C. bracarensis* eragindako kandidiasia Europan, Amerikan eta Asian deskribatu da, eta berriro ere, Europan deskribatu dira kasu gehienak (Malek et al., 2019). *C. bracarensis* lisina nitrogeno iturri bakar gisa erabiltzeko gai da; *C. glabrata*, berriz, ez da asimilatzeko gai eta egitekotan, oso modu ahulean egiten du (Correia et al., 2006). *C. bracarensis* eta *C. nivariensis* espezieek agar kromogeno CHROMAGAR Candidan kolonia zuri bereizezinak sortzen dituzten arren, *C. bracarensis*ek positibo ematen du trehalosako proba azkarrean.

C. bracarensis, *Candida* beste espezie batzuk bezala, ingurumen-laginetatik eta giza gorputzaren lagin klinikoetatik isolatu da, bereziki mukosetatik eta odoletik; isolamendu guztiak antzekoak izanda (Warren et al., 2010). Kandidiasi inbaditzaile gehienak infekzio endogenoak diren arren, isolamendu klinikoak eta ingurumen-isolamenduak berdinak

izateak erakusten du infekzio horiek iturri exogenoa izan dezaketela (Alcoba-Flórez et al., 2005; Malek et al., 2019).

*C. bracarensis*aren birulentzia-faktoreei buruzko ezagutza, hala nola biofilmen edo entzima hidrolitikoen ekoizpena edo farmako antifungikoekiko in vitro sentsibilitatea, oso mugatua da. Espezie hori biofilmak sortzeko gai da, eta ez dago alderik *C. glabrata*k sortzen dituenekin. Biofilm horien matrizeak karbohidratoz eta proteinez osatuta daude batez ere, *C. albicans* eta *C. glabrata*ren kasuan bezala, karbohidratoen proportzioa *C. glabrata*k sortutako biofilmetan handiagoa den arren (Moreira et al., 2015). Bestalde, entzima hidrolitikoen ekoizpena anduiren araberakoa dela ikusi da; badira proteasa eta fosfolipasa asko ekoizten duten anduiak eta jarduera entzimatikorik erakusten ez duten beste andui batzuk. Gainera, beti argitaratu izan da *C. bracarensis* espezieak jarduera hemolitikoa duela baina ez duela esterasak ekoizteko gaitasunik (Moreira et al., 2015; Treviño-Rangel et al., 2018).

Farmako antifungikoen in vitro sentikortasun-azterketei dagokionez, *C. bracarensis* espeziearekin egindako ikerketak ere oso mugatuak dira. Espezie horren isolamenduak argitaratu dira, B anfoterizinarekiko, flukonazolarekiko, itrakonazolarekiko, borikonazolarekiko eta posakonazolarekiko erresistentziarekin (Bishop et al., 2008; Lockhart et al., 2009; Warren et al., 2010; Hou et al., 2017). *C. bracarensis* espeziearen benetako intzidentzia ezezaguna da kandidiasi inbaditzaileetan (Warren et al., 2010; Morales-Lopez et al., 2016; Malek et al., 2018; Asadzadeh et al., 2019).

1.3. *Candida auris*

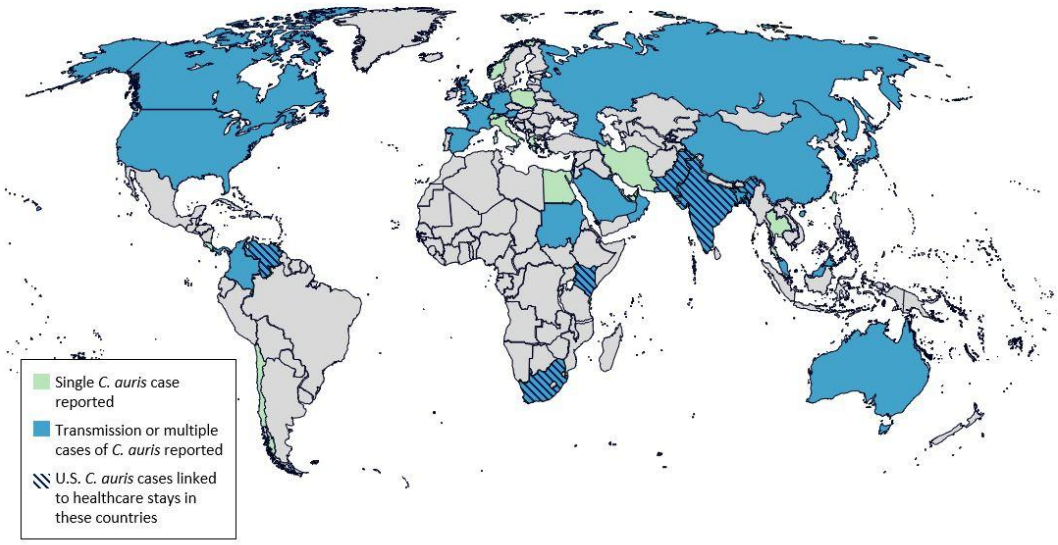
C. auris Candida generoaren barruan deskribatutako espezie berriena da, 2009an isolatu zen lehen aldiz Japoniako otitis ertaina zuen paziente baten kanpoko entzumen-hodian (Satoh et al., 2009). Isolamendu horren analisi genetikoak, zehazki 26S rADN genearen D1/D2 domeinuak eta DNA erribosomal nuklearraren ITS zatiaren sekuentzien azterketa, eta analisi kimiotaxonomikoek espezie berri bat irudikatzen duela adierazi zuten.

Metschnikowiaceae barruan kokatuta eta *Candida ruelliae* eta *Candida haemulonii* espezieekin harreman filogenetiko estua duen espeziea (Satoh et al., 2009).

C. auris espeziearen taxonomia hurrengo hau da: Erresuma *Fungi*, Suberresuma *Dikarya*, Dibisioa (Phylum) *Ascomycota*, Subdibisioa (Subphylum) *Saccharomycotina*, Klasea *Saccharomycetes*, Ordena *Saccharomycetales*, Familia *Metschnikowiaceae* Generoa *Clavispora*, Kladoa *Clavispora*/*Candida* (NCBI:txid498019).

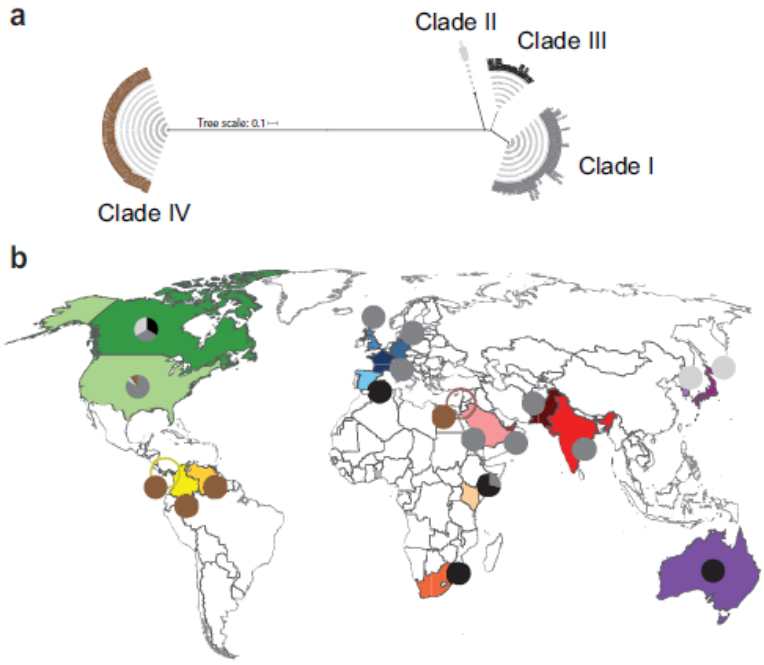
C. auris nolabaiteko tolerantzia termikoa duen legamia haploidea da. Ondo hazten da 37-40 ºC-tan eta 42 ºC-tan hazteko gai da, modu motel eta ahulean, baina ezin da 45 ºC-tan hazi. CHROMagar Candida agar kromogenoan arroxa-beix koloreko koloniak sortzen ditu, eta arto- edo arroza-irineko Tween 80 agarrean ez ditu hifak edo pseudohifak garatzen (Satoh et al., 2009; Kumar et al., 2017).

C. auris eragindako kandidiasien epidemiologia ez da ondo ezagutzen. Espezie hori 2009an Japonian lehen aldiz deskribatu zenetik, urte horretan bertan Hego Korean otitisa zuten 15 pazientetan *C. auris* isolatu zela jakinarazi zen. Hala ere, Hego Koreako *Candida* isolamenduen atzera begirako azterlan batean *C. auris*en lehen anduia, 1996koa zela argitaratu zen (Kim et al., 2009). *SENTRY Antimicrobial Surveillance Program* atzera begirako azterlanean hainbat herrialdetatik etorritako 15000 isolamendu klinikoren analisia egin zen eta ikusi zen espezie horren intzidentzia txikia zela 2009 baino lehenagoko isolamenduen artean (Pfaller et al., 2019). Mundu osoko hainbat herrialdeko *C. auris*en infekzioen jakinarazpenak jaso dira eta gaur egun, *C. auris* bost kontinentetako kandidiasi inbaditzailearen agerraldi nosokomialei lotutako patogeno emergentea bihurtu da (Borman et al., 2016; Arendrup eta Patterson, 2017; Lepak et al., 2017; Lockhart et al., 2017; Arikan-Akdagil et al., 2018; Quindós et al., 2018). Onddo patogeno horrek eragindako agerraldi ugari direla eta 2016 urtean, osasun-erakunde nagusiek osasun larrialdi egoera global deklaratu zuten, *The Centers for Disease Control and Prevention*ek (CDC), Osasunaren Mundu Erakundeak (OME), *Pan American Health Organization*ek (PAHO) eta *South African National Institute of Communicable Disease*sek (NICD) (9. Irudia) (Lone eta Ahmad, 2018).



9. Irudia: 2020ko martxoaren 31n *C. auris* kasuak jakinarazi diren herrialdeak (CDC)

Orain arte, Hego Korea, India, Pakistan, Banglades, Kuwait, Israel, Oman, Malaysia, Txina, Arabiar Emirerri Batuak, Saudi Arabia, Iran, Singapur, Thailandia, Hegoafrika, Kenya, Panama, Kolonbia, Venezuela, AEB, Kanada, Australia, Erresuma Batua, Norvegia, Frantzia, Austria, Belgika, Suitza, Holanda, Errusia, Alemania eta Espainia dira *C. auris*en infekzio kasuen berri eman duten herrialdeak. *C. auris* ikerketa filogenetikoetan oinarrituta klado geografikotan banatuta dago: hegoaldeko Asiako kladoak (I. kladoa), ekialdeko Asia (II. kladoa), Hegoafrika (III. kladoa) eta Hego Amerika (IV. kladoa) (10. Irudia) (Jeffery-Smith et al., 2018; Lone and Ahmad, 2018; Rhodes and Fisher, 2019; Chow et al., 2020). Berriki, Iraneko *C. auris* isolamenduak bosgarren klado bat osatzea proposatu da (Chow et al., 2018; Ademe eta Girma, 2020).



10. Irudia: *C. auris* kladoen munduko banaketa (Chow et al., 2020)

*C. auris*ek sortzen dituen infekzio inbaditzaileek hilkortasun handia dute (%30-50). Espezie horren suntsipena oso zaila dela argitaratu da, asteak eta hilabeteak iraun dezake ospitalean nahiz eta desinfekzio-prozedura oso eraginkorrak erabili (Iguchi et al., 2019). Gainera, birulentzia faktore desberdinak deskribatu dira. Atxikitzeko gaitasun handia dauka eta biofilmak garatu ditzake, bai kateter zentraletan bai beste gailu biomediko batzuetan; hala ere, *C. albicans* sortutako biofilmak baino askoz sinpleagoak dira (Lockhart et al., 2017; Welsh et al., 2017; Arastehfar et al., 2018; Arikan-Akdagil et al., 2018). Duela gutxiko ikerketa batean, txerri larruan ex vivo ereduan *C. auris* biofilm sendoak garatzeko gaitasuna jakinarazi zen (Horton et al., 2020). Beste birulentzia-faktoreak ere deskribatu dira, hala nola fosfolipasa, proteasa eta hemolisinen ekoizpena, ostalariaren ehunak inbaditzeko eta infektatzeko aukera ematen diona (Jeffery-Smith et al., 2018; Rossato and Colombo, 2018). Horretaz gain, frogatu da gizakien neutrofiloek ez dutela *C. auris* zelulak neutralizatzen *C. albicans* bezain modu eraginkorrean (Johnson et al., 2018).

C. auris beste *Candida* generoko espezieetan ikusi ez den fenotipo agregatzailea erakutsi du. Borman eta laguntzaileek (2016) deskribatu zuten haien azterlanean erabilitako isolamendu batzuen hazkuntza agregatuak sortuz gertatzen zela, zelulek haien artean estuki hazteko gaitasuna baitzuten eta horien arteko banaketa oso zaila zen. Fenotipo agregatzaile honek fármako antifúngikoekiko erresistentsia edo sentikortasun murriztuarekin, birulentziarekin eta bizirauteko ahalmen handiagoarekin erlazionatuta dago (Borman et al., 2016; Short et al., 2019; Brown et al., 2020). Hala ere, orain arte egindako azterlanetan ikusi da fenotipo agregatzailea zuten isolamenduek birulentzia baxuagoa erakutsi zutela, fenotipo ez-agregatzailea zuten isolamenduekin alderatuta (Borman et al., 2016; Sherry et al., 2017; Arias et al., 2020).

Identifikazio proba eraginkorrak behar dira *C. auris* detektatzeko, gaizki identifikatzen baita proba metabolikoetan oinarritutako ohiko identifikazio metodoekin, eta harreman filogenetiko estua duen beste *Candida* espezie batzuekin nahastu daiteke, *Candida haemulonii* kasu. Bere identifikazioa PCR edo masen desorzio/ionizazio espektrofotometrian (MALDI-TOF) oinarritutako metodo molekularrekin egin behar da (Arendrup eta Patterson., 2017).

*C. auris*en ezaugarririk garrantzitsuena eta nabarmenena farmako antifungiko askoekiko erresistentzia handia da. Multierresistentzia hori *C. auris*ak eragindako infekzio inbaditzaileen intzidentzia altuarekin lotuta dago, batez ere (Lockhart et al., 2017; Welsh et al., 2017 Kenters et al., 2019). Deskribatu denez, isolamenduen %93a flukonazolarekiko erresistenteak ziren, %54a borikonazolarekiko erresistenteak, %35a B anfoterizinarekiko erresistenteak, %7a ekinoandinekiko erresistenteak eta %50a aldi berean bi farmako antifungikorekiko erresistenteak ziren (Lockhart et al., 2017). Flukonazola kandidiasien tratamenduan aukeratzen den farmako antifungikoetako bat da baina *C. auris*ek flukonazolarekiko erresistentzia handia duenez espezie horrek eragiten dituen infekzioen tratamendua oztopatzen du (Lockhart et al., 2017; Welsh et al., 2017; Eldesouky et al., 2018). Ekinokandinekiko erresistentzia berriz txikia denez, *C. auris* eragindako kandidiasi inbaditzaileen tratamenduan gehien erabiltzen diren farmakoak dira, B anfoterizinarekin konbinatuta edo konbinatu gabe (Lockhart et al., 2017; Ruiz-

Gaitán et al., 2017; Kenters et al., 2019; Chamdramati et al., 2020; Mulet Bayona et al., 2020). Ikusi da geografikoki bereizitak dauden *C. auris*en klonek farmako antifungikoen aurkako erresistentzia-mekanismo desberdinak garatu dituztela (Jeffery-Smith et al., 2018).

1.4. Eredu esperimental alternatiboak

Doktorego tesia egin dudan denbora honetan liburu kapitulu baten egileetako bat izan naiz. Kapituloaren izenburua "Modelos experimentales in vivo para el estudio de las infecciones" da eta "Actualizaciones en Biomedicina" izeneko liburuan argitaratu da (UPV/EHU argitaletxea, 2020, ISBN: 978-84-1319-234-5). Kapitulu osoa 1. eranskinean aurki daiteke, eta, jarraian, horren laburpen bat aurkezten da.

Eredu esperimentalek mikroorganismo askok eragindako gaixotasunen patogenia eta terapia ezagutzeko aukera ematen digute, gizakien erabilera saihestuz. Patogenoaren, eta zehazki legamien, patogenizitate jokaera aldatu egin daiteke in vitro edo in vivo azterketetan, emaitza desberdinak erakutsiz. Beraz, in vitro jasotako emaitzak in vivo analisiekin berretsi behar dira. In vivo ikerketekin ostalari eta patogenoen arteko elkarrekintzen kausa-efektu harremanen azterketa zehatza egin daiteke, mikroorganismoen benetako patogenotasuna ezagutuz. Horrela, teknika diagnostiko eta farmako antifungiko berriak gara daitezke.

Eredu animalien artean sagua (*Mus musculus*), untxia (*Oryctolagus cuniculus*), arratoia (*Rattus norvegicus*) edo kobaiak (*Cavia porcellus*) infekzio mikrobianoak aztertzeko método ohikoenak dira. Hala ere, alderdi ekonomikoek, logistikoek eta etikoek animalia ornodunen erabilera murrizten dute. Beraz, ordezko ereduak, animalia ornogabeen ereduak, besteak beste, etorkizun handiko aukerak dira gaixotasun infekziosoak aztertzeko. Ondooak, bakterioak eta birusak aztertu dira dagoeneko *Danio rerio* zebra arrainean, *Acanthamoeba castellani* ameban, *Caenorhabditis elegans* nematodoan,

Drosophila melanogaster fruitu-eulian, *Galleria mellonella* lepidopteroan, *Bombix mori* zeta-harrean edo *Tenebrio molitor* koleopteroan (3. Taula) (Glavis-Bloom et al., 2012).

1.4.1. *Caenorhabditis elegans*

C. elegans nematodoa 1960ko hamarkadan erabili zen lehen aldiz ikerketan (Brenner et al., 1974). Lurzoruan bizi den organismo hermafrodita bat da, *Rhabditidae* familiakoa. Bere luzera milimetro bat ingurukoa da, eta bere gorputza gardena denez ez da sakrifikatu behar behaketa esperimentala egiteko. Bere ugalketa zikloa laburra da, 2-4 egun artekoa, eta bere bizitza 2-3 aste ingurukoa.

Nematodo hori laborategian erraz haz daiteke 15 °C-tan *Escherichia coli* andui ez patogenoak dituzten agar-plaketan, mikroorganismoz elikatzen baita. Elikatze ezaugarri horri esker, andui ez patogenoekin elikatu beharrean, beste bakterio edo onddo patogeno batzuekin ordezka daitezke, infekzioa sortzeko. Bestalde, bere genoma erabat sekuentziatuta dago eta Wormbase datu-basean (http://www.wormbase.org/) bere gene, mutante eta fenotipoei buruzko informazio guztia aurki daiteke (Breger et al., 2007; Desalermos et al., 2011; Elkabti et al., 2018; Segal eta Frenkel, 2018). Gaur egun mutante ugari dauden arren, *C. elegans* AU37 anduia erabiltzen da gehien mikrobiologiako saiakuntzetan (Ortega-Riveros et al., 2017). Andui horrek mutazio bikoitza du: i) *glp-4* genean aurkitzen den mutazioak nematodoaren gonaden garapenari eragiten dio eta 25 °C-tan indibiduoak antzuak dira, beraz, esperimentuetan zehar nematodo kopuru bera erabiltzeko aukera ematen du; ii) *sek-1* genean dagoen mutazioaren ondorioz, nematodoek infekzioak izateko aukera handiagoa dute (Breger et al., 2007).

Eredu esperimental hori bakterioen birulentzia eta tratamendu desberdinak aztertzeko asko erabili da, hala nola, *E. coli*, *Listeria monocytogenes*, *Pseudomonas aeruginosa* edo *Staphylococcus aureus* (Thompson et al., 2014; Fuchs et al., 2016; Schifano et al., 2019; Vasquez-Rifo et al., 2019; Yang et al., 2019). Animalia eredu hori birusak aztertzeko erabili da ere, birusen eta ostalariaren arteko elkarrekintzak eta antibiralen eragina aztertzeko besteak beste (Chen et al., 2017; Gammon et al., 2017; Martin et al., 2017; Jiang et al., 2017). Onddoekin egindako ikerketak ere ugariak dira, hala nola *Candida* edo

Criptococcus neoformans (Desalermos et al., 2015). *Candida albicans* espezieari buruz azterlan asko argitaratu dira, birulentzia, hifen eraketa, erantzun immunitarioa eta patogeno horren aurkako konposatu antifungikoen eragina aztertu dirarelarik (Pukkila-Worley et al., 2009; Pukkila-Worley et al., 2011; Sun et al., 2015; Ortega-Riveros et al., 2017; Mohammad et al., 2019; Kim et al., 2020). Beste *Candida* espezie desberdinak ere aztertu dira, hala nola, *C. parapsilosis*, *C. metapsilosis*, *C. orthopsilosis*, *C. glabrata*, *C. auris*, *C. krusei* edo *C. tropicalis*, bere patogenesia, antifungiko eta molekula berrien eragina eta erantzun immunitarioa ikertzeko (Scorzoni et al., 2013; Brilhante et al., 2016; Ortega-Riveros et al., 2017; Souza et al., 2018; Eldesouky et al., 2018; Eldesouky et al., 2019; Eldesouky et al., 2020a, 2020b, 2020c; Lima et al., 2020). Ikerkuntzan erabiltzeko eredu egokia den arren, baditu mugak ere, hala nola berezko immunitate-sistemaren eta giza sistemaren arteko aldeak, edo farmakoen jarduera eta ugaztunengan dituzten kontzentrazio eraginkorrak iragartzeko zailtasuna (Giacomotto eta Ségalat, 2010).

1.4.2. *Galleria mellonella*

Argizari-sits handia bezala ezaguten da *Pyralidae* familiaren barruan kokatzen den *G. mellonella* lepidopteroa. Horren larba egoera azken urteotan ostalari-patogeno elkarrekintzak aztertzeko asko erabiltzen den animalia eredu berri bat da. Hainbat abantaila ditu, hala nola tenperatura-tarte zabal batean (15-42 °C) bizirauteko duen gaitasuna, giza gorputzaren tenperatura barne dagoela, erraz manipulatzen dira 1,5-2,5 cm tamainakoak dira eta, eta mikrobio-inokulu edo tratamendu farmakologikoa dosi zehatzetan emateko aukera. Gainera, erraz ikusten da patogenoak eragindako infekzioaren eragina, kolore beltz, melanizazio masiboa dela eta, eta mugimendurik gabe geratzen dira hiltzean. Larben erantzun immuneak antzekotasunak dauzka ugaztunen berezko erantzun immunearekin. Hala ere, *G. mellonella*ren larbek ez dute ugaztunek daukaten hartutako immunitaterik. Larben immunitate zelularrean eta immunitate humoralean hainbat zelulak eta molekulak hartzen dute parte; baina horren erantzun immunea batez ere hemolinfan aurkitzen diren zelula espezializatuen mende dago, hemozitoak deritzenak. Hemozitoek patogenoak ezagutzen eta fagozitatzen dituzte, ugaztun neutrofiloek egiten duten bezala. Beraz, hemolinfako hemozitoen dentsitatea eta patogeno fagozitatuak dituzten

hemozitoen ehunekoak kontrolatzen dira, patogenoen birulentzia ebaluatzeko parametro gisa. Bestalde, eredu horren genoma duela gutxi sekuentziatua izan den arren, *G. mellonella*ren desabantaila nagusietako bat gaur egun dagoen mutante kopuru mugatua da, honen kostua handia delarik.

*G. mellonella*ren larbak *Acinetobacter baumannii*, *L. monocytogenes*, *S. aureus*, *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *E. coli* edo *Salmonella enterica* bakterioen birulentziaren eta horien aurkako farmako antibakterianoen eraginkortasun azterketetan erabili dira (Hernandez et al., 2019). Halaber, onddoak *Aspergillus*, *Candida*, *Cryptococcus* edo *Fusarium* aztertzeko ere (Trevijano-Contador eta Zaragoza, 2019).

G. mellonella ereduan *Candida* generoko hainbat espezieren azterketak egin dira. Berriro ere, ikerketa lan gehienak *C. albicans* espezieari buruzkoak dira. Horietan, besteak beste, birulentzia, patogenoaren eta ostalariaren arteko elkarrekintzak, hifen garapenaren eragina edo infekzioaren aurkako tratamendua aztertu dira (Fuchs et al., 2010; Li et al., 2013; Frenkel et al., 2016; Sheehan et al., 2019; Vertyporokh et al., 2020). *C. parapsilosis* konplexua osatzen duten espezieen birulentzia, ostalari-patogeno elkarrekintzak eta tratamendu antifungikoaren eraginkortasuna ere ikertu dira, *C. krusei* eta *C. tropicalis* espezieetan bezala (Mesa-Arango et al., 2013; Scorzoni et al., 2013; Gago et al., 2014; Perini et al., 2019; Binder et al., 2020; García-Carnero et al., 2020; Marcos-Zambrano et al., 2020). *G. mellonella* ereduan *C. glabrata* espeziearekin egindako azterlanak murritzagoak dira, baina bere birulentzia, antifungikoekiko sentikortasuna eta garatutako erresistentziak aztertu dira (Ames et al., 2017; Bordallo-Cardona et al., 2018; Marcos-Zambrano et al., 2020). Orain arte, ordezko eredu hori ez da erabili *C. nivariensis* eta *C. bracarensis* espezieen azterketa egiteko. Aldiz, azken urteetan *C. auris* espezie multierresistentearen birulentzia, fenotipoaren eragina eta tratamendu berrien eraginkortasuna hainbat ikerketa lanetan aztertu dira (Borman et al., 2016; Szekeley et al., 2019; Barreto et al., 2020; Muñoz et al., 2020; Romera et al., 2020; Fan et al., 2021).

1.4.3. *Drosophila melanogaster*

Drosophila melanogaster Drosophilidae familiako dipteroa da eta fruitu-eulia edo ozpina-eulia esaten zaio. Frutaren eulia edo ozpinaren eulia bezala ezagutzen da. Bere tamaina 2-3 mm-koa da, belaunaldi berriak denbora laburrean sortzen ditu eta erantzun immunea ugaztunen berezko erantzun immunearekin antzekotasunak ditu. Beraz, immunitate-sistema aztertzeko asko erabili den eredu esperimentala da. *G. mellonella*k bezala, hemozitoak ditu immunitate zelularraren osagai nagusitzat, eta peptido antimikrobianoak, berriz, immunitate humoralaren osagai nagusitzat. Bestalde, bere genoma erabat sekuentziatua dago, mutante bilduma zabal bat eta manipulazio genetikorako tresna desberdinak daudelarik (http://www.flybase.org /). (Tzelepis et al., 2013). Eredu horren desabantaila nagusietako bat mantentzeko tenperatura da, 25-29 °C-koa da, eta, beraz, gizakientzat patogenoak diren tenperaturan, 37 °C-an alegia, ezin da mantendu eta patogenoen birulentziaren azterketa zailagoa izan daiteke. Hala ere, oso eredu baliagarria izan da infekzioa errazten duten birulentzia-faktoreak eta antimikrobianoen eraginkortasuna aztertzeko.

D. melanogaster askotan erabili da: i) gizakientzako birus patogenoak aztertzeko, hala nola, B hepatitisaren birusa, dengearen birusa, Epstein-Barr birusa, giza immunoeskasiaren birusa, koronabirusa, eta zitomegalobirusa; ii) bakterio patogenoak, adibidez *Chlamydia*, *Enterococcus faecalis*, *P. aeruginosa*, *Serratia marcescens*, *S. aureus* eta *Vibrio cholerae*; eta iii) onddoak *Aspergillus*, *Candida*, *Cryptococcus* edo *Fusarium* (Tzelepis et al., 2013).

1.4.4. *Bombyx mori*

Bombyx mori zeta harra duela 5.000 urte baino gehiagotik zetaren ekoizpenean erabiltzen den lepidoptero bat da. Bere genoma erabat sekuentziatua dago, eta bioteknologia, genomika eta mikrobiologia ikerketetan animalia eredu garrantzitsu bihurtu da (Mita et al., 2004). Ornogabeetan dauden gainerako animalia-ereduak bezala, errazak dira hazten eta laborategian mantentzen, oso txikiak direlako. Tamaina egokia dute inokuluak eta tratamenduak kontzentrazio ezagunetan injektatzeko. Eredu horren kasuan, gainera,

bereizi daitezke administrazio intrahemokelikoa eta intraintestinala, gizakien zain barneko administrazioari eta ahozkoari dagozkienak, hurrenez hurren. Mantentze temperatura zabala da eta esperimentuak giza gorputzaren 37 °C-an egin daitezke. *B. mori*ren immunitate zelularrean hemozitoak patogenoak fagozitatzeaz eta desagerrarazteaz arduratzen dira, eta immunitate humoralean péptido antimikrobianoak dira nagusiak (Panthee et al., 2017).

Animalia-eredu hau bakterio eta onddo askoren birulentziaren eta potentzial antimikrobianoa duten molekula berrien azterketan erabili da, hala nola *E. coli*, *P. aeruginosa*, *S. aureus*, *V. cholerae*, *A. fumigatus*, *Candid*a edo *C. neoformans* (Panthee et al., 2017; Matsumoto eta Sekimizu, 2019).

1.4.5. *Tenebrio molitor*

Tenebrio molitor irinaren harra esaten zaio eta mundu osoan aurkitzen den intsektu koleoptero bat da. Larbak zerealez eta ale ehoz elikatzen dira, eta askotan izurriteak sortzen dituzte. Lehen aipatutako animalietan bezala, *G. mellonella* eredua barne, *T. molitor* ere 37 °C-tan bizi daiteke, tamaina egokia dauka inokuluak eta farmakoen kontzentrazio ezagunetan injektatzeko, eta hildako larbak melanizatzen dira eta erraz bereiz daitezke (Canteri de Souza et al., 2018).

Eredu horren azterlanak mugatuak diren arren, *S. aureus*, *C. albicans* eta *C. neoformans* giza patogenoei buruzko ikerlanak egiteko erabili da (de Souza et al., 2015; McGonigle et al., 2016).

1.4.6. *Danio rerio*

Zebra-arraina esaten zaion teleosteo bat da eta askotan infekzioak eta farmako antimikrobianoen bidezko tratamenduak aztertzeko erabili den ordezko eredua da. Hainbat abantailak ditu, hala nola, ugalketa-tasa handia, tamaina txikia, mantentze-kostu txikia eta manipulazio genetikoak egiteko aukera ematen digun genoma guztiz sekuentziatua. Gainera, ezaugarri garrantzitsuena da berezko immunitatea eta hartutakoa

dituela eta antzekotasunak dauzkala ugaztunen erantzun immunearekin (Meeker eta Trede, 2008).

Danio rerio gardena da enbrioi- eta larba-estadioan, eta horrek ostalari-patogeno elkarrekintzak in vivo irudien bidez aztertzeko oso estimatua izan da eta da ikerketan. Gainera, mikroorganismo gabeko enbrioiak lortzeko metodologiak garatu dira, ezaugarri hori ere oso baliotsua da elkarekintzak aztertzeko (Chao et al., 2010; Meijer eta Spanik, 2011). Bestalde, eredu horretako mutanteak daude bere sistema immunitarioko zelulak molekula fluoreszenteekin markatuta dituztenak; horri esker, ostalariaren erantzun immunearen eta patogenoaren jarraipen egokia egin daiteke.

Eredu horren enbrioiekin lan egiteko desabantaila handiena zen inokuloak mikroinjekzio bidez eman behar zirela eta oso teknika neketsua zela. Hala ere, mikroinjekzio horiek egiteko sistema automatizatu bat garatzeko lanean ari dira gaur egun (Carvalho et al., 2011).

Ordezko eredu hori mikrobiologiari buruzko ikerlan askotan erabili da, horren makrofagoek ugaztunen makrofagoekin duten antzekotasunagatik. Birusak, hala nola chikungunya birusa, herpes birus sinplea eta a influenza birusa aztertu dira (Varela et al., 2017). Era berean, bakterio gram-negatiboak, gram-positiboak, mikobakterioak eta *Candida* bezalako onddoak ere. Ikerketa guzti horiek asko lagundu dute mikroorganismo beraren ezagueran eta ostalariaren immunitate-sistemaren estrategiak ezagutzen (Levraud et al., 2009; Chao et al., 2010; Li y Hu, 2012; Oksanen et al., 2013; Díaz-Pascual et al., 2017; Varas et al., 2017). Infekzio automatizatuak eskala handian garatu dira eredu honetarako; hori oso aukera erakargarria da efektu antimikrobianoa izan dezaketen molekulak azkar aztertzeko eta, aldi berean, toxikotasunari buruzko informazioa lortzeko (Varela et al., 2017). Mikrobiologiaren eta immunologiaren arloetan ez ezik, ordezko eredu hori ikerketa farmakologikoetan, giza gaixotasunen azterketetan, neurologian, toxikologian edo bioindikatzaile gisa ere erabili da, besteak beste (Froehlicher et al., 2009; El-Amrani et al., 2012;Blaser and Vira, 2014; Bailey et al., 2015; Sanz-Landaluze

et al., 2015; Vargas et al., 2015; Kent et al., 2020; Shen et al., 2020; van de Venter et al., 2020).

3. Taula. Ordezko eredu esperimentalak

Eredua	Ezaugarriak	Infekzio-metodoak	Ikertutako mikroorganismoak	Bibliografia
Caenorhabditis elegans	1 mm, gardena, hermafrodita, saiakuntzak 15 edo 25 °C-tan, genoma sekuentziatua, berezko immunitate-sistema	Ingestioa	Birusak, bakterioak eta onddoak	Pukkila-Worley et al., 2009; Thompson et al., 2014; Gammon et al., 2017; Ortega-Riveros et al., 2017
Galleria mellonella (Eztiaren sits handia)	1,5-2,5 cm, saiakuntzak 37 °Ctan, larba hilen melanizazio korporala, genoma sekuentziatua, berezko immunitate-sistema hemozitoekin	Injekzioa	Birusak, bakterioak eta onddoak	Mesa-Arango et al., 2014; Hernandez et al., 2019; Maurer et al., 2019; Trevijano-Contador y Zaragoza, 2019
Drosophila melanogaster (Frutaren eulia)	2-3 mm, saiakuntzak 25-29 °C-tan, genoma sekuentziatua, berezko immunitate-sistema hemozitoekin	Injekzioa, ingestioa eta topikoa	Birusak, bakterioak eta onddoak	Tzelepis et al., 2013; Sampaio et al., 2018; Segal y Frenkel, 2018; Lee et al., 2018
Bombyx mori (Zetaren harra)	4-5 cm, saiakuntzak 37 °C-tan, genoma sekuentziatua, berezko immunitate-sistema hemozitoekin	Injekzioa	Birusak, bakterioak eta onddoak	Saha et al., 2017; Panthee et al., 2017; Matsumoto y Sekimizu, 2019
Tenebrio molitor (Irinaren harra)	2-3 cm, saiakuntzak 37 °C-tan, larba hilen melanizazio korporala, berezko immunitate-sistema hemozitoekin	Injekzioa	Birusak, bakterioak eta onddoak	de Souza et al., 2015; McGonigle et al., 2016
Danio rerio (Zebra-arraina)	5-6 cm, saiakuntzak 24-28 °C-tan, genoma sekuentziatua, immunitate-sistema berezkoa eta adaptatiboa	Injekzioa	Birusak, bakterioak eta onddoak	Varela et al., 2017; Levraud et al., 2009; Chao et al., 2010; Li y Hu, 2012; Varas et al., 2017

2. JUSTIFIKAZIOA ETA HELBURUAK

2. Justifikazioa eta helburuak

Candida generoko espezieak infekzio fungiko inbaditzaileen erantzule nagusiak dira, eta horien %70-90a eragiten dituzte. Nahiz eta *Candida albicans* infekzio horien eragile etiologiko nagusia izan, faktore batzuek, profilaxi bezala farmako antifungikoen erabilera barne, sentikortasun txikia duten espezieak edo berez erresistenteak diren beste espezie batzuk hautatzen lagundu dute.

Candida glabrata kandidiasi inbaditzailearen bigarren edo hirugarren agente etiologikoa da AEBetan, Australian eta Erdialdeko eta Iparraldeko Europan (Astvad et al., 2018; Lamoth et al., 2018; Quindós et al., 2018; Pfaller et al., 2019). Flukonazola kandidiasien tratamenduan erabiltzen den farmako antifungiko nagusietako bat da eta espezie horrek sentikortasun txikia du edo erresistentea da flukonazolarekiko. *C. glabrata sensu strict*oek ezaugarri fenotipiko eta genetiko asko partekatzen ditu *Candida nivariensis* eta *Candida bracarensis* espezieekin. *C. glabrata sensu stricto* baino antifungikoekiko sentikortasun baxuagoagoa duten bi espezie horien isolamendu klinikoak deskribatu dira (Lopez-Soria et al., 2013; Aznar-Marin et al., 2016; Astvad et al., 2018). Aldaketa etiologikoek ondorio larriak izan ditzakete kandidiasiaren diagnostikoan, tratamenduan edo pronostikoan, besteak beste, patogeniekiko edo antifungikoekiko sentikortasuna oso desberdina izan daitekeelako. Beraz, beharrezkoa da harreman filogenetiko estua duten *C. glabrata*, *C. nivariensis* eta *C. bracarensis* espezieen birulentzia aztertzea eta farmako antifungikoek haien aurka duten eraginkortasuna ezagutzea, gero terapia optimoak ezartzeko kandidiasia duten pazienteengan, dosi egokiak erabiliz eraginkortasunik handiena lortzeko.

Bestalde, *Candida auris* duela gutxi deskribatu den *Candida* generoko espezie bat da, eta gaitasun handia du farmako antifungikoekiko erresistentzia garatzeko. Izan ere, *C. auris* isolamendu klinikoak askotan erresistenteak izaten dira zelula-diana desberdina duten farmako antifungiko familia desberdinei (Lockhart et al., 2017). Multierresistentea den espezie hori osasun-larrialdi globala bezala deklaratu da, eta patogeno fungiko nagusietako bat bihurtu da. Gainera, *C. auris* espezieak garrantzi kliniko berezia du, ospitaleko ingurunean irauteko ahalmen handia duelako eta oso zaila delako bertatik

eliminatzea, baita tratamendua konplexua delako ere. Ezaugarri horiek *C. auris*ak eragiten duen kandidiasi inbaditzaileen hilkortasun handiari laguntzen diote (%30-70).

*Candida*ren birulentzia eta farmako antifungikoen eraginkortasun terapeutikoa in vivo aztertzeko, kandidiasi inbaditzaile animalia-eredu ohikoak ornodunak izan dira. Hala ere, alderdi etikoengatik, logistikoengatik eta ekonomikoengatik, ordezko ereduak bilatzeak eta erabiltzeak garrantzi berezia hartu du. Eredu bezala animalia ornogabeak, besteak beste, erabiltzea hainbat abantaila eskaintzen dituzte: hala nola prezioa eta mantentze erraza, ugalketa-ziklo laburra, saiakuntzak 37 °C-tan egiteko aukera, giza gorputzaren antzeko tenperatura, eta entsegu bakoitzean banako kopuru handia erabiltzea. Horregatik, ordezko eredu horiek erakargarriak dira ostalari eta patogenoen arteko elkarrekintzak aztertzeko eta tratamendu antifungiko berrien eraginkortasuna ebaluatzeko.

Doktorego tesi honen lan hipotesia honako hau izan zen:

1. HIPOTESIA: ***Candida*k eragindako infekzioak kontrolatzeko, tratamendu antifungikoen eraginkortasunari eta birulentziari buruzko azterlanak egin behar dira, *Caenorhabditis elegans* eta *Galleria mellonella* kandidiasi-inbaditzaile ordezko ereduetan egin daitezkeenak.** Hipotesi hori frogatzeko planteatutako **helburua** *Caenorhabditis elegans* nematodoaren eta *Galleria mellonella* intsektu lepidopteroaren larbaren erabilgarritasuna ikertzea izan zen. Kandidiasi-inbaditzaile eredu horiek probatuko ziren: i) *Candida* espezie emergenteen birulentzia aztertzeko, zehazki *C. auris*ena eta harreman filogenetiko estua duten *C. glabrata*, *C. nivariensis* eta *C. bracarensis* espezieena; ii) espezie horiek sortzen dituzten kandidiasien aurkako tratamendu antifungikoen eraginkortasuna ebaluatzeko.

Helburu orokor hori **bigarren mailako helburu** hauetan bana daiteke:

1. Helburua: *Candida glabrata*, *Candida nivariensis* eta *Candida bracarensis* espezie emergenteen birulentzia aztertzea eta konparatzea *Caenorhabditis elegans* eta *Galleria mellonella* kandidiasi-ereduetan.

2. Helburua: *Candida glabrata*, *Candida nivariensis* eta *Candida bracarensis* espezieek eragindako infekzioen aurkako tratamendu antifungikoen eraginkortasuna ebaluatzea *Caenorhabditis elegans* eta *Galleria mellonella* kandidiasi-ereduetan.

3. Helburua: *Candida auris* espezie emergentearen birulentzia aztertzea *Caenorhabditis elegans* eta *Galleria mellonella* kandidiasi-ereduetan.

4. Helburua: *Candida auris* espezieak sortutako infekzioen aurkako tratamendu antifungikoen eraginkortasuna ebaluatzea *Caenorhabditis elegans* eta *Galleria mellonella* kandidiasi-ereduetan.

3. DISEINU ESPERIMENTALA

3. Diseinu esperimentala

Proposatutako helburuak lortzeko, diseinu esperimental hau egin zen:

Lehenengo eta bigarren helburuak hiru azterlanetan landu ziren. Horietan, *Caenorhabditis elegans* eta *Galleria mellonella* kandidiasi-ereduen erabilgarritasuna ebaluatu zen, harreman filogenetiko estua duten *Candida glabrata*, *Candida nivariensis* eta *Candida bracarensis* espezieen birulentzia aztertzeko. Hiru espezie horien birulentzia-faktoreak in vitro ere aztertu ziren. Bestalde, bi kandidiasi-ereduak erabili ziren espezie horiek eragindako infekzioen aurkako farmako antifungiko nagusien eraginkortasuna zehazteko. Alde batetik, *C. elegans* kandidiasi-ereduan erabilitako farmako antifungikoak B anfoterizina, ekinoandinak (anidulafungina, kaspofungina eta mikafungina) eta zenbait azol (flukonazola, posakonazola eta borikonazola) izan ziren. Horrez gain, *G. mellonella* kandidiasi-ereduan hiru ekinoandinen (anidulafungina, kaspofungina eta mikafungina) eraginkortasuna frogatu zen. Azken eredu horretan, gainera, hiru *Candida* espezie horien eta *G. mellonella* larben arteko elkarrekintzak aztertu ziren, hemozitoen dentsitatea eta hemozitoen jarduera fagozitikoa aztertuz. In vivo lortutako espezie horien antifungikoekiko sentikortasun emaitzak in vitro lortutakoekin alderatu ziren.

1. **Azterlana.-** *Ekaia* aldizkarian argitaratua

   «***Galleria mellonella* insektua eta *Caenorhabditis elegans* nematodoa, infekzio eredu boteretsuak *Candida glabrata* eta erlazionatutako espezieen birulentzia ikertzeko**»

   «Experimental candidiasis models in the insect *Galleria mellonella*and the nematode *Caenorhabditis elegans* are useful to evaluate the virulence of *Candida glabrata* and related species»

2. Azterlana- *Antimicrobial Agents* and *Chemotherapy* aldizkarian argitaratua

«*Caenorhabditis elegans* kandidiasi-eredu gisa *Candida glabrata, Candida nivariensis* eta *Candida bracarensis* espezieen birulentzia eta horien aurkako tratamendu antifungikoaren eraginkortasuna ebaluatzeko»

«*Caenorhabditis elegans* as a model system to assess *Candida glabrata*, *Candida nivariensis* and *Candida bracarensis* virulence and antifungal efficacy»

3. Azterlana- Prestaketa prozesuan

«*Candida glabrata, Candida nivariensis* eta *Candida bracarensis* espezieen patogenesia eta sentikortasun antifungikoaren azterketa *Galleria mellonella* ostalari ornogabean»

«Pathogenesis and antifungal susceptibility of *Candida glabrata*, *Candida nivariensis* and *Candida bracarensis* in the invertebrate host *Galleria mellonella*»

Hirugarren eta laugarren helburuak bi azterlanetan landu ziren. Horietan *C. elegans* eta *G. mellonella* kandidiasi-ereduen erabilgarritasuna ebaluatu zen, *C. auris* espezie multierresistentearen patogenizia aztertzeko. Bi kandidiasi-ereduetan konparatu zen *C. auris* espezieak aurkezten dituen bi fenotipoen birulentzia, fenotipo agregatzailea eta fenotipo ez-agregatzailea. Gainera, *C. auris* isolamendu klinikoen birulentziaren, biopelikulak sortzeko ahalmenaren eta jatorri klinikoaren (odola, gernua eta orofaringea) arteko lotura posiblea ebaluatu zen.

Tratamendu antifungikoaren eraginkortasuna hemokultibotik zetozen *C. auris* isolamendu klinikoen aurka aztertu zen. In vitro probatutako farmako antifungikoak B anfoterizina eta ekinoandinak (anidulafungina, kaspofungina eta mikafungina) izan ziren. Farmakoak monoterapian zein horien arteko konbinaketak eginez xake-taularen metodologia erabiliz aztertu ziren. Gainera, *C. elegans* kanididasi-eredua erabili zen in vivo aztertzeko B anfoterizinaren eta hiru ekinoandinen arteko konbinazioen eraginkortasuna *C. auris* isolamendu kliniko horiek eragindako infekzioen aurka.

4. Azterlana- *Virulence* aldizkarian argitaratua

«*Candida auris*en birulentzia *Caenorhabditis elegans* eta *Galleria mellonella* ostalari ez-konbentzionaletan»

«Virulence of *Candida auris* in the nonconventional hosts *Caenorhabditis elegans* and *Galleria mellonella*»

5. Azterlana- Prestaketa prozesuan

«B anfoterizinaren eta ekinoandinen arteko konbinazioa *Candida auris* espeziearen aurka in vitro eta in vivo *Caenorhabditis elegans* ostalari ez-konbentzionalean»

«Combination of amphotericin B with echinocandins against *Candida auris in vitro* and *in vivo* in the nonconventional host *Caenorhabditis elegans*»

4. MATERIALAK ETA METODOAK

4. Materialak eta metodoak

4.1. Mikroorganismoak

Doktorego-tesi honetan erabili diren *Candida glabrata*, *Candida. nivariensis*, *Candida bracarensis* eta *Candida auris* anduien eta isolamenduen informazioa 4. eta 5. Tauletan laburbiltzen da:

4. Taula: Doktorego-tesi honetan erabilitako *Candida glabrata*, *Candida bracarensis* eta *Candida nivariensis* anduien informazioa

Espeziea	Anduia/ Isolamendua	Jatorria	Bilduma/Iturria
C. glabrata	ATCC 90030	Odola	*American Type Culture Collection*
	NCPF 3203	Odola	*National Collection of Pathogenic Fungi*
C. bracarensis	NCYC 3397	Kateter	*National Collection of Yeast Cultures*
	NCYC 3133	Odola	*National Collection of Yeast Cultures*
C. nivariensis	CBS 9984	Odola	*Colección Española de Cultivos Tipo*
	CECT 11998	Garbiketa bronkoalbeolarra	*Westerdijk Fungal Biodiversity Institute*

5. Taula: Doktorego-tesi honetan erabilitako *Candida auris* isolamenduen informazioa

Anduia/ Isolamendua	Jatorria	Bilduma/Iturria
CJ94	Odola	Hospital La Fe (Valencia)
CBS 15605	Odola	*Westerdijk Fungal Biodiversity Institute* / Hospital La Fe (Valentzia)
CBS 15606	Odola	*Westerdijk Fungal Biodiversity Institute* / Hospital La Fe (Valentzia)
CBS 15607	Odola	*Westerdijk Fungal Biodiversity Institute* / Hospital La Fe (Valentzia)
JMRC:NRZ 1101	Odola	*Jena Microbial Resource Collection* / *Institut für Hygiene und Mikrobiologie* (Würzburg, Alemania)
CR243	Orofaringea	Hospital La Fe (Valentzia)
CR312	Orofaringea	Hospital La Fe (Valentzia)
CR201	Gernua	Hospital La Fe (Valentzia)
CR220	Gernua	Hospital La Fe (Valentzia)
CR424	Gernua	Hospital La Fe (Valentzia)
CR440	Gernua	Hospital La Fe (Valentzia)
CR14	Gernua	Hospital La Fe (Valentzia)

4.2. Mikroorganismoen mantentzea

*Candida*ren isolamendu guztiak -80 °C-tan krioboletan eta giro-tenperaturan ur destilatu esterila zuten bialetan mantendu ziren. Esperimentuak Sabouraud agar glukosatuan (SDA) 37 °C-tan 24 orduz hazitako legamia kultibotatik abiatu ziren.

4.3 Entzima hidrolitikoen ekoizpena

4.3.1. Fosfolipasak

Fosfolipasen jarduera Polak-ek aurrez deskribatutako metodologia jarraituz aztertu zen (Polak, 1992), baina Price eta laguntzaileek deskribatutako gorringoa duen agarra erabilita (Price et al., 1982). Agarra prestatu zen, eta horri arrautza-gorringoa gehitu zitzaion. Ondoren, 0,7 MacFarland esekidura prestatu zen erabilitako andui bakoitzerako. eta horren 10 µl-ko tanta bat agarraren gainazalean utzi zen. Andui bakoitza hirukoiztuta erein zen plaka desberdinetan eta *Candida albicans* NCPF 3153 anduia kontrol positibo gisa erabili zen. Plakak 37 °C-tan inkubatu ziren sei egunetan zehar, eta kolonia inguruan sortutako haloak neurtu ziren bigarren, hirugarren eta seigarren egunetan. Fosfolipasa jarduera (Pz) seigarren egunean lortutako neurriarekin kalkulatu zen, honako formula hau erabiliz:

Pz= koloniaren diametroa/haloaren diametroa

Pz-ren balioa zenbat eta txikiagoa izan, orduan eta handiagoa izango da fosfolipasa jarduera. Beraz, Pz = 0 balioak jarduera oso altua adierazten du eta Pz = 1 balioak, fosfolipasa jarduerarik ez dagoela adierazten du.

4.3.2. Proteasak

Proteasen ekoizpena aztertzeko, Cassone eta laguntzaileek deskribatutako behi-seroalbumina (BSA) duen ingurune solidoa erabili zen (Cassone et al., 1987). Jarduera fosfolipasa neurtzeko erabilitako protokoloan bezala, 0,7 McFarland esekidura prestatu zen aztertutako andui bakoitzeko, eta horren 10 µl-ko tanta bat utzi zen agarrean. Tantak lehortu ondoren, plakak 37 °C-tan inkubatu ziren sei egunetan zehar. Laginak hirukoiztuta erein ziren plaka desberdinetan, eta kontrol positibo gisa erabilitako anduia *Candida dubliniensis* UPV 00-135 izan zen. Proteasen ekoizpena zehazteko, koloniaren eta prezipitatu opakoen artean sortu zen halo gardena neurtu zen.

Halorik ikusi ez zenean, ez zen proteasa-jarduerarik detektatu. Sortutako haloa 1-2 mm ingurukoa zenean, anduia proteasa-ekoizletzat kontsideratu zen. Sortutako haloa 2 mm-tik gorakoa zenean, anduia proteasa-ekoizle handia bezala kontsideratu zen.

4.3.3. Hemolisinen ekoizpena

Gaitasun hemolitikoaren azterketa Manns eta laguntzaileek aurrez deskribatutako metodologia jarraituz aztertu zen (Manns et al., 1994), baina Luo eta laguntzaileek egindako aldaketekin (Luo et al., 2001). Kasu honetan, 1 McFarland esekidura prestatu zen andui bakoitzeko eta horren 10 µl-ko tanta bat erein zen agarraren gainazalean. Saiakuntza hirukoiztuta egin zen eta kontrol positibo gisa *C. albicans* ATCC 90028 anduia erabili zen. Plakak 37 °C-tan inkubatu ziren 48 orduz %5 CO_2rekin. Denbora hori igarota, plakak irakurri ziren jarduera hemolitikoa (Hz) zehazteko. Horretarako, honako formula hau erabili zen:

Hz= haloaren diametroa/koloniaren diametroa

Hz-ren balioa 1etik gorakoa zenean jarduera hemolitikoa zegoela interpretatu zen. Gainera, aztertzen zen zein hemolisi mota egiten zen ,osoa edo partziala.

4.4. Biofilmaren garapena

Biofilmak 100 putzutako poliestirenozko mikrotitulazio-plaketan prestatu ziren, hondo lauak zituztenak eta egoera esterilean (Labsystems, Finlandia). Kontrol positibo modura *C. albicans* SC5314 anduia erabili zen. *Candida*ren hazitako zelulak hiru alditan garbitu ziren esterila zen fosfato gatzezko disoluzio indargetzaile batekin (PBS, Sigma-Aldrich, AEB), eta 1 x 10^6 zelula/ml-ko kontzentrazioa zuen zelula esekidura prestatu zen 1640 RPMI-an, L-glutaminarekin eta pH-a 7ra doitu zen 3-(N-morfolina) propanesulfoniko azidoarekin, MOPS (Sigma-Aldrich)(0,165 M). Esekidura zelular bakoitzetik 100 µl utzi ziren plakaren putzu bakoitzean eta mikroplakak 37 °C-tan inkubatu ziren 24 edo 48

orduz. Ondoren, gutxi atxikitutako zelula planktonikoak kontu handiz garbitu ziren soluzioarekin.

Biofilmen biomasaren kuantifikazioa kristal-bioleta (CV) tindatzailearekin tindatu ondoren egin zen (Peeters et al., 2008). Aurrez garbitutako mikroplakak 30 minutuz lehortzen egon eta gero, 100 µl (CV) (%0,4) disoluzioa gehitu zen putzu bakoitzean, eta 20 minutuz inkubatu ziren giro-tenperaturan. Ondoren, putzu bakoitza birritan garbitu zen 250 µl ur destilatu esterilarekin, eta, azkenik, %33ko azido azetiko disoluzioa gehitu zitzaion CV tindatzailearen bidez tindatutako biomasari.

Biopelekularen aktibitate metabolikoa 2,3-bis (2-metoxi- 4-nitro-5-sulfophenyl) -5-[(fenilaminoa) -carbonil] - 2H-tetrazolium hydroxide (XTT, Sigma-Aldrich) murrizketarekin ebaluatu zen (Ramage et al., 2001). Soluzio 1 µM menadionarekin aurrez garbitutako putzu bakoitzean 100 µl XTT gehitu ziren, eta mikroplakak bi ordu 37 ºC-tan inkubatu ziren iluntasunean.

Bai biofilmen biomasaren kuantifikazioa zein horien aktibitate metabolikoa espektrofotometriaren bidez kalkulatu zen. Absorbantzia emaitzak BioScreen C MBR (Growth Curves Ltd, Finlandia) mikroplaka irakurgailuarekin neurtu ziren, 600 nm-ko uhin luzeran biomasa kuantifikatzeko eta 492 nm-ko uhin luzeran aktibitate metabolikoa zehazteko. Esperimentuak hiru bider egin ziren hiru egun desberdinetan.

4.5. Farmako antifungikoen jarduera ebaluatzeko in vitro metodologiak

4.5.1. *Clinical & Laboratory Standards Institut*ak proposatutako metodoa

*Candida*ren sentsibilitate antifungikoa aztertzeko, *Clinical and Laboratory Standards Institut*ak (CLSI) garatutako M27-A3 eta M27-A3/S4 protokoloak erabili ziren (CLSI, 2012). Bertan, *Cryptococcus neoformans* eta *Candida* generoko legamiek farmako antifungikoekiko duten sentsibilitatea ebaluatzeko jarraitu beharreko metodoa deskribatzen da. Ikerketa honetan farmako antifungiko desberdinak erabili ziren: B

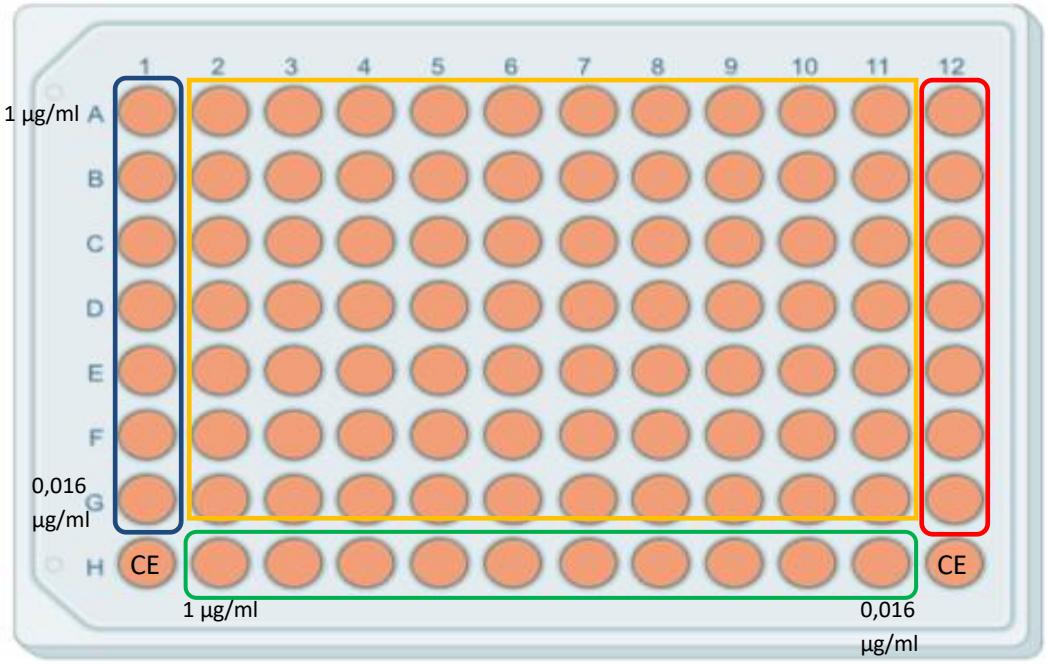
anfoterizina (AmB) (Sigma-Aldrich Inc., USA), anidulafungina (AND) (Pfizer SA, Madrid, Spain), kaspofungina (CAS) (Merck and Com Inc., NJ, USA), mikafungina (MCF) (Astellas Pharma Inc., Japan), posakonazola (PCZ) (Merck & Com Inc., NJ, USA), borikonazola (VCZ)(Pfizer SA, Madrid, Spain) eta flukonazola (FCZ) (Pfizer SA, Madril, Spain). Saiakuntzak egin ziren farmako antifungikoen diluzio bikoitz seriatuekin aurrez prestatutako 96 putzuko mikroplaketan. Erabilitako kontzentrazio-tartea 0,03 eta 16 µg/ml artekoa izan zen, baina FCZren kasuan kontzentrazio-tartea 0,12 eta 64 µg/ml artekoa izan zen. Saiakuntzak egiteko, 24 orduko *Candida* hazkuntzatik abiatuta, 0,5-2,5 x 10^3 UFC/ml-ko legamia-kontzentrazioa zuen esekidura prestatu zen. Kontrol modura erabilitako anduiak *C. krusei* ATCC 6258 eta *C. parapsilosis* ATCC 22019 izan ziren. Antifungiko bakoitzerako kontzentrazio minimo inhibitzailea (MIC) zehaztu zen CLSIaren aurretik aipatutako protokoloetan deskribatutakoaren arabera.

4.5.2. *European Committee on Antimicrobial Susceptibility Testing*ak proposatutako metodoa

Odol-jatorria duten *C. auris*eko bost isolamenduek ekinokandinekiko duten sentikortasuna aztertzeko, *European Committee on Antimicrobial Susceptibility Testing*ak (EUCAST) garatutako E.DEF 7.3.2 protokoloa erabili zen, legamien sentikortasun antifungikoa aztertzeko erabiltzen dena (Arendrup et al., 2020). Saiakuntzak 96 putzuko mikroplaketan egin ziren, aldez aurretik, AND, CAS eta MCF ekinoandinen diluzio bikoitz seriatuekin prestatuta zeudenak, kontzentrazio-tartea 0,008 eta 4 µg/ml artekoa izan zelarik. Saiakuntzak egiteko 24 orduko *C. auris* hazkuntzatik abiatuta 1-5 x 10^5 UFC/ml-ko kontzentrazioa zuen esekidura prestatu zen, zeinetik 100 µl inokulatu ziren. Kontrol modura erabilitako anduiak *Candida krusei* ATCC 6258 eta *Candida parapsilosis* ATCC 22019 izan ziren. Hazkunde zelularraren %50a murrizten zuen kontzentrazio minimo inhibitzailea (MIC_1) edo %90a (MIC_0) 35 °C-tan inkubatu eta 24 ordu igaro eta gero zehaztu zen. Plaken irakurketa espektofotometro baten bidez egin zen (Tekan, Suitza). Isolamenduak sentikortzat edo erresistentetzat sailkatzeko, *Centers for Disease Control and Prevention* (CDC) erakundeak proposatutako ebaketa-puntuak hartu ziren kontuan (CDC, 2018).

4.5.3. Farmakoen aktibitate konbinatuaren azterketa xake-taularen metodoaren bitartez

Odol-jatorrizko *C. auris*eko bost isolamenduek antifungikoekiko duten sentikortasuna aztertzeko saiakuntzak xake-taula izeneko metodologia erabiliz zen. Teknika hori jarraituz, AmB polienoa eta AND, CAS eta MCF ekinoandinak konbinatu ziren. Saiakuntzan AmB farmakoa aztertzeko erabilitako kontzentrazio-tartea 0,016 eta 1 µg/ml artekoa izan zen. Hiru ekinokandinen kasuan, erabilitako kontzentrazio-tartea 0,016tik 8 µg/ml-ra bitartekoa izan zen. Hurrengo irudian zehazten dira antifungikoen eta hazkunde- eta esterilitate-kontrolen antolaketa (11. Irudia):



11. Irudia: Mikroplakaren antolaketa antifungikoen konbinaketaren azterketa egiteko, xake-taularen metodoaren bidez

4.5.4. Loeweren teoria gehigarria

Antifungiko konbinatuen arteko elkarrekintza aztertzeko kalkuluak Loeweren teoria gehigarriaren bidez egin ziren, honako formula hau erabiliz (FICI = *fractional inhibitory concentration index*):

$$FICI = \frac{\text{MIC A konbinaketan}}{\text{MIC A monoterapian}} + \frac{\text{MIC B konbinaketan}}{\text{MIC B monoterapian}}$$

Kasu honetan, MIC_0 balioa erabili zen. Elkarrekintzaren sailkapena ondorengo balioen arabera kalifikatu zen (Te Dorsthorst et al, 2002):

FICI ≤0,5 → Efektu Sinergikoa
0,5< FICI ≥ 1 → Efektu Gehigarria
1< FICI ≥ 4 → Efektu Indiferentea
FICI > 4 → Efektu Antagonista

4.6. *Caenorhabditis elegans* in vivo eredua

4.6.1. *Caenorhabditis elegans* anduia eta mantentze baldintzak

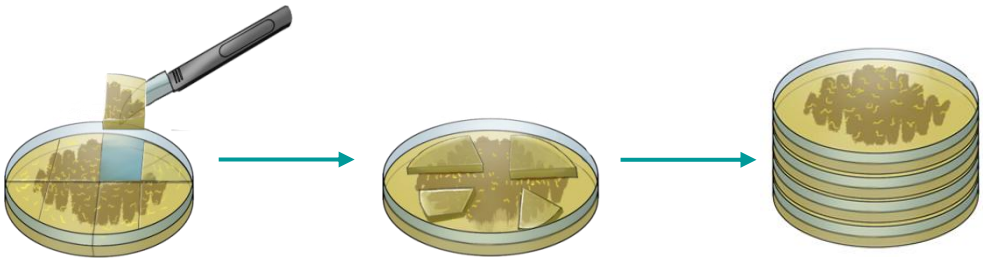
C. elegans in vivo modeloarekin egindako saiakuntzak AU37 andui mutante bikoitzarekin egin ziren. Andui hori Minnesotako Unibertsitatean (AEB) Caenorhabditis zentrotik (CGC) lortu zen. Bi mutazio horiek dituzten nematodoak 15 ºC-tan mantentzen direnean normalak dira bai tamainari dagokionez bai morfologikoki. Hala ere, ale bakoitza 25 ° C-tan hazi eta garatzen direnean, *glp-4* geneko mutazioak gonadak garatzeko gai ez izatea eragiten du, eta, beraz, ez dira ugaltzeko gai, saiakuntzetan zehar ale kopuru berarekin etengabe lan egitea ziurtatuz. Bestalde, *sek-1* geneko mutazioak nematodoak infekzioarekiko sentikorragoak izatea eragiten du, eta, beraz, errazagoa da saiakuntzetan infekzioak sortzea. *C. elegans* andui horrek 25 °C-tan inkubatzeak erduaren erabilera eta jarraipen egokia ahalbidetzen du saiakuntzan zehar (Breger et al., 2007, Pukkila-Worley et al., 2009).

Kandidiasi-eredu hori erabiltzeko medioak eta diluzioak hurrengo hauek dira:

- Nematodoak hazteko kultibo-ingurumena (NGM, *Nematode Growth Media*): 2,4 g NaCl, 13,6 g agar bakteriologiko, 2 g peptona, 800 µl $CaCl_2$ (1 M), 800 µl 5 mg/ml kolesterol etanolean, 800 µl $MgSO_4$ (1M), 20 ml KPO_4, 777 ml H_2O destilatua.
- M9 indargetzailea: 3 g KH_2PO_4, 6 g Na_2HPO_4, 5 g NaCl, 1 ml $MgSO_4$, 1 L H_2O destilatua.
- Garbiketa disoluzioa: 1 ml sodio hipoklorito (37 g/L), 0,5 ml NaOH (5M).

Nematodoak *Escherichia coli*ren OP50 anduiarekin ereindutako NGM duten plaketan 15 °C-tan mantentzen dira, erreproduzitu ahal izateko. *E. coli* anduia hori ez-patogenoa da, urazilorako auxotrofoa da eta bere hazkuntza NGM ingurumenean motela da. Horregatik, nematodoarentzako elikagai bezala balio du, eta, gainera, sortzen den soropil mugatuak, aleak erraz behatzea ahalbidetzen du. NGM ingurumeneko osagaiek nematodoak urgeruza mehe batez estalita mantentzea ahalbidetzen dute, eta horrek azaleko tentsioa mantentzen laguntzen du, agarrean askatasunez mugitzen laguntzen dielarik (Breger et al. 2007; Pukkila-Worley et al. 2009).

Nematodoen mantentze-prozedura laburbilduz azaltzen da: NGM plaketako elikagaia agortzen denean, nematodoak *E. coli* duten NGM agar plaka berrietara transferitzen dira. Prozesu hori, nematodoak dituen NGM agarra xerra batekin 16 zatitan zatitzean datza, eta, ondoren, horietako lau zati plaka berri bakoitzera transferitzea. Zatien gainazalak plaka berriaren gainazalarekin kontaktuan egon behar du, nematodoak plaka berrira mugitu ahal izateko elikagaiaren bila, eta 10-15 minutu igaro ondoren agar-zatiak kentzen dira. Horrela plaka batetik lau plaka berri izango ditugu nematodo kopurua handituz (12. Irudia).



12. Irudia: C. elegans nematodoen mantentze-prozedura NGM eta *E. coli* OP50 anduia erabiliz

4.6.2. *Caenorhabditis elegans* hazkuntza-faseen sinkronizazioa

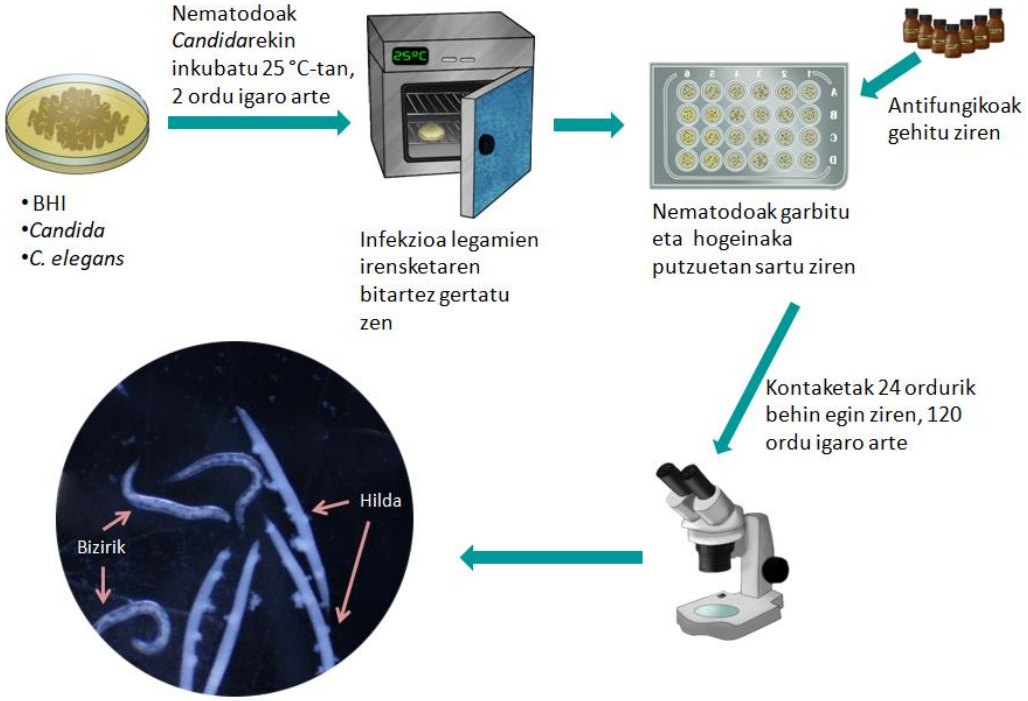
Nematodoekin egindako saiakuntzak arrautzetatik abiatuta egin ziren. Hori lortzeko prozedura honakoa izan zen. NGM plakak, nematodoak dutenak eta 15 °C-tan mantendutakoak, gutxi gorabehera 3,5 ml ur destilaturekin garbitu ziren. Nematodoak beirazko Pasteur pipeta batekin bildu ziren eta 15 ml-ko hondo konikoa duten hodietan sartu ziren. Hodi horiek bi minutuz 1200 rpm-tan zentrifugatu ziren, nematodoak sedimentatzeko eta hodiaren hondoan kontzentratu bat sor zezala. Ondoren, soberakina kendu zen 3,5 ml utzi arte eta 1,5 ml garbiketa-soluzio gehitu ziren. Garbiketa-soluzioa 8 minutuz jardun zen, lagina behin eta berriz astinduz, nematodoen gorputza apurtzen laguntzeko. Denbora hori igarotakoan, nematodoen gorputza desintegratzen zen, eta arrautzez osatutako prezipitatu bat baino ez zen geratzen. Lagina 3500 rpm-tan 30 segundo zehar zentrifugatu ondoren, garbiketa-soluzioa kentzen zen arrautzen prezipitatua bakarrik uzteko. Baldintza antzuetan, 12 ml M9 indargetzaile gehitu ziren eta lagina 30 segunduz 3500 rpm-tan zentrifugatu zen. Azken prozesu hori berriro errepikatu zen, prezipitatua behar bezala garbitzen zela ziurtatzeko. Ondoren, soberakina kendu zen, 500 µl inguru utziz, eta, prezipitatua homogeneizatu ondoren, arrautzak *E. coli* gabeko NGM plaketan erein ziren. Azkenik, plakak 15 °C-tan inkubatu ziren gau osoan zehar, eta denbora horretan, arrautzek eklosionatzen ziren eta, elikagai faltaren ondorioz, C. elegans larben hazkuntza L1 fasean gelditu zen. L1 fasean zeuden nematodo gazteak *E. coli* OP50 soropila zuten NGM plaketara transferitu ziren, mantentze-prozeduran erabilitako metodo bera erabiliz. Une horretan, saiakuntzan erabiliko ziren ale guztiak sinkronizatuta zeuden

L1 hazkuntza-fasean. Plakak 25 ºC-tan inkubatu ziren 72 orduz gutxi gorabehera, eta, ondoren, saiakuntza hasteko nematodo helduak (L4 hazkuntza-fasean) lortu ziren.

4.6.3. *Caenorhabditis elegans* nematodoaren infekzio-prozedura

Infekzio-prozesu osoa fluxu laminarreko kanpaian egin zen (13. Irudia). Saiakuntza baino 24 ordu lehenago, bihotz-garuna infusio agar (BHI) plakak prestatu ziren, kanamizinarekin gehigarrituta (45 µg/ml) (bakterio-kutsadura saihesteko) eta *Candida*ren hazkuntzarekin (erabilitako plakek 50 mm-ko diametroa zuten). Horretarako, saiakuntzan erabiliko zen *Candida* anduiaren 24 orduko hazkuntza erabili zen. Hazkuntza horretatik abiatuta, 2 McFarland esekidura prestatu zen eta horren 50 µl erein ziren BHI agarrean. Plakak 37 ºC-tan inkubatu ziren 24 orduz.

Saiakuntzaren egunean, aldez aurretik sinkronizatutako nematodo helduak zituzten NGM plakak 3,5 ml kanamizinarekin gehigarritutako (45 µg/ml) M9 indargetzailarekin garbitu ziren. Nematodoak biltzeko, beirazko Pasteur pipeta bat erabili zen eta 15 ml-ko hodietan utzi ziren. Lagina 1200 rpm-tan 2 minutuz zentrifugatu zen eta nematodoak sedimentatu egin ziren. Ondoren, gain-jalkina kendu zen mililitro bat inguru utzi arte, eta suspentzioa homogeneizatu ondoren, nematodoak legamia zuen BHI agar plakara transferitu ziren. Plaka hori 25 ºC-tan inkubatu zen bi orduz nematodoek legamiak irents zitzaten. Denbora hori igaro ondoren, BHI agar plaka garbitu zen 3,5 ml M9 indargetzailearekin, eta beirazko Pasteur pipeta batekin jaso ziren nematodoak. Nematodoak hondoan sedimentatzeko, 15 ml-ko hodi batean isuri ziren eta 10 minutuz mugitu gabe utzi ziren. Ondoren, soberakina kendu eta kanamizinarekin osagarritutako M9 indargetzailea gehitu zen. Lagina 1200 rpm-tan zentrifugatu zen bi minutuz eta soberakina kendu zen. Azkenik, nematodoen kutikulari itsatsita egon zitezkeen *Candida* zelula guztiak ezabatzeko asmoz, *E coli* gabeko NGM agar plaketan utzi ziren marruskaduraren bidez ezabatzeko.



13. Irudia: *C. elegans* nematodoen infekzio-prozesua *Candida*rekin eta tratamendua farmako antifungikoekin

4.6.4. *Caenorhabditis elegans* erabiliz *Candida* infekzioaren aurkako tratamendua

Konposatu antifungikoen eraginkortasuna ebaluatzeko, nematodoak 24 putzutako plaketan sartu ziren. Putzu bakoitza, kanamizinaz eta 10 µg/ml kolesterol-etanolarekin osagarritutako M9 indargetzailearekin alikuotatu zen (13. Irudia). Nematodoak 20 aleko taldetan sartu ziren putzu bakoitzeko, NGM plaketatik banan-banan jaso ondoren lupa erabiliz ereiteko euskarriarekin. Saiakuntza bakoitzean antifungiko eta nematodoen kontrol bakoitzerako hiru putzu erabili ziren. Hau da, saiakuntza bakoitzean 60 ale erabili ziren. Kontrol gisa, tratamendu antifungikorik gabe infektatutako nematodoak eta nematodo osasuntsuak erabili ziren. Doktorego tesi honetan egindako azterketen arabera erabilitako antifungikoak eta horien kontzentrazioak aldatu ziren, eta hurrengo ataletan zehazten dira. Plakak, antifungikoak gehitu ondoren, 25 °C-tan inkubatu ziren 120 orduz,

eta nematodo biziak eta hilak 24 ordutik behin zenbatu ziren mikroskopio esteroskopiko baten bidez (13. Irudia). Saiakuntzak aztertutako andui bakoitzarekin hirutan egin ziren, egun desberdinetan.

4.6.4.1. Bigarren azterlana: Candida glabrata, Candida bracarensis *eta* Candida nivariensis *espezieek eragindako infekzioaren aurkako tratamendu antifungikoaren eraginkortasunaren azterketa*

Ikerketa lan horretan erabilitako antifungikoak eta kontzentrazioak ondokoak izan ziren: AmB, VCZ eta PCZ 1 eta 2µg/ml-ko kontzentrazioetan, FCZ 32, 64 eta 128 µg/ml-ko kontzentrazioetan, CAS eta MCF 4 eta 8 µg/ml-ko kontzentrazioetan eta AND 8 µg/ml-ko kontzentrazioan.

4.6.4.2. Bostgarren azterlana: Candida auris *eragindako infekzioaren aurkako antifungikoen konbinazioaren eraginkortasunaren azterketa*

Azterlan horretan, erabilitako antifungikoak AmB eta hiru ekinoandinak izan ziren. Saiakuntzetan erabilitako kontzentrazioak *C. auris* isolamenduaren arabera aldatu ziren, in vitro konbinazio saiakuntzetan lortutako emaitzak erabili baitziren (6. Taula).

6. Taula: *C. elegans* in vivo ereduan egindako saiakuntzetan erabilitako *C. auris* isolamenduen aurkako antifungikoen konbinaketen kontzentrazioak

Isolamenduak	AmB/AND (µg/ml)	AmB/CAS (µg/ml)	AmB/MCF (µg/ml)
CJ94	0,03/1	0,03/0,5	0,03/0,25
CBS 15605	0,03/0,5	0,03/0,5	0,03/0,5
CBS 15606	0,06/2	0,06/1	0,06/2
CBS 15607	0,03/1	0,03/1	0,06/0,5
JMRC:NRZ 1101	0,25/2	0,25/4	0,5/0,5

4.7. *Galleria mellonella* in vivo eredua

4.7.1. *Galleria mellonella* larbak eta mantentze baldintzak

*G. mellonella*ren larbak Bichosa enpresatik (Espainia) lortu ziren. Laborategira iritsitako ale guztiak gari-zahaiaz eta eztiaz hornituriko zulozko plastikozko kutxetan banatu ziren eta bertan 48 orduz 25 °C-tan eta iluntasunean ingurugirora egokitzen utzi ziren. Saiakuntza egunean larba osasuntsuenak eta tamainaz antzekoak, 0,3 eta 0,5 g artekoak, hautatu ziren eta Petri plaka esterilizatu bakoitzean bost edo 20 larba sartu ziren. Plakak iluntasunean inkubatu ziren 37 °C-tan eta elikagairik gabe.

4.7.2. *Candida* inokuluen prestaketa eta *Galleria mellonella*ren infekzioa

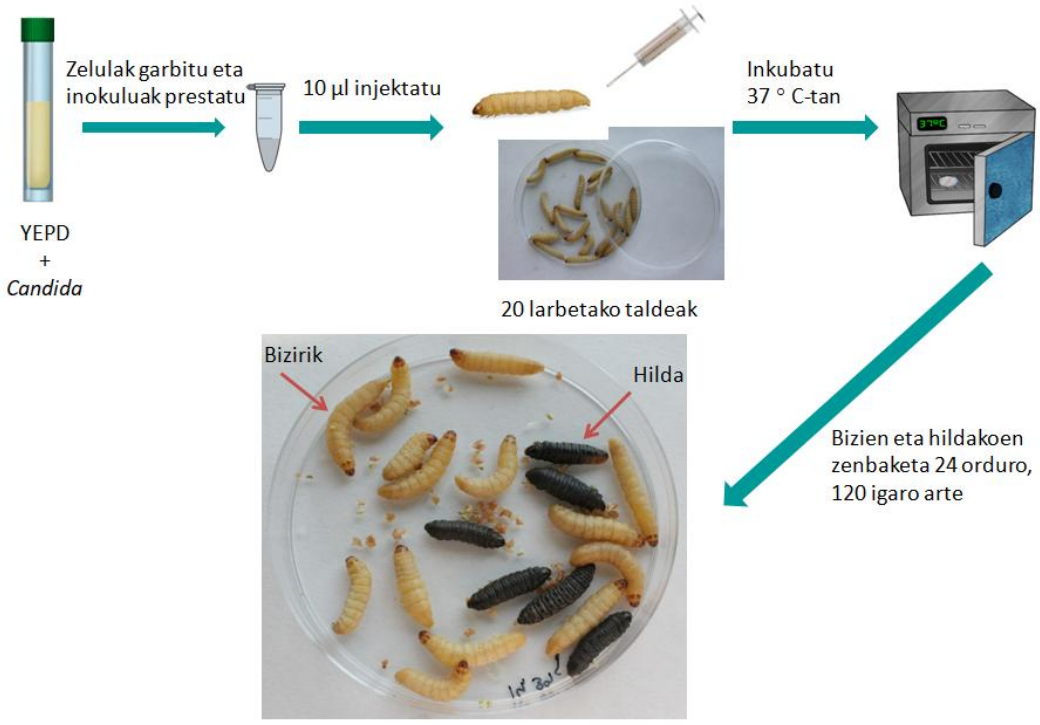
*Candida*ren inokuluak prestazeko aurrez YEPD (% 1 legamia estraktua, % 2 peptona bakteriologikoa, % 2 D-glucosa; Panreac, España) ampizilina (20 µg/ml) gehigarrizko saldan hazi ziren, bakterioen kutsadura ekiditeko. Hazitako Candida zelulak hiru aldiz garbitu ziren PBS indargetzailearekin 2500 rpm-tan 10 minutuz zentrifugatuz. eta hemozitometroan zenbaketak egin ziren. Hemozitometro baten bidez legami-zelulen zenbaketak egin ziren mikroskopioan eta ondoren, 1:10eko diluzio seriatuak egin ziren PBSn 10^7, 10^8 eta 10^9 zelula/ml kontzentrazioak prestatzeko. *Candida*ren kontzentrazio bakoitzetik 10µl injektatu ziren larba bakoitzean, beraz, sartutako kontzentrazio zelularra 10^5, 10^6 eta 10^7 zelula/larba-takoa izan zen, hurrenez hurren.

Honez gain esperimentu bakoitzean bi kontrol erabili ziren, kontrol batean 20 larba erabili ziren injekziorik jasoko ez zutenak eta beste kontrolean beste 20 larba PBS 20 µg/ml anpizilina gehigarrizko soluzio bat injektatuta.

Infekzio prozesuaren abiapuntua, larba mahaian buruz gora pausatzea izan zen eta bere ezkerraldeko azken pro-hanka %70eko etanolean bustiriko torunda batekin garbitzea, injekzio gunea desinfektatzeko. Doitasun handiko xiringa baten bidez larbei legamien inokulu-bolumena edo PBS 20 µg/ml anpizilina gehigarrizko soluzioa injektatu zitzaien. Xiringa erabili aurretik, hiru aldiz etanolez eta ondoren beste hiru aldiz PBS-z garbitu zen eta prozesu hori berriz ere errepikatu zen Petri plaka bakoitzean zeuden 20 larbak

injektatzeaz amaitzen zen bakoitzean, hau da, kontzentrazio eta andui aldaketa bat egitea egokitzen zenean.

Infekzio prozesuarekin amaituta, larbak Petri plaketan 37°C-tan eta ilunpean inkubatu ziren. *G. mellonella*-ren biziraupenaren jarraipena egin zen 24 orduro 120 ordu igaro arte. Larbak hiltzerakoan mugimendu gabe geratzen ziren eta marroi ilun kolorea hartzen zuten, bere gorputzaren melanizazioagatik (14. Irudia). Esperimentu guztiak hirutan egin ziren egun desberdinetan.

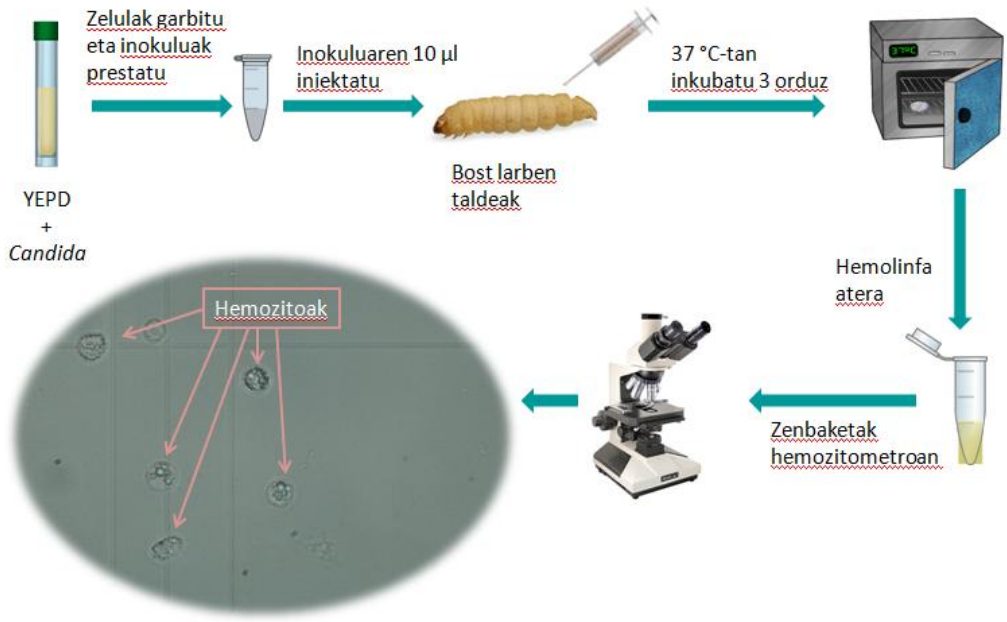


14. Irudia: *G. mellonella* larben infekzio-prozedura

4.7.3. *Galleria mellonella*ren hemozitoen ekoizpena

*Candida*ren zelulak aurreko atalean bezala hazi eta garbitu ziren. Berriz ere 10^7, 10^8 eta 10^9 zelula/ml inokuluak prestatu ziren eta larba bakoitzean horietako 10 µl injektatu ziren doitasun-xiringa batekin (15. Irudia).

Saiakuntza horiek egiteko, 0,3-0,5 g arteko pisua zuten larbak erabili ziren bosnakako taldetan, kontrol modura injekziorik jasoko ez zuten larbak eta larba PBS 20 µg/ml anpizilina gehigarrizko soluzio bat injektatuta erabili ziren. *G. mellonella*ren larbak hiru orduz inkubatu ziren iluntasunean 37 °C-tan eta ondoren horien hemolinfa bildu zen. Intsektuetarako gatz indargetzaile (IPS, 150 mM sodio kloruroa, 5 mM potasio kloruroa, 10 mM Tris-HCl pH 6,9, 10 mM EDTA eta 30 mM sodio zitratoa) soluzioaren 1:1 diluzioarekin, larben melanizazioa ekiditeko. Hemozitoen ekoizpena hemozitometro batekin mikroskopioan egindako zenbaketen bidez aztertu zen eta saiakuntza guztiak hirutan egin ziren egun desberdinetan.

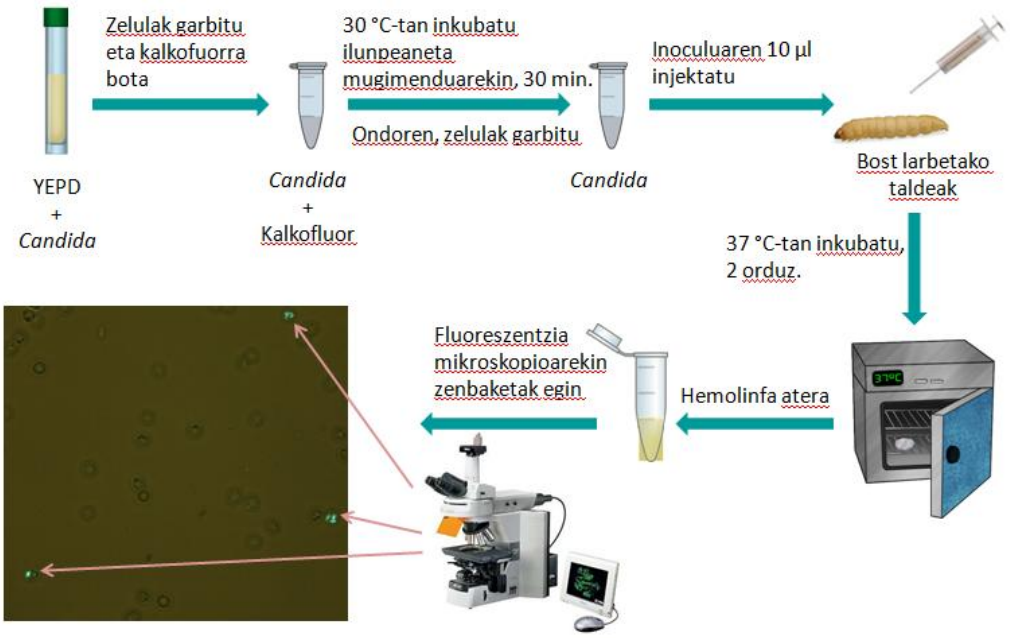


15. Irudia: *G. mellonella*ren larben infekzio prozesua eta hemolinfa ateratzeko prozesua, hemozitoen zenbaketak egiteko

4.7.4. *Galleria mellonella*ren hemozitoen gaitasun fagozitikoa

Larben hemozitoen gaitasun fagozitikoaren azterketa egiteko, bost larbaz osatutako taldeak erabili ziren. Kasu honetan, *Candida*ren 10^8 zelula/ml inokuloarekin egin zen infekzioa eta ezkerreko azken propatan 10 µl injektatu ziren (16. Irudia). *Candida* zelulak

aldez aurretik 0,4 mg kalkofluor zuriko tindatzailearekin (Sigma Aldrich, St. Louis, Mo, AEB) tindatu ziren 30 minutuz 30 ºC-tan, gero PBSrekin garbituz. Tindatutako *Candida*ren zelulak injektatu ondoren, infektatutako larbak bi orduz inkubatu ziren iluntasunean 37 ºC-tan. Denbora hori igaro ondoren, larben hemolinfa bildu zen eta melanizazioa saihesteko 1:1 diluzioa egin zen IPSn. *Candida* zelulak fagozitatuta zituzten hemozitoak zenbatzeko Nikon Eclipse 80i (Melville, NY, AEB) fluoreszentzia-mikroskopioa erabili zen. Gutxienez 100 hemozito zenbatu ziren, zelula fagozitatuekin eta zelula fagozitatu gabe. Esperimentuak hirutan egin ziren egun desberdinetan.



16. Irudia: *G. mellonella* larben infekzio prozesua eta hemolinfa ateratzeko prozesua, *Candida* zelulak fagozitatutako hemozitoen zenbaketak egiteko

4.7.5. *Galleria mellonella* erabiliz *Candida* infekzioaren aurkako tratamendua

*Candida*ren hazkuntza zelularra YEPDan eta 10^8 zelula/ml inokuloaren prestaketa aurretik deskribatu bezala egin ziren. Larbak 20 aleko taldetan banatu ziren eta

inokuloaren 10 µl injektatu zitzaien, azken kontzentrazioa 10^6 zelula/larba izanik, berriz ere bi larba kontrol taldeak aztertu ziren.

4.7.5.1. *Hirugarren azterlana: Ekinokandinekin egindako tratamendu antifungikoaren eraginkortasunaren azterketa* Candida glabrata, Candida bracarensis *eta* Candida nivariensis *espezieek eragindako infekzioaren aurka*

Azterlan hori garatzeko hiru ekinokandinak aztertu ziren *Candida glabrata*, *Candida bracarensis* eta *Candida nivariensis* espezieek sortzen zuten infekzioak tratatzeko. Frogatutako kontzentrazioak 4 eta 8 µg/g larbakoa izan ziren. Konposatu antifungiko horien efektu toxikoa ere aztertu zen eta horretarako infektatu gabeko larbei tratamendu antifungikoa injektatu zitzaien. Larbak 37 °C-tan inkubatu ziren iluntasunean 120 orduz, 24 ordutik behin horien biziraupena aztertuz. Esperimentu guztiak hirutan egin ziren egun desberdinetan.

4.8. Analisi estatistikoa

Biofilmen produkzioaren emaitzak Student´s t-testarekin aztertu ziren SPSS v24.0 (IBM, Chicago, IL, USA) estatistika-programarekin.

Biziraupen-kurbak Kaplan-Meier estatistikoaren bitartez kalkulatu ziren GraphPad Prism 5 programa erabilita (GraphPad Software, La Jolla, CA, USA). Log-rank estatistikoa *Candida* espezieren edota anduien arteko desberdintasunak eta tratamendu antifungikoen arteko desberdintasunak estimatzeko erabili zen. Estatistika-azterketak SPSS v24.0 programarekin garatu ziren eta adierazpen maila 0,05 balioan ezarri zen ($p < 0,05$). Bai hemozitoen ekoizpena eta baita horien ahalmen fagozitikoa ere ANOVA unifaktorialaren bidez aztertu zen, SPSS estatistika-programarekin ($p < 0,05$ estatistikoki adierazgarritzat jo zen).

5. EMAITZAK

1. Azterlana

Galleria mellonella insektua eta *Caenorhabditis elegans* nematodoa, infekzio eredu boteretsuak *Candida glabrata* eta erlazionatutako espezieen birulentzia ikertzeko

«Experimental candidiasis models in the insect *Galleria mellonella*and the nematode *Caenorhabditis elegans* are useful to evaluate the virulence of *Candida glabrata* and related species»

Ainara Hernando-Ortiz, Estibaliz Mateo, Marcelo Ortega-Riveros, Iker De-la-Pinta, Guillermo Quindós, Elena Eraso

Mikologia Medikoko laborategia, UFI 11/25, Immunologia, Mikrobiologia eta Parasitologia Saila, Medikuntza eta Erizaintza Fakultatea, (UPV/EHU)

Ekaia 2019, 36; 175-190

Laburpena

Kandidiasia *Candida* generoko espezieek eragindako infekzio mikotikoa da. *Candida albicans* agente etiologiko nagusia da baina gero eta gehiagotan *Candida* generoko beste espezie batzuk agertzen ari dira kandidiasiaren eragile bezala eta, hauen artean, *Candida glabrata* espeziea. Espezie honekin lotuta beste bi espezie daude, *Candida bracarensis* eta *Candida nivariensis*, teknika molekularrek ondo desberdindu ditzaketenak. Aldaketa etiologiko hauek ondorio larriak izan ditzakete kandidiasiaren diagnostikoan, tratamenduan edota pronostikoan, besteak beste, bere patogenia edo antifungikoekiko sentikortasuna oso desberdina izan daitezkeelako. Ordezko eredu esperimentalek erabiliz mikroorganismoek eragindako gaixotasunen patogenia eta terapia ezagutzeko ezinbesteko aukera ematen dizkigute, eta hauen artean, *Caenorhabditis elegans* nematodoa eta *Galleria mellonella* lepidopteroa ditugu. Ikerketa lan honetan konbentzionalak ez diren bi animalia eredu hauen erabilgarritasuna ebaluatu nahi izan da *Candida glabrata*, *Candida bracarensis* eta *Candida nivariensis* harreman filogenetiko estua duten hiru espezie hauen birulentzia in vivo aztertzeko.

Hitz gakoak: *Candida glabrata, Candida bracarensis, Candida nivariensis*, birulentzia, ordezko ereduak.

Abstract

Candidiasis is a mycotic infection caused by species of *Candida* genus. *Candida albicans* is the major aetiological agent, although other species of *Candida*, such as *Candida glabrata*, are considered emerging causes of this disease. The species, *Candida bracarensis* and *Candida nivariensis*, are phylogenetically similar to *Candida glabrata* and can be correctly differentiated by molecular techniques. These changes in the aetiology have serious implications for diagnosis, treatment and prognosis; considering that yeast pathogenesis or susceptibilities to current antifungal drugs may be different. Invertebrate models, such as the nematode *Caenorhabditis elegans* and the lepidopter *Galleria mellonella*, are an attractive alternative for the study of fungal pathogenesis and antifungal therapy. The aim of this research study was to evaluate the usefulness of these two non-convetional model to assess the in vivo virulence of the phylogenetically close-related species, *Candida glabrata, Candida bracarensis* and *Candida nivariensis.*

Keywords: *Candida glabrata, Candida bracarensis, Candida nivariensis*, virulence, non-conventional models.

1. SARRERA

1.1. Kandidiasia eta *Candida glabrata*

Kandidiasia gizakiak jasaten duen mikosi oportunista ohikoena da. Oro har, ahoko eta genitaletako mukosako, larruazalpeko eta azazkaleko gaixotasun arinak dira, tratamenduak oso gutxitan desagerrarazi ezin dituenak. Sistemikoak edo inbasoreak diren kandidiasiak, berriz, bakanetan gertatzen dira, baina gaixotasun larriak eta heriotza-tasa altuak eragiten dituzte. Halako infekzioak jasaten dituzten pertsonek azpiko gaixotasun larriak, neutropenia edo defentsa gutxituak izan ohi dituzte, eta sendatzea zailagoa izaten da. Azken hogei urteotan mikosi inbasoreek gorakada nabarmena izan dute, eta osasun arazo garrantzitsu bihurtu dira [1,2].

Kandidiasi terminoak *Candida albicans* espezieak eta *Candida* generoaren barruan dauden beste espezieek eragindako infekzioa definitzen du. *Candida* AEBko eta Europako infekzio nosokomialen laugarren kausa ohikoena da, eta kandidiasi inbasorearen 72,8 kasu gertatzen dira urtero milioi bat biztanleko. *C. albicans* agente etiologiko nagusia da, baina gero eta gehiagotan *Candida* generoko beste espezie batzuk agertzen ari dira kandidiasiaren eragile gisa; besteak beste, *Candida parapsilosis*, *Candida glabrata*, *Candida tropicalis*, *Candida krusei* eta, berriki, *Candida auris*. *Candida glabrata*ren intzidentzia, espezie horien artean, nabarmen handitu da, eta AEBko eta erdialdeko zein iparraldeko Europako kandidemietan bigarren espezie isolatuena da [1]. Ohikoa da isolatzea jaioberrietan, adineko pazienteengan, sabeleko kirurgian, transplantean, eta kortikoide edo antibiotiko tratamenduak aldez aurretik jaso dituzten pazienteengan, baita bulbobaginitisa duten emakumeengan ere [3].

*Candida glabrata*ren taxonomiak, *Candida albicans* eta *Candida parapsilosis* espezieenak bezala, aldaketa garrantzitsuak izan ditu azken urteotan. Espezie horiek *Candida* generoko beste espezie berriekin harreman filogenetiko estua dute, eta espezie kriptiko deritze. Espezie kriptikoek ezaugarri morfologiko oso antzekoak dituzte, eta

espezie nagusietatik bereizten zailak dira, baina taxonomikoki desberdinak dira, eta patogenia edo antifungikoekiko sentikortasuna ere oso desberdina izan daiteke [2,4]. *Candida bracarensis*ek eta *Candida nivariensis*ek ezaugarri fenotipiko asko partekatzen dituzte *Candida glabrata*rekin. Beraz, oker identifika daitezke, teknika molekular zehatzak erabiltzen ez badira [5,6,7]. Hiru espezie horien datu zehatzak (intzidentziari, birulentziari edo antifungikoekiko sentikortasunari dagokienez) oraindik ezezagunak dira [8,9].

1.2. Eredu esperimentalak

Eredu esperimentalek bakterioek eta onddoek eragindako gaixotasunen patogenia eta terapia ezagutzeko aukera ematen digute, gizakien erabilera saihestuz. Animalia eta beraren ingurunea kontrola ditzakegunez, ostalari eta patogenoen arteko elkarrekintzen kausa-efektu harremanen azterketa zehatza egin daiteke.

Patogeniaren, farmakologiaren eta immunologiaren aurrerapenak, batez ere, ugaztun ereduetan egiaztatu dira, hala nola sagu, arratoi, untxi eta tximinoetan. Arratoiak, *Mus musculus*, baliatzea da infekzio fungikoak aztertzeko metodo ohikoena, besteak beste, anatomiako eta erantzun immunologikoko antzekotasunak direla medio. Hala ere, alderdi ekonomikoek, logistikoek eta etikoek animalia ornodunen erabilera murrizten dute, batez ere andui kopuru handia aztertzea beharrezkoa denean. Horregatik, ordezko ereduak erabiltzen dira: eredu konputazionalak, landareak edota mikroorganismoak, baina baita animalia ornogabeak eta odol hotzeko animaliak ere. Ondooak aztertu dira dagoeneko *Arabidopsis thaliana* landarean, *Acanthamoeba castellanii* eta *Dictyostelium discoideum* amebetan, *Caenorhabditis elegans* nematodoan, *Bombyx mori* zeta-harrean, *Culex quinquefasciatus* eltxoan, *Drosophila melanogaster* fruitu-eulian, *Blattella germanica* **labezomorro arrean,** *Galleria mellonella* lepidopteroan eta *Danio rerio* arrainean [10,11,12,13].

Candida aztertzeko eredu aproposenek zenbait ezaugarri bete behar dituzte, besteak beste: sarbidean kolonizazio eta inbasio prozesuak zehatz erreproduzitu behar ditu, eta

infekzioarekin lotutako immunitate-sistema edo hormonen baldintzak berdindu behar ditu. Gainera, infekzio esperimentala nahiko luzea izango da *Candida*ren birulentzia faktoreak pausoz pauso aktibatzeko eta ostalariaren defentsak abian jartzeko.

1.2.1. Caenorhabditis elegans

Caenorhabditis elegans nematodoa lurzoruan bizi den *Rhabditidae* familiako organismo hermafrodita da. Eredu egokia da eragile infekziosoen eraginak ezagutzeko, mikroorganismoz elikatzen baita eta ugaztun eta gizakiak kutsa ditzaketen bakterio eta onddo patogeno asko jasan baititzake [12]. Mutante ugari daude eskuragarri, eta, horien artean, *Caenorhabditis elegans* AU37 anduia. Horrek *glp-4* genean daukan mutazioak 25 ºC-an indibiduo antzuak ekoizten ditu. Horrela, kopuru finkoa mantentzen da esperimentuan zehar, eta *sek-1* genean daukan mutazioak berezko erantzun immunea eragiten du, eta infekzioarekiko sentikorrak dira [14,15]. Lan batzuetan, eredu horrekin *Candida*ren zenbait espezieren birulentzia eta horien kontrako antifungikoen eragina ikertu dira [15,16,17].

1.2.2. *Galleria mellonella*

Lepidoptero hau *Pyralidae* familiaren barruan kokatzen da, eta argizari-sits handia esaten zaio. Haren larba egoera *Candida*k sortutako infekzioan gertatzen diren ostalari-patogeno elkarrekintzak ikertzeko eredu berri bat da.

*Galleria mellonella*ren larbak erraz eta ekonomikoki lortzen dira, eta espeziea eredu erakargarria da abantaila argiak dituelako onddoen infekzioak ikertzeko, erabiltzen diren beste ereduekin konparatuta. Esate baterako, erraz manipulatzen dira, eta erraz ikusten da infekzioaren eragina larbak beltz eta mugimendurik gabe gelditzen direlako. Ugaztunak ez diren eredu asko ez bezala, gizakientzat *Candida* patogenoa den tenperaturan, 37 °C-an alegia, mantendu daiteke, eta beraren erantzun immuneak antzekotasunak dauzka ugaztunen berezko erantzun immunearekin [12, 18].

Oraindik ere gutxi dira *Candida*ren ikerketan *Galleria mellonella* eredua erabili duten lanak. Alde batetik, *Candida albicans*en andui basati eta mutanteen arteko birulentzia konparatu da [18]; *Candida parapsilosis* eta harreman filogenetiko estua duten beste bi espezieren arteko birulentzia aztertu da [19]; *Candida auris* eta beste *Candida* espezieen birulentzia ikertu da [10], eta *Candida glabrata* andui baten birulentzia eredu honetan eta arratoiaren ereduan ere konparatu da [4]. Bestalde, *Candida*ren zenbait espezieren kontrako tratamendu antifungikoaren eraginkortasuna egiaztatu da [4,15,18,20].

2. HELBURUA

Ikerketa honen helburua da *Galleria mellonella* eta *Caenorhabditis elegans* konbentzionalak ez diren animalia esperimentalen erabilgarritasuna aztertzea eta bi ordezko eredu horiek erabiliz harreman filogenetiko estua duten *Candida glabrata*, *Candida bracarensis* eta *Candida nivariensis* espezieen birulentzia aztertzea eta konparatzea.

3. METODOLOGIA

3.1. *Candida*: anduiak eta hazkuntza baldintzak

Candida generoko harreman filogenetiko estua duten hiru espezie hauek erabili ziren, kultura bilduma batetik baino gehiagotatik lortuta: NCPF (*National Collection of Pathogenic Fungi*) bildumatik, *Candida glabrata* NCPF 3203 anduia (Salisbury, Erresuma Batua); CECT (*Colección Española de Cultivos Tipo*) bidumatik *Candida nivariensis* CECT 11998 anduia (Valentzia, Espainia) eta NCYC (*National Collection of Yeast Cultures*) bildumatik *Candida bracarensis* NCYC 3133 anduia (Norwich, Erresuma Batua).

Hiru andui horiek Sabouraud dextrosa-agar hazkuntza-ingurunean (Difco, AEB) hazi ziren 37 °C-an 24 orduz.

3.2. *Candida*ren infekzio eredua *Caenorhabditis elegans* nematodoan

Candida infekzioa aztertzeko *Caenorhabditis elegans* nematodoaren eredu esperimentala erabiliz egin behar izan ziren pausoak 17. Irudian ikus daitezke eskematikoki.

3.2.1. Anduia eta hazkuntza baldintzak

Caenorhabditis elegans nematodoaren mutazio bikoitza duen AU37 anduia erabili zen. Horrela, esperimentu osoan zehar indibiduo kopuru berarekin lan egitea eta infekzioa egoki garatzea ziurtatu zen.

Eredua beste ikertzaile batzuek azaldutakoaren arabera garatu zen [14,16]. NGM (*Nematode Growth Media*) agar hazkuntza-ingurunean [21] nematodoa hedatu, eta *Escherichia coli* OP50 anduia elikagaitzat erabili zen. Urazilorako auxotrofoa den bakterio andui hau NGM agarrean erein zen, eta, behin haziz gero, nematodoak hazkuntza-ingurune horretan sartu ziren inkubagailuan15 °C-an ugaltzeko.

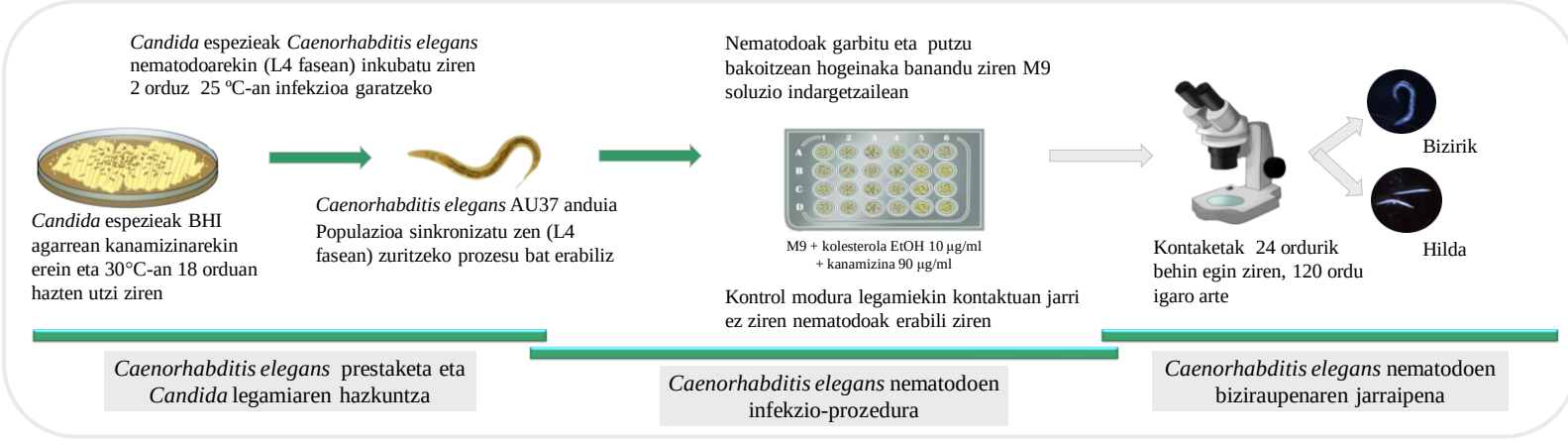
3.2.2. Nematodoen L4 hazkuntza fasea

Nematodo-populazioa sinkronizatua lortu zen, baldintza esteriletan zuritzeko prozesu bat erabiliz. Laburbilduz, NGM hazkuntza-inguruneko Petri kutxetan zeuden nematodo guztiak, 3,5 ml ur distilatuz eta Pasteur pipeta batez baliatuz, 15 ml-ko saio-hodietan batu ziren. Laginak 1200 rpm-an 2 minutuz zentrifugatu eta gero, alkohola hipokloritozko garbiketa soluzio batean utzi ziren, eta nematodoen gorputzak desegin ziren beren barruan zeuden arraultzak askatuz. Laginak 3500 rpm-an 30 segundoz zentrifugatu ondoren gain-jalkina kendu eta M9 soluzio indargetzailea (3 g KH_2PO_4, 6 g Na_2HPO_4, 5 g NaCl, 1 ml 1 M $MgSO_4$ eta H_2O-tik 1 L-ra) erabiliz arrautza multzo garbia berreskuratu eta NGM hazkuntza inguruneko Petri kutxetan banatu zen. Gau osoa 15 °C-an inkubatu eta gero, arrautzak eklosionatu eta L1 hazkuntza fasean zeuden nematodo berriak *E. coli* OP50 anduiaren kultura zeukan NGM hazkuntza-inguruneko Petri

kutxetara pasatu ziren. Nematodo guztiak L4 hazkuntza fase berean egotea lortu zen 25 °C-an 48 orduko inkubazioa igaro eta gero.

3.2.3. Nematodoen infekzio-prozedura eta biziraupena

Kandidiasi inbasorea garatu zen Breger eta beraren laguntzaileek [14] azaldu zuten bezala. Laburbilduz, L4 hazkuntza faseko nematodoak Petri kutxetatik 15 ml-ko saio-hodietara pasatu ziren 45 µg/ml kanamizina zuen M9 soluzio indargetzailea erabiliz, eta 1200 rpm-an 2 minutuz zentrifugatu ondoren nematodoak saio-hodiaren beheko partean geratu ziren. *Candida* anduiaren kultura zeukan BHI (*Brain Hearth Infusion*) hazkuntza-inguruneko Petri kutxetan zabaldu ziren horiek 2 orduz 25 °C-an, baldintza horietan nematodoek legamiak jan eta infekzioa gara zezaten. Denbora hori igarota, nematodoak berreskuratu eta garbitu ziren M9 soluzio indargetzailearekin, 45 µg/ml kanamizina gehiturik. Jarraian 1200 rpm-ko zentrifugazioarekin lortutako nematodo-multzoa NGM hazkuntza-inguruneko Petri kutxetan utzi zen, nematodoen kutikulan egon zitezkeen legamiak marruskaduraz kentzeko. Azkenik, mikrotiter plaketan nematodoak hogeinaka banatu ziren, eta 25 °C-an inkubatu. 60 nematodo prestatu ziren *Candida* espezie bakoitzaren heriotza aztertzeko. Biziraupena 24 ordurik behin estereomikroskopio batez (Nikon SMZ-745, Japonia) baliatuz ikuskatu zen, 120 ordu igaro arte. Nematodoa hilda zegoela jotzen zen zurrun eta mugimendurik gabe geratzen zenean. Kontrol modura, legamiak jan ez zituzten nematodoak erabili ziren. Esperimentua hiru aldiz gutxienez errepikatu zen *Candida* espezie bakoitzarentzat denboraldi desberdinetan.



17. Irudia: *Candida*ren infekzio eredua *Caenorhabditis elegans* nematodoan

3.3. *Candida*ren infekzio eredua *Galleria mellonella* lepidopteroan

Candida infekzioa aztertzeko *Galleria mellonella* lepidopteroaren eredu esperimentala erabiliz egin behar izan ziren pausoak 18. irudian ikus daitezke eskematikoki.

3.3.1. Candida inokuluaren prestaketa

*Candida*ren anduiak YPD hazkuntza-inguruneko saldan (%1 legamia estraktu, %2 peptona bakteriologiko, %2 D-glucosa) (Panreac, Espainia) hazi ziren 30 °C-an eta agitazioarekin inkubatuz. Gau osoa igaro ondoren, legamiak bildu, eta bi aldiz garbitu ziren fosfato gatzezko disoluzio indargetzaile batekin (PBS, pH 7,2), 1200 rpm-an 2 minutuz zentrifugatuz.

Hemozitometro batez baliatuz, legamia-zelulen kontaketak egin ziren. Ondoren, 1 x 10^7, 1 x 10^6 eta 1 x 10^5 zelula mililitroko kontzentrazioak zituzten legamia suspentsioak prestatu ziren *Candida* andui bakoitzeko. Suspentsioak PBS 20 µg/ml anpizilina gehigarri erabiliz egin ziren, bakterioen kutsadura ekiditeko.

3.3.2. Galleria mellonella*ren infekzio-prozedura eta biziraupena*

Galleria mellonella larbak hogeiko taldetan Petri kutxa hutsetan antolatu ziren, eta inokuluak injektatu ziren beste ikertzaileek deskribatu zuten bezala [15,19,20]. Inokuluak aipatutako legamien kontzentrazio ezberdinekin prestatu ziren 10 µl-ko bolumen batean. Inokulu-bolumen hori doitasun handiko xiringa baten bidez larben ezkerraldeko azken pro-hankan injektatu zen. Pro-hanka, aldez aurretik, %70eko etanol soluzio batekin garbitu zen. Infekzio-prozeduraren ondoren, larbak 37 °C-an eta ilunpean inkubatu ziren. Larben biziraupena 24 orduro aztertu zen 120 ordu igaro arte. Larbak bere kolorea marroi ilunera aldatzen zuenean eta mugimendurik gabe geratzen zenean hilda zegoela jotzen zen. Proba guztietan, bi kontrol erabili ziren; alde batetik, infektatu gabeko hogeiko larba-talde bat eta, bestetik, beste hogeiko larba-talde bat 10 µl PBS 20 µg/ml anpizilina gehigarrizko soluzio bat injektatuta. Esan bezala, legamia kontzentrazio bakoitzean

hogeiko larba-talde bat erabili zen, eta esperimentua hiru aldiz gutxienez errepikatu zen denboraldi desberdinetan *Candida* espezie bakoitzarentzat.

3.4. Estatistika

Biziraupen-kurbak Kaplan-Meier estatistikoaren bitartez kalkulatu ziren. Log-rank *Candida* espezieren arteko birulentzia desberdintasunak estimatzeko erabili zen. Estatistika-azterketak SPSS v21.0 (IBM, AEB) eta Stata v12.0 (StataCorp, AEB) programekin garatu ziren. Adierazpen maila 0,05 balioan ezarri zen.

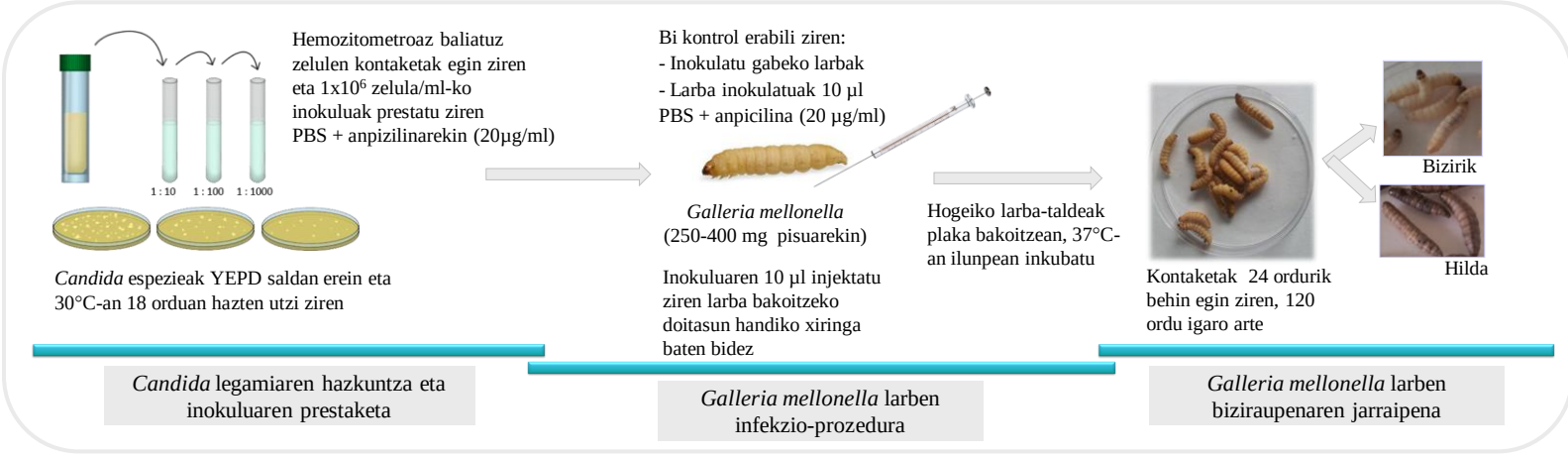
4. EMAITZAK

4.1. *Candida*ren infekzio eredua *Caenorhabditis elegans* nematodoan

Caenorhabditis elegans nematodoak *Candida glabrata*, *Candida bracarensis* eta *Candida nivariensis* espezieekin infektatu ziren, eta kandidiasi inbasorea garatu zuten. Eredu egokia izan zen filogenetikoki oso antzekoak diren hiru espezie horien birulentzia aztertzeko. Nematodoek jandako legamia zelulen kopurua kontrolatu ezin izan arren, infekzio baldintza berdinak erabilita nematodoek biziraupen desberdina aurkeztu zuten hiru *Candida* espezie hauen infekzioekin.

Guztira, 740 nematodo aztertu ziren, 560 infektatu eta 180 kontrol. Lehenengo 48 orduko biziraupen kontaketetan nematodo guztiak mugitzen ziren mikrotiter plaketetan, baina, gero, zurrunak eta mugimendurik gabekoak agertzen hasi ziren. Infekzio gabeko nematodoen biziraupen-tasa ia aldaketarik gabe mantendu zen 120 orduko esperimentuaren amaierara arte, baina *Candida* espezieekin infektatutako nematodoen biziraupen-tasak, berriz, behera egiten zuen 48 ordutik aurrera (19-a. Irudia). Kontrol eta infektatuen emaitzak konparatuz, desberdintasunak estatistikoki adierazgarriak izan ziren %95eko konfiantza-tarteaz (p=0).

Nahiz eta *Candida nivariensis* CECT 11998 anduiaren birulentzia lehenengo 48 orduetan ageri, birulentzia ertaina azaldu zuen azkenik; nematodoen biziraupen-tasa 120 ordu igarota %73koa izan zen. Birulentzia handiena *Candida glabrata* NCPF 3203 anduiak erakutsi zuen: 72 ordu igaro eta gero agertu ziren lehenengo nematodoen hilotzak, eta biziraupen-tasa 120 orduren ondoren %63koa izan zen. Birulentzia txikienekoa, berriz, *Candida bracarensis* NCYC 3133 anduiarena izan zen, nematodoen %89ko biziraupenarekin. Hiru espezieekin infektatutako nematodoen biziraupenak konparatuz, soilik desberdintasun estatistikoki adierazgarriak aurkitu ziren *Candida bracarensis* NCYC 3133 anduiarekin, %95eko konfiantza-tarteaz (p=0).



18. Irudia: *Candida*ren infekzio eredua *Galleria mellonella* lepidopteroan.

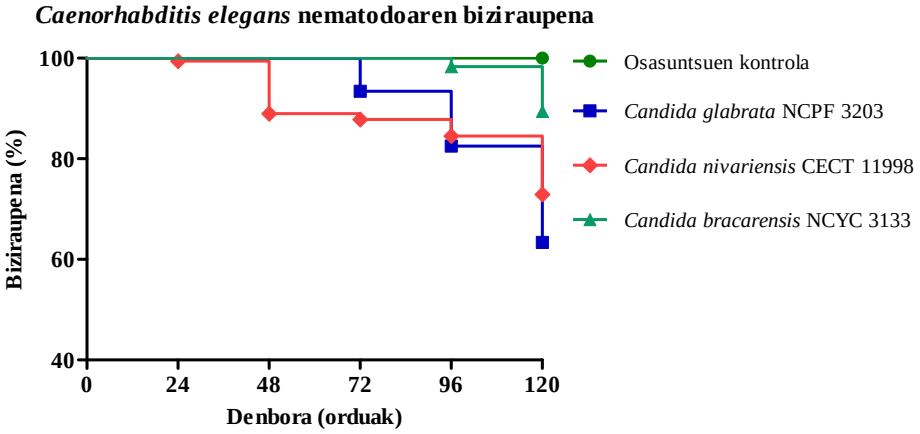
4.2. *Candida*ren infekzio eredua *Galleria mellonella* lepidopteroan

Hiru *Candida* espezieak, *Candida glabrata*, *Candida bracarensis* eta *Candida nivariensis*, gai izan ziren *Galleria mellonella* infektatzeko eta kandidiasi inbasorea garatzeko. Beste ereduan bezala, hemen ere espezie hauen birulentzia aztertu ahal izan zen.

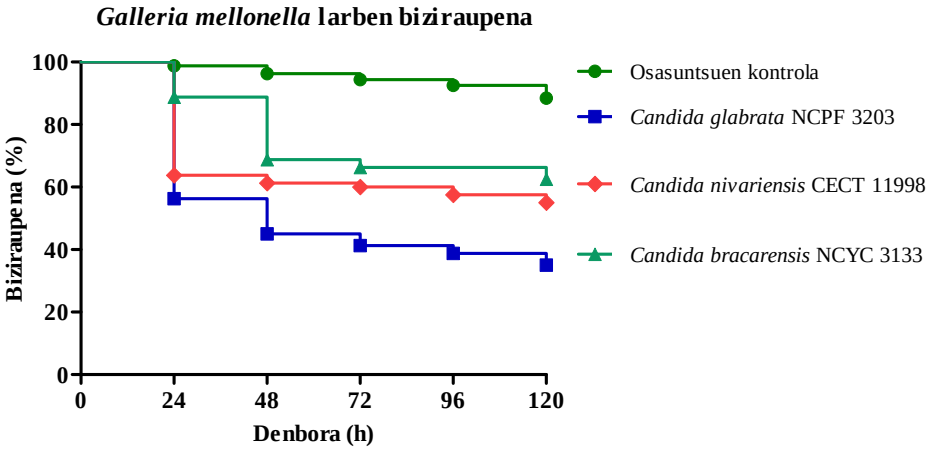
Gure ikerketa lan honetan, 660 larba aztertu ziren, 540 infektatu eta 120 kontrol. *Galleria mellonella* ereduan inokulu zehatza sartzea posible denez, infekzioa kontrolatuagoa da. Hiru legamia kontzentrazioen artean desberdintasunak ikusi ziren, baina 1 x 10^6 zelula/ml izan zen kontzentrazio egokiena *Candida* espezieen birulentzia konparatzeko (19-b. Irudia). Kontrol larben biziraupena 120 ordu igaro eta gero %88koa izan zen. *Candida* espezieekin infektatutako larbak, berriz, 24 ordutik aurrera hasi ziren hiltzen, eta hiru espezieekin biziraupen-tasek joera berdina izan zuten denboran zehar. Kontroleko eta infektatutako taldeen emaitzak konparatuz, desberdintasunak estatistikoki adierazgarriak izan ziren %95eko konfiantza-tarteaz (p=0).

Birulentzia altuena *Candida glabrata* NCPF 3203 anduiak adierazi zuen, 120 ordu igarota larben %35eko biziraupenarekin. Birulentzia ertaina *Candida nivariensis* CECT 11998 anduiari zegokion (%55eko biziraupena 120 ordu eta gero). Azkenik, *Candida bracarensis* NCYC 3133 anduiak birulentzia baxuena izan zuen, eta larben %62,5ek bizirik jarraitzen zuen 120 ordu pasatu eta gero ere. Legamiekin infektaturiko larben biziraupenak alderatuz, soilik *Candida glabrata* NCPF 3203 anduiarekin infektatutako larben biziraupena beste bi anduiekin lortutako emaitzekin konparatuz topatu ziren estatistikoki adierazgarriak ziren desberdintasunak, %95eko konfiantza-tarteaz ($p \leq 0{,}014$).

a)



b)



19. Irudia: *Candida glabrata* NCPF 3203, *Candida bracarensis* NCYC 3133 eta *Candida nivariensis* CECT 11998 anduiekin infektatutako *Caenorhabditis elegans* nematodoen (a) eta *Galleria mellonella* lepidopteroen (b) biziraupen-kurbak. *Galleria mellonella* lepidopteroan agertzen diren biziraupen-kurbak *Candida* legamiaren 1 x 10^6 zelula mililitroko kontzentrazioko inokuluarekin infektatutako larbenak dira.

5. EZTABAIDA

Kandidiasia mikosis ohikoena da, eta kandidiasi inbasore edo sistemikoak morbiditate eta heriotza-tasa handiak eragiten ditu, gaixotasun larriak edota defentsak gutxituak dituzten gaixoen artean batez ere [1]. Kandidiasiaren etiologia aldatuz doa, eta, nahiz eta *Candida albicans* espezieak nagusi izaten jarraitu, *Candida albicans* ez diren beste espezieen presentzia gero eta nabarmenagoa da. Izan ere, espezie horiek 1970eko-1990eko hamarkadetan kandidiasi sistemikoen %10-40 kasuetan isolatu ziren, eta azken hogei urteetan proportzio hori %35-65era igo da [2]. Gero eta gehiago hedatzen ari diren espezieen artean *Candida glabrata* daukagu, eta horri arreta jarri behar zaio ohiko antifungikoekiko sentikortasun murriztua duelako, besteak beste [8]. *Candida glabrata* espezietik *Candida bracarensis* eta *Candida nivariensis* espezieak bereizteak berebiziko garrantzia du, azken espezie horiek antifungikoekiko duten sentikortasuna desberdina izan daitekeelako eta okerreko aukera eginez gero tratamenduak huts egin dezakeelako [2,4,7,16].

Beraz, kandidiasiaren etiologia ezagutzeak eta *Candida* espezieen karakterizazio edota patogenia ikertzeak tratamendu egokia aukeratzea ahalbidetuko du. Horretarako, *in vivo* eredu esperimentalak erabiltzea oso lagungarria da, eta *Candida* espezieek eragiten dituzten infekzioen ezagueran aurrerapen garrantzitsuak lortzen ari dira [13]. Animalia ornogabeen artean, nematodoak eta intsektuak infekzio eredu esperimental gisa ditugu, eta haien erabiliera egokia da birulentzia, ostalariaren eta patogenoaren arteko elkarrekintzak edota mikrobioen aurkako efektua duten molekula berriak ikertzeko. Animalia mota hauek infekzio eredu gisa erabiltzeak hainbat onura eskaintzen ditu. Halakoen artean, esate baterako, hauek aipa ditzakegu: i) ornodunekin dauden arazo etikoak saihestea, ii) kostu baxua eta laborategian erraz mantentzea, edo iii) esperimentuak 37 °C-an egitea [4,13,15,16,19,20].

Ikerketa lan honetan bi ordezko eredu esperimental erabili dira, *Caenorhabditis elegans* nematodoa eta *Galleria mellonella* lepidopteroa, *Candida glabrata*, *Candida bracarensis* eta *Candida nivariensis* espezieen birulentzia aztertzeko. Guk dakigula,

Candida bracarensis eta *Candida nivariensis* espezieei buruz orain arte ez da *in vivo* ereduetan eginiko ikerketarik argitaratu, eta *Candida glabrata*k eragindako infekzioak ordezko eredu esperimentalak erabiliz azaltzen dituzten ikerketak gutxi dira [4,11,16].

Gure ikerketan, hiru *Candida* espezieek bi eredu esperimentaletan antzeko birulentzia erakutsi zuten. Bai *Caenorhabditis elegans* nematodoan eta bai *Galleria mellonella* lepidopteroan, *Candida glabrata* izan zen espezie birulentoena; *Candida nivariensis* espezieak birulentzia ertaina erakutsi zuen, eta apalena, *Candida bracarensis*ek. Halere, lortutako biziraupen-portzentajeak desberdinak izan ziren bi ereduetan. *Caenorhabditis elegans* nematodoan 120 orduren buruan bizirik irauten zuten banakoen kopurua altuagoa zela ikusi zen *Galleria mellonella* lepidopteroarekin konparatuz. Hori zenbait arrazoirengatik izan daiteke, besteak beste, ereduetan sartutako legamia kopuruarengatik. Hau da, *Galleria mellonella* larbetan sartu zen inokulua zehatza eta ezaguna izan zen, eredu horren tamainak doitasun handiko xiringa bitartez zelula kopuru jakina inokulatzeko aukera ematen baitu [15], baina *Caenorhabditis ellegans* nematodoaren kasuan, aldiz, beraren tamaina txikia dela eta, ezin da inokulatu eta legamia zelulak elikatze prozesuaren bitartez barneratzen ditu. Elikatze prozesuaren denborak berak eragin zuzena eduki dezake [16]. Berez, gure esperimentuetan elikatze prozesuaren denbora bi ordukoa izan zen, baina Scorzonik eta beraren laguntzaileek hiru ordu erabili zituzten, eta, beraz, haiek lortu zituzten biziraupen-portzentajeak gureak baino txikiagoak izan ziren [15].

Bi ereduetan lortutako biziraupen tasen arteko desberdintasuna inkubazio tenperaturan egon daiteke ere. *Caenorhabditis ellegans* 25 °C-an inkubatzen da, eta *Galleria mellonella*, 37 °C-an. Kontuan badugu *Candida* legamiaren hazkuntza tenperatura egokiena 37 °C dela, tenperatura horretan azkarrago haziko da, eta, beraz, infekzioa birulentoagoa izango da eta, hortaz, biziraupena txikiagoa.

Candida glabrata espeziearekin infektatutako *Caenorhabditis elegans* nematodoan lortutako biziraupen-tasa beste autoreen emaitzekin alderaturik altuagoa da: %63 Ortega-Riverosek eta laguntzaileek aurkitutako < %50en aldean [16]. Gauza bera gertatzen da

Galleria mellonella ereduan, larben biziraupena baxuagoa izan zen (%35) beste ikertzaileek lortutako emaitzekin alderaturik (biziraupena: >%60) [4]. Esperimentuak egiteko erabili ziren *Candida glabrata*ren anduiak desberdinak izateagatik gerta daitezke desberdintasun horiek.

Beste bi espezieei buruz, *Candida bracarensis* edota *Candida nivariensis* espezieei buruz, gutxi ezagutzen da, bai epidemiologiari bai birulentziari dagokionez. Izatez, Lockhartek eta beraren laguntzaileek egindako ikerketaren arabera, *Candida bracarensis* eta *Candida nivariensis* espezieek *Candida glabrata*ren isolamendu klinikoen %0,2 soilik osatzen dute [22]. Aldiz, Argentinan egindako lan baten arabera, *Candida nivariensis* espeziea hasiera batean *Candida glabrata* gisa identifikatutako isolamenduen %2,56ri zegokion [23]. Ikerketa berriak behar dira bi espezie horien ezagueran aurrera egiteko, tartean eredu esperimentalekin egindako ikerketak.

Gure lan honetan lortutako emaitzekin erakusten da bai *Galleria mellonella* bai *Caenorhabditis elegans* infekzio eredu boteretsuak direla *Candida* patogenoa ikertzeko. Biek emaitza onak eman zituzten *Candida glabrata*ren eta berarekin harreman filogenetiko estua duten beste bi espezieen birulentzia aztertzean eta konparatzean. Farmakoekiko sentikortasuna iker daiteke etorkizun handiko ordezko eredu hauekin. Sentikortasun horretan *Candida* espezieek edo anduiek ekoiztutako birulentzia faktoreek duten efektua ere azter daiteke. Birulentzia faktore hoien artean biopelikulak eta entzima hidrolitikoak aipa daitezke. Biopelikulak antifungikoekiko erresistentzia eta ostalariaren immunitate sistemarekiko babespenarekin erlazionatuta daude; entzima hidrolitikoak, berriz, legamiaren atxikimenduarekin, ehunetan zehar sartzeko gaitasunarekin edota inbasio berarekin [16,17]. Farmakoekiko sentikortasuna iker daiteke etorkizun handiko ordezko eredu hauekin, eta, horrekin lotuta, espezie edo andui bakoitzek birulentzia faktore ekoizteko gaitasuna kontutan izanik. Biopelikulak garatzeak antifungikoekiko erresistentzia eta ostalariaren immunitate sistemarekiko babespenarekin erlazionatuta baitaude; entzima hidrolitikoak *Candida* espezieek sortu dezaketen beste birulentzia faktore honek legamiaren atxikimenduarekin, ehunetan zehar sartzeko gaitasuna edota inbasio berarekin lotuta daude [16,17]. Gainera, *Galleria mellonella* larben hemolinfa

eskura daiteke, eta bertan dauden immunitate-sistemak ekoizten dituen berezko zelulak (hemozitoak, alegia) iker ditzakegu. Horrela, patogeno gisa joka dezaketen mikroorganismoen aurka larben immunitate-sistemak nola lan egiten duen iker dezakegu, hemozitoen ekoizpeneko aldaketak edota hemozitoek patogenoak fagozitatzeko gaitasuna aztertuz [15,19,20]. Bide zabala dago oraindik eredu esperimental hauekin egiteko, eta *Candida* espezie eta anduien birulentzia eta haiek eragindako gaixotasunen patogenia eta terapia ezagutzeko.

ESKER ONAK

Eskerrak eman nahi dizkiogu Eusko Jaurlaritzako Hezkuntza, Unibertsitate eta Ikerketa Sailari lan hau finantzatzeagatik (GIC15/78 IT-990-16). Ainara Hernando-Ortizek UPV/EHUko ikertzaileak prestatzeko kontratazio laguntza dauka (PIF 16/39).

6. BIBLIOGRAFIA

[1] QUINDÓS G., MARCOS-ARIAS C., SAN-MILLÁN R., MATEO E. eta ERASO E. 2018. «The continuous changes in the aetiology and epidemiology of invasive candidiasis: from familiar *Candida albicans* to multiresistant *Candida auris*». *International Microbiology*, **21**, 107-119.

[2] SADEGHI G., EBRAHIMI-RAD M., MOUSAVI S.F., SHAMS-GHAHFAROKHI M. eta RAZZAGHI-ABYANEH M. 2018. «Emergence of non-*Candida albicans* species: epidemiology, phylogeny and fluconazole susceptibility profile». *Journal de Mycologie Medicale*, **28**, 51-58.

[3] MENDLING W., BRASCH J., CORNELY O.A., EFFENDY I., FRIESE K., GINTER-HANSELMAYER G., HOF H., MAYSE P., MYLONAS I., RUHNKE M., SCHALLER M. eta WEISSENBACHER E.R. 2015. «Guideline: vulvovaginal candidosis (AWMF 015/072), S2k (excluding chronic mucocutaneous candidosis)» *Mycoses*, **58**, Suppl 1:1-15.

[4] AMES L., DUXBURY S., PAWLOWSKA B., HO H.L., HAYNES K. eta BATES S. 2017. «*Galleria mellonella* as a host model to study *Candida glabrata* virulence and antifungal efficacy». *Virulence*, **8**, 1909-1917.

[5] ALCOBA-FLÓREZ J., MÉNDEZ-ÁLVAREZ S., CANO J., GUARRO J., PÉREZ-ROTH E. eta DEL PILAR ARÉVALO M. 2005. «Phenotypic and molecular characterization of *Candida nivariensis* sp. nov., a possible new opportunistic fungus». *Journal of Clinical Microbiology*, **43**, 4107-4111.

[6] BISHOP J.A., CHASE N., LEE R., KURTZMAN C.P. eta MERZ W.G. 2008. «Production of white colonies on CHROMagar *Candida* medium by members of the *Candida glabrata* clade and other species with overlapping phenotypic traits». *Journal of Clinical Microbiology*, **46**, 3498-3500.

[7] ROMEO O., SCORDINO F., PERNICE I., LO PASSO C. eta CRISEO G. 2009. «A multiplex PCR protocol for rapid identification of *Candida glabrata* and its phylogenetically related species *Candida nivariensis* and *Candida bracarensis*». *Journal of Microbiolical Methods*, 71, 117-120.

[8] AZNAR-MARIN P., GALAN-SANCHEZ F., MARIN- CASANOVA P., GARCÍA-MARTOS P. eta RODRÍGUEZ-IGLESIAS M. 2016. «*Candida nivariensis* as a new emergent agent of vulvovaginal candidiasis: description of cases and review of published studies». *Mycopathologia*, 181, 445-449.

[9] LÓPEZ-SORIA L.M., BERECIARTUA E., SANTAMARÍA M., SORIA L.M., HERNÁNDEZ-ALMARAZ J.L., MULARONI A., NIETO J. eta MONTEJO M. 2013. «Primer caso de fungemia asociada a catéter por *Candida nivariensis* en la Península Ibérica». Revista Iberoamericana de Micología, 30,69-71.

[10] BORMAN A.M., SZEKELY A. eta JOHNSON, E.M. 2016. «Comparative pathogenicity of United Kingdom isolates of the emerging pathogen *Candida auris* and

other key pathogenic *Candida* species». *Clinical Science and Epidemiology*, **1**, e00189-16.

[11] COTTER G., DOYLE S. eta KAVANAGH K. 2000. «Development of an insect model for the in vivo pathogenicity testing of yeasts». *FEMS Immunology* & *Medical Microbiology,* **27**, 163-169.

[12] GLAVIS-BLOOM J., MUHAMMED M. eta MYLONAKIS E. 2012. «Of model hosts and man: using *Caenorhabditis elegans*, *Drosophila melanogaster* and *Galleria mellonella* as model hosts for infectious disease research». *Advances in Experimental Medicine and Biology,* **710**, 11-17.

[13] SEGAL E. eta FRENKEL M. J. 2018. «Experimental in vivo models of candidiasis». *Journal of Fungi*, **4**, 21.

[14] BREGER J, FUCHS BB, APERIS G, MOY T.I., AUSUBEL F.M. eta MYLONAKIS E. 2007. «Antifungal chemical compounds identified using a *C. elegans* pathogenicity assay». *PLoS Pathogen*, **3**. 0168-0178.

[15] SCORZONI L., DE LUCAS M.P., MESA-ARANGO A.C., FUSCO-ALMEIDA A.M., LOZANO E., CUENCA-ESTRELLA M., MENDES- GIANNINI M.J. eta ZARAGOZA O. 2013. «Antifungal efficacy during *Candida krusei* infection in non-conventional models correlates with the yeast in vitro susceptibility profile». *PLoS One*; **8**, e60047.

[16] ORTEGA-RIVEROS M., DE-LA-PINTA I., MARCOS-ARIAS C., EZPELETA G., QUINDÓS G. eta ERASO E. 2017. «Usefulness of the non-conventional *Caenorhabditis elegans* model to assess *Candida* virulence». *Mycopathologia*, **182**, 785–795.

[17] OLSON M. L., JAYARAMAN A. eta KAO K C. 2018. «Relative abundances of *Candida albicans* and *Candida glabrata* in in vitro coculture biofilms impact biofilm structure and formation». *Applied and Environmental Microbiology*, **84**, e02769-17.

[18] BORMAN A.M., SZEKELY A., LINTON C.J., PALMER M.D., BROWN, P. eta JOHNSON, E.M. 2013. «Epidemiology, antifungal susceptibility, and pathogenicity of *Candida africana* isolates from the United Kingdom». *Journal of Clinical Microbiology*, **51**, 967-972.

[19] GAGO S., GARCIA-RODAS R., CUESTA I., MELLADO E. eta ALASTRUEY-IZQUIERDO A. 2014. «*Candida parapsilosis*, *Candida orthopsilosis* and *Candida metapsilosis* virulence in the non-conventional host *Galleria mellonella*». *Virulence*, **5**, 278-85.

[20] MESA-ARANGO A.C., FORASTIERO A., BERNAL-MARTINEZ L., CUENCA- ESTRELLA M., MELLADO E. eta ZARAGOZA O. 2013. «The non-mammalian host *Galleria mellonella* can be used to study the virulence of the fungal pathogen *Candida tropicalis* and the efficacy of antifungal drugs during infection by this pathogenic yeast». *Medical Mycology*, **51**, 461-72.

[21] STIERNAGLE T. 2006. «Maintenance of *C. elegans*». *WormBook*, **11**, 1–11.

[22] LOCKHART S.R., MESSER S.A., GHERNA M., BISHOP J.A., MERZ W.G., PFALLER M.A eta DIEKEMA D.J. 2009. «Identification of *Candida nivariensis* and *Candida bracarensis* in a large global collection of *Candida glabrata* isolates: comparison to the literature». *Journal of Clinical Microbiology*, **47**, 1216–7.

[23] MORALES-LOPEZ S.E., TAVERNA C.G., BOSCO-BORGEAT M.E. , MALDONADO I., VIVOT W., SZUSZ W., GARCIA-EFFRON G. eta CORDOBA S.B. 2016. «*Candida glabrata* species complex prevalence and antifungal susceptibility testing in a culture collection: first description of *Candida nivariensis* in Argentina». *Mycopathologia*,**181**,871–878

2. Azterlana (2. Eranskina/Anexo 2)

Caenorhabditis elegans eredu sistema _Candida glabrata_, _Candida nivariensis_ eta _Candida bracarensis_ espezieen birulentzia eta antifungikoen eraginkortasuna ebaluatzeko

«_Caenorhabditis elegans_ as a model system to assess _Candida glabrata_, _Candida nivariensis_ and _Candida bracarensis_ virulence and antifungal efficacy»

Ainara Hernando-Ortiz, Estibaliz Mateo, Marcelo Ortega-Riveros, Iker De-la-Pinta, Guillermo Quindós, Elena Eraso

Mikologia Medikoko laborategia, UFI 11/25, Immunologia, Mikrobiologia eta Parasitologia Saila, Medikuntza eta Erizaintza Fakultatea, (UPV/EHU)

Antimicrobial Agents Chemotheraphy 2020, 21; 64:e00824-20

Laburpena

Candida albicans kandidiasi inbaditzailearen agente etiologiko nagusia izaten jarraitzen duen arren, *Candida glabrata* eta sortzen ari diren *Candida* generoko beste espezie batzuk gero eta gehiago agertzen ari dira. *Candida glabrata* espeziea munduko herrialde askotan kandidiasiaren bigarren kausarik ohikoena da. Hala ere, *Candida nivariensis* eta *Candida bracarensis* espezieek harreman filogenetiko estua daukate *C. glabrata*rekin eta isolamendu klinikoak *C. glabrata* bezala oker identifika daitezke, teknika molekular zehatzak erabiltzen ez badira. Gutxi ezagutzen da bi espezie kriptiko horiei buruz. Beraz, patogenesi-azterketak behar dira horien birulentzia-ezaugarriak eta farmako antifungikoekiko sentikortasuna ulertzeko. *Caenorhabditis elegans* nematodoa eredu aproposa da *Candida*k sortutako infekzioan gertatzen diren ostalari-patogeno elkarrekintzak aztertzeko. Ikerketa lan honetan *C. glabrata*, *C. nivariensis* eta *C. bracarensis* espezieen birulentzia aztertzeko *C. elegans* ordezko ereduaren erabilgarritasuna ikertu zen. Hiru espezieek kandidiasia eragin zuten eta *C. glabrata*k birulentziarik handiena sortzen zuela baieztatu zen. Bestalde, gaur egun erabiltzen diren farmako antifungikoen eraginkortasuna zehaztu zen espezie horiek *C. elegans* ereduan eragindako infekzioaren aurka. AmB eta azolak *C. glabrata*k eta *C. bracarensis*ek sortutako infekzioen aurkako eraginkorrenak izan ziren, ekinonkandinak, berriz, *C. nivariensis*ek eragindakoak tratatzeko aktiboagoak izan ziren. *C. elegans* infekzio ereduak harreman filogenetiko estua duten hiru *Candida* espezie horien patogenotasuna ikertzeko baliagarritasuna frogatu zen.

Hitz gakoak: kandidiasia, *Caenorhabditis elegans*, ordezko eredu ez-konbentzionala, patogenotasuna, antifungikoekiko sentikortasuna.

Bigarren azterlana *Antimicrobial Agents Chemotheraphy* aldizkarian argitaratutako lana da eta 2. eranskinean zehaztuta dago. Ondoren, emaitza nabarmenenen laburpena aurkezten da.

1. HELBURUA

Azterlana honen helburua *C. elegans* ordezko eredua erabiliz *C. glabrata*, *C. nivariensis* eta *C. bracarensis* espezieen patogenotasuna ikertzen da. Alde batetik, harreman estua duten hiru espezie horien potentzial birulentoa zehazten da *C. elegans* ordezko ereduan. Bestalde, B anfoterizina, ekinoandinak eta hainbat azol antifungikoekiko sentikortasuna aztertzen eta konparatzen da in vitro eta in vivo, *C. elegans* eredua erabiliz.

2. EMAITZEN ETA EZTABAIDAREN LABURPENA

2.1. *Candida* anduien karakterizazioa: aktibitate entzimatikoa eta in vitro antifungikoekiko sentikortasuna

*Candida*ren erreferentziazko sei andui erabili ziren, espezie bakoitzaren erreferentziazko bi andui barne: *C. glabrata* ATCC 90030 eta NCPF 3203, *C. nivariensis* CBS 9984 eta CECT 11998, eta *C. bracarensis* NCYC 3397 eta NCYC 3133 (7. Taula).

Fosfolipasa eta proteasen ekoizpena eta jarduera hemolitikoa aztertu ziren, eta sei anduietako bat ere ez zen fosfolipasa edo proteasaren ekoizlea izan, baina alfa hemolisia (partziala) egin zuten, *C. glabrata* ATCC 90030 anduia izan ezik, azken horrek jarduera gamma hemolitikoa (hemolisirik ez) erakutsi baitzuen.

Beste birulentzia-faktore batzuen artean, entzima hidrolitikoak (hemolisinak edo fosfolipasa sekretuak) eta aspartil-proteinasak sortzea, adibidez, birulentzia-faktore garrantzitsuak dira kandidiasiaren patogenesia eragile bezala (Cassone et al., 1987; Luo et al., 2001; Taniguchi et al., 2009). Faktore horiek aztertzeko, hainbat protokolo argitaratu dira eta horien zuzendu beharreko zenbait aspektuak ere (Taniguchi et al., 2009; Fakhim et al., 2018). *C. glabrata*ren eta beste bi espezie horien birulentzia-faktoreei dagokienez, gutxi ezagutzen da eta birulentzia-faktore horien gabezia analisi gehigarrien bidez

baieztatu beharko litzateke. Hala ere, frogatu da adhesinak, Epa familiako proteinak barne, birulentzian inplikatuta daudela eta oso presente daudela *C. glabrata*ren zelula-mintzean (Ames et al., 2017). *C. nivariensis* eta *C. bracarensis* espezieen potentzial patogenoa azal daiteke, besteak beste, bi espezie horietan detektatutako *EPA* geneen kopuru handiagatik. Gene horiek ez daude patogenoak ez diren beste espezie batzuetan, *Nakaseomyces* kladoaren barruan (Frenkel et al., 2016; Frankowski et al., 2013).

Farmako antifungikoen eraginkortasuna aztertu zen; uretan disolbatutakoak (Kaspofungina (CAS), flukonazola (FCZ) eta micafungina (MCF)) eta %1 dimethyl sulfoxide (DMSO) konposatuan disolbatutakoak (B anfoterizina (AmB), anidulafungina (AND), posakonazola (PCZ) eta borikonazola (VCZ)). *C. glabrata*, *C. nivariensis* eta *C. bracarensis* farmako antifungikoekiko in vitro aztertutako sentikortasunaren emaitzak 7. Taulan laburbiltzen da. Aztertutako andui guztiak sentikorrak izan ziren farmako antifungikoen aurrean. Emaitza horiek *C. elegans* ereduan in vivo esperimentuetan lortutakoekin alderatu ziren.

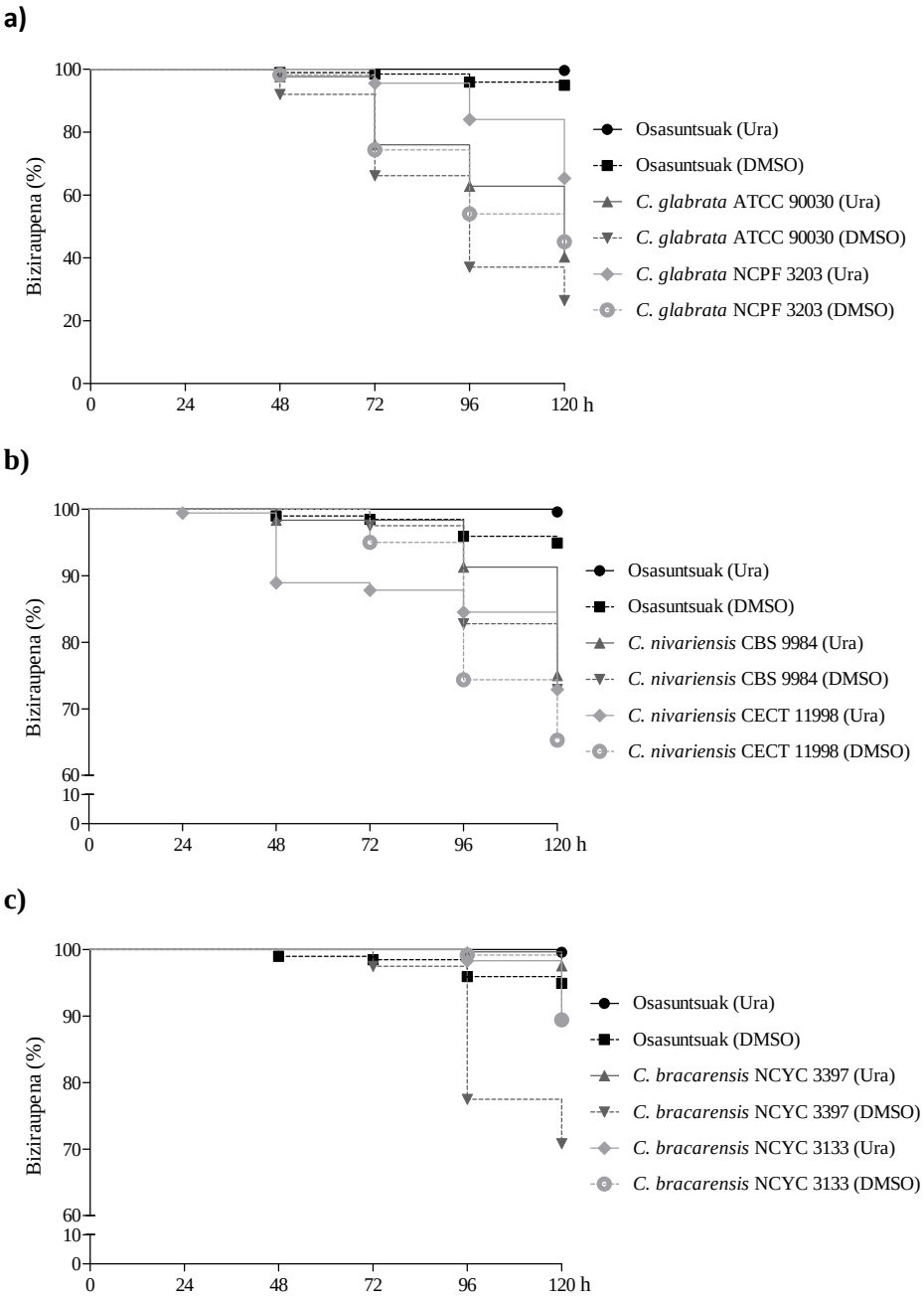
7. Taula: In vitro aktibitate antifungikoa *C. glabrata, C. nivariensis* eta *C. bracarensis* espezieen aurka.

	MIC (µg/ml)						
***Candida* anduia**	**CAS**	**MCF**	**AND**	**AmB**	**PCZ**	**VCZ**	**FCZ**
***C. glabrata* ATCC 90030**	0.5	0.03	0.06	1	1	0.5	8
***C. glabrata* NCPF 3203**	0.25	0.03	0.06	1	0.5	0.25	4
***C. nivariensis* CBS 9984**	0.25	0.03	0.06	2	0.5	0.06	8
***C. nivariensis* CECT 11998**	0.25	0.03	0.06	2	0.5	0.12	4
***C. bracarensis* NCYC 3397**	0.25	0.03	0.06	1	1	0.12	4
***C. bracarensis* NCYC 3133**	0.25	0.03	0.06	2	1	0.12	4

2.2. *Caenorhabditis elegans* nematodoaren biziraupena *Candida* infekzioaren aurrean

*Candida*ren hiru espezie horiek kandidiasi inbaditzailea sortzeko gai izan ziren *C. elegans* nematodoan, eta kontrol taldearekin, infektatu gabeko nematodoekin, estatistikoki esanguratsuak diren aldeak ikusi ziren. *C. glabrata* ATCC 90030 anduia espezie bortitzena izan zen esperimentuak amaitzean (120h), nematodoen %50 baino gehiagoko hilkortasuna eragin zuen bakarra izan zelarik. Bestalde, hilkortasun txikiena eragin zuen espeziea *C. bracarensis* izan zen, hiru espezieen artean birulentziarik txikiena erakutsiz (20. Irudia). Lortutako emaitza horiek bat datoz literaturan dauden espezie horien kasuen eraginarekin (Fujita et al., 2007; Borman et al., 2008; Moreira et al., 2015; Mariné et al., 2006).

DMSOren %1 konposatuak *Candida*ren infekzioa izan ondoren *C. elegans* nematodoen biziraupenean izan zezakeen eragina ere aztertu zen. DMSOren eragina, neurri handiagoan edo txikiagoan, *Candida*ren sei anduiekin infektatutako nematodoen biziraupenean ikusi zen, baita kontrol gisa erabilitako infektatu gabeko nematodoetan ere. Desberdintasun nabarmenak hauteman ziren infektatu gabeko nematodoen artean, DMSOren presentzian eta absentzian 120 ordu pasa eta gero (%95,2 eta %99,6ko biziraupena 120h, hurrenez hurren) ($p = 0$). *C. glabrata*ren bi anduiekin eta *C. bracarensis* NCYC 3397 anduiarekin infektatutako nematodoen biziraupenean antzeman ziren aldeak DMSOren absentzia eta presentzian, biziraupena gutxituz DMSO ingurunean zegoenean. Konposatu horren presentzian, desberdintasunak ikusi ziren infektatu gabeko nematodoen eta *Candida*ren hiru espezieekin infektatuen artean, *C. bracarensis* NCYC anduiarekin izan ezik ($p = 0,98$). Nematodoen biziraupenaren murrizketa horren arrazoia izan liteke DMSO konposatuak *C. elegans*en har helduen mintzaren jariakortasuna alda dezakeelako, metabolitoen eta kanpoko molekulen elkartrukean eragina izanik (Wahyuningsih et al., 2008).



20. Irudia: *Caenorhabditis elegans* nematodoaren biziraupen-kurbak *Candida glabrata* (a), *Candida nivariensis* (b) eta *Candida bracarensis* (c) infekzioaren aurrean %1 dimethyl sulfoxide (DMSO) konposatuaren presentzian eta absentzian (ura).

2.3. Tratamendu antifungikoekiko eraginkortasuna kandidiasiaren aurka *Caenorhabditis elegans* nematodoa erabiliz

Farmako antifungikoek *Candida* infekzioaren aurka duten eragina ebaluatzeko, aurrez infektatutako *C. elegans* nematodoak tratatu ziren, 8 µg/ml AND, 4 eta 8 µg/ml CAS eta MCF, 32, 64 eta 128 µg/ml FCZ, edo 1 eta 2 µg/ml VCZ, PCZ eta AmB kontzentrazioekin. *C elegans* ereduan aztertutako *C. glabrata*, *C. nivariensis* eta *C. bracarensis* espezieen aurkako farmako antifungiko eraginkorrenak 8. Taulan agertzen dira.

Tratamendu guztiak eraginkorrak izan ziren *C. glabrata*k eragindako infekzioaren aurka, eta nematodoen hilkortasunean murrizketa handiagoa ikusi zen DMSOn disolbatutako farmako antifungikoekin, konposatu hori toxikoa izan arren. Beste bi espezieen, *C. nivariensis* eta *C. bracarensis*, aurkako tratamenduaren eraginkortasuna erabilitako andui eta farmako antifungikoaren araberakoa izan zen.

AmB, DMSOn disolbatutako farmakoa, oso eraginkorra izan zen *C. glabrata* eta *C. bracarensis* anduien aurka, nematodoen ondoriozko hilkortasunaren murrizketa-ehuneko handienak lortu baitziren. Andui horiek guztiak sentikorrak izan ziren bai in vitro bai in vivo AmB farmakoaren aurrean, non kontzentrazio berdinak frogatu ziren. *C. glabrata* espezieak sortutako infekzioaren aurrean, AmB 1 edo 2 µg/ml-rekin tratatutako nematodoek lortu zituzten biziraupen-ehuneko handienak, eta nematodoen hilkortasuna %51,2ra murriztu zen. Polietileno horrek ere emaitza onak erakutsi zituen in vitro *C. glabrata*ren eta *C. bracarensis*en aurkako beste ikerketa batzuetan (Astvad et al., 2018; Carrillo-Muñoz et al., 2010) eta *C. glabrata*ren infekzioaren tratamenduan *G. mellonella*n (Mariné et al., 2006) eta arratoia ereduetan (Wahyuningsih et al., 2008).

DMSOn ere prestatutako VCZ eta PCZ farmako antifungikoek *C. glabrata*ren anduien eta *C. bracarensis* NCYC 3397 anduiaren aurka eragin ona izan zuten; bi farmakoek antzeko igoera lortu zuten *C elegans* nematodoaren biziraupen-ehunekoetan. VCZren 1 µg/ml kontzentrazioarekin egindako tratamendua izan zen *C. bracarensis* NCYC 3397 anduiaren aurka DMSOn disolbatutakoen artean eraginkorrena. Arratoi ereduetan

egindako in vivo azterketek ere hobekuntza bera erakutsi zuten bi farmako antifungikoetan *C. glabrata* infekzioaren aurka (Morales-Lopéz et al., 2017; Figueiredo-Carvalho et al., 2016). Literaturan argitaratuta dago bi espezie horiek daukaten azol berri horiekiko in vitro sentikortasuna, baina *C. glabrata* (Astvad et al., 2017; Pham et al., 2014) eta *C. bracarensis* (Correia et al., 2006; Angoulvant et al., 2016) isolamenduen sentikortasun murriztua ere deskribatu da.

C. glabrata infekzioen aurka MCF (8 µg/ml) bidezko tratamendua izan zen uretan disolbatutako antifungikoen artean eraginkorrena, eta nematodoen biziraupen-ehunekoak %96,8tik gorakoak izan ziren. CAS farmako antifungikoak ere eragin bortitza izan zuen *C. glabrata* infekzioaren aurka. Hala ere, uretan disolbatutako bi farmako horien eragina ebaluatzea zaila izan zen *C. bracarensis* anduiek *C. elegans* ereduan eragindako infekzio baxua izan zelako. Ekinoandinak dira *C. glabrata* infekzio inbaditzaileen aurrean aukerako tratamendua, baina farmako horiekiko erresistenteak diren isolamenduak argitaratu dira (Fuller et al., 2019; Spreghini et al., 2012; Basas et al., 2019). Erresistentzia ekinoandinarekiko esposizioarekin eta praktika klinikoan erabilera areagotzearekin lotu da (Quindós et al., 2018; Wright et al., 2019; Desalermos et al., 2011; Fernandez-Silva et al., 2014). Bestalde, eta garrantzitsua da aipatzea FCZ kandidiasi inbaditzailearen tratamenduan sarritan erabiltzen den farmako antifungikoa dela; baina jakinarazi da *C. glabrata*k gero eta gehiagotan farmako horiekiko erresistentzia eskuratu duela (Quindós et al., 2018). Gure ikerketa lan honetan, *Candida* andui guztiek izan zuten in vitro FCZ farmakoekiko sentikortasuna, eta in vivo FCZ dosi handienak behar izan ziren (64 edo 128 µg/ml) *C. glabrata* eta *C. nivariensis* espeziearekin infektatutako *C. elegans* nematodoaren biziraupena nabarmen handitu zela detektatzeko.

C. nivariensis espezieari dagokionez, CAS and MCF, uretan disolbatutako ekinokandinak, eraginkorrenak izan ziren *C. elegans* eredua erabiliz. Uretan disolbatutako edozein farmako antifungikoekin aztertutako tratamendua eraginkorra izan zen *C. nivariensis* espezieak eragindako infekzioaren aurka; CECT 11998 anduiarekin infektatutakoen kasuan izan ezik, infektatutako nematodo horiek FCZ dosi altuena behar

izan baitzuten tratatu gabeko nematodo infektatuekiko aldeak behatzeko. Bestalde, DMSO disolbatutako tratamenduetako bakar batek ere ez zuen CECT 1198 anduiarekin infektatutako nematodoen hilkortasuna murriztu, tratatu gabeko infektatuekin alderatuta. AmB (2 µg/ml) eta VCZ (1 µg/ml) farmakoen tratamenduak bakarrak izan ziren CBS 9984 anduiarekin infektatutako nematodoen hilkortasuna nabarmen murriztu zutenak. Zenbait in vitro ikerketek frogatu zuten *C. nivariensis* Amb, AND, PCZ eta VCZ farmakoekiko duen sentikortasuna (Correia et al., 2006; Borman et al., 2008; Dudiuk et al., 2017; Castanheira et al., 2017; Beyda et al., 2014). Hala ere, badira espezie horretako anduiak ere, FCZ, VCZ edo PCZ farmakoekiko sentikortasun murriztua eta erresistentzia dutenak.

8. Taula: *Caenorhabditis elegans* nematodoaren biziraupena 120 orduko infekzioaren ondoren *C. glabrata*, *C. nivariensis* y *C. bracarensis* espezieekin.

Candida anduia	*C. elegans* biziraupenaren ehunekoak, DMSOren presentzian / absentzian	Farmako antifungiko eraginkorrenak (*C. elegans* biziraupenaren ehunekoak 120 orduko infekzioaren ondoren)	
		Uretan disolbatua	DMSOn disolbatua
Candida glabrata			
ATCC 90030	%40.3 / %26.5	Mikafungina, 8 µg/ml (%91.5)	Anfotericina B 1 µg/ml (%82.4)
NCPF 3203	%65.4 / %45.1	Mikafungina, 8 µg/ml (%96.8)	Anfotericina B 2 µg/ml (%85.2)
Candida nivariensis			
CECT 11998	%72.9 / %65.3	Mikafungina, 8 µg/ml (%88.3)	Borikonazol 1 µg/ml (%64.8)
CBS 9984	%75 / %73	Kaspofungina, 8 µg/ml (%94.9)	Anfotericina B 2 µg/ml (%83.2)
Candida bracarensis			
NCYC 3133	%89.4 / %89.4	Kaspofungina, 8 µg/ml (%94.5)	Anfotericina B 1 µg/ml (%80.1)
NCYC 3397	%97.6 / %70.8	Kaspofungina, 8 µg/ml (%100)	Borikonazol 1 µg/ml (%96.3)

3. ONDORIOA

Ondorioz, *C. elegans* nematodoa infekzio eredu egokia eta erraza izan zen *C. glabrata*, *C. nivariensis* eta *C. bracarensis* birulentzia ikertzeko. *C. glabrata* espezierik bortitzena izan zen. Gainera, *C. elegans* sistema eredu arrakastatsua izan zen *C. glabrata*k eta erlazionatutako espezieek eragindako infekzioen aurkako farmako antifungikoak in vivo aztertzeko. Probatutako farmako antifungiko guztiak eraginkorrak izan ziren C. *glabrata*k eragindako infekzioaren aurka. Konkretuki, AmB eta azol berriak eraginkorrenak izan ziren, eta ondoren, ekinoandinak, ordena honetan: MCF, CAS eta AND. Ekinokandinak, MCF eta CAS, ere izan ziren aktiboak *C. nivariensis* infekzioaren aurka, eta ondoren, FLZ kontzentrazio handietan. Azol berriek, AmB eta AND eragin terapeutiko onak izan zituzten *C. bracarensis* NCYC 3397 anduiak *C. elegans*en eragindako infekzioaren aurka. Hala ere, *C. bracarensis* anduien aurkako tratamendu antifungikoen eragina batzuetan ez zen oso zehatza izan, espezie horren birulentzia baxua zelako, beraz, infekzio-dosia modu zehatzagoan inokulatu daitekeen beste eredu batzuk behar dira. Gure azterlan honek *C. glabrata*, *C. nivariensis* eta *C. bracarensis* espezieen patogenotasuna eta espezie horiek sortzen dituzten infekzioen aurkako tratamenduak hobeto ezagutzen laguntzen du.

3. Azterlana (3. Eranskina/Anexo)

Candida glabrata, Candida nivariensis eta _Candida bracarensis_ espezieek eragindako kandidiasia _Galleria mellonella_ eredua erabiliz: anduien birulentzia eta erantzun terapeutikoa ekinokandinei

«Candidiasis by *Candida glabrata*, *Candida nivariensis* and *Candida bracarensis* in *Galleria mellonella*: strain virulence and therapeutic responses to echinocandins»

Ainara Hernando-Ortiz, Elena Eraso, Guillermo Quindós, Estibaliz Mateo

Mikologia Medikoko laborategia, UFI 11/25, Immunologia, Mikrobiologia eta Parasitologia Saila, Medikuntza eta Erizaintza Fakultatea, (UPV/EHU)

Prestatzen ari den artikulua

Laburpena

Candida albicans kandidiasi inbaditzailearen eragile etiologiko nagusia da, baina arreta berezia merezi du *Candida* espezie emergenteen prebalentzia gora egiten doala; *Candida glabrata*, *Candida nivariensis* eta *Candida bracarensis* espezieena, besteak beste. *C. glabrata*k garrantzi klinikoa hartu du, normalean erabiltzen diren farmako antifungikoekiko, flukonazolarekiko bereziki, sentsibilitate txikia duelako. Ekinokandinak kandidiasi kasu gehienetan aukerako tratamendua izan arren, hiru espezie horietan *FKS* geneen mutazioei lotutako erresistentziaren berri eman da. Ikerketa lan gutxi daude hiru espezie horien birulentzia eta erantzun terapeutiko ugaztunak ez diren in vivo ereduak erabiliz aztertzen dituztenak. Beraz, azterlan honetan *C. glabrata*, *C. nivariensis* eta *C. bracarensis* espezieak ikertu ziren *G. mellonella* ereduan. Larben biziraupena eta ostalari eta patogeno arteko interakzioak (hemozitoen produkzioa eta horien gaitasuna legamia fagozitatzeko) aztertu ziren. *Candida*ren hiru espezie horiek infekzio hilgarria eragin zuten *G. mellonella*n; *C. glabrata* izan zen espezie bortitzena eta *C. bracarensis* gutxiena. *G. mellonella*ren hemozitoek *C. bracarensis* espeziearen zelulak beste bi espezieenak baino eraginkortasun handiagoz fagozitatu zituzten. Bestalde, *G. mellonella* larbak anidulafungina, kaspofungina eta mikafungina farmako antifungikoekin tratatu ziren. Kaspofungina eta mikafungina eraginkorragoak izan ziren larbak babesteko *C. glabrata* eta *C. nivariensis* eragindako infekzioetatik; anidulafungina, berriz, *C. bracarensis* infekzioetatik. *G. mellonella* kandidiasi-eredua erraza eta egokia da *Candida* espezieen birulentzia eta erantzun terapeutikoa ebaluatzeko. Gainera, larben immunitate-sisteman patogenoen birulentziaren arabera dauden desberdintasunak arrakastaz detektatzeko aukera ematen du.

Hitz gakoak: patogeno emergenteak, patogenotasuna, tratamendu antifungikoaren eraginkortasuna, eredu ornogabea

Hirugarren azterlanari dagokion artikulua prestaketa prozesuan dago eta 3. eranskinean zehaztuta dago. Ondoren, emaitza nabarmenenen laburpena aurkezten da.

1. HELBURUA

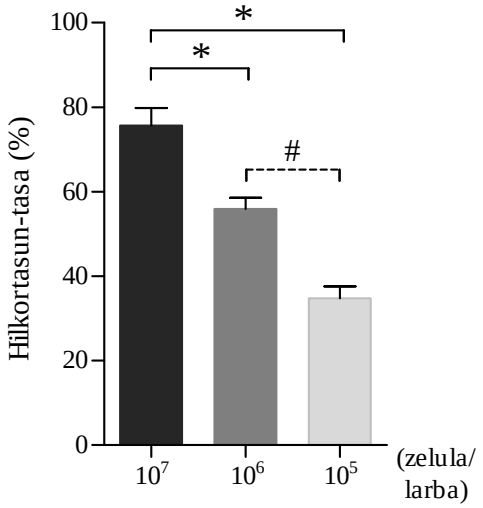
Azterlana honen helburua *G. mellonella* ordezko eredua erabiliz *C. glabrata*, *C. nivariensis* eta *C. bracarensis* espezieen birulentzia ikertzea da. Alde batetik, larben biziraupena eta horien immunitate sistemak infekzioari aurka egiteko ahalmena ikertu zen. Bestalde, anidulafungina, kaspofungina eta mikafungina antifungikoekiko sentikortasuna aztertu zen *G. mellonella* eredua erabiliz.

2. EMAITZEN ETA EZTABAIDAREN LABURPENA

2.1. *Candida glabrata, Candida nivariensis eta C. bracarensis* espezieen birulentziaren azterketa *Galleria mellonella* eredua erabiliz

G. mellonella eredua garrantzia lortzen ari du bere tamaina egokia delako infekzio kontrolatuak garatzeko, gainera, bai mikroorganismoen bai tratamenduen suspentsioak kontzentrazio jakinetan injektatzea baimentzen baitu (Killiny et al., 2018). Animalia eredu hori *Candida* espezie desberdinen azterketan erabili da (Mesa-Arango et al., 2012; Scorzoni et al., 2013*;* Gago et al., 2014; Borman et al., 2016; Marcos-Zambrano et al.,2020; Huang et al., 2020; Muñoz et al., 2020). Baina, *C. glabrata*, *C. nivariensis* eta *C. bracarensis* espeziekin azterlan gutxi argitaratu dira ugaztunak ez diren in vivo animalia ereduak erabiliz (Ames et al., 2017; Hernando-Ortiz et al., 2020). Azterlan honetan erabilitako anduiak 9. Taulan deskribatuta daude. Erabilitako hiru espezieak *G. mellonella* eredu alternatiboan infekzio inbasiboa garatzeko gai izan ziren. Gainera, kontrol modura erabilitako larben eta infektatutako larben artean desberdintasun esanguratsua ikusi zen ($p \leq 0.003$). Larben hilkortasuna injektatutako inokuluaren mendekoa izan zen; zenbat eta inokulo handiagoa, orduan eta hilkortasun handiagoa ikusi zen eta inokuluen artean desberdintasun adierazgarriak lortuz ($p \leq 0.001$) (21. Irudia). Emaitza horiek aldez aurretik behatu dira *G. mellonella* ereduan *Candida* espezie

desberdinekin egindako beste azterlan batzuetan (Mesa-Arango et al., 2012; Scorzoni et al., 2013; Ames et al., 2017; Sherry et al., 2017; Maurer et al., 2019).



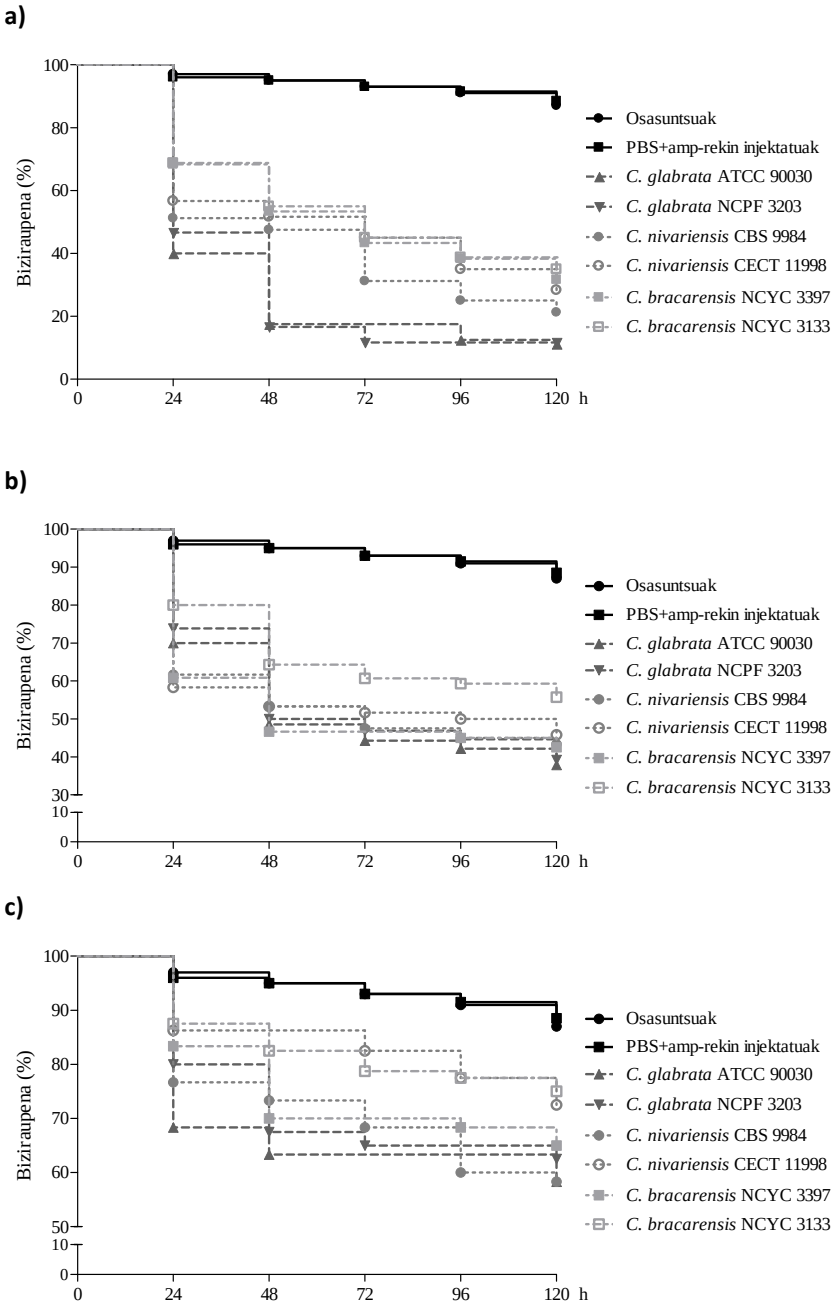
21. Irudia: Infektatutako *G. mellonella* larben heriotza-tasa: 1 × 10^7, 1 × 10^6 eta 1 × 10^5 zelula/larva inokuluekin (*C. glabrata*, *C. nivariensis* eta *C. bracarensis*). *Estatistikoki desberdintasun adierazgarriak 1 x 10^7 zelula/larba inokuluarekin; #estatistikoki desberdintasun adierazgarriak 1 x 10^6 eta 1 x 10^5 zelula/larva inokuluen artean.

Inokulu altuenarekin (1 × 10^7 zelula/larba) infektatutako larben biziraupenean desberdintasun adierazgarriak ikusi ziren *C. glabrata* eta beste bi espezieen artean ($p \leq 0,024$) (22a. Irudia). *C. glabrata* espezieak hilkortasun altuena sortu zuen. Emaitza horiek bat datoz *C. elegans* animalia ereduan lortutakoekin, bertan *C. glabrata* espezieak ere nematodo portzentai altuena hil baitzituen (Hernando-Ortiz et al., 2020). Legami-inokulu horrekin ikusi zen esperimentua hasi eta 48 ordu igaro ondoren, *C. glabrata*k eragindako *G. mellonella*ren hilkortasun-ratioa %80koa izan zen, eta *C. nivariensis* eta *C. bracarensis* eragindakoa aldiz %50-60 bitartekoa. Beste ikertzaile batzuek ere *C. glabrata*ren birulentzia aztertzeko inokulu altuak (7,5 × 10^6 zelula/larba) erabili zuten (Ames et al., 2017). Hala ere, beste *Candida* espezie batzuekin egindako azterlanetan, *C. albicans*, *C. auris*, *C. tropicalis*, *C. krusei* eta *C. parapsilosis* konplexua kasu, inokulu

baxuagoak nahikoak izan ziren kandidiasia garatzeko (Mesa-Arango et al., 2012; Scorzoni et al., 2013; Gago et al., 2014; Borman et al., 2016; Sherry et al., 2017; Marcos-Zambrano et al., 2020; Huang et al., 2020; Muñoz et al., 2020).

Injektatutako beste bi inokuluekin (1×10^6 eta 1×10^5 zelula/larba) aldiz, ez zen hiru espezieen artean desberdintasun esanguratsurik ikusi, *C. bracarensis* NCYC 3133 anduiarekin infektatutako larbetan izan ezik (22b eta c Irudiak). Andui honek 120 ordu igaro ondoren larben biziraupen-tasarik handiena lortu baitzuten (%55,7 eta %75, hurrenez hurren).

G. mellonella larben biziraupena 1×10^6 zelula/larba inokuluarekin %55 baino txikiagoa izan zen 48 ordu igaro ondoren, *C. bracarensis* NCYC 3133 anduiarekin izan ezik, azken horrek 120 ordu baino gehiago behar izan baitzituen % 44ko heriotza-tasa sortzeko (22b Irudia). Azken andui horrek eta *C. nivariensis* CECT 11998 anduiak ez zuten larben %28 baino gehiago hil 120 ordu igaro ondoren 1×10^5 zelula/larba inokuloarekin, eta *Candida*ren gainerako anduiek ez zuten lortu larben %42a baino gehiago hil (22c Irudia).



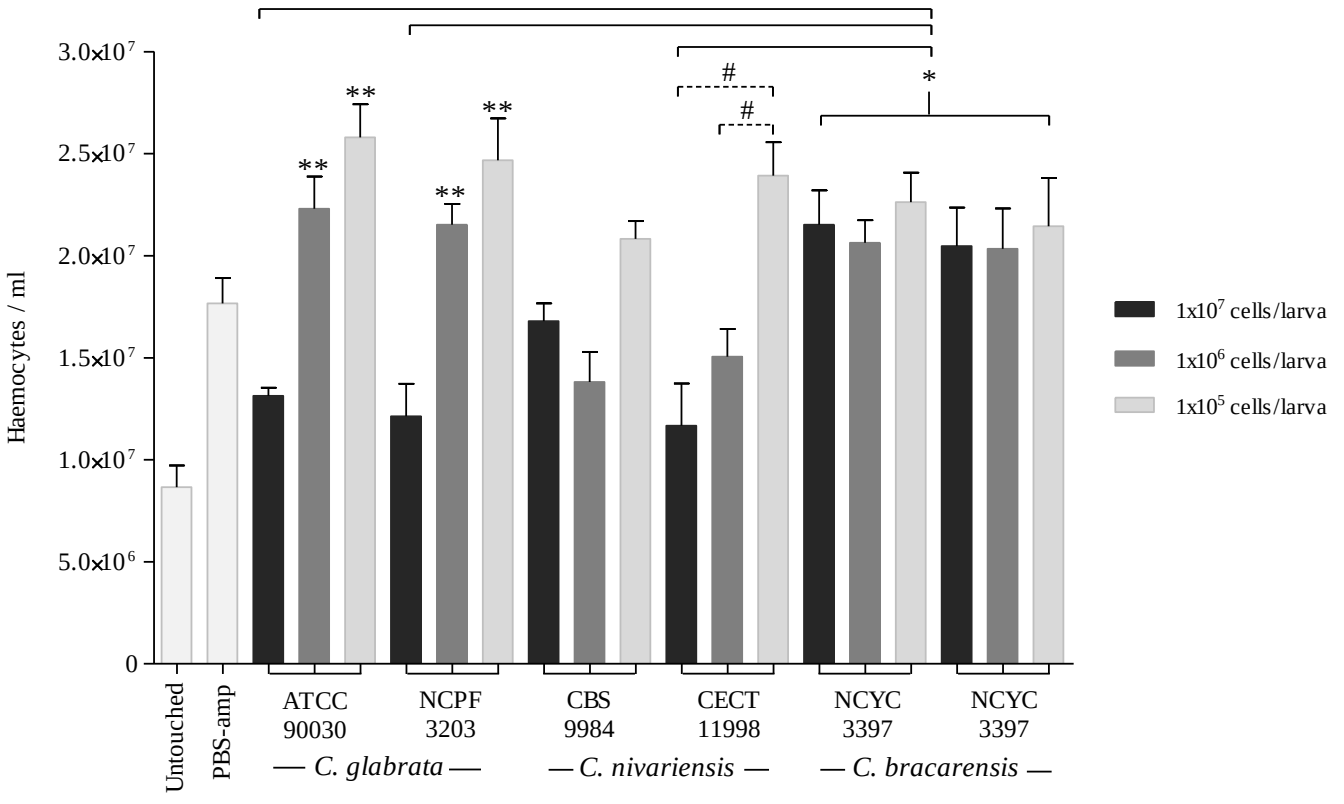
22. irudia: *Candida glabrata, Candida nivariensis* eta *Candida bracarensis* espeziekin infektatutako *Galleria mellonella*ren biziraupena (a)1 x 10^7, (b)1 x 10^6 eta(c) 1 x 10^5 zelula/larba inokuluekin

2.2. Hemozitoen ekoizpenean eta aktibitate fagozitikoa kandidiasis infekzioan

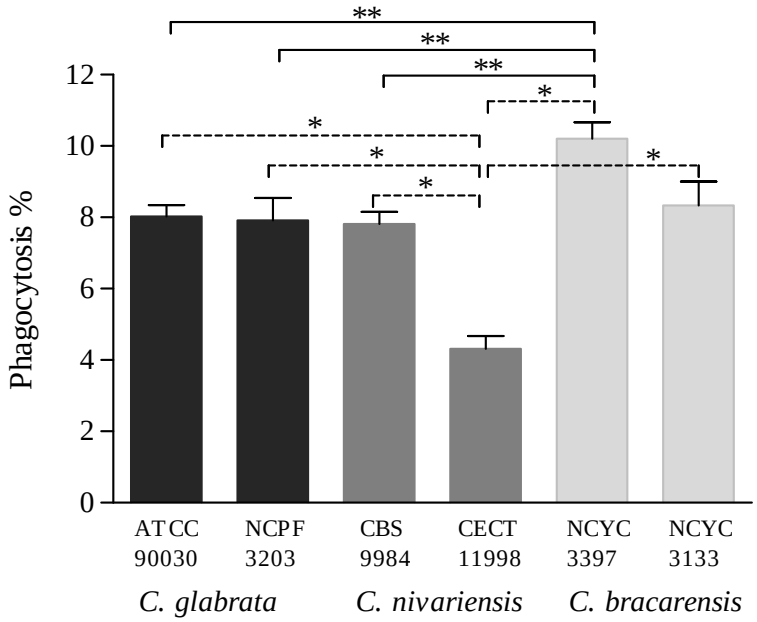
G. mellonella ereduaren bidez ostalariaren eta patogenoaren arteko elkarrekintzak aztertzeko posibilitatea dago, larbaren immunitate-sistemaren eraginkortasuna aztertuz. Lepidoptero honek patogenoei aurre egiteko hemozitoen ekoizpena handitzen du eta horien bitartez patogenoak fagozitatzen ditu. Fagozitazioa patogenoen birulentzia-ahalmenaren eta larbaren immunitate-sistematik ihes egiteko gaitasunaren araberakoa izango da (Bergin et al, 2003). Hemozitoen ekoizpena aztertzeko larbak 1×10^5, 1×10^6 eta 1×10^7 zelula/larba inokuluekin infektatu ziren eta hemozitoen dentsitatea 3 ordu igaro ondoren neurtu zen (23a. Irudia). Espezie guztietako bi inokulu baxuenek (*C. nivariensis* anduiek 1×10^6 zelula/larba inokuluarekin izan ezik ($p \leq 0,49$) eta PBS-amp kontrol taldeak hemozitoen kopurua nabarmen handitu zuten osasuntsuen kontrol-taldearekin alderatuta ($p \leq 0,005$),. *Candida*ren inokulu altuenarekin, lortutako emaitzen arabera, *C. glabrata* eta *C. nivariensis* CECT 11998 anduiekin infektatutako larbetan hemozito dentsitate baxuagoa ekoiztu zen, *C. bracarensis* anduiekin alderatuta ($p \leq 0,041$). Beste ikerketa batzuetan *C. albicans*, *C. krusei*, *C. tropicalis*, *C. parapsilosis* edo *C. orthopsilosis* espezieek eragindako infekzioan zehar behatutako hemozitoen ekoizpena are txikiagoa izan zen, ziurrenik espezie horien birulentzia handiagoaren ondorioz (Mesa-Arango et al., 2012; Scorzoni et al., 2013; Gago et al., 2014; Perini et al., 2019). *C. bracarensis* anduiekin infektatutako larbek hemozitoen ekoizpena handitzeko gaitasuna erakutsi zuten eta hiru inokuluen artean ez ziren desberdintasun adierazgarririk egon.

*G. mellonell*an aktibitate fagozitiko altuena *C. bracarensis* NCYC 3397 anduiaren aurka behatu zen (%11,26 ± 0,91), eta desberdintasun adierazgarriak hauteman ziren *C. glabrata* eta *C. nivariensis* espeziekin ($p \leq 0.041$) (23b. Irudia). Aldiz, *C. nivariensis* CECT 11998 anduia izan zen larben hemozitoek gutxien fagozitatu zutena (%5,31 ± 0,71) eta gainerako anduiekin alderatuta desberdintasunak ikusi ziren ($p \leq 0.001$). *C. parapsilosis* konplexuko espezieetan ere ikusi zen *C. metapsilosis* espeziea, birulentzia gutxien zeukan espeziea, izan zela larbek gehien fagozitatu zutena (Gago et al., 2014).

a)


Haemocytes / ml
3.0x10^7
2.5x10^7
2.0x10^7
1.5x10^7
1.0x10^7
5.0x10^6
0
**
**
**
**
#
#
*
Untouched
PBS-amp
ATCC 90030
NCPF 3203
CBS 9984
CECT 11998
NCYC 3397
NCYC 3397
— C. glabrata —
— C. nivariensis —
— C. bracarensis —
1x10^7 cells/larva
1x10^6 cells/larva
1x10^5 cells/larva

b)



23. irudia: **a)** *C. glabrata*, *C. nivariensis* eta *C. bracarensis* espezien 1 x 10^5, 1 x 0^6 eta 1 x 10^7zelua/larba inokuluekin infektatutako larben hemozitoen ekoizpena. *Estatistikoki desberdintasun adierazgarriak *C. bracarensis* espeziearekin; **Estatistikoki desberdintasun adierazgarriak *C. glabrata* 1 x 10^7 zelula/larba inokuluarekin infektatutako larbekin; #Estatistikoki desberdintasun adierazgarriak *C. nivariensis* CECT 1198 1 x 10^7 eta 1 x 10^6 zelula/larba inokuluekin infektatutako larben artean. **b)** *C. glabrata*, *C. nivariensis* and *C. bracarensis* espezien 1 x 10^6 zelula/larba inokuluarekin infektatutako larben hemozitoen jarduera fagozitikoa. *Estatistikoki desberdintasun adierazgarriak *C. nivariensis* CECT 11998 anduiarekin; ** Estatistikoki desberdintasun adierazgarriak *C. bracarensis* NCYC 3397 anduiarekin.

2.3. Ekinokandinen eraginkortasuna kandidiasi inbaditzailea tratatzeko *Galleria mellonella* ereduan

C. glabrata espezieak eragindako kandidiasiaren tratamendurako ekinokandinak gomendatzen dira (Katiyar et al., 2012; Pham et al., 2014; Morales-Lopez et al., 2017). Hala ere, azken urteetan deskribatu da ekinokandinekiko sentikortasun murriztua duten edo erresistenteak diren isolamenduak areagotu direla, batez ere farmako horiekiko esposizioaren ondorioz (Beyda et al., 2014; Pham et al., 2014; Domán et al., 2015; Bordallo-Cardona et al., 2018; Coste et al., 2020; Al-Baqsami et al., 2020). *C. nivariensis* eta *C. bracarensis* espezieetan ere azolekiko eta b anfoterizinarekiko sentikortasun murriztua edota erresistentziak deskribatu dira (Pfaller et al., 2012; Alexander et al., 2013; Faria-Ramos et al., 2014; Ames et al., 2017; Astvad et al., 2018; Ko et al., 2018; Fujita et al., 2007; Bishop et al., 2008; Borman et al., 2008; Lockhart et al., 2009; Cuenca-Estrella et al., 2011; Gil-Alonso et al., 2015; Figueiredo-Carvalho et al., 2016; Hou et al., 2017; Morales-Lopez et al., 2017; Arastehfar et al., 2019; Alobaid et al., 2020; Shi et al., 2020). Gure azterlanean hiru *Candida* espezieen *C. glabrata*, *C. nivariensis* eta *C. bracarensis*, 1 x 10^6 zelula/larba inokuluak eragindako kandidiasia AND, CAS eta MCF ekinokandinen bi kontzentrazioekin tratatu zen (4 eta 8 µg/larba) (9. Taula). CAS eta MCF antifungikoak *C. glabrata* eta *C. nivariensis* espezieen aurka eraginkorrenak izan ziren, eta *C. bracarensis* espeziearen aurka berriz, AND farmakoa. Emaitza horiek aurrez *C. elegans* ereduan ikusitakoaren ildoan daude (Hernando-Ortiz et al., 2020).

9. Taula: *C. glabrata*, *C. nivariensis* eta *C. bracarensis* espezieekin infektatutako *Galleria mellonella*ren biziraupena ekinokandinen tratamenduarekin eta tratamendurik gabe.

Anduia	*G. mellonella*ren biziraupen-ehunekoak 120 ordu igaro ondoren ($1x10^6$ zelula/larba inokuluarekin infektatuta)	Farmako antifungiko tratamendu eraginkorrenak (*G. mellonella*ren biziraupen-ehunekoaren handipena 120 ordu igaro ondoren)
Candida glabrata		
ATCC 90030	%38,7	Mikafungina, 8 µg/larba (%34,6) Mikafungina, 4 µg/ larba (%33)
NCPF 3203	%39.2	Anidulafungina, 4 µg/larba (%45,8) Mikafungina, 8 µg/ larba (%42,5)
Candida nivariensis		
CBS 9984	%43.3	Mikafungina, 4 µg/larba (%41,7) Kaspofungina, 8 µg/ larba(%38,4)
CECT 11998	%45.8	Kaspofungina, 4 µg/larba (%)35,9 Kaspofungina, 8 µg/ larba (%34,2)
Candida bracarensis		
NCYC 3397	%42.5	Anidulafungina, 4 µg/larba (%29,2) Kaspofungina, 8 µg/ larba (%19,2)
NCYC 3133	%55.7	Micafungina 4 µg/larba (%24,3) Anidulafungina, 8 µg/ larba (%21)

C. glabrata andui biak oso sentikorrak izan ziren MCF-rekiko, saiatutako bi kontzentrazioetan, eta larben biziraupena nabarmen handitzea lortu zen infektatu eta tratatu gabeko larba taldearekiko ($p \leq 0,0001$)(9. Taula). Zehazki, MCF tratamendu altuenak (8 µg/larba) *C. glabrata* anduiekin infektatutako larben biziraupena %73,3 eta %81,7ra igo zuen (ATCC 90030 eta NCPF 3203, hurrenez hurren), infektatu eta tratatu gabekoen biziraupena %39,2 baino baxuagoa zela. Beste ikertzaile batzuek ere ikusi zuten MCF eraginkorra zela *C. glabrata* espezieak sortarazitako infekzioaren aurka eredu murinoetan (Mariné et al., 2006; Howard et al., 2011). Hala ere, MCF-rekiko

erresistenteak diren eta *FSK2* genean mutazioak dituzten *C. glabrata* anduien hautaketa ikusi zen in vitro antifungiko horren dosi baxuen esposizioaren ondorioz (Bordallo-Cardona et al., 2018).

Gure ikerketa lan honetan CAS bidezko tratamendua ere eraginkorra izan zen *C. glabrata* espeziearen aurka; *G. mellonella*ren biziraupena nabarmen handitu zelako infektatu eta tratatu gabeko larbekin alderatuta ($p \leq 0,004$). *C. glabrata* anduiekin infektatutako larben biziraupena CAS tratamendu altuenarekin (8 µg/g larba) %81,7raino igo zen eta kontzentrazio baxuenarekin (4 µg/g larba) %71,7raino. CAS antifungikoaren eraginkortasuna Ames eta laguntzaileek (2017) ikusi zuten ere, *C. glabrata*rekin infektatutako *G. mellonella* larben biziraupena %80raino igo baitzen 4 µg/g larba kontzentrazioarekin tratatu ondoren (Ames et al., 2017). Gainera, CAS farmakoaren eraginkortasuna *C. glabrata*k eredu murinoan sortutako kandidiasiaren aurka ere frogatu da (Spreghini et al., 2012; Fernandez-Silva et al., 2014; Domán et al., 2015; Wiederhold et al., 2016). Hala ere, CAS kontzentrazio altuak (20 mg/kg) erabiltzeak ekinokandinekiko erresistenteak ziren *C glabrata* anduien hautaketa eragiten zuela ikusi zen eredu murinoan, andui horiek FKS mutazioak zituztenak (Healey et al., 2017).

Azkenik, AND bidezko tratamenduak eragina izan zuen soilik *C. glabrata* NCPF 3203 anduiaren infekzioaren aurka (4 µg/g larba), eta larben biziraupena %45,8a igo zen ($p \leq 0,011$, kutsatutako eta tratatu gabeko kontrol-taldearekin alderatuta). *C. glabrata* ATCC 90030 anduiarekin infektatutako *G. mellonella* larbek ez zuten hobekuntza adierazgarririk izan farmako antifungiko horrekin. Emaitza hauek bat datoz Spregini eta laguntzaileek (2012) deskribatutakoarekin, AND tratamenduak hiru ekinokandinen artean eraginkortasun txikiena izan zuen *C. glabrata*k eredu murinoan eragindako infekzioaren aurka.

C. nivariensis eragindako infekzioak arrakastaz tratatu ziren MCF eta CAS farmako antifungikoekin (9. Taula). CAS tratamendua *C. nivariensis* andui biekin infektatutako larben biziraupena %34,2-38,4 artean igo zuen, eta desberdintasun adierazgarriak egon ziren infektatu eta tratatu gabeko taldearekin ($p \leq 0,0001$). López-Soria eta laguntzaileek

(2013) ikusi zuten CAS oso eraginkorra zela *C. nivariensis* eragindako kateterrari lotutako fungemia tratatzeko. In vitro saiakuntzetan ere *C. nivariensis* espeziearen sentikortasuna CAS-rekiko deskribatu zen (Wahyuningsih et al., 2008; Lockhart et al., 2009; Chowdhary et al., 2010; Sharma et al., 2013; Tay et al., 2014; Hou et al., 2017; Hernando-Ortiz et al., 2020).

Gure azterlanean MCF ere *C. nivariensis* espezieak sortzen duen kandidiasia tratatzeko oso eraginkorra izan zen, infektatutako larben biziraupena estatistikoki igoera adierazgarria hauteman zuen ($p \leq 0,001$). Izatez, *C. nivariensis* CBS 9984 anduiarekin infektatutako larben kasuan biziraupena %41,7 igo zen MCF 4 µg/g larba tratamenduarekin. *C. nivariensis* espezieak MCF-rekiko duen sentikortasuna in vitro saiakuntzetan ere frogatu zen (Lockhart et al., 2009; Sharma et al., 2013; Hou et al., 2017; Morales-Lopez et al., 2017; Hernando-Ortiz et al., 2020). AND tratamenduak *C. nivariensis* eragindako infekzioan eraginik txikiena izan zuen. Soilik 4 µg/g larba kontzentrazioak murriztu zuen adierazgarriki *C. nivariensis* CBS 9984 anduiarekin infektatutako larben hilkortasuna ($p \leq 0,0001$). *C. nivariensis* CECT 11998 anduiaren infekzioaren aurka aldiz, AND tratamenduak hilkortasuna areagotu zuen (%28,3a 4 µg/g larba kontzentrazioarekin eta %38,3a 8 µg/g larba kontzentrazioarekin).

C. bracarensis izan zen, hiru espezieen artean, *G. mellonella* ereduan ekinoandinekiko sentikortasun txikiena izan zuena. Hiru ekinokandinen artean AND eraginkorrena izan zen. *C. bracarensis* NCYC 3397 anduiarekin infektatutako larben biziraupen altuena behatu zen AND 4 µg/g larba tratamenduarekin, %71,7koa izan baitzen. Era berean, AND (8 µg/g larba) eta MCF (4 µg/g larba) tratamenduek adierazgarriki babestu zituzten larbak *C. bracarensis* NCYC 3133 anduiak sortarazitako infekziotik, horien biziraupena %76,7 ($p = 0,008$) eta %80 ($p = 0,002$) izan baitziren hurrenez hurren. CAS tratamenduari dagokiola, soilik *C. bracarensis* NCYC 3397 anduiarekin infektatutako larbetan behatu zen hilkortasunaren murrizketa adierazgarria, %19,2ko murrizketa hain zuzen ere ($p = 0,015$). Hala ere, hainbat ikerketa lanetan *C. bracarensis* espezieak AND-rekiko zuen sentikortasunaz gain, beste bi ekinokandinekiko sentsibilitatea ere deskribatu da (Lockhart et al., 2009; Warren et al., 2010; Hou et al., 2017; Hernando-Ortiz et al., 2020).

3. ONDORIOA

Azterlan honetan ondoriozta dezakegu *G. mellonella* eredua *C. glabrata*, *C. nivariensis* eta *C. bracarensis* espezieen birulentzia eta ostalari eta patogenoaren arteko interakzioak aztertzeko egokia dela. Kandidiasi-eredu honetan, *C. grabata* espeziearen birulentzia handiena eta *C. bracarensis* aldiz txikiena berretsi ahal izan zen. Gainera, *C. glabrata* eta *C. nivariensis* espezieekin infektatutako larbetan hemozitoen ekoizpena murriztu egin zen, eta *C. bracarensis* zelulak izan ziren hemozitoek hobeto fagozitatu zituztenak. Tratamenduan CAS eta MCF farmakoak eraginkorrenak izan ziren *C. glabrata* eta *C. nivariensis* espezieen aurka, *C. bracarensis* espeziearen aurka berriz AND izan zen.

4. Azterlana (4. Eranskina/Anexo)

Caenorhabditis elegans eta _Galleria mellonella_ ereduak erabiliz jatorri kliniko desberdinetatik datozen _Candida auris_ isolamenduen birulentziaren azterketa

«Virulence of *Candida auris* from different clinical origins in *Caenorhabditis elegans* and *Galleria mellonella* host models»

Ainara Hernando-Ortiz[1], Estibaliz Mateo[1], Aitzol Perez-Rodriguez[1], Piet W.J. de Groot, Guillermo Quindós[1], Elena Eraso[1]

[1]Mikologia Medikoko laborategia, UFI 11/25, Immunologia, Mikrobiologia eta Parasitologia Saila, Medikuntza eta Erizaintza Fakultatea, (UPV/EHU)

[2]Centro Regional de Investigación Biomédica, Parque Científico y Tecnológico Castilla La Mancha, Universidad de Castilla La Mancha, Albacete.

Virulence 12 (2021); 1063-1075

Laburpena

Candida auris farmako antifungikoekiko erresistentzia handia duen patogeno fungiko emergente bat da, eta kandidiasi inbaditzaileen agerraldi nosokomialen erantzulea da. Espezie horren patogenizitateari buruzko zenbait ikerketa argitaratu diren arren, *C. auris* espeziearen birulentziari buruzko ezagutza oraindik mugatua da. Azterlan honen helburua, jatorri kliniko desberdineko (odol-laginak, gernu-laginak eta orofaringe-laginak) *C. auris* isolamenduen patogenizitatea aztertzea izan zen bi kandidiasi-eredu desberdinetan, *Caenorhabditis elegans* eta *Galleria mellonella*. Isolamendu horietako batek fenotipo agregatzailea zuen eta beste 11 isolamenduek ez-agregatzailea. Gainera, saiatu zen *C. auris* isolamenduen birulentzia, agregatuak eratzeko gaitasunarekin, biofilmak sortzeko gaitasunarekin eta jatorri klinikoarekin egon ahal diren lotura posibleak ebaluatzea. Fenotipo agregatzailea zuen isolamendua ez zen hain birulentoa izan in vivo ornogabe infekzio-eredu bietan, isolamendu ez-agregatzaileekin konparatuz, baina biofilmak sortzeko gaitasun handiagoa erakutsi zuen. Odol-laginetako isolamenduak, gernu- eta arnas laginetatik isolatutakoak baino askoz birulentoagoak izan ziren *G. mellonella* kandidiasi-ereduan. Ikerketa lan honekin, bi kandidiasi-ereduek abantailak eta desabantailak dituztela, baina *C. auris*aren birulentzia in vivo ebaluatzeko baliagarriak direla ondorioztatzen dugu. Bestalde, bi ereduek agerian uzten dute espezie horrek izan dezakeen birulentzia desberdintasunak, isolamenduek izan dezaketen fenotipo agregatzailearen eta jatorri klinikoaren ondorioz.

Hitz gakoak: *C. auris*, patogeno emergenteak, candidiasia, birulentzia, eredu ornogabeak.

Laugarren azterlanari dagokion artikulua *Virulence* aldizkarian argitaratu da eta 4. eranskinean zehaztuta dago. Ondoren, emaitza nabarmenenen laburpena aurkezten da.

1. HELBURUA

Azterlan honen helburua lagin kliniko desberdinetatik lortutako *C. auris* isolamenduen birulentzia aztertzea izan zen, horien fenotipo agregatzailea eta ez-agregatzailea kontutan edukita. In vitro ebaluatu zen *C. auris* isolamenduek biofilmak sortzeko gaitasuna eta baita horien aktibitate hemolitikoa eta entzimatikoa ere. Gainera, isolamendu horien birulentzia in vivo aztertu zen *C. elegans* eta *G. mellonella* animalia eredu ornogabeak erabiliz.

2. EMAITZEN ETA EZTABAIDAREN LABURPENA

2.1. *Candida auris* isolamenduen in vitro azterketa: biofilmen eraketa eta aktibitate hemolitikoa eta entzimatikoa

Azterlan honetan hamabi *C. auris* isolamendu erabili ziren, isolamendu batek fenotipo agregatzailea zuen (Alemaniako *Institut für Hygiene und Mikrobiologie*-n isolatutakoa) eta beste 11 isolamenduek fenotipo ez agregatzailea (Espainiako *Hospital Universitario y Politécnico La Fe*-n isolatutakoak). Horietako bost *C. auris* isolamenduak odol laginetatik isolatu ziren (fenotipo agregatzaileduna barne), bost gernu laginetatik eta beste bi orofaringe laginetatik.

Hamabi isolamendu horiek inongo klado zehatz batean sartuta ez badaude ere, Espainiako 11 isolamenduak Hego Afrikako kladoaren (III. kladoaren) isolamenduetatik filogenetikoki hurbil zeudela ikusi zen (Ruiz-Gaitán et al., 2018; Chow et al., 2020). Klado hori odol-uharraren infekzioekin eta agregatuen sorrerarekin erlazionatzen da (Szekely et al., 2019).

Biofilmen eraketa aztertu zenean, *C. albicans* SC5314 kontrol anduiak biofilm dentsoena ekoiztu zuen, *C. auris* 12 isolamenduak ekoiztutakoarekin konparatuz adierazgarriki

dentsoagoa izanda ($p < 0.0001$) (10. Taula). Biofilma eratzeko gaitasuna patogenizitate faktore garrantzitsua da *Candida*n, biofilmek legamia zelulak farmako antifungikoetatik eta sistema immunetik babesten baititu. Odol-laginatik lortutako JMRC: NRZ 1101 isolamenduak, fenotipo agregatzailea zuena, biofilma sortzeko gaitasun handiena erakutsi zuen, biomasa eta aktibitate metaboliko handienarekin ($p<0.0001$). Fenotipo ez-agregatzaileen artean CR14 isolamenduak biomasaren ekoizpenean ($p < 0.0001$) eta aktibitate metabolikoan ($p < 0.02$) baliorik altuena erakutsi zuen. Nahiz eta gure emaitzetan fenotipo agregatzailea zuen isolamendua (JMRC: NRZ 1101 isolamendua) biofilmaren ekoizle hoberena izan, Sherry eta laguntzaileek (2017) deskribatu zuten fenotipo ez-agregatzailea zuten isolamenduak biofilm-ekoizle hobeagoak zirela, fenotipo agregatzailea zuten isolamenduekin alderatuta (Sherry et al., 2017). Hala ere, *C. auris* biofilma ekoizteko ahalmena frogatu da bai fenotipo agregatzailea zein ez-agregatzailea duten isolamenduetan (Brown et al., 2020). Gainera, deskribatu da *C. auris* espezieak biofilma sortzeko gaitasuna duenean sentikortasun txikiagoa duela antifungikoekiko, biofilmak babestu egiten dutelako legami-zelulak antifungikoen eraginetik (Kean et al., 2020; Romera et al., 2019).

C. auris espeziean, beste *Candida* espezie batzuetan bezala, birulentzia-faktore garrantzitsu gisa deskribatu dira entzima hidrolitikoen ekoizpena eta aktibitate hemolitikoa. Izan ere, biofilmen ekoizpenarekin eta entzima hidrolitikoen ekoizpenarekin erlazionatutako geneak deskribatu dira *C. auris* espeziean. Baita legamiaren-atxikimenduan inplikatutako geneak (*ALS* eta *SAP* familiakoak), adierazpen handiagoa erakutsi dutenak isolatu agregatzaileetan ez-agregatzaileetan baino (Muñoz et al., 2018; Brown et al., 2020; Ben-Ami et al., 2017; Chatterjee et al., 2015). Hala ere, gure azterlanean, *C. auris* isolamenduetako bat ere ez zen fosfolipasa, proteasa edo aktibitate hemolitikoaren ekoizlea izan. Izan ere, *C. auris* espeziearen birulentzian eragiten duten faktoreetan heterogeneotasun handia deskribatu da bai klado desberdinen artean zein klado bakoitzaren barruan (Brown et al., 2020).

10 Taula: *C. auris* isolamendu bakoitzaren neurketa biofilmaren biomasa eta aktibitate metabolikoa Cristal violeta (CV) eta XTT erredukzioaren bitartez, hurrenez hurren.

Espeziea eta isolaketa lekua	Isolamendua	Biofilmaren biomasa		Biofilmaren aktibitate metabolikoa	
		OD (24 h)	OD (48 h)	OD (24 h)	OD (48 h)
Candida albicans					
	SC5314	0.719 ± 0.066	0.696 ± 0.071	1.159 ± 0.154	1.247 ± 0.172
Candida auris					
Odola	JMRC:NRZ 1101	0.508 ± 0.047	0.361 ± 0.039	0.421 ± 0.147	0.357 ± 0.075
	CJ94	0.068 ± 0.038	0.080 ± 0.046	0.145 ± 0.070	0.122 ± 0.031
	CBS15605	0.074 ± 0.049	0.079 ± 0.048	0.156 ± 0.086	0.124 ± 0.033
	CBS15606	0.068 ± 0.042	0.083 ± 0.051	0.135 ± 0.062	0.103 ± 0.028
	CBS15607	0.086 ± 0.079	0.077 ± 0.043	0.132 ± 0.074	0.098 ± 0.023
Gernua	CR14	0.227 ± 0.034	0.179 ± 0.054	0.237 ± 0.095	0.114 ± 0.060
	CR201	0.080 ± 0.036	0.057 ± 0.023	0.118 ± 0.035	0.122 ± 0.067
	CR220	0.103 ± 0.038	0.062 ± 0.030	0.119 ± 0.020	0.102 ± 0.048
	CR424	0.101 ± 0.024	0.082 ± 0.026	0.057 ± 0.039	0.056 ± 0.045
	CR440	0.090 ± 0.027	0.074 ± 0.025	0.121 ± 0.033	0.115 ± 0.055
Orofaringea	CR243	0.065 ± 0.033	0.084 ± 0.035	0.146 ± 0.056	0.119 ± 0.042
	CR312	0.081 ± 0.024	0.085 ± 0.046	0.085 ± 0.030	0.087 ± 0.047

OD: dentsitate optikoa

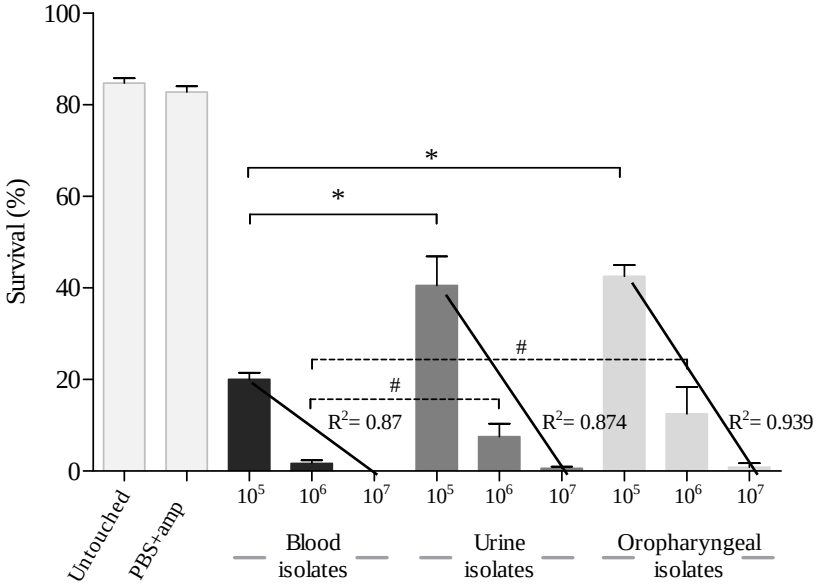
2.2. *C. auris*aren birulentzia *Caenorhabditis elegans* eta *Galleria mellonella* animalia ereduak erabiliz

*C. auris*aren birulentzia hainbat eredutan aztertu bada ere, hala nola saguetan (Ben-Ami et al., 2017; Fakhim et al., 2018; Yue et al., 2018; Torres et al., 2020; Wang et al., 2018), arrainetan (Johnson et al., 2018) edo *D. melanogaster* eulian (Wurster et al., 2019), gutxi batzuk bakarrik erabili dute *C. elegans* nematodoa (Eldesouky et al., 2018; Lima et al., 2020) edo *G. mellonella*ren larba (Borman et al., 2016; Sherry et al., 2017; Wang et al., 2018; Arias et al., 2020; Romera et al., 2020). Azterlan honetan, aztertutako 12 *C. auris* isolamenduek, nematodoen eta larben %47,7a gutxienez hil zituzten 120 ordu igaro ondoren, baina lehen 24-48 orduetan *G. mellonella* ereduan larben hilkortasuna handiagoa izan zen. Gu bezala, beste autore batzuk ere ikusi dituzte desberdintasunak heriotza-denboran bi animalia-eredu horien artean, non *G. mellonella* larben heriotza 48 ordu igaro ondoren gertatu zen (Borman et al., 2016; Sherry et al., 2017) eta *C. elegans* ereduan aldiz 48-96 ordu igaro ondoren (Eldesouky et al., 2018; Lima et al., 2020). *C. auris*en birulentzia *C. albicans* espezienarekin alderatu daitekeela eta *C. haemulonii* konplexuko espezieena baino handiagoa dela frogatu da in vitro biofilmen azterketen bidez eta baita in vivo animalia eredu ez konbentzionalak, *G. mellonella* eta *C. elegans* ereduetan alegia, edota eredu murinoak erabiliz ere (Muñoz et al., 2020; Borman et al., 2016; Sherry et al., 2017; Lima et al., 2020). Hala ere, *C. auris* isolamenduen artean birulentzia aldakortasun handia deskribatu da ziurrenik, euren dibertsitate genomikoarekin lotuta (Brown et al., 2020). Gure azterlanean nematodo osasuntsuen %100 bizirik ailegatu ziren saiakuntzen amaierara (120 ordu). Infektatuen heriotza-tasa aldiz, %76,6 eta %92,4 artekoa izan zen, fenotipo agregatzailea duen JMRC:NRZ 1101 isolamenduarekin infektatutako nematodoena izan ezik. JMRC:NRZ 1101 isolamenduak bakarrik nematodoen %47,7a hiltzea lortu zuen 120 ordu ondoren. JMRC:NRZ 1101 isolamenduak agregatuak sortu zituen *C. elegans* zein *G. mellonella* ereduetan. *C. auris* espezieak agregatuak sortzeko duen gaitasuna Borman eta laguntzaileek (2016) deskribatu zuten lehen aldiz *G. mellonella* eredua erabiliz, eta ondoren beste ikertzaile batzuk berretsi zuten (Ben-Ami et al., 2017; Sherry et al., 2017; Muñoz et al., 2020).

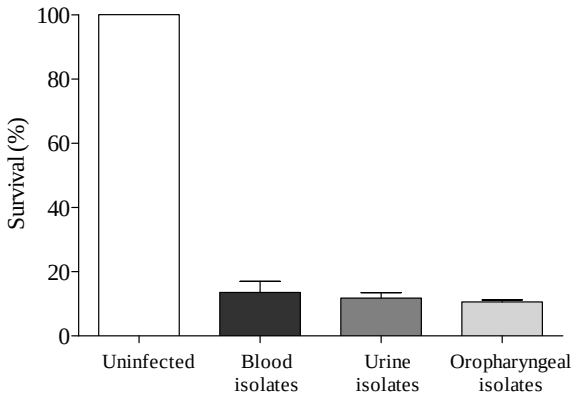
G. mellonella ereduan birulentzia-saiakuntzak egiteko *C. auris* isolamendu bakoitzaren hiru inokulu erabili ziren (1×10^5, 1×10^6 eta 1×10^7 zelula/larba). *G. mellonella*n kontrol-taldeen biziraupena altua izan zen: bai larba osasuntsuen taldean (%85), zein PBS-anpizilinarekin inokulatutako taldean ere (%82,3). Infektatutako larben biziraupena berriz murriztu zen injektatutako inokulua zenbat eta handiagoa izan zenean eta inokuluen artean korrelazio sendoa ikusi zen ($R^2 > 0.87$) (24a Irudia). Biziraupenaren eta injektatutako inokuluaren arteko erlazio hori beste *Candida* espezie batzuetan ikusi da ere (Ames et al., 2017; Mesa-Arango et al., 2013; Scorzoni et al., 2013). Gainera, *G. mellonella* labak 1 x 10^5 zelula/larba inokuluarekin infektatu ondoren isolamenduen jatorrian desberdintasunak ikusi genituen. Izatez, odol-jatorriko isolamenduen birulentzia gernu- eta orofaringe-jatorrikoenak baino adierazgarriki handiagoa izan zen ($p < 0,009$) (24. Irudia). 1 x 10^6 zelula/larba inokuloarekin lortutako biziraupen-emaitzek ere desberdintasunak erakutsi zituzten, fenotipo ez-agregatzailea zuten odol-jatorriko isolamenduak gernu- eta orofaringe-jatorrikoak baino adierazgarriki birulentoagoak izan baitziren.

Inokulu baxuenarekin (1 x 10^5 zelula/larba) infektatutako larben hilkortasuna %60 baino txikiagoa izan zen 48 ordura arte, eta %43,7 eta %83,3 artekoa 120 ordura arte. Inokulu handienarekin aldiz (1×10^7 zelula/larba), larben hilkortasuna %60 baino gehiago izan zen 24 ordu igaro ondoren, eta %98,3 baino gehiago 120 ordu igarota. Azkenik, tarteko inokuluarekin (1 x 10^6 zelula/larba) lortutako emaitzak egokienak izan ziren *C. auris*en birulentzia aztertzeko eta isolamenduen arteko desberdintasunak nabarmentzeko.

a)



b)



24. Irudia: a) *G. mellonella* larben biziraupena 120 ordu igaro ondoren, odol-jatorria, gernu-jatorria eta jatorri orofaringeoa duten hamabi *C. auris* isolamendu klinikoekin infektatuta (1 × 10^5, 1 × 10^6 eta 1 × 10^7 zelula/larba). Barrarik ez egoteak %0ko biziraupena adierazten du. Asteriskoak *C. auris* odol-jatorriko isolamenduetako 1 x 10^5 zelula/larba inokuluarekin infektatutako larbekin estatistikoki esanguratsua den diferentzia adierazten du. b) *C. elegans* nematodoen biziraupena 120 ordu igaro ondoren, odol-jatorria, gernu-jatorria eta orofaringe-jatorria duten hamabi *C. auris* isolamendu klinikoekin infektatuta

2.3. *C. auris* isolamenduen birulentzia laginen jatorri klinikoaren arabera

Jatorri kliniko desberdineko (odol-, gernu- eta orofaringe-laginak) *C. auris* isolamenduen birulentzia aztertu eta konparatu zen *C. elegans* eta *G. mellonella* ereduak erabiliz. Horretarako, 1 x 10^6 zelula/larba inokuluarekin infektatutako *G. mellonella* ereduaren emaitzak erabili genuen. Inokulu hori *C. auris* espeziearekin aurretik egindako ikerketa lan batzuetan erabili zen (Borman et al., 2016; Sherry et al., 2017; Wang et al., 2018; Arias et al., 2020; Romera et al., 2020) eta beste *Candida* espezie batzuen birulentziaren analisirako ere (Gago et al., 2014; Mesa-Arango et al., 2013;Scorzoni et al., 2013).

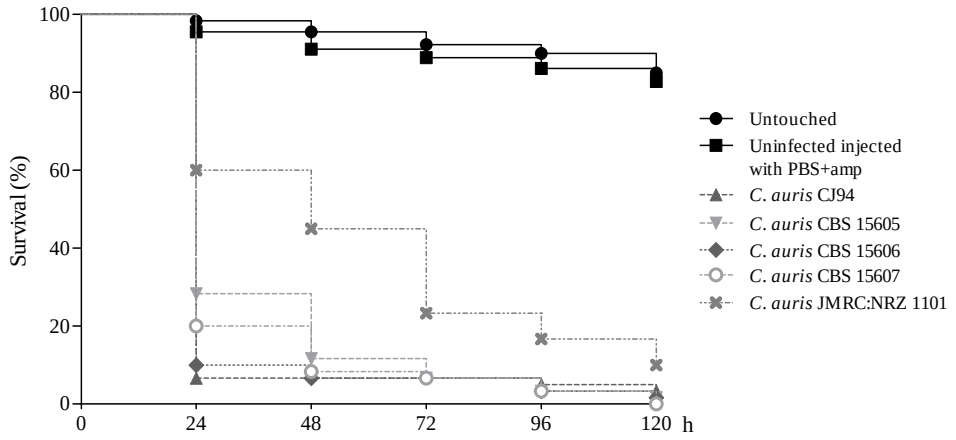
C. auris isolamendu ez-agregatzaileek birulentzia desberdina erakutzi zuten *G. mellonella* ereduan, horien jatorri klinikoaren arabera. Ondoko *C. auris* isolamenduen birulentziaren kategorizazioa ikusi zen: odol-isolamenduak > gernu-isolamenduak > orofaringe-isolamenduak. *G. mellonella* larben biziraupenaren batez bestekoa %1,7koa izan zen odol-jatorriko isolamenduen kasuan, %7,5ekoa gernu-jatorriko isolamenduen kasuan eta %12,5ekoa orofaringe-isolamenduen kasuan (24a Irudia). Lehenago ere ikusi da odol-laginetatik datozen *Candida* isolamenduak are birulentoagoak direla (Oksuz et al., 2007; D'Eça et al., 2011; L`Ollivier et al., 2012; Atalay et al., 2015). *C. elegans* infekzio-ereduan, ordea, ezin izan genuen *C. auris* isolamenduen birulentzia horien jatorri klinikoarekin lotu. *C. auris* isolamendu ez-agregatzaileekin infektatutako nematodoen batez besteko biziraupena oso antzekoa izan baitzen hiru jatorri klinikoetan: %13,6 odol-jatorriko isolamenduetan, %11,8 gernu-jatorriko isolamenduetan eta %10,6 orofaringe-jatorriko isolamenduetan (24b Irudia).

Gainera, esan dezakegu *C. auris* denbora gehiago behar izan zuela *C. elegans* ereduan hilkortasuna sortarazteko *G. mellonella* ereduan baino. Nabarmendu behar dugu legamia inokulua emateko bidea, irensketa (*C. elegans*) eta parenterala (*G. mellonella*), guztiz desberdinak direla bi ereduetan eta infekzioaren garapenean eragina eduki ahal duela.

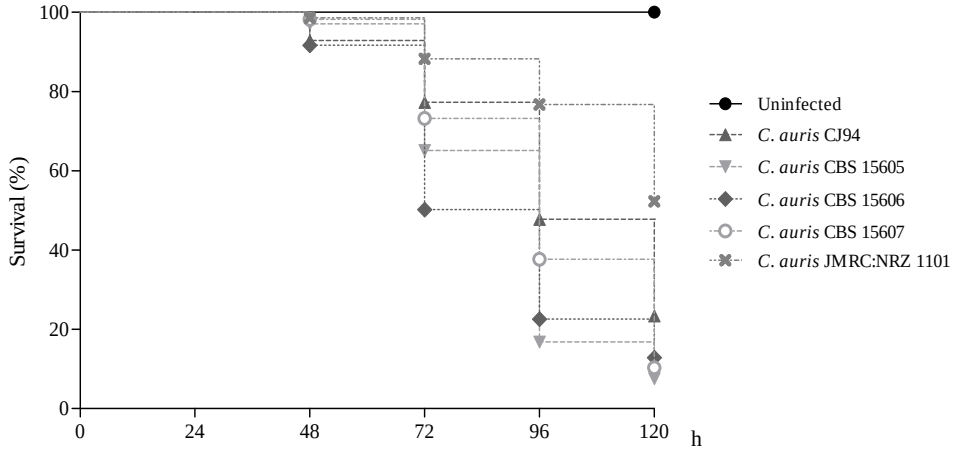
Odol-jatorriko isolamenduekin infektatutako *G. mellonella* larben biziraupena %0-10 artekoa izan zen saiakuntzaren amaieran (120 ordu) eta isolamendu gehienek larben %70etik gorako hilkortasuna eragin zuten 24 ordu igaro ondoren (25. Irudia). CBS15607

odol-isolamenduak larben %100 hil zuen 120 ordu igaro ondoren, baina ez zen desberdintasun adierazgarririk ikusi beste odol-isolamendu ez-agregatzaileekin (25. Irudia). Aitzitik, desberdintasun adierazgarriak hauteman ziren fenotipo ez-agregatzailea duten odol-jatorriko lau isolamenduen eta gainerako isolamenduen artean (gernu- zein orofaringe-jatorriko isolamenduak). *C. elegans* ereduan, berriz, biziraupena %7,6-52,3 artekoa izan zen eta 96 ordu baino gehiago beharrezkoak izan ziren %70ko hilkortasuna sortzeko. CBS15605 isolamenduak hildako nematodoen ehuneko handiena eragin zuen saiakuntzaren amaieran, %92,7 alegia. Kasu honetan, ez zen ikusi desberdintasun adierazgarririk birulentzian CBS15606 odol-isolamenduarekin eta hiru gernu-jatorriko isolamenduekin (CR220, CR440 eta CR14).

a)



b)

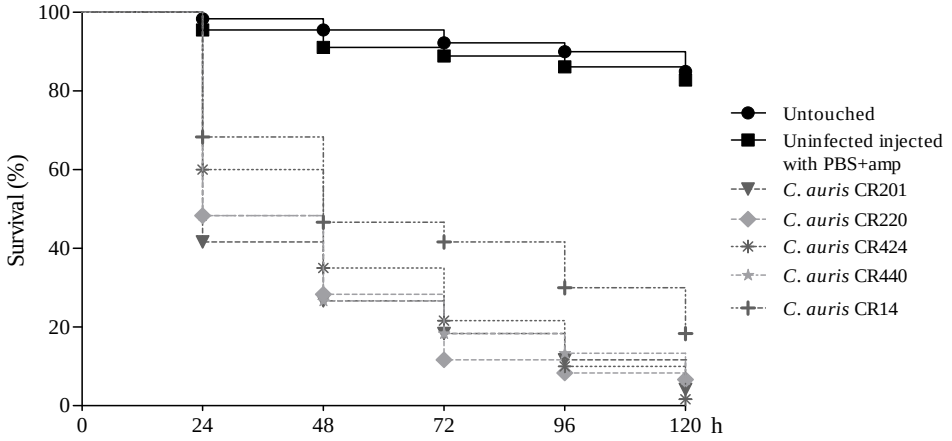


25. irudia: Biziraupen-kurbak, odol-jatorriko *C. auris* isolamenduekin infektatu ondoren a) *G. mellonella* ereduan (1x10^6 zelula/larba inokulua) eta b) *C. elegans* ereduan.

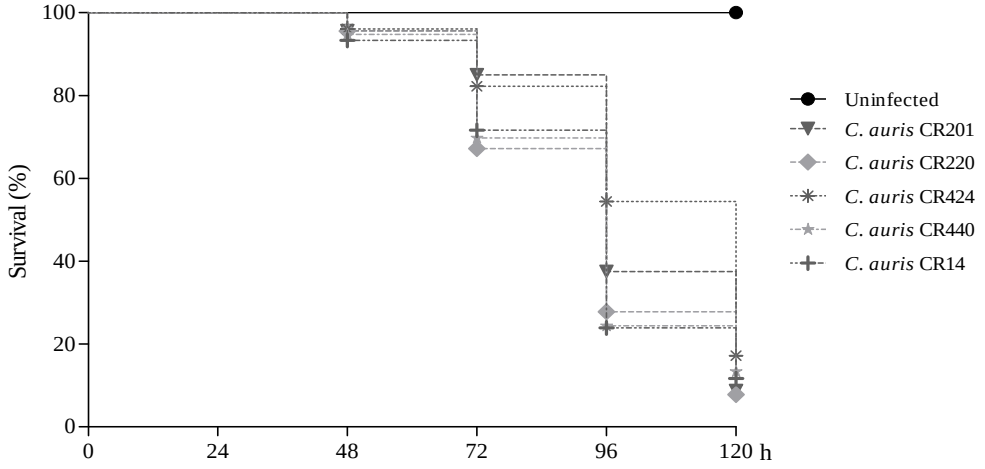
Gernu-jatorriko isolamenduekin infektatutako *G. mellonella* larben biziraupena %1,7-18,3 artean egon zen 120 ordu igaro ondoren. *C. elegans* ereduan, berriz, %7,8-17,2 artean (26. Irudia). Odol-laginetatik isolatutakoekin bezala, gernu-laginetatik lortutako isolamenduek *G. mellonella* ereduan denbora laburragoa behar izan zuten larben %50eko hilkortasuna eragiteko (48 ordu). *C. elegans* ereduan aldiz, gutxienez 96 ordu behar izan zituzten hilkortasun bera sortarazteko. *G. mellonella* ereduan bost gernu-isolamenduetatik lau birulentzia handia erakutsi zuten eta larben hilkortasuna %80-93,3 bitartean egon zen 72-120 ordu igarota (26. Irudia). Haien artean ez ziren egon desberdintasun adierazgarririk, baina bai bestelako *C. auris* isolamenduekin ($p \leq 0,021$). *C. elegans* ereduan gernu-isolamenduetatik, CR220 isolamenduak eragin zuen nematodoen hilkortasuna handiena (%92,2), CBS15605 odol-isolamenduak eragindakoarekin batera ehuneko hilkortasun handienetarikoak izanik. Hala ere, ez zen egon desberdintasun adierazgarririk bi odol-isolamenduen (CBS 15605 eta CBS15606) edota bi gernu-isolamenduen (CR440 eta CR14) birulentzien artean.

Bestalde, orofaringe-jatorriko bi *C. auris* isolamenduek, %6,7-18,3 *G. mellonella* larben biziraupena eragin zuten 120 ordu igaro ondoren eta %60ko hilkortasuna 48 ordu igarota (27. Irudia). *C. elegans* nematodoaren biziraupena, berriz, %10-11,2koa izan zen 120 ordu igaro ondoren eta *C. auris*ek 96 ordu behar izan zituzten %60ko hilkortasuna sortzeko. Ez zen desberdintasun adierazgarririk ikusi bi isolamendu horiek sortarazitako *G. mellonella* eta *C. elegans* hilkortasunaren artean. Orofaringe-jatorriko CR312 isolamenduak larben %93,3 hil zituen 120 ordu ondoren (27. Irudia). Hala ere, isolamendu horrek (CR312) eragin zuen hilkortasun-zinetika birulentzia txikiagoko bi isolamenduen antzekoa izan zen (CR14 gernu-isolamendua eta CR243 orifaringe-isolamendua). Aitzitik, desberdintasun adierazgarriak egon ziren azken bi isolamendu horien (CR14 eta CR243) eta lehen aipatutako birulentoagoak izan ziren lau gernu-jatorriko isolamenduen artean ($p \leq 0,006$ eta $p \leq 0,045$, hurrenez hurren).

a)

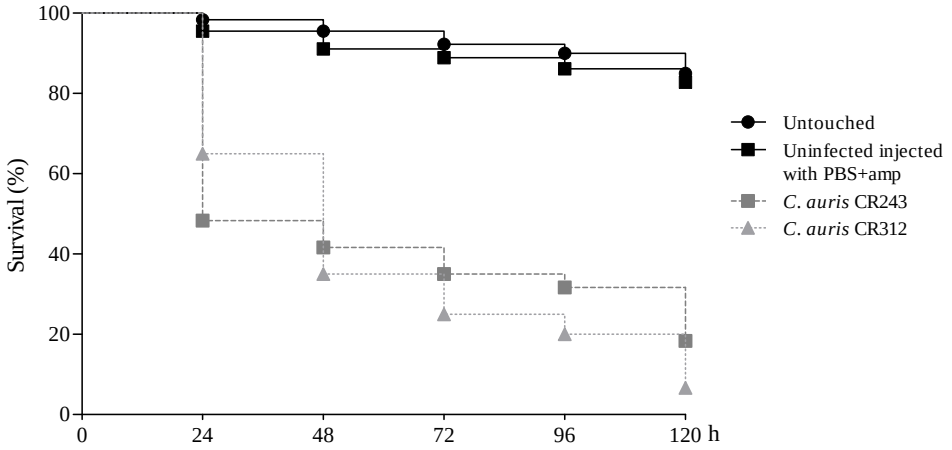


b)

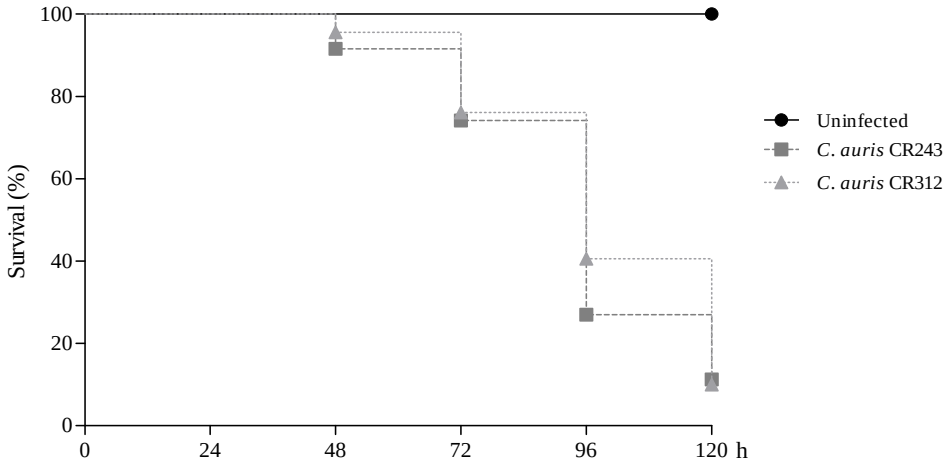


26. Irudia: Biziraupen-kurbak, gernu-jatorriko *C. auris* isolamenduekin infektatu ondoren a) *G. mellonella* ereduan (1 x 10^6 zelula/larba inokulua) eta b) *C. elegans* ereduan.

a)



b)



27. Irudia: Biziraupen-kurbak, orofaringe-jatorriko *C. auris* isolamenduekin infektatu ondoren a) *G. mellonella* ereduan (1 x 10^6 zelula/larba inokulua) eta b) *C. elegans* ereduan.

Fenotipo agregatzailea edo ez-agregatzailea edukitzea *C. auris* espeziearen birulentzian duen eragina aztertu zen ere bi kandidiasi-ereduetan. Odol-jatorriko fenotipo agregatzailea duen JMRC: NRZ 1101 isolamenduak, odol-jatorriko isolamenduen artean, birulentzia txikiena aurkeztu zuen *G. mellonella* ereduan ($p < 0.0001$), eta larben %80ko hilkortasuna eragin zuen 96 ordu igarota. Izan ere, eredu horretan ikusi zen, fenotipo agregatzailea duen isolamendu horren birulentzia (JMRC: NRZ 1101) gernu- eta orofaringe-jatorriko isolamendu gehienekin aldera daitekela. *C. elegans* ereduan aldiz, isolamendu agregatzaile horrek (JMRC: NRZ 1101) %52,3ko heriotza-tasa baino ez zuen eragin saiakuntzaren amaieran (120 ordu). Bere birulentzia nabarmen baxuagoa izan zen gainerako isolamenduen birulentziarekin alderatuta ($p < 0.0001$) (25. Irudia). Beste ikertzaile batzuek ere fenotipo agregatzailea duten isolamenduetan birulentzia txikiagoa ikusi dute (Borman et al., 2016; Sherry et al., 2017). Arias eta laguntzaileek (2020) *G. mellonella* ereduan iradoki zuten *C. auris* isolamendu ez-agregatzaileek gaitasun handiagoa izan zezaketela larben ehunetan mugitzeko eta barreiatzeko, eta gu horrekin bat gatoz. *C. elegans* ereduan, ordea, nematodoek agregatuak irensteko zailtasun handiagoa izan lezakete. Ondorioz, irentsitako karga fungikoa txikiagoa izango litzateke eta garatutako infekzioa arinagoa izatea ekarriko luke.

Oro har, gure emaitzek bi ereduetan frogatu dute *C. auris* espeziearen birulentzia-potentziala handia dela, beste *Candida* espezie batzuen antzekoa edo handiagoa izan zena (Muñoz et al., 2020; Gago et al., 2014; Ortega-Riveros et al., 2017; Hernando-Ortiz et al., 2020; Scorzoni et al., 2013).

3. ONDORIOA

Laburbilduz, azterlan honetan frogatu dugu *C. elegans* eta *G. mellonella* kandidiasi-ereduak egokiak direla *C. auris* isolamenduen birulentzia aztertzeko. Halaber, *G. mellonella* eredua baliagarria izan zen jatorri kliniko desberdineko isolamenduen birulentzian desberdintasunak detektatzeko. Azterlan honetan odol-jatorriko isolamenduek birulentzia handiena erakutsi zuten. Gainera, animalia-eredu biak

baliagarriak izan ziren *C. auris*en isolamendu batek fenotipo agregatzailea edukitzeak bere birulentzian izan dezakeen eragina aztertzeko ere. Hala ere, fenotipo agregatzailea duten *C. auris* isolamendu gehiago aztertu beharko ziren espezie honen birulentzia in vivo ereduetan zehaztasun handiagoz ebaluatzeko.

5. Azterlana (5. Eranskina/Anexo 5)

B anfoterizinaren eta ekinokandinen arteko konbinazioaren eraginkortasuna *Candida auris* espeziearen aurka in vitro eta in vivo *Caenorhabditis elegans* ereduan.

«Effectiveness of the combination amphotericin B with echinocandins against *Candida auris* in vitro and in *Caenorhabditis elegans* host model»

Ainara Hernando-Ortiz, Elena Eraso, Guillermo Quindós eta Estibaliz Mateo

Mikologia Medikoko laborategia, UFI 11/25, Immunologia, Mikrobiologia eta Parasitologia Saila, Medikuntza eta Erizaintza Fakultatea, (UPV/EHU)

Prestaketan dagoen azterlana

Laburpena

Candida auris onddo patogeno multierresistentea da, kandidiasi inbaditzaileen agerraldien arduraduna eta osasun larrialdi bezala deklaratu dena mundu osoan zehar. Aukera terapeutiko gutxi daude patogeno horren aurrean, beraz, farmako antifungiko desberdinen konbinazioak etorkizun handiko alternatiba terapeutikotzat hartzen dira. Animalia-eredu ornogabeak aukera interesgarriak dira, *Caenorhabditis elegans* nematodoa besteak beste, eredu murinoek eragindako murrizketa etikoak gainditzen baitituzte. *Candida*ren aurkako tratamendu antifungikoen eraginkortasuna ebaluatzeko. Lan honen helburua B anfoterizina eta ekinokandinen konbinazioekiko sentikortasuna ebaluatzea izan zen odol-jatorriko *C. auris*en bost isolamendu klinikoen aurka, horietako isolamendu bat fenotipo agregatzailea zuena. Horretarako, azterketak bai in vitro zein in vivo *C. elegans* ereduan egin ziren. In vitro lortutako emaitzetan B anfoterizinaren eta ekinoandinen artean interakzio sinergikoak ikusi ziren *C. auris*en isolamendu gehienekin, baina isolamendu agregatzailearekin MIC balioak altuagoak izan ziren agregatzaileak ez ziren isolamenduekin alderatuta. In vivo, *C. auris*en infekzioa tratatzeko konbinaketa eraginkorrena B anfoterizinaren eta kaspofunginaren artekoa izan zen, *C. elegans*en biziraupena %99raino iritsiz. Aldiz, B anfoterizinaren eta mikafunginaren arteko konbinazioak eraginkortasun txikiena izan zuen. Lortutako emaitzek erakutsi zuten B anfoterizina eta ekinokandinen arteko konbinazioak estrategia aproposa dela odol-jatorriko *C. auris* isolamenduek eragindako kandidiasiaren tratamendurako. Gainera, *C. elegans* ordezko eredua egokia da *C. auris* multierresistenteen infekzioen tratamenduak ikertzeko.

Hitz gakoak: *Candida auris*, patogeno emergenteak, antifungikoen konbinazioa, *Caenorhabditis elegans*, kandidiasia.

Bosgarren azterlanari dagokion artikulua prestaketa prozesuan dago eta 5. eranskinean zehaztuta dago. Ondoren, emaitza nabarmenenen laburpena aurkezten da.

1. HELBURUA

Azterlan honen helburua odol-jatorriko *C. auris* isolamenduen aurka B anfoterizina eta ekinokandinen arteko konbinazioen eraginkortasuna aztertzea zen, bai in vitro zein in vivo *C. elegans* ereduan. Horretarako, in vitro B anfoterizinarekiko, anidulafunginarekiko, caspofunginarekiko eta micafunginarekiko aktibitatea aztertu zen monoterapian eta farmakoen konbinaketan *C. auris* isolamendu horien aurka. In vitro aukeratutako tratamendurik eraginkorrenak in vivo ikertu ziren *C. elegans* eredua erabiliz, eta monoterapiako tratamenduen eraginkortasuna farmakoen konbinaketen tratamenduenarekin alderatu zen *C. auris* eragindako infekzioak tratatzeko

2. EMAITZEN ETA EZTABAIDAREN LABURPENA

2.1. Odol-jatorriko *C. auris* isolamendu klinikoen in vitro sentikortasuna B anfoterizinarekiko eta ekinokandinekiko.

Odol-jatorriko *C. auris*en bost isolamenduen sentikortasuna aztertu zen B anfoterizinarekiko (AmB), anidulafunginarekiko (AND), kaspofunginarekiko (CAS) eta mikafunginarekiko (MCF) bai monoterapian zein horien konbinaketen bidez (11.Taula). *C. auris*aren flukonazolarekiko duen erresistentzia altua da, isolamenduen %90etik %99-100etaraino deskribatu da ikerketa lan batzuetan (Lockhart et al., 2017; Chowdhary et al., 2018; Khan et al., 2018; Ahmad et al., 2020; Zhu et al., 2020). AmB-rekiko erresistentziak ere deskribatu dira, baina datuak %8 eta 43 artean kokatzen dira (Lockhart et al., 2017; Tsay et al., 2017; Chowdhary et al., 2018; Khan et al., 2018; Ninan et al., 2020; Pfaller et al., 2021). Gure azterlanean *C. auris*en isolamendu guztiak AmB-rekiko sentikorrak izan ziren. Ekinokandinekiko, aldiz, soilik fenotipo ez agregatzailea zuten isolamenduak (CJ94, CBS 15605, CBS 15606, CBS 16507) izan ziren sentikorrak.

Fenotipo agregatzailea zuen isolamendua (JMRC:NRZ 1101), berriz, hiru ekinokandinekiko erresistentea izan zen. Nahiz eta bibliografian ekinokandinekiko erresistentzia tasa %4 baino baxuagoa dela deskribatu (Tsay et al., 2017; Chowdhary et al., 2018; Khan et al., 2018; Ninan et al., 2020; Zhu et al., 2020), ekinokandinekiko MIC balio altuak ere deskribatu dira. Hala nola, Erresuma Batuan egondako agerraldi baten ikerketa epidemilogikoan *C. auris* isolamendu bat deskribatu zen hiru ekinokandinekiko erresistentea zena (Rhodes et al., 2018). Beste hainbat ikerketa klinikoetan ekinokandinekiko erresistenteak diren *C. auris* isolamenduak ere deskribatu dira (Biagi et al., 2019; Woodworth et al., 2019). Gainera, azolekiko, AmB-rekiko eta ekinokandinekiko erresistenteak diren isolamendu klinikoak deskribatu dira, isolamendu pan-erresistenteak. Ekinokandinak *C. auris* espeziearen infekzioak tratatzeko aukeratzen den tratamendua dela kontuan hartuta, isolamendu pan-erresistenteak agertzeak kandidiasiaren tratamenduaren arazoa areagotu dezake (Lockhart et al., 2019; Ostrowsky et al., 2020). Horregatik, *C. auris* espeziearen aurkako farmako antifungiko eta tratamendu eraginkorrak lortzea gaur eguneko helburua da (Bruno et al., 2020). Kasu kliniko desberdinetan deskribatu den bezala, antifungikoen konbinazioaren bidezko tratamenduak aukera aproposa dira *C. auris* espezieak eragindako infekzioen aurka; besteak beste, AmB eta ekinokandinen arteko konbinazioa (Ruiz-Gaitán et al., 2017; Chamdramati et al., 2020; Ostrowsky et al., 2020) edo isabukonazola eta ekinokandinen artekoa (Mulet Bayona et al., 2020).

11. Taula: B anfoterizina (AmB) eta ekinokandinen (anidulafungina (AND), kaspofungina (CAS) eta mikafungina (MCF)) in vitro aktibitate antifungikoa odol-jatorriko bost *C. auris* isolamendu klinikoen aurka

C. auris isolamenduaren erreferentzia	Jatorria	Fenotipoa	MIC_0(µg/ml)[a]	MIC_1(µg/ml)[b]		
			AmB	AND	CAS	MCF
CJ94	Hospital La Fe (Espainia)	Ez agregatzailea	0,5	0,25	0,25	0,12
CBS 15605	Hospital La Fe (Espainia) Westerdijk Fungal Biodiversity Institute	Ez agregatzailea	0,5	0,12	0,25	0,12
CBS 15606	Hospital La Fe (Espainia) Westerdijk Fungal Biodiversity Institute	Ez agregatzailea	0,5	0,12	0,25	0,12
CBS 15607	Hospital La Fe (Espainia) Westerdijk Fungal Biodiversity Institute	Ez agregatzailea	0,5	0,12	0,25	0,12
JMRC:NRZ 1101*	Institut für Hygiene und Mikrobiologie (Alemania) Jena Microbial Resource Collection	Agregatzailea	1	4	>8	4

a→ MIC_0: Hazkuntza zelularraren %90 murrizten duen kontzentrazio minimo inhibitzailea
b→ MIC_1: Hazkuntza zelularraren %50 murrizten duen kontzentrazio minimo inhibitzailea
*→ Fenotipo agregatzailea

Gure azterlanean AmB eta ekinokandinen arteko konbinazioen aktibitatea in vitro aztertzeko xake taularen metodologia jarraitu zen. Lortutako emaitzak 12. Taulan laburtuta agertzen dira. Aztertutako hiru konbinaketek elkarrekintza sinergikoa erakutsi zuten *C. auris* isolamenduen aurka, AmB eta MCFren arteko konbinazioa izan ezik; horrek fenotipo agregatzailea zuen isolamenduaren (JMRC: NRZ 1101) aurka elkarrekintza gehigarria erakutsi baitzuen. Oro har, MIC balio baxuagoak lortu ziren konbinazioetan monoterapian baino, eta AmB eta MCFren konbinazioa aktiboena izan zen *C. auris* isolamenduen aurka. Antifungikoen arteko konbinaketen eraginkortasuna *C. auris* espeziearen aurka ikerketa lan desberdinetan aztertu da (Valentín et al., 2016; Bidaud et al., 2019; Bidaud et al., 2020; Caballero et al., 2021; Fakhim et al., 2017; O´Brien et al., 2020; Pfaller et al., 2021; Schwarz et al., 2020; Wu et al. 2020). Era berean, ekinokandinen eta hainbat antifungikoen arteko konbinazioak *Candida* espezie desberdinen aurka aztertu dira (Oliveira et al., 2005; Nishi et al., 2009; Tobudic et al., 2010; Chaturvedi et al., 2011; Valentín et al., 2016).

12. Taula: B anfoterizina (AmB) eta ekinokandinen (anidulafungina (AND), kaspofungina (CAS) and mikafungina (MCF)) arteko konbinazioen in vitro aktibitate antifungikoa odol-jatorriko bost *C. auris* isolamendu klinikoen aurka

	MIC_0 (µg/ml)					MIC_0 (µg/ml)					MIC_0 (µg/ml)				
***C. auris* isolamendua**	**AmB**	**AND**	**AmB/ AND**	**FICI**	**E***	**AmB**	**CAS**	**AmB/ CAS**	**FICI**	**E***	**AmB**	**MCF**	**AmB/ MCF**	**FICI**	**E***
CJ94	0,5	>8	0,03/1	0,122	S	0,5	>8	0,03/1	0,122	S	0,5	>8	0,03/0,25	0,076	S
CBS 15605	0,5	>8	0,03/0.5	0,091	S	0,5	>8	0,03/0.5	0,091	S	0,5	>8	0,03/0,5	0,091	S
CBS 15606	0,5	>8	0,06/2	0,245	S	0,5	>8	0,06/1	0,128	S	0,5	>8	0,06/2	0,245	S
CBS 15607	0,5	>8	0,03/1	0,122	S	0,5	>8	0,03/1	0,122	S	0.5	>8	0,06/0,5	0,151	S
JMRC:NRZ 1101	1	>8	0,25/2	0,375	S	1	>8	0,25/4	0,5	S	1	>8	0,5/0,5	0,562	Ad
GM	0,574	>8	0,054/ 1,148	-	-	0,574	>8	0,054/ 1,148	-	-	0,574	>8	0,071/ 0,574	-	-
Tartea	0,5-1	>8	0,03-0,25/ 0,5-2	-	-	0,5-1	>8	0,03-0,25/ 0,5-4	-	-	0,5-1	>8	0,03-0,5/ 0,25-2	-	-

FICI (fractional inhibitory concentration index)
*Elkarekintzaren efektua: S= Sinergikoa; Ad= Gehigarria interaction.
GM (Batezbesteko geometrikoa)
MIC_0 (Hazkuntza zelularraren %90 murrizten duen kontzentrazio minimo inhibitzailea)

2.2. B anfoterizina eta ekinokandinen arteko konbinazioen eragina *C. elegans* kandidiasi ereduan

C. elegans in vivo eredua erabili zen odol-jatorriko *C. auris*en bost isolamendu klinikoek eragindako kandidiasiaren aurkako tratamenduaren eraginkortasuna ebaluatzeko. *C. elegans* ereduaren erabilgarritasuna *C. auris* espeziea aztertzeko jada deskribatu da (Eldesouky et al.2018; Lima et al., 2020; Hernando-Ortiz et al., 20201). Dakigunez, animalia-eredu hori orain arte ez da erabili AmB eta ekinokandinen konbinazioen eraginkortasuna aztertzeko *C. auris* espezieak eragindako infekzioen aurka. Hala ere, beste farmako desberdinen konbinazioak bai aztertu dira, hala nola, azolen konbinazioa sulfamethoxazolarekin, lopinavir GIB proteasen inhibitzailearekin, aprepitant agente antiemetikoarekin edota ospemifene konposatu estilbenikoarekin (Eldesouky et al., 2018; 2020a; 2020b; 2020c). Gure azterlanean in vivo ereduan frogatutako farmakoen kontzentrazioak in vitro azterketetan lortutako emaitzak kontuan hartuta izan ziren (12. Taula). *C. elegans* nematodoen biziraupenen emaitzak 28-32. Irudietan laburtuta daude.

Infektatutako nematodoen biziraupena ez zen adierazgarriki areagotu monoterapian erabilitako AmB tratamenduarekin, infektatutako eta tratatu gabeko nematodoekin alderatuta. Baina, AmB eta AND edo AmB eta CAS konbinazio tratamenduek *C. auris* isolamendu guztiek eragindako infekzioaren aurkako babesa erakutsi zuten, nematodoen biziraupena adierazgarriki areagotu baitzen (28-32 Irudiak). AmB eta MCF konbinazioak aldiz, soilik CBS 15606 eta CBS 15607 isolamenduek eraginkako infekzioen aurka babestu zituen nematodoak, infektatu eta tratatu gabeko nematodoekin alderatuta (30-31 Irudiak).

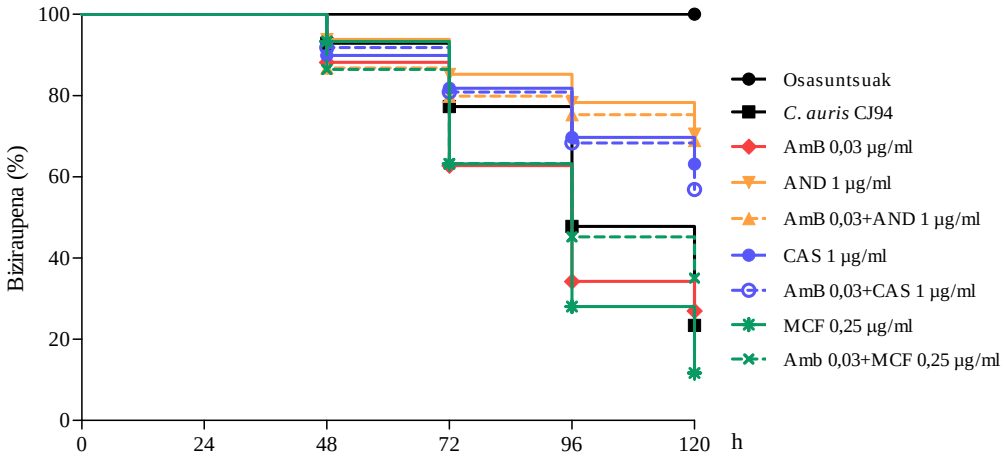
AmB eta CAS konbinazioarekin nematodoen biziraupena %56,8-99 tartean egon zen eta nematodoen hilkortasunaren batez besteko murrizketa %53,48koa izan zen. *C. auris* isolamendu agregatzaileak (JMRC:NRZ 1101) eta hiru isolamendu ez-agregatzaileek (CBS 15605, CBS 15606, CBS 15607) eragindako infekzioaren aurka AmB eta CAS konbinazioa tratamendu eraginkorrena izan zen (29-32. Irudiak). Izan ere, hilkortasunaren murrizpena %46,7 eta 69,6 artean egon zen. CAS monoterapiarekin aldiz nematodoen

hilkortasuna %9,4 eta 56,9 artean murriztu zen, konbinaketarekin lortutako biziraupen emaitzak adierazgarriki hobeagoak izanda ($p \leq 0,02$). *C. auris*en CJ94 isolamenduak eragindako infekzioaren aurka ez zen desberdintasun adierazgarririk behatu konbinaketa eta monoterapia emaitzak alderatzean, biziraupena %33,4 eta %39,7 handitu baitzen, hurrenez hurren (28. Irudia).

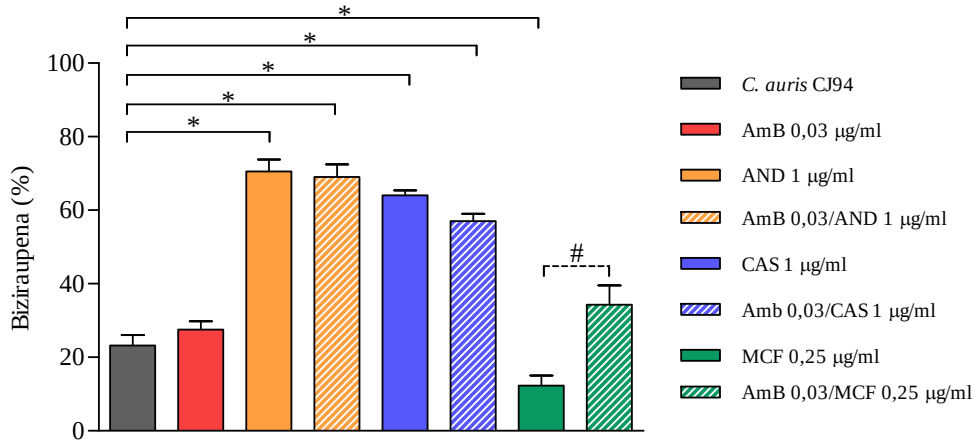
AmB eta AND konbinazioa adierazgarriki hobeto babestu zituen *C. auris* CBS 15605 eta CBS 15607 isolamenduekin infektatutako nematodoak, monoterapiarekin alderatuta ($p \leq 0,032$) (29 eta 31. Irudiak). Gainerako *C. auris* isolamenduen aurka aldiz, eragin bera izan zuten konbinaketa tratamenduak eta monoterapiakoak. AmB eta AND konbinazioaren eraginkortasuna Ruiz-Gaitán eta laguntzaileek (2017) deskribatutako kasu klinikoan frogatu zuten *C. auris* eragindako infekzioaren aurka.

AmB eta MCF konbinazioak eraginkortasun baxuena izan zuen *C. elegans* ereduan. Nematodoen biziraupena %68,7raino igo zen eta horien hilkortasunaren murrizketa ez zen %17tik gorakoa izan *C. auris*en lau isolamenduek (CJ94, CBS 15605, CBS 15607, JMRC:NRZ 1101) eragindako inkekzioen kasuan. Izan ere, soilik CBS 15607 isolamenduak eragindako infekzioak monoterapiarekin tratatu zirenean desberdintasun adierazgarriak behatu ziren *C. elegans* nematodoen biziraupenean, infektatu eta tratatu gabeko nematodoekin alderatuta ($p < 0,001$). *C. auris*en CBS 15606 isolamenduarekin infektatutako nematodoen kasuan, animalien biziraupenak %55,8ra eta %59,9ra igo ziren farmako horien konbinaketarekin eta monoterapiarekin, hurrenez hurren, eta ez ziren desberdintasun adierazgarriak behatu horien artean. Chamdramati eta laguntzaileek (2020) egindako atzera begirako ikerketan deskribatu zuten jaioberrietan *C. auris* eragindako infekzioaren aurka AmB eta MCF konbinazioaren eraginkortasuna, horien biziraupena %83 areagotu baitzen.

a)

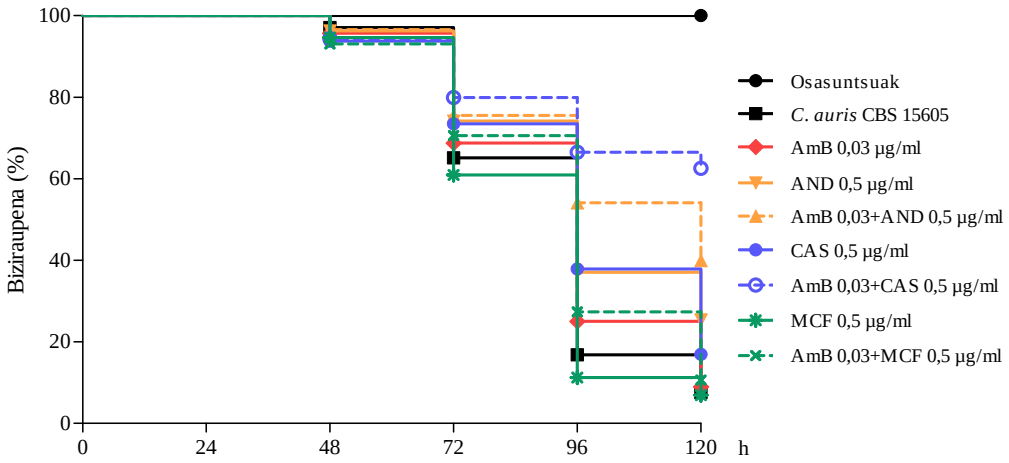


b)

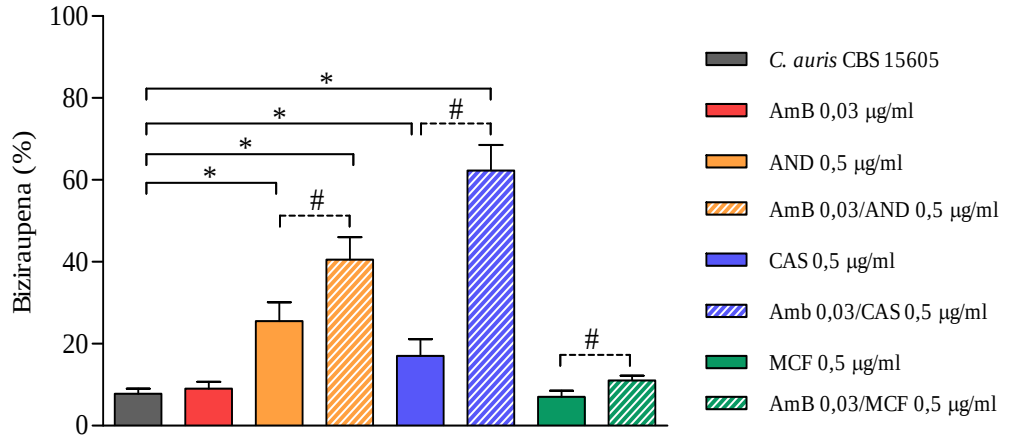


28. Irudia: *C. auris* CJ94 isolamenduarekin infektatutako *C. elegans*en biziraupen-kurbak (a) eta 120 ordu igaro ondoren lortutako biziraupenaren portzentaia (b). Estatistikoki adierazgarriak diren desberdintasunak infektatutako eta tratatu gabeko nematodoekin alderatuta (*) eta infektatutako eta dagozkien ekinokandina monoterapiarekin tratatutako nematodoekin alderatuta (#)

a)

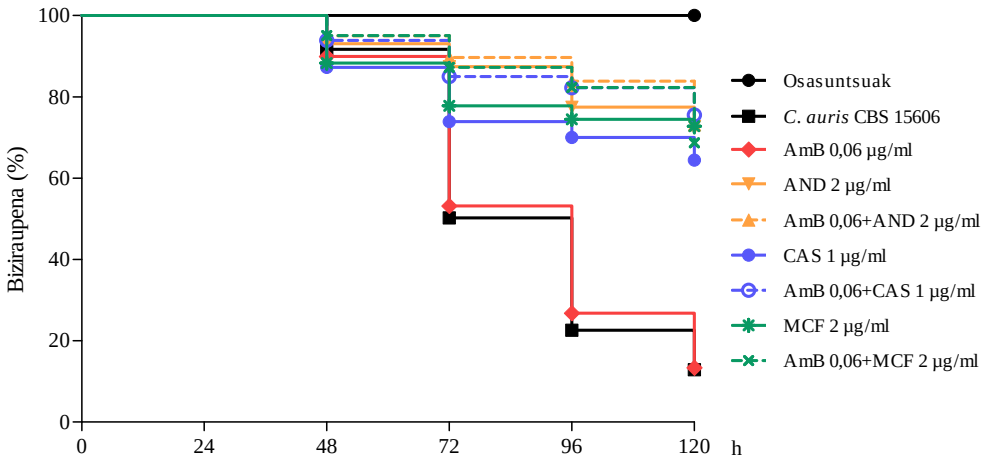


b)

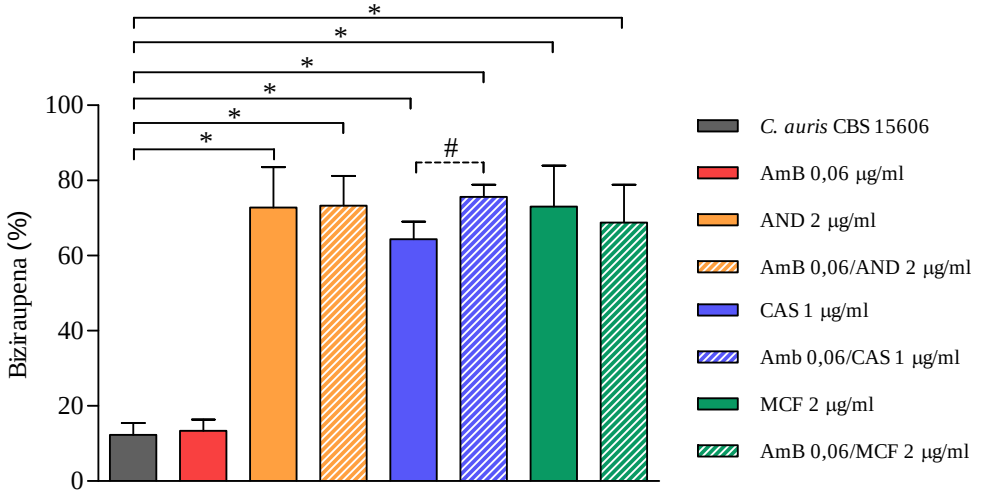


29. Irudia: *C. auris* CBS 15605 isolamenduarekin infektatutako *C. elegans*en biziraupen-kurbak (a) eta 120 ordu igaro ondoren lortutako biziraupenaren portzentaia (b). Estatistikoki adierazgarriak diren desberdintasunak infektatutako eta tratatu gabeko nematodoekin alderatuta (*) eta infektatutako eta dagozkien ekinokandina monoterapiarekin tratatutako nematodoekin alderatuta (#)

a)

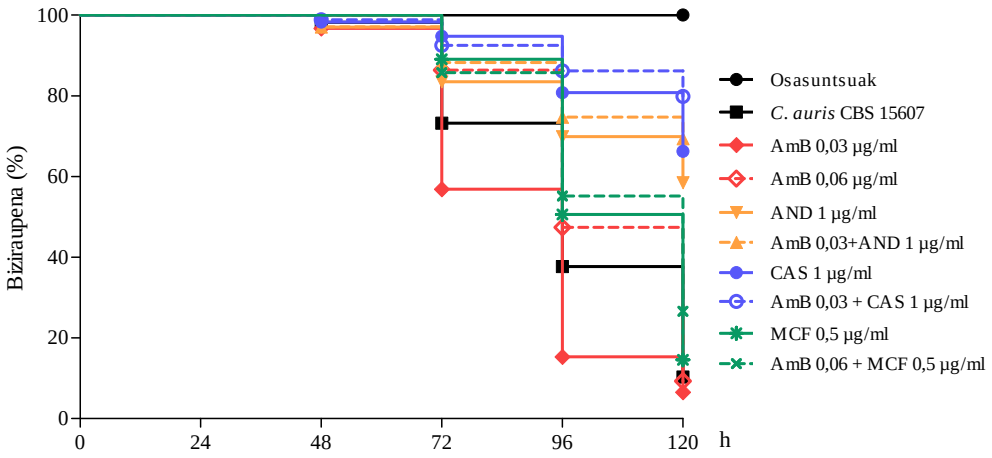


b)

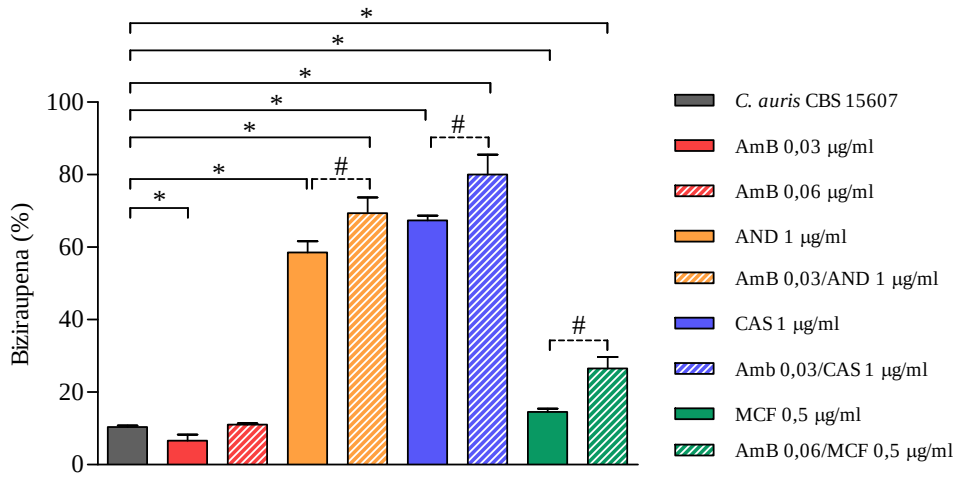


30. Irudia: *C. auris* CBS 15606 isolamenduarekin infektatutako *C. elegans*en biziraupen-kurbak (a) eta 120 ordu igaro ondoren lortutako biziraupenaren portzentaia (b). Estatistikoki adierazgarriak diren desberdintasunak infektatutako eta tratatu gabeko nematodoekin alderatuta (*) eta infektatutako eta dagozkien ekinokandina monoterapiarekin tratatutako nematodoekin alderatuta (#)

a)

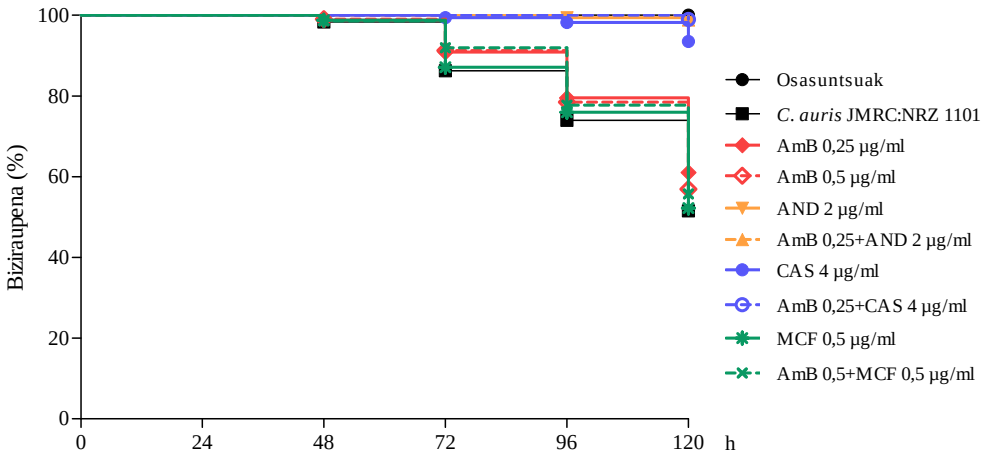


b)

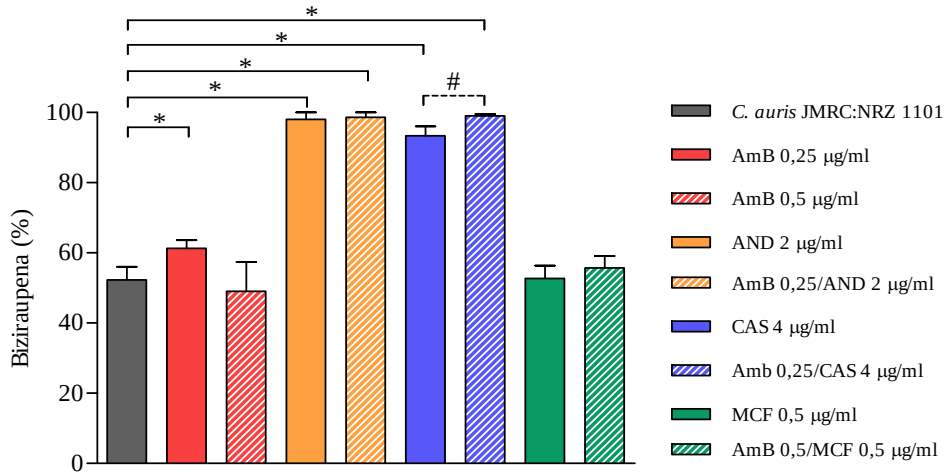


31. Irudia: *C. auris* CBS 15607 isolamenduarekin infektatutako *C. elegans*en biziraupen-kurbak (a) eta 120 ordu igaro ondoren lortutako biziraupenaren portzentaia (b). Estatistikoki adierazgarriak diren desberdintasunak infektatutako eta tratatu gabeko nematodoekin alderatuta (*) eta infektatutako eta dagozkien ekinokandina monoterapiarekin tratatutako nematodoekin alderatuta (#)

a)



b)



32. Irudia: *C. auris* JMRC:NRZ 1101 isolamenduarekin infektatutako *C. elegans*en biziraupen-kurbak (a) eta 120 ordu igaro ondoren lortutako biziraupenaren portzentaia (b). Estatistikoki adierazgarriak diren desberdintasunak infektatutako eta tratatu gabeko nematodoekin alderatuta (*) eta infektatutako eta dagozkien ekinokandina monoterapiarekin tratatutako nematodoekin alderatuta (#)

3. ONDORIOA

Ikerketa lan honetan AmB eta ekinokandinen arteko konbinaketen efektu sinergikoa *C. auris* espeziearen aurka frogatu zen, bai in vitro zein in vivo *C. elegans* ereduan. Fenotipo agregatzailearen isolamenduak MIC balio altuagoak erakutsi zituen in vitro, bai monoterapian bai farmakoen konbinazioetan, isolamendu ez-agregatzaileekin alderatuta. In vivo saiakuntzetan, aztertutako hiru konbinazioetatik, AmB eta CAS konbinazioa eraginkorrena izan zen nematodoen hilkortasuna murrizteko. AmB eta MCF konbinazioak aldiz, eraginkortasun baxuena erakutsi zuen.

6. EZTABAIDA OROKORRA

6. Eztabaida orokorra/Discusión

Candida es el principal agente etiológico de las micosis invasivas. La incidencia de las candidiasis invasivas ha experimentado un aumento en los últimos años. En algunos países, es la cuarta causa de las infecciones nosocomiales invasivas que se diseminan por el torrente sanguíneo y la tercera causa más común de estas en la UCI (Lamoth et al., 2018; Pais et al., 2019). Estas infecciones se relacionan con estancias prolongadas en el hospital y una alta morbilidad y mortalidad, siendo superior esta última al 30% (Cornistein et al., 2013; Quindós et al., 2018; Raja et al., 2020).

La epidemiología de las candidiasis varía en función de la región geográfica e, incluso, entre hospitales, pero en la mayoría de los casos, *Candida albicans* es la causa más común de candidiasis invasiva (Sadeghi et al., 2018). Sin embargo, otras especies como *Candida glabrata*, *Candida parapsilosis*, *Candida krusei*, *Candida tropicalis* y, la de más reciente aparición, *Candida auris*, se han convertido en patógenos oportunistas importantes, y pueden aislarse en hasta dos tercios de los pacientes con candidemia (Borman et al., 2019; Falces-Romero et al., 2020). De hecho, la tendencia que se observa es un decrecimiento de las candidiasis por *C. albicans* y un aumento de las causadas por *C. parapsilosis* y *C. glabrata* (Hou et al., 2017; Lamoth et al., 2018).

Concretamente, *C. glabrata* es la segunda causa de candidemia en EE.UU., Canadá, Australia y norte y este Europa, ocupando el tercer lugar en los países del sur de Europa, como España (Puig-Asensio et al., 2014; Sadeghi et al., 2018; Quindós et al., 2018). Además, la mortalidad asociada a la candidiasis invasiva por *C. glabrata* puede ser superior a la de *C. albicans* (Ryan et al., 2019). Muchos autores relacionan el incremento mundial de esta especie con la profilaxis realizada con fluconazol, ya que muchos aislamientos clínicos de *C. glabrata* tienen sensibilidad reducida o son resistentes a este fármaco (Whaley and Rogers, 2016; Astvad et al., 2018; Healey et al., 2017; Gale et al., 2020). Aunque la proporción de aislamientos de *C. glabrata* resistentes al fluconazol varía, se puede decir que en términos generales está entre el 8 y el 10% (Quindós et al., 2018). Esta resistencia genera problemas en los tratamientos de las candidiasis, ya que el

fluconazol es uno de los fármacos antifúngicos elegidos preferentemente para tratar muchas candidiasis, por ser barato, con toxicidad limitada y estar disponible para su administración oral e intravenosa (Falces-Romero et al., 2020).

Las especies *Candida nivariensis* y *Candida bracarensis* están filogenéticamente muy relacionadas con *C. glabrata* y no se pueden diferenciar por las técnicas fenotípicas utilizadas en los laboratorios. Además, el conocimiento sobre la virulencia y sensibilidad antifúngica de estas dos especies es limitado (Lopez-Soria et al., 2013; Aznar-Marin et al., 2016; Astvad et al., 2017).

Por otra parte, *C. auris* es un patógeno emergente con una alta capacidad para desarrollar multirresistencia a los fármacos antifúngicos, especialmente resistencia al fluconazol, y un elevado potencial de transmisión nosocomial. Además, su virulencia es similar a la de *C. albicans* y se ha descrito una mortalidad del 30-50% en las infecciones invasivas causadas por *C. auris* (Arastehfar et al., 2018; Colombo et al., 2017; Lockhart et al., 2017). Uno de los principales factores relacionados con la adquisición de una infección por *C. auris* es la estancia en UCI o en unidades de reanimación dentro de los cuidados postoperatorios. Hay factores que facilitan que *C. auris* colonice y persista en el entorno nosocomial, entre los que destacan su capacidad para adherirse a las superficies de diferentes materiales, su potencial para formar biopelículas o la tolerancia a la sal (Ademe y Girma, 2020). Un factor relevante en *C. auris* es el fenotipo agregante observado por primera vez por Borman et al. (2016) en algunos aislamientos clínicos, que se asocia con una menor virulencia pero con una mayor resistencia a los fármacos antifúngicos (Borman et al., 2016; Hernando-Ortiz et al., 2021). Sin embargo, no hay unas condiciones clínicas concretas asociadas a la infección por *C. auris*, por lo que diferenciar la infección causada por esta especie de otras infecciones sistémicas es difícil (Cortegiani et al., 2018). Debido a esto, es necesaria la identificación rápida de *C. auris* para aplicar precozmente tratamientos antifúngicos apropiados y evitar esta alta tasa de mortalidad asociada. No obstante, al igual que en el caso de las tres especies de *Candida* antes citadas, los métodos fenotípicos de identificación no son suficientes para la correcta identificación de *C. auris*. Por ello, se necesitan métodos moleculares para identificar la

especie *C. auris* y diferenciarla de especies cercanas como *Candida haemulonii, Candida duobushaemulonii* o *Candida lusitaniae* (Kordalewska et al., 2017).

El estudio de las especies emergentes de *Candida*, como *C. glabrata*, *C. nivariensis*, *C. bracarensis* y *C. auris*, permite tener un mejor control de las infecciones que causan, mejorar en su diagnóstico y en la elección del tratamiento antifúngico más adecuado. El conocimiento de estas especies incluye la caracterización de los perfiles de virulencia y el análisis de la sensibilidad a los fármacos antifúngicos tanto in vitro como in vivo. Este trabajo de Tesis doctoral se plantea bajo la hipótesis de que los modelos alternativos de candidiasis invasora en *C. elegans* y *G. mellonella* son modelos in vivo apropiados para conocer el potencial de virulencia de las especies emergentes de *Candida* y la eficacia de los tratamientos antifúngicos contra las infecciones causadas por estas.

Para demostrar esta hipótesis se analizó inicialmente la producción in vitro de los factores de virulencia de estos patógenos: la producción de fosfolipasas y proteasas, la actividad hemolítica y la capacidad para formar biopelículas. Los ensayos realizados mostraron que ninguna de las cepas de *C. glabrata*, *C. nivariensis* o *C. bracarensis* producían fosfolipasas ni proteasas. Sin embargo, en todas ellas se observó actividad alfa-hemolítica (hemólisis parcial), excepto en la cepa *C. glabrata* ATCC 90030 que mostró una actividad gamma-hemolítica (sin hemolisis). Entre los numerosos estudios sobre *C. glabrata* algunos autores tampoco refieren producción de fosfolipasas, proteasas o actividad hemolítica en los aislamientos de *C. glabrata* que analizan (Ortega-Riveros et al., 2017; Kaan et al., 2021). En otros trabajos, sin embargo, esta especie es capaz de presentar una actividad hemolítica y de secretar proteasas pero no fosfolipasas (Ghannoum et al., 2000; Seneviratne et al., 2011; Figueiredo-Carvalho et al., 2017; Subramanya et al., 2017; Kalaiarasan et al., 2018; Lotfalikhani et al., 2018).

Respecto a las especies *C. nivariensis* y *C. bracarensis* existe un número menor de estudios sobre sus factores de virulencia. Entre ellos, Figueiredo-Carvalho et al. (2016) describieron que *C. nivariensis* no era productora de fosfolipasas ni de hemolisinas. Moreira et al. (2015) observaron que los aislamientos de *C. bracarensis* producían

proteasas y mostraban actividad hemolítica. También Treviño-Rangel et al. (2018) detectaron la capacidad de producir tanto fosfolipasas como proteasas y de presentar actividad hemolítica en un aislamiento de *C. bracarensis* causante de candidiasis vulvovaginal (Treviño-Rangel et al., 2018).

En el caso de *C. auris* se han descrito aislamientos productores de enzimas hidrolíticas o actividad hemolítica. Kumar et al. (2015) publicaron el caso de un aislamiento de *C. auris* proveniente de una paciente con vulvovaginitis que era productor de fosfolipasas, proteasas y que desarrollaba actividad hemolítica. Shaban et al. (2020) encontraron que el 96% de los aislamientos de *C. auris* secretaban proteasas, pero ninguno producía fosfolipasas; mientras que Larkin et al. (2017) observaron que la producción de fosfolipasas y proteasas era dependiente de cepa, y que el 37,5% y el 64% de las cepas eran productoras de fosfolipasas y proteasas, respectivamente. En nuestro trabajo los 12 aislamientos clínicos de *C. auris* analizados no mostraron producción de fosfolipasas o proteasas ni actividad hemolítica.

Existen varios estudios donde se comparan diferentes protocolos para analizar la producción de enzimas como las fosfolipasas y en los que se destacan las limitaciones en su detección (Echeverría et al., 2002; Taniguchi et al., 2009). Por lo tanto, sería conveniente profundizar en la investigación de estos factores de virulencia para conocer mejor el grado de patogenicidad de las especies patógenas de *Candida*.

Entre los estudios de virulencia de *Candida* se encuentra el análisis de la capacidad de adherirse a superficies, tanto bióticas como abióticas, y de formar biopelículas. En la especie *C. glabrata* las proteínas Epa y otras adhesinas juegan un papel importante tanto en la adhesión como en la formación de biopelículas en diferentes sustratos (Weig et al., 2004; de Groot et al., 2008; Gomez-Molero et al., 2015). *C. glabrata* tiene una alta capacidad para formar biopelículas en catéteres tanto urinarios como vasculares, en válvulas protésicas y en marcapasos que lleva a la necesidad de tener que retirarlos para la correcta recuperación del paciente, hecho que prolonga la estancia en el hospital (Timmermans et al., 2018). La formación de biopelículas está relacionada con la

protección del agente patógeno frente al sistema inmunitario del hospedador y a los tratamientos antifúngicos (Gabaldón et al., 2013; Tam et al., 2015; d'Enfert et al., 2016; Rodrigues et al., 2016; Gabaldón & Fairhead, 2019; Galocha et al., 2019; Kumar et al., 2019; Valotteau et al., 2019; Widiasih Widiyanto et al., 2019). En el caso de *C. auris*, la formación de biopelículas también se ha relacionado con una disminución en la eficacia de los tratamientos antifúngicos contra las candidiasis causada por esta especie que, de por sí, presenta multirresistencias (Dominguez et al., 2019; Romera et al., 2019; Kean et al., 2020). Diferentes estudios han demostrado que *C. auris* tiene una capacidad similar a la de *C. glabrata* para generar biopelículas, e incluso que algunos de los aislamientos clínicos de *C. auris* son capaces de producir una biomasa comparable a la de los aislamientos de *C. albicans* (Sherry et al., 2017; Romera et al., 2019). Además, los aislamientos de *C. auris* con fenotipo no agregante se han descrito como mayores productores de biopelículas que los de fenotipo agregante (Sherry et al., 2017). Sin embargo, en este trabajo de Tesis doctoral se ha observado lo contrario, puesto que el aislamiento de *C. auris* con fenotipo agregante (JMRC:NRZ 1101) mostró una mayor actividad metabólica y una mayor producción de biomasa que el resto de aislamientos con fenotipo no agregante. Esta diferencia de resultados puede deberse a la diversidad genética descrita entre los diferentes clados de *C. auris* y a la heterogeneidad que esta especie presenta en relación a su patogenia (Brown et al., 2020). Como ya se ha mencionado en el estudio 4 (Anexo 4), es necesario realizar más estudios utilizando aislamientos de *C. auris* entre los que se incluyan aislamientos con fenotipo agregante y provenientes de diferentes clados geográficos, para evaluar con mayor precisión la virulencia de esta especie.

Para analizar la virulencia de *Candida*, además de las técnicas in vitro, es necesario utilizar modelos in vivo ya que nos ofrecen mayor información gracias a la posibilidad de estudiar las interacciones entre el hospedador y el patógeno. Los modelos animales más utilizados para los estudios de las candidiasis invasivas siguen siendo los vertebrados, principalmente los ratones, en los que se han estudiado diversas presentaciones clínicas, entre las que destacan las infecciones orales o vaginales, gastrointestinales y sistémicas o

diseminadas (Segal and Frenkel, 2018). El modelo de candidiasis invasiva en ratón se ha utilizado ampliamente para estudiar los mecanismos de patogenicidad o para comparar la virulencia de diferentes especies de *Candida* o de cepas dentro de una misma especie y para el análisis de la eficacia del tratamiento contra la infección (Frenkel et al., 2016; De Bernardis et al., 2018; Fakhim et al., 2018; Hirayama et al., 2020; Muñoz et al., 2020). Sin embargo, debido a las restricciones éticas y a la necesidad de instalaciones adecuadas para su mantenimiento, el uso de estos modelos de candidiasis en animales vertebrados se está viendo limitado. Es por ello que los modelos alternativos de candidiasis en los animales invertebrados están adquiriendo una relevancia especial, siendo los más utilizados la mosca de la fruta *Drosophila melanogaster*, la larva de la polilla grande de la miel *Galleria mellonella* y el nematodo *Caenorhabditis elegans*.

Concretamente, los modelos de candidiasis *C. elegans* y *G. mellonella* ofrecen interesantes ventajas como su fácil manejo en el laboratorio, y poder observar y seguir fácilmente la evolución de la infección producida por el patógeno. En el caso del nematodo *C. elegans* su pequeño tamaño permite el almacenamiento de un gran número de individuos en un espacio pequeño, tiene un ciclo reproductivo corto, y al estar su genoma totalmente secuenciado hay disponibles mutantes útiles para diferentes fines. Sin embargo, en este modelo de candidiasis no es posible inyectar concentraciones controladas de inóculos ni ajustar las dosis terapéuticas de los fármacos que se desean ensayar. Esta limitación no se observa con la larva de la polilla de la miel *G. mellonella* ya que su tamaño (1,5-2,5 cm) permite inyectar inóculos de concentraciones conocidas (Jemel et al., 2020). Además, este insecto sobrevive a un amplio rango de temperatura (15-42 °C), que incluye la temperatura habitual del cuerpo humano (36-37 °C), lo que permite analizar la virulencia y la patogenia de un amplio número de microorganismos. El sistema inmunitario innato de este invertebrado permite obtener información valiosa de las interacciones entre hospedador y patógeno a través de la producción de hemocitos y la capacidad fagocítica de dichos hemocitos. Entre los inconvenientes de este modelo destaca la limitada disponibilidad de larvas con una producción controlada. A este respecto, y hasta el momento, solo existe en el mercado la empresa BioSystems

Technology (Inglaterra) que comercializa larvas de *G. mellonella* con su genoma secuenciado y una producción controlada, hecho que encarece significativamente el coste de este modelo. En este trabajo de Tesis doctoral ambos modelos aportaron resultados interesantes sobre el conocimiento de las especies emergentes *C. glabrata*, *C. nivariensis*, *C. bracarensis* y *C. auris*.

En el estudio 2 (Anexo 2), el análisis de la virulencia de *C. glabrata*, *C. nivariensis* y *C. bracarensis* realizado en el modelo de candidiasis en *C. elegans* mostró que *C. glabrata* era la especie más virulenta, mientras que *C. bracarensis* fue la menos virulenta. Estos resultados coinciden con los datos epidemiológicos que muestran que, de estas tres especies, *C. glabrata* es la causante de un número mayor de candidiasis que *C. nivariensis* y *C. bracarensis* (Lockhart et al., 2009; Swoboda-Kopec et al., 2014; Dudiuk et al., 2017; Asadzadeh et al., 2019). La virulencia de estas tres especies analizada en el modelo en *G. mellonella* y descrita en el estudio 3 (Anexo 3) del apartado resultados también corroboraron lo anteriormente descrito. Además, en este modelo se utilizaron diferentes inóculos microbianos (1×10^5; 1×10^6; 1×10^7 células de *Candida* por larva) y, como se describe en otros estudios, la letalidad fue dependiente del inóculo (Mesa-Arango et al., 2013; Scorzoni et al., 2013; Ames et al., 2017; Sherry et al., 2017; Maurer et al., 2019).

En el estudio de la respuesta innata mediada por los hemocitos y la evaluación de su capacidad fagocítica de las larvas de *G. mellonella* infectadas con *Candida*, se observó que *C. glabrata* y *C. nivariensis* indujeron la producción de un número menor de hemocitos que *C. bracarensis*. Son varios los trabajos que también describen una disminución en la respuesta de hemocitos contra la infección causada por *C. tropicalis*, *C. krusei* o las especies del complejo *C. parapsilosis* aunque, en la mayoría de los casos, esta disminución fue menor a la observada con las especies ensayadas en este trabajo de Tesis; esta diferencia posiblemente esté ligada a su distinto potencial virulento (Mesa-Arango et al., 2013; Scorzoni et al., 2013; Gago et al., 2014; Perini et al., 2019). Además, en nuestro trabajo se observó que los hemocitos fagocitaron con mayor eficacia las células de *C. bracarensis* que las de las otras dos especies, siendo las tasas de fagocitosis

de *C. nivariensis* las más bajas. Esta diferencia en la capacidad fagocítica de los hemocitos de *G. mellonella* podría estar asociada con la diferente capacidad de evasión de las especies de *Candida*. Algunas especies fúngicas como *Criptococcus neoformans*, *C. albicans* o *C. glabrata* tienen mecanismos activos para evadir el sistema inmunitario del hospedador y evitar su eliminación por los fagocitos. Entre los mecanismos que desarrolla el patógeno se incluye la producción de inhibidores de la quimiotaxis o de la fagocitosis, la secreción de moléculas señuelo, la inducción de vías de protección contra el estrés, la interferencia con el proceso de maduración del fagosoma y la capacidad de escapar de los fagocitos mediante la expulsión de las células fúngicas o la lisis de la célula fagocítica (Erwig and Gow, 2016). Concretamente, se ha descrito que *C glabrata* está bien adaptada a la supervivencia intracelular dentro de los macrófagos y es capaz de reproducirse dentro de fagosomas en fase endosomal tardía y no acidificados. Por el contrario, cuando se emplean células de *C. glabrata* muertas por calor, estas se observan dentro de fagolisosomas completamente maduros, lo que sugiere que las células vivas bloquean la acidificación del fagosoma (Kasper et al., 2014). Además, Kaur et al. (2007) describieron un aumento en la expresión de los genes de proteasas secretadas por yapsinas en células de *C. glabrata* fagocitadas por macrófagos como mecanismo de escape. Seider et al. (2014) identificaron 23 genes relacionados con la capacidad de *C. glabrata* para resistir la muerte intracelular inducida por los macrófagos. Sin embargo, no hay datos sobre las estrategias que *C. nivariensis* y *C. bracarensis* pueden desarrollar para evadir al sistema inmunitario del hospedador, lo cual sería de gran interés investigar para conocer mejor la capacidad de infección de estas dos especies desconocidas de *Candida*.

La virulencia de los aislamientos clínicos de *C. auris* (sangre, orina y orofaríngeo) no mostró diferencias significativas entre sus distintos orígenes en el desarrollo de la infección en el nematodo *C. elegans*. Sin embargo, en el modelo de candidiasis en *G. mellonella* se observó una virulencia significativamente mayor de los aislamientos de sangre respecto a los aislamientos de orina y orofaríngeos de *C. auris*; excepto para el aislamiento de origen sanguíneo, *C. auris* JMRC:NRZ 1101, con fenotipo agregante que

fue el que causó menor mortalidad tanto en *C. elegans* como en *G. mellonella*. Estos resultados de menor virulencia en el aislamiento con fenotipo agregante coinciden con lo descrito en otros estudios realizados in vivo (Borman et al., 2016; Sherry et al., 2017; Muñóz et al., 2020). Sin embargo, en el estudio de Romera et al. (2020) realizado en el modelo en *G. mellonella* no se observaron diferencias entre la virulencia de aislamientos de *C. auris* con fenotipo agregante y no agregante. Con la intención de explicar la diferencia observada en este trabajo de Tesis en relación con la virulencia de *C. auris* con ambos modelos, cabe recordar que la vía de inoculación de *Candida* en estos dos modelos es diferente y esto podría condicionar el desarrollo de la infección. La administración parenteral de *Candida* en el modelo en *G. mellonella* es más directa y controlada que la ingestión del patógeno en el modelo de *C. elegans*. En este último caso los nematodos podrían tener dificultades para ingerir los agregados que forman las células de *C. auris* en el caso del fenotipo agregante y, por tanto, el número de células fúngicas ingeridas en un tiempo determinado podría ser menor que cuando se trata de las células con fenotipo no agregante. Además, se ha descrito que las diferencias en la virulencia de *C. auris* podrían tener relación con la plasticidad fenotípica que caracteriza a esta especie en respuesta a las diferentes condiciones, como puede ser el paso por un cuerpo de mamífero o las variaciones de temperatura en la colonización de un nicho específico.

Ambos modelos de candidiasis in vivo ensayados en este trabajo se han utilizado también para el análisis de la virulencia de otras especies de *Candida*. Así, se determinó en el nematodo *C. elegans* el efecto de la formación de biopelículas en la virulencia de *C. auris* y de las especies filogenéticamente cercanas a esta, *C. haemulonii* y *C. duobushaemulonii*, y se describió que en estas dos últimas especies la formación de biopelículas podría tener un efecto claro en dicha virulencia (Lima et al., 2020). Por otro lado, no se detectaron diferencias significativas en la virulencia entre las tres especies del complejo *C. parapsilosis* (Souza et al., 2015) o se describió una mayor virulencia de las especies *C. albicans* y *C. krusei*, mientras que la especie *C. dubliniensis* mostró la menor virulencia (Ortega-Riveros et al., 2017). Pukkila et al. (2009) también utilizaron este modelo de candidiasis en *C. elegans* para estudiar el efecto del fenotipo de levadura e hifa

de *C. albicans* en su virulencia y observaron que la generación de hifas aumentó significativamente su virulencia. El modelo de candidiasis en *G. mellonella* se ha utilizado para el estudio de la virulencia y la patogenia de las especies *C. tropicalis* (*Mesa-Arango et al., 2013*), *C. krusei* (Scorzoni et a., 2013), del complejo *C. parapsilosis* (Gago et al., 2014) *C. albicans* y *C. auris* (García-Carnero et al., 2020; Marcos-Zambrano et al., 2020).

Por otro lado, en este trabajo de Tesis doctoral también se estudió la sensibilidad de *C. glabrata*, *C. nivariensis*, *C. bracarensis* y *C. auris* a varios fármacos antifúngicos. Se observó que la sensibilidad in vitro de *C. glabrata*, *C. nivariensis* y *C. bracarensis* a siete fármacos antifúngicos (anfotericina B, fluconazol, posaconazol, voriconazol, anidulafungina, caspofungina y micafungina) fue muy alta y no se detectó ninguna resistencia ni sensibilidad reducida. En el estudio in vitro de la sensibilidad a anfotericina B, anidulafungina, caspofungina y micafungina de los cinco aislamientos de sangre de *C. auris*, el aislamiento con fenotipo agregante fue resistente a las tres equinocandinas.

La capacidad de *C. glabrata* y *C. auris* para desarrollar resistencia, especialmente al fluconazol, está demostrada, y en muchos casos, los aislamientos que no son resistentes presentan sensibilidad reducida o son dosis dependientes a dicho antifúngico (Ben-Ami et al., 2016; Dellière et al., 2016; Lamonth et al., 2018; Sadeghi et al., 2018; Sardi et al., 2013) y, en menor medida, al posaconazol y voriconazol (Al-Baqsami et al., 2020; Astvad et al., 2017; Carrillo-Muñoz et al., 2010). También se han descrito fracasos terapéuticos en infecciones causadas por aislamientos de *C. nivariensis* y *C. bracarensis* que mostraron una sensibilidad reducida o eran resistentes a fluconazol, voriconazol o posaconazol (Aznar-Marin et al., 2016; Borman et al., 2008; Figueiredo-Carvalho et al., 2016; Fujita et al., 2007; Lockhart et al., 2009; Malek et al., 2019; Warren et al., 2010). En el caso de *C. auris*, las tasas de resistencia al fluconazol en algunos estudios pueden llegar al 99% de los aislamientos analizados y en la mayoría se mantiene alrededor del 90%, lo cual dificulta el tratamiento de las infecciones causadas por este patógeno (Ahmad et al., 2020; Chowdhary et al., 2018; Khan et al., 2018; Lockhart et al., 2017; Ruiz-Gaitan et al., 2019; Zhu et al., 2020). La resistencia o sensibilidad reducida de

C. auris a voriconazol y posaconazol también se ha descrito previamente (Ahmad et al., 2020; Kathuria et al., 2015; Khan et al., 2018; Lockhart et al., 2017), llegando a ser resistentes a voriconazol hasta el 54% de los aislamientos analizados en el estudio realizado por Lockhart et al. (2017).

La anfotericina B ha demostrado ser eficaz in vitro contra *C. glabrata*, *C. nivariensis* y *C. bracarensis* (Astvad et al., 2017; Hull et al., 2012; Mashaly et al., 2019; Moreira et al., 2015). Sin embargo, se han descrito resistencias a este fármaco antifúngico hasta en el 6,8% de los aislamientos de *C. glabrata* y de fracasos terapéuticos en algunos casos clínicos de candidiasis causadas por esta especie (Khan et al., 2008; Krogh-Madsen et al., 2006; Al-Baqsami et al., 2020). También se han descrito aislamientos de *C. auris* con resistencia a la anfotericina B (Lockhart et al., 2017; Khan et al., 2018; Ninan et al., 2020; Pfaller et al., 2021), con tasas de resistencia que van desde el 8% (Chowdhary et al., 2018) hasta el 43% (Tsay et al., 2017). Escandón et al. (2019) realizaron un estudio epidemiológico de aislamientos de *C. auris* con resistencias a anfotericina B en diferentes hospitales de Colombia y observaron que la resistencia de este patógeno a la anfotericina B variaba en función de la región en la que se encontraba el hospital. Además, también se vio que la tasa de resistencia en los aislamientos obtenidos del ambiente hospitalario en la región norte de Colombia fue del 67%, mientras que en la zona central del país era nula. Estas diferencias se debieron a una significativa diferenciación genética entre los aislamientos de *C. auris* provenientes de las dos zonas, generándose un subclado correspondiente a la zona norte y otro a la zona central (Escandón et al., 2019).

La limitación en el uso del fluconazol como tratamiento contra las infecciones de *C. glabrata* o *C. auris* debido a las elevadas tasas de resistencia, han propiciado el uso de las equinocandinas como opción terapéutica de las candidiasis causadas por estas especies. Sin embargo, su utilización, muchas veces sin un protocolo terapéutico definido, puede estar relacionado con la selección de aislamientos de *Candida* con sensibilidad reducida o resistencia antifúngica, sobre todo en el caso de *C. glabrata* (Al- Baqsami et al., 2020). En el estudio prospectivo realizado por Fuller et al. (2019) se describió una resistencia a la micafungina en el 2,5% de los aislamientos clínicos de *C. glabrata*

provenientes de hospitales canadienses, resistencias que no se habían descrito en estudios anteriores en este país. En varios estudios realizados en EE.UU. se observó un aumento significativo de los aislamientos de *C. glabrata* resistentes a las equinocandinas en pacientes con candidiasis invasiva, con tasas que oscilaban entre el 7,8% y el 12,3% (Pfaller et al., 2009; Zimbeck et al., 2010; Cleveland et al., 2012; Pfaller et al., 2012; Pham et al., 2014; Vallabhaneni et al., 2015; Astvad et al., 2017; Castanheira et al., 2017; Healey and Perlin, 2018). Además, Pham et al. (2014) observaron que una alta proporción (36%) de los aislamientos de *C. glabrata* resistentes a equinocandinas también mostraron resistencia a los azoles, siendo aislamientos multirresistentes. En varios estudios se describen infecciones invasivas de brecha o intercurrentes por *C. glabrata* habiendo recibido los pacientes un tratamiento previo con caspofungina o micafungina (Cleary et al., 2008; Alexander et al., 2013; Dannaoui et al., 2012; Lockhart et al., 2012; Pfaller et al., 2019; Saraya et al., 2014).

La resistencia clínica a las equinocandinas en *C. glabrata* se relaciona con sustituciones de aminoácidos en las regiones *hot-spot* 1 y 2 de las dos subunidades del complejo 1,3-β-D-glucano sintasa, codificadas por los genes *FKS1* y *FKS2*. Se ha descrito una relación muy estrecha entre la detección de mutaciones en los genes *FKS*, los valores de MIC elevados sugerentes de resistencia, y el fracaso del tratamiento clínico en los pacientes con candidiasis por *C. glabrata* con una exposición previa o prolongada al tratamiento con equinocandinas. Por lo que la detección de mutaciones en los genes *FKS* se ha descrito como un método preciso para predecir el fracaso terapéutico (Alexander et al., 2013; Lewis et al., 2013; Saraya et al., 2014; Kolaczkowska et al., 2016; Fuller et al., 2019).

La tasa de resistencia a las equinocandinas de *C. auris* se mantiene baja, entre el 2-3% (Tsay et al., 2017; Chowdhary et al., 2018; Zhu et al., 2020). Sin embargo, en un estudio realizado en la India se detectaron valores de MIC altas de caspofungina en el 37% de los aislamientos de *C. auris* y el 24% de los mismos se consideraron resistentes (Kathuria et al. 2015). Rhodes et al. (2018) también observaron valores de MIC de caspofungina altos en los aislamientos de *C. auris* provenientes de un brote en Reino Unido, al igual que

otros autores lo detectaron en EE.UU. (Rhodes et al., 2018; Biagi et al., 2019; Woodworth et al., 2019). Además, *C. auris* se ha convertido en un grave problema en el ámbito hospitalario por su capacidad para desarrollar multirresistencias y se han descrito aislamientos clínicos panresistentes con resistencia a azoles, polienos y equinocandinas (Lockhart et al., 2019; Ostrowsky et al., 2020). Incluso se ha declarado que nos enfrentamos a un número cada vez mayor de infecciones invasivas causadas por especies diferentes a *C. albicans* con multirresistencia, la mayoría de ellas causadas por *C. glabrata* y *C. auris* (Colombo et al., 2017).

Son varias las estrategias que se han propuesto para realizar un tratamiento eficaz de las candidiasis causadas por aislamientos multirresistentes. Entre ellas se encuentra la búsqueda de nuevas moléculas o compuestos eficaces contra estas especies y contra las biopelículas que estas pueden producir (Fernández-Calderón et al., 2021; Mishra et al., 2021). La mayoría de las infecciones por *Candida* asociadas a biopelículas surgen de células que colonizan las superficies de los dispositivos médicos implantados (Coad et al., 2015). Por este motivo, el empleo de biomateriales antifúngicos está ganando interés como estrategia eficaz para combatir estas infecciones asociadas a biopelículas. El desarrollo de dichos biomateriales está relacionado con la funcionalización de la superficie de los dispositivos empleando fármacos antifúngicos u otras moléculas y polímeros con actividad antifúngica. De esta forma se evita la adhesión de *Candida* a las superficies y se bloquea su capacidad para formar una biopelícula (Vera-González and Shukla, 2020). En diferentes estudios, se observó que los discos de titanio con caspofungina en su superficie (fármaco antifúngico más utilizado en el anclaje directo a la superficie) inhibían más del 89% de la biopelícula formada por *C. albicans* respecto a los discos no funcionalizados con caspofungína (Coad et al., 2015; Kucharíková et al., 2016; Michl et al., 2017). Por lo que esta técnica podría ser una herramienta poderosa para combatir las infecciones causadas por especies que se caracterizan por ser capaces de desarrollar resistencias como lo son *C. glabrata* y *C. auris*. Además, se ha descrito la eficacia de moléculas como los lipopéptidos cíclicos en la inhibición de la formación de biopelículas de *C. auris* (Ramachandran et al., 2018). Incluso el uso de nuevas

aproximaciones terapéuticas, como el uso de células madre de cuello uterino humano que han demostrado ser eficaces en la inhibición in vitro del crecimiento de diferentes especies de *Candida* (Schneider et al., 2019).

Otra de las estrategias para tratar las candidiasis causadas por aislamientos multirresistentes es la encapsulación de fármacos antifúngicos en nanopartículas. Un ejemplo es la anfotericina B liposómica que ha demostrado conseguir disminuir su toxicidad en comparación a su formulación libre, permitiendo así el uso de dosis más altas (Soo Hoo, 2017). Además, mediante la encapsulación de fármacos también se evitan los problemas de penetración que son tan comunes, y la concentración intracelular del fármaco antifúngico es mayor. En varios estudios se observó la eficacia de la encapsulación de anfotericina B en nanopartículas para tratar macrófagos infectados por *C. glabrata* (Aparna et al., 2018; Sandhya et al., 2018). El uso de las nanopartículas de plata también se ha aplicado contra *C. auris* y se ha demostrado su eficacia en la inhibición de la formación de biopelículas en superficies del ambiente hospitalario (Lara et al., 2020; Vazquez-Munoz et al., 2020).

Entre las estrategias más extendidas en la práctica clínica nos encontramos con la combinación de diferentes fármacos antifúngicos, especialmente si la diana de acción de los fármacos es distinta. En este trabajo de Tesis doctoral se analizó la eficacia de la combinación de anfotericina B con equinocandinas contra cinco aislamientos de *C. auris* de origen sanguíneo. Los resultados mostraron interacciones sinérgicas en las tres combinaciones contra cuatro de los cinco aislamientos con fenotipo no agregante. Sin embargo, contra el aislamiento con fenotipo agregante (JMRC:NRZ 1101) las combinaciones entre anfotericina B y anidulafungina o anfotericina B y caspofungina mostraron interacciones sinérgicas, mientras que la combinación entre anfotericina B y micafungina solo mostró una interacción aditiva.

Son muy pocos los estudios que analizan la eficacia contra *C. auris* de la combinación de fármacos antifúngicos o la combinación de estos con otro tipo de fármacos (Fakhim et al., 2017; Bidaud et al., 2019; Bidaud et al., 2020; O´Brien et al., 2020; Schwarz et al., 2020;

Shaban et al., 2020; Wu et al., 2020; Caballero et al., 2021; Nagy et al., 2021; Pfaller et al., 2021). Se han descrito interacciones sinérgicas in vitro al combinar voriconazol con micafungina o con caspofungina (Fakhim et al., 2017; Pfaller et al., 2021). Caballero et al. (2021) describieron interacciones sinérgicas en la combinación de isavuconazol con equinocandinas. Esta sinergia terapéutica también fue observada por Nagy et al. (2021) en su estudio de combinación de isavuconazol y caspofungina. Además, las combinaciones de anidulafungina y caspofungina con flucitosina o colistina han sido sinérgicas, mientras que la combinación con micafungina era indiferente (Bidaud et al., 2019; Bidaud et al., 2020; O´Brien et al., 2020). Los investigadores Shaban et al. (2020) describieron la eficacia in vitro de la combinación de carvacrol con diferentes fármacos antifúngicos al observar interacciones sinérgicas o aditivas contra más del 64% de los aislamientos de *C. auris* analizados. Concretamente, estas interacciones se observaron en un 68% de los aislamientos al combinar el carvacrol con fluconazol, en un 64% al combinarlo con anfotericina B y en un 96% al combinarlo con nistatina. Sin embargo, al combinar carvacrol con caspofungina solo se observó ese tipo de interacciones contra el 28% de los aislamientos. También se han descrito diferentes casos clínicos sobre la eficacia del tratamiento de equinocandinas combinado con anfotericina B (Ruiz-Gaitán et al., 2017; Chamdramati et al., 2020; Ostrowsky et al., 2020) o con isavuconazol (Mulet-Bayona et al., 2020) para las candidiasis causadas por *C. auris*.

La terapia combinada también ha demostrado ser eficaz contra aislamientos de *C. glabrata* con resistencia a varios fármacos antifúngicos. Alves et al. (2012) observaron interacciones sinérgicas entre anfotericina B y flucitosina contra el 61,77% de los aislamientos de *C. glabrata* sensibles al fluconazol y el 76,47% de los aislamientos de *C. glabrata* resistentes al fluconazol. Además, son varios los estudios que han descrito la eficacia de la combinación de fármacos antifúngicos con otro tipo de compuestos para tratar las infecciones causadas por *C. glabrata*. Entre ellos, Tome et al. (2018) probaron la eficacia de la combinación de los fármacos inmunomoduladores, tacrólimus y/o ciclosporina A con fluconazol, itraconazol o anfotericina B, y observaron interacciones sinérgicas en el 93% de los aislamientos analizados. Sin embargo, este porcentaje fue

menor en el estudio realizado por Denardi et al. (2015), puesto que no observaron interacciones sinérgicas entre tacrólimus y fluconazol, ketaconazol, itraconazol o voriconazol en más del 77% de los aislamientos de *C. glabrata* resistentes al fluconazol, porcentaje que fue incluso inferior contra los aislamientos sensibles al fluconazol. Mashaly y Shrief (2019) describieron interacciones sinérgicas entre el antibacteriano polimixina B y el fluconazol contra el 68,9% de los aislamientos de *C. glabrata, C. nivariensis* y *C. bracarensis* analizados.

En este trabajo de Tesis doctoral los modelos animales resultaron apropiados para estudiar la efectividad de los tratamientos antifúngicos contra las especies emergentes de *Candida*. Así, el modelo de candidiasis en *C. elegans* fue útil para evaluar la eficacia del tratamiento antifúngico contra las infecciones causadas por *C. glabrata*, *C. nivariensis* y *C. bracarensis*. La eficacia de los tratamientos dependió de la especie de *Candida* y los fármacos anfotericina B y voriconazol fueron los mejores tratamientos contra las candidiasis causadas por *C. glabrata* y *C. bracarensis*, mientras que la micafungina y caspofungina lo fueron para tratar las infecciones causadas por *C. nivariensis*. Estos resultados concuerdan con lo descrito por Ames et al. (2017) con el modelo de candidiasis en *G. mellonella*, en el que el tratamiento con anfotericina B protegía a las larvas de la infección por *C. glabrata*, hecho que también coincide con los resultados descritos de sensibilidad in vitro de *C. glabrata* y *C. bracarensis* a la anfotericina B (Moreira et al., 2015; Astvad et al., 2017). La efectividad del tratamiento con caspofungina se ha descrito en un enfermo que padecía una candidemia asociada a catéter causada por *C. nivariensis* (López-Soria et al., 2013) que concuerda con la sensibilidad in vitro de *C. nivariensis* a caspofungina y micafungina (Lockhart et al., 2009; Tay et al., 2014; Morales-López et al., 2017).

De igual manera, el modelo de candidiasis en *G. mellonella* permitió valorar la eficacia del tratamiento con equinocandinas contra la infección causada por *C. glabrata, C. nivariensis* y *C. bracarensis*. Se observó que caspofungina y micafungina fueron las equinocandinas más eficaces en el tratamiento de las candidiasis causadas por *C. glabrata* y *C. nivariensis*, mientras que anidulafungina lo fue en las infecciones por *C. bracarensis*

en *G. mellonella*. Ames et al. (2017) también observaron un aumento en la supervivencia de las larvas de *G. mellonella* infectadas con *C. glabrata* tras el tratamiento con caspofungina. Sin embargo, en el modelo de candidiasis en *C. elegans* el tratamiento con anidulafungina fue el menos eficaz de las tres equinocandinas. Esto puede deberse a que la preparación de esta equinocandina se realizó con DMSO, mientras que las otras dos se disolvieron en agua, y como se describe en el estudio 2, se demostró que el DMSO producía un efecto negativo en la supervivencia de los nematodos.

El modelo invertebrado de candidiasis en *G. mellonella* ha demostrado ser útil en el estudio de la eficacia de los tratamiento basados en los fármacos antifúngicos de uso habitual (Mesa-Arango et al., 2013; Scorzoni et al., 2013; Souza et al., 2015; Ames et al., 2017), pero también para el análisis de la actividad antifúngica de otras moléculas y compuestos (Browne et al., 2014; Sa et al., 2018; Ajdidi et al., 2019; Nile et al., 2019; Osmanov et al., 2019) o para la evaluación de diferentes formas para vehiculizar los fármacos, como las nanopartículas descritas como un sistema de encapsulación que mejora la actividad antifúngica (Spadari et al., 2019; Moya-Andérico et al., 2021). Además, este modelo se ha aplicado para evaluar la efectividad de la combinación de fármacos antifúngicos o de estos con otro tipo de fármacos, especialmente en las infecciones causadas por *C. albicans* (Li et al., 2013; Li et al., 2017; Gu et al., 2018; Lu et al., 2018). Concretamente, Li et al. (2013) observaron la eficacia de la combinación de fluconazol y anfotericina B contra *C. albicans*. Otros autores observaron también la eficacia de la combinación del fluconazol con otros compuestos como el fármaco mucolítico ambroxol hidrocloruro, o como los antibacterianos tetraciclinas o gentamicina contra la infección causada por *C. albicans* (Li et al., 2017; Gu et al., 2018; Lu et al., 2018).

En el estudio de la eficacia de las combinaciones para tratar las candidiasis, el modelo en *C. elegans* fue apropiado para analizar la efectividad del tratamiento de combinación de anfotericina B con equinocandinas en las infecciones causadas por cinco aislamientos de *C. auris* de origen sanguíneo. Como se presenta en el estudio 5 (Anexo 5), se tuvieron en cuenta los datos de sensibilidad in vitro a estos fármacos antifúngicos y se utilizaron las

CMI resultantes como orientación para definir las pautas de tratamiento de los nematodos infectados. Aunque en los ensayos in vitro la combinación de anfotericina B con micafungina causaba la mayor inhibición del crecimiento de *C. auris*, esta combinación tuvo la menor eficacia terapéutica en los ensayos in vivo. Sin embargo, la combinación de anfotericina B y caspofungina aumentó significativamente la supervivencia de los nematodos infectados por cuatro de los cinco aislamientos, incluido el aislamiento con fenotipo agregante, en comparación a la observada en monoterapia. Esta combinación de fármacos se ha utilizado como estrategia para el tratamiento de algunos pacientes con infecciones causadas por *C. auris* (Ruiz-Gaitán et al., 2017; Chamdramati et al., 2020; Mulet-Bayona et al., 2020; Ostrowsky et al., 2020). Además, cuatro estudios realizados en el modelo de candidiasis en *C. elegans* han comprobado la eficacia de la combinación de azoles con otros fármacos como sulfametoxazol (Eldesouky et al., 2018), lopinavir (Eldesouky et al., 2020[a]), aprepitant (Eldesouky et al., 2020[b]) y ospemifeno (Eldesouky et al., 2020[c]), demostrando así que este modelo es una herramienta muy útil para el estudio de nuevas moléculas antimicrobianas (Moy et al., 2009; Breger et al., 2007; Desalermos et al., 2011; Maglioni et al., 2016; Peterson and Pukkila-Worley, 2018).

El conocimiento sobre las especies emergentes de *Candida* es aún limitado. Aunque son muchos los estudios realizados sobre las especies patógenas de *Candida*, algunos aspectos relacionados con su capacidad de evasión del sistema inmune, su grado de patogenicidad o los tratamientos antifúngicos eficaces contra las infecciones que causan siguen sin estar claros. La investigación presentada en esta Tesis doctoral pretende mostrar la eficacia de los modelos alternativos de candidiasis invasora en *C. elegans* y *G. mellonella* para avanzar en el conocimiento de la patogenia de *Candida*, de las interacciones hospedador-patógeno y de la búsqueda de nuevas terapias antifúngicas contra las candidiasis invasivas.

7. ONDORIOAK

7. Conclusiones

1. Los modelos de candidiasis invasiva en *Caenorhabditis elegans* y *Galleria mellonella* fueron útiles tanto para el estudio de la virulencia de las especies emergentes de *Candida* como para evaluar la terapia antifúngica contra las candidiasis.
2. La especie *Candida glabrata* fue más virulenta que sus especies filogenéticamente relacionadas, *Candida nivariensis* y *Candida bracarensis*, en los modelos de candidiasis en *C. elegans* y *G. mellonella*.
3. El estudio de la respuesta de hemocitos de *G. mellonella* y la evaluación de su capacidad fagocítica han aportado información relevante sobre las interacciones entre el hospedador y el patógeno durante las infecciones causadas por *C. glabrata*, *C. nivariensis* y *C. bracarensis*.
4. La respuesta innata mediada por los hemocitos en el modelo de candidiasis en *G. mellonella* fue más eficaz contra *C. bracarensis* que contra *C. glabrata* y *C. nivariensis*.
5. Los aislamientos de la especie emergente *Candida auris* con fenotipo no agregante fueron más virulentos que el aislamiento con fenotipo agregante en los modelos de candidiasis en *C. elegans* y *G. mellonella*.
6. Los aislamientos de *C. auris* de sangre fueron más virulentos que los de otros orígenes clínicos en el modelo de candidiasis en *G. mellonella*.
7. La anfotericina B y los azoles fueron eficaces contra las infecciones causadas por *C. glabrata* y *C. bracarensis* en el modelo de candidiasis en *C. elegans*. Mientras que el tratamiento con cualquiera de las tres equinocandinas lo fue contra *C. nivariensis*.
8. Los tratamientos con caspofungina y micafungina fueron los más eficaces contra la infección por *C. glabrata* y *C. nivariensis* en el modelo de candidiasis en *G. mellonella*. Sin embargo, el tratamiento con anidulafungina fue el más efectivo contra la infección causada por *C. nivariensis*.

9. La combinación de anfotericina B y caspofungina fue el tratamiento más eficaz contra la infección causada por *C. auris* en el modelo de candidiasis en *C. elegans*.

8. BIBLIOGRAFIA

8. Bibliografia

Ademe M, Girma F. *Candida auris*: From multidrug resistance to pan-resistant strains. *Infect Drug Resist* 2020; 13:1287-1294. doi:10.2147/IDR.S249864

Adler-Moore JP, Gangneux JP, Pappas PG. Comparison between liposomal formulations of amphotericin B. *Med Mycol* 2016; 54(3):223-231. doi:10.1093/mmy/myv111

Ahmad S, Khan Z, Al-Sweih N, Alfouzan W, Joseph L. *Candida auris* in various hospitals across Kuwait and their susceptibility and molecular basis of resistance to antifungal drugs. *Mycoses* 2020; 63(1):104-112. doi:10.1111/myc.13022

Ajdidi A, Sheehan G, Abu Elteen K, Kavanagh K. Assessment of the in vitro and in vivo activity of atorvastatin against *Candida albicans*. *J Med Microbiol* 2019; 68(10):1497-1506. doi:10.1099/jmm.0.001065

Al-Baqsami ZF, Ahmad S, Khan Z. Antifungal drug susceptibility, molecular basis of resistance to echinocandins and molecular epidemiology of fluconazole resistance among clinical *Candida glabrata* isolates in Kuwait. *Sci Rep* 2020; 10(1):6238. doi:10.1038/s41598-020-63240-z

Alcoba-Flórez J, Méndez-Álvarez S, Cano J, Guarro J, Pérez-Roth E, Arévalo MP. Phenotypic and molecular characterization of *Candida nivariensis* sp. nov., a possible new opportunistic fungus. *J Clin Microbiol*. 2005; 43(8):4107-4111.

Alexander BD, Johnson MD, Pfeiffer CD, et al. Increasing echinocandin resistance in *Candida glabrata*: Clinical failure correlates with presence of FKS mutations and elevated minimum inhibitory concentrations. *Clin Infect Dis* 2013; 56(12):1724-1732. doi:10.1093/cid/cit136

Allen D, Wilson D, Drew R, Perfect J. Azole antifungals: 35 years of invasive fungal infection management. *Expert Rev Anti Infect Ther* 2015; 13(6):787-798. doi:10.1586/14787210.2015.1032939

Alobaid K, Asadzadeh M, Bafna R, Ahmad S. First isolation of *Candida nivariensis*, an emerging fungal pathogen, in Kuwait. *Med Princ Pract* 2021; 30(1):80-84. doi:10.1159/000511553

Alonso MF, Gow NAR, Erwig LP, Bain JM. Macrophage migration is impaired within *Candida albicans* biofilms. *J Fungi* (Basel) 2017; 3(3):31. doi:10.3390/jof3030031

Alves IA, Bandeira LA, Mario DA, et al. Effects of antifungal agents alone and in combination against *Candida glabrata* strains susceptible or resistant to fluconazole. *Mycopathologia* 2012; 174(3):215-21. doi:10.1007/s11046-012-9538-7

Amani D, Emira N, Ismail T, et al. Extracellular enzymes and adhesive properties of medically important *Candida* spp. strains from landfill leachate. *Microb Pathog* 2018; 116:328-334. doi:10.1016/j.micpath.2018.01.042

Ames L, Duxbury S, Pawlowska B, Lui Ho H, Haynes K, Bates S. *Galleria mellonella* as a host model to study *Candida glabrata* virulence and antifungal efficacy. *Virulence* 2017; 8:1909-1917. doi.org/10.1080/21505594.2017.1347744

Angoulvant A, Guitard J, Hennequin C. Old and new pathogenic Nakaseomyces species: epidemiology, biology, identification, pathogenicity and antifungal resistance. *FEMS Yeast Res* 2016; 16(2):fov114. doi: 10.1093/femsyr/fov114

Aparna V, Melge AR, Rajan VK, et al. Carboxymethylated ι-carrageenan conjugated amphotericin B loaded gelatin nanoparticles for treating intracellular *Candida glabrata* infections. *Int J Biol Macromol* 2018; 110:140-149. doi:10.1016/j.ijbiomac.2017.11.126

Arastehfar A, Fang W, Badali H, et al. Low-cost tetraplex PCR for the global spreading multi-drug resistant fungus, *Candida auris* and its phylogenetic relatives. *Front Microbiol* 2018; 9:1119. doi:10.3389/fmicb.2018.01119

Arastehfar A, Daneshnia F, Salehi MR, et al. Molecular characterization and antifungal susceptibility testing of *Candida nivariensis* from blood samples – an Iranian multicentre study and a review of the literature. *J Med Microbiol* 2019; 68(5):770-777. doi:10.1099/jmm.0.000963

Arendrup MC, Meletiadis J, Mouton JW, et al. EUCAST DEFINITIVE DOCUMENT E.DEF 7.3.2. Method for the determination of broth dilution minimum inhibitory concentrations of antifungal agents for yeasts. 2020; 1-21

Arendrup MC, Patterson TF. Multidrug-resistant *Candida*: Epidemiology, molecular mechanisms, and treatment. *J Infect Dis* 2017; 216(3):S445-S451. doi:10.1093/infdis/jix131

Arias LS, Butcher MC, Short B, et al. Chitosan ameliorates *Candida auris* virulence in a *Galleria mellonella* infection model. *Antimicrob Agents Chemother* 2020; 64(8):e00476-20. doi:10.1128/AAC.00476-20

Arikan-Akdagli S, Ghannoum M, Meis JF. Antifungal resistance: specific focus on multidrug resistance in *Candida auris* and secondary azole resistance in *Aspergillus fumigatus*. *J Fungi* 2018; 4:129. doi:10.3390/jof4040129

Asadzadeh M, Alanazi AF, Ahmad S, Al-Sweih N, Khan Z. Lack of detection of *Candida nivariensis* and *Candida bracarensis* among 440 clinical *Candida glabrata* sensu lato isolates in Kuwait. *PLoS One* 2019; 14(10):1-16. doi:10.1371/journal.pone.0223920

Astvad KMT, Hare RK, Arendrup MC. Evaluation of the in vitro activity of isavuconazole and comparator voriconazole against 2635 contemporary clinical *Candida* and *Aspergillus* isolates. *Clin Microbiol Infect* 2017; 23(11):882-887. doi:10.1016/j.cmi.2017.03.023

Astvad KMT, Johansen HK, Røder BL, et al. Update from a 12-year nationwide fungemia surveillance: Increasing intrinsic and acquired resistance causes concern. *J Clin Microbiol* 2018; 56(4):e01564-17 doi:10.1128/JCM.01564-17

Atalay MA, Koc AN, Demir G, Sav H. Investigation of possible virulence factors in *Candida* strains isolated from blood cultures. *Niger J Clin Pract* 2015; 18(1):52-5. doi:10.4103/1119-3077.146979

Aznar-Marin P, Galan-Sanchez F, Marin-Casanova P, et al. *Candida nivariensis* as a new emergent agent of vulvovaginal candidiasis: description of cases and review of published studies. *Mycopathologia* 2016; 181(5-6):445-9. doi:10.1007/s11046-015-9978-y

Bailey JM, Oliveri AN, Levin ED. Pharmacological analyses of learning and memory in zebrafish (*Danio rerio*). *Pharmacol Biochem Behav* 2015; 139:103-11. doi:10.1016/j.pbb.2015.03.006

Barreto TL, Rossato L, de Freitas ALD, et al. Miltefosine as an alternative strategy in the treatment of the emerging fungus *Candida auris*. *Int J Antimicrob Agents* 2020; 56(2):106049. doi:10.1016/j.ijantimicag.2020.106049

Basas J, Palau M, Gomis X, Almirante B, Gavaldà J. Efficacy of liposomal amphotericin B and anidulafungin using an antifungal lock technique (ALT) for catheter-related *Candida albicans* and *Candida glabrata* infections in an experimental model. *PLoS One* 2019; 14(2):e0212426. doi:10.1371/journal.pone.0212426

Bellmann R, Smuszkiewicz P. Pharmacokinetics of antifungal drugs: practical implications for optimized treatment of patients. *Infection* 2017; 45(6):737-779. doi:10.1007/s15010-017-1042-z

Ben-Ami R, Zimmerman O, Finn T, et al. Heteroresistance to fluconazole is a continuously distributed phenotype among *Candida glabrata* clinical strains associated with in vivo persistence. *mBio* 2016; 7(4):e00655-16. doi:10.1128/mBio.00655-16

Ben-Ami R, Berman J, Novikov A, et al. Multidrug-Resistant *Candida haemulonii* and *C. auris*, Tel Aviv, Israel. *Emerg Infect Dis* 2017; 23(1):195–203. doi:10.3201/eid2302.161486

Bergin D, Brennan M, Kavanagh K. Fluctuations in haemocyte density and microbial load may be used as indicators of fungal pathogenicity in larvae of *Galleria mellonella*. *Microbes Infect* 2003; 5:1389–1395. doi.org/10.1016/j.micinf.2003.09.019

Beyda ND, John J, Kilic A, Alam MJ, Lasco TM, Garey KW. FKS mutant *Candida glabrata*: risk factors and outcomes in patients with candidemia. *Clin Infect Dis* 2014; 59(6):819-25. doi:10.1093/cid/ciu407

Biagi MJ, Wiederhold NP, Gibas C, et al. Development of high-level echinocandin resistance in a patient with recurrent *Candida auris* candidemia secondary to chronic candiduria. *Open Forum Infect Dis* 2019; 6(7):1-5. doi:10.1093/ofid/ofz262

Bialková A, Šubík J. Biology of the pathogenic yeast *Candida glabrata*. *Folia Microbiol (Praha)* 2006; 51(1):3-20. doi:10.1007/BF02931443

Bidaud AL, Botterel F, Chowdhary A, Dannaouia E. In vitro antifungal combination of flucytosine with amphotericin B, voriconazole, or micafungin against *Candida auris* shows no antagonism. *Antimicrob. Agents Chemother* 2019; 63:1–7. doi.org/10.1128/AAC.01393-19

Bidaud AL, Djenontin E, Botterel F, Chowdhary A, Dannaoui E. Colistin interacts synergistically with echinocandins against *Candida auris*. *Int J Antimicrob Agents* 2020; 55(3):105901. doi:10.1016/j.ijantimicag.2020.105901

Binder U, Arastehfar A, Schnegg L, et al. Efficacy of LAMB against emerging azole- and multidrug-resistant *Candida parapsilosis* isolates in the *Galleria mellonella* model. *J Fungi* (Basel) 2020; 6(4):377. doi:10.3390/jof6040377

Bishop JA, Chase N, Magill SS, Kurtzman CP, Fiandaca MJ, Merz WG. *Candida bracarensis* detected among isolates of *Candida glabrata* by peptide nucleic acid fluorescence in situ hybridization: Susceptibility data and documentation of presumed infection. *J Clin Microbiol* 2008; 46(2):443-446. doi.org/10.1128/JCM.01986-07

Blaser RE, Vira DG. Experiments on learning in zebrafish (*Danio rerio*): a promising model of neurocognitive function. *Neurosci Biobehav Rev* 2014; 42:224-31. doi:10.1016/j.neubiorev.2014.03.003

Bordallo-Cardona MÁ, Escribano P, Marcos-Zambrano LJ, et al. Low and constant micafungin concentrations may be sufficient to lead to resistance mutations in FKS2 gene of *Candida glabrata*. *Med Mycol* 2018; 56(7):903–906. doi.org/10.1093/mmy/myx124

Borman AM, Petch R, Linton CJ, Palmer MD, Bridge PD, Johnson EM. *Candida nivariensis*, an emerging pathogenic fungus with multidrug resistance to antifungal agents. *J Clin Microbiol* 2008; 46(3):933-938. doi:10.1128/JCM.02116-07

Borman AM, Szekely A, Johnson EM. Comparative pathogenicity of United Kingdom isolates of the emerging pathogen *Candida auris* and other key pathogenic *Candida* species. *mSphere* 2016; 1(4):4-6. doi:10.1128/mSphere.00189-16

Borman AM, Muller J, Walsh-Quantick J, et al. Fluconazole resistance in isolates of uncommon pathogenic yeast species from the United Kingdom. *Antimicrob Agents Chemother* 2019; 63(8):1-12. doi:10.1128/AAC.00211-19

Breger J, Fuchs BB, Aperis G, Moy TI, Ausubel FM, Mylonakis E. Antifungal chemical compounds identified using a *C. elegans* pathogenicity assay. *PLoS Pathog* 2007; 3:e18. doi:10.1371/journal.ppat.0030018

Brenner S. The genetics of *Caenorhabditis elegans*. *Genetics* 1974; 77:71-94

Brilhante RSN, Oliveira JS, Evangelista AJJ, et al. *Candida tropicalis* from veterinary and human sources shows similar in vitro hemolytic activity, antifungal biofilm susceptibility and pathogenesis against *Caenorhabditis elegans*. *Vet Microbiol* 2016; 192:213-219. doi:10.1016/j.vetmic.2016.07.022

Brown JL, Delaney C, Short B, et al. *Candida auris* phenotypic heterogeneity determines pathogenicity *in vitro*. *mSphere* 2020; 5(3):e00371-20. doi:10.1128/mSphere.00371-20

Browne N, Hackenberg F, Streciwilk W, Tacke M, Kavanagh K. Assessment of in vivo antimicrobial activity of the carbene silver(I) acetate derivative SBC3 using *Galleria mellonella* larvae. *Biometals* 2014; 27(4):745-52. doi:10.1007/s10534-014-9766-z

Bruno M, Kersten S, Bain JM, et al. Transcriptional and functional insights into the host immune response against the emerging fungal pathogen *Candida auris*. *Nat Microbiol* 2020; 5(12):1516-1531. doi:10.1038/s41564-020-0780-3

Caballero U, Kim S, Eraso E, Quindós G, Vozmediano V, Schmidt S, Jauregizar N. In vitro synergistic interactions of isavuconazole and echinocandins against *Candida auris*. *Antibiotics* 2021; 10(4):355. doi:10.3390/antibiotics10040355

Campos-Garcia L, Jimenez-valdes RJ, Hernandez-bello R et al. *Candida albicans* and non- *albicans* isolates from bloodstream have different capacities to induceneutrophil estracellular traps. *J Fungi* 2019; 5:28. doi:10.3390/jof5020028

Canteri de Souza P, Custódio Caloni C, Wilson D, Sergio Almeida R. An invertebrate host to study fungal infections, mycotoxins and antifungal drugs: *Tenebrio molitor*. *J Fungi* (Basel) 2018; 4(4):125. doi:10.3390/jof4040125

Carrillo-Muñoz AJ, Tur-Tur C, Hernández-Molina JM, et al. Antifungal activity of posaconazole against *Candida* spp. and non-*Candida* clinical yeasts isolates. *Rev Esp Quimioter* 2010; 23(3):122-5. PMID: 20844842

Carvalho R. A High-throughput screen for tuberculosis progression. *PLoS One* 2011; 6:e16779. doi:10.1371/journal.pone.0016779

Cassone A, De Bernardis F, Mondello F, Ceddia T, Agatensi L. Evidence for a correlation between proteinase secretion and vulvovaginal candidosis. *J Infect Dis* 1987; 156:777–783. doi.org/10.1093/infdis/156.5.777

Castanheira M, Deshpande LM, Davis AP, Rhomberg PR, Pfaller MA. Monitoring antifungal resistance in a global collection of invasive yeasts and molds: application of CLSI epidemiological Cutoff values and whole-genome sequencing analysis for detection of azole resistance in *Candida albicans*. *Antimicrob Agents Chemother* 2017; 61(10):e00906-17. doi:10.1128/AAC.00906-17

Cavalheiro M, Teixeira MC. *Candida* Biofilms: threats, challenges, and promising strategies. *Front Med* 2018; 13;5:28. doi:10.3389/fmed.2018.00028

Chaabane F, Graf A, Jequier L, Coste AT. Review on Antifungal Resistance Mechanisms in the Emerging Pathogen *Candida auris*. *Front Microbiol* 2019; 10:1-8. doi:10.3389/fmicb.2019.02788

Chandramati J, Sadanandan L, Kumar A, Ponthenkandath S. Neonatal *Candida auris* infection: Management and prevention strategies. A single centre experience. *J Paediatr Child Health* 2020; doi:10.1111/jpc.15019

Chao C. Hsu PC, Jen CF, et al. Zebrafish as a model host for *Candida albicans* infection. *Infect Immun* 2010; 78:2512-21. doi:10.1128/IAI.01293-09

Chapman B, Slavin M, Marriott D, et al. Changing epidemiology of candidaemia in Australia. *J Antimicrob Chemother* 2017; 72(4):1103-1108. doi:10.1093/jac/dkw422

Chatterjee S, Alampalli SV, Nageshan RK, Chettiar ST, Joshi S, Tatu US. Draft genome of a commonly misdiagnosed multidrug resistant pathogen *Candida auris*. *BMC Genomics* 2015; 16(1):686. doi:10.1186/s12864-015-1863-z

Chaturvedi V, Ramani R, Andes D, et al. Multilaboratory testing of two-drug combinations of antifungals against *Candida albicans*, *Candida glabrata*, and *Candida parapsilosis*. *Antimicrob Agents Chemother* 2011; 55(4):1543-8. doi:10.1128/AAC.01510-09

Chen K, Franz CJ, Jiang H, Jiang Y, Wang D. An evolutionarily conserved transcriptional response to viral infection in *Caenorhabditis nematodes*. *BMC Genomics* 2017; 18(1):303. doi:10.1186/s12864-017-3689-3

Chow NA, De Groot T, Badali H, Abastabar M, Chiller TM, Meis JF. Potential fifth clade of *Candida auris*, Iran, 2018. *Emerg Infect Dis* 2019; 25(9):1780-1781. doi:10.3201/eid2509.190686

Chow NA, Muñoz JF, Gade L, et al. Tracing the evolutionary history and global expansion of *Candida auris* using population genomic analyses. *MBio* 2020; 11(2): e03364-19. doi:10.1128/mBio.03364-19

Chowdhary A, Randhawa HS, Khan ZU, et al. First isolations in India of *Candida nivariensis*, a globally emerging opportunistic pathogen. *Med Mycol* 2010; 48(2):416-420. doi:10.1080/13693780903114231

Chowdhary A, Sharma C, Meis JF. *Candida auris*: A rapidly emerging cause of hospital-acquired multidrug-resistant fungal infections globally. *PLoS Pathog* 2017; 13(5):1-10. doi:10.1371/journal.ppat.1006290

Chowdhary A, Prakash A, Sharma C, et al. A multicentre study of antifungal susceptibility patterns among 350 *Candida auris* isolates (2009-17) in India: Role of the ERG11 and FKS1 genes in azole and echinocandin resistance. *J Antimicrob Chemother* 2018; 73(4):891-899. doi:10.1093/jac/dkx480

Clark NM, Grim SA, Lynch JP. Posaconazole: Use in the prophylaxis and treatment of fungal infections. *Semin Respir Crit Care Med* 2015; 36(5):767-785. doi:10.1055/s-0035-1562902

Cleary JD, Garcia-Effron G, Chapman SW, Perlin DS. Reduced *Candida glabrata* susceptibility secondary to an FKS1 mutation developed during candidemia treatment. *Antimicrob Agents Chemother* 2008; 52(6):2263-5. doi:10.1128/AAC.01568-07

Cleveland AA, Farley MM, Harrison LH, et al. Changes in incidence and antifungal drug resistance in candidemia: results from population-based laboratory surveillance in Atlanta and Baltimore, 2008-2011. *Clin Infect Dis* 2012; 55(10):1352-61. doi:10.1093/cid/cis697

Cleveland AA, Harrison LH, Farley MM, et al. Declining incidence of candidemia and the shifting epidemiology of *Candida* resistance in two US metropolitan areas, 2008-2013: Results from population-based surveillance. *PLoS One* 2015; 10(3):2008-2013. doi:10.1371/journal.pone.0120452

CLSI. Reference method for broth dilution antifungal susceptibility testing of yeasts; Fourth informational Supplement. CLSI documents M27-A3. CLSI, Wayne, PA: Clinical and Laboratory Standards Institute; USA; 2010.

CLSI. Reference method for broth dilution antifungal susceptibility testing of yeasts; Fourth informational Supplement. CLSI documents M27-A3 S4. CLSI, Wayne, PA: Clinical and Laboratory Standards Institute; USA, 2012.

Coad BR, Lamont-Friedrich SJ, Gwynne L, et al. Surface coatings with covalently attached caspofungin are effective in eliminating fungal pathogens. *J Mater Chem B* 2015; 3(43):8469-8476. doi:10.1039/c5tb00961h

Colombo AL, Júnior JNA, Guinea J. Emerging multidrug-resistant *Candida* species. *Curr Opin Infect Dis* 2017, 30(6):528-538. doi:10.1097/QCO.0000000000000411

Cornistein W, Mora A, Orellana N, Capparelli FJ, Del Castillo M. *Candida*: epidemiología y factores de riesgo para especies no *albicans. Enferm Infecc Microbiol Clin* 2013; 31(6):380-384. doi:10.1016/j.eimc.2012.09.011

Correia A, Sampaio P, James S, Pais C. *Candida bracarensis* sp. nov., a novel anamorphic yeast species phenotypically similar to *Candida glabrata*. *Int J Syst Evol Microbiol* 2006; 56:313–317. doi:10.1099/ijs.0.64076-0

Cortegiani A, Misseri G, Fasciana T, Giammanco A, Giarratano A, Chowdhary A. epidemiology, clinical characteristics, resistance, and treatment of infections by *Candida auris*. *J Intensive Care* 2018; 6 (69):1–13. doi:10.1186/s40560-018-0342-4

Costa-de-Oliveira S, Rodrigues AG. *Candida albicans* ntifungal resistance and tolerance in bloodstream infections: The Triad Yeast-Host-Antifungal. *Microorganisms* 2020, 8(2):154. doi:10.3390/microorganisms8020154

Coste AT, Kritikos A, Li J, et al. Fungal Infection Network of Switzerland (FUNGINOS). Emerging echinocandin-resistant *Candida albicans* and *glabrata* in Switzerland. *Infection* 2020; 48(5):761-766. doi:10.1007/s15010-020-01475-8

Cuenca-Estrella M, Gomez-Lopez A, Isla G, et al. Prevalence of *Candida bracarensis* and *Candida nivariensis* in a Spanish collection of yeasts: Comparison of results from a reference centre and from a population-based surveillance study of candidemia. *Med Mycol* 2011; 49(5):525-529. doi:10.3109/13693786.2010.546373

Dannaoui E, Desnos-Ollivier M, Garcia-Hermoso D, et al. *Candida* spp. with acquired echinocandin resistance, France, 2004-2010. *Emerg Infect Dis* 2012; 18(1):86-90. doi:10.3201/eid1801.110556

D'Eça Júnior A, Silva AF, Rosa FC, et al. In vitro differential activity of phospholipases and acid proteinases of clinical isolates of *Candida*. *Rev Soc Bras Med Trop* 2011; 44(3):334-8. doi:10.1590/s0037-86822011005000036

d'Enfert C, Janbon G. Biofilm formation in *Candida glabrata*: What have we learnt from functional genomics approaches? *FEMS Yeast Res* 2016; 16(1):fov111. doi:10.1093/femsyr/fov111

Dellière S, Healey K, Gits-Muselli M, et al. Fluconazole and echinocandin resistance of *Candida glabrata* correlates better with antifungal drug exposure rather than with MSH2 mutator genotype in a french cohort of patients harboring low rates of resistance. *Front Microbiol* 2016; 7:2038. doi:10.3389/fmicb.2016.02038

Denardi LB, Mario DA, Loreto ÉS, Santurio JM, Alves SH. Synergistic effects of tacrolimus and azole antifungal compounds in fluconazole-susceptible and fluconazole-resistant *Candida glabrata* isolates. *Braz J Microbiol* 2015; 46(1):125-9. doi:10.1590/S1517-838246120120442

Desalermos A, Muhammed M, Glavis-Bloom J, Mylonakis E. Using *Caenorhabditis elegans* for antimicrobial drug discovery. *Expert Opin Drug Discov* 2011; 6(6):645-652. doi:10.1517/17460441.2011.573781

Desalermos A, Tan X, Rajamuthiah R, et al. A multi-host approach for the systematic analysis of virulence factors in *Cryptococcus neoformans*. *J Infect Dis* 2015; 211(2):298-305. doi:10.1093/infdis/jiu441

De Bernardis F, Graziani S, Tirelli F, Antonopoulou S. *Candida* vaginitis: virulence, host response and vaccine prospects. *Med Mycol* 2018; 56(suppl_1):26-31. doi:10.1093/mmy/myx139

de Groot PW, Kraneveld EA, Yin QY, et al. The cell wall of the human pathogen *Candida glabrata*: differential incorporation of novel adhesin-like wall proteins. *Eukaryot Cell* 2008; 7(11):1951-64. doi:10.1128/EC.00284-08

de Souza PC, Morey AT, Castanheira GM, et al. *Tenebrio molitor* (Coleoptera: Tenebrionidae) as an alternative host to study fungal infections. *J Microbiol Methods* 2015; 118:182-6. doi:10.1016/j.mimet.2015.10.004

Díaz-Pascual F, Ortíz-Severín J, Varas MA, Allende ML, Chávez FP. In vivo host-pathogen interaction as revealed by global proteomic profiling of zebrafish larvae. *Front Cell Infect Microbiol* 2017; 7(JUL):1-11. doi:10.3389/fcimb.2017.00334

Domán M, Kovács R, Perlin DS, Kardos G, Gesztelyi R, Juhász B, Bozó A, Majoros L. Dose escalation studies with caspofungin against *Candida glabrata*. *J Med Microbiol* 2015; 64(9):998–1007. doi.org/10.1099/jmm.0.000116

Dominguez EG, Zarnowski R, Choy HL, et al. Conserved role for biofilm matrix polysaccharides in *Candida auris* drug resistance. *mSphere* 2019; 4(1):e00680-18. doi:10.1128/mSphereDirect.00680-18

Douglas LJ. *Candida* biofilms and their role in infection. *Trends Microbiol* 2003, 11: 30-36. doi:10.1016/s0966-842x(02)00002-1

Dudiuk C, Theill L, Gamarra S, Garcia-Effron G. Detection of cryptic *Candida* species recognized as human pathogens through molecular biology techniques. *Curr Fungal Infect Rep* 2017; 11:176-183. doi:10.1007/s12281-017-0294-5

Echeverría A, Durante AG, Arechavala A, Negroni R. Estudio comparativo de dos medios de cultivo para la detección de la actividad fosfolipasa en cepas de *Candida albicans* y *Cryptococcus neoformans*. *Rev Iberoam Micol* 2002; 19:95–98

El-Amrani S, Pena-Abaurrea M, Sanz-Landaluze J, et al Bioconcentration of pesticides in zebrafish eleutheroembryos (*Danio rerio*). *Sci Total Environ* 2012; 425:184-90. doi:10.1016/j.scitotenv.2012.02.065

Eldesouky HE, Li X, Abutaleb NS, Mohammad H, Seleem MN. Synergistic interactions of sulfamethoxazole and azole antifungal drugs against emerging multidrug-resistant *Candida auris*. *Int J Antimicrob Agents* 2018; 52:754–761. doi:10.1016/j.ijantimicag.2018.08.016

[a]Eldesouky HE, Salama EA, Lanman NA, Hazbun TR, Seleem MN. Potent synergistic interactions between Lopinavir and azole antifungal drugs against emerging multidrug-resistant *Candida auris*. *Antimicrob Agents Chemother* 2020; 65(1):e00684-20. doi:10.1128/AAC.00684-20

[b]Eldesouky HE, Lanman NA, Hazbun TR, Seleem MN. Aprepitant, an antiemetic agent, interferes with metal ion homeostasis of *Candida auris* and displays potent synergistic interactions with azole drugs. *Virulence* 2020; 11(1):1466-1481. doi:10.1080/21505594.2020.1838741

[c]Eldesouky HE, Salama EA, Hazbun TR, Mayhoub AS, Seleem MN. Ospemifene displays broad-spectrum synergistic interactions with itraconazole through potent interference with fungal efflux activities. *Sci Rep* 2020; 10(1):6089. doi:10.1038/s41598-020-62976-y

Elkabti AB, Issi L, Rao RP. *Caenorhabditis elegans* as a model host to monitor the *Candida* infection processes. *J Fungi* 2018; 4:123. doi:10.3390/jof4040123

Erwig LP, Gow NA. Interactions of fungal pathogens with phagocytes. *Nat Rev Microbiol* 2016; 14(3):163-76. doi:10.1038/nrmicro.2015.21

Escandón P, Chow NA, Caceres DH, et al. Molecular epidemiology of *Candida auris* in Colombia reveals a highly related, countrywide colonization with regional patterns

in amphotericin B resistance. *Clin Infect Dis* 2019; 68(1):15-21. doi:10.1093/cid/ciy411. PMID: 29788045

Fakhim H, Chowdhary A, Prakash A, et al. *In Vitro* Interactions of echinocandins with triazoles against multidrug-resistant *Candida auris*. *Antimicrob Agents Chemother* 2017; 61(11):e01056-17. doi:10.1128/AAC.01056-17

Fakhim H, Vaezi A, Dannaoui E, Chowdhary A, Nasiry D, Faeli L, Meis JF, Badali H. Comparative virulence of *Candida auris* with *Candida haemulonii*, *Candida glabrata* and *Candida albicans* in a murine model. *Mycoses* 2018; 61:377–382. doi.org/10.1111/myc.12754.

Falces-Romero I, Romero-Gómez MP, Moreno-Ramos F, et al. Epidemiology of bloodstream *Candida* species in a Spanish tertiary care hospital as a guide for implementation of T2MR (T2CANDIDA®) for rapid diagnosis of candidemia. *Med Mycol* 2021; 59(4):350-354. doi:10.1093/mmy/myaa056

Fan S, Yue H, Zheng Q, et al. Filamentous growth is a general feature of *Candida auris* clinical isolates. *Med Mycol* 2021; 23:myaa116. doi:10.1093/mmy/myaa116

Faria-Ramos I, Neves-Maia J, Ricardo E, et al. Species distribution and in vitro antifungal susceptibility profiles of yeast isolates from invasive infections during a Portuguese multicenter survey. *Eur J Clin Microbiol Infect Dis* 2014; 33(12):2241-2247. doi:10.1007/s10096-014-2194-8

Fernández-Calderón MC, Hernández-González L, Gómez-Navia C, Blanco-Blanco MT, Sánchez-Silos R, Lucio L, Pérez-Giraldo C. Antifungal and anti-biofilm activity of a new Spanish extract of propolis against *Candida glabrata*. *BMC Complement Med Ther* 2021; 21(1):147. doi:10.1186/s12906-021-03323-0

Fernández-Silva F, Lackner M, Capilla J, et al. In vitro antifungal susceptibility of *Candida glabrata* to caspofungin and the presence of FKS mutations correlate with treatment response in an immunocompromised murine model of invasive infection. *Antimicrob Agents Chemother* 2014; 58(7):3646-9. doi:10.1128/AAC.02666-13

Fidel PL, Vazquez JA, Sobel JD. *Candida glabrata*: Review of epidemiology, pathogenesis, and clinical disease with comparison to *C. albicans*. *Clin Microbiol Rev* 1999; 12(1):80-96. doi:10.1128/cmr.12.1.80

Figueiredo-Carvalho MHG, de Souza Ramos L, Barbedo LS, et al. First description of *Candida nivariensis* in Brazil: Antifungal susceptibility profile and potential virulence attributes. *Mem Inst Oswaldo Cruz* 2016; 111(1):51-58. doi:10.1590/0074-02760150376

Figueiredo-Carvalho MHG, Ramos LS, Barbedo LS, et al. Relationship between the antifungal susceptibility profile and the production of virulence-related hydrolytic enzymes in brazilian clinical strains of *Candida glabrata*. *Mediators Inflamm* 2017; 2017:8952878. doi:10.1155/2017/8952878

Frankowski H, Alavez S, Spilman P, Mark KA, Nelson JD, Mollahan P, Rao RV, Chen SF, Lithgow GJ, Ellerby HM. Dimethyl sulfoxide and dimethyl formamide increase lifespan of *C. elegans* in liquid. *Mech Ageing Dev* 2013; 134:69-78. doi.org/10.1016/j.mad.2012.10.002

Frenkel M, Mandelblat M, Alastruey-Izquierdo A, et al. Pathogenicity of *Candida albicans* isolates from bloodstream and mucosal candidiasis assessed in mice and *Galleria mellonella*. *J Mycol Med* 2016; 26(1):1-8. doi:10.1016/j.mycmed.2015.12.006

Frías-De-León MG, Hernández-Castro R, Vite-Garín T, et al. Antifungal resistance in *Candida auris*: molecular determinants. *Antibiotics* (Basel) 2020, 9(9):568. doi:10.3390/antibiotics9090568

Froehlicher M, Liedtke A, Groh KJ, et al. Zebrafish (*Danio rerio*) neuromast: promising biological endpoint linking developmental and toxicological studies. *Aquat Toxicol* 2009; 95(4):307-19. doi:10.1016/j.aquatox.2009.04.007

Fuchs BB, Eby J, Nobile CJ, El Khoury JB, et al. Role of filamentation in *Galleria mellonella* killing by *Candida albicans*. *Microbes Infect* 2010; 12(6):488-96. doi:10.1016/j.micinf.2010.03.001

Fuchs BB, RajaMuthiah R, Souza AC, et al. Inhibition of bacterial and fungal pathogens by the orphaned drug auranofin. *Future Med Chem* 2016; 8(2):117-32. doi:10.4155/fmc.15.182

Fujita SI, Senda Y, Okusi T, et al. Catheter-related fungemia due to fluconazole-resistant *Candida nivariensis*. *J Clin Microbiol* 2007; 45(10):3459-3461. doi:10.1128/JCM.00727-07

Fuller J, Dingle TC, Bull A, et al. Species distribution and antifungal susceptibility of invasive *Candida* isolates from Canadian hospitals: results of the CANWARD 2011–16 study. *J Antimicrob Chemother* 2019; 74(4):iv48-iv54. doi:10.1093/jac/dkz287

Gabaldón T, Martin T, Marcet-Houben M, et al. Comparative genomics of emerging pathogens in the *Candida glabrata* clade. *BMC Genomics* 2013; 14:623. doi:10.1186/1471-2164-14-623

Gabaldón T, Carreté L. The birth of a deadly yeast: tracing the evolutionary emergence of virulence traits in *Candida glabrata*. *FEMS Yeast Res* 2016; 16(2):fov110. doi:10.1093/femsyr/fov110

Gabaldón T, Fairhead C. Genomes shed light on the secret life of *Candida glabrata*: not so asexual, not so commensal. *Curr Genet* 2019; 65(1):93-98. doi:10.1007/s00294-018-0867-z

Gabaldón T, Gómez-Molero E, Bader O. Molecular Typing of *Candida glabrata*. *Mycopathologia* 2020; 185(5):755-764. doi:10.1007/s11046-019-00388-x

Gago S, García-Rodas R, Cuesta I, Mellado E, Alastruey-Izquierdo A. *Candida parapsilosis*, *Candida orthopsilosis* and *Candida metapsilosis* virulence in the non-conventional host *Galleria mellonella*. *Virulence* 2014; 5:278–285. doi.org/10.4161/viru.26973

Gale AN, Sakhawala RM, Levitan A, et al. Identification of essential genes and fluconazole susceptibility genes in *Candida glabrata* by profiling hermes transposon insertions. *G3* (Bethesda) 2020; 10(10):3859-3870. doi:10.1534/g3.120.401595

Galocha M, Pais P, Cavalheiro M, Pereira D, Viana R, Teixeira MC. Divergent approaches to virulence in *C. albicans* and *C. glabrata*: two sides of the same coin. *Int J Mol Sci* 2019; 20(9):2345. doi:10.3390/ijms20092345

Gammon DB. *Caenorhabditis elegans* as an emerging model for virus-host interactions. *J Virol* 2017; 91:e00509-17. doi:10.1128/JVI.00509-17

García-Carnero LC, Clavijo-Giraldo DM, Gómez-Gaviria M, et al. Early virulence predictors during the *Candida* Species-*Galleria mellonella* interaction. *J Fungi* (Basel) 2020; 6(3):152. doi:10.3390/jof6030152

Ghannoum MA. Potential role of phospholipases in virulence and fungal pathogenesis. *Clin Microbiol Rev* 2000; 13(1):122-143. doi:10.1128/CMR.13.1.122-143.2000

Giacomotto J, Ségalat L. High-throughput screening and small animal models, where are we? *Br J Pharmacol* 2010; 160:204-16. doi:10.1111/j.1476-5381.2010.00725.x

Gil-Alonso S, Jauregizar N, Cantón E, Eraso E, Quindósa G. In Vitro fungicidal activities of anidulafungin, caspofungin, and micafungin against *Candida glabrata*, *Candida bracarensis*, and *Candida nivariensis* evaluated by time-kill studies. *Antimicrob Agents Chemother* 2015; 59(6):3615-3618. doi:10.1128/AAC.04474-14

Glavis-Bloom J, Muhammed M, Mylonakis E. Of model hosts and man: using *Caenorhabditis elegans*, *Drosophila melanogaster* and *Galleria mellonella* as model hosts for infectious disease research. *Adv Exp Med Biol* 2012; 710:11-7. doi:10.1007/978-1-4419-5638-5_2

Gómez-Molero E, de Boer AD, Dekker HL, et al. Proteomic analysis of hyperadhesive *Candida glabrata* clinical isolates reveals a core wall proteome and differential incorporation of adhesins. *FEMS Yeast Res* 2015; 15(8):fov098. doi:10.1093/femsyr/fov098

Grazziotin LR, Moreira LB, Ferreira MAP. Comparative effectiveness and safety between amphotericin B lipid formulations: A systematic review. *Int J Technol Assess Health Care* 2018; 34(3):343-351. doi:10.1017/S026646231800034X

Gu W, Yu Q, Yu C, Sun S. In vivo activity of fluconazole/tetracycline combinations in *Galleria mellonella* with resistant *Candida albicans* infection. *J Glob Antimicrob Resist* 2018; 13:74-80. doi:10.1016/j.jgar.2017.11.011

Hamill RJ. Amphotericin B formulations: A comparative review of efficacy and toxicity. *Drugs* 2013; 73(9):919-934. doi:10.1007/s40265-013-0069-4

Healey KR, Nagasaki Y, Zimmerman M, et al. The gastrointestinal tract is a major source of echinocandin drug resistance in a murine model of *Candida glabrata* colonization and systemic dissemination. *Antimicrob Agents Chemother* 2017; 61(12):1-12. doi:10.1128/AAC.01412-17

Healey KR, Perlin DS. Fungal resistance to echinocandins and the MDR phenomenon in *Candida glabrata*. *J Fungi* (Basel) 2018; 4(3):105. doi:10.3390/jof4030105

Hernandez RJ, Hesse E, Dowling AJ, et al. Using the wax moth larva *Galleria mellonella* infection model to detect emerging bacterial pathogens. *PeerJ* 2019; 6:e6150. doi:10.7717/peerj.6150

Hernando-Ortiz A, Mateo E, Ortega-Riveros M, De-la-Pinta I, Quindós G, Eraso E. *Caenorhabditis elegans* as a model system to assess *Candida glabrata*, *Candida nivariensis*, and *Candida bracarensis* virulence and antifungal efficacy. *Antimicrob Agents Chemother* 2020; 64(10):e00824-20. doi:10.1128/AAC.00824-20

Hernando-Ortiz A, Mateo E, Perez-Rodriguez A, de Groot PWJ, Quindós G, Eraso E. Virulence of *Candida auris* from different clinical origins in *Caenorhabditis elegans* and *Galleria mellonella* host models. *Virulence* 2021; 12(1):1063-1075. doi:10.1080/21505594.2021.1908765

Hirayama T, Miyazaki T, Ito Y, et al. Virulence assessment of six major pathogenic *Candida* species in the mouse model of invasive candidiasis caused by fungal translocation. *Sci Rep* 2020; 10(1):3814. doi:10.1038/s41598-020-60792-y

Horton M V., Johnson CJ, Kernien JF, et al. *Candida auris* forms high-burden biofilms in skin niche conditions and on porcine skin. *mSphere* 2020; 5(1):1-8. doi:10.1128/msphere.00910-19

Hou X, Xiao M, Chen SCA, et al. Identification and antifungal susceptibility profiles of *Candida nivariensis* and *Candida bracarensis* in a multi-center Chinese collection of yeasts. *Front Microbiol* 2017; 8:5. doi:10.3389/fmicb.2017.00005

Howard SJ, Livermore J, Sharp A, et al. Pharmacodynamics of echinocandins against *Candida glabrata*: Requirement for dosage escalation to achieve maximal antifungal activity in neutropenic hosts. *Antimicrob Agents Chemother* 2011; 55(10): 4880-4887. doi.org/10.1128/AAC.00621-11

Huang XW, Xu MN, Zheng HX, et al. Pre-exposure to *Candida glabrata* protects *Galleria mellonella* against subsequent lethal fungal infections. *Virulence* 2020; 11(1):1674-1684. doi:10.1080/21505594.2020.1848107

Hull CM, Bader O, Parker JE, et al. Two clinical isolates of *Candida glabrata* exhibiting reduced sensitivity to amphotericin B both harbor mutations in ERG2. *Antimicrob Agents Chemother* 2012; 56(12):6417-21. doi:10.1128/AAC.01145-12

Iguchi S, Itakura Y, Yoshida A, et al. *Candida auris* : A pathogen difficult to identify, treat, and eradicate and its characteristics in Japanese strains. *J Infect Chemother* 2019; 25(10):743-749. doi:10.1016/j.jiac.2019.05.034

Jeffery-Smith A, Taori SK, Schelenz S, et al. *Candida auris*: A review of the literature. *Clin Microbiol Rev* 2018; 31(1):1-18. doi:10.1128/CMR.00029-17

Jensen RH, Astvad KMT, Silva LV, et al. Stepwise emergence of azole, echinocandin and amphotericin B multidrug resistance in vivo in *Candida albicans* orchestrated by multiple genetic alterations. *J Antimicrob Chemother* 2015; 70(9):2551-2555. doi:10.1093/jac/dkv140

Jiang H, Chen K, Sandoval LE, Leung C, Wang D. An evolutionarily conserved pathway essential for orsay virus infection of *Caenorhabditis elegans*. *mBio* 2017; 8(5):e00940-17. doi:10.1128/mBio.00940-17

Johnson CJ, Davis JM, Huttenlocher A, Kernien JF, Nett JE. Emerging fungal pathogen *Candida auris* evades neutrophil attack. *mBio* 2018; 9(4):e01403-18. doi:10.1128/mBio.01403-18

Kaan Ö, Koç AN, Atalay MA, Mutlu Sarigüzel F. Molecular epidemiology, antifungal susceptibility and virulence factors of *Candida glabrata* complex strains in Kayseri/Turkey. *Microb Pathog* 2021; 154:104870. doi:10.1016/j.micpath.2021.104870

Kalaiarasan K, Singh R, Chaturvedula L. Changing virulence factors among vaginal non-*albicans Candida* species. *Indian J Med Microbiol* 2018; 36(3):364-368. doi:10.4103/ijmm.IJMM_18_94

Kalantar E, Assadi M, Pormazaheri H, et al. *Candida* non *albicans* with a high amphotericin B resistance pattern causing candidemia among cancer patients. *Asian Pacific J Cancer Prev* 2014; 15(24):10933-10935. doi:10.7314/APJCP.2014.15.24.10933

Karkowska-Kuleta J, Rapala-Kozik M, Kozik A. Fungi pathogenic to humans: Molecular bases of virulence of *Candida albicans*, *Cryptococcus neoformans* and *Aspergillus fumigatus*. *Acta Biochim Pol* 2009; 56(2):211-224. doi:10.18388/abp.2009_2452

Kasper L, Seider K, Hube B. Intracellular survival of *Candida glabrata* in macrophages: Immune evasion and persistence. *FEMS Yeast Res* 2015; 15(5):1-12. doi:10.1093/femsyr/fov042

Kasper L, Seider K, Gerwien F, et al. Identification of *Candida glabrata* genes involved in pH modulation and modification of the phagosomal environment in macrophages. *PLoS One* 2014; 9(5):e96015. doi:10.1371/journal.pone.0096015

Kathuria S, Singh PK, Sharma C, et al. Multidrug-Resistant *Candida auris* Misidentified as *Candida haemulonii*: Characterization by Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry and DNA Sequencing and its antifungal susceptibility profile variability by Vitek 2, CLSI Broth Microdilution,

and Etest Method. *J Clin Microbiol* 2015; 53(6):1823-30. doi:10.1128/JCM.00367-15

Katiyar SK, Alastruey-Izquierdo A, Healey KR, Johnson ME, Perlin DS, Edlind TD. Fks1 and Fks2 are functionally redundant but differentially regulated in *Candida glabrata*: implications for echinocandin resistance. *Antimicrob Agents Chemother* 2012; 56(12):6304-6309. doi:10.1128/AAC.00813-12

Kaur R, Ma B, Cormack BP. A family of glycosylphosphatidylinositol-linked aspartyl proteases is required for virulence of *Candida glabrata*. *Proc Natl Acad Sci U S A* 2007; 104(18):7628-33. doi:10.1073/pnas.0611195104

Kean R, Brown J, Gulmez D, Ware A, Ramage G. *Candida auris*: A decade of understanding of an enigmatic pathogenic yeast. *J Fungi* (Basel) 2020; 6(1):30. doi:10.3390/jof6010030

Kent ML, Sanders JL, Spagnoli S, Al-Samarrie CE, Murray KN. Review of diseases and health management in zebrafish *Danio rerio* (Hamilton 1822) in research facilities. *J Fish Dis* 2020; 43(6):637-650. doi:10.1111/jfd.13165

Kenters N, Kiernan M, Chowdhary A, et al. Control of *Candida auris* in healthcare institutions: Outcome of an International Society for Antimicrobial Chemotherapy expert meeting. *Int J Antimicrob Agents* 2019; 54(4):400-406. doi:10.1016/j.ijantimicag.2019.08.013

Khan ZU, Ahmad S, Al-Obaid I, Al-Sweih NA, Joseph L, Farhat D. Emergence of resistance to amphotericin B and triazoles in *Candida glabrata* vaginal isolates in a case of recurrent vaginitis. *J Chemother* 2008; 20(4):488-491. doi:10.1179/joc.2008.20.4.488

Khan Z, Ahmad S, Al-Sweih N, Joseph L, Alfouzan W, Asadzadeh M. Increasing prevalence, molecular characterization and antifungal drug susceptibility of serial *Candida auris* isolates in Kuwait. *PLoS One* 2018; 13(4):1-12. doi:10.1371/journal.pone.0195743

Killiny N. Generous hosts: Why the larvae of greater wax moth, *Galleria mellonella* is a perfect infectious host model?. *Virulence* 2018; 9(1):860-865. doi:10.1080/21505594.2018.1454172

Kim MN, Shin JH, Sung H, et al. *Candida haemulonii* and closely related species at 5 university hospitals in Korea: identification, antifungal susceptibility, and clinical features. *Clin Infect Dis* 2009, 48: e57–61. doi:10.1086/597108

Kim GH, Rosiana S, Kirienko NV, Shapiro RS. A simple nematode infection model for studying *Candida albicans* pathogenesis. *Curr Protoc Microbiol* 2020; 59(1):e114. doi:10.1002/cpmc.114

Ko JH, Peck KR, Jung DS, et al. Impact of high MIC of fluconazole on outcomes of *Candida glabrata* bloodstream infection: a retrospective multicenter cohort study.

Diagn Microbiol Infect Dis 2018; 92(2):127-132. doi:10.1016/j.diagmicrobio.2018.05.001

Kołaczkowska A, Kołaczkowski M. Drug resistance mechanisms and their regulation in non-*albicans Candida* species. *J Antimicrob Chemother* 2016; 71(6):1438-50. doi:10.1093/jac/dkv445

Kordalewska M, Zhao Y, Lockhart SR, Chowdhary A, Berrio I, Perlin DS. Rapid and accurate molecular identification of the emerging multidrug-resistant pathogen *Candida auris*. *J Clin Microbiol* 2017; 55:2445–2452. doi:10.1128/JCM.00630-17

Kramer A, Schwebke I, Kampf G. How long do nosocomial pathogens persist on inanimate surfaces? A systematic review. *BMC Infect Dis* 2006; 6:1-8. doi:10.1186/1471-2334-6-130

Krcmery V, Barnes AJ. Non-*albicans Candida* spp. causing fungaemia: pathogenicity and antifungal resistance. *J Hosp Infect* 2002; 50(4):243-260. doi:10.1053/jhin.2001.1151

Krogh-Madsen M, Arendrup MC, Heslet L, Knudsen JD. Amphotericin B and caspofungin resistance in *Candida glabrata* isolates recovered from a critically ill patient. *Clin Infect Dis* 2006; 42(7):938-944. doi:10.1086/500939

Kucharíková S, Gerits E, De Brucker K, et al. Covalent immobilization of antimicrobial agents on titanium prevents Staphylococcus aureus and *Candida albicans* colonization and biofilm formation. *J Antimicrob Chemother* 2016; 71, 936–945. doi:10.1093/jac/dkv437

Kumar D, Banerjee T, Pratap CB, Tilak R. Itraconazole-resistant *Candida auris* with phospholipase, proteinase and hemolysin activity from a case of vulvovaginitis. *J Infect Dev Ctries* 2015; 9(4):435-7. doi:10.3855/jidc.4582

Kumar A, Sachu A, Mohan K, Vinod V, Dinesh K, Karim S. Simple low cost differentiation of *Candida auris* from *Candida haemulonii* complex using CHROMagar Candida medium supplemented with Pal's medium. *Rev Iberoam Micol* 2017, 34:109-111. doi:10.1016/j.riam.2016.11.004

Kumar K, Askari F, Sahu MS, Kaur R. *Candida glabrata*: A lot more than meets the eye. *Microorganisms* 2019; 7(2):39. doi:10.3390/microorganisms7020039

L'Ollivier C, Labruère C, Jebrane A, et al. Using a Multi-Locus Microsatellite Typing method improved phylogenetic distribution of *Candida albicans* isolates but failed to demonstrate association of some genotype with the commensal or clinical origin of the isolates. *Infect Genet Evol* 2012; 12(8):1949-57. doi:10.1016/j.meegid.2012.07.025

Lamoth F, Lockhart SR, Berkow EL, Calandra T. Changes in the epidemiological landscape of invasive candidiasis. *J Antimicrob Chemother* 2018; 73:i4-i13. doi:10.1093/jac/dkx444

Laniado-Laborín R, Cabrales-Vargas MN. Amphotericin B: Side effects and toxicity. *Rev Iberoam Micol* 2009; 26: 223-227. doi:10.1016/j.riam.2009.06.003

Lara HH, Ixtepan-Turrent L, Jose Yacaman M, Lopez-Ribot J. Inhibition of Candida *auris* biofilm formation on medical and environmental surfaces by silver nanoparticles. *ACS Appl Mater Interfaces* 2020; 12(19):21183-21191. doi:10.1021/acsami.9b20708

Larkin E, Hager C, Chandra J, et al. The Emerging Pathogen *Candida auris*: growth phenotype, virulence factors, activity of antifungals, and effect of SCY-078, a novel glucan synthesis inhibitor, on growth morphology and biofilm formation. *Antimicrob Agents Chemother* 2017; 61(5):e02396-16. doi:10.1128/AAC.02396-16

Ledoux MP, Denis J, Nivoix Y, Herbrecht R. Isavuconazole: A new broad-spectrum azole. Part 2: pharmacokinetics and clinical activity. *J Mycol Med* 2018; 28(1):15-22. doi:10.1016/j.mycmed.2018.02.002

Lee YJ, Jang HJ, Chung IY, Cho YH. *Drosophila melanogaster* as a polymicrobial infection model for *Pseudomonas aeruginosa* and *Staphylococcus aureus*. *J Microbiol* 2018; 56(8):534-541. doi:10.1007/s12275-018-8331-9

Lepak AJ, Zhao M, Berkow EL, Lockhart SR, Andes DR. Pharmacodynamic optimization for treatment of invasive *Candida auris* infection. *Antimicrob Agents Chemother* 2017; 61(8):1-5. doi:10.1128/AAC.00791-17

Levraud JP, Disson O, Kissa K, et al. Real-time observation of *Listeria monocytogenes*-phagocyte interactions in living zebrafish larvae. *Infect Immun* 2009; 77(9):3651-3660. doi:10.1128/IAI.00408-09

Lewis JS 2nd, Wiederhold NP, Wickes BL, Patterson TF, Jorgensen JH. Rapid emergence of echinocandin resistance in *Candida glabrata* resulting in clinical and microbiologic failure. *Antimicrob Agents Chemother* 2013; 57(9):4559-61. doi:10.1128/AAC.01144-13

Li YJ, Hu B. Establishment of multi-site infection model in zebrafish larvae for studying *Staphylococcus aureus* infectious disease. *J Genet Genomics* 2012; 39(9):521-534. doi:10.1016/j.jgg.2012.07.006

Li DD, Deng L, Hu GH, et al. Using *Galleria mellonella-Candida albicans* infection model to evaluate antifungal agents. *Biol Pharm Bull* 2013; 36(9):1482-7. doi:10.1248/bpb.b13-00270

Li J, Shan Y, Fan S, Liu X. Prevalence of *Candida nivariensis* and *Candida bracarensis* in vulvovaginal candidiasis. *Mycopathologia* 2014; 178(3-4):279-283. doi:10.1007/s11046-014-9800-2

Li X, Zhao Y, Huang X, Yu C, Yang Y, Sun S. Ambroxol hydrochloride combined with fluconazole reverses the resistance of *Candida albicans* to fluconazole. *Front Cell Infect Microbiol* 2017; 7:124. doi:10.3389/fcimb.2017.00124

Lima SL, Rossato L, Salles de Azevedo Melo A. Evaluation of the potential virulence of *Candida haemulonii* species complex and *Candida auris* isolates in *Caenorhabditis elegans* as an in vivo model and correlation to their biofilm production capacity. *Microb Pathog* 2020; 148:104461. doi:10.1016/j.micpath.2020.104461

Lockhart SR, Messer SA, Gherna M, et al. Identification of *Candida nivariensis* and *Candida bracarensis* in a large global collection of *Candida glabrata* isolates: Comparison to the literature. *J Clin Microbiol* 2009; 47(4):1216-7. doi:10.1128/JCM.02315-08

Lockhart SR, Iqbal N, Cleveland AA, Farley MM, et al. Species identification and antifungal susceptibility testing of *Candida* bloodstream isolates from population-based surveillance studies in two U.S. cities from 2008 to 2011. *J Clin Microbiol* 2012; 50(11):3435-42. doi:10.1128/JCM.01283-12

Lockhart SR, Etienne KA, Vallabhaneni S, et al. Simultaneous emergence of multidrug-resistant *Candida auris* on 3 continents confirmed by whole-genome sequencing and epidemiological analyses. *Clin Infect Dis* 2017; 64:134-140. doi:10.1093/cid/ciw691

Lockhart SR. *Candida auris* and multidrug resistance: Defining the new normal. *Fungal Genet Biol* 2019; 131:103243. doi:10.1016/j.fgb.2019.103243

Lone SA, Ahmad A. *Candida auris*—the growing menace to global health. *Mycoses* 2019; 62(8):620-637. doi:10.1111/myc.12904

López-Ávila K, Dzul-Rosado KR, Lugo-Caballero C, et al. Mecanismos de resistencia antifúngica de los azoles en *Candida albicans*. *Rev Biomed* 2016; 27(490):127-136.

López-Soria LM, Bereciartua E, Santamaría M, et al. Primer caso de fungemia asociada a catéter por *Candida nivariensis* en la Península Ibérica. *Rev Iberoam Micol* 2013; 30(1):69-71. doi:10.1016/j.riam.2012.09.001

Lotfalikhani A, Khosravi Y, Sabet NS, Na SL, Ng KP, Tay ST. Genetic diversity, antifungal susceptibility and enzymatic characterisation of Malaysian clinical isolates of *Candida glabrata*. *Trop Biomed* 2018; 35(4):1123-1130. PMID: 33601859

Lu M, Yu C, Cui X, Shi J, Yuan L, Sun S. Gentamicin synergises with azoles against drug-resistant Candida albicans. *Int J Antimicrob Agents* 2018; 51(1):107-114. doi:10.1016/j.ijantimicag.2017.09.012

Luo G, Samaranayake LP, Yau JY. *Candida* species exhibit differential in vitro hemolytic activities. *J Clin Microbiol* 2001; 39:2971–2974. doi.org/10.1128/JCM.39.8.2971-2974.2001.

Maertens JA. History of the development of azole derivatives. *Clin Microbiol Infect* 2004; 10(1):1-10. doi:10.1111/j.1470-9465.2004.00841.x

Maglioni S, Arsalan N, Ventura N. *C. elegans* screening strategies to identify pro-longevity interventions. *Mech Ageing Dev* 2016; 157:60-9. doi:10.1016/j.mad.2016.07.010

Małek M, Mrowiec P, Klesiewicz K, et al. Prevalence of human pathogens of the clade *Nakaseomyces* in a culture collection—the first report on *Candida bracarensis* in Poland. *Folia Microbiol* (Praha) 2019; 64:307-312. doi:10.1007/s12223-018-0655-7

Manns JM, Mosser DM, Buckley HR. Production of a hemolytic factor by *Candida albicans*. *Infect Immun* 1994; 62:5154-5156. doi.org/10.1128/IAI.62.11.5154-5156.1994

Marcos-Zambrano LJ, Bordallo-Cardona MÁ, Borghi E, et al. *Candida* isolates causing candidemia show different degrees of virulence in *Galleria mellonella*. *Med Mycol* 2020; 58(1):83-92. doi:10.1093/mmy/myz027

Mariné M, Serena C, Pastor FJ, Guarro J. Combined antifungal therapy in a murine infection by *Candida glabrata*. *J Antimicrob Chemother* 2006; 58:1295-1298. doi.org/10.1093/jac/dkl395.

Martin A, Rex EA, Ishidate T, Lin R, Gammon DB. Infection of *Caenorhabditis elegans* with vesicular stomatitis virus via microinjection. *Bio Protoc* 2017; 7(22):e2617. doi:10.21769/BioProtoc.2617

Mashaly G, Shrief R. *Candida glabrata* complex from patients with healthcare-associated infections in Mansoura University Hospitals, Egypt: distribution, antifungal susceptibility and effect of fluconazole and polymyxin B combination. *Germs* 2019; 9(3):125-132. doi:10.18683/germs.2019.1167

Matsumoto Y, Semikizu K. Silkworm as an experimental animal for research on fungal infections. *Microbiol Immunol* 2019; 63:41-50. doi:10.1111/1348-0421.12668

Maurer E, Hörtnagl C, Lackner M, et al. *Galleria mellonella* as a model system to study virulence potential of mucormycetes and evaluation of antifungal treatment. *Med Mycol* 2019; 57(3):351-362. doi:10.1093/mmy/myy042

McGonigle JE, Purves J, Rolff J. Intracellular survival of *Staphylococcus aureus* during persistent infection in the insect *Tenebrio molitor*. *Dev Comp Immunol* 2016; 59:34-8. doi:10.1016/j.dci.2016.01.002

Meeker ND, Trede NS. Immunology and zebrafish: Spawning new models of human disease. *Dev Comp Immunol* 2008; 32:745-57. doi:10.1016/j.dci.2007.11.011

Meijer AH, Spaink HP. Host-pathogen interactions made transparent with the zebrafish model. *Curr Drug Targets* 2011; 12:1000-17. doi:10.2174/138945011795677809

Mesa-Arango AC, Forastiero A, Bernal-Martínez L, et al. The non-mammalian host *Galleria mellonella* can be used to study the virulence of the fungal pathogen *Candida tropicalis* and the efficacy of antifungal drugs during infection by this pathogenic yeast. *Med Mycol* 2013; 51:461–472. doi.org/10.3109/13693786.2012.737031

Michl TD, Giles C, Mocny P, et al. Caspofungin on ARGET-ATRP grafted PHEMA polymers: enhancement and selectivity of prevention of attachment of *Candida albicans*. *Biointerphases* 2017; 12:05G602. doi:10.1116/1.4986054

Miranda-Zapico I, Eraso E, Hernández-Almaraz JL, et al. Prevalence and antifungal susceptibility patterns of new cryptic species inside the species complexes *Candida parapsilosis* and *Candida glabrata* among blood isolates from a Spanish tertiary hospital. *J Antimicrob Chemother* 2011; 66(10):2315-2322. doi:10.1093/jac/dkr298

Mishra P, Gupta P, Pruthi V. Cinnamaldehyde incorporated gellan/PVA electrospun nanofibers for eradicating *Candida* biofilm. *Mater Sci Eng C Mater Biol Appl* 2021; 119:111450. doi:10.1016/j.msec.2020.111450

Mita K, Kasahara M, Sasaki S, et al. The genome sequence of silkworm, *Bombyx mori*. *DNA Res* 2004; 11:27-35. doi:10.1093/dnares/11.1.27

Mohammad H, Eldesouky HE, Hazbun T, Mayhoub AS, Seleem MN. Identification of a Phenylthiazole small molecule with dual antifungal and antibiofilm activity against *Candida albicans* and *Candida auris*. *Sci Rep* 2019; 9(1):18941. doi:10.1038/s41598-019-55379-1

Morales-López S, Dudiuk C, Vivot W, Szusz W. Phenotypic and molecular evaluation of echinocandin susceptibility of *Candida*. *Antimicrob Agents Chemother* 2017; 61(7):7-10. doi:10.1128/AAC.00170-17

Moreira A, Silva S, Botelho C, Sampaio P, Pais C, Henriques M. *Candida bracarensis*: Evaluation of virulence factors and its tolerance to amphotericin B and fluconazole. *Mycopathologia* 2015; 180:305–315. doi:10.1007/s11046-015-9925-y

Moy TI, Conery AL, Larkins-Ford J, et al. High-throughput screen for novel antimicrobials using a whole animal infection model. *ACS Chem Biol* 2009; 4(7):527-33. doi:10.1021/cb900084v

Moya-Andérico L, Vukomanovic M, Cendra MDM, et al. Utility of *Galleria mellonella* larvae for evaluating nanoparticle toxicology. *Chemosphere* 2021; 266:129235. doi:10.1016/j.chemosphere.2020.129235

Mukherjee PK, Sheehan DJ, Hitchcock CA, Ghannoum MA. Combination treatment of invasive fungal infections. *Clin Microbiol Rev* 2005; 18(1):163-194. doi:10.1128/CMR.18.1.163

Mulet Bayona JV, Tormo Palop N, Salvador García C, et al. Characteristics and Management of Candidaemia Episodes in an Established *Candida auris* Outbreak. *Antibiotics* (Basel) 2020; 9(9):E558. doi:10.3390/antibiotics9090558

Muñoz JF, Gade L, Chow NA, Loparev VN, Juieng P, Berkow EL, Farrer RA, Litvintseva AP, Cuomo CA. Genomic insights into multidrug-resistance, mating and virulence in *Candida auris* and related emerging species. *Nat Commun* 2018; 9(1):5346. doi:10.1038/s41467-018-07779-6

Muñoz JE, Ramirez LM, Dias LDS, et al. Pathogenicity levels of Colombian strains of *Candida auris* and Brazilian strains of *Candida haemulonii* species complex in both murine and *Galleria mellonella* experimental models. *J Fungi* (Basel). 2020, 6(3):E104. doi:10.3390/jof6030104

Nagy F, Tóth Z, Nyikos F, et al. In vitro and in vivo interaction of caspofungin with isavuconazole against *Candida auris* planktonic cells and biofilms. *Med Mycol* 2021; 22:myab032. doi:10.1093/mmy/myab032

Nile C, Falleni M, Cirasola D, et al. Repurposing pilocarpine hydrochloride for treatment of *Candida albicans* infections. *mSphere* 2019; 4(1):e00689-18. doi:10.1128/mSphere.00689-18

Ninan MM, Sahni RD, Chacko B, Balaji V, Michael JS. *Candida auris:* Clinical profile, diagnostic challenge and susceptibility pattern: Experience from a tertiary-care centre in South India. *J Glob Antimicrob Resist* 2020; 21:181-185. doi:10.1016/j.jgar.2019.10.018

Nishi I, Sunada A, Toyokawa M, Asari S, Iwatani Y. In vitro antifungal combination effects of micafungin with fluconazole, voriconazole, amphotericin B, and flucytosine against clinical isolates of *Candida species. J Infect Chemother* 2009; 15(1):1-5. doi:10.1007/s10156-008-0653-9

O'Brien B, Chaturvedi S, Chaturvedi V. In vitro evaluation of antifungal drug combinations against multidrug-resistant *Candida auris* isolates from New York outbreak. *Antimicrob Agents Chemother* 2020; 64(4):1-14. doi:10.1128/AAC.02195-19

Oksanen KE, Halfpenny NJA, Sherwood E, et al. An adult zebrafish model for preclinical tuberculosis vaccine development. *Vaccine* 2013; 31(45):5202-5209. doi:10.1016/j.vaccine.2013.08.093

Oksuz S, Sahin I, Yildirim M, et al. Phospholipase and proteinase activities in different *Candida* species isolated from anatomically distinct sites of healthy adults. *Jpn J Infect Dis* 2007; 60(5):280-3. PMID: 17881867

Oliveira ER, Fothergill AW, Kirkpatrick WR, Coco BJ, Patterson TF, Redding SW. In vitro interaction of posaconazole and caspofungin against clinical isolates of *Candida glabrata*. *Antimicrob Agents Chemother* 2005; 49(8):3544-5. doi:10.1128/AAC.49.8.3544-3545.2005

Ortega-Riveros M, De-la-Pinta I, Marcos-Arias C, Ezpeleta G, Quindós G, Eraso E. Usefulness of the non-conventional *Caenorhabditis elegans* model to assess *Candida* virulence. *Mycopathologia* 2017; 182:785-95. doi:10.1007/s11046-017-0142-8

Osmanov A, Wise A, Denning DW. In vitro and in vivo efficacy of miramistin against drug-resistant fungi. *J Med Microbiol* 2019; 68(7):1047-1052. doi:10.1099/jmm.0.001007

Ostrowsky B, Greenko J, Adams E, et al. *C. auris* Investigation Work Group. *Candida auris* isolates resistant to three classes of antifungal medications - New York, 2019. *MMWR Morb Mortal Wkly Rep* 2020; 69(1):6-9. doi:10.15585/mmwr.mm6901a2

Padder SA, Prasad R, Shah AH. Quorum sensing: A less known mode of communication among fungi. *Microbiol Res* 2018, 210:51-58. doi:10.1016/j.micres.2018.03.007

Pais P, Galocha M, Viana R, et al. Microevolution of the pathogenic yeasts *Candida albicans* and *Candida glabrata* during antifungal therapy and host infection. *Microb Cell* 2019; 6(3):142-159. doi:10.15698/mic2019.03.670

Panthee S, Paudel A, Hamamoto H, Semikizu K. Advantages of the silkworm as an animal model for developing novel antimicrobial agents. *Front Microbiol* 2017; 8:373. doi:10.3389/fmicb.2017.00373

Peeters E, Nelis HJ, Coenye T. Comparison of multiple methods for quantification of microbial biofilms grown in microtiter plates. *J Microbiol Methods* 2008; 72(2):157-165. doi:10.1016/j.mimet.2007.11.010

Pemán J, Cantón E, Camarena Miñana JJ, et al. Changes in the epidemiology of fungaemia and fluconazole susceptibility of blood isolates during the last 10 years in Spain: Results from the FUNGEMYCA study. *Rev Iberoam Micol* 2011; 28(2):91-99. doi:10.1016/j.riam.2011.02.005

Pemán J, Cantón E, Quindós G, et al. Epidemiology, species distribution and in vitro antifungal susceptibility of fungaemia in a Spanish multicentre prospective survey. *J Antimicrob Chemother* 2012; 67(5):1181-1187. doi:10.1093/jac/dks019

Pemán J, Salavert M. Epidemiología general de la enfermedad fúngica invasora. *Enferm Infecc Microbiol Clin* 2012; 30(2):90-98. doi:10.1016/j.eimc.2011.09.004

Perini HF, Moralez ATP, Almeida RSC, et al. Phenotypic switching in *Candida tropicalis* alters host-pathogen interactions in a *Galleria mellonella* infection model. *Sci Rep* 2019; 9(1):12555. doi:10.1038/s41598-019-49080-6

Perlin DS, Rautemaa-Richardson R, Alastruey-Izquierdo A. The global problem of antifungal resistance: prevalence, mechanisms, and management. *Lancet Infect Dis* 2017; 17(12):e383-e392. doi:10.1016/S1473-3099(17)30316-X

Peterson ND, Pukkila-Worley R. *Caenorhabditis elegans* in high-throughput screens for anti-infective compounds. *Curr Opin Immunol* 2018; 54:59-65. doi:10.1016/j.coi.2018.06.003

Pfaller MA, Messer SA, Hollis RJ, Boyken L, Tendolkar S, Kroeger J, Diekema DJ. Variation in susceptibility of bloodstream isolates of *Candida glabrata* to fluconazole according to patient age and geographic location in the United States in 2001 to 2007. *J Clin Microbiol* 2009; 47(10):3185-90. doi:10.1128/JCM.00946-09

Pfaller MA, Castanheira M, Lockhart SR, Jones RN. *Candida glabrata*: Multidrug resistance and increased virulence in a major opportunistic fungal pathogen. *Curr Fungal Infect Rep* 2012; 6:154-164. doi:10.1007/s12281-012-0091-0

Pfaller MA, Messer SA, Woosley LN, Jones RN, Castanheira M. Echinocandin and triazole antifungal susceptibility profiles for clinical opportunistic yeast and mold isolates collected from 2010 to 2011: Application of new CLSI clinical breakpoints and epidemiological cutoff values for characterization of geographic and temporal trends of antifungal resistance. *J Clin Microbiol* 2013; 51(8):2571-2581. doi:10.1128/JCM.00308-13

Pfaller MA, Diekema DJ, Turnidge JD, Castanheira M, Jones RN. Twenty years of the SENTRY Antifungal Surveillance Program: Results for *Candida* species from 1997-2016. *Open Forum Infect Dis* 2019; 6(1):79. doi:10.1093/ofid/ofy358

Pfaller MA, Messer SA, Deshpande LM, Rhomberg PR, Utt EA, Castanheira M. Evaluation of synergistic activity of isavuconazole or voriconazole plus anidulafungin and the occurrence and genetic characterization of *Candida auris* detected in a surveillance program. *Antimicrob Agents Chemother* 2021; 65(4):e02031-20. doi:10.1128/AAC.02031-20

Pham CD, Iqbal N, Bolden CB, et al. Role of FKS mutations in *Candida glabrata*: MIC values, echinocandin resistance, and multidrug resistance. *Antimicrob Agents Chemother* 2014; 58(8):4690-4696. doi:10.1128/AAC.03255-14

Pierce CG, Chaturvedi AK, Lazzell AL, et al. A novel small mole-cule inhibitor of *Candida albicans* biofilm formation, filamentation and virulence with low potential for the development of resistance. *NPJ Biofilms Microbiomes* 2015; 1:15012. doi:10.1038/npjbiofilms.2015.12

Polak A. Virulence of *Candida albicans* mutants. *Mycoses* 1992; 35(1-2):9-16. doi:10.1111/j.1439-0507.1992.tb00813.x

Price MF, Wilkinson ID, Gentry LO. Plate method for detection of phospholipase activity in *Candida albicans*. *Sabouraudia* 1982; 20(1):7-14. doi:10.1080/00362178285380031

Pristov KE, Ghannoum MA. Resistance of *Candida* to azoles and echinocandins worldwide. *Clin Microbiol Infect* 2019; 25(7):792-798. doi:10.1016/j.cmi.2019.03.028

Puig-Asensio M, Padilla B, Garnacho-Montero J, et al. Epidemiology and predictive factors for early and late mortality in *Candida* bloodstream infections: A population-based surveillance in Spain. *Clin Microbiol Infect* 2014; 20(4). doi:10.1111/1469-0691.12380

Pukkila-Worley R, Peleg AY, Tampakakis E, Mylonakis E. *Candida albicans* hyphal formation and virulence assessed using a *Caenorhabditis elegans* infection model. *Eukaryot Cell* 2009; 8:1750-8. doi:10.1128/EC.00163-09

Quindós G. Epidemiology of candidaemia and invasive candidiasis. A changing face. *Rev Iberoam Micol* 2014; 31(1):42-8. doi:10.1016/j.riam.2013.10.001

Quindós G, Marcos-Arias C, San-Millán R, et al. The continuous changes in the aetiology and epidemiology of invasive candidiasis: from familiar *Candida albicans* to multiresistant *Candida auris*. *Int Microbiol* 2018; 21(3):107-119. doi:10.1007/s10123-018-0014-1

Quindós G, Gil-Alonso S, Marcos-Arias C, et al. Therapeutic tools for oral candidiasis: Current and new antifungal drugs. *Med Oral Patol Oral Cir Bucal* 2019, 24(2):e172-e180. doi:10.4317/medoral.22978

Raja NS. Epidemiology, risk factors, treatment and outcome of *Candida* bloodstream infections because of *Candida albicans* and *Candida* non-*albicans* in two district general hospitals in the United Kingdom. *Int J Clin Pract* 2021; 75(1):e13655. doi:10.1111/ijcp.13655

Ramachandran R, Shrivastava M, Narayanan NN, Thakur RL, Chakrabarti A, Roy U. Evaluation of antifungal efficacy of three new cyclic lipopeptides of the class bacillomycin from *Bacillus subtilis* RLID 12.1. *Antimicrob Agents Chemother* 2017; 62(1):e01457-17. doi:10.1128/AAC.01457-17

Ramage G, VandeWalle K, Wickes BL, López-Ribot JL. Characteristics of biofilm formation by *Candida albicans*. *Rev Iberoam Micol* 2001; 18(4):163-170.

Ramos L de S, Barbedo LS, Braga-Silva LA, Santos ALS dos, Pinto MR, Sgarbi DB da G. Protease and phospholipase activities of *Candida* spp. isolated from cutaneous candidiasis. *Rev Iberoam Micol* 2015; 32(2):122-125. doi:10.1016/j.riam.2014.01.003

Reyes-Montes MDR, Acosta-Altamirano G, Duarte-Escalante E, et al. Usefulness of a multiplex PCR for the rapid identification of *Candida glabrata* species complex in mexican clinical isolates. *Rev Inst Med Trop Sao Paulo* 2019; 61:1-7. doi:10.1590/s1678-9946201961037

Rhodes J, Abdolrasouli A, Farrer RA, et al. Genomic epidemiology of the UK outbreak of the emerging human fungal pathogen *Candida auris*. *Emerg Microbes Infect* 2018; 7(1):43. doi:10.1038/s41426-018-0045-x

Rhodes J, Fisher MC. Global epidemiology of emerging *Candida auris. Curr Opin Microbiol* 2019; 52:84-89. doi:10.1016/j.mib.2019.05.008

Roberts JK, Stockmann C, Constance JE, et al. Pharmacokinetics and pharmacodynamics of antibacterials, antifungals, and antivirals used most frequently in neonates and infants. *Clin Pharmacokinet* 2014; 53(7):581-610. doi:10.1007/s40262-014-0147-0

Rodrigues CF, Silva S, Azeredo J, Henriques M. *Candida glabrata*'s recurrent infections: biofilm formation during Amphotericin B treatment. *Lett Appl Microbiol* 2016; 63(2):77-81. doi:10.1111/lam.12600

Romeo O, Scordino F, Pernice I, Lo Passo C, Criseo G. A multiplex PCR protocol for rapid identification of *Candida glabrata* and its phylogenetically related species

Candida nivariensis and *Candida bracarensis*. *J Microbiol Methods* 2009; 79(1):117-120. doi:10.1016/j.mimet.2009.07.016

Romera D, Aguilera-Correa JJ, Gadea I, et al. *Candida auris*: a comparison between planktonic and biofilm susceptibility to antifungal drugs. *J Med Microbiol* 2019; 68(9):1353-1358. doi:10.1099/jmm.0.001036.

Romera D, Aguilera-Correa JJ, García-Coca M, et al. The *Galleria mellonella* infection model as a system to investigate the virulence of *Candida auris* strains. *Pathog Dis* 2020; 78(9):ftaa067. doi:10.1093/femspd/ftaa067

Rossato L, Colombo AL. *Candida auris*: What have we learned about its mechanisms of pathogenicity?. *Front Microbiol* 2018; 9:3081. doi:10.3389/fmicb.2018.03081

Ruiz Gaitán AC, Moret A, López Hontangas JL, et al. Fungemia nosocomial por *Candida auris*: primeros cuatro casos en Europa continental. *Rev Iberoam Micol* 2017; 34(1):23-27. doi:10.1016/j.riam.2016.11.002

Ruiz-Gaitán A, Moret AM, Tasias-Pitarch M, et al. An outbreak due to *Candida auris* with prolonged colonisation and candidaemia in a tertiary care European hospital. *Mycoses* 2018; 61(7):498-505. doi:10.1111/myc.12781

Ruiz-Gaitán A, Martínez H, Moret AM, et al. Detection and treatment of *Candida auris* in an outbreak situation: risk factors for developing colonization and candidemia by this new species in critically ill patients. *Expert Rev Anti Infect Ther* 2019; 17(4):295-305. doi:10.1080/14787210.2019.1592675

Ryan P, Motherway C, Powell J, et a. Candidaemia in an Irish intensive care unit setting between 2004 and 2018 reflects increased incidence of *Candida glabrata*. *J Hosp Infect* 2019; 102(3):347-350. doi:10.1016/j.jhin.2019.01.017

Sá NP, Lima CM, A Dos Santos JR, et al. A phenylthiazole derivative demonstrates efficacy on treatment of the cryptococcosis & candidiasis in animal models. *Future Sci OA* 2018; 4(6):FSO305. doi:10.4155/fsoa-2018-0001

Sadeghi G, Ebrahimi-Rad M, Mousavi SF, et al. Emergence of non-*Candida albicans* species: Epidemiology, phylogeny and fluconazole susceptibility profile. *J Mycol Med* 2018; 28:51-58. doi:10.1016/j.mycmed.2017.12.008

Saha SS, Suzuki J, Uda A, Watanabe K, Shimizu T, Watarai M. Silkworm model for Francisella novicida infection. *Microb Pathog* 2017; 113:94-101. doi:10.1016/j.micpath.2017.10.036

Sandhya M, V A, Maneesha K S, Raja B, R J, S S. Amphotericin B loaded sulfonated chitosan nanoparticles for targeting macrophages to treat intracellular *Candida glabrata* infections. *Int J Biol Macromol* 2018; 110:133-139. doi:10.1016/j.ijbiomac.2018.01.028

Sanz-Landaluze J, Pena-Abaurrea M, Muñoz-Olivas R, Cámara C, Ramos L. Zebrafish (*Danio rerio*) eleutheroembryo-based procedure for assessing bioaccumulation. *Environ Sci Technol* 2015; 49(3):1860-9. doi:10.1021/es504685c

Saraya T, Tanabe K, Araki K, et al. Breakthrough invasive *Candida glabrata* in patients on micafungin: a novel FKS gene conversion correlated with sequential elevation of MIC. *J Clin Microbiol* 2014; 52(7):2709-12. doi:10.1128/JCM.03593-13

Sardi JCO, Scorzoni L, Bernardi T, et al. *Candida* species: Current epidemiology, pathogenicity, biofilm formation, natural antifungal products and new therapeutic options. *J Med Microbiol* 2013; 62(1):10-24. doi:10.1099/jmm.0.045054-0

Satoh K, Makimura K, Hasumi Y, Nishiyama Y, Uchida K, Yamaguchi H. *Candida auris* sp. nov., a novel ascomycetous yeast isolated from the external ear canal of an inpatient in a Japanese hospital. *Microbiol Immunol* 2009; 53:41-44. doi:10.1111/j.1348-0421.2008.00083.x.

Schifano E, Marazzato M, Ammendolia MG, et al. Virulence behavior of uropathogenic *Escherichia coli* strains in the host model *Caenorhabditis elegans*. *Microbiologyopen* 2019; 8(6):e00756. doi:10.1002/mbo3.756

Schneider J, Mateo E, Marcos-Arias C, et al. Antifungal activity of the human uterine cervical stem cells conditioned medium (hUCESC-CM) against *Candida albicans* and other medically relevant species of *Candida*. *Front Microbiol* 2018; 9:2818. doi:10.3389/fmicb.2018.02818

Schwarz P, Bidaud AL, Dannaoui E. In vitro synergy of isavuconazole in combination with colistin against *Candida auris*. *Sci Rep* 2020; 10(1):21448. doi.org/10.1038/s41598-020-78588-5

Scorzoni L, de Lucas MP, Mesa-Arango AC, et al. Antifungal efficacy during *Candida krusei* infection in non-conventional models correlates with the yeast in vitro susceptibility profile. *PLoS One* 2013; 8:3. doi.org/10.1371/journal.pone.0060047

Segal E, Frenkel M. Experimental in vivo models of candidiasis. *J Fungi* (Basel) 2018; 4.pii:E21. doi:10.3390/jof4010021

Seider K, Gerwien F, Kasper L, et al. Immune evasion, stress resistance, and efficient nutrient acquisition are crucial for intracellular survival of *Candida glabrata* within macrophages. *Eukaryot Cell* 2014; 13(1):170-83. doi:10.1128/EC.00262-13

Seneviratne CJ, Wong SS, Yuen KY, et al. Antifungal susceptibility and virulence attributes of bloodstream isolates of *Candida* from Hong Kong and Finland. *Mycopathologia* 2011; 172(5):389-95. doi:10.1007/s11046-011-9444-4

Shaban S, Patel M, Ahmad A. Improved efficacy of antifungal drugs in combination with monoterpene phenols against *Candida auris*. *Sci Rep* 2020; 10(1):1162. doi:10.1038/s41598-020-58203-3

Sharma C, Wankhede S, Muralidhar S, et al. *Candida nivariensis* as an etiologic agent of vulvovaginal candidiasis in a tertiary care hospital of New Delhi, India. *Diagn Microbiol Infect Dis* 2013; 76(1):46-50. doi:10.1016/j.diagmicrobio.2013.02.023

Sheehan G, Kavanagh K. Proteomic Analysis of the responses of *Candida albicans* during infection of *Galleria mellonella* larvae. *J Fungi* (Basel) 2019; 5(1):7. doi:10.3390/jof5010007

Shen C, Zuo Z. Zebrafish (*Danio rerio*) as an excellent vertebrate model for the development, reproductive, cardiovascular, and neural and ocular development toxicity study of hazardous chemicals. *Environ Sci Pollut Res Int* 2020; 27(35):43599-43614. doi:10.1007/s11356-020-10800-5

Sherry L, Ramage G, Kean R, et al. Biofilm-forming capability of highly virulent, multidrug-resistant *Candida auris*. *Emerg Infect Dis* 2017; 23(2):328-331. doi:10.3201/eid2302.161320

Shi Y, Zhu Y, Fan S, Vitagliano A, Liu X, Liao Y, Liang Y, Vitale S, G: Clinical characteristics and antifungal susceptibility of *Candida nivariensis* from vulvovaginal candidiasis. *Gynecol Obstet Invest* 2020; 85:88-93. doi.org/10.1159/000504095

Short B, Brown J, Delaney C, et al. *Candida auris* exhibits resilient biofilm characteristics in vitro: implications for environmental persistence. *J Hosp Infect* 2019; 103(1):92-96. doi:10.1016/j.jhin.2019.06.006

Soo Hoo L. Fungal fatal attraction: a mechanistic review on targeting liposomal amphotericin B (AmBisome®) to the fungal membrane. *J Liposome Res* 2017; 27(3):180-185. doi:10.1080/08982104.2017.1360345

Souza AC, Fuchs BB, Pinhati HM, et al. *Candida parapsilosis* resistance to fluconazole: molecular mechanisms and in vivo impact in infected *Galleria mellonella* larvae. *Antimicrob Agents Chemother* 2015; 59(10):6581-7. doi:10.1128/AAC.01177-15

Spadari CC, de Bastiani FWMDS, Lopes LB, Ishida K. Alginate nanoparticles as non-toxic delivery system for miltefosine in the treatment of candidiasis and cryptococcosis. *Int J Nanomedicine* 2019; 14:5187-5199. doi:10.2147/IJN.S205350

Spreghini E, Orlando F, Sanguinetti M, et al. Comparative effects of micafungin, caspofungin, and anidulafungin against a difficult-to-treat fungal opportunistic pathogen, *Candida glabrata*. *Antimicrob Agents Chemother* 2012; 56(3):1215-22. doi:10.1128/AAC.05872-11

Subramanya SH, Baral BP, Sharan NK, et al. Antifungal susceptibility and phenotypic virulence markers of *Candida* species isolated from Nepal. *BMC Res Notes* 2017; 10(1):543. doi:10.1186/s13104-017-2852-x

Sun L, Liao K, Wang D. Effects of magnolol and honokiol on adhesion, yeast-hyphal transition, and formation of biofilm by *Candida albicans*. *PLoS One*. 2015; 10(2):e0117695. doi:10.1371/journal.pone.0117695

Swoboda-Kopeć E, Sikora M, Golas M, Piskorska K, Gozdowski D, Netsvyetayeva I. *Candida nivariensis* in comparison to different phenotypes of *Candida glabrata*. *Mycoses* 2014; 57(12):747-753. doi:10.1111/myc.12264

Szekely A, Borman AM, Johnson EM. *Candida auris* isolates of the Southern Asian and South African Lineages exhibit different phenotypic and antifungal susceptibility profiles *In Vitro*. *J Clin Microbiol* 2019; 57(5):e02055-18. doi:10.1128/JCM.02055-18

Tam P, Gee K, Piechocinski M, Macreadie I. *Candida glabrata*, friend and foe. *J Fungi* 2015; 1(2):277-292. doi:10.3390/jof1020277

Taniguchi L, de Fatima Faria B, Rosa RT, et al. Proposal of a low-cost protocol for colorimetric semi-quantification of secretory phospholipase by *Candida albicans* grown in planktonic and biofilm phases. *J Microbiol Methods* 2009; 78:171–174. doi.org/10 .1016/j.mimet.2009.05.012.

Tay ST, Lotfalikhani A, Sabet NS, et al. Occurrence and characterization of *Candida nivariensis* from a culture collection of *Candida glabrata* clinical isolates in Malaysia. *Mycopathologia* 2014; 178:307-314. doi:10.1007/s11046-014-9778-9

Te Dorsthorst DTA, Verweij PE, Meletiadis J, et al. In vitro interaction of flucytosine combined with amphotericin B or fluconazole against thirty-five yeast isolates determined by both the fractional inhibitory concentration index and the response surface approach. *Antimicrob Agents Chemother* 2002; 46(9):2982-2989. doi:10.1128/AAC.46.9.2982-2989.2002

Thompson T, Brown PD. Comparison of antibiotic resistance, virulence gene profiles, and pathogenicity of methicillin-resistant and methicillin-susceptible *Staphylococcus aureus* using a *Caenorhabditis elegans* infection model. *Pathog Glob Health* 2014; 108:283-91. doi:10.1179/2047773214Y.0000000155

Timmermans B, Peñas AD Las, Castaño I, Van Dijck P. Adhesins in *Candida glabrata*. *J Fungi* 2018; 4(2):1-16. doi:10.3390/jof4020060

Tobudic S, Kratzer C, Lassnigg A, Graninger W, Presterl E. In vitro activity of antifungal combinations against *Candida albicans* biofilms. *J Antimicrob Chemother*.2010; 65(2):271-4. doi:10.1093/jac/dkp429

Tome M, Zupan J, Tomičić Z, Matos T, Raspor P. Synergistic and antagonistic effects of immunomodulatory drugs on the action of antifungals against *Candida glabrata* and *Saccharomyces cerevisiae*. *PeerJ* 2018; 6:e4999. doi:10.7717/peerj.4999

Torres SR, Pichowicz A, Torres-Velez F, et al. Impact of *Candida auris* infection in a neutropenic murine model. *Antimicrob Agents Chemother* 2020; 64(3):e01625-19. doi:10.1128/AAC.01625-19

Trevijano-Contador N, Zaragoza O. Immune response of *Galleria mellonella* against human fungal pathogens. *J Fungi* (Basel) 2019; 5.pii:E3. doi:10.3390/jof5010003

Treviño-Rangel R de J, Espinosa-Pérez JF, Villanueva-Lozano H, et al. First report of *Candida bracarensis* in Mexico: hydrolytic enzymes and antifungal susceptibility pattern. *Folia Microbiol* (Praha) 2018; 63(4):517-523. doi:10.1007/s12223-018-0592-5

Trouvé C, Blot S, Hayette MP, et al. Epidemiology and reporting of candidaemia in Belgium: a multi-centre study. *Eur J Clin Microbiol Infect Dis* 2017; 36(4):649-655. doi:10.1007/s10096-016-2841-3

Tsay S, Welsh RM, et al. Notes from the Field: Ongoing transmission of *Candida auris* in health care facilities - United States, June 2016–May 2017. *MMWR Morb Mortal Wkly Rep* 2017; 66(19):514-515. doi:10.15585/mmwr.mm6619a7

Tzelepis I, Kapsetaki SE, Panayidou S, Apidianakis Y. *Drosophila melanogaster*: a first step and a stepping-stone to anti-infectives. *Curr Opin Pharmacol* 2013; 13:763-8. doi:10.1016/j.coph.2013.08.003

Valentín A, Cantón E, Pemán J, Fernandez-Rivero ME, Tormo-Mas MA, Martínez JP. In vitro activity of anidulafungin in combination with amphotericin B or voriconazole against biofilms of five *Candida* species. *J Antimicrob Chemother* 2016; 71(12):3449-3452. doi:10.1093/jac/dkw316

Vallabhaneni S, Cleveland AA, Farley MM, et al. Epidemiology and risk factors for echinocandin nonsusceptible *Candida glabrata* bloodstream infections: data from a large multisite populationbased candidemia surveillance program, 2008–2014. *Open Forum Infect Dis* 2015; 2:ofv163. doi.org/10.1093/ofid/ofv163

Valotteau C, Prystopiuk V, Cormack BP, Dufrêne YF. Atomic force microscopy demonstrates that *Candida glabrata* uses three epa proteins to mediate adhesion to abiotic surfaces. *mSphere* 2019; 4(3):e00277-19. doi:10.1128/mSphere.00277-19

van de Venter M, Didloff J, Reddy S, et al. Wild-Type Zebrafish (*Danio rerio*) larvae as a vertebrate model for diabetes and comorbidities: A Review. *Animals* (Basel) 2020; 30;11(1):54. doi:10.3390/ani11010054

Varas M, Fariña A, Díaz-Pascual F, et al. Live-cell imaging of *Salmonella typhimurium* interaction with zebrafish larvae after injection and immersion delivery methods. *J Microbiol Methods* 2017; 135:20-25. doi:10.1016/j.mimet.2017.01.020

Varela M. Modelling viral infections using zebrafish: Innate immune response and antiviral research. *Antiviral Res* 2017; 139:59-68. doi:10.1016/j.antiviral.2016.12.013

Vargas RA, Sarmiento K, Vásquez IC. Zebrafish (*Danio rerio*): A potential model for toxinological studies. *Zebrafish* 2015; 12(5):320-6. doi:10.1089/zeb.2015.1102

Vasquez-Rifo A, Veksler-Lublinsky I, Cheng Z, et al. The *Pseudomonas aeruginosa* accessory genome elements influence virulence towards *Caenorhabditis elegans*. *Genome Biol* 2019; 20(1):270. doi:10.1186/s13059-019-1890-1

Vatanshenassan M, Boekhout T, Meis JF, et al. *Candida auris* identification and rapid antifungal susceptibility testing against echinocandins by MALDI-TOF MS. *Front Cell Infect Microbiol* 2019; 9:1-9. doi:10.3389/fcimb.2019.00020

Vazquez-Munoz R, Lopez FD, Lopez-Ribot JL. Silver nanoantibiotics display strong antifungal activity against the emergent multidrug-resistant yeast *Candida auris*

under both planktonic and biofilm growing conditions. *Front Microbiol* 2020; 11:1673. doi:10.3389/fmicb.2020.01673

Vera-González N, Shukla A. Advances in biomaterials for the prevention and disruption of *Candida* biofilms. *Front Microbiol* 2020; 11:538602. doi:10.3389/fmicb.2020.538602

Vertyporokh L, Wojda I. Immune response of *Galleria mellonella* after injection with non-lethal and lethal dosages of *Candida albicans*. *J Invertebr Pathol* 2020; 170:107327. doi:10.1016/j.jip.2020.107327

Wahyuningsih R, Sahbandar IN, Theelen B, et al. *Candida nivariensis* isolated from an Indonesian human immunodeficiency virus infected patient suffering from oropharyngeal candidiasis. *J Clin Microbiol* 2008; 46:388-391. doi.org/10.1128/JCM.01660-07

Wang X, Bing J, Zheng Q, et al. The first isolate of *Candida auris* in China: clinical and biological aspects. *Emerg Microbes Infect* 2018; 7(1):93. doi:10.1038/s41426-018-0095-0

Warren TA, McTaggart L, Richardson SE, Zhang SX. *Candida bracarensis* bloodstream infection in an immunocompromised patient. *J Clin Microbiol* 2010; 48(12):4677-4679. doi:10.1128/JCM.01447-10

Weig M, Jänsch L, Gross U, De Koster CG, Klis FM, De Groot PW. Systematic identification in silico of covalently bound cell wall proteins and analysis of protein-polysaccharide linkages of the human pathogen *Candida glabrata*. *Microbiology* 2004; 150:3129-44. doi:10.1099/mic.0.27256-0

Welsh RM, Bentz ML, Shams A, et al. Survival, Persistence, and Isolation of the emerging multidrug-resistant pathogenic yeast *Candida auris* on a plastic health care surface. *J Clin Microbiol* 2017; 55(10):2996-3005. doi.org/10.1128/JCM.00921-17

Whaley SG, Rogers PD. Azole Resistance in *Candida glabrata*. *Curr Infect Dis Rep* 2016; 18(12):41. doi:10.1007/s11908-016-0554-5

Widiasih Widiyanto T, Chen X, Iwatani S, Chibana H, Kajiwara S. Role of major facilitator superfamily transporter Qdr2p in biofilm formation by *Candida glabrata*. *Mycoses* 2019; 62(12):1154-1163. doi:10.1111/myc.13005

Wiederhold NP, Najvar LK, Fothergill AW, et al. The novel arylamidine T-2307 demonstrates in vitro and in vivo activity against echinocandin-resistant *Candida glabrata*. *J Antimicrob Chemother* 2016; 71:692-695. doi:10.1093/jac/dkv398

Woodworth MH, Dynerman D, Crawford ED, et al. Sentinel case of *Candida auris* in the Western United States following prolonged occult colonization in a returned traveler from India. *Microb Drug Resist* 2019; 25(5):677-680. doi:10.1089/mdr.2018.0408

Wright WF, Bejou N, Shields RK, Marr K, McCarty TP, Pappas PG. Amphotericin B induction with voriconazole consolidation as salvage therapy for *FKS*-Associated

echinocandin resistance in *Candida glabrata* septic arthritis and osteomyelitis. *Antimicrob Agents Chemother* 2019; 63(8):e00512-19. doi:10.1128/AAC.00512-19

Wu Y, Totten M, Memon W, Ying C, Zhang SX. In vitro antifungal susceptibility of the emerging multidrug- resistant pathogen *Candida auris* to miltefosine alone and in combination with amphotericin B. *Antimicrob Agents Chemother* 2020; 64(2):2019-2021. doi:10.1128/AAC.02063-19

Wurster S, Bandi A, Beyda ND, et al. *Drosophila melanogaster* as a model to study virulence and azole treatment of the emerging pathogen *Candida auris*. *J Antimicrob Chemother* 2019; 74(7):1904-1910. doi:10.1093/jac/dkz100

Yang KH, Yun B, Choi HJ, et al. Simple evaluation of *Listeria monocytogenes* pathogenesis using *Caenorhabditis elegans* animal mdel. *Food Sci Anim Resour* 2019; 39(1):84-92. doi:10.5851/kosfa.2019.e6

Yue H, Bing J, Zheng Q, et al. Filamentation in *Candida auris*, an emerging fungal pathogen of humans: passage through the mammalian body induces a heritable phenotypic switch. *Emerg Microbes Infect* 2018; 7(1):188. doi:10.1038/s41426-018-0187-x

Zhu YC, O'Brien B, Leach L, et al. Laboratory analysis of an outbreak of *Candida auris* in New York from 2016 to 2018: Impact and lessons learned. *J Clin Microbiol* 2020; 58(4):1-16. doi:10.1128/JCM.01503-19

Zimbeck AJ, Iqbal N, Ahlquist AM, et al. FKS mutations and elevated echinocandin MIC values among *Candida glabrata* isolates from U.S. population-based surveillance. *Antimicrob Agents Chemother* 2010; 54:5042–5047. doi.org/10.1128/AAC.00836-10

Zonios DI, Bennett JE. Update on azole antifungals. *Semin Respir Crit Care Med* 2008; 29(2):198-210. doi:10.1055/s-2008-1063858

9. ERANSKINAK

1. Eranskina/Anexo 1

Modelos experimentales in vivo para el estudio de las infecciones

Estibaliz Mateo, Iker de la Pinta, Ainara Hernando, Irene Jurado y Elena Eraso

[1]Departamento de Inmunología, Microbiología y Parasitología, UFI 11/25, Facultad de Medicina y Enfermería, Universidad del País Vasco/Euskal Herriko Unibertsitatea UPV/EHU, Bilbao

Actualizaciones en Biomedicina (2020) ISBN: 978-84-1319-234-5)

Resumen: Los modelos animales son una herramienta útil para estudiar la patogénesis microbiana, la respuesta inmune provocada en el hospedador, la evaluación de la eficacia de los antimicrobianos y el desarrollo de nuevas terapias. Sin embargo, la experimentación animal está asociada a importantes problemas éticos, por lo que además de reducir el número de animales y refinar los procedimientos realizados en ellos para minimizar el dolor y/o el estrés, se intenta reemplazar el uso de los animales vertebrados por otros modelos (regla de las 3R). Se está fomentando el desarrollo de modelos innovadores en animales invertebrados o que no utilicen animales para impulsar la investigación futura. Estos organismos alternativos son muy útiles para investigar rasgos específicos de virulencia del patógeno y su papel en la infección ya que, aunque no están estrechamente relacionados con los vertebrados superiores desde un punto de vista evolutivo, comparten aspectos importantes de la respuesta innata frente a los microorganismos.

Palabras clave: Modelos experimentales alternativos; inmunidad innata; virulencia microbiana; terapia antimicrobiana; *Caenorhabditis elegans*; *Galleria mellonella*; *Drosophila melanogaster*; *Danio rerio*.

1. Introducción

Los modelos animales de infección están diseñados para simular con la mayor fidelidad posible la infección humana. Estos modelos nos permiten avanzar en el conocimiento de la patogenia, anatomía patológica y terapéutica de las enfermedades causadas por virus, bacterias y hongos que afectan cada vez más frecuentemente al ser humano.

En el estudio de las interacciones hospedador-patógeno utilizando animales en lugar de seres humanos, el animal y su entorno se pueden controlar, lo que permite un preciso análisis de la relación de causa y efecto de estas interacciones. La elección de un modelo apropiado es la clave para conseguir la máxima fidelidad en la simulación de la infección

en el ser humano y son varios los factores a tener en cuenta a la hora de seleccionar dicho modelo. Entre estos factores influye la selección de i) la cepa a estudiar, ya que su virulencia determinará el proceso de infección y la respuesta del hospedador; ii) la especie animal, que actuará como hospedador debido a la diferente susceptibilidad a la infección que existe entre ellas; iii) el estado inmunológico y otros factores de riesgo del hospedador, que condicionarán el curso de la infección y iv) la vía de inoculación del patógeno en el hospedador.

El modelo ideal para el estudio de la patogénesis no sólo debe reproducir lo más fielmente posible el proceso de colonización e invasión en la puerta anatómica específica de entrada, sino que también debe asemejar las condiciones del sistema inmunitario u hormonales asociadas con la infección en ese sitio particular. Además, la infección experimental debe ser lo suficientemente prolongada para revelar de forma secuencial la implicación de los factores de virulencia del patógeno y la puesta en marcha de las defensas del hospedador.

Por otra parte, los fallos terapéuticos y el incremento de las tasas de resistencia detectadas en los patógenos hacen necesario la búsqueda de nuevas dianas y el desarrollo de nuevas estrategias terapéuticas. Los métodos convencionales para el desarrollo de nuevas drogas implican i) la selección computacional de potenciales genes diana en base a la información del genoma secuenciado y el cribado de colecciones de compuestos químicos para conseguir moléculas que inhiban la función de ese gen diana, ii) el cribado de colecciones de pequeñas moléculas con capacidad de inducir un fenotipo específico en proteínas diana purificadas o en cultivos celulares. Después de la selección de algunos prometedores compuestos, éstos son probados in vivo en modelos animales (Moy. y cols., 2009; Giacomotto y Ségalat, 2010).

Los avances en la patogénesis, farmacología e inmunología de las infecciones se han llevado a cabo tradicionalmente en modelos mamíferos como ratones, ratas, conejos, conejillos de indias y monos. Los modelos murinos y más concretamente en el ratón, *Mus musculus*, son el método más común de estudio de las infecciones. Estos modelos son ventajosos debido, entre otros, a que presentan cierta similitud con la anatomía y la

respuesta inmune humana. Sin embargo, aspectos económicos relacionados con la obtención y mantenimiento de un número suficiente de ratones, logísticos como el tiempo de reproducción y éticos por el sufrimiento animal, están limitando cada vez más el uso de animales vertebrados en la experimentación.

2. Modelos experimentales alternativos

Se consideran modelos alternativos, según la definición de la OTA (*Office of Technologies Assessment* de EE.UU.), todo aquel que reemplaza el uso de animales de laboratorio, reduce el número de animales requeridos en los experimentos o retira procedimientos o protocolos preexistentes para minimizar el nivel de sufrimiento o angustia que puedan experimentar los animales (regla de las "3R"). En el sentido más estricto del término, un modelo alternativo sustituye el uso de un animal vivo por un sistema no animal y utiliza modelos computacionales, vegetales o microorganismos. Sin embargo, una interpretación más amplia de este término dejaría espacio al uso de animales invertebrados y de sangre fría con sistemas neurales poco desarrollados en los que la sensación de dolor es casi inexistente. Estos animales se pueden obtener en grandes cantidades a un bajo coste, son de fácil manejo y su pequeño tamaño y corta vida facilita la experimentación en el laboratorio.

Los estudios llevados a cabo previamente en modelos mamíferos han sido últimamente complementados con la introducción de una variedad de modelos. Entre los vertebrados, se están utilizando dos modelos alternativos en la etapa embrionaria del desarrollo para reducir los problemas éticos asociados con el uso de individuos adultos: embriones de pez cebra, *Danio rerio*, y huevos de gallina embrionados. Entre los animales invertebrados han sido ampliamente utilizados tres grupos: amebas, insectos y nematodos. Las amebas como *Acanthamoeba castellanii* y *Dictyostelium discoideum* son un modelo óptimo para investigar la actividad fagocítica por ser depredadores ambientales. Estos modelos son interesantes especialmente para el estudio de los patógenos intracelulares, ya que los mecanismos que resultan en la patogénesis intracelular parecen estar conservados desde las amebas hasta las células fagocíticas de

los mamíferos (Hoffmann y cols., 2014; Varas y cols., 2018). Otros invertebrados en los que se ha analizado la patogénesis, la eficacia de los compuestos antimicrobianos y la respuesta innata contra los patógenos son el nematodo *Caenorhabditis elegans*, la cucaracha *Blattella germanica*, el gusano de seda *Bombyx mori*, el mosquito *Culex quinquefasciatus*, la mosca de la fruta *Drosophila melanogaster*, el lepidóptero *Galleria mellonella* y el coleóptero *Tenebrio molitor* (Glavis-Bloom y cols., 2012).

Por último, se ha descrito que las plantas también son útiles para modelar la patogénesis microbiana, revelando en algunos casos mecanismos de infección ampliamente conservados. Al igual que los animales, se ha demostrado que las plantas poseen un sistema inmunológico innato que responde a los virus, bacterias y hongos invasores. Los modelos de infección de plantas, como *Arabidopsis thaliana, Lemna minor* o *Medicago sativa*, a menudo producen resultados más rápidos, son más convenientes y menos costosos que muchos modelos de infección animal (Kamal y cols., 2019).

2.1. *Caenorhabditis elegans*

El nematodo *Caenorhabditis elegans* es un organismo hermafrodita de la familia *Rhabditidae* que habita en el suelo y fue utilizado por primera vez en la década de 1960 como modelo para estudiar el desarrollo y el sistema nervioso de los animales (Brenner, 1974). Desde entonces, este invertebrado ha sido útil en el descubrimiento de mecanismos moleculares como la muerte celular, el envejecimiento, el desarrollo y la función neuronal. En el ámbito de la microbiología ha permitido estudiar tanto los componentes conservados evolutivamente de las interacciones hospedador-patógeno, como la eficacia de nuevos fármacos con actividad antimicrobiana (Peterson y cols., 2018).

Caenorhabditis elegans es apropiado para conocer los efectos de los agentes infecciosos, ya que se alimenta de microorganismos y es susceptible a muchos de los patógenos bacterianos y fúngicos que pueden infectar a mamíferos y al ser humano. En el

laboratorio, *C. elegans* puede cultivarse fácilmente en placas con agar o medio líquido y se alimenta generalmente de cepas no patógenas de *Escherichia coli*, pudiendo ser sustituidas por bacterias u hongos patógenos para generar una infección. Este nematodo es un buen modelo para la observación experimental ya que presenta un cuerpo transparente, cilíndrico no segmentado de aproximadamente 1 mm de longitud y 80 µm de diámetro que permite detectar ópticamente los cambios funcionales y morfológicos que se producen sin tener que matarlo o diseccionarlo (Figura 1). Además, en el laboratorio se puede obtener una progenie genéticamente idéntica por auto-fertilización de hasta 300 larvas por cada individuo. Presentan una simplicidad fisiológica y anatómica de aproximadamente 1.000 células, incluyendo unas 300 neuronas. Su genoma está completamente secuenciado y se ha mostrado que comparte más del 50% de sus genes con el ser humano. Además, permite su almacenamiento indefinido por crioconservación en nitrógeno líquido, por lo que se pueden mantener colecciones de miles de cepas genéticamente modificadas que pueden ser de interés en diferentes ensayos (Giacomotto y Ségalat, 2010). En internet, la base de datos *Wormbase* (http://www.wormbase.org/) contiene información completa sobre genes, mutantes y fenotipos, y otros detalles adicionales acerca de *C. elegans* (Breger y cols., 2007). Existen gran cantidad de mutantes disponibles y, entre ellos, la cepa doble mutante *glp-4*;*sek-1* de *C. elegans* (cepa AU37) destaca en estudios microbiológicos y de inmunidad (Thompson y cols., 2014; Ortega-Riveros y cols., 2017). La mutación en el gen *glp-4* convierte a los nematodos en estériles al incubarlos a 25 °C, ya que se interrumpe su morfogénesis gonadal. De este modo, se puede mantener constante el número de individuos durante los ensayos. La mutación en el gen *sek-1* impide la correcta expresión de una proteína quinasa activada por mitógenos, la cual interviene en la cascada de señalización que media la expresión de genes de defensa frente a patógenos, haciéndolo así más susceptible a la infección (Breger y cols., 2007).

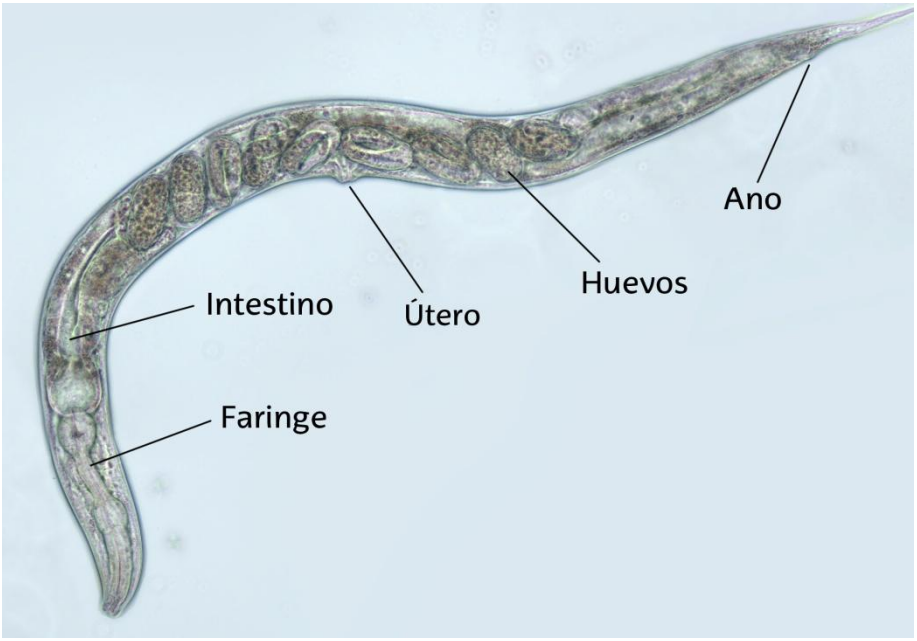


Figura 1. *Caenorhabditis elegans*. Microscopía de contraste de fases.

Los estudios de infección de *C. elegans* con bacterias y hongos han constatado semejanzas con la patogénesis de estas infecciones en mamíferos. Por ello, este nematodo es comúnmente empleado en estudios de virulencia de todo tipo de microorganismos, desde bacterias como *Coxiella burnetii*, *E. coli*, *Listeria monocytogenes*, *Pseudomonas aeruginosa* o *Staphylococcus aureus*, hasta dos de los hongos patógenos más prevalentes como *Candida albicans* y *Cryptococcus neoformans* (Figura 2). Además, en los últimos años se ha comenzado a implementar este modelo animal en el estudio de los virus (Pukkila-Worley y cols., 2009; Thompson y cols., 2014; Gammon y cols., 2017).

Por otro lado, se ha utilizado este animal para identificar la actividad potencial de diferentes compuestos. El estudio de esta actividad in vivo permite, además de ver qué compuestos bloquean el crecimiento de los patógenos, analizar la toxicidad de un compuesto dado e, incluso, se pueden identificar compuestos que mejoren la inmunidad del hospedador. Los sistemas automatizados, como el análisis automático de imágenes, han facilitado la identificación de compuestos activos frente a diferentes patógenos en este modelo (Moy y cols., 2009).

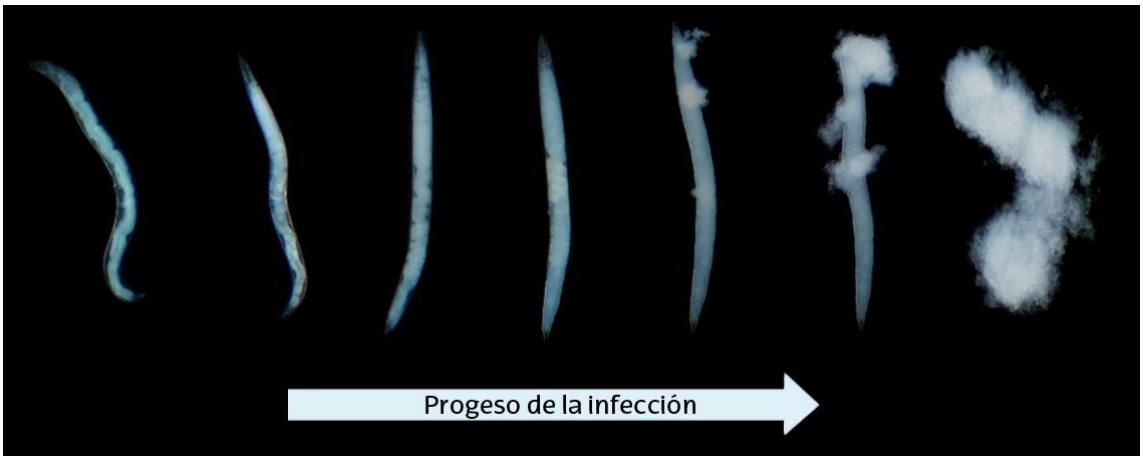


Figura 2. Infección de *Caenorhabditis elegans* por *Candida albicans*. El proceso comienza con el colapso de la luz intestinal por parte de la levadura y culmina en el desarrollo de micelios a lo largo del cuerpo del nematodo y la perforación de su cutícula.

A pesar de las ventajas expuestas, la utilización de *C. elegans* como modelo animal plantea también algunas limitaciones. Las principales son que algunas enfermedades no se pueden reproducir en el modelo, ya que el animal no tiene los genes u órganos correspondientes, y que el sistema inmune innato del nematodo presenta diferencias respecto al de los mamíferos. En el caso de la evaluación de compuestos, puede ser difícil predecir la actividad en mamíferos y las concentraciones efectivas de los compuestos identificados, debido a que la gruesa cutícula del nematodo bloquea la absorción y su pequeño tamaño hace que sea imposible medir la cantidad del compuesto que ha sido absorbida (Giacomotto y Ségalat, 2010).

2.2. *Galleria mellonella*

El lepidóptero *Galleria mellonella* pertenece a la familia *Pyralidae* y es conocido como la polilla grande de la cera. Su estado larval es un modelo emergente para estudiar las interacciones hospedador-patógeno que tienen lugar durante la infección.

Las larvas de *G. mellonella* se obtienen de modo fácil y económico y presentan claras ventajas frente a otros modelos utilizados en el estudio de las infecciones. En primer lugar, destaca su capacidad de sobrevivir a temperaturas entre 15-42 °C, permitiendo así

estudiar la infección producida por microorganismos patógenos para el ser humano a 37 °C. Además, son de fácil manejo y la administración de la carga microbiana o el tratamiento puede realizarse por vía tópica, oral o por inyección, siendo esta última la más adecuada ya que la larva recibe una dosis precisa. El tamaño de las larvas es suficiente (1,5-2,5 cm) para determinar la tasa de mortalidad sin requerir de equipamiento especial, puesto que la muerte de éstas provoca además de inmovilidad, una melanización masiva en respuesta a la infección tornándolas de color negro (Figura 3).

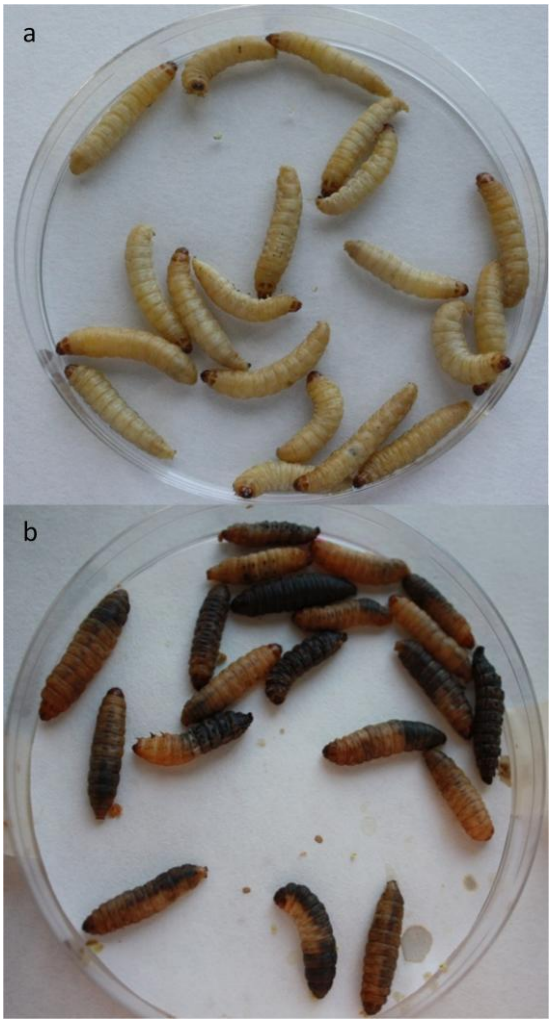


Figura 3. Larvas de *Galleria mellonella*. (**a**) Ejemplares vivos. (**b**) Ejemplares muertos por la infección con *Candida albicans*.

El sistema inmune de la larva presenta similitudes tanto funcionales como estructurales con el sistema inmune innato de los mamíferos ya que está muy conservado en insectos y mamíferos (Hernandez y cols., 2019). Sin embargo, no puede simular el papel frente a la infección de la respuesta inmune adaptativa de los mamíferos. La respuesta inmune de la larva consiste tanto en barreras estructurales como en respuestas innatas celular y humoral desarrolladas por los hemocitos, células especializadas que forman parte de la hemolinfa. Los patógenos son reconocidos por receptores de membrana y son fagocitados al interior de los hemocitos, de manera equivalente a la actividad de los neutrófilos de los mamíferos. La respuesta inmune de los insectos que actúa frente a bacterias y hongos implica la melanización de las larvas, cambios en la población de hemocitos circulando en la hemolinfa y síntesis de nuevas proteínas en la hemolinfa (Trevijano-Contador y Zaragoza, 2019). Además, se han identificado factores homeostáticos activos para reparar las heridas de la cutícula y limitar el desarrollo de la infección. La densidad de hemocitos y el porcentaje de fagocitosis en *G. mellonella* se utilizan como parámetros para evaluar la virulencia de los patógenos.

Galleria mellonella se ha utilizado para estudiar la patogenia y la efectividad de antibacterianos en monoterapia o combinación frente a bacterias resistentes a algunos de ellos. Algunas de las bacterias estudiadas son *Acinetobacter baumannii,* por su capacidad de desarrollar resistencia a muchos de los antibacterianos de los que se dispone, *L. monocytogenes, S. aureus, Streptococcus pneumoniae, Streptococcus pyogenes, Vibrio parahaemolyticus,* y enterobacterias como *E. coli*, *Proteus mirabillis* o *Salmonella enterica* (Hernandez y cols., 2019). Además, se ha usado este modelo para estudiar la capacidad patógena de hongos como *Aspergillus fumigatus, Blastomyces dermatitidis, Candida, C. neoformans, Fusarium, Histoplasma capsulatum, Paracoccidioides brasiliensis, Paracoccidioides lutzii* y *Rhizopus* (Maurer y cols., 2019; Trevijano-Contador y Zaragoza, 2019).

Es aún necesario desarrollar herramientas que permitan estudiar a nivel celular y molecular el modelo en *G. mellonella,* puesto que una de las limitaciones de este modelo es que su genoma aún no ha sido completamente secuenciado y que no se han

desarrollado métodos bien establecidos para generar mutantes (Trevijano-Contador y Zaragoza, 2019). La disponibilidad de datos genómicos permitirá desarrollar micromatrices y métodos para su manipulación genética. Además, es necesario definir líneas de *G. mellonella* y describir métodos estandarizados sobre su propagación y mantenimiento para poder así potenciar el uso de este modelo y permitir comparar los resultados obtenidos en diferentes laboratorios.

2.3. *Drosophila melanogaster*

El díptero *Drosophila melanogaster* pertenece a la familia *Drosophilidae* y es conocido como la mosca de la fruta o la mosca del vinagre (Figura 4).

Las principales ventajas que presenta este insecto como modelo experimental son su pequeño tamaño (2-3 mm), corto tiempo de generación, un genoma secuenciado en su totalidad y la disponibilidad tanto de colecciones de mutantes como de diversas herramientas genéticas para su manipulación (http://www.flybase.org/). Los estudios con *D. melanogaster* han puesto de manifiesto que muchos de los mecanismos de la inmunidad innata de los insectos son similares a los de organismos superiores, con genes y vías semejantes a las encontradas en los mamíferos. De hecho, la mosca de la fruta presenta homólogos funcionales para el 75% de los genes relacionados con enfermedades humanas, más que cualquier otro modelo de invertebrado usado actualmente. Al igual que en *G. mellonella*, la respuesta inmune innata en *D. melanogaster* presenta tanto componentes celulares, que implican hemocitos que realizan la fagocitosis de microorganismos extraños, como componentes humorales, compuestos por péptidos antimicrobianos producidos en la grasa corporal, el equivalente al hígado de mamíferos, que luego son secretados en la hemolinfa. Esta actividad antimicrobiana puede persistir durante varios días y reconoce una amplia variedad de patógenos Las vías de señalización que controlan la producción de estos péptidos antimicrobianos se activan por la interacción de los receptores de reconocimiento de patrones con moléculas de la superficie de bacterias y hongos, o por factores de virulencia secretados (Tzelepis y cols., 2013).

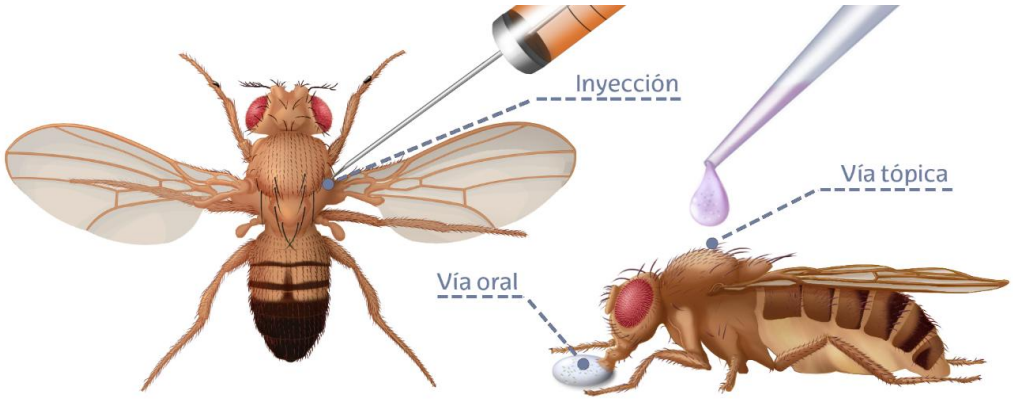


Figura 4. Principales vías de infección en *Drosophila melanogaster*

A pesar de las numerosas ventajas que presenta este modelo para el estudio de las interacciones patógeno-hospedador, el hecho de que sea infectado y mantenido a 25-29 ºC de temperatura, puede suponer un problema para estudiar patógenos o factores de virulencia que requieran la temperatura corporal de mamíferos. No obstante, la utilización de este díptero en un amplio rango de estudios con distintos microorganismos patógenos para el ser humano ha ayudado a comprender algunos mecanismos de la respuesta inmune innata, el papel que juegan en el proceso infectivo ciertos factores de virulencia y a estudiar la eficacia de compuestos antimicrobianos.

Un amplio número de estudios realizados en este modelo in vivo nos ha permitido conocer la función que realizan durante la infección ciertos genes, y sus correspondientes proteínas, de importantes virus que afectan al ser humano, tales como el citomegalovirus humano, coronavirus, virus del dengue, virus de Epstein-Barr, virus de la hepatitis B o virus de la inmunodeficiencia humana (VIH). Por ejemplo, en el caso del VIH-1, se ha visto que la expresión de una proteína asociada a membrana (Nef) del virus durante la infección contribuye al declive de las células T del sistema inmune humano, principal característica que presentan los pacientes con síndrome de inmunodeficiencia adquirida (Hughes, 2012). También se han obtenido resultados exitosos en diversos estudios realizados con bacterias como *Chlamydia*, *Enterococcus faecalis*, *P. aeruginosa*, *Serratia marcescens*, *S. aureus* o *Vibrio cholerae* (Tzelepis y cols., 2013).

El detonante de utilizar pequeños modelos como hospedadores en el estudio de las infecciones fúngicas comenzó cuando se descubrió que la ruta de señalización de los receptores Toll en *D. melanogaster* era indispensable como defensa contra los patógenos fúngicos (Lemaitre y cols., 1996). Es por eso que las cepas mutantes de *D. melanogaster* deficientes en receptores Toll son análogas en muchos aspectos a los mamíferos inmunodeficientes que presentan riesgo de infección por hongos oportunistas. Esta analogía ha permitido realizar diversos estudios que han demostrado que ciertas características de *C. albicans*, como la capacidad de formar hifas, implican una mayor virulencia del hongo. Estos resultados concuerdan con los obtenidos en el modelo murino, reflejando la utilidad de la mosca de la fruta como modelo para el estudio de la patogénesis de estas infecciones fúngicas (Segal y Frenkel, 2018). Los estudios realizados en *D. melanogaster* con otros hongos patógenos, incluyendo *A. fumigatus, C. neoformans, Fusarium* y *Scedosporium*, también han ayudado a comprender algunos mecanismos de la respuesta inmune innata y ciertos factores de virulencia (Tzelepis y cols., 2013).

Por otro lado, se ha utilizado *D. melanogaster* como modelo de hospedador para la evaluación in vivo de la eficacia de compuestos antimicrobianos. Aunque *D. melanogaster* no se puede cultivar en medio líquido, lo que impide la automatización del proceso, la detección de posibles compuestos se puede hacer manualmente (Giacomotto y Ségalat, 2010). Además, la administración del fármaco se puede realizar, no solo mezclándolo en el alimento, sino también mediante inyecciones localizadas de dosis más precisas. Se ha descrito que los fármacos antituberculosos rifampicina e isoniacida son eficaces en el tratamiento de la infección por el microorganismo modelo de la tuberculosis (*Mycobacterium marinum*) en *D. melanogaster*, demostrando la existencia de similitudes entre el modelo y los mamíferos (Tzelepis y cols., 2013). También se ha evaluado la respuesta de moscas infectadas con *A. fumigatus* y *C. albicans*, encontrando que es un modelo adecuado para probar compuestos antifúngicos. Estos resultados sugieren que se trata de un modelo potencial para realizar evaluaciones farmacológicas y para estudiar combinaciones de fármacos. Sin embargo, los análisis farmacocinéticos realizados en *D. melanogaster* han sido problemáticos, ya que los métodos actuales que

existen para medir el nivel de fármaco son técnicamente poco precisos y el metabolismo de los insectos puede ser muy distinto al de mamíferos. En consecuencia, por el momento, los parámetros farmacológicos críticos como la absorción, distribución, metabolización, excreción, toxicidad e interacción de los compuestos han de ser necesariamente probados en modelos mamíferos que son filogenéticamente más cercanos a los seres humanos (Tzelepis y cols., 2013).

2.4. *Bombyx mori*

Bombyx mori, conocido comúnmente como gusano de la seda, es un lepidóptero que se utiliza en la producción de la seda desde hace más de 5.000 años. Desde que su genoma se secuenció se han descrito más de 400 mutaciones, por lo que se ha convertido en un modelo animal relevante en biotecnología, genómica y estudios moleculares microbiológicos (Mita y cols., 2004).

Al igual que el resto de modelos animales invertebrados, son fáciles de criar y mantener en el laboratorio por su tamaño reducido. Además, la cría y alimentación de estos insectos están bien establecidas, por lo que los individuos crecen homogéneamente, a diferencia de otros modelos lepidópteros como *G. mellonella*. Esta uniformidad es importante para la obtención de resultados reproducibles en los ensayos en los que se utilice como modelo (Panthee y cols., 2017).

Tanto *B. mori* como *G. mellonella* son invertebrados con un tamaño adecuado para la inyección directa de inóculos, fármacos antimicrobianos o moléculas nuevas a una concentración conocida. En el caso de *B. mori*, además, se puede diferenciar entre la administración intrahemocélica y la intraintestinal, que corresponden a la administración intravenosa en humanos y la oral, respectivamente (Matsumoto y Sekimizu, 2019). Otra característica en común es que este modelo resiste un rango amplio de temperaturas y los ensayos se pueden realizar a 37 °C, lo que facilita el estudio de infecciones por microorganismos patógenos para el ser humano.

La inmunidad innata de *B. mori* consta de respuesta humoral y celular. La primera está mediada por la producción de diferentes proteínas como la fenoloxidasa, péptidos

antimicrobianos o lisozimas. La respuesta celular, en cambio, la realizan los hemocitos que se encargan de la fagocitosis, de la encapsulación o de formar nódulos para eliminar los microorganismos (Panthee y cols., 2017).

El gusano de la seda se ha utilizado en estudios de patogenia de las infecciones que producen bacterias como *E. coli, L. monocytogenes, P. aeruginosa, S. marcescens, S. aureus, S. pyogenes* o *V. cholerae* y las toxinas extracelulares de algunas de ellas. El uso de *B. mori* como modelo animal en microbiología también se extiende al estudio de las infecciones por hongos como *A. fumigatus, Candida* o *C. neoformans*. Además, se ha utilizado como modelo animal para probar nuevas moléculas con potencial antimicrobiano y desarrollar nuevos agentes antibióticos como son la lisocina E, la nosocomicina y el nuevo compuesto antifúngico ASP2397 (Panthee y cols., 2017; Matsumoto y Sekimizu, 2019).

2.5. *Tenebrio molitor*

Tenebrio molitor, habitualmente conocido como gusano de la harina, es un insecto coleóptero que se encuentra en todo el mundo, principalmente en las regiones templadas del hemisferio norte. Este insecto se considera una plaga de cereales o granos molidos almacenados ya que las larvas se alimentan de ellos. Estas larvas constituyen, a su vez, una excelente fuente de proteínas animales y se utilizan como alimento.

En comparación con otros modelos alternativos de hospedadores utilizados en el estudio de patógenos humanos, *T. molitor* presenta algunas ventajas importantes. El gusano de la harina puede incubarse a 37 °C, temperatura adecuada para que muchos patógenos expresen factores de virulencia. Además, el tamaño de las larvas es suficiente para realizar inoculaciones precisas por inyección y para poder extraer un volumen considerable de hemolinfa (5-10 μl/larva). Por último, las larvas muertas se identifican fácilmente porque se vuelven marrones a consecuencia de la melanización (Canteri de Souza y cols., 2018).

Tenebrio molitor tiene un complejo sistema inmunológico innato con respuestas humorales y celulares. Aunque el número de estudios en que se ha utilizado es limitado,

este insecto es susceptible a la infección por una serie de microorganismos de importancia médica como *S. aureus*, *C. albicans* o *C. neoformans*, por lo que puede ser un buen candidato para el estudio de la virulencia microbiana, las respuestas inmunes del hospedador a las infecciones y la actividad de los compuestos antimicrobianos (de Souza y cols., 2015; McGonigle y cols., 2016).

2.6. *Danio rerio*

El pez cebra (*Danio rerio*) es un teleósteo con un sistema neurológico pobremente desarrollado. Su alta tasa de reproducción (300 huevos por semana), el pequeño tamaño de los adultos (3-5 cm) y el bajo coste de mantenimiento de una gran población, han convertido al pez cebra en el modelo vertebrado de elección para el estudio de las enfermedades infecciosas y del efecto de los antimicrobianos (Figura 5a). Asimismo, la disponibilidad de la secuencia del genoma completo y de herramientas eficientes para su manipulación genética también contribuyen a la utilidad del modelo. Por otro lado, a diferencia de los modelos animales invertebrados anteriormente mencionados, *D. rerio* es más similar en términos genéticos, fisiológicos y anatómicos a los mamíferos y, lo más importante, posee mecanismos inmunitarios tanto innatos como adaptativos que presentan una notable similitud con los de mamíferos (Meeker y Trede, 2008).

Otra de las principales fortalezas del pez cebra como modelo para el estudio de las enfermedades infecciosas de los vertebrados se encuentra en las excelentes posibilidades que ofrece para la obtención de imágenes in vivo de las interacciones hospedador-patógeno, en combinación con herramientas avanzadas para el análisis genómico y para la obtención de mutantes a gran escala. Por esta razón, muchos estudios de enfermedades infecciosas en el modelo del pez cebra se han concentrado, sobre todo, en los períodos embrionario y larval del desarrollo, cuando las ventajas del modelo son máximas, ya que los embriones se desarrollan externamente y siguen siendo transparentes durante varios días de desarrollo (Figura 5b). El estudio de embriones en desarrollo permite evaluar la contribución de diferentes tipos de células del sistema inmunitario en la progresión de la enfermedad. Debido a la separación temporal de las respuestas de la inmunidad innata respecto a la adaptativa, los embriones de pez cebra y sus larvas son particularmente

útiles para el estudio de los factores genéticos del hospedador implicados en la patología (Meijer y Spaink, 2011). Además, algunas líneas de pez cebra transgénico contienen células del sistema inmunitario marcadas constitutivamente con moléculas fluorescentes, por lo que se puede seguir el progreso de los patógenos y de las células inmunitarias que responden frente a ellos simultáneamente. El seguimiento del comportamiento de las células inmunes del embrión también se puede realizar tomando imágenes de microscopía de contraste de fases. Por último, otra de las contribuciones importantes para el estudio de las interacciones hospedador-microorganismo en un ambiente controlado ha sido el establecimiento de métodos para obtener embriones libres de microorganismos (Chao y cols., 2010; Meijer y Spanik, 2011).

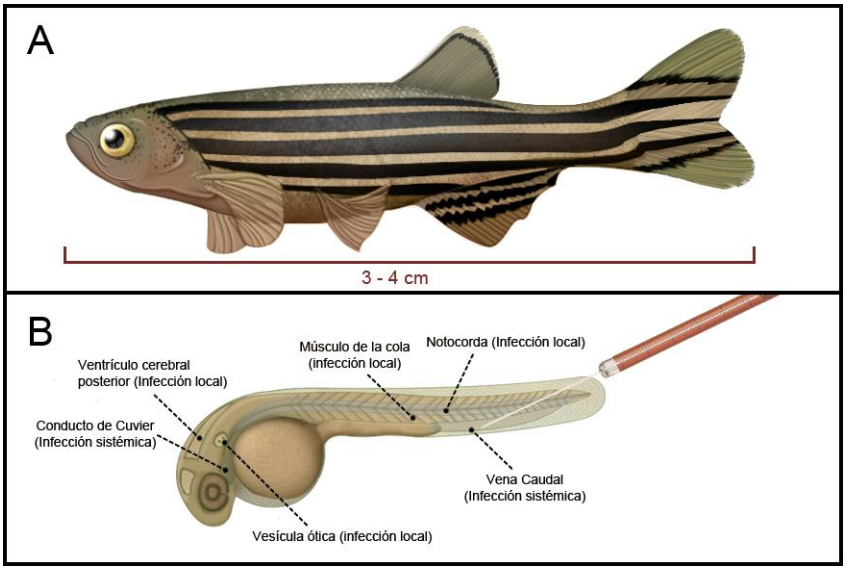


Figura 5. *Danio rerio* (**a**) Adulto. (**b**) Zonas de microinyección de la larva.

Desde el descubrimiento en 1999 de la presencia de macrófagos en el embrión del pez cebra, con una respuesta a las infecciones microbianas muy similar a la de los macrófagos de embriones de mamíferos, numerosas investigaciones han empleado este modelo (Herbomel, 1999). Se ha utilizado este pez para examinar el proceso infectivo de distintos virus, como son el virus Chikungunya, el virus herpes simple y el virus influenza A (Varela y cols., 2017). En lo referente a bacterias, se ha utilizado este modelo para estudiar un amplio rango de infecciones producidas por microrganismos Gram-negativos

(*P. aeruginosa*, *Salmonella* Typhimurium o *Shigella flexneri*), Gram-positivos (*L. monocytogenes* o *S. aureus*) y micobacterias (*Mycobacterium tuberculosis* o *Mycobacterium leprae*). Los resultados de estos estudios han contribuido notoriamente en el avance de nuestro conocimiento sobre la patogenia de estas infecciones víricas y bacterianas y la respuesta inmune desencadenada por el hospedador frente a ellas, respaldando la utilidad del pez cebra como modelo animal vertebrado.

También se han estudiado con este modelo las infecciones por hongos. Una gran ventaja que presenta es la posibilidad de estudiar el dimorfismo de hongos dependiente de la temperatura durante la infección. Se ha demostrado que *C. albicans* puede colonizar e invadir varias zonas del cuerpo del pez cebra y es capaz de matar adultos dependiendo de la dosis infectiva. Se ha examinado, también, la implicación de diferentes factores de virulencia en la progresión de la infección y en la letalidad (Chao y cols., 2010).

Además, el uso de embriones y larvas de pez cebra para el cribado de alto rendimiento de fármacos es también una alternativa muy atractiva. Los embriones pueden incorporar fácilmente los compuestos a través de la piel y los ensayos se pueden realizar en placas de 96 pocillos. Una gran ventaja del uso de este modelo durante el estudio de la actividad antimicrobiana es que posibilita las infecciones automatizadas a gran escala (hasta 2000 embriones por hora), lo que permite probar varios compuestos en un periodo corto de tiempo (Varela y cols., 2016). No obstante, el principal problema es el desarrollo de sistemas de infección, ya que, para la mayoría de los agentes patógenos bacterianos probados, la infección de los embriones por inmersión ha demostrado ser ineficaz y es necesaria la microinyección. Sin embargo, en los últimos años se ha dado un paso importante con el desarrollo de un sistema automatizado de inyección de embriones para su infección con *M. marinum* (Carvalho y cols., 2011). Por último, cabe señalar que la realización del cribado de fármacos en un organismo vertebrado en desarrollo presenta una gran ventaja, ya que se puede obtener simultáneamente información sobre la eficacia del compuesto y sobre la teratogenicidad o toxicidad general del mismo.

3. Conclusiones

El uso de modelos alternativos a la utilización de mamíferos en experimentación ha generado resultados científicos sólidos, ya que reproducen algunas de las condiciones de la enfermedad observadas en animales superiores. Son especialmente útiles para modelar infecciones microbianas, son baratos, generalmente requieren menos manipulaciones y son más aceptables desde el punto de vista ético que la experimentación con organismos superiores. Estos modelos se han utilizado para evaluar las interacciones de los microorganismos con el hospedador, los factores de virulencia de los patógenos, comparar la virulencia entre cepas o especies, evaluar la respuesta inmunitaria y la eficacia y toxicidad de los tratamientos antimicrobianos. Sin embargo, es importante señalar que ningún modelo invertebrado disponible ha sido capaz de sustituir completamente a los modelos de vertebrados. En un futuro, es posible que los avances tecnológicos y científicos disminuyan aún más la necesidad de modelos mamíferos.

Agradecimientos

EM y EE agradecen la financiación recibida de la Consejería de Educación, Universidades e Investigación del Gobierno Vasco-Eusko Jaurlaritza (GIC15/78 IT-990-16) y del Ministerio de Ciencia, Innovación y Universidades (SAF2017-86188-P). AH tiene un contrato predoctoral de la Universidad del País Vasco/Euskal Herriko Unibertsitatea UPV/EHU (PIF 2016).

Conflicto de interés

Los autores declaran no tener ningún conflicto de interés.

Bibliografía

1. Breger J, Fuchs BB, Aperis G, Moy TI, Ausubel FM, Mylonakis E. Antifungal chemical compounds identified using a *C. elegans* pathogenicity assay. PLoS Pathog. 2007;3:e18.
2. Brenner S. The genetics of *Caenorhabditis elegans*. Genetics. 1974;77:71-94.

3. Canteri de Souza P, Custódio Caloni C, Wilson D, Sergio Almeida R. An invertebrate host to study fungal infections, mycotoxins and antifungal drugs: *Tenebrio molitor*. J Fungi (Basel). 2018;4. pii: E125.
4. Carvalho R. A High-throughput screen for tuberculosis progression. PLoS One. 2011;6:e16779.
5. Chao C. Zebrafish as a model host for *Candida albicans* infection. Infect Immun. 2010;78:2512-21.
6. de Souza PC, Morey AT, Castanheira GM, Bocate KP, Panagio LA, Ito FA, Furlaneto MC, Yamada-Ogatta SF, Costa IN, Mora-Montes HM, Almeida RS. *Tenebrio molitor* (Coleoptera: Tenebrionidae) as an alternative host to study fungal infections. J Microbiol Methods. 2015;118:182-6.
7. Gammon DB. *Caenorhabditis elegans* as an emerging model for virus-host interactions. J Virol. 2017;91:e00509-17.
8. Giacomotto J, Ségalat L. High-throughput screening and small animal models, where are we? Br J Pharmacol. 2010;160:204-16.
9. Glavis-Bloom J, Muhammed M, Mylonakis E. Of model hosts and man: using *Caenorhabditis elegans*, *Drosophila melanogaster* and *Galleria mellonella* as model hosts for infectious disease research. Adv Exp Med Biol. 2012;710:11-7.
10. Herbomel P. Ontogeny and behaviour of early macrophages in the zebrafish embryo. Development. 1999;126:3735-45.
11. Hernandez RJ, Hesse E, Dowling AJ, Coyle NM, Feil EJ, Gaze WH, Vos M. Using the wax moth larva *Galleria mellonella* infection model to detect emerging bacterial pathogens. PeerJ. 2019;6:e6150.
12. Hoffmann C, Harrison CF, Hilbi H. The natural alternative: protozoa as cellular models for *Legionella* infection. Cell Microbiol. 2014;16:15-26.
13. Hughes TT. *Drosophila* as a genetic model for studying pathogenic human viruses. Virology. 2012;423:1-5.
14. Kamal F, Radziwon A, Davis CM, Dennis JJ. Duckweed (*Lemna minor*) and alfalfa (*Medicago sativa*) as bacterial infection model systems. Methods Mol Biol. 2019;1898:191-8.

15. Lemaitre B, Nicolas E, Michaut L, Reichhart JM, Hoffmann JA. The dorsoventral regulatory gene cassette spatzle/Toll/cactus controls the potent antifungal response in *Drosophila* adults. Cell. 1996;86:973-83.
16. Matsumoto Y, Semikizu K. Silkworm as an experimental animal for research on fungal infections. Microbiol Immunol. 2019; 63:41-50.
17. Maurer E, Hörtnagl C, Lackner M, Grässle D, Naschberger V, Moser P, Segal E, Semis M, Lass-Flörl C, Binder U. *Galleria mellonella* as a model system to study virulence potential of mucormycetes and evaluation of antifungal treatment. Med Mycol. 2019;57:351-62.
18. McGonigle JE, Purves J, Rolff J. Intracellular survival of *Staphylococcus aureus* during persistent infection in the insect *Tenebrio molitor*. Dev Comp Immunol. 2016;59:34-8.
19. Meeker ND, Trede NS. Immunology and zebrafish: Spawning new models of human disease. Dev Comp Immunol. 2008;32:745-57.
20. Meijer AH, Spaink HP. Host-pathogen interactions made transparent with the zebrafish model. Curr Drug Targets. 2011;12:1000-17.
21. Mita K, Kasahara M, Sasaki S, Nagayasu Y, Yamada T, Kanamori H, Namiki N, Kitagawa M, Yamashita H, Yasukochi Y, Kadono-Okuda K, Yamamoto K, Ajimura M, Ravikumar G, Shimomura M, Nagamura Y, Shin-i T, Abe H, Shimada T, Morishita S , Sasaki T. The genome sequence of silkworm, *Bombyx mori*. DNA Res. 2004;11:27-35.
22. Moy TI, Conery AL, Larkins-Ford J, Wu G, Mazitschek R, Casadei G, Lewis K, Carpenter AE, Ausubel FM. High-throughput screen for novel antimicrobials using a whole animal infection model. ACS Chem Biol. 2009;4:527-33.
23. Ortega-Riveros M, De-la-Pinta I, Marcos-Arias C, Ezpeleta G, Quindós G, Eraso E. Usefulness of the non-conventional *Caenorhabditis elegans* model to assess *Candida* virulence. Mycopathologia. 2017;182:785-95.
24. Panthee S, Paudel A, Hamamoto H, Semikizu K. Advantages of the silkworm as an Animal model for developing novel antimicrobial agents. Front Microbiol. 2017;8:373.

25. Peterson ND, Pukkila-Worley R. *Caenorhabditis elegans* in high-throughput screens for anti-infective compounds. Curr Opin Immunol. 2018;54:59-65.
26. Pukkila-Worley R, Peleg AY, Tampakakis E, Mylonakis E. *Candida albicans* hyphal formation and virulence assessed using a *Caenorhabditis elegans* infection model. Eukaryot Cell. 2009;8:1750-8.
27. Segal E, Frenkel M. Experimental in vivo models of candidiasis. J Fungi (Basel). 2018;4.pii:E21.
28. Thompson T, Brown PD. Comparison of antibiotic resistance, virulence gene profiles, and pathogenicity of methicillin-resistant and methicillin-susceptible *Staphylococcus aureus* using a *Caenorhabditis elegans* infection model. Pathog Glob Health. 2014;108:283-91.
29. Trevijano-Contador N, Zaragoza O. Immune response of *Galleria mellonella* against human fungal pathogens. J Fungi (Basel). 2019;5.pii:E3.
30. Tzelepis I, Kapsetaki SE, Panayidou S, Apidianakis Y. *Drosophila melanogaster*: a first step and a stepping-stone to anti-infectives. Curr Opin Pharmacol. 2013;13:763-8.
31. Varas MA, Riquelme-Barrios S, Valenzuela C, Marcoleta AE, Berríos-Pastén C, Santiviago CA, Chávez FP. Inorganic polyphosphate is essential for *Salmonella* Typhimurium virulence and survival in *Dictyostelium discoideum*. Front Cell Infect Microbiol. 2018;8:8.
32. Varela M. Modelling viral infections using zebrafish: Innate immune response and antiviral research. Antiviral Res. 2017;139:59-68.

2. Eranskina/Anexo 2

Caenorhabditis elegans as a model system to assess *Candida glabrata, Candida nivariensis* and *Candida bracarensis* virulence and antifungal efficacy

Ainara Hernando-Ortiz, Estibaliz Mateo, Marcelo Ortega-Riveros, Iker De-la-Pinta, Guillermo Quindós and Elena Eraso

Department of Immunology, Microbiology and Parasitology, Faculty of Medicine and Nursery, University of the Basque Country (UPV/EHU), Bilbao, Spain

Antimicrobial Agents and Chemotherapy 64 (2020) e00824-20

Abstract

Although *Candida albicans* remains the major etiological agent of invasive candidiasis, *Candida glabrata* and other emerging species of *Candida* are increasingly isolated. This species is the second most prevalent cause of candidiasis in many regions of the world. However, clinical isolates of *Candida nivariensis* and *Candida bracarensis* can be misidentified and are underdiagnosed due to shared phenotypic traits with *C. glabrata*. Little is known about both cryptic species. Pathogenesis studies are therefore needed to understand their virulence traits and their susceptibility to antifungal drugs. The susceptibility of *Caenorhabditis elegans* to different *Candida* species makes this nematode an excellent model for assessing host–fungal interactions. We evaluated the usefulness of *C. elegans* as a nonconventional host model to analyze the virulence of *C. glabrata*, *C. nivariensis* and *C. bracarensis*. The three species caused candidiasis and the highest virulence of *C. glabrata* was confirmed. Furthermore, we determined the efficacy of current antifungal drugs against the infection caused by these species in the *C. elegans* model. Amphotericin B and azoles showed the highest activity against *C. glabrata* and *C. bracarensis* infections, while echinocandins were more active for treating those caused by *C. nivariensis*. *C. elegans* proved to be a useful model system for assessing the pathogenicity of these closely related species.

Keyboards: candidiasis, *Caenorhabditis elegans*, nonconventional host model, pathogenesis, antifungal susceptibility

1. Introduction

Invasive candidiasis is the most frequent mycosis, mainly in patients suffering from immunodeficiency. Although *Candida albicans* remains the predominant etiological agent, there is an increase in infections caused by other *Candida* species, such as *Candida parapsilosis*, *Candida glabrata*, *Candida krusei* and *Candida auris*, which has been associated with reduced antifungal susceptibility or even increased rates of resistance (1, 2, 3). Among these species, *C. glabrata* has been considered the second or third most isolated species of *Candida* from blood cultures according to geographical distribution. This species is a frequent cause of candidemia in the USA, Australia and North and Central Europe, and there is a trend toward an etiological rise in Spain and Portugal (2, 4, 5, 6). *C. glabrata* invasive infection treatment is often a clinical challenge due to the increasing prevalence of azole resistance. Although echinocandins are considered the treatment of choice (7), *C. glabrata* is also the species most likely to be resistant to echinocandins (8, 9).

C. glabrata sensu stricto shares high phenotypic similarities and genetic closeness with *Candida bracarensis* and *Candida nivariensis*. As of yet, the reported incidence of *C. bracarensis* and *C. nivariensis* is low, and data about their virulence and antifungal susceptibility are unclear (5, 10, 11). Among several virulence factors, production of hydrolytic enzymes such as hemolysins or secreted phospholipases and aspartyl proteinases are considered important virulence factors contributing to the pathogenesis of candidiasis (12, 13, 14).

Invertebrate models are promising alternatives to mammals in the study of invasive candidiasis because they provide great advantages considering ethical issues, costs and physiological simplicity. The nematode *Caenorhabditis elegans* is one of these models successfully applied to advance the knowledge of *Candida* infection pathogenesis. This worm is approximately 1 mm in length, and transparent, and has a short reproductive cycle of 2-4 days and life span of 2-3 weeks. Moreover, its genome has been sequenced, and a wide variety of mutant strains are available (15, 16, 17). However, few studies have

analyzed the utility of this model host to assess the virulence of *Candida* species and antifungal efficacy for candidiasis (18, 19, 20, 21). In particular, to the best of our knowledge, this nonconventional model has never been applied to study the pathogenesis of candidiasis caused by *C. glabrata* and other phylogenetically closely-related species.

Within this framework, we were interested in assessing the utility of the *C. elegans* host model to study, for the first time, the pathogenesis of *C. glabrata, C. nivariensis* and *C. bracarensis*. For this purpose, we determined that this nonconventional infection model can be applied to determine the virulence behavior of these three phylogenetically related species in vivo. Furthermore, we evaluated the in vivo antifungal efficacies of amphotericin B, echinocandins and several azoles using the *C. elegans* model and tried to correlate them with their in vitro susceptibility profiles.

2. Materials and methods

2.1. Microorganisms and growth conditions

Reference strains of *Candida* used to carry out the experiments are detailed in Table 1. They include two reference strains of each species of the *C. glabrata* complex: *C. glabrata* ATCC 90030 and NCPF 3203, *C. nivariensis* CBS 9984 and CECT 11998, and *C. bracarensis* NCYC 3397 and NCYC 3133. These strains were cultured in yeast extract peptone dextrose (YEPD; 1% yeast extract, 2% bacteriological peptone, 2% D-glucose) liquid medium (Panreac, Spain) at 30 ºC for 18 h under shaking conditions. The double mutant *C. elegans* AU37 strain (*glp-4*(*bn2*); *sek-1*(*km4*)) used in this study was obtained from the Caenorhabditis Genetics Center (University of Minnesota, USA). This double mutation increases the susceptibility to microbial infections (*sek-1*) and maintains a constant number of sterile worms at 25 ºC (*glp-4*). The *C. elegans* strain was propagated at 15 ºC on nematode growth medium (NGM) agar plates previously seeded with the nonpathogenic strain OP50 of *Escherichia coli*, which was used as a food source for the nematodes. The experiments were performed with a synchronous population of

worms in the L4 larval stage obtained as previously described by Ortega-Riveros et al. (20).

Table 1. Survival of *Caenorhabditis elegans* infected with each of the six *Candida* strains used in this study and evaluation of antifungal treatment.

Strain	Origin	Collection reference	Survival of *C. elegans* at 120 h in absence / presence of DMSO	The three most effective antifungal drugs (survival of *C. elegans* at 120 h)	
				Dissolved in water	Dissolved in DMSO
Candida glabrata					
ATCC 90030	Blood	American Type Culture Collection	40.3% / 26.5%	Micafungin, 8 µg/ml (91.5%) Micafungin, 4 µg/ml (90.6%) Caspofungin, 4 µg/ml (89.6%)	Amphotericin B, 1 µg/ml (82.4%) Voriconazole, 2 µg/ml (82.1%) Posaconazole, 2 µg/ml (81.5%)
NCPF 3203	Blood	National Collection of Pathogenic Fungi	65.4% / 45.1%	Micafungin, 8 µg/ml (96.8%) Fluconazole, 128 µg/ml (94.6%) Caspofungin, 8 µg/ml (91.8%)	Amphotericin B, 2 µg/ml (85.2%) Voriconazole, 2 µg/ml (83.8%) Voriconazole, 1 µg/ml (83.7%)

Candida nivariensis					
CECT 11998	Blood	Colección Española de Cultivos Tipo	72.9% / 65.3%	Micafungin, 8 µg/ml (88.3%) Caspofungin, 8 µg/ml (86.4%) Caspofungin, 4 µg/ml (86.2%)	Voriconazole, 1 µg/ml (64.8%) Voriconazole, 2 µg/ml (63.5%) Amphotericin B, 1 µg/ml (57.2%)
CBS 9984	Bronchoalveolar lavage	Westerdijk Fungal Biodiversity Institute	75% / 73%	Caspofungin, 8 µg/ml (94.9%) Micafungin, 8 µg/ml (94%) Micafungin, 4 µg/ml (92.7%)	Amphotericin B, 2 µg/ml (83.2%) Voriconazole, 1 µg/ml (81.2%) Amphotericin B, 2 µg/ml (74.7%)
Candida bracarensis					
NCYC 3133	Catheter	National Collection of Yeast Cultures	89.4% / 89.4%	Caspofungin, 8 µg/ml (94.5%) Caspofungin, 4 µg/ml (94.4%) Micafungin, 8 µg/ml (92.6%)	Amphotericin B, 1 µg/ml (80.1%) Voriconazole, 2 µg/ml (76%) Amphotericin B, 2 µg/ml (75.8%)
NCYC 3397	Blood	National Collection of Yeast Cultures	97.6% / 70.8%	Caspofungin, 8 µg/ml (100%) Micafungin, 4 µg/ml (99.6%) Micafungin, 8 µg/ml (99.5%)	Voriconazole, 1 µg/ml (96.3%) Posaconazole, 2 µg/ml (96.1%) Amphotericin B, 2 µg/ml (95.4%)

DMSO: 1% dimethyl sulfoxide

2.2. Production of phospholipase, proteinase and hemolytic activity

The production of phospholipases and proteinases and the hemolytic activity of these phylogenetically related species were analyzed. Phospholipase activity was tested following the method described by Polak (22), but using malt agar plates containing 1 M NaCl, 5 mM $CaCl_2$ and 8% sterile egg-yolk emulsion (23). To evaluate the production of aspartyl proteinase, solid medium containing bovine serum albumin (Sigma-Aldrich Inc., USA) was used as described by Cassone et al. (12). The phospholipase activity was defined as the ratio of the diameter of the colony to the total diameter of the colony plus the precipitation zone. The proteinase activity was established as the estimated diameter of the lytic area around the growth of the strain. Finally, the hemolytic activity was studied using the methodology described by Luo et al. (13) but using the plate assay described by Manns et al. (24).

2.3. In vitro antifungal susceptibility

The antifungal efficacy of seven antifungal drugs against the six strains of *C. glabrata*, *C. nivariensis* and *C. bracarensis* was tested. The drug concentration ranged from 0.03 to 16 µg/ml for amphotericin B (AmB) (Sigma-Aldrich Inc., USA), anidulafungin (AND) (Pfizer SA, Madrid, Spain), caspofungin (CAS) (Merck and Com Inc., NJ, USA), micafungin (MCF) (Astellas Pharma Inc., Japan), posaconazole (PCZ) (Merck & Com Inc., NJ, USA) and voriconazole (VCZ) (Pfizer SA, Madrid, Spain). The concentration ranged from 0.12 to 64 µg/ml for fluconazole (FCZ) (Pfizer SA, Madrid, Spain). The minimum inhibitory concentration (MIC) of the antifungal drugs against each strain was determined according to the methodology described for yeasts in documents M27-A3 and M27-A3/S4 from the Clinical Laboratory Standards Institute (25, 26). Type strains obtained from the American Type Culture Collection (ATCC), *C. krusei* ATCC 6258 and *C. parapsilosis* ATCC 22019 were used as quality controls for in vitro antifungal susceptibility testing.

2.4. *Caenorhabditis elegans* infection

The assays were performed as previously described by Breger et al. (27). *C. elegans* populations were placed for 2 h at 25 °C on brain heart infusion (BHI) agar plates (Panreac, Spain) seeded with lawns of the different *Candida* strains, allowing the worms to ingest them. Afterward, the nematodes were washed with M9 buffer (3 g of KH_2PO_4, 6 g of Na_2HPO_4, 5 g of NaCl, 1 ml of 1 M $MgSO_4$ and H_2O to 1 l) supplemented with kanamycin (90 µg/ml) and placed on NGM agar plates to remove the yeast cells from their cuticles. Then, the nematodes were transferred in groups of 20 worms to each well of microtiter plates that contained M9 buffer supplemented with kanamycin and 10 µg/ml cholesterol in ethanol. Sixty nematodes were used to study the mortality caused by each strain of *Candida*, and groups of uninfected nematodes were included as controls in each experiment. Microtiter plates were incubated at 25 °C and visually scored as live or dead nematodes every 24 h using a stereomicroscope (Nikon SMZ-745, Japan) for the subsequent 120 h. All experiments were conducted at least in triplicate on different days.

2.5. Antifungal treatments

To evaluate the effect of antifungal drugs against *Candida* infection, previously infected L4 nematodes were treated with concentrations of 8 µg/ml AND, 4 and 8 µg/ml CAS and MCF, 32, 64 and 128 µg/ml FCZ, or 1 and 2 µg/ml VCZ, PCZ and AmB.

The stock solutions of CAS, FCZ and MCF were prepared in water, while AmB, AND, PCZ and VCZ were prepared in 1% dimethyl sulfoxide (DMSO) following the instructions of the manufacturer. Different concentrations of antifungal drugs were prepared and added to the microtiter plates, and in each condition, 60 nematodes were included. In each experiment, 14 different treatments were assessed for each strain, and groups of infected but untreated nematodes were also analyzed in the presence and absence of 1% DMSO as controls to test the effect of DMSO. At least 960 nematodes were assayed for each strain and experiment. Microtiter plates with nematodes under different conditions were incubated at 25°C, and survival was visually monitored every

24 h for the subsequent 120 h. All experiments were conducted at least in triplicate on different days.

2.6. Statistics

Survival analysis curves were prepared by the Kaplan-Meier method with GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA). The long-rank test with the statistical program SPSS v24.0 (IBM, Chicago, IL, USA) was applied to estimate the differences in the survival of *C. elegans* infected with the different *Candida* strains and conditions ($p<0.05$ was considered statistically significant).

3. Results

3.1. Characterization of *Candida* strains: enzymatic activity and in vitro antifungal susceptibility

Phospholipase and proteinase production and hemolytic activity were studied to analyze the virulence traits of these species. No phospholipase or proteinase activity was detected in any of the strains tested. However, alpha (partial) hemolysis was observed in all strains, except the *C. glabrata* ATCC 90030 strain, which showed gamma hemolysis (no hemolysis).

The in vitro antifungal activities against *C. glabrata, C. nivariensis* and *C. bracarensis* are summarized in Table 2. All six strains of these three closely related species were susceptible to all antifungal drugs tested. MICs of the quality controls were within the published ranges.

Table 2. In vitro antifungal activity of caspofungin (CAS), micafungin (MCF), anidulafungin (AND), amphotericin B (AmB), posaconazole (PCZ), voriconazole (VCZ) and fluconazole (FCZ) against *Candida glabrata*, *Candida nivariensis* and *Candida bracarensis* strains.

Strain	MIC (µg/ml)						
	CAS	**MCF**	**AND**	**AmB**	**PCZ**	**VCZ**	**FCZ**
C. glabrata ATCC 90030	0.5	0.03	0.06	1	1	0.5	8
C. glabrata NCPF 3203	0.25	0.03	0.06	1	0.5	0.25	4
C. nivariensis CBS 9984	0.25	0.03	0.06	2	0.5	0.06	8
C. nivariensis CECT 11998	0.25	0.03	0.06	2	0.5	0.12	4
C. bracarensis NCYC 3397	0.25	0.03	0.06	1	1	0.12	4
C. bracarensis NCYC 3133	0.25	0.03	0.06	2	1	0.12	4

MIC: minimum inhibitory concentration

3.2. Survival of *Caenorhabditis elegans* infected with *Candida*

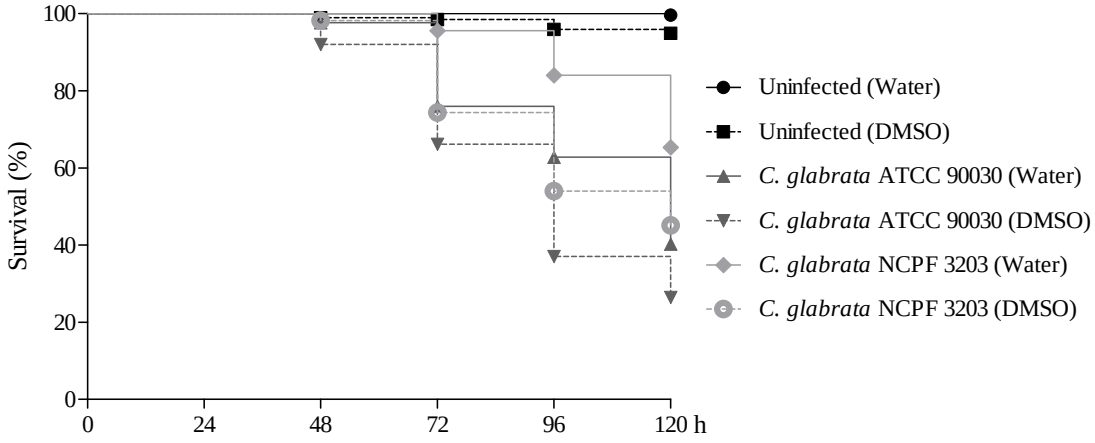
The ability of the three closely related species to develop infection in *C. elegans* was assessed (Figure 1). All six strains of *Candida* were able to kill *C. elegans* and showed statistically significant differences with the survival of uninfected nematodes, which remained nearly constant throughout the experiment (99.6% survival at 120 h). It took at least two days to detect nematodes killed by any of the three *Candida* species. Although the *C. nivariensis* CECT 11998 strain caused higher initial mortality, the *C. glabrata* ATCC 90030 strain was the most lethal at 120 h. Only the *C. glabrata* ATCC 90030 strain achieved a mortality rate of more than 50% at 120 h. Moreover, the *C. glabrata* ATCC 90030 strain was significantly more virulent than the NCPF 3203 strain (p=0.001). There were also differences between the two strains of *C. bracarensis* (p=0), which is the species that killed the lowest percentage of nematodes, but not between the two strains of *C. nivariensis* (Table 1).

We also evaluated the ability of these six *Candida* strains to cause infection in the presence of DMSO in the medium. A significant 4.4% reduction in the viability of

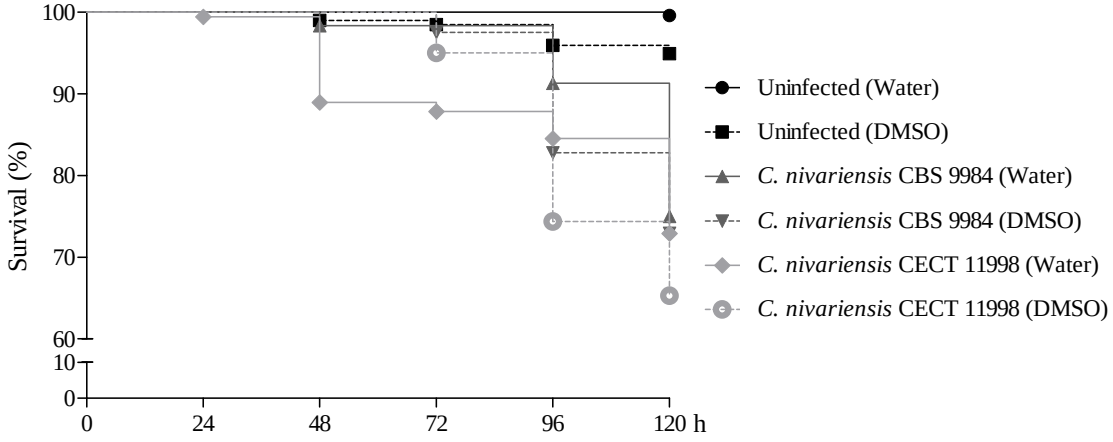
uninfected nematodes in the presence of DMSO was detected compared to that in the absence of DMSO (95.2% and 99.6% survival at 120 h, respectively, p=0). The survival percentages of *C. elegans* infected with *Candida* were also lower in the presence of DMSO. However, the presence of DMSO in the medium, with respect to its absence, resulted in significantly lower survival of the nematodes at 120 h infected with either strain of *C. glabrata* ($p<0.001$) or with the *C. bracarensis* NCYC 3397 strain (p=0), but not with the remaining strains (Table 1). Moreover, when DMSO was in the medium, significant survival differences were detected between the uninfected nematodes and those infected with any of the *Candida* strains except for the *C. bracarensis* NCYC 3133 strain (p=0.98).

Overall, our findings indicate the following virulence categorization of these three *Candida* species in the *C. elegans* model: *C. glabrata* > *C. nivariensis* > *C. bracarensis*.

a)


Survival (%)
100
80
60
40
20
0
0
24
48
72
96
120 h
Uninfected (Water)
Uninfected (DMSO)
C. glabrata ATCC 90030 (Water)
C. glabrata ATCC 90030 (DMSO)
C. glabrata NCPF 3203 (Water)
C. glabrata NCPF 3203 (DMSO)

b)


Survival (%)
100
90
80
70
60
10
0
0
24
48
72
96
120 h
Uninfected (Water)
Uninfected (DMSO)
C. nivariensis CBS 9984 (Water)
C. nivariensis CBS 9984 (DMSO)
C. nivariensis CECT 11998 (Water)
C. nivariensis CECT 11998 (DMSO)

c)

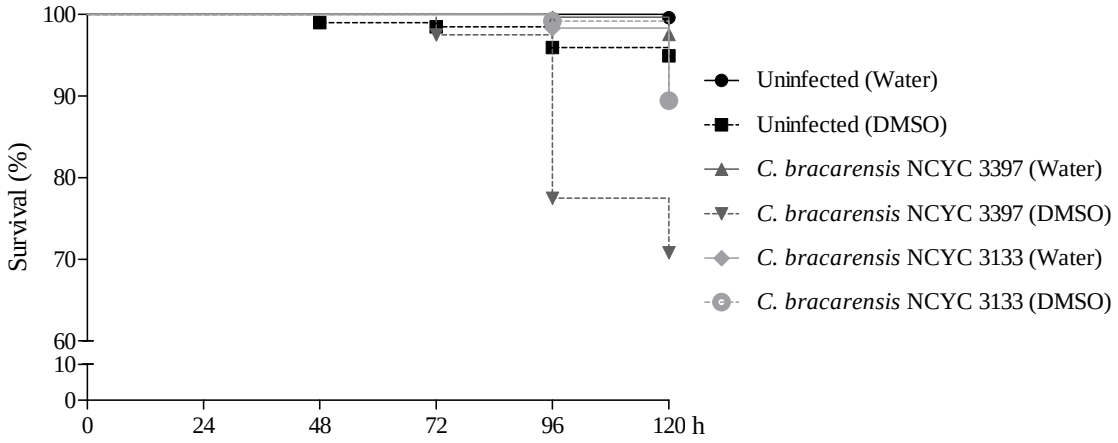


Figure 1. Survival curves of *Caenorhabditis elegans* infected with strains of *Candida glabrata* (a), *Candida nivariensis* (b) or *Candida bracarensis* (c) in the absence (water) or presence of 1% dimethyl sulfoxide (DMSO).

3.3. Antifungal therapy efficacy for candidiasis in *Caenorhabditis elegans*

Nematodes infected with each of the six *Candida* strains were treated with three antifungal drugs prepared in water (CAS, FCZ and MCF) and with four prepared in DMSO (AmB, AND, PCZ and VCZ) at different concentrations.

We detected that, with respect to that of infected and untreated *C. elegans*, all antifungal drugs significantly reduced the mortality of *C. elegans* during *C. glabrata* infection. However, the reduction in nematode mortality during infections caused by *C. nivariensis* and *C. bracarensis* was drug- and strain-dependent (Figure 2).

The antifungal drugs prepared in water achieved a nematode survival of up to 96.8% (value obtained with 8 µg/ml MCF) in the treatment of *C. glabrata* infection, and those prepared in DMSO reached a nematode survival of no more than 85.2% (with 2 µg/ml AmB) (Table 1). Nevertheless, these antifungal drugs in DMSO managed to reduce the mortality for a higher percentage of nematodes. When the nematodes following infection with the *C. glabrata* ATCC 90030 strain were treated with 8 µg/ml MCF or 1 µg/ml

AmB, a higher worm mortality reduction was obtained (51.2% for both). For nematodes infected with the NCPF 3203 strain and treated with 8 µg/ml MCF or 2 µg/ml AmB, the mortality was reduced by 31.4% and 40.1%, respectively (Figure 2).

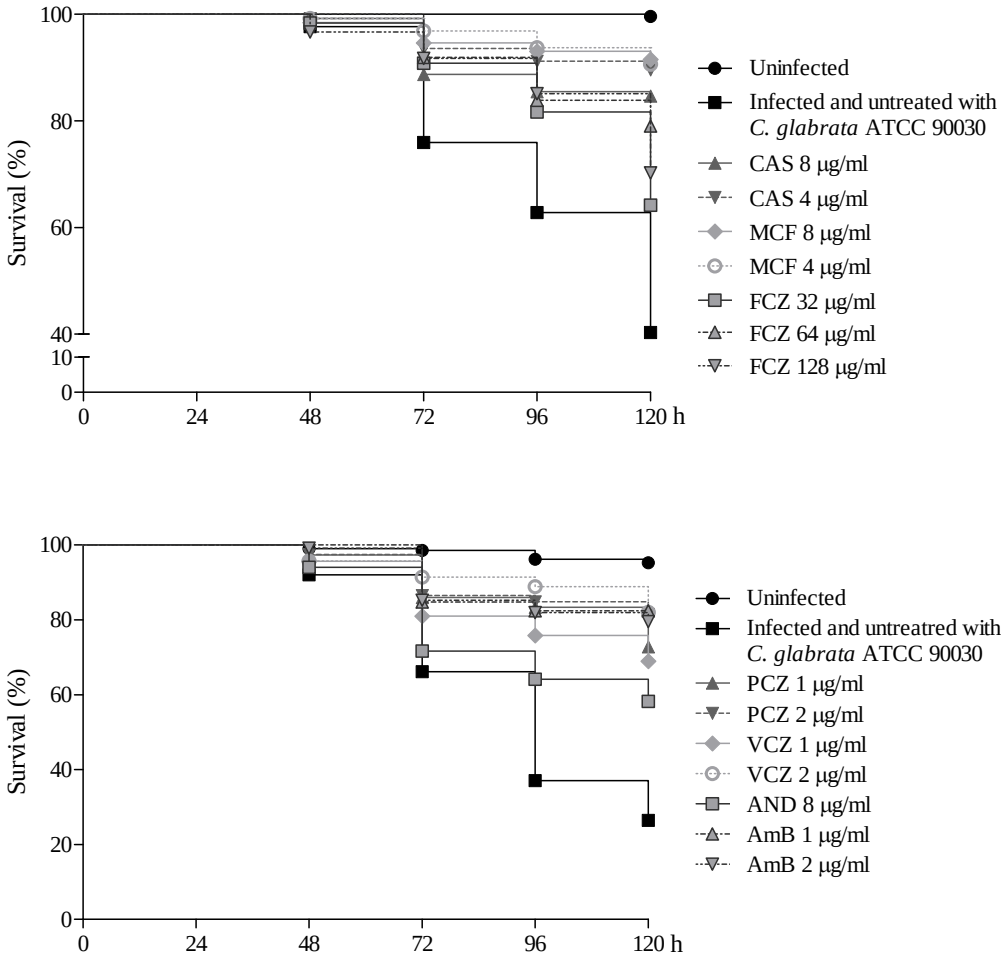
Among the antifungal drugs prepared in water, 8 µg/ml MCF was the most effective for treating infections caused by either *C. glabrata* strain (Table 1). During ATCC 90030 strain infection, significant differences in survival were detected when the worms were treated with 8 µg/ml MCF or FCZ at all concentrations tested (p=0.006) but not when comparing to other drugs (4 µg/ml MCF and both concentrations of CAS) that also resulted in effective treatments. However, against NCPF 3203 strain infection, MCF (8 µg/ml) was significantly more effective than all the other antifungal drugs except FCZ (128 µg/ml), which was similarly effective (96.8% and 94.6% survival at 120 h, respectively). AmB (1 and 2 µg/ml) resulted in the highest percentage of *C. elegans* survival. Nevertheless, no significant differences were observed between these and the other antifungal drugs prepared in DMSO against the infection of either *C. glabrata* strain, except for the treatment with VCZ (1 µg/ml) (p=0.015) or AND (8 µg/ml) (p=0) against the infection of the ATCC 90030 strain and AND (8 µg/ml) (p=0.001) against NCPF 3203 strain infection. These latter drugs allowed the least number of worms to survive (Figure 2).

MCF and CAS (8 µg/ml) were effective in protecting against *C. nivariensis* and *C. bracarensis* infections (Table 1). Although the treatment using any of the antifungal drugs prepared in water significantly protected against *C. nivariensis* CBS 9984 strain infection, echinocandins produced the highest *C. elegans* survival results. The same was observed for treating *C. nivariensis* CECT 11998 strain infection, except that with the lowest doses of FCZ (32 and 64 µg/ml), no differences were detected in worm survival compared to that of infected and untreated *C. elegans* (Figure 2). AmB (2 µg/ml), among those prepared in DMSO, was the most effective against *C. nivariensis* CBS 9984 strain infection, and together with VCZ (1 µg/ml), these drugs significantly increased worm survival compared to the survival of infected and untreated worms. The other drugs did not reduce the mortality of *C. elegans* (Figure 2). No treatment prepared in DMSO

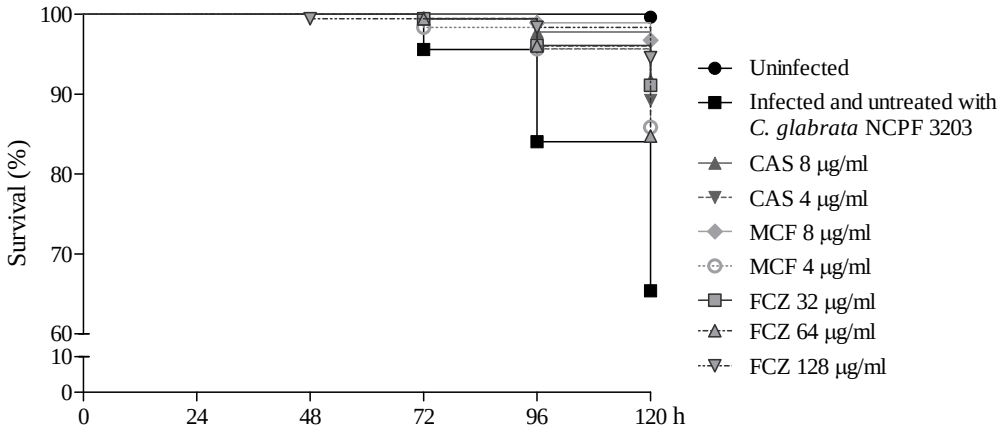
significantly increased the survival rate of *C. elegans* infected with the *C. nivariensis* CECT 11998 strain compared to that of infected and untreated worms.

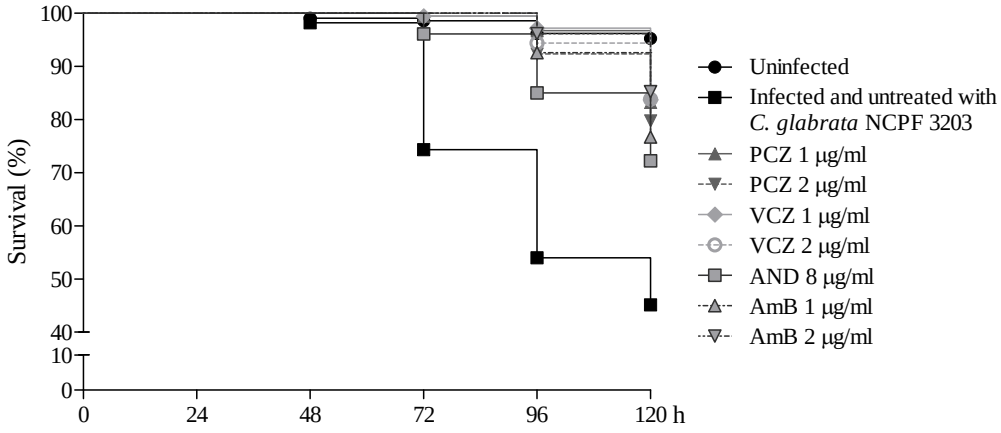
C. bracarensis was the least virulent of the three *Candida* species, and the survival of nematodes infected with either strain was so high in the absence of DMSO that it was difficult to evaluate the efficacy of some treatments. Treatment with the antifungal drugs prepared in water increased the survival of nematodes infected with either strain but did not achieve a significant improvement in worm survival, likely due to the low effect of the infection. On the other hand, the antifungal drugs prepared in DMSO resulted in increased survival of *C. elegans* infected with the NCYC 3397 strain, with 1 µg/ml VCZ achieving the highest worm survival (96.3%) ($p=0$). However, no drugs achieved protection against *C. elegans* infection with the *C. bracarensis* NCYC 3133 strain.

a) *Caenorhabditis elegans* infection with *C. glabrata* strain ATCC 90030

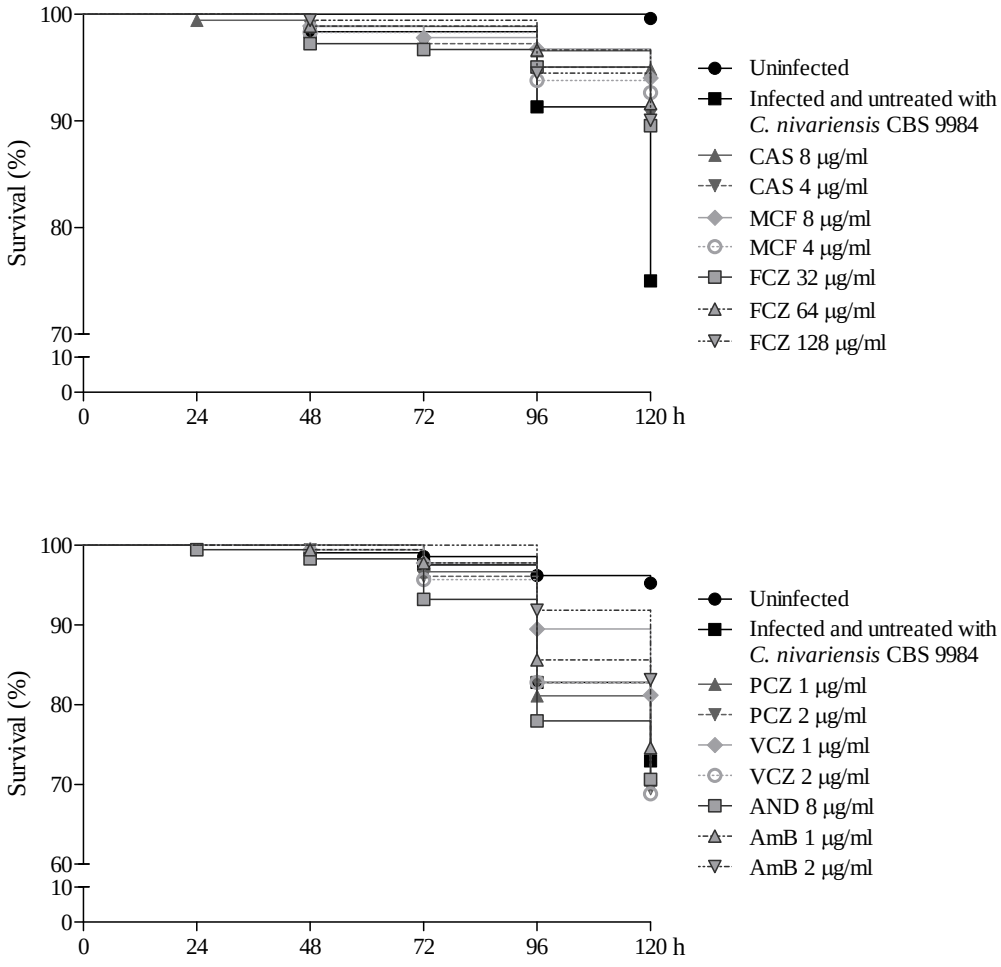


b) *Caenorhabditis elegans* infection with *C. glabrata* strain NCPF 3203

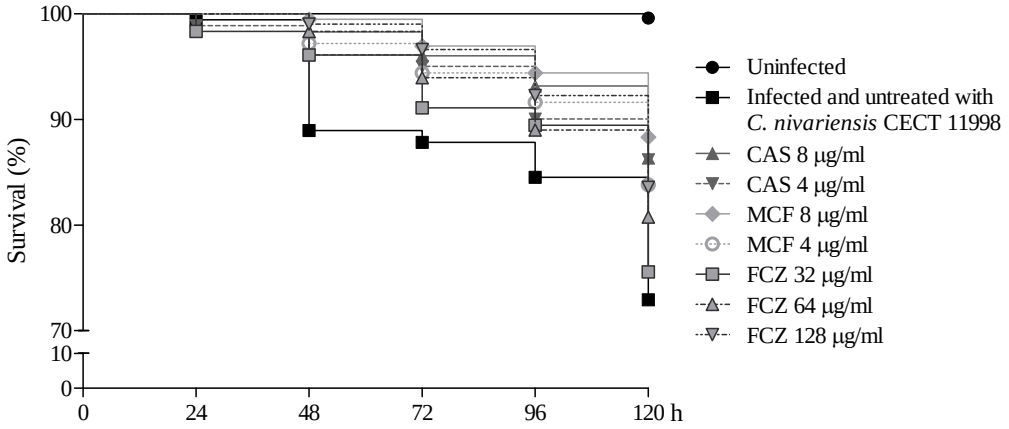


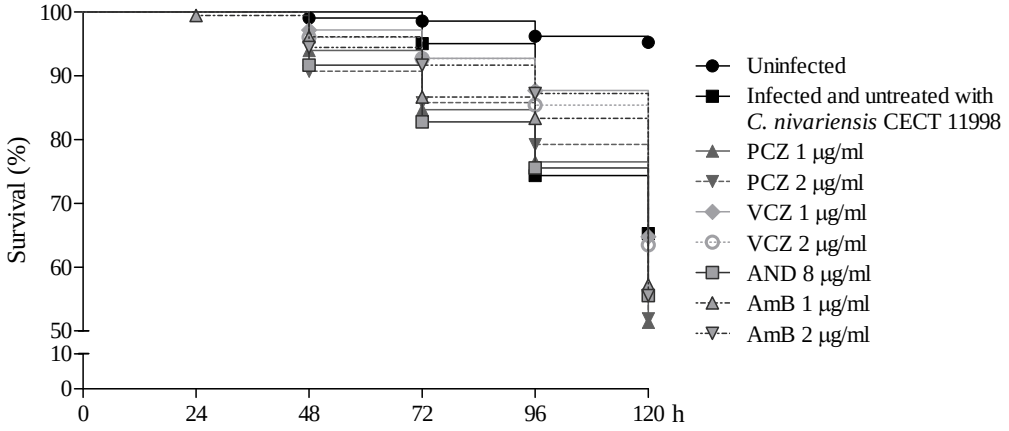


c) *Caenorhabditis elegans* infection with *C. nivariensis* strain CBS 9984



d) *Caenorhabditis elegans* infection with *C. nivariensis* strain CECT 11998





e) *Caenorhabditis elegans* infection with *C. bracarensis* strain NCYC 3397

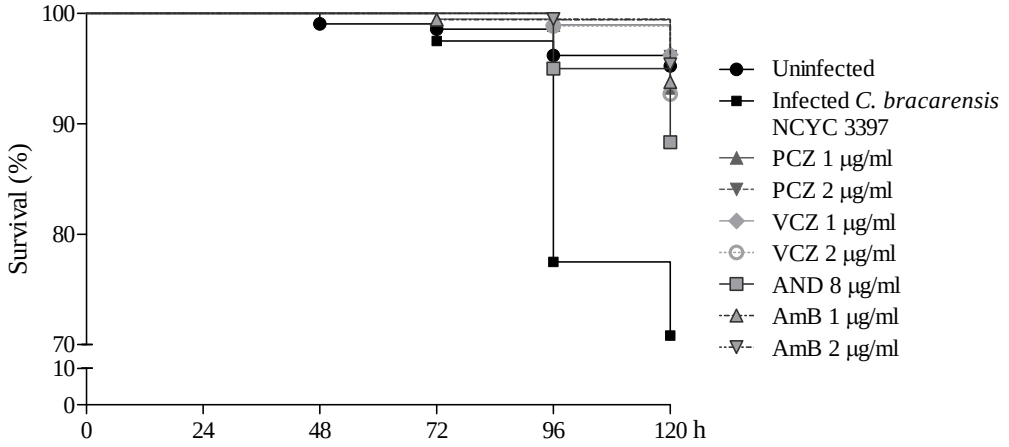


Figure 2. Efficacy of the antifungal drugs at different concentrations during *Caenorhabditis elegans* infection with *Candida glabrata* ATCC 90030 (a), *Candida glabrata* NCPF 3203 (b), *Candida nivariensis* CBS 9984 (c), *Candida nivariensis* CECT 11998 (d) or *Candida bracarensis* NCYC 3397 (e). The antifungal drugs fluconazole (FCZ), caspofungin (CAS) and micafungin (MCF) were prepared in water, while amphotericin B (AmB), anidulafungin (AND), posaconazole (PCZ) and voriconazole (VCZ) were prepared in 1% dimethyl sulfoxide (DMSO).

4. Discussion

C. elegans has been explored as an alternative model for characterizing host–fungal interactions. Most studies of invasive candidiasis using this host model focus on the infection caused by *C. albicans* (15, 28), and few studies involve other *Candida* species (18, 19, 20, 21). The emergence of *C. parapsilosis*, *C. glabrata*, *C. krusei* and *C. auris*, among others responsible for invasive candidiasis, makes both the study of the pathogenesis and worldwide surveillance of these species necessary (2). The actual epidemiology of emergent species that cause candidiasis, such as *C. glabrata* and the phylogenetically closely related species *C. nivariensis* and *C. bracarensis* is still unknown. Multiple studies have reported misidentified isolates of these cryptic species (29, 30, 31, 32, 33). Molecular approaches based on PCR, sequencing or MALDI-TOF MS are increasingly being applied because of their success in identifying rare *Candida*

species. Therefore, enhanced knowledge of *C. glabrata* and closely related species improves the diagnosis and choice of the most appropriate antifungal treatment.

The six strains of *C. glabrata*, *C. nivariensis* and *C. bracarensis* used in this study were able to infect and kill *C. elegans*. Despite current knowledge of the pathogenic potential of *Candida* using in vivo models, no studies have yet been published examining the pathogenesis of *C. nivariensis* and *C. bracarensis*. Nevertheless, there are sound data on the capacity of these cryptic species to cause infection in humans (10, 11, 29, 34, 35, 36, 37, 38, 39). Fairly little is known about virulence factors of *C. glabrata*. We did not detect proteinase, phospholipase or hemolytic activities in any of the six *Candida* strains tested. Several studies compared different protocols for analyzing the production of enzymes, such as phospholipase, highlighting the limitations in their detection (14, 40). Therefore, the absence of these virulence factors would need to be confirmed by further analysis. However, it has been demonstrated that adhesins, including proteins of the Epa family, are involved in virulence and are highly present at the cell surface of *C. glabrata* (41). The pathogenic potential of *C. nivariensis* and *C. bracarensis* could be explained, among others reasons, by the high numbers of *EPA* genes detected in these two species but not in other nonpathogenic species of the *Nakaseomyces* clade to which these species belong (42, 43).

In our study, *C. glabrata* was the most virulent species, and *C. bracarensis* was the least virulent species in *C. elegans*, which is coincident with the incidence of cases of these species in the literature (30, 31, 44, 45). Virulence studies of *C. glabrata* developed in in vivo models reported that approximately 30% of mice infected with 1×10^5 CFU per mouse survived up to 19 days post infection (46). However, no more than three days were necessary to kill *Galleria mellonella* larvae with an infective dose of 2.5×10^6 cells per larva (47). This difference highlighted the effect of the infective dose, although the specific characteristics of each host model also have to be considered (48). One of the limitations in the *C. elegans* model is not being able to control the precise infective dose administered, so the time employed in the infection of nematodes is one of the factors to consider (20). In the present work, the survival percentages of *C. elegans* at 120 h were

lower in the presence of 1% DMSO. This effect of DMSO was observed, to a greater or lesser extent, in the survival of nematodes infected with the six *Candida* strains and even in uninfected nematodes used as controls. The addition of DMSO when the eggs hatch has been shown to have a beneficial effect on the longevity of *C. elegans*. However, nematodes should be in this first stage of life and not in the adult stage after egg laying because DMSO could alter the membrane fluidity of worms, affecting the exchange of metabolites and external molecules (49). This phenomenon could be a potential explanation for the decrease in nematode survival observed.

Notably, the antifungal drugs prepared in DMSO managed to reduce the mortality of a high percentage of nematodes infected with either strain of *C. glabrata* or with the *C. bracarensis* NCYC 3397 strain, indicating their effectiveness despite the toxicity of the drug. AmB was very effective against these strains of *C. glabrata* and *C. bracarensis*, as the highest percentages of reduction in nematodes mortality were achieved. This polyene also showed good results in other studies in vitro against *C. glabrata* and *C. bracarensis* (5, 50) and in treating *C. glabrata* infection in *G. mellonella* (47) and murine models (51). Moreover, AmB was also effective in combination with VCZ for the treatment of persistent *C. glabrata* candidemia (9). In our study, the activities of VCZ and PCZ against these three *Candida* strains were similar; both drugs achieved a similar increase in worm survival percentage in each *Candida* strain infection. In vivo studies conducted in murine models also exhibited the same improvement for both antifungal drugs against *C. glabrata* infection (52, 53). In vitro susceptibility of *C. glabrata* and *C. bracarensis* to these second-generation triazoles has been reported, although reduced susceptibility of *C. glabrata* (5, 54) and *C. bracarensis* (30, 38) isolates has also been described.

Regarding *C. nivariensis*, none of the drugs in DMSO increased the survival of nematodes infected with either of the two strains studied by more than 10%; despite the susceptibility detected in vitro. Several in vitro studies showed the susceptibility of this species to AmB, AND, PCZ and VCZ (30, 32, 39, 55, 56); however, *C. nivariensis*

isolates with reduced susceptibility or resistance to FCZ, VCZ or PCZ have also been reported (36, 37, 57).

Echinocandins are the treatment of choice for *C. glabrata* invasive infections, but there are reports of *C. glabrata* isolates with resistance to these drugs (3, 58, 59). Resistance has been associated with echinocandin exposure and increased use in clinical practice (2, 9, 56, 60). In time-kill studies, all echinocandins were less active against *C. nivariensis* (61). However, our findings showed that in the *C. elegans* model, CSF and MCF were the most effective treatments against *C. nivariensis* infections and were also very effective against *C. glabrata* infections. The low virulence of *C. bracarensis* strains in this host model made it difficult to assess the effect of these two drugs. Nevertheless, AND showed good results against infection with both *C. bracarensis* NCYC 3397 and *C. glabrata* NCPF 3203 strains. AND was reported as the least effective echinocandin against *C. glabrata* infection in mice (62). Conversely, in a rabbit model of candidiasis, AND was more effective than liposomal AmB for treating *C. glabrata* infection associated with catheter colonization (63). The effectiveness of CSF was reported as an adequate treatment for *C. nivariensis* catheter-related fungemia (10), and it was also the most effective echinocandin in a murine model of *C. glabrata* infection with a dose of 1 mg/kg or 20 mg/kg (62, 64, 65, 66, 67). The treatment with CSF at this last dose in a murine model showed the appearance of *C. glabrata* strains harboring *FKS* mutations after five to nine days of treatment (8). Moreover, in a *G. mellonella* model of *C. glabrata* infection, 4 µg/g CSF increased larval survival by 60% (47). MCF presents a low interlaboratory MIC variability compared to that of CSF, and its clinical use is widespread compared to that of AND (68). However, *C. glabrata* isolates resistant to MCF have been reported (3, 9). Likewise, susceptible isolates of *C. glabrata*, *C. nivariensis* and *C. bracarensis* were also detected in several in vitro studies (30, 38, 39, 56).

Finally, FCZ is an antifungal drug frequently used in the treatment of invasive candidiasis, but increasing acquired resistance of *C. glabrata* has been reported (2). No protective effect against *C. glabrata* infection was observed in *G. mellonella* treated with

3, 6 or 12 µg/mg FCZ (47). However, in a murine model, treatment with high doses of FCZ (from 30 to 100 mg/kg FCZ) was required to achieve a significant decrease in the fungal burden (51, 52, 69). Although all strains were susceptible to FCZ in vitro, the highest doses of FCZ (64 or 128 µg/ml) were needed to detect a significant increase in the survival of *C. elegans* infected with *C. glabrata* or *C. nivariensis*. FCZ-resistant *C. nivariensis* isolates have been reported (36, 37), as well as susceptible isolates (39).

In conclusion, *C. elegans* was an appropriate and simple infection model to study the virulence of *C. glabrata* and the closely related species *C. nivariensis* and *C. bracarensis*. *C. glabrata* was the most virulent species. Moreover, this model system was successfully used for in vivo screening of antifungal drugs against infections caused by these three *Candida* species. However, the effect of antifungal treatments against *C. bracarensis* strains was sometimes compromised due to the low virulence of this species, and therefore, other models are needed where the infective dose can be more accurate.

Acknowledgements

This work was supported by the Consejería de Educación, Universidades e Investigación (GIC15/78 IT-990-16) of Gobierno Vasco-Eusko Jaurlaritza. A. Hernando-Ortiz was funded by a Ph.D. grant from the University of the Basque Country (PIF 16/39).

5. Bibliography

1. Sadeghi G, Ebrahimi-Rad M, Mousavi SF, Shams-Ghahfarokhi M, Razzaghi-Abyaneh M. 2018. Emergence of non-*Candida albicans* species: Epidemiology, phylogeny and fluconazole susceptibility profile. J Mycol Med **28**:51-58. https://doi.org/10.1016/j.mycmed.2017.12.008
2. Quindós G, Marcos-Arias C, San-Millán R, Mateo E, Eraso E. 2018. The continuous changes in the aetiology and epidemiology of invasive candidiasis: from familiar *Candida albicans* to multiresistant *Candida auris*. Int Microbiol **21**(3):107–19. https://doi.org/10.1007/s10123-018-0014-1

3. Fuller J, Dingle TC, Bull A, Shokoples S, Laverdière M, Baxter MR, Adam HJ, Karlowsky JA, Zhanel GG. 2019. Species distribution and antifungal susceptibility of invasive *Candida* isolates from Canadian hospitals: results of the CANWARD 2011-16 study. J Antimicrob Chemother **74**(4): iv48-iv54. https://doi.org/10.1093/jac/dkz287
4. Lamoth F, Lockhart SR, Berkow EL, Calandra T. 2018. Changes in the epidemiological landscape of invasive candidiasis. J Antimicrob Chemother **73**(1): 4-13. https://doi.org/10.1093/jac/dkx444
5. Astvad KMT, Johansen HK, Røder BL, Rosenvinge FS, Knudsen JD, Lemming L, Schønheyder HC, Hare RK, Kristensen L, Nielsen L, Gertsen JB, Dzajic E, Pedersen M, Østergård C, Olesen B, Søndergaard TS, Arendrup MC. 2018. Update from a 12-year nationwide fungemia surveillance: Increasing intrinsic and acquired resistance causes concern. J Clin Microbiol **56**(4):1-15. https://doi.org/10.1128/JCM.01564-17
6. Pfaller MA, Diekema DJ, Turnidge JD, Castanheira M, Jones RN. 2019. Twenty years of the SENTRY Antifungal Surveillance Program: Results for *Candida* species from 1997-2016. Open Forum Infect Dis **6**(1):79-94. https://doi.org/10.1093/ofid/ofy358
7. Pappas PG, Kauffman CA, Andes DR, Clancy CJ, Marr KA, Ostrosky-Zeichner L, Reboli AC, Schuster MG, Vazquez JA, Walsh TJ, Zaoutis TE, Sobel JD. 2016. Executive summary: clinical practice guideline for the management of candidiasis: 2016 update by the Infectious Diseases Society of America. Clin Infect Dis **62**:409–417. https://doi.org/10.1093/cid/civ1194
8. Healey KR, Nagasaki Y, Zimmerman M, Kordalewska M, Park S, Zhao Y, Perlin DS. 2017. The gastrointestinal tract is a major source of echinocandin drug resistance in a murine model of *Candida glabrata* colonization and systemic dissemination. Antimicrob Agents Chemother **61**(12):1-12. https://doi.org/10.1128/AAC.01412-17

9. Wright WF, Bejou N, Shields RK, Marr K, McCarty TP, Pappas PG. 2019. Amphotericin B induction with voriconazole consolidation as salvage therapy for FKS -associated echinocandin resistance *in Candida glabrata* septic arthritis and osteomyelitis. Antimicrob Agents Chemother **63**(8):1-6. https://doi.org/10.1128/aac.00512-19
10. López-Soria LM, Bereciartua E, Santamaría M, Soria LM, Hernández-Almaraz JL, Mularoni A, Nieto J, Montejo M. 2013. Primer caso de fungemia asociada a catéter por *Candida nivariensis* en la Península Ibérica. Rev Iberoam Micol **30**(1):69-71. http://dx.doi.org/10.1016/j.riam.2012.09.001
11. Aznar-Marin P, Galan-Sanchez F, Marin-Casanova P, García-Martos P, Rodríguez-Iglesias M. 2016. *Candida nivariensis* as a New Emergent Agent of Vulvovaginal Candidiasis: Description of Cases and Review of Published Studies. Mycopathologia **181**(5-6):445-9. https://doi.org/10.1007/s11046-015-9978-y
12. Cassone A, De Bernardis F, Mondello F, et al. 1987. Evidence for a correlation between proteinase secretion and vulvovaginal candidosis. J Infect Dis **156**:777–83. https://doi.org/10.1093/infdis/156.5.777
13. Luo G, Samaranayake LP, Yau JY. 2001. *Candida* species exhibit differential in vitro hemolytic activities. J Clin Microbiol **39**:2971-4. http://doi.org/10.1128/JCM.39.8.2971–2974.2001
14. Taniguchi L, de Fátima Faria B, Rosa RT, de Paula e Carvalho A, Gursky LC, Elifio-Esposito SL, Parahitiyawa N, Samaranayake LP, Rosa EAR. 2009. Proposal of a low-cost protocol for colorimetric semi-quantification of secretory phospholipase by *Candida albicans* grown in planktonic and biofilm phases. J Microbiol Methods **78(**2):171-174. doi:10.1016/j.mimet.2009.05.012
15. Elkabti AB, Issi L, Rao RP. 2018. *Caenorhabditis elegans* as a model host to monitor the *Candida* infection processes. J Fungi **4**:123. https:// doi:10.3390/jof4040123
16. Segal E, Frenkel M. 2018. Experimental in vivo models of candidiasis. J Fungi **4**:21. https://doi.org/10.3390/jof4010021

17. Desalermos A, Muhammed M, Glavis-Bloom J, Mylonakis E. 2011. Using *Caenorhabditis elegans* for antimicrobial drug discovery. Expert Opinion on Drug Discovery **6**(6): 645-652. https://doi.org/10.1517/17460441.2011.573781
18. Scorzoni L, de Lucas MP, Mesa-Arango AC, Fusco-Almeida AM, Lozano E, Cuenca-Estrella M, Mendes-Giannini MJ, Zaragoza O. 2013. Antifungal efficacy during *Candida krusei* infection in non-conventional models correlates with the yeast *in vitro* susceptibility profile. PLoS One **8**(3):e60047. https://doi.org/10.1371/journal.pone.0060047
19. Ford CB, Funt JM, Abbey D, Issi L, Guiducci C, Martinez DA, Delorey T, Li BY, White TC, Cuomo C, Rao RP, Berman J, Thompson DA, Regev A. 2015. The evolution of drug resistance in clinical isolates of *Candida albicans*.. eLife **4**:e00662. https://doi.org/10.7554/eLife.00662
20. Ortega-Riveros M, De-la-Pinta I, Marcos-Arias C, Ezpeleta G, Quindós G, Eraso E. 2017. Usefulness of the Non-conventional *Caenorhabditis elegans* Model to Assess *Candida* Virulence. Mycopathologia **182**(9-10):785-95. https://doi.org/10.1007/s11046-017-0142-8
21. Souza ACR, Fuchs BB, Alves V, Jayamani E, Colombo AL, Mylonakis E. 2018. Pathogenesis of the *Candida parapsilosis* complex in the model host *Caenorhabditis elegans*. Genes **9**(8):401. https://doi.org/10.3390/genes9080401
22. Polak A. 1992. Virulence of *Candida albicans* mutants. Mycoses **35**:9-16. https://doi.org/10.1111/j.1439-0507.1992.tb00813.x
23. Price MF, Wilkinson ID, Gentry LO. 1982. Plate method for detection of phospholipase activity in *Candida albicans*. Sabouraudia **20**:7-14. https://doi.org/10.1080/00362178285380031
24. Manns JM, Mosser DM, Buckley HR. 1994. Production of a hemolytic factor by *Candida albicans*. Infect Immun **62**:5154-6.
25. CLSI. Reference method for broth dilution antifungal susceptibility testing of yeasts; Fourth informational Supplement. CLSI documents M27-A3. CLSI, Wayne, PA: Clinical and Laboratory Standards Institute; USA; 2010.

26. CLSI. Reference method for broth dilution antifungal susceptibility testing of yeasts; Fourth informational Supplement. CLSI documents M27-A3 S4. CLSI, Wayne, PA: Clinical and Laboratory Standards Institute; USA, 2012
27. Breger J, Fuchs BB, Aperis G, Moy TI, Ausubel FM, Mylonakis E. 2007. Antifungal chemical compounds identified using a *C. elegans* pathogenicity assay. *PLoS Pathog* **3**(2):0168-0178. doi:10.1371/journal.ppat.0030018
28. Kumar A, Baruah A, Tomioka M, Iino Y, Kalita MC, Khan M. 2019. *Caenorhabditis elegans*: a model to understand host-microbe interactions. Cell Mol Life Sci. https://doi.org/10.1007/s00018-019-03319-7
29. Bishop JA, Chase N, Magill SS, Kurtzman CP, Fiandaca MJ, Merz WG. 2008. *Candida bracarensis* detected among isolates of *Candida glabrata* by peptide nucleic acid fluorescence in situ hybridization: Susceptibility data and documentation of presumed infection. J Clin Microbiol **46**(2): 443-446. https://doi.org/10.1128/JCM.01986-07
30. Lockhart SR, Messer SA, Gherna M, Bishop JA, Merz WG, Pfaller MA, Diekema DJ. 2009. Identification of *Candida nivariensis* and *Candida bracarensis* in a large global collection of *Candida glabrata* isolates: Comparison to the literature. J Clin Microbiol **47**(4):1216-7. https://doi.org/10.1128/JCM.02315-08
31. Swoboda-Kopeć E, Sikora M, Golas M, Piskorska K, Gozdowski D, Netsvyetayeva I. 2014. *Candida nivariensis* in comparison to different phenotypes of *Candida glabrata*. Mycoses **57**:747-753. https://doi.org/10.1111/myc.12264
32. Morales-López SE, Taverna CG, Bosco-Borgeat ME, Maldonado I, Vivot W, Szusz W, Garcia-Effron G, Córdoba SB. 2016. *Candida glabrata* species complex prevalence and antifungal susceptibility testing in a culture collection: First description of *Candida nivariensis* in Argentina. Mycopathologia **181**(11-12):871-8. https://doi.org/10.1007/s11046-016-0052-1
33. Małek M, Mrowiec P, Klesiewicz K, Skiba-Kurek I, Szczepański A, Białecka J, Żak I, Bogusz B, Kędzierska J, BudakA, Karczewska E. 2018. Prevalence of

human pathogens of the clade *Nakaseomyces* in a culture collection—the first report on *Candida bracarensis* in Poland. Folia Microbiol. https://doi.org/10.1007/s12223-018-0655-7

34. Alcoba-Flórez J, Méndez-Álvarez S, Cano J, Guarro J, Pérez-Roth E, Del Pilar Arévalo M. 2005. Phenotypic and molecular characterization of Candida nivariensis sp. nov., a possible new opportunistic fungus. J Clin Microbiol **43**(8):4107-11. https://doi.org/10.1128/JCM.43.8.4107-4111.2005
35. Correia A, Sampaio P, James S, Pais C. 2006. *Candida bracarensis* sp. nov., a novel anamorphic yeast species phenotypically similar to *Candida glabrata*. Int J Syst Evol Microbiol **56**(1):313-7. https://doi.org/10.1099/ijs.0.64076-0
36. Fujita SI, Senda Y, Okusi T, Ota Y, Takada H, Yamada K, Kawano M. 2007. Catheter-related fungemia due to fluconazole-resistant *Candida nivariensis*. J Clin Microbiol **45**(10):3459-61. https://doi.org/10.1128/JCM.00727-07
37. Borman AM, Petch R, Linton CJ, Palmer MD, Bridge PD, Johnson EM. 2008. *Candida nivariensis*, an emerging pathogenic fungus with multidrug resistance to antifungal agents. J Clin Microbiol **46**(3):933–938. https://doi.org/10.1128/JCM.02116-07
38. Warren TA, McTaggart L, Richardson SE, Zhang SX. 2010. *Candida bracarensis* bloodstream infection in an immunocompromised patient. J Clin Microbiol **48**(12): 4677-4679. https://doi.org/10.1128/JCM.01447-10
39. Tay ST, Lotfalikhani A, Sabet NS, Ponnampalavanar S, Sulaiman S, Na SL, Ng K P. 2014. Occurrence and characterization of *Candida nivariensis* from a culture collection of *Candida glabrata* clinical isolates in Malaysia. Mycopathologia **178**:307-314. https://doi.org/10.1007/s11046-014-9778-9
40. Echeverría A, Durante AG, Arechavala A, Negroni R. 2002. Estudio comparativo de dos medios de cultivo para la detección de la actividad fosfolipasa en cepas de *Candida albicans* y *Cryptococcus neoformans*. Rev Iberoam Micol **19**(2):95-98.
41. Gómez-Molero E, de Boer AD, Dekker HL, Moreno-Martínez A, Kraneveld EA, Ichsan, Chauhan N, Weig M, de Soet JJ, de Koster CG, Bader O, deGroot PWJ. 2015. Proteomic analysis of hyperadhesive candida glabrata clinical isolates

reveals a core wall proteome and differential incorporation of adhesins. FEMS Yeast Res **15**(8):1-10. https://doi.org/10.1093/femsyr/fov098

42. Gabaldón T, Martin T, Marcet-Houben M, Durrens P, Bolotin-Fukuhara M, Lespinet O, Arnaise S, Boisnard S, Aguileta G, Atanasova R, Bouchier C, Couloux A, Creno S, Almeida Cruz J, Devillers H, Enache-Angoulvant A, Guitard J, Jaouen L, Ma L, Marck C, Neuvéglise C, Pelletier E, Pinard A, Poulain J, Recoquillay J, Westhof E, Wincker P, Dujon B, Hennequin C, Fairhead C. 2013. Comparative genomics of emerging pathogens in the *Candida glabrata* clade. BMC Genomics **14**:623. http://www.biomedcentral.com/1471-2164/14/623
43. Angoulvant A, Guitard J, Hennequin C. 2016. Old and new pathogenic *Nakaseomyces* species: Epidemiology, biology, identification, pathogenicity and antifungal resistance. FEMS Yeast Research **16**(2):1-13. https://doi.org/10.1093/femsyr/fov114
44. Dudiuk C, Theill L, Gamarra S, Garcia-Effron G. 2017. Detection of Cryptic *Candida* Species Recognized as Human Pathogens Through Molecular Biology Techniques. Current Fungal Infection Reports **11**:176-183. https://doi.org/10.1007/s12281-017-0294-5
45. Asadzadeh M, Alanazi AF, Ahmad S, Al-Sweih N, Khan Z. 2019. Lack of detection of *Candida nivariensis* and *Candida bracarensis* among 440 clinical *Candida glabrata* sensu lato isolates in Kuwait. PLoS One **14**(10): e0223920. https://doi.org/10.1371/journal.pone.0223920
46. Fakhim H, Vaezi A, Dannaoui E, Chowdhary A, Nasiry D, Faeli L, Meis JF, Badali H. 2018. Comparative virulence of *Candida auris* with *Candida haemulonii*, *Candida glabrata* and *Candida albicans* in a murine model. Mycoses **61**:377-382. https://doi.org/10.1111/myc.12754
47. Ames L, Duxbury S, Pawlowska B, Ho H lui, Haynes K, Bates S. 2017. *Galleria mellonella* as a host model to study *Candida glabrata* virulence and antifungal efficacy. Virulence **8**(8): 1909–1917. https://doi.org/10.1080/21505594.2017.1347744

48. Frenkel M, Mandelblat M, Alastruey-Izquierdo A, Mendlovic S, Semis R, Segal E. 2016. Pathogenicity of *Candida albicans* isolates from bloodstream and mucosal candidiasis assessed in mice and *Galleria mellonella*. J Mycol Med **26**:1-8. https://doi.org/10.1016/j.mycmed.2015.12.006

49. Frankowski H, Alavez S, Spilman P, Mark KA, Nelson JD, Mollahan P, Rao RV, Chen SF, Lithgow GJ, Ellerby HM. 2013. Dimethyl sulfoxide and dimethyl formamide increase lifespan of *C. elegans* in liquid. Mech Ageing Dev **134**:69-78. https://doi.org/10.1016/j.mad.2012.10.002

50. Moreira A, Silva S, Botelho C, Sampaio P, Pais C, Henriques M. 2015. *Candida bracarensis*: Evaluation of Virulence Factors and its Tolerance to Amphotericin B and Fluconazole. Mycopathologia **180**(5-6):305-15. https://doi.org/10.1007/s11046-015-9925-y

51. Mariné M., Serena C, Pastor FJ, Guarro J. 2006. Combined antifungal therapy in a murine infection by *Candida glabrata*. J Antimicrob Chemother. **58**(6):1295-1298. https://doi.org/10.1093/jac/dkl395

52. Spreghini E, Maida C M, Tomassetti S, Orlando F, Giannini D, Milici M E, Scalise G, Barchiesi F. 2008. Posaconazole against *Candida glabrata* isolates with various susceptibilities to fluconazole. Antimicrobial Agents and Chemotherapy 52(6):1929-1933. https://doi.org/10.1128/AAC.00130-08

53. Sanchis M, Capilla J, Castanheira M, Martin-Vicente A, Sutton DA, Fothergill AW, Wiederholdc NP, Guarro J. 2016. Voriconazole minimum inhibitory concentrations are predictive of treatment outcome in experimental murine infections by *Candida glabrata*. Int J Antimicrob Agents **47**(4):286-8. http://dx.doi.org/10.1016/j.ijantimicag.2015.12.020

54. Carrillo-Muñoz AJ, Tur-Tur C, Hernández-Molina JM, Quindós G, Marcos-Arias C, Eraso E, Cárdenes D, Ortiz-Maestro O, Santos P, Estivill D, Guardia C, Giusiano G. 2010. Antifungal activity of posaconazole against *Candida* spp. and non-*Candida* clinical yeasts isolates. Rev Esp Quimioter **23**(3):122-125

55. Wahyuningsih R, Sahbandar IN, Theelen B, Hagen F, Poot G, Meis JF, Rozalyani A, Sjam R, Widodo D, Djauzi S, Boekhout T. 2008. *Candida nivariensis* isolated from an Indonesian human immunodeficiency virus-infected patient suffering from oropharyngeal candidiasis. J Clin Microbiol. **46**: 388-391. https://doi.org/10.1128/JCM.01660-07

56. Morales-López S, Dudiuk C, Vivot W, Szusz W, Córdoba SB, Garcia-Effron G. 2017. Phenotypic and molecular evaluation of echinocandin susceptibility of *Candida glabrata*, *Candida bracarensis*, and *Candida nivariensis* strains isolated during 30 years in Argentina. Antimicrob Agents Chemother **61**(7):7-10. https://doi.org/10.1128/AAC.00170-17

57. Figueiredo-Carvalho M H G, de Souza Ramos L, Barbedo L S, da Silva Chaves A L, Muramoto I A, dos Santos A L S, Almeida-Paes G, Zancopé-Oliveira R M. 2016. First description of *Candida nivariensis* in Brazil: Antifungal susceptibility profile and potential virulence attributes. Memorias Do Instituto Oswaldo Cruz **111**(1):51-58. https://doi.org/10.1590/0074-02760150376

58. Pham CD, Iqbal N, Bolden CB, Kuykendall RJ, Harrison LH, Farley MM, Schaffner W, Beldavs ZG, Chiller TM, Park BJ, Cleveland AA, Lockhart SR. 2014. Role of FKS mutations in *Candida glabrata*: MIC values, echinocandin resistance, and multidrug resistance. Antimicrob Agents Chemother **58**:4690-4696. https://doi.org/10.1128/AAC.03255-14

59. Castanheira M, Deshpande LM, Davis AP, Rhomberg PR, Pfaller MA. 2017. Monitoring antifungal resistance in a global collection of invasive yeasts and molds: Application of CLSI epidemiological cutoff values and whole-genome sequencing analysis for detection of azole resistance in *Candida albicans*. Antimicrob Agents Chemother **61**(10):1-20. https://doi.org/10.1128/AAC.00906-17

60. Beyda N D, John J, Kilic A, Alam M J, Lasco T M, Garey K W. 2014. FKS mutant *Candida glabrata*: Risk factors and outcomes in patients with candidemia. Clinical Infectious Diseases **59**(6):819–825. https://doi.org/10.1093/cid/ciu407

61. Gil-Alonso S, Jauregizar N, Cantón E, Eraso E, Quindós G. 2015. In vitro fungicidal activities of anidulafungin, caspofungin, and micafungin against *Candida glabrata*, *Candida bracarensis*, and *Candida nivariensis* evaluated by time-kill studies. Antimicrob Agents Chemother **59**(6):3615-8. https://doi.org/10.1128/AAC.04474-14
62. Spreghini E, Orlando F, Sanguinetti M, Posteraro B, Giannini D, Manso E, Barchiesi F. 2012. Comparative effects of micafungin, caspofungin, and anidulafungin against a difficult-to-treat fungal opportunistic pathogen, *Candida glabrata*. Antimicrob Agents Chemother **56**(3):1215-22. https://doi.org/10.1128/AAC.05872-11
63. Basas J, Palau M, Gomis X, Almirante B, Gavaldà J. 2019. Efficacy of liposomal amphotericin B and anidulafungin using an antifungal lock technique (ALT) for catheter-related *Candida albicans* and *Candida glabrata* infections in an experimental model. PLoS One **14**(2):1-11. https://doi.org/10.1371/journal.pone.0212426
64. Fernández-Silva F, Lackner M, Capilla J, Mayayo E, Sutton D, Castanheira M, Fothergill AW, Lass-Flörl C, Guarro J. 2014. In vitro antifungal susceptibility of *Candida glabrata* to caspofungin and the presence of FKS mutations correlate with treatment response in an immunocompromised murine model of invasive infection. Antimicrob Agents Chemother **58**(7): 3646–3649. https://doi.org/10.1128/AAC.02666-13
65. Domán M, Kovács R, Perlin DS, Kardos G, Gesztelyi R, Juhász B, Bozó A, Majoros L. 2015. Dose escalation studies with caspofungin against *Candida glabrata*. J Med Microbiol **64**(9):998–1007. https://doi.org/10.1099/jmm.0.000116
66. Howard SJ, Livermore J, Sharp A, Goodwin J, Gregson L, Alastruey-Izquierdo A, Perlin DS, Warn PA, Hope WW. 2011. Pharmacodynamics of echinocandins against *Candida glabrata*: Requirement for dosage escalation to achieve maximal antifungal activity in neutropenic hosts. Antimicrob Agents Chemother **55**(10): 4880-4887. https://doi.org/10.1128/AAC.00621-11

67. Wiederhold NP, Najvar LK, Fothergill AW, Bocanegra R, Olivo M, McCarthy DI, Fukuda Y, Mitsuyama J, Patterson T F. 2016. The novel arylamidine T-2307 demonstrates in vitro and in vivo activity against echinocandin-resistant *Candida glabrata*. J Antimicrob Chemother **71**: 692-695. https://doi.org/10.1093/jac/dkv398

68. Bienvenu AL, Leboucher G, Picot S. 2019. Comparison of *FKS* gene mutations and minimum inhibitory concentrations for the detection of *Candida glabrata* resistance to micafungin: A systematic review and meta-analysis. Mycoses **62**:835-846. https://doi.org/10.1111/myc.12929

69. Ben-Ami R, Zimmerman O, Finn T, Amit S, Novikov A, Wertheimer N, Lurie-Weinberger M, Berman J. 2016. Heteroresistance to fluconazole is a continuously distributed phenotype among *Candida glabrata* clinical strains associated with in vivo persistence. MBio **7**(4):e00655-16. https://doi.org/10.1128/mBio.00655-16

AMERICAN SOCIETY FOR MICROBIOLOGY | Antimicrobial Agents and Chemotherapy®

SUSCEPTIBILITY

Caenorhabditis elegans as a Model System To Assess *Candida glabrata*, *Candida nivariensis*, and *Candida bracarensis* Virulence and Antifungal Efficacy

Ainara Hernando-Ortiz,[a] Estibaliz Mateo,[a] Marcelo Ortega-Riveros,[a] Iker De-la-Pinta,[a] Guillermo Quindós,[a] Elena Eraso[a]

[a]UFI 11/25 Microbios y Salud, Departamento de Inmunología, Microbiología y Parasitología, Facultad de Medicina y Enfermería, Universidad del País Vasco/Euskal Herriko Unibertsitatea, UPV/EHU, Bilbao, Spain

ABSTRACT Although *Candida albicans* remains the major etiological agent of invasive candidiasis, *Candida glabrata* and other emerging species of *Candida* are increasingly isolated. This species is the second most prevalent cause of candidiasis in many regions of the world. However, clinical isolates of *Candida nivariensis* and *Candida bracarensis* can be misidentified and are underdiagnosed due to phenotypic traits shared with *C. glabrata*. Little is known about the two cryptic species. Therefore, pathogenesis studies are needed to understand their virulence traits and their susceptibility to antifungal drugs. The susceptibility of *Caenorhabditis elegans* to different *Candida* species makes this nematode an excellent model for assessing host-fungus interactions. We evaluated the usefulness of *C. elegans* as a nonconventional host model to analyze the virulence of *C. glabrata*, *C. nivariensis*, and *C. bracarensis*. The three species caused candidiasis, and the highest virulence of *C. glabrata* was confirmed. Furthermore, we determined the efficacy of current antifungal drugs against the infection caused by these species in the *C. elegans* model. Amphotericin B and azoles showed the highest activity against *C. glabrata* and *C. bracarensis* infections, while echinocandins were more active for treating those caused by *C. nivariensis*. *C. elegans* proved to be a useful model system for assessing the pathogenicity of these closely related species.

KEYWORDS candidiasis, *Caenorhabditis elegans*, nonconventional host model, pathogenesis, antifungal susceptibility, antifungal susceptibility testing

Invasive candidiasis is the most frequent mycosis, mainly in patients suffering from immunodeficiency. Although *Candida albicans* remains the predominant etiological agent, there is an increase in infections caused by other *Candida* species, such as *Candida parapsilosis*, *Candida glabrata*, *Candida krusei*, and *Candida auris*, which has been associated with reduced antifungal susceptibility or even increased rates of resistance (1–3). Among these species, *C. glabrata* has been considered the second or third most isolated species of *Candida* from blood cultures according to geographical distribution. This species is a frequent cause of candidemia in the United States, Australia, and North and Central Europe, and there is a trend toward an etiological rise in Spain and Portugal (2, 4–6). *C. glabrata* invasive infection treatment is often a clinical challenge due to the increasing prevalence of azole resistance. Although echinocandins are considered the treatment of choice (7), *C. glabrata* is also the species most likely to be resistant to echinocandins (8, 9).

C. glabrata sensu stricto shares high phenotypic similarities and genetic closeness with *Candida bracarensis* and *Candida nivariensis*. As of yet, the reported incidence of *C. bracarensis* and *C. nivariensis* is low, and data about their virulence and antifungal

Citation Hernando-Ortiz A, Mateo E, Ortega-Riveros M, De-la-Pinta I, Quindós G, Eraso E. 2020. *Caenorhabditis elegans* as a model system to assess *Candida glabrata*, *Candida nivariensis*, and *Candida bracarensis* virulence and antifungal efficacy. Antimicrob Agents Chemother 64:e00824-20. https://doi.org/10.1128/AAC.00824-20.

Address correspondence to Estibaliz Mateo, estibaliz.mateo@ehu.eus.

Received 27 April 2020
Returned for modification 23 June 2020
Accepted 19 July 2020
Accepted manuscript posted online 27 July 2020
Published 21 September 2020

3. Eranskina/Anexo 3

Candidiasis by *Candida glabrata*, *Candida nivariensis* and *Candida bracarensis* in *Galleria mellonella*: strain virulence and therapeutic responses to echinocandins

Ainara Hernando-Ortiz, Elena Eraso, Guillermo Quindós and Estibaliz Mateo

Department of Immunology, Microbiology and Parasitology, Faculty of Medicine and Nursery, University of the Basque Country (UPV/EHU), Bilbao, Spain

Manuscript in preparation

Abstract

Candida albicans is the major etiological agent of invasive candidiasis but the increasing prevalence of emerging species of *Candida*, such as *Candida glabrata* and phylogenetically closely-related species, *Candida nivariensis* and *Candida bracarensis*, requires special attention. *C. glabrata* has gained clinical relevance due to its reduced susceptibility to commonly used antifungal drugs, particularly fluconazole. Although, echinocandins are considered the treatment of choice for most cases of candidaemia, resistance associated with *FKS* genes mutations has been reported in these three *Candida* species. Differences in virulence among these species and their therapeutic responses using in vivo non-mammalian models are scarcely analyzed. Therefore, in this study, the survival of *G. mellonella* and host-pathogen interactions (variation in haemocyte production and their ability to phagocytise yeast) during infection by *C. glabrata*, *C. nivariensis* and *C. bracarensis* were analyzed. These three species produced lethal infection in *G. mellonella*; *C. glabrata* was the most virulent species and *C. bracarensis* the less. Haemocytes of *G. mellonella* phagocytised *C. bracarensis* cells more effectively than those of the other two species. Moreover, therapeutic responses to echinocandins were also assessed in the *G. mellonella* model of candidiasis. Treatment with caspofungin and micafungin was most effective to protect larvae during *C. glabrata* and *C. nivariensis* infections while anidulafungin was during *C. bracarensis* infection. The model of candidiasis in *G. mellonella* is simple and appropriate to assess the virulence and therapeutic response of these emerging *Candida* species, moreover successfully allows detecting differences in the immune system of the host depending on the virulence of pathogens.

Keyboards: emerging pathogen, pathogenesis, antifungal susceptibility, invertebrate models

1. INTRODUCTION

There is a substantial change in the aetiology of candidiasis worldwide, with an increasing prevalence of non-*Candida albicans* species, such as *Candida parapsilosis, Candida glabrata, Candida tropicalis, Candida krusei* and *Candida auris* (Pappas et al., 2018). *C. glabrata* is an emerging pathogen and the second cause of candidaemia in the USA, Canada, Australia and Northern and Eastern Europe. In Spain, candidiasis due to *C. glabrata* are the third most frequent, behind those caused by *C. albicans* and *C. parapsilosis* (Quindós et al., 2018). *Candida bracarensis* and *Candida nivariensis* are species phylogenetically close to *C. glabrata* that should be identified by molecular methods, including MALDI-TOF MS (Matrix Assisted Laser Desorption/Ionization Mass Spectrometry) (Johnson, 2009; Jamal et al., 2014; Asadzadeh et al., 2019; Alobaid et al., 2020). *C. glabrata* often develops resistance to antifungal drugs, especially fluconazole (Alexander et al., 2013; Beyda et al., 2014; Astvad et al., 2018; Asadzadeh et al., 2019; Pfaller et al., 2019). Infections caused by *C. nivariensis* and *C. bracarensis* with reduced susceptibility or even resistance to azoles has also been reported (Fujita et al., 2007; Borman et al., 2008; Bishop et al., 2008; Lockhart et al., 2009; Warren et al., 2010; Figueiredo-Carvalho et al., 2016; Hou et al., 2017; Cartier et al., 2020). The echinocandins, anidulafungin (AND), caspofungin (CAS) and micafungin (MCF) are the treatment of choice for *C. glabrata* invasive infections but echinocandin resistance associated with *FKS* genes mutations has been reported (Cornelly et al., 2012; Kołaczkowska and Kołaczkowski, 2016; McCarty et al., 2018; Coste et al., 2020; Al-Baqsami et al., 2020). Frequency of *C. nivariensis* and *C. bracarensis* candidiasis is low (0.05-0.2 %) and evidence on echinocandins activities against these species is scarce (Lockhart et al., 2009; Astvad et al., 2018; Arastehfar et al., 2019). However, it is necessary a deeper knowledge to avoid therapeutic failures (Ames et al., 2017).

Common mammalian models of infection are linked to ethical concerns that recommend limiting their use. Models in invertebrate animals, such as those developed in *Caenorhabditis elegans* and *Galleria mellonella*, are being increasingly introduced for studying host-pathogen interactions and for evaluating antimicrobial efficacy of old

antimicrobial agents and new ones (Ames et al., 2017; Elkabti et al., 2018; Hernando-Ortiz et al., 2020; Jemel et al., 2020). *G. mellonella*, the greater wax moth, is a short live cycle lepidopteron that facilitates experiments at 37 °C, simulating human body temperature (Mesa-Arango et al., 2012; Cook et al., 2013; Scorzoni et al., 2013; Gago et al., 2014; Killiny et al., 2018). Larval size allows injecting the yeast inoculum and the drugs for treatment at specific concentrations. In addition, it is a useful model for studying host-pathogen interactions because its immune system has conserved similarities with the mammal innate defences (Mesa-Arango et al., 2012; Cook et al., 2013; Browne et al., 2014; Gago et al., 2014; Killiny et al. 2018; Segal et al., 2018; Marcos-Zambrano et al., 2019). These interactions can be evaluated by assessing the larvae response against pathogens and the phagocytic capacity of haemocytes present in the haemolymph of the larvae (Gago et al., 2014; Scorzoni et al., 2013).

In this work, we analyze the different virulence of *C. glabrata*, *C. nivariensis* and *C. bracarensis* in *G. mellonella* and the interactions between these *Candida* species and the *G. mellonella* haemocyte density and phagocytic response. Moreover, the effectiveness of echinocandins, AND, CAS, and MCF, for treating invasive candidiasis caused by these *Candida* species was evaluated in *G. mellonella* host model.

2. METHODOLOGY

2.1 *Candida* strains and growth conditions

Six commercially available reference strains, including two strains of each species *C. glabrata*, *C. nivariensis* and *C. bracarensis*, were obtained from different culture collections (Table 1).

Yeasts were cultured overnight in yeast extract peptone dextrose broth (YEPD; 1% yeast extract, 2% bacteriological peptone, 2% D-glucose) liquid medium (Panreac, Spain) at 30 °C under shaking conditions. Then, yeast cells were washed three times with phosphate-buffered saline solution (PBS) and resuspended in PBS supplemented with ampicillin (20 mg/L) to avoid a possible bacterial contamination. Cell counting was performed by microscopy using a Burker haemocytometer and three concentrations of 1×10^7, 1×10^8 and 1×10^9 yeast cells/ml were prepared in PBS-ampicillin (20 mg/L) to use as inocula.

Table 1. Survival of *Galleria mellonella* larvae infected with *C. glabrata*, *C. nivariensis* and *C. bracarensis* with and without treatment with echinocandins.

Strain	Origin	Collection reference	Survival percentages of *G. mellonella* infected with $1x10^6$ cells/larva at 120 h	Most effective antifungal treatments (survival rate increase of *G. mellonella* at 120 h)
Candida glabrata				
ATCC 90030	Blood	American Type Culture Collection	38.7%	Micafungin, 8 µg/ larva (34.6%) Micafungin, 4 µg/ larva (33%) Caspofungin, 4 µg/ larva (33%)
NCPF 3203	Blood	National Collection of Pathogenic Fungi	39.2%	Anidulafungin, 4 µg/ larva (45.8%) Micafungin, 8 µg/ larva (42.5 %) Caspofungin, 8 µg/ larva (42.5%)
Candida nivariensis				
CBS 9984	Bronchoalveolar lavage	Westerdijk Fungal Biodiversity Institute	43.3%	Micafungin, 4 µg/ larva (41.7%) Caspofungin, 8 µg/ larva (38.4%) Caspofungin, 4 µg/ larva (35%)
CECT 11998	Blood	Colección Española de Cultivos Tipo	45.8%	Caspofungin, 4 µg/ larva (35.9%) Caspofungin, 8 µg/ larva (34.2%) Micafungin, 4 µg/ larva (29.2%)
Candida bracarensis				
NCYC 3397	Blood	National Collection of Yeast Cultures	42.5%	Anidulafungin, 4 µg/ larva (29.2%) Caspofungin, 8 µg/ larva (19.2%) Anidulafungin, 8 µg/ larva (14.2%)
NCYC 3133	Catheter	National Collection of Yeast Cultures	55.7%	Micafungin, 4 µg/ larva (24.3%) Anidulafungin, 8 µg/ larva (21%) Caspofungin, 8 µg/ larva (14.6%)

2.2 Survival of *Galleria mellonella*

Larvae of *G. mellonella* weighing between 0.3 and 0.5 g (Bichosa, Spain) were placed in groups of 20 individuals in Petri plates to perform the experiments. The last left pro-leg of larvae was cleaned with ethanol 70% before injecting 10 µl of *Candida* suspension into the larvae haemocele with a precision syringe (Agilent, USA). The final inocula tested were 1×10^5, 1×10^6 and 1×10^7 cells/larva. Two uninfected larvae groups were used as controls in all trials: a group of untouched larvae and a group of larvae injected with 10 µl of PBS-ampicillin to control the possible effect of the injection on larvae survival (sham group). The larvae were incubated at 37 °C in dark for 120 h, and survival was monitored every 24 h by visual inspection of melanisation and the absence of movement. Each trial was performed at least three times on different days. A total of 180 larvae were used to assess the infection caused for each of the six *Candida* strains and 40 larvae were used as control in each trial.

2.3 Haemocyte density determination

Groups of five larvae of *G. mellonella* were inoculated with 1×10^5, 1×10^6 and 1×10^7 cells/larva. As control, a group of uninfected larvae inoculated with 10 µl PBS-ampicillin was used (sham group). Larvae were incubated at 37 °C in dark for 3 h and then, 50 µl of haemolymph was collected from each larva and mixed with insect physiological saline buffer (IPS buffer; 150 mM sodium chloride, 5 mM potassium chloride, 10 mM Tris-HCl pH 6.9, 10 mM EDTA and 30 mM sodium citrate) to avoid melanisation and coagulation of haemolymph. Haemocyte density was determined by microscopy counting using a haemocytometer. Each assay was performed at least three times on different days.

2.4 Phagocytic activity of haemocytes

Five *G. mellonella* larvae were used for each *Candida* strain. Yeast cells were stained with 0.4 mg/ml of Calcofluor white (Sigma Aldrich, St. Louis, MO, USA) for 30 min at 30 ºC and washed twice with PBS before inoculated in each larva 1×10^6 cells/larva. Larvae were incubated at 37 °C for 2 h and afterwards, 50 µl of haemolymph of each

larva was collected in the same volume of IPS buffer. Phagocytosis quantification was performed by fluorescence microscope Nikon Eclipse 80i (Melville, NY, USA) counting a minimum of 100 haemocytes with and without yeast phagocytised from each larva. Trials were performed at least three times on different days.

2.5 Antifungal treatments with echinocandins

Groups of 20 larvae of *G. mellonella* were inoculated with 1×10^6 cells/larva. These infected larvae were immediately treated with AND (Pfizer SA, Madrid, Spain), CAS (Merk & Com Inc, NJ, USA) and MCF (Astellas Pharma Inc, Japan) at concentrations of 4 and 8 μg/g larva. Three control groups with uninfected larvae were included, a group of untouched larvae, a group of larvae injected with PBS-ampicillin (sham group), and a third group of larvae injected with each antifungal drug to evaluate their possible toxicity. The larvae were incubated at 37 °C in dark for 120 h, and survival was monitored every 24 h by visual inspection of melanisation and the absence of movement. Each trial was performed at least three times on different days. A total of 780 larvae were used to assess the effect of the different treatments against each of the six *Candida* strains and 100 larvae were used as control in each trial.

2.6 Statistics

The results obtained of haemocytes production and phagocytic activity were analysed using one-way ANOVA of the statistical program SPSS v24.0 (IBM, Chicago, IL, USA). Survival analysis curves were prepared with the Kaplan-Meier method using GraphPad Prism 5 software (GraphPad Software, La Jolla, CA, USA). Differences in *G. mellonella* survival infected with the *Candida* strains and exposed to the different antifungal treatments were analysed by the log-rank test using SPSS v24.0. The value of $p < 0.05$ was considered as statistically significant.

3. RESULTS

3.1. Virulence of *Candida* in the *Galleria mellonella* model

C. glabrata, *C. nivariensis* and *C. bracarensis* caused invasive candidiasis in the *G. mellonella* model. Significant differences were observed in the survival of infected and uninfected larvae used as controls ($p \leq 0.003$). Survivals of untouched larvae and those PBS-ampicillin injected larvae were 87 % ± 1.45 % and 88.5 % ± 1.5 %, respectively, without significant differences among them ($p=0.663$).

Three inocula were assayed to evaluate the virulence of the strains of *C. glabrata*, *C. nivariensis* and *C. bracarensis* observing that larval mortality was inoculum-dependent. Mortality rate was directly proportional to the injected inoculum: 34.7 % ± 7.1 %, 55.9 % ± 6.4 % and 76.8 % ± 10.3 % from lowest to highest inocula, respectively. Moreover, there were significant differences among the three species ($p \leq 0.001$) (Figure 1). The virulence of these species in the *G. mellonella* model was categorized as *C. glabrata* > *C. nivariensis* > *C. bracarensis*, regardless of the inoculum applied. However, there were mortality rate differences among the injected inocula with the strains of each species.

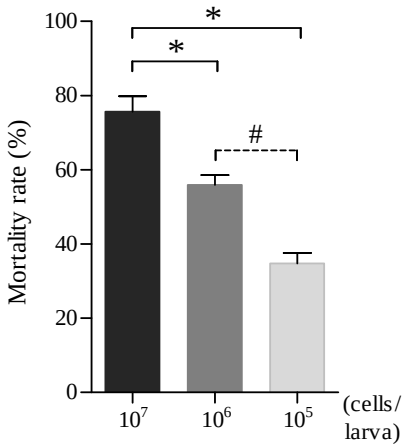


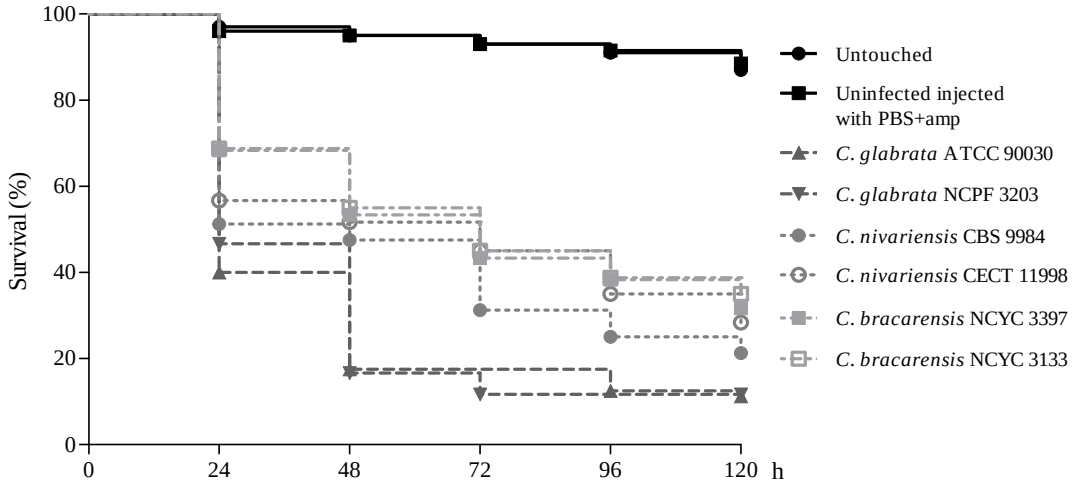
Figure 1. Mortality rate of *G. mellonella* larvae infected with 1×10^7, 1×10^6 and 1×10^5 cells/larva of *C. glabrata*, *C. nivariensis* and *C. bracarensis*.

The infection caused by 1×10^7 cells/larva showed that *C. glabrata* was significantly more virulent than *C. nivariensis* ($p \leq 0.024$) and *C. bracarensis* ($p \leq 0.001$), showing differences in their killing kinetics. At 48 h of infection, larvae inoculated with *C. glabrata* achieved mortality rates above 80 %, while the mortality rate was 50-60 % for those inoculated with *C. nivariensis* and *C. bracarensis*. However, during the next 72 h of infection, *C. glabrata* killed 8% more larvae, and *C. nivariensis* and *C. bracarensis* killed more than 20% (Figure 2a). There were also significant differences between the *C. nivariensis* CBS 9984 strain and the *C. bracarensis* NCYC 3133 strain, being the last one the least virulent of tested strains ($p=0.048$).

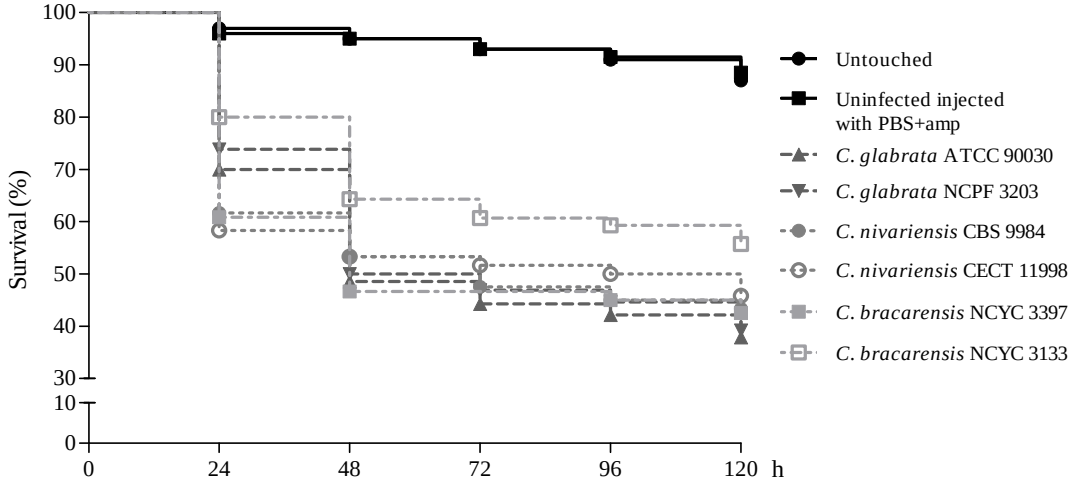
Strikingly, there were no significant differences in virulence among the three species when larvae were inoculated with 1×10^6 and 1×10^5 cells/larva, except for the *C. bracarensis* NCYC 3133 strain that achieved the highest survival rate (55.7 % and 75 %, respectively) at 120 h post-infection. Survival of *G. mellonella* inoculated with 1×10^6 cells/larva was lower than 55% at 48 h post-infection except with the *C. bracarensis* NCYC 3133 strain, which it took more than 120 h to reach a mortality rate of 44% (Figure 2b). This latter and the *C. nivariensis* CECT 11998 strains did not kill more than 28% of the larvae after 120 h with the inoculum of 1×10^5 cells/larva, and the remaining *Candida* strains failed to kill more than 42% of the larvae (Figure 2c).

No differences in the larvae survival were found within the two *C. glabrata* strains or within both *C. nivariensis* strains, regardless of the inoculum used. However, the survival for larvae infected with 1×10^6 cells of *C. bracarensis* strain NCYC 3397 was significantly lower than that of larvae infected with the *C. bracarensis* NCYC 3133 strain ($p=0.013$).

a)


100
80
60
40
20
0
Survival (%)
0
24
48
72
96
120
h
Untouched
Uninfected injected with PBS+amp
C. glabrata ATCC 90030
C. glabrata NCPF 3203
C. nivariensis CBS 9984
C. nivariensis CECT 11998
C. bracarensis NCYC 3397
C. bracarensis NCYC 3133

b)


100
90
80
70
60
50
40
30
10
0
Survival (%)
0
24
48
72
96
120
h
Untouched
Uninfected injected with PBS+amp
C. glabrata ATCC 90030
C. glabrata NCPF 3203
C. nivariensis CBS 9984
C. nivariensis CECT 11998
C. bracarensis NCYC 3397
C. bracarensis NCYC 3133

c)

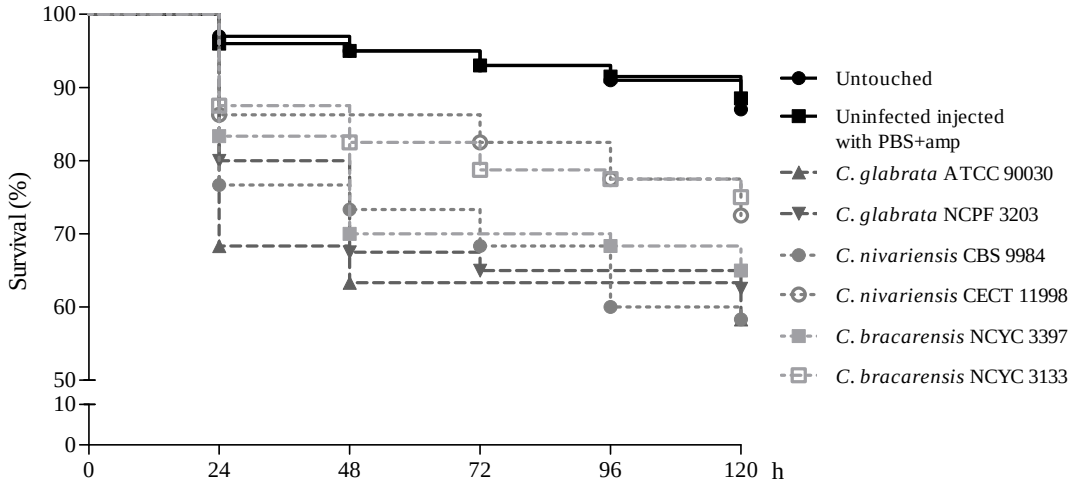


Figure 2. Survival curves of *G. mellonella* infected with 1×10^7 (a), 1×10^6 (b), and 1×10^5 (c) cells/larva of *C. glabrata*, *C. nivariensis* and *C. bracarensis*

3.2. Haemocyte production during candidiasis

G. mellonella larvae were injected with 1×10^5, 1×10^6 and 1×10^7 cells/larva and haemocytes density was calculated at 3 h post-infection (Figure 3). The injection of PBS-ampicillin and the two lowest inocula of all species, except 1×10^6 cells/larva of both *C. nivariensis* strains ($p \leq 0.492$), induced a significant increase ($p \leq 0.005$) in the number of haemocytes compared with the control group of untouched larvae. It is noteworthy that the density of haemocytes from larvae infected with the highest inoculum (1×10^7 cells/larva) of both strains of *C. glabrata*, and *C. nivariensis* strain CECT 11998 was significantly lower in comparison to that from larvae infected with both *C. bracarensis* strains ($p \leq 0.041$).

Larvae infected with *C. bracarensis* showed the highest haemocyte density to overcome the infection caused with any of the three inocula tested, without significant differences among them. Neither was difference between the haemocyte densities of the larvae infected with the three inocula of the *C. nivariensis* CBS 9984 strain. However, larvae

infected with 1×10^5 and 1×10^6 cells of *C. glabrata* showed a higher haemocyte number than those infected with 1×10^7 yeasts ($p \leq 0.004$).

3.3. Phagocytic activity of *Galleria mellonella* haemocytes during candidiasis

Host-pathogen interactions during candidiasis were also analyzed by assessing the phagocytic ability of the haemocytes of *G. mellonella*. Phagocytosis was evaluated with larvae infected with 1×10^6 *Candida* cells/larva, which was considered, according to the results of hemocyte density determination, the most suitable inoculum because of the adequate number of hemocytes and yeasts.

The three *Candida* species were phagocytosed after 2 h of the infection (Figure 4). *C. bracarensis* was more effectively phagocytized than *C. glabrata* and *C. nivariensis*. The percentage of haemocytes that phagocytosed *C. bracarensis* strain NCYC 3397 was the highest observed (11.26±0.91 %) and it was significantly different to the other two species ($p \leq 0.041$). In contrast, cells of *C. nivariensis* strain CECT 11998 were the least phagocytosed (5.31±0.71 %, $p \leq 0.001$).

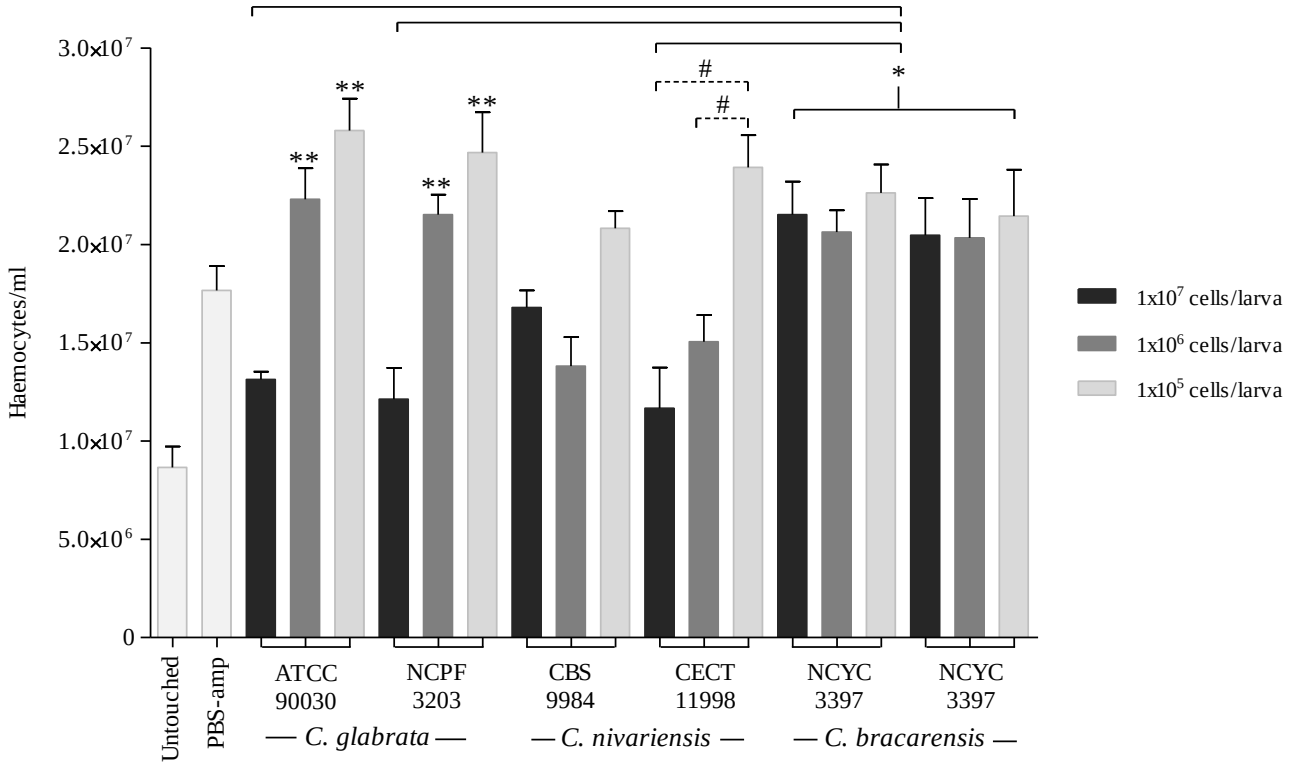


Figure 3. Haemocytes production from *G. mellonella* larvae infected with 1×10^7, 1×10^6 and 1×10^5 cells/larva of *C. glabrata*, *C. nivariensis* and *C. bracarensis*. Statistically significant differences compared to larvae infected with *C. bracarensis* (*), 1×10^7 cells/larva of *C. glabrata* (**), and 1×10^7 and 1×10^6 cells/larva of the *C. nivariensis* CECT 1198 strain (#).

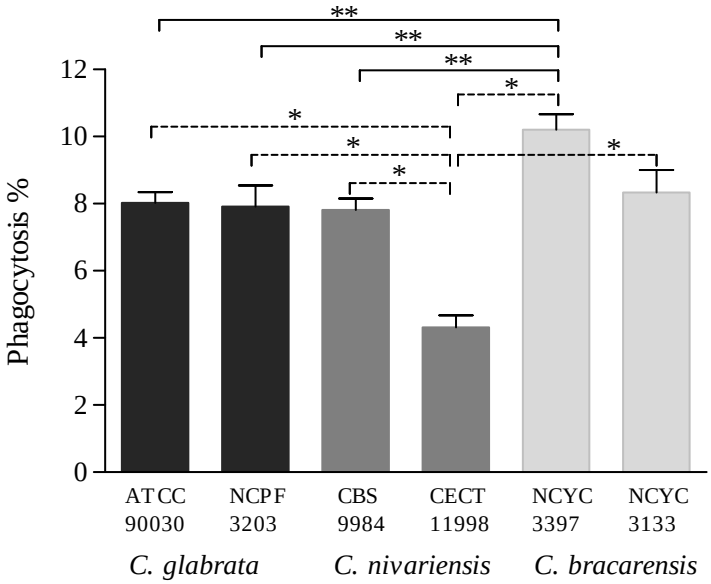


Figure 4. Phagocytic activity of *G. mellonella* at 2 h post-infection with 1 × 10^6 cells/ml of *C. glabrata*, *C. nivariensis* and *C. bracarensis*. Statistically significant differences compared to larvae infected with *C. nivariensis* strain CECT 11998 (*) and *C. bracarensis* strain NCYC 3397 (**).

3.4. Efficacy of echinocandins treatment of invasive candidiasis in *Galleria mellonella*

Treatment with three different echinocandins was analyzed after the injection of 1 × 10^6 cells/larva. This inoculum was selected as the most appropriate because the larvae survival was similar during the infection by all six strains, so the effect of the antifungal treatment could be more accurately assessed.

Echinocandin treatment was very effective for *C. glabrata* candidiasis. After 120 h there was a reduction in larvae mortality of between 33% and 45.8% (Table 1). However, the efficacies of these echinocandins against *C. glabrata* infection were strain-dependent (Table 1 and Figure 5a-b).

Candidiasis caused bay both *C. glabrata* strains responded to MCF at the two concentrations tested, achieving a significant increased larvae survival with respect to infected and untreated larvae (p=0). Specifically, treatment with MCF (8 μg/larva) against ATCC 90030 strain infection increased larvae survival from 38.7% to 73.3%. During treatment against CBS 3203 strain candidiasis, survival increased from 39.2% to 81.7% compared to the survival of infected and untreated larvae.

CAS was also effective against *C. glabrata* candidiasis: the larvae survival was significantly higher than that of infected and untreated larvae ($p \leq 0.004$). Treatment of NCPF 3203 strain infection with 4 μg/g larva of CAS reached a 39.1% increase survival of larvae. This survival increase was higher (42.5%) using 8 μg/g larva of CAS. However, when candidiasis caused by ATCC 90030 was treated with 8 μg/g larva of CAS the survival of *G. mellonella* increased to 18%, while with 4 μg/g larva of CAS, it increased up to 33%.

C. glabrata NCPF 3203 infection only responded adequately to 4 μg/g larva of AND. An increase of larvae survival of 45.8% with respect to infected and untreated control group was achieved ($p \leq 0{,}011$). The *G. mellonella* larvae infected with the ATCC 90030 strain showed no improvement with AND treatment. Although the mortality of larvae infected with this strain and treated with 8 μg/g larva of AND increased up to 7%, there were no differences in the survival when compared with infected and untreated control larvae group (Figure 5a).

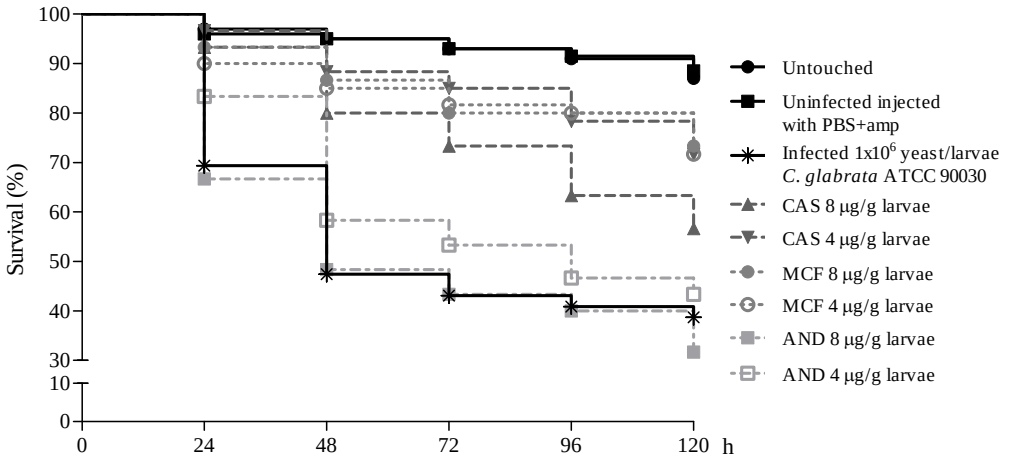
C. nivariensis infection was successfully treated with MCF and CAS (Figure 5c and d). The survival rates of larvae infected with both strains of *C. nivariensis* and treated with CAS ranged from 61.6% to 65.8%, and there were significant differences compared to those infected and untreated larvae (p=0). A statistically significant increase in larvae survival was also detected when *G. mellonella* infected with *C. nivariensis* was treated with MCF ($p \leq 0.001$). Larvae survival increased up to 41.7% with 4 μg/g larva of MCF against the infection by the *C. nivariensis* CBS 9984 strain. Strikingly, this significant difference was not detected in larvae infected with the *C. nivariensis* CBS 9984 strain and

treated with MCF at the highest concentration (8 µg/g larva) that only got a survival increase of 8.4%.

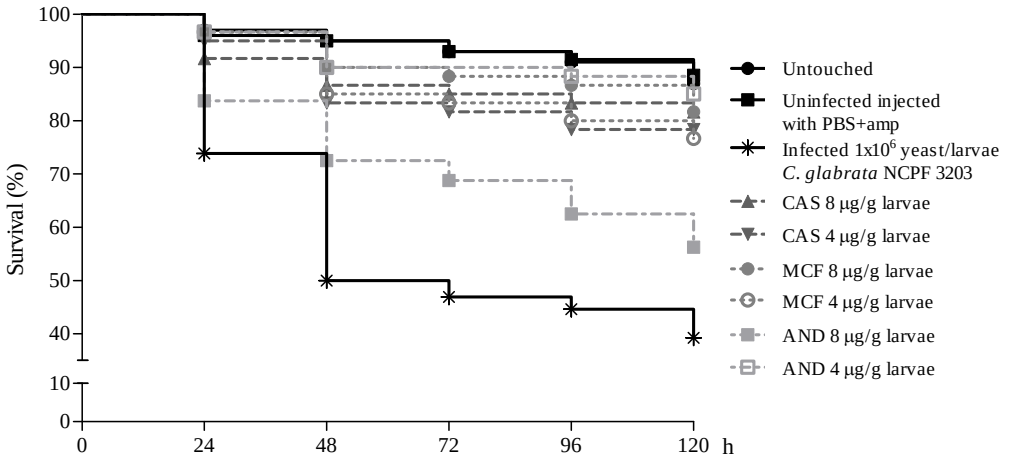
AND treatment of *C. nivariensis* infection was the least effective. Only 4 µg/g larva of AND significantly increased *G. mellonella* survival (33.4%) during infection with the C. *nivariensis* CBS 9984 strain (p=0) (Figure 5c). The treatment of candidiasis by *C. nivariensis* strain CECT 11998 with AND even increased the larvae mortality (28.3% with 4 µg/g larva of AND, and 38.3% with 8 µg/g larva of AND) (Figure 5d).

C. bracarensis infection was the least susceptible to treatment with echinocandins (Figure 5e and f). It is noteworthy that AND was the most effective during *G. mellonella* infection with this *Candida* species. The concentration of 4 µg/g larva of AND presented the highest protective effect during larvae infection with the *C. bracarensis* NCYC 3397 strain: infected and untreated larvae survival was 42.5% and treatment with AND increased survival to 71.7%. The therapeutic agents MCF (4 µg/g larva) and AND (8 µg/g larva) reached also high effect against *C. bracarensis* NCYC 3133 infection: larvae survival rate significantly increased by 24.3% (p=0.002) and 21% (p=0.008), respectively. Although the rest of MCF treatments achieved mortality reductions between 5.8% and 11%, there were no differences with the infected and untreated control group. The antifungal agent CAS during *C. bracarensis* infection reduced the larvae mortality rate between 6.3% and 19.2%, and there were also no differences with untreated larvae, except in larvae infected with the *C. bracarensis* NCYC 3397 strain and treated with 8 µg/g larva of CAS (19.2% of survival increase) (p=0.015).

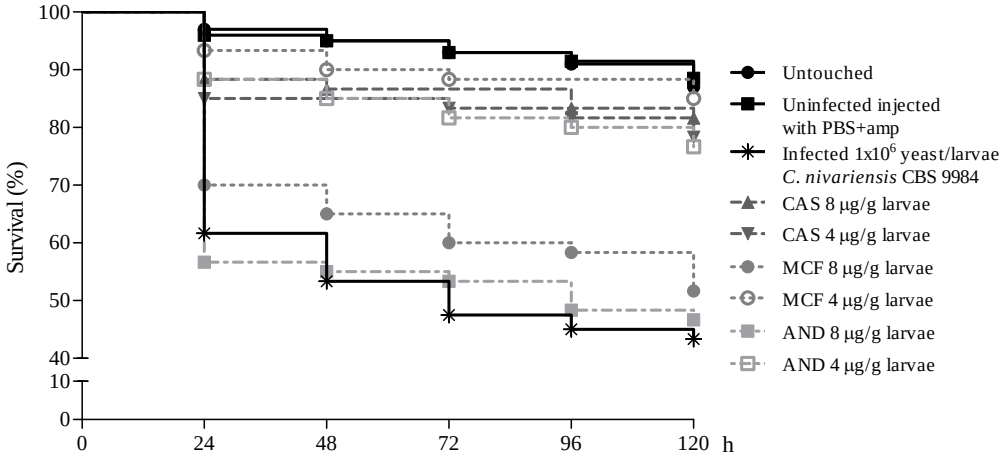
a)


Survival (%)
100
90
80
70
60
50
40
30
10
0
0
24
48
72
96
120
h
Untouched
Uninfected injected with PBS+amp
Infected 1x10^6 yeast/larvae C. glabrata ATCC 90030
CAS 8 µg/g larvae
CAS 4 µg/g larvae
MCF 8 µg/g larvae
MCF 4 µg/g larvae
AND 8 µg/g larvae
AND 4 µg/g larvae

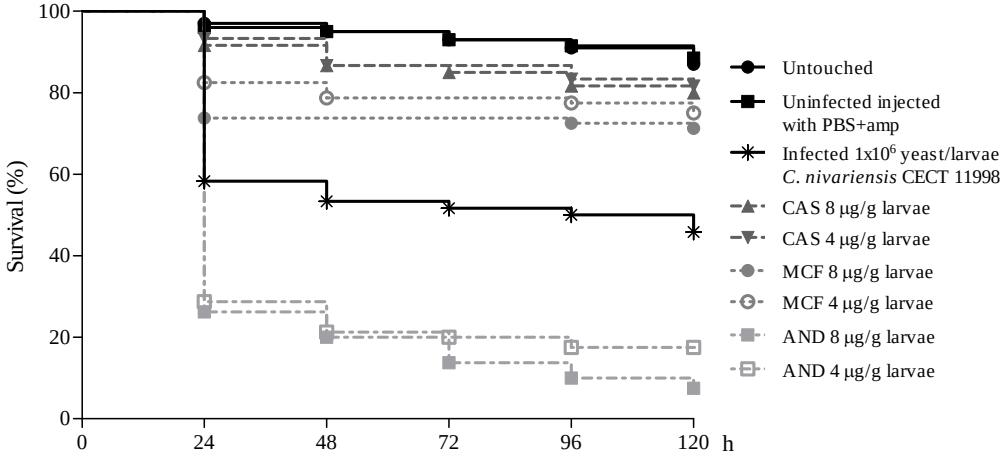
b)


Survival (%)
100
90
80
70
60
50
40
10
0
0
24
48
72
96
120
h
Untouched
Uninfected injected with PBS+amp
Infected 1x10^6 yeast/larvae C. glabrata NCPF 3203
CAS 8 µg/g larvae
CAS 4 µg/g larvae
MCF 8 µg/g larvae
MCF 4 µg/g larvae
AND 8 µg/g larvae
AND 4 µg/g larvae

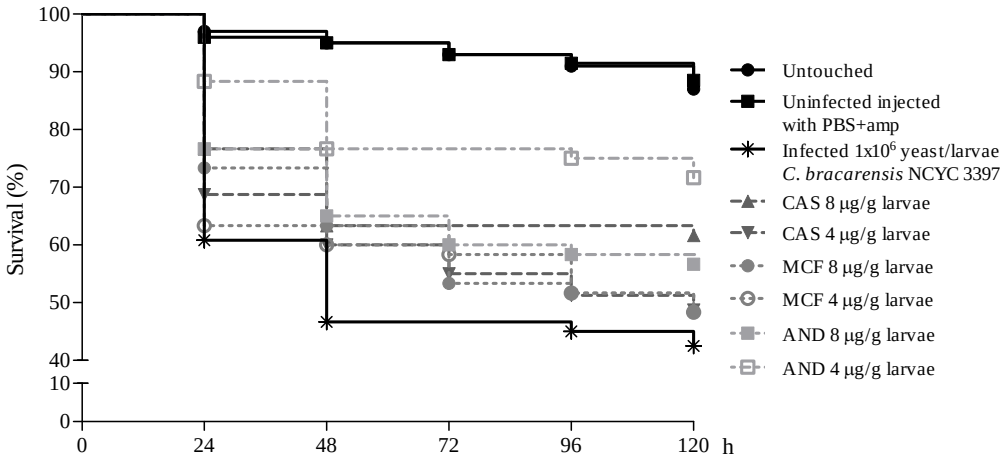
c)


100
90
80
70
60
50
40
10
0
Survival (%)
0
24
48
72
96
120
h
Untouched
Uninfected injected with PBS+amp
Infected 1x10^6 yeast/larvae C. nivariensis CBS 9984
CAS 8 µg/g larvae
CAS 4 µg/g larvae
MCF 8 µg/g larvae
MCF 4 µg/g larvae
AND 8 µg/g larvae
AND 4 µg/g larvae

d)


100
80
60
40
20
0
Survival (%)
0
24
48
72
96
120
h
Untouched
Uninfected injected with PBS+amp
Infected 1x10^6 yeast/larvae C. nivariensis CECT 11998
CAS 8 µg/g larvae
CAS 4 µg/g larvae
MCF 8 µg/g larvae
MCF 4 µg/g larvae
AND 8 µg/g larvae
AND 4 µg/g larvae

e)



f)

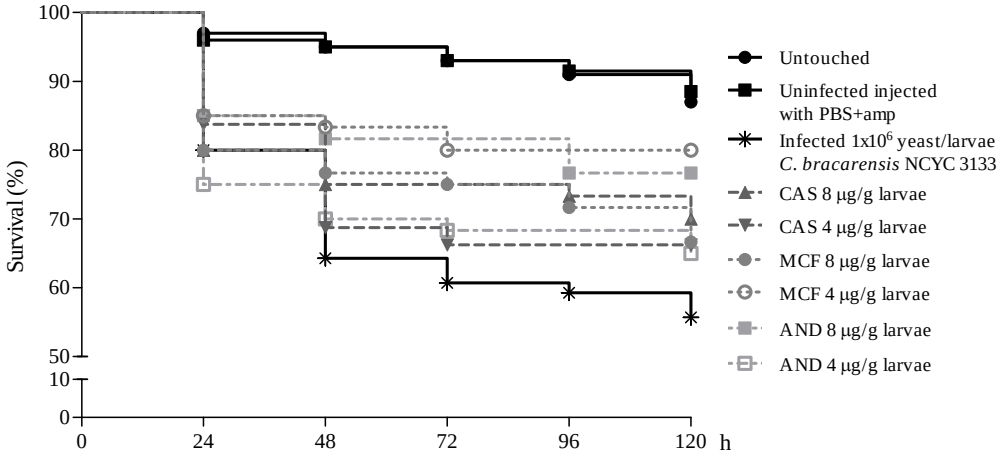


Figure 5: Echinocandis susceptibility of *C. glabrata* ATCC 90030 (a), *C. glabrata* NCPF 3203 b), *C. nivariensis* CBS 9984 (c), *C. nivariensis* CECT 11998 (d), *C. bracarensis* NCYC 3397 (e), and *C. bracarensis* NCYC 3133 (f) strains during *G. mellonella* infection. Larvae were inoculated with $1x10^6$ cells/larva and treated with anidulafungin (AND), caspofungin (CAS) and micafungin (MCF) at concentrations of 4 and 8 µg/g larva.

4. DISCUSSION

Alternative animal models, such as those in *C. elegans* and *G. mellonella*, have been explored as a useful option to study the pathogenesis and treatment of invasive candidiasis (Elkabti et al., 2018; Jemel et al., 2020). The use of *G. mellonella* has acquired relevance as larva size makes possible to control the infection development and treatment more easily, since it allows the injection of microorganism suspensions and antimicrobial treatments at specific concentrations (Killiny et al., 2018). The model of invasive candidiasis in *G. mellonella* has been successfully used in several studies with different species of *Candida* and filamentous fungi (Mesa-Arango et al., 2012; Scorzoni et al., 2013; Gago et al., 2014; Borman et al., 2016; Sherry et al., 2017; Maurer et al., 2019; Marcos-Zambrano et al.,2019; Huang et al., 2020; Muñoz et al., 2020). However, there are few studies about *C. glabrata*, *C. nivariensis* and *C. bracarensis* candidiasis using in vivo non-mammalian models (Ames et al., 2017; Hernando-Ortiz et al., 2020). Although the incidence of candidiasis caused by *C. nivariensis* and *C. bracarensis* is low, several authors consider that the difficulty in achieving a correct identification may be causing a misjudgment of the real medical importance of these species. (Bishop et al, 2008, Lockhart et al, 2009, Swoboda-Kopec et al., 2014; Morales-Lopez et al., 2016; Malek et al, 2018; Asadzadeh et al., 2019). In addition, the emergence of isolates resistant to antifungal drugs, such as amphotericin B, fluconazole, voriconazole and even echinocandins, makes it necessary to deepen the knowledge on *C. glabrata* and these two closely related species (Fujita et al., 2007; Borman et al., 2008; Bishop et al., 2008; Coste et al., 2020; Lockhart et al., 2009; Warren et al., 2010; Figuereido-Carvalho et al., 2016; Hou et al., 2017; Alobaid et al., 2020; Shi et al., 2020).

Therefore, in the present study the usefulness of *G. mellonella* model to assess the pathogenicity of *C. glabrata*, *C. nivariensis* and *C. bracarensis* was analyzed. The six strains studied of these *Candida* species developed invasive candidiasis in larvae of *G. mellonella*: *C. glabrata* and *C. bracarensis* were the most and less virulent species, respectively, just as we had previously observed in a model of candidiasis in *C. elegans* in

which *C. glabrata* killed the highest percentage of nematodes followed by *C. nivariensis* and *C. bracarensis* (Hernando-Ortiz et al., 2020).

A notable difference between these two models, *C. elegans* and *G. mellonella*, is the yeast inoculum administered. The inoculum is unknown in the case of *C. elegans* model since the route of infection is oral and is only controlled by the time used for infection (Ortega-Riveros et al., 2017). However, in the case of *G. mellonella* model, *Candida* cells are injected into haemolymph, allowing a more precise control and better knowledge of the effect of yeast inocula (Jemel et al., 2020). In an attempt to detect the most appropriate inoculum, three different *Candida* concentrations were evaluated. The highest concentration (1×10^7 cells/larva) showed significant virulence differences between the three *Candida* species. Ames et al. (2017) also reported that the highest injected dose tested (7.5×10^6 cells/larva) was the best for studying *C. glabrata* virulence in *G. mellonella*. Nevertheless, other *Candida* species such as *C. albicans*, *C. tropicalis*, *C. krusei* and *C. parapsilosis* complex required lower yeast inocula to cause candidiasis in this model, even detecting higher larvae mortality rates than those observed for *C. glabrata* (Mesa-Arango et al., 2012; Scorzoni et al., 2013; Gago et al., 2014; Borman et al., 2016; Sherry et al., 2017; Marcos-Zambrano et al., 2019; Huang et al., 2020; Muñoz et al., 2020). Furthermore, and in agreement with previous studies, an increase in mortality was observed in *G. mellonella* as the injected fungal load was higher (Mesa-Arango et al., 2012; Scorzoni et al., 2013; Ames et al., 2017; Sherry et al., 2017; Maurer et al., 2019).

An additional highlight of the *G. mellonella* model of invasive candidiasis is the analysis of host-pathogen interactions. This lepidopteran uses different mechanisms to combat pathogens, such as a variable production of haemocytes and phagocytosis according to the virulence of pathogens (Bergin et al, 2003). Larvae infected with *C. glabrata* and *C. nivariensis* produced fewer haemocytes than those infected with *C. bracarensis*, and the latter species was the most effectively phagocytized by haemocytes. This was consistent with the ability of these three species to develop infection in *G. mellonella*. Several authors have attributed the decrease of haemocytes in haemolymph to the

formation of nodules at the sites of infection in order to contain the spread of pathogens (Bergin et al., 2003; Mesa-Arango et al., 2012; Scorzoni et al., 2013). The haemocyte production observed in other studies during the infection caused by *C. albicans*, *C. krusei*, *C. tropicalis*, *C. parapsilosis* or *C. orthopsilosis* was even lower, likely due to a higher virulence of these species (Mesa-Arango et al., 2012; Scorzoni et al., 2013; Gago et al., 2014; Perini et al., 2019). *C. bracarensis* as well as other not so virulent species, such as *C. metapsilosis*, generated a low *G. mellonella* haemocyte response. Although the phagocytic rate detected in larvae infected with *C. parapsilosis* complex was higher than that detected with *C. glabrata*, a significant difference was observed in the less virulent species, *C. metapsilosis* and *C. bracarensis*, respectively, compared to other close-related species (Gago et al., 2014).

In vitro antifungal susceptibility has been widely studied in *C. glabrata*, *C. nivariensis* and *C. bracarensis*, indicating a reduced susceptibility or even resistance to azoles and amphotericin B (Pfaller et al., 2012; Alexander et al., 2013; Faria-Ramos et al., 2014; Ames et al., 2017; Astvad et al., 2018; Ko et al., 2018 Fujita et al., 2007; Bishop et al., 2008; Borman et al., 2008; Lockhart et al., 2009; Cuenca-Estrella et al., 2011; Gil-Alonso et al., 2015; Figueiredo-Carvalho et al., 2016; Hou et al., 2017; Morales-Lopez et al., 2016; Arastehfar et al., 2019; Alobaid et al., 2020; Shi et al., 2020). Specifically, the six strains of these three closely related species, used in the current study, were in vitro susceptible to amphotericin B, azoles (posaconazole and voriconazole) and all three echinocandins (Hernando-Ortiz et al., 2020).

Echinocandins are mainly indicated for the treatment of candidiasis caused by *C. glabrata* (Katiyar et al., 2012; Pham et al., 2014; Morales-Lopez et al., 2017). However, an increase in echinocandin resistance has been described, mainly due to acquired *FKS* mutations associated to previous exposure to these drugs, although this incidence is still low, it could be useful to know the local resistance patterns to establish adequate empirical treatment strategies (Beyda et al., 2014; Pham et al., 2014; Domán et al., 2015; Bordallo-Cardona et al., 2018; McCarty et al., 2018Coste et al., 2020; Al-Baqsami et al., 2020). Echinocandins treatment was effective against the infection caused by these three

Candida species in *G. mellonella* host model. CAS and MCF showed in vivo efficacy during *C. glabrata* and *C. nivariensis* infections while AND during *C. bracarensis* candidiasis. These findings are strongly consistent with those previously observed in the *C. elegans* model of candidiasis (Hernando-Ortiz et al., 2020). Treatment with CAS protected the larvae during *C. glabrata* infection, maintaining survival above 72% after 120 h infection. Ames et al. (2017) also detected up to 80% survival rate in *C. glabrata* infection treated with 4 μg/g larva of CAS. This effectiveness of CAS has also been reported in *C. glabrata* murine models of invasive candidiasis (Spreghini et al., 2012; Fernandez-Silva et al., 2014; Domán et al., 2015; Wiederhold et al., 2016). However, Healy et al. (2017) reported that administration of high doses of CAS (20 mg/kg) in murine models of candidiasis for 5-9 days selected *C. glabrata* strains with *FKS* mutations resistant to echinocandins. Although in time-kill studies echinocandins showed a lower effect against *C. nivariensis* (Gil-Alonso et al., 2015), we observed that CAS achieved a reduction in larval mortality during *C. nivariensis* infection. Lopez-Soria et al. (2013) observed that CAS was very effective in resolving a catheter-associated fungemia caused by *C. nivariensis*. Moreover, *C. nivariensis* and *C. bracarensis* have been susceptible to CAS in vitro (Wahyuningsih et al., 2008; Lockhart et al., 2009; Chowdhary et al., 2010; Sharma et al., 2013; Tay et al.,2014; Hou et al., 2017).

The effectiveness of treatment with MCF of candidiasis caused by *C. glabrata* was also reported in murine model (Mariné et al., 2006; Howard et al., 2011). However, in these studies a higher MCF concentration than those used of CAS was required to achieve the same effect. *C. glabrata* resistant strains can be selected when antifungal drugs are used in low doses, as *C. glabrata* strains exposed for 2-4 days to low and constant MCF concentrations developed resistance due to mutations in the *FKS2* gene (Bordallo-Cardona et al., 2018). In the present study, MCF was also effective against *C. nivariensis* infection confirming the in vitro susceptibility of this species to the MCF (Lockhart et al., 2009; Warren et al., 2010; Sharma et al., 2013; Hou et al., 2017; Morales-Lopez et al., 2017; Hernando-Ortiz et al., 2020).

Treatment with AND was the least effective against infection caused by *C. glabrata* and *C. nivariensis* in *G. mellonella*. Strikingly, larvae infected with *C. glabrata* ATCC 90030 and *C. nivariensis* CECT 11998 strains and treated with AND showed an increase in mortality up to six times higher than that of infected and untreated larvae. This lower effect of AND treatment has also been reported in a murine model of *C. glabrata* infection (Spregini et al., 2012). However, infected larvae treated with AND showed a reduction in mortality rates higher than with CAS and MCF in *C. bracarensis* infection in *G. mellonella*. This is consistent with observations reported in other *C. bracarensis* candidiasis models and in vitro susceptibility testing (Lockhart et al., 2009; Hou et al., 2017; Hernando-Ortiz et al., 2020).

In conclusion, our findings demonstrate that *G. mellonella* is a suitable model to study virulence and host-pathogen interactions caused by the emerging species of *C. glabrata*, *C. nivariensis* and *C. bracarensis*. In this non-mammalian model of candidiasis it was possible to corroborate the higher virulence of *C. glabrata* compared to its two closely related species, being *C. bracarensis* the less virulent. These differences in virulence were also detected analyzing haemocyte larval production and phagocytosis, being *C. bracarensis* the best phagocyted species. Echinocandins, considered first-line therapy in human candidiasis, were effective during *G. mellonella* infection with these three *Candida* species: CAS and MCF were most effective for *C. glabrata* and *C. nivariensis* infections, while AND was for *C. bracarensis* infection. This study contributes to encourage future research to extend this model in the screening of promising therapeutic agents for infections caused by *C. albicans* and other emerging *Candida* species.

5. BIBLIOGRAPHY

1. Al-Baqsami ZF, Ahmad S, Khan Z. Antifungal drug susceptibility, molecular basis of resistance to echinocandins and molecular epidemiology of fluconazole resistance among clinical Candida glabrata isolates in Kuwait. Sci Rep. 2020 Apr 10;10(1):6238. doi: 10.1038/s41598-020-63240-z. PMID: 32277126; PMCID: PMC7148369
2. Alexander BD, Johnson MD, Pfeiffer CD, et al. 2013.Increasing echinocandin resistance in *Candida glabrata*: Clinical failure correlates with presence of FKS mutations and elevated minimum inhibitory concentrations. Clin Infect Dis **56**:1724-1732. doi:10.1093/cid/cit136
3. Alobaid K, Asadzadeh M, Bafna R, Ahmad S. 2020. First isolation of *Candida nivariensis*, an emerging fungal pathogen, in Kuwait. *Med Princ Pract*. http://doi.org/10.1159/000511553
4. Ames L, Duxbury S, Pawlowska B, Ho H lui, Haynes K, Bates S. 2017. *Galleria mellonella* as a host model to study *Candida glabrata* virulence and antifungal efficacy. *Virulence* **8**(8): 1909–1917. https://doi.org/10.1080/21505594.2017.13477440014-1
5. Arastehfar A, Daneshnia F, Salehi MR, et al. 2019. Molecular characterization and antifungal susceptibility testing of *Candida nivariensis* from blood samples - an Iranian multicentre study and a review of the literature. *J Med Microbiol* **68**(5):770-777. doi: 10.1099/jmm.0.000963. Erratum in: *J Med Microbiol*. 2019; **68**(11):1695. PMID: 30924763
6. Asadzadeh M, Alanazi AF, Ahmad S, Al-Sweih N, Khan Z. 2019. Lack of detection of *Candida nivariensis* and *Candida bracarensis* among 440 clinical *Candida glabrata* sensu lato isolates in Kuwait. PLoS One **14**(10):1-16. doi:10.1371/journal.pone.0223920
7. Astvad KMT, Johansen HK, Røder BL, Rosenvinge FS, Knudsen JD, Lemming L, Schønheyder HC, Hare RK, Kristensen L, Nielsen L, Gertsen JB, Dzajic E, Pedersen M, Østergård C, Olesen B, Søndergaard TS, Arendrup MC. 2018.

Update from a 12-year nationwide fungemia surveillance: Increasing intrinsic and acquired resistance causes concern. *J Clin Microbiol* **56**(4):1-15. https://doi.org/10.1128/JCM.01564-17

8. Bergin D, Brennan M, Kavanagh K. 2003. Fluctuations in haemocyte density and microbial load may be used as indicators of fungal pathogenicity in larvae of *Galleria mellonella*. *Microbes Infect* **5**:1389–1395. https://doi.org/10.1016/j.micinf.2003.09.019
9. Beyda ND, John J, Kilic A, Alam MJ, Lasco TM, Garey KW. 2014. FKS mutant *Candida glabrata*: Risk factors and outcomes in patients with candidemia. Clin Infect Dis **56**:819-825. doi:10.1093/cid/ciu407
10. Bishop JA, Chase N, Magill SS, Kurtzman CP, Fiandaca MJ, Merz WG. 2008. *Candida bracarensis* detected among isolates of *Candida glabrata* by peptide nucleic acid fluorescence in situ hybridization: Susceptibility data and documentation of presumed infection. J Clin Microbiol **46**(2): 443-446. https://doi.org/10.1128/JCM.01986-07
11. Bordallo-Cardona MÁ, Escribano P, Marcos-Zambrano LJ, Díaz-García J, De La Pedrosa EG, Cantón R, Bouza E, Guinea J. 2018. Low and constant micafungin concentrations may be sufficient to lead to resistance mutations in FKS2 gene of *Candida glabrata*. Med Mycol **56**(7):903–906. https://doi.org/10.1093/mmy/myx124
12. Borman AM, Petch R, Linton CJ, Palmer MD, Bridge PD, Johnson EM. 2008. *Candida nivariensis*, an emerging pathogenic fungus with multidrug resistance to antifungal agents. J Clin Microbiol **46**(3):933–938. https://doi.org/10.1128/JCM.02116-07
13. Borman AM, Szekely A, Johnson EM. 2016. Comparative pathogenicity of United Kingdom isolates of the emerging pathogen *Candida auris* and other key pathogenic *Candida* species. mSphere **1**(4):4-6. doi:10.1128/mSphere.00189-16
14. Cartier N, Chesnay A, N'diaye D, Thorey C, Ferreira M, Haillot O, Bailly É, Desoubeaux G. 2020. *Candida nivariensis*: Identification strategy in mycological laboratories. *J Mycol Med* **30**(4):101042. doi: 10.1016/j.mycmed.2020.101042

15. Chowdhary A, Randhawa HS, Khan ZU, et al. 2010. First isolations in India of *Candida nivariensis*, a globally emerging opportunistic pathogen. Med Mycol **48**(2):416-420. doi:10.1080/13693780903114231

16. Cook SM, McArthur JD, 2013. Developing *Galleria mellonella* as a model host for human pathogens. Virulence **4**:5, 350-353. https://doi.org/10.4161/viru.25240
17. Cornely OA, Bassetti M, Calandra T, Garbino J, Kullberg BJ, Lortholary O, et al. ESCMID* guideline for the diagnosis and management of *Candida* diseases 2012: non-neutropenic adult patients. Clin Microbiol Infect. 2012;18:19–37. doi: 10.1111/1469-0691.12039.
18. Coste AT, Kritikos A, Li J, Khanna N, Goldenberger D, Garzoni C, Zehnder C, Boggian K, Neofytos D, Riat A, Bachmann D, Sanglard D, Lamoth F; Fungal Infection Network of Switzerland (FUNGINOS). Emerging echinocandin-resistant Candida albicans and glabrata in Switzerland. Infection. 2020 Oct;48(5):761-766. doi: 10.1007/s15010-020-01475-8. Epub 2020 Jul 13. PMID: 32661647; PMCID: PMC7518979
19. Cuenca-Estrella M, Gomez-Lopez A, Isla G, et al. 2011. Prevalence of *Candida bracarensis* and *Candida nivariensis* in a Spanish collection of yeasts: Comparison of results from a reference centre and from a population-based surveillance study of candidemia. Med Mycol **49**(5):525-529. doi:10.3109/13693786.2010.546373
20. Domán M, Kovács R, Perlin DS, Kardos G, Gesztelyi R, Juhász B, Bozó A, Majoros L. 2015. Dose escalation studies with caspofungin against *Candida glabrata*. J Med Microbiol **64**(9):998–1007. https://doi.org/10.1099/jmm.0.000116
21. Elkabti AB, Issi L, Rao RP. *Caenorhabditis elegans* as a Model Host to Monitor the *Candida* Infection Processes. J Fungi (Basel). 2018 Nov 7;4(4):123. doi: 10.3390/jof4040123. PMID: 30405043; PMCID: PMC6309157
22. Faria-Ramos I, Neves-Maia J, Ricardo E, et al. 2014. Species distribution and in vitro antifungal susceptibility profiles of yeast isolates from invasive infections

during a Portuguese multicenter survey. Eur J Clin Microbiol Infect Dis **33**(12):2241-2247. doi:10.1007/s10096-014-2194-8

23. Fernańdez-Silva F, Lackner M, Capilla J, Mayayo E, Sutton D, Castanheira M, Fothergill AW, Lass-Flörl C, Guarro J. 2014. In vitro antifungal susceptibility of *Candida glabrata* to caspofungin and the presence of FKS mutations correlate with treatment response in an immunocompromised murine model of invasive infection. Antimicrob Agents Chemother **58**(7): 3646–3649. https://doi.org/10.1128/AAC.02666-13
24. Figueiredo-Carvalho MHG, de Souza Ramos L, Barbedo LS, et al. 2016. First description of *Candida nivariensis* in Brazil: Antifungal susceptibility profile and potential virulence attributes. Mem Inst Oswaldo Cruz. **111**(1):51-58. doi:10.1590/0074-02760150376
25. Fujita SI, Senda Y, Okusi T, Ota Y, Takada H, Yamada K, Kawano M. 2007. Catheter-related fungemia due to fluconazole-resistant *Candida nivariensis*. J Clin Microbiol **45**(10):3459-61. https://doi.org/10.1128/JCM.00727-07
26. Gago S, García-Rodas R, Cuesta I, Mellado E, Alastruey-Izquierdo A. 2014. *Candida parapsilosis*, *Candida orthopsilosis* and *Candida metapsilosis* virulence in the non-conventional host *Galleria mellonella*. Virulence **5**:278–285. https://doi.org/10.4161/viru.26973
27. Gil-Alonso S, Jauregizar N, Cantón E, Eraso E, Quindós G. 2015. In Vitro fungicidal activities of anidulafungin, caspofungin, and micafungin against *Candida glabrata*, *Candida bracarensis*, and *Candida nivariensis* evaluated by time-kill studies. Antimicrob Agents Chemother **59**(6):3615-8. https://doi.org/10.1128/AAC.04474-14
28. Healey KR, Nagasaki Y, Zimmerman M, et al. 2017. The gastrointestinal tract is a major source of echinocandin drug resistance in a murine model of *Candida glabrata* colonization and systemic dissemination. Antimicrob Agents Chemother **61**(12):1-12. doi:10.1128/AAC.01412-17
29. Hou X, Xiao M, Chen SCA, Wang H, Yu SY, Fan X, Kong F, Xu YC. 2017. Identification and antifungal susceptibility profiles of *Candida nivariensis* and

Candida bracarensis in a multi-center Chinese collection of yeasts. Front Microbiol **8**:5. https://doi.org/10.3389/fmicb.2017.00005

30. Howard SJ, Livermore J, Sharp A, Goodwin J, Gregson L, Alastruey-Izquierdo A, Perlin DS, Warn PA, Hope WW. 2011. Pharmacodynamics of echinocandins against *Candida glabrata*: Requirement for dosage escalation to achieve maximal antifungal activity in neutropenic hosts. Antimicrob Agents Chemother **55**(10): 4880-4887. https://doi.org/10.1128/AAC.00621-11
31. Huang XW, Xu MN, Zheng HX, Wang ML, Li L, Zeng K, Li DD. 2020. Pre-exposure to *Candida glabrata* protects *Galleria mellonella* against subsequent lethal fungal infections. *Virulence* **11**(1):1674-1684. doi: 10.1080/21505594.2020.1848107
32. Jamal WY, Ahmad S, Khan ZU, Rotimi VO. 2014. Comparative evaluation of two matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) systems for the identification of clinically significant yeasts. *Int J Infect Dis* **26**:167–170. https://doi.org/10.1016/j.ijid.2014.05
33. Jemel S, Guillot J, Kallel K, Botterel F, Dannaoui E. *Galleria mellonella* for the Evaluation of Antifungal Efficacy against Medically Important Fungi, a Narrative Review. Microorganisms. 2020 Mar 11;8(3):390. doi: 10.3390/microorganisms8030390. PMID: 32168839; PMCID: PMC7142887
34. Johnson EM. 2009. Rare and emerging *Candida* species. Curr Fungal Infect Rep **3**:152–159. https://doi.org/10.1007/s12281-009-0020-z
35. Katiyar SK, Alastruey-Izquierdo A, Healey KR, Johnson ME, Perlin DS, Edlind TD. 2012. Fks1 and Fks2 are functionally redundant but differentially regulated in *Candida glabrata*: implications for echinocandin resistance. Antimicrob Agents Chemother **56**(12):6304-6309. doi:10.1128/AAC.00813-12
36. Killiny N. 2018. Generous hosts: Why the larvae of greater wax moth, *Galleria mellonella* is a perfect infectious host model? Virulence **9**(1):860-865. doi:10.1080/21505594.2018.1454172
37. Ko JH, Peck KR, Jung DS, et al. 2018. Impact of high MIC of fluconazole on outcomes of *Candida glabrata* bloodstream infection: a retrospective multicenter

cohort study. Diagn Microbiol Infect Dis **92**(2):127-132. doi:10.1016/j.diagmicrobio.2018.05.001

38. Kołaczkowska A, Kołaczkowski M. Drug resistance mechanisms and their regulation in non-albicans *Candida* species. J. Antimicrob. Chemother. 2016;71:1438–1450. doi: 10.1093/jac/dkv445.
39. Lockhart SR, Messer SA, Gherna M, Bishop JA, Merz WG, Pfaller MA, Diekema DJ. 2009. Identification of *Candida nivariensis* and *Candida bracarensis* in a large global collection of *Candida glabrata* isolates: Comparison to the literature. J Clin Microbiol **47**(4):1216-7. https://doi.org/10.1128/JCM.02315-08
40. McCarty TP, Lockhart SR, Moser SA, Whiddon J, Zurko J, Pham CD, Pappas PG. 2018. Echinocandin resistance among *Candida* isolates at an academic medical centre 2005–15: analysis of trends and outcomes. J Antimicrob Chemother **73(**6):1677–1680. https://doi.org/10.1093/jac/dky059
41. Małek M, Mrowiec P, Klesiewicz K, Skiba-Kurek I, Szczepański A, Białecka J, Żak I, Bogusz B, Kędzierska J, Budak A, Karczewska E. 2018. Prevalence of human pathogens of the clade *Nakaseomyces* in a culture collection—the first report on *Candida bracarensis* in Poland. Folia Microbiol. https://doi.org/10.1007/s12223-018-0655-7
42. Marcos-Zambrano LJ, Bordallo-Cardona MÁ, Borghi E, et al. 2020. *Candida* isolates causing candidemia show different degrees of virulence in *Galleria mellonella*. Med Mycol **0**:1-10. doi:10.1093/mmy/myz027
43. Mariné M., Serena C, Pastor FJ, Guarro J. 2006. Combined antifungal therapy in a murine infection by *Candida glabrata*. J Antimicrob Chemother. **58**(6):1295-1298. https://doi.org/10.1093/jac/dkl395
44. Maurer E, Hörtnagl C, Lackner M, et al. 2019. *Galleria mellonella* as a model system to study virulence potential of mucormycetes and evaluation of antifungal treatment. Med Mycol **57**(3):351-362. doi:10.1093/mmy/myy042
45. Mesa-Arango AC, Forastiero A, Bernal-Martínez L, Cuenca-Estrella M, Mellado E, Zaragoza O. 2013. The non-mammalian host *Galleria mellonella* can be used

to study the virulence of the fungal pathogen *Candida tropicalis* and the efficacy of antifungal drugs during infection by this pathogenic yeast. Med Mycol **51**:461–472. https://doi.org/10.3109/13693786.2012.737031

46. Miranda-Zapico I, Eraso E, Hernández-Almaraz JL, López-Soria LM, Carrillo Muñoz AJ, Hernández-Molina JM, Quindós G. 2011. Prevalence and antifungal susceptibility patterns of new cryptic species inside the species complexes *Candida parapsilosis* and *Candida glabrata* among blood isolates from a Spanish tertiary hospital. J Antimicrob Chemother **66**:2315–2322. https://doi.org/10.1093/jac/dkr298
47. Morales-López S, Dudiuk C, Vivot W, Szusz W. 2017. Phenotypic and molecular evaluation of echinocandin susceptibility of *Candida*. Antimicrob Agents Chemother **61**(7):7-10. doi:10.1128/AAC.00170-17
48. Morales-López SE, Taverna CG, Bosco-Borgeat ME, Maldonado I, Vivot W, Szusz W, Garcia-Effron G, Córdoba SB. 2016. *Candida glabrata* species complex prevalence and antifungal susceptibility testing in a culture collection: First description of *Candida nivariensis* in Argentina. Mycopathologia **181**(11-12):871-8. https://doi.org/10.1007/s11046-016-0052-1
49. Muñoz J, Ramirez L, Dias L dos S, et al. 2020. Pathogenicity levels of Colombian strains of *Candida auris* and brazilian strains of *Candida haemulonii* species complex in both murine and *Galleria mellonella* Experimental Models. J Fungi **6**(3):104. doi:10.3390/jof6030104
50. Pappas PG, Lionakis MS, Arendrup MC, Ostrosky-Zeichner L, Kullberg BJ. 2018. Invasive candidiasis. *Nat Rev Dis Primers* **11**(4):18026. doi: 10.1038/nrdp.2018.26. PMID: 29749387.
51. Pfaller MA, Castanheira M, Lockhart SR, Jones RN. 2012. *Candida glabrata*: Multidrug resistance and increased virulence in a major opportunistic fungal pathogen. Curr Fungal Infect Rep **6**:154-164. doi:10.1007/s12281-012-0091-0
52. Pfaller MA, Diekema DJ, Turnidge JD, Castanheira M, Jones RN. 2019. Twenty years of the SENTRY Antifungal Surveillance Program: Results for *Candida*

species from 1997-2016. Open Forum Infect Dis **6**(S1):79-94. doi:10.1093/ofid/ofy358

53. Pham CD, Iqbal N, Bolden CB, et al. 2014. Role of FKS mutations in *Candida glabrata*: MIC values, echinocandin resistance, and multidrug resistance. Antimicrob Agents Chemother **58**(8):4690-4696. doi:10.1128/AAC.03255-14
54. Quindós G, Marcos-Arias C, San-Millán R, Mateo E, Eraso E. 2018. The continuous changes in the aetiology and epidemiology of invasive candidiasis: from familiar *Candida albicans* to multiresistant *Candida auris*. Int Microbiol **21**(3):107–19. https://doi.org/10.1007/s10123-018-
55. Scorzoni L, de Lucas MP, Mesa-Arango AC, Fusco-Almeida AM, Lozano E, Cuenca-Estrella M, Mendes-Giannini MJ, Zaragoza O, 2013. Antifungal Efficacy during *Candida krusei* Infection in Non-Conventional Models Correlates with the Yeast In Vitro Susceptibility Profile. PLoS One **8**:3. https://doi.org/10.1371/journal.pone.0060047
56. Segal E, Frenkel M. 2018. Experimental in vivo models of Candidiasis. J Fungi **4**:21. https://doi.org/10.3390/jof4010021
57. Sharma C, Wankhede S, Muralidhar S, et al. 2013. *Candida nivariensis* as an etiologic agent of vulvovaginal candidiasis in a tertiary care hospital of New Delhi, India. Diagn Microbiol Infect Dis **76**(1):46-50. doi:10.1016/j.diagmicrobio.2013.02.023
58. Sherry L, Ramage G, Kean R, et al. 2017. Biofilm-forming capability of highly virulent, multidrug-resistant *Candida auris*. Emerg Infect Dis **23**(2):328-331. doi:10.3201/eid2302.161320
59. Shi Y, Zhu Y, Fan S, Vitagliano A, Liu X, Liao Y, Liang Y, Vitale S, G: 2020. Clinical characteristics and antifungal susceptibility of *Candida nivariensis* from vulvovaginal candidiasis. *Gynecol Obstet Invest*; **85**:88-93. http://doi.org/10.1159/000504095
60. Spreghini E, Orlando F, Sanguinetti M, Posteraro B, Giannini D, Manso E, Barchiesia F. 2012. Comparative effects of micafungin, caspofungin, and anidulafungin against a difficult-to-treat fungal opportunistic pathogen, *Candida*

glabrata. Antimicrob Agents Chemother **56**(3):1215-22. https://doi.org/10.1128/AAC.05872-11

61. Swoboda-Kopeć E, Sikora M, Golas M, Piskorska K, Gozdowski D, Netsvyetayeva I. 2014. *Candida nivariensis* in comparison to different phenotypes of *Candida glabrata*. Mycoses **57**:747-753. https://doi.org/10.1111/myc.12264
62. Tay ST, Lotfalikhani A, Sabet NS, et al. 2014. Occurrence and Characterization of *Candida nivariensis* from a culture collection of *Candida glabrata* clinical isolates in Malaysia. Mycopathologia **178**:307-314. doi:10.1007/s11046-014-9778-9
63. Wahyuningsih R, Sahbandar IN, Theelen B, et al. 2008. *Candida nivariensis* isolated from an Indonesian human immunodeficiency virus-infected patient suffering from oropharyngeal candidiasis. J Clin Microbiol **46**:388-391. doi:10.1128/JCM.01660-07
64. Warren TA, McTaggart L, Richardson SE, Zhang SX. 2010. *Candida bracarensis* bloodstream infection in an immunocompromised patient. J Clin Microbiol **48**: 4677–4679 https://doi.org/10.1128/JCM.01447-10
65. Warren TA, McTaggart L, Richardson SE, Zhang SX. 2010. *Candida bracarensis* bloodstream infection in an immunocompromised patient. J Clin Microbiol **48**:4677– 4679. https://doi.org/10.1128/JCM.01447-10.-10
66. Wiederhold NP, Najvar LK, Fothergill AW, et al. 2016. The novel arylamidine T-2307 demonstrates in vitro and in vivo activity against echinocandin-resistant *Candida glabrata*. J Antimicrob Chemother **71**:692-695. doi:10.1093/jac/dkv398

4. Eranskina/Anexo 4

Virulence of *Candida auris* from different clinical origins in *Caenorhabditis elegans* and *Galleria mellonella* host models

Ainara Hernando-Ortiz[1], Estibaliz Mateo[1], Aitzol Perez-Rodriguez[1], Piet W.J. de Groot[2], Guillermo Quindós[1] and Elena Eraso[1]

[1]Department of Immunology, Microbiology and Parasitology, Faculty of Medicine and Nursery, University of the Basque Country (UPV/EHU), Bilbao, Spain

[2]Regional Center for Biomedical Research, Castilla-La Mancha Science & Technology Park, University of Castilla-La Mancha, Albacete, Spain

Virulence 12 (2021) 1063-1075

ABSTRACT

Candida auris is an emerging multidrug-resistant fungal pathogen responsible for nosocomial outbreaks of invasive candidiasis. Although several studies on the pathogenicity of this species have been reported, the knowledge on *C. auris* virulence is still limited. This study aims to analyze the pathogenicity of *C. auris,* using one aggregating isolate and eleven non-aggregating isolates from different clinical origins (blood, urine and oropharyngeal specimens) in two alternative host models of candidiasis: *Caenorhabditis elegans* and *Galleria mellonella*. Furthermore, possible associations between virulence, aggregation, biofilm-forming capacity, and clinical origin were assessed. The aggregating phenotype isolate was less virulent in both *in vivo* invertebrate infection models than non-aggregating isolates but showed higher capacity to form biofilms. Blood isolates were significantly more virulent than those isolated from urine and respiratory specimens in the *G. mellonella* model of candidiasis. We conclude that both models of candidiasis present pros and cons but prove useful to evaluate the virulence of *C. auris in vivo*. Both models also evidence the heterogeneity in virulence that this species can develop, which may be influenced by the aggregative phenotype and clinical origin.

Keyboards: *Candida auris*, emerging pathogen, candidiasis, virulence, invertebrate models

1. INTRODUCTION

Candida albicans is the predominant etiological agent of invasive candidiasis [1, 2]. However, recently *Candida auris* is emerging as a major cause of invasive nosocomial infections with multidrug-resistance and high morbidity and mortality [3, 4]. Since this species was first described in 2009, several clinical presentations, such as colonization, mucosal infection or bloodstream infection have been reported worldwide, mostly associated with outbreaks in surgical and medical intensive care units (ICU) [5, 6]. Phenotypic and genotypic characterization studies of *C. auris* isolates from different countries have revealed the high diversity of this species identifying up to five possible different phylogeographical clades, four clearly distinct and a potential fifth clade, around the world [6, 7].

A wide array of virulence mechanisms is encoded in the genome of *C. auris* [8]. Expression of phospholipase, proteinase, and hemolytic activities, adherence and biofilm formation, antifungal drug and environmental stress resistance are described among the most relevant virulence traits [9-11]. However, the knowledge on *C. auris* virulence remains scarce and further studies are required to understand its pathogenic behavior, which has been reported to be substantially variable among clades [12]. Currently, about 30 studies have incorporated *in vivo* models in the analysis of *C. auris* virulence, murine models being the most widely used [13-18]. However, ethical and economical issues for using these models of candidiasis are encouraging the use of alternative systems such as invertebrate models [19, 20]. Several virulence studies of *C. auris* infections have been reported in invertebrate models using *Caenorhabditis elegans*, *Drosophila melanogaster*, *Danio rerio* or *Galleria mellonella* [11, 21-29]. Moreover, non-mammalian hosts have been successfully implemented for studying host-pathogen interactions and invasive candidiasis caused by other *Candida* species, and to evaluate the effectiveness of new therapeutic approaches [11, 30-33].

Advances in the knowledge of the virulence potential of *C. auris* will contribute to the control of infections by this emerging pathogen. Recently, two different phenotypes,

aggregating and non-aggregating (single-cell phenotype), have been described among *C. auris* clinical isolates; the latter presenting a higher virulence [21, 22, 26]. Transcriptional profiles were evidently different between isolates with these two different phenotypes, and differences in the host response, depending on whether there is loss of tissue integrity, were also reported [12]. Although attempts have been made to correlate the site of origin of clinical isolates with their virulence in different *Candida* species [34-37], no association has been reported between the pathogenicity of *C. auris* and the origin of clinical specimens.

Therefore, the aim of this study was to analyze the virulence of *C. auris* isolates including both, aggregating and non-aggregating phenotypes that were retrieved from different clinical specimens. The ability of several *C. auris* isolates to form biofilms and produce hemolytic and enzymatic activity was assessed *in vitro* and the virulence traits of these isolates were probed *in vivo* using the invertebrate model hosts, *C. elegans and G. mellonella*. Our results demonstrate that both model organisms can be killed by aggregative as well as non-aggregative *C. auris* isolates. Moreover, this study reaffirmed that the variability among *C. auris* isolates as well as the site of infection and the different immune response of the host could affect this species virulence; being the *C.auris* blood isolates the most virulent.

2. MATERIALS AND METHODS

2.1. *Candida auris* isolates and growth conditions

Twelve clinical *C. auris* isolates from different patients suffering from candidiasis were analyzed (Table 1). These isolates included five from blood, five from urine, and two from oropharynx. All but one of the isolates came from patients of the Hospital Universitario y Politécnico La Fe of Valencia, Spain (Dr. Alba Ruiz Gaitán and Dr. Javier Pemán), three of them being registered in the CBS-KNAW culture collection of Westerdijk Fungal Biodiversity Institute. One blood isolate, *Candida auris* JMRC:NRZ

1101 (Jena Microbial Resource Collection) was recovered from a patient attended at the Institut für Hygiene und Mikrobiologie, Würzburg, Germany (Dr. Oliver Kurzai).

C. auris isolates were stored in vials containing sterile distilled water at room temperature and cultured on Sabouraud dextrose agar (Difco, Becton Dickinson, USA) at 37 °C for 24 h before use. The assays in the *C. elegans* model were performed by culturing *C. auris* on brain heart infusion (BHI, Panreac, Spain) agar plates supplemented with kanamycin (90 µg/ml) and incubated at 37 °C for 24 h. *C. auris* isolates used for the *G. mellonella* model assay and for biofilm studies were cultured in yeast extract peptone dextrose broth (YEPD, Panreac) and incubated overnight at 30 °C in shaking conditions.

2.2. Microscopic visualization

The cellular morphology of the twelve clinical *C. auris* isolates was observed microscopically. *C. auris* isolates were grown in YEPD broth for 24 h at 30 °C in shaking conditions, washed three times with sterile phosphate-buffered saline solution (PBS, Sigma-Aldrich, USA) and collected by centrifugation at 2500 rpm for 10 min. Cell suspensions of each *C. auris* isolate were adjusted to a final concentration of 1×10^8 cells/ml with sterile PBS after cell counting by microscopy using a Burker hemocytometer. Microscopic appearance of each sample was visualized using a Nikon Eclipse 80i fluorescence microscope (Melville, NY, USA).

2.3. Biofilm development

The ability of *C. auris* isolates to develop biofilms was assessed with *C. albicans* strain SC5314 being included as control. Biofilms were produced in sterile, flat-bottomed honeycomb 100-well polystyrene microtiter plates (Labsystems, Finland). Precultured cells were harvested and washed three times with sterile PBS. After cell counting by microscopy, suspensions at a concentration of 1×10^6 cells/ml were prepared in RPMI 1640 medium supplemented with L-glutamine and buffered at pH 7 with 0.165 M 3-(N-morpholine) propanesulfonic acid, MOPS (Sigma-Aldrich). A volume of 100 µl of each cell suspension was dispensed into the wells of the plate. All outer wells from the first

and last column of the plate were kept empty to avoid the “edge-effect”. Microplates were incubated at 37 °C for 24 or 48 h. Afterwards, to carefully eliminate planktonic and poorly adhered cells, spent RPMI was removed and the biofilms were washed three times by adding and removing 100 µl of sterile PBS

Quantification of biofilm biomass was performed by Crystal violet (CV) staining [38]. After drying the washed biofilms at room temperature for 30 min, 100 µl of 0.4% CV solution (Merck, Germany) was added to each well and incubated for 20 min at room temperature. Each well was then washed twice with 250 µl of sterile distilled water, and finally 150 µl of 33% acetic acid was added to solubilize the CV-stained biomass for spectrophotometric measurement.

Biofilm metabolic activity was evaluated by reduction of 2,3-bis (2-methoxy- 4-nitro-5-sulfophenyl)-5-[(phenylamino)-carbonyl]- 2H-tetrazolium hydroxide (XTT, Sigma-Aldrich) [39]. A volume of 100 µl of XTT with 1 µM of menadione was added to each washed well, and microplates were incubated in darkness for 2 h at 37 ºC.

Absorbance measurements were conducted with a BioScreen C MBR microplate reader (Growth Curves Ltd, Finland) at a wavelength of 600 nm for biomass quantification and at 492 nm for metabolic activity determination. Each experiment was performed with biological triplicates on separate days.

2.4. Determination of phospholipase, proteinase and hemolytic activities

Phospholipase activity was determined using a precipitation assay as described by Polak (1992) [40] with malt agar plates supplemented with egg yolk [41]. Phospholipase activity was defined as the ratio of the colony diameter compared to the total diameter of colony plus precipitation zone. *C. albicans* isolate UPV/EHU 04-125 was used as a control with high phospholipase activity.

Aspartyl proteinase activity was assayed as described by Cassone et al. (1987) [42]. Proteinase activity was determined as the diameter of the lytic area around growing

colonies. High aspartyl proteinase producer strain *Candida dubliniensis* UPV/EHU 00-134 was used as a control.

Hemolytic activity was analyzed as described by Luo et al. (2001) [43] by performing the plate assay described by Manns et al. (1994) [44]. *C. albicans* ATCC 90028 with high hemolytic activity was used as a control.

Enzymatic and hemolytic activities were analyzed at least with biological triplicates on separate days.

2.5. Survival assays in *Caenorhabditis elegans*

C. elegans strain AU37 (*glp-4*(*bn2*); *sek-1*(*km4*)) was obtained from the Caenorhabditis Genetics Center (University of Minnesota, USA). The double mutation in this strain generates nematodes that are not able to reproduce at 25 °C (*glp-4*) and are more susceptible to infection (*sek-1*). The nematodes were kept in the laboratory at 15 °C in plates with nematode growth medium (NGM, 3 g of NaCl, 17 g of agar, 2.5 g of peptone, 1 ml of 1 M $CaCl_2$, 1 ml of 5 mg/ml cholesterol in ethanol, 1 ml of 1 M $MgSO_4$, 25 ml 1 M KPO_4, 975 ml H_2O) seeded with the non-pathogenic *Escherichia coli* strain OP50 as nourishment. Prior to survival experiments, nematodes were synchronized to the same growth stage, as described by Ortega-Riveros et al. (2017) [32]. Nematodes were then placed on BHI agar plates supplemented with kanamycin (90 µg/ml) and seeded with the isolate of *C. auris* to be assayed to feed the nematodes with the yeast [45]. After incubating the plates at 25 °C for 2 h, the nematodes were washed with M9 buffer (3 g of KH_2PO_4, 6 g of Na_2HPO_4, 5 g of NaCl, 1 ml of 1 M Mg SO_4 and H_2O to 1 l) supplemented with kanamycin (90 µg/ml) and placed in plates with NGM agar for 15 min to eliminate, by friction, *C. auris* cells that might have adhered to the nematode cuticle. Nematodes were placed in groups of 20 individuals in 24-well plates containing M9 buffer supplemented with 10 µg/ml of cholesterol in ethanol and kanamycin (90 µg/ml). Nematode survival was monitored every 24 h until 120 h with a stereomicroscope (Nikon SMZ-745, Japan). A minimum of 60 nematodes were used in each experiment for each

C. auris isolate, and groups of uninfected nematodes were included as controls. The experiments were carried out in triplicate on different days.

2.6. Survival tests in *Galleria mellonella*

G. mellonella larvae with a weight between 0.3 and 0.5 g were obtained from Bichosa (Spain). Experiments were started one day after caterpillars arrived, which were placed in groups of 20 individuals in Petri dishes. The last left pro-leg of each larvae was cleaned with 70% ethanol and 10 µl of a *C. auris* suspension were inoculated with a precision syringe (Agilent, USA). Inoculum of *C. auris* cells was prepared by washing overnight yeast cultures with PBS supplemented with ampicillin (20 µg/ml) to remove remnants of YEPD and prevent bacterial contamination. Concentrations of 1×10^5, 1×10^6 and 1×10^7 cells/larva were assayed to monitor the virulence of each *C. auris* isolate. In all trials, two uninfected groups were used as controls; one with untouched larvae and a sham group with larvae inoculated with 10 µl PBS-ampicillin to observe a possible effect of the injection. Survival counts of alive and dead larvae were determined by visual inspection of movement and melanization every 24 h until 120 h after infection. Trials were conducted in triplicate on three different days.

2.7. Statistics

The quantitative results obtained in biofilm production were analyzed using the Student's *t* test of the statistical program SPSS v24.0 (IBM, Chicago, IL, USA). The analysis of the virulence of *C. auris* isolates according to the origin of clinical specimens was analyzed by one-way ANOVA using SPSS v24.0. Survival curves were prepared with the Kaplan-Meier method using GraphPad Prism 5 software (GraphPad Software, La Jolla, CA, USA). Differences between the survival rates in both invertebrate models infected with *C. auris* were analyzed by the log-rank test of SPSS v24.0. For all statistical analyses values of $p<0.05$ were considered statistically significant.

3. RESULTS AND DISCUSSION

In the present study, we characterized virulence traits of twelve clinical isolates of *C. auris in vitro* and compared their capability to infect and kill *C. elegans* and *G. mellonella in vivo*. These *C. auris* isolates, that were recovered from patients at two different locations (Spain and Germany), were observed microscopically, and only the blood isolate JMRC:NRZ 1101 from Germany displayed an aggregating phenotype, which was not observed in the other *C. auris* isolates from Spain (Figure S1).

3.1. Biofilm formation, enzymatic activity, and hemolytic activity of *Candida auris*

The ability of the twelve *C. auris* clinical isolates to develop biofilms is summarized in Table 1. None of the twelve isolates produced a biofilm denser than the *C. albicans* SC5314 control strain (Table 1). The ability to form a biofilm is an important factor in *Candida* pathogenicity because fungal cells within biofilms are protected from the action of the immune system and antifungal agents. The lower *C. auris* biofilm production compared to *C. albicans* SC5314 has been reported previously [9]. Blood isolate JMRC:NRZ 1101 with aggregating phenotype showed the highest biofilm biomass production and metabolic activity ($p \leq 0.0001$). The non-aggregating urine isolate CR14 produced the second most dense biofilm with a significantly higher biomass ($p \leq 0.0001$) and metabolic activity ($p \leq 0.02$, except at 48 h for most of isolates) than the other *C. auris* isolates. Although *C. auris* biofilms are thinner than those of *C. albicans* [46], the capacity of *C. auris* to form a biofilm has been clearly associated with a lower antifungal susceptibility [16, 47]. The ability of *C. auris* to form a biofilm has been reported for both aggregating and non-aggregating phenotypes [12]. Biomass values obtained in the current study were similar to those reported by Sherry et al. (2017) [22]. However, these authors reported the highest biofilm-forming capacity for non-aggregating isolates. Brown and co-workers [12] also detailed differences in gene expression between both phenotypes: genes related to cellular components (membrane and cell wall constituents) were upregulated in isolates with aggregating phenotype whereas genes related to biological processes and metabolic functions were expressed higher in non-aggregating isolates.

Moreover, Short et al. (2019) [48] suggested that the ability of *C. auris* to form cellular aggregates increases survival of the yeast, which coincided with the upregulation of biofilm-associated genes. In addition, in *C. albicans*, the ability to produce biofilm has been associated with the expression of secreted aspartyl proteinases [49]. Genes involved in biofilm formation and in the production of phospholipase, proteinase and hemolysin activity have been reported in *C. auris*, with genes involved in cell adhesion and invasion (*ALS* and *SAP* families) showing stronger expression in aggregating than in non-aggregating isolates [12, 13, 50, 51]. However, in the present study, phospholipase and proteinase activities were not detected in any clinical isolate nor any hemolytic activity was observed (Table S1).

Table 1. Biofilm biomass and metabolic activity levels of each *C. auris* isolate measured with Crystal violet and XTT reduction assays, respectively.

Species	Origin site	Isolate	Biomass of biofilm		Metabolic activity of biofilm	
			OD (24 h)	OD (48 h)	OD (24 h)	OD (48 h)
Candida albicans		SC5314	0.719 ± 0.066	0.696 ± 0.071	1.159 ± 0.154	1.247 ± 0.172
Candida auris	blood	JMRC:NRZ 1101*	0.508 ± 0.047	0.361 ± 0.039	0.421 ± 0.147	0.357 ± 0.075
		CJ94	0.068 ± 0.038	0.080 ± 0.046	0.145 ± 0.070	0.122 ± 0.031
		CBS15605	0.074 ± 0.049	0.079 ± 0.048	0.156 ± 0.086	0.124 ± 0.033
		CBS15606	0.068 ± 0.042	0.083 ± 0.051	0.135 ± 0.062	0.103 ± 0.028
		CBS15607	0.086 ± 0.079	0.077 ± 0.043	0.132 ± 0.074	0.098 ± 0.023
	urine	CR14	0.227 ± 0.034	0.179 ± 0.054	0.237 ± 0.095	0.114 ± 0.060
		CR201	0.080 ± 0.036	0.057 ± 0.023	0.118 ± 0.035	0.122 ± 0.067
		CR220	0.103 ± 0.038	0.062 ± 0.030	0.119 ± 0.020	0.102 ± 0.048
		CR424	0.101 ± 0.024	0.082 ± 0.026	0.057 ± 0.039	0.056 ± 0.045
		CR440	0.090 ± 0.027	0.074 ± 0.025	0.121 ± 0.033	0.115 ± 0.055
	oropharyngeal	CR243	0.065 ± 0.033	0.084 ± 0.035	0.146 ± 0.056	0.119 ± 0.042
		CR312	0.081 ± 0.024	0.085 ± 0.046	0.085 ± 0.030	0.087 ± 0.047

OD: optical density

* The *C. auris* JMRC:NRZ 1101 blood isolate displayed an aggregating phenotype

3.2. Virulence of *Candida auris* in *Caenorhabditis elegans* and *Galleria mellonella*

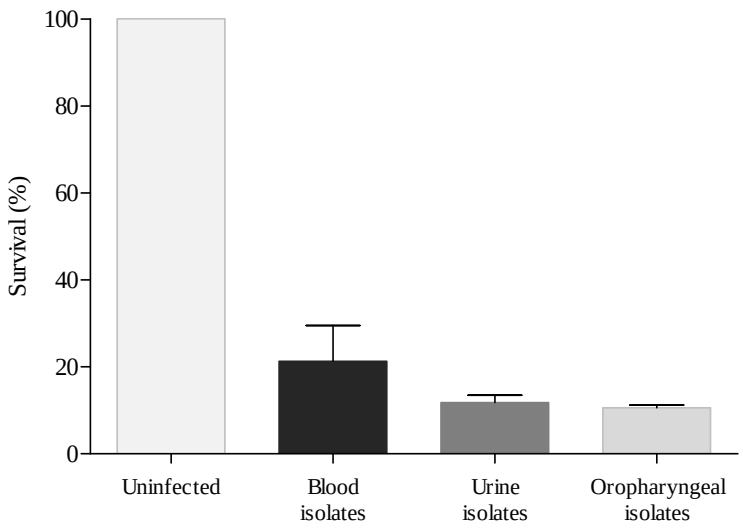
C. auris virulence has been studied in murine [13-15, 17, 24], fish [28], and fly models [25]. However, there have only been a few studies in the nematode *C. elegans* [23, 29] and the moth larvae *G. mellonella* [21, 22, 24, 26, 27]. In the present study, all *C. auris* isolates were able to cause the death of at least 47.7% of *C. elegans* and *G. mellonella* individuals after 120 h (Figure 1).

Uninfected *C. elegans* nematodes used as controls remained viable (100% survival) during the 120 h. A high amount of non-aggregating yeast cells was observed at 120 h post-infection for all *C. auris* isolates, except for the *C. auris* JMRC:NRZ 1101 isolate that caused yeast aggregates at 120 h post-infection in *C. elegans* (Figure S2). This cellular pattern was also observed in infected *G. mellonella* (data not shown). These results are consistent with those reported by Borman et al. (2016) [21] who first described the formation of aggregates by some *C. auris* isolates, which later was corroborated by Sherry et al. (2017) [22] and Muñoz et al. (2020) [11]. These studies reported that *C. auris* did not form hyphae unlike *C. albicans* in the *G. mellonella* model of candidiasis, and that non-aggregating *C. auris* isolates were more virulent, even when compared to some *C. albicans* isolates [11, 21, 22]. In a murine model, Ben-Ami et al. (2017) [13] also identified yeast cells during the infection and recovered aggregates from murine tissues. Yue et al. (2018) [15] observed a filamentous morphology of *C. auris* in cultures on YEPD medium from liver, kidney, brain, lung, and spleen specimens of mice suffering from invasive candidiasis. However, two days of incubation at 30 °C followed by five additional days at 25 °C were needed to observe this morphological switch from yeast to hyphae.

Killing assays in *G. mellonella* were performed with inocula of 1×10^5, 1×10^6 and 1×10^7 cells/larva for each *C. auris* isolate. In both control groups, most larvae were alive by 120 h post-infection with survival averages of 85% ± 3.2% in the case of the untouched group control and 82.3% ± 3.6% in larvae inoculated with PBS-ampicillin (Figure 1). Survival of *G. mellonella* significantly decreased when a higher inoculum of *C. auris* was administered, as reported for other species of *Candida* [31, 52, 53]. There was a strong correlation ($R^2 \geq$

0.87) between the injected inocula of *C. auris* and the survival of *G. mellonella*. Interestingly, there were statistically significant differences between the virulence of *C. auris* blood isolates and that of urine and oropharyngeal isolates using the inoculum of 1×10^5 cells/larva ($p \leq 0.009$). Differences were also detected with the inoculum of 1×10^6 cells/larva without considering the isolate with aggregating phenotype ($p \leq 0.032$) (Figure 1). The inoculum of 1×10^6 cells/larva appeared the most appropriate for analyzing the virulence of *C. auris* in the *G. mellonella* model based on the findings with all the *C. auris* isolates at the three different inocula and the mortality rates obtained. Therefore, the results obtained with 1×10^6 cells/larva are presented and discussed in detail in the main text while those obtained with other inocula are presented in Figure S3. Inocula of 1×10^7 cells/larva caused the death of more than 60% of the larvae during the first 24 h of infection, and of more than 98.3% at 120 h (Figure S3). On the opposite, with the lowest inoculum of 1×10^5 cells/larva, none of the *C. auris* isolates had killed more than 60% of *G. mellonella* larvae at 48 h, with mortality ranging from 43.7% to 83.3% at 120 h (Figure S3). In this *in vivo* model the highest mortality was observed for blood isolates (Figure 1).

a)



b)

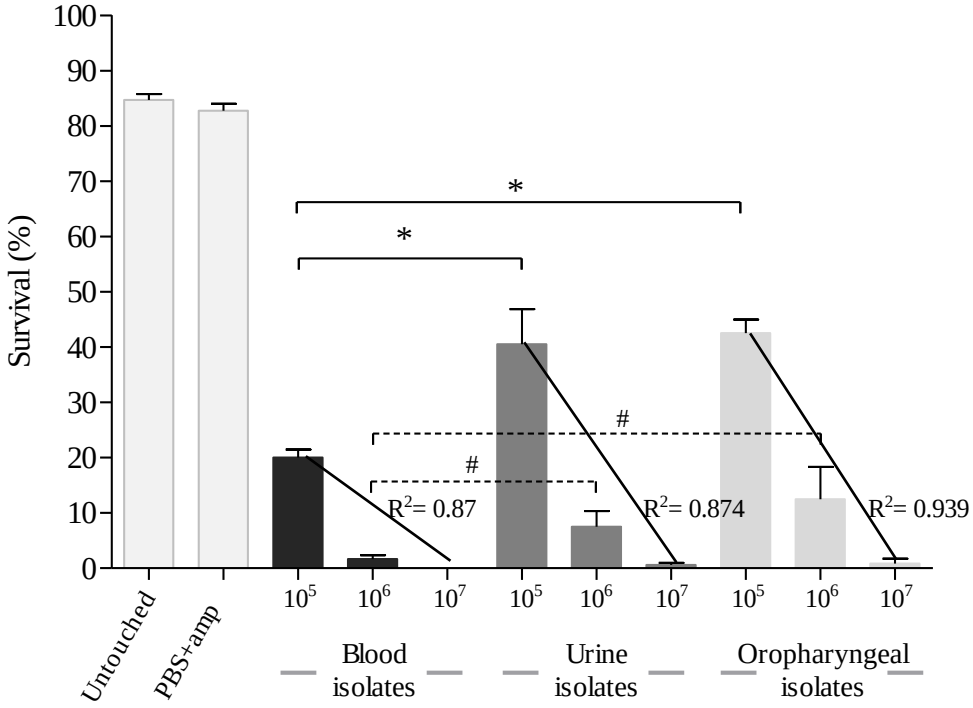


Figure 1. Survival average of *C. elegans* (a) and *G. mellonella* (b) at 120 hours post-infection with twelve different *C. auris* isolates according to the origin of the clinical specimen. Untouched control groups of nematodes and larvae and a group of larvae inoculated with PBS and ampicillin were also included. Larvae of *G. mellonella* were infected with three inocula of *C. auris* isolates (1×10^5, 1×10^6 and 1×10^7 cells/larva) and correlation coefficients were calculated. Absent bars indicate 0% survival rate of *G. mellonella*. Error bars represent standard errors. Solid lines with asterisks denote statistically significant differences of the inoculum 1×10^5 cells/larva between *C. auris* blood isolates and the other *C. auris* isolates from urine and oropharyngeal. Dotted lines with hashtag denote statistically significant differences of the inoculum 1×10^6 cells/larva between *C. auris* blood isolates, without including the aggregative German isolate, and the other *C. auris* isolates from urine and oropharyngeal. The statistical analysis was performed using the one-way ANOVA test ($p<0.05$).

3.2.1 Virulence of *C. auris* isolates according to the origin of the clinical specimens

Differences in the expression of virulence factors have been observed between *C. auris* isolates from different geographical origins, such as the lack of adhesion or few biofilm production associated with clade II isolates (East Asian clade), which frequently cause otitis [12, 54, 55]. Several studies have associated the expression of specific virulence factors of *Candida* with the site of infection [34-37]. In the present study, *C. auris* isolates were not grouped within any specific clade but the eleven isolates from the Spanish outbreak are phylogenetically close to clade III isolates (South African clade) [5, 6]. This clade III, as well as clade II, is associated with bloodstream infections and with aggregate formation [56].

We compared the virulence of the *C. auris* isolates according to their clinical origin in both *in vivo* models. The applied inoculum size of 1×10^6 cells/larva or lower ($2.5 - 5 \times 10^5$ cells/larva) has also previously been used to establish the virulence of *C. auris* [21, 22, 24, 26, 27] and other species of *Candida* [30, 52, 53]. Strikingly, the *C. auris* isolates presented different virulence potential according to their clinical origin. In the *G. mellonella* model, the following general virulence categorization was observed when comparing the eleven non-aggregating isolates: blood > urine > oropharyngeal isolates with average survival percentages of infected larvae after 120 h of 1.7% for blood, 7.5% for urine, and 12.5% for oropharyngeal isolates. In contrast, mortality of *C. elegans* caused by infection with *C. auris* isolates was not clearly associated to their clinical origin. The average survival of nematodes infected with the non-aggregating *C. auris* isolates was very similar for the three groups of clinical isolates: 13.6% for the infection with blood isolates, 11.8% with urine isolates, and 10.6% with oropharyngeal isolates (Figure 1).

The virulence of the five individual blood isolates, the five urine isolates and the two oropharyngeal isolates is presented in Figures 2, 3 and 4, respectively. The survival rates of *G. mellonella* infected with blood isolates ranged from 0% to 10% at 120 h, and from 7.6% to 52.3% in the *C. elegans* model. Moreover, most of the *C. auris* isolates killed more than 70% of the *G. mellonella* larvae at 24 h but took more than 96 h in the *C. elegans* model (Figure 2). Survival rates for *G. mellonella* infected with urine isolates were between 1.7%

and 18.3% at 120 h (7.8% - 17.2% in *C. elegans*). However, all urine isolates killed more than 50% of the *G. mellonella* larvae at 48 h, while in the case of *C. elegans* at least 96 h were required (Figure 3). Finally, for the two oropharyngeal isolates there were no significant differences in virulence between the two non-mammalian models: survival rates of *G. mellonella* at 120 h ranged from 6.7% to 18.3%, whereas those of *C. elegans* were between 10% and 11.2%. Once again, in *G. mellonella* both isolates killed more than 60% of the larvae after 48 h but in *C. elegans* they took 96 h to eliminate the same percentage of host organisms (Figure 4). In all cases, in the first 24 - 48 h post-infection *C. auris* caused higher mortality in the *G. mellonella* model than in the *C. elegans* model (Figures 2-4). These results are comparable to those obtained in other studies, which observed that *C. auris* caused death of *G. mellonella* within 48 h [21, 22] and of *C. elegans* between 48 and 96 h post-infection [23, 29]. Our findings corroborate the idea that both models successfully demonstrate the virulence potential of *C. auris*, which was similar to or higher than that of other *Candida* species [11, 30, 32, 33, 53]. The observed virulence of *C. auris* was comparable to *C. albicans* [21, 22, 29] and higher than isolates from *C. haemulonii* complex species [11, 29]. However, also within the species *C. auris* differences in virulence between isolates from different clades have been noted, probably associated with their genomic diversity [12].

The *C. auris* JMRC:NRZ 1101 blood isolate, with aggregating phenotype, was significantly less virulent than the other blood isolates in both models ($p \leq 0.0001$) (Table S2 and S3). This isolate killed more than 80% of the *G. mellonella* larvae in 96 h, but it took 120 h to achieve a mortality rate of 52.3% of *C. elegans* nematodes (Figure 2). Association of an aggregative phenotype of *C. auris* with lower pathogenicity compared to non-aggregating counterparts has also already been described earlier [21, 22]. Furthermore, the virulence of this aggregating isolate in *C. elegans* was also significantly lower than that of all non-aggregating isolates from different infection sites ($p \leq 0.0001$) (Figures 3 and 4; Table S3). In *G. mellonella*, the aggregating isolate caused a mortality comparable to that of most isolates from other clinical origins (Figure 2 and Table S2) and only the mortality caused by this isolate was significantly lower than of urine isolate CR201 ($p=0.016$).

Regarding non-aggregating *C. auris* isolates, blood isolate CBS15607 killed 100% of *G. mellonella* at 120 h but no significant differences were observed with other non-aggregating blood isolates (Figure 2). High virulence was observed for four out of five urine isolates (80%), which killed *G. mellonella* with a mortality rate ranging from 80% to 93.3% between 72 and 120 h without significant differences among them (Figure 3). The CR312 oropharyngeal isolate also killed 93.3% of larvae at 120 h (Figure 4), however, its killing kinetics were similar to the two isolates with the lowest virulence, urine isolate CR14 and oropharyngeal isolate CR243, which achieved 81.7% mortality at 120 h. Conversely, there were significant differences between the latter two isolates and the aforementioned four urine isolates ($p \leq 0.006$ and $p \leq 0.045$, respectively). A higher virulence of *Candida* isolates from blood specimens has previously been reported [35-37]. *C. elegans* infection by non-aggregating isolates caused a mortality rate of more than 76.6% at 120 h and *C. auris* isolates took more than 72 h to exceed a mortality of 50% (Figures 2-4). Eldesouky et al. (2018) [23] employed 30 min in the infection of worms and reported similar killing kinetics, whereas Lima et al. (2020) [29] infected *C. elegans* for four hours and detected a mortality rate of more than 70% at 72 h. Blood isolate CBS15605 and urine isolate CR220 killed the highest percentage of nematodes at 120 h, 92.7% and 92.2%, respectively. However, no significant virulence differences were observed among them and the CBS15606 blood isolate and two urine isolates (CR440 and CR14) (Table S3). Five out of eleven isolates (45.5%) showed the same killing kinetics. Moreover, the oropharyngeal isolates also showed high lethality, causing a mortality rate of 88.8% at 120 h without significant differences with the mortality caused by the CBS15607 blood isolate. Strikingly, the less virulent isolates in the *C. elegans* model were the CJ94 blood isolate and the CR424 urine isolate with similar killing kinetics, both being significantly different to the rest of the isolates (Table S3). However, both isolates were among the five most virulent against *G. mellonella*, and their mortality rates in the *C. elegans* and *G. mellonella* models differed: 82.8% versus 98.3% for the blood isolate and 76.6% versus 96.7% for the urine isolate, respectively.

It is noteworthy that non-aggregating isolates were more pathogenic than the aggregating isolate and that the aggregating isolate used in this study formed more biofilm than the non-

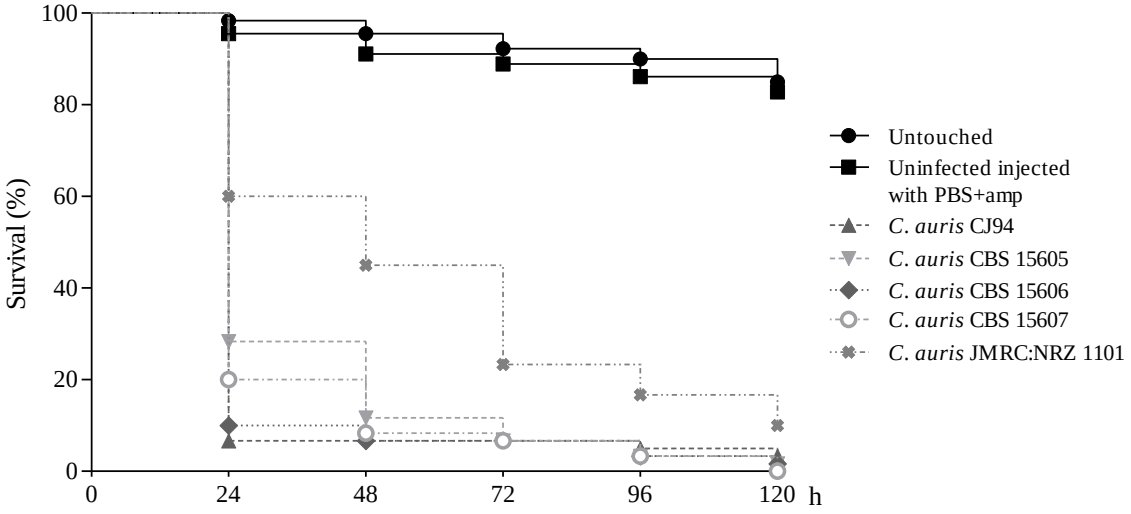
aggregating isolates. Sherry et al. (2017) [22] reported the opposite, a higher biofilm formation capacity in non-aggregating than in aggregating isolates. The aggregating *C. auris* JMRC:NRZ 1101 blood isolate could be expressing genes implicated in biofilm formation and also genes involved with other virulence factors. The expression of these virulence genes could render a virulence of this isolate similar to that of some non-aggregating isolates, yielding similar killing kinetics during infection of *G. mellonella*. Transcriptional analysis of *C. auris* isolates revealed a high number of upregulated genes involved in biofilm formation and other pathogenic traits, which can be associated with the high resistance of this species to antifungal drugs [10, 12]. In addition, differences in the infection profile and the host response to infection were observed for aggregating and non-aggregating phenotypes using a three-dimensional skin epithelial model. Although both phenotypes were more virulent in presence of a wound, an aggregating isolate was more cytotoxic and proinflammatory than a non-aggregating one with more ability to evade the immune system [12]. Trying to explain the differences found between both models of candidiasis, it is important to mention that in *G. mellonella C. auris* inoculum was injected parenterally, achieving higher concentrations in the tissues. Moreover, a higher lethal capacity of the non-aggregating isolates versus the aggregating isolate was noted in this model, likely due to the differential immune response of the host. Arias et al. (2020) [26] suggested that, due to a higher ability to move and disseminate within *G. mellonella* tissues, yeast cells might be more virulent than aggregates of cells, with which we concur. In addition, *C. elegans* infection occurs by yeast ingestion during a defined time. Variations in exposure time consequently might result in a higher or lower fungal burden, leading to variations in the killing kinetics [32]. It may also be possible that *C. elegans* has greater difficulty in ingesting cellular aggregates than individual yeasts. This fact could lead to a lower intake of fungal burden resulting in a milder infection rather than an aggregating isolate being less virulent. A stronger cytokine response and lower macrophage lysis capacity and neutrophil recruitment have been reported for *C. auris* compared to *C. albicans* [18, 28]. Interestingly, structurally unique *C. auris* mannoproteins contribute to a strong innate host defense in all five *C. auris* clades, although clade-specific differences were observed, clade V being the least immunogenic [28]. Both infection models, *C. elegans* and *G. mellonella*, present mechanisms and responses of the innate

immune system that are conserved in mammals and may contribute to a better understanding of host-*C. auris* interactions.

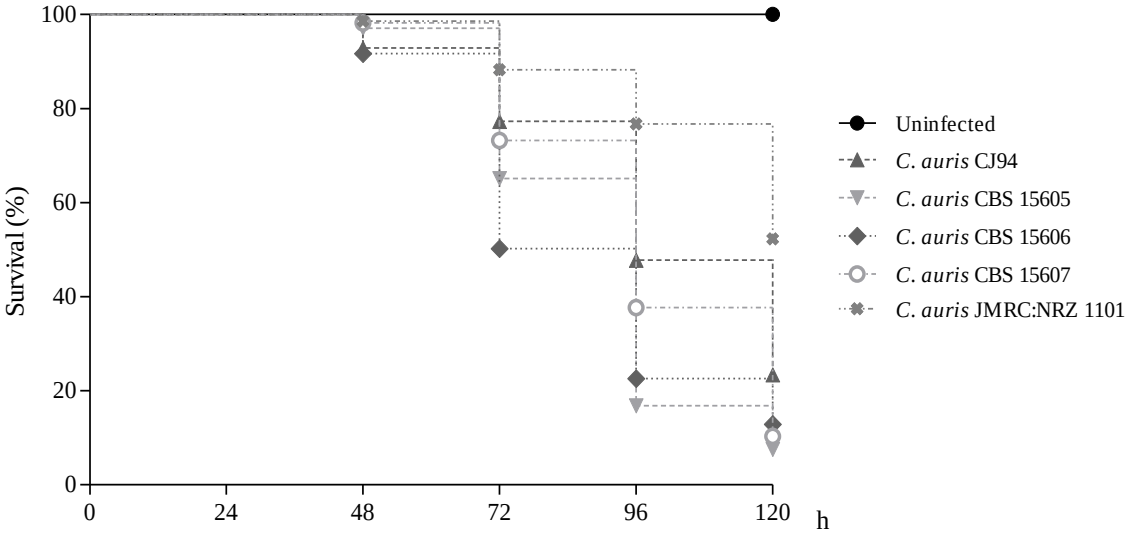
Overall, in both models, isolates of *C. auris* behaved differently depending on their clinical origin; although the response to *C. auris* infection of the two models of candidiasis was also different. These differences in the virulence of *C. auris* according to the site of infection seem consistent with reports about the phenotypic plasticity of this species of *Candida* in response to environmental conditions, such as the passage through a mammalian body and variations in temperature to colonize a specific niche [15]. However, further studies are required using other *C. auris* isolates with aggregating phenotype, if possible, from different geographic clades, to assess more accurately their virulence *in vivo*. There may be a level of heterogeneity in virulence among aggregating *C. auris* isolates similar to that observed for non-aggregating isolates in this and other studies [12, 18].

In conclusion, we demonstrated that both *C. elegans* and *G. mellonella* models of candidiasis are simple and clearly appropriate to assess the virulence of *C. auris* isolates. Likewise, these models are useful to detect variations in the virulence of clinical isolates with different origin and/or capacity to form cell aggregates. The model host *G. mellonella*, which allows a more precise and direct inoculation of the pathogen into the host tissues, revealed a significantly higher virulence for *C. auris* isolates from blood specimens. Among the successful strategies of *C. auris* to cause infection are its ability to evade neutrophil attack and to resist treatments with commonly used antifungal drugs. Therefore, obtaining a more effective and accurate therapy is one of the main targets on which to focus actions against this pathogen. These low-cost and manageable *in vivo* models are promising tools to analyze host-pathogen interactions or the effectiveness of current and new antifungal drugs against *C. auris*.

a)


Survival (%)
100
80
60
40
20
0
0
24
48
72
96
120
h
Untouched
Uninfected injected with PBS+amp
C. auris CJ94
C. auris CBS 15605
C. auris CBS 15606
C. auris CBS 15607
C. auris JMRC:NRZ 1101

b)


Survival (%)
100
80
60
40
20
0
0
24
48
72
96
120
h
Uninfected
C. auris CJ94
C. auris CBS 15605
C. auris CBS 15606
C. auris CBS 15607
C. auris JMRC:NRZ 1101

c)

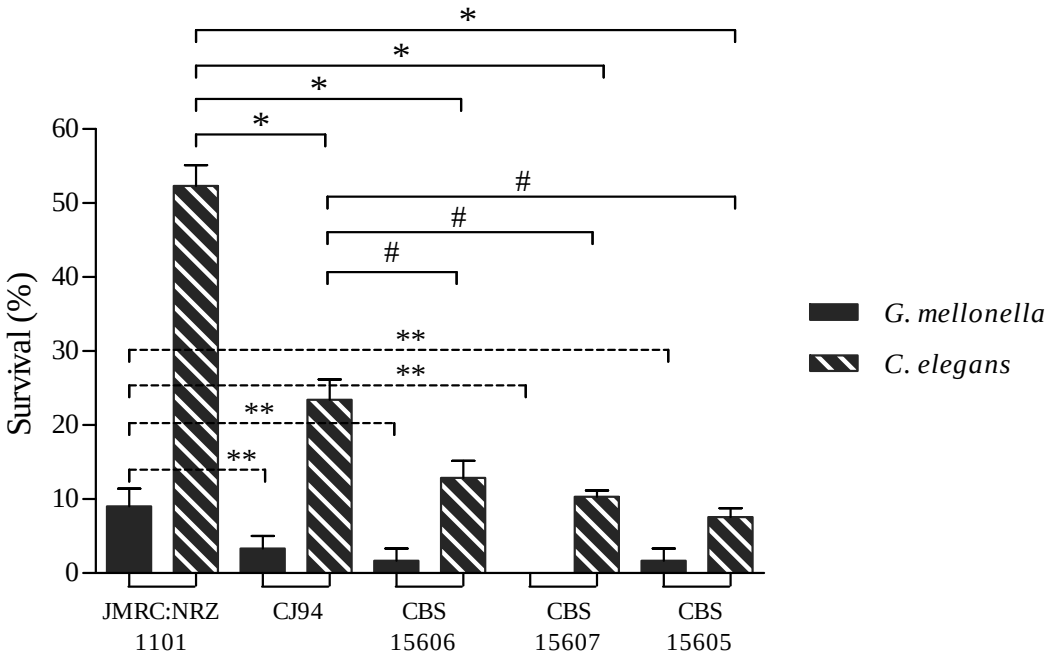
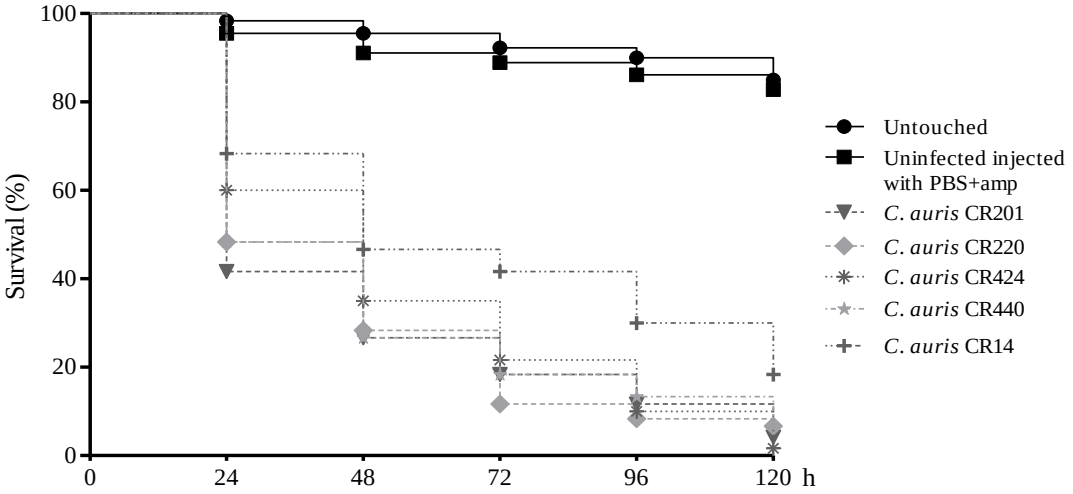
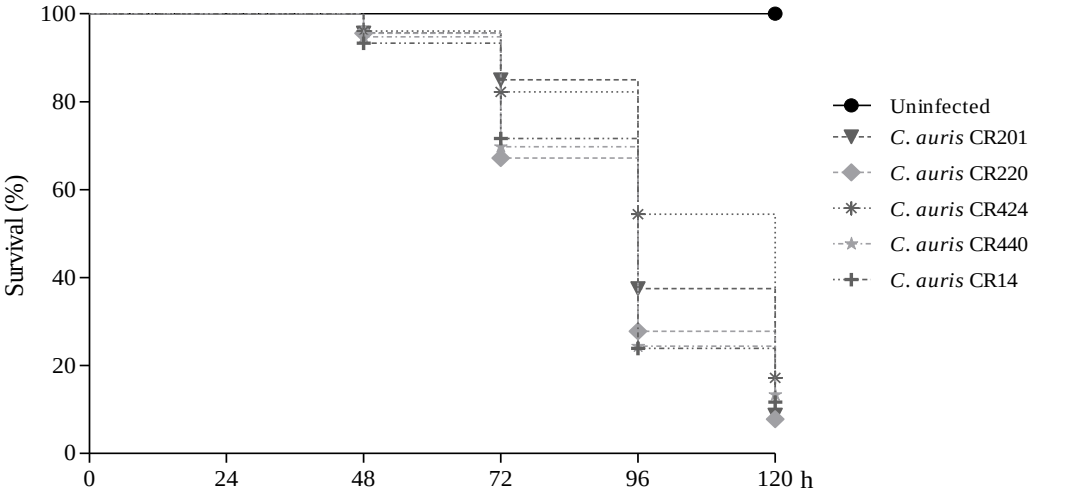


Figure 2. Survival curves of *G. mellonella* (a) and *C. elegans* (b) infected with *C. auris* blood isolates. Larvae of *G. mellonella* were infected with 1×10^6 cells/larva and the control groups used were a group of untouched larvae and larvae inoculated with PBS and ampicillin (PBS+amp) as a puncture (sham) control group. *C. elegans* worms were infected by *C. auris* cell ingestion for 2 h. c) Survival percentages at 120 h post-infection of *G. mellonella* and *C. elegans* infected with *C. auris* blood isolates. The *C. auris* isolates were sorted from highest to lowest survival percentages of *C. elegans*. Statistically significant differences in pathogenicity of *C. auris* blood isolates compared to the least virulent isolate JMRC:NRZ 1101 (* *C. elegans*; ** *G. mellonella*) and the second least virulent isolate CJ94 (#) calculated using the log-rank test ($p<0.05$) are indicated. Absent bars indicate 0% survival rate.

a)


100
80
60
40
20
0
Survival (%)
0
24
48
72
96
120 h
Untouched
Uninfected injected with PBS+amp
C. auris CR201
C. auris CR220
C. auris CR424
C. auris CR440
C. auris CR14

b)


100
80
60
40
20
0
Survival (%)
0
24
48
72
96
120 h
Uninfected
C. auris CR201
C. auris CR220
C. auris CR424
C. auris CR440
C. auris CR14

c)

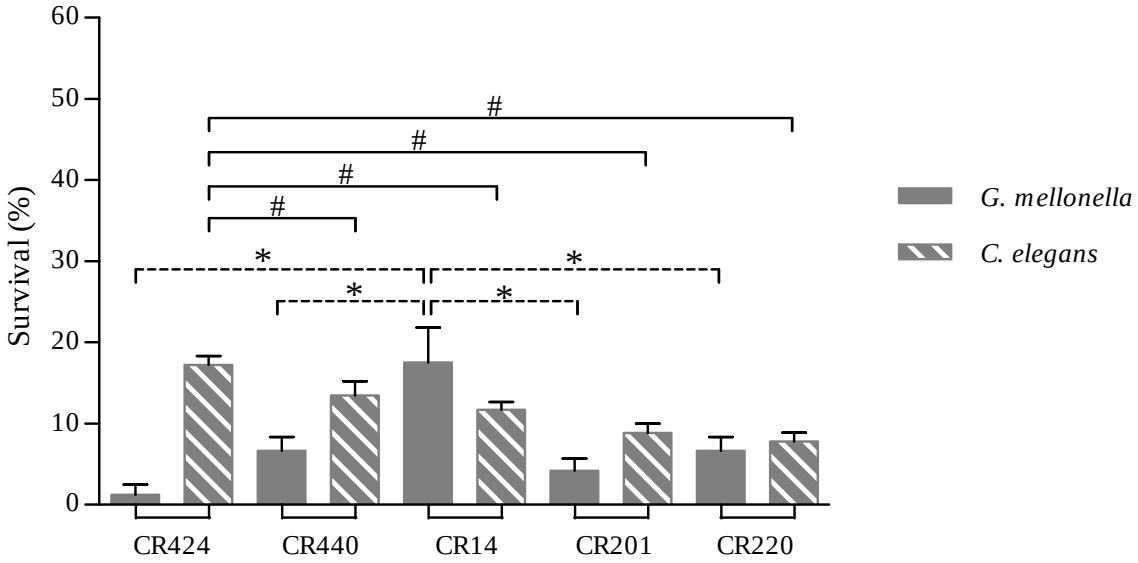
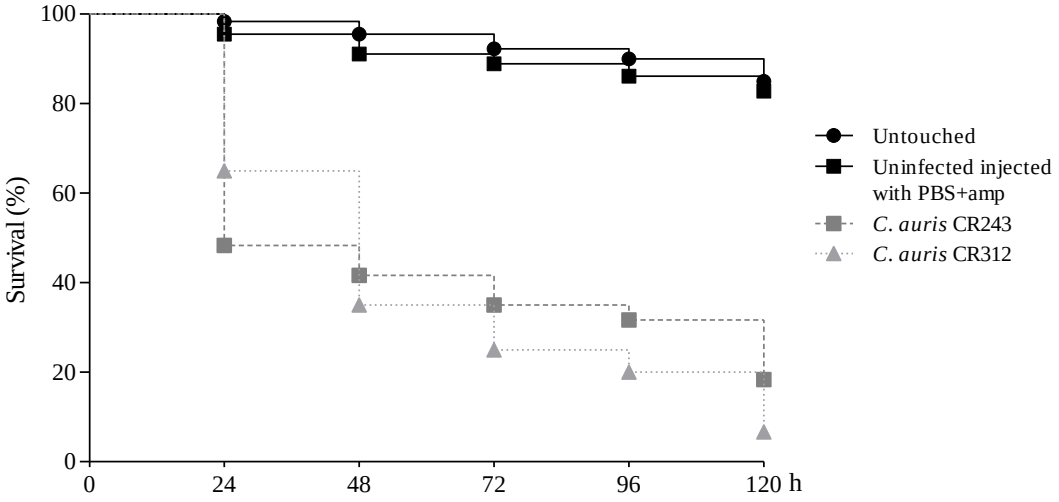
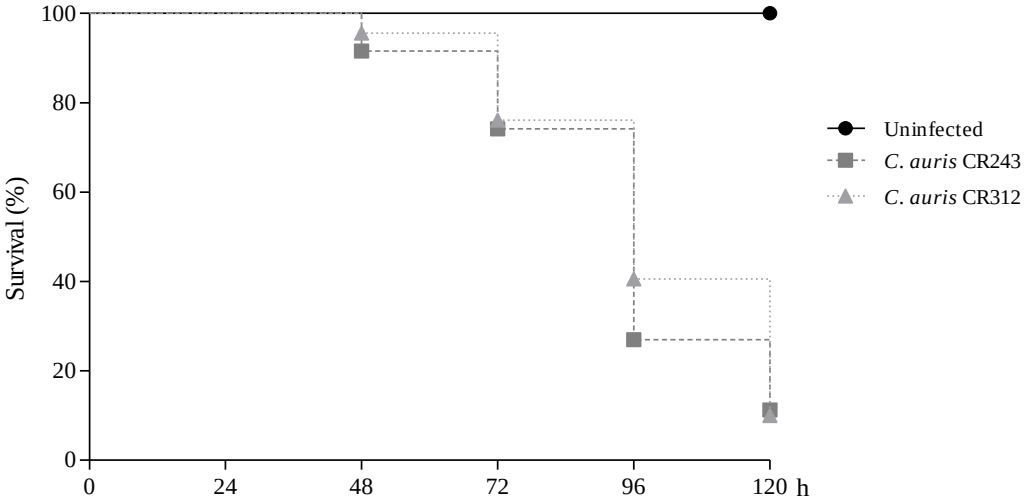


Figure 3. Survival curves of *G. mellonella* (a) and *C. elegans* (b) infected with *C. auris* urine isolates. Larvae of *G. mellonella* were infected with 1×10^6 cells/larva and the control groups used were a group of untouched larvae and larvae inoculated with PBS and ampicillin (PBS+amp) as a puncture (sham) control group. *C. elegans* worms were infected by *C. auris* cell ingestion for 2 h. c) Survival percentages at 120 hours post-infection of *G. mellonella* and *C. elegans* infected with *C. auris* urine isolates. The *C. auris* isolates were sorted from highest to lowest survival percentages of *C. elegans*. Statistically significant differences in pathogenicity of *C. auris* urine isolates compared to the least virulent isolate in *G. mellonella*, *C. auris* CR14 (*), and the highest virulent isolate in *C. elegans*, *C. auris* CR424 (#), calculated using the log-rank test ($p<0.05$) are indicated.

a)


100
80
60
40
20
0
Survival (%)
0
24
48
72
96
120 h
Untouched
Uninfected injected with PBS+amp
C. auris CR243
C. auris CR312

b)


100
80
60
40
20
0
Survival (%)
0
24
48
72
96
120 h
Uninfected
C. auris CR243
C. auris CR312

c)

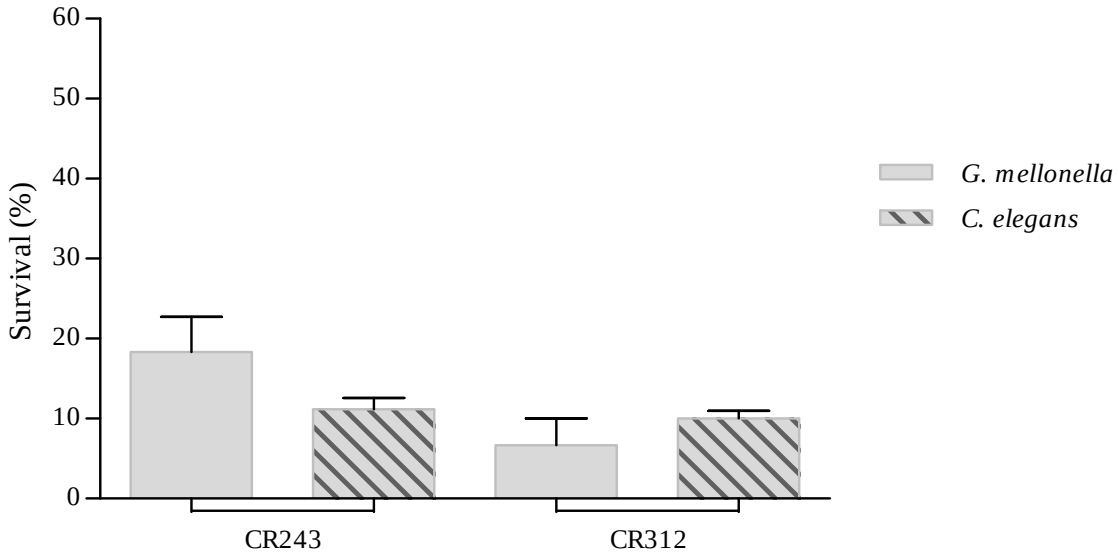


Figure 4. Survival curves of *G. mellonella* (a) and *C. elegans* (b) infected with *C. auris* oropharyngeal isolates. Larvae of *G. mellonella* were infected with 1×10^6 cells/larva and the control groups used were a group of untouched larvae and larvae inoculated with PBS and ampicillin (PBS+amp) as a puncture (sham) control group. *C. elegans* worms were infected by *C. auris* cell ingestion for 2 h. c) Survival percentages at 120 hours post-infection of *G. mellonella* and *C. elegans* infected with *C. auris* oropharyngeal isolates. The *C. auris* isolates were sorted from highest to lowest survival percentages of *C. elegans*.

Acknowledgements

Alba Ruiz Gaitán and Javier Pemán (Hospital Universitario y Politécnico La Fe of Valencia, Spain) and Oliver Kurzai (Institut für Hygiene und Mikrobiologie, Würzburg, Germany) are thanked for kindly providing clinical isolates. This work was supported by grants from the Spanish Ministry of Economy and Competitiveness (MINECO) [SAF2017-86188-P] and from the Consejería de Educación, Universidades e Investigación of Gobierno Vasco-Eusko Jaurlaritza [GIC15/78 IT-990-16]. Ainara Hernando-Ortiz and Aitzol Perez-Rodriguez were funded by Ph.D. grants from the University of the Basque Country (PIF 16/39 and PIF17/167, respectively).

4. BIBLIOGRAPHY

1. Quindós G, Marcos-Arias C, San-Millán R, et al. The continuous changes in the aetiology and epidemiology of invasive candidiasis: from familiar *Candida albicans* to multiresistant *Candida auris*. Int Microbiol. 2018;21(3):107-119. doi:10.1007/s10123-018-0014-1
2. Sabino R, Veríssimo C, Pereira ÁA, et al. Candida auris, an agent of hospital-associated outbreaks: Which challenging issues do we need to have in mind? Microorganisms. 2020;8(2). doi:10.3390/microorganisms8020181
3. Lockhart SR, Etienne KA, Vallabhaneni S, et al. 2017. Simultaneous emergence of multidrug-resistant *Candida auris* on 3 continents confirmed by whole-genome sequencing and epidemiological analyses. Clin Infect Dis. 2017;64:134-140. https://doi.org/10.1093/cid/ciw691
4. Arikan-Akdagli S, Ghannoum M., Meis JF. Antifungal resistance: specific focus on multidrug resistance in *Candida auris* and secondary azole resistance in *Aspergillus fumigatus*. J Fungi. 2018;4:129. https://doi.org/10.3390/jof4040129
5. Ruiz-Gaitán A, Moret AM, Tasias-Pitarch M, et al. An outbreak due to *Candida auris* with prolonged colonisation and candidaemia in a tertiary care European hospital. Mycoses. 2018;61(7):498-505. doi:10.1111/myc.12781

6. Chow NA, Muñoz JF, Gade L, et al. Tracing the evolutionary history and global expansion of *Candida auris* using population genomic analyses. MBio. 2020; 11:e03364-19. doi:10.1128/mBio.03364-19
7. Chow NA, de Groot T, Badali H, et al. Potential fifth clade of *Candida auris*, Iran, 2018. Emerg Infect Dis. 2019; 25: 1780–1781. doi: 10.3201/eid2509.190686
8. Muñoz JF, Gade L, Chow NA, et al. Genomic insights into multidrug-resistance, mating and virulence *in Candida auris* and related emerging species. Nat Commun. 2018;9(1):1-13. doi:10.1038/s41467-018-07779-6
9. Chybowska AD, Childers DS, Farrer RA. Nine things genomics can tell us about *Candida auris*. Front Genet. 2020;11(April):1-18. doi:10.3389/fgene.2020.00351
10. Kean R, Delaney C, Sherry L, et al. Transcriptome assembly and profiling of *Candida auris* reveals novel insights into biofilm-mediated resistance. mSphere. 2018;3(4):e00334-18. doi: 10.1128/mSphere.00334-18
11. Muñoz JE, Ramirez LM, Dias LDS, Rivas LA, Ramos LS, Santos ALS, Taborda CP, Parra-Giraldo CM. Pathogenicity levels of Colombian strains of *Candida auris* and Brazilian strains of *Candida haemulonii* species complex in both murine and *Galleria mellonella* experimental models. J Fungi (Basel). 2020;6(3):E104. doi: 10.3390/jof6030104
12. Brown JL, Delaney C, Short B, et al. *Candida auris* phenotypic heterogeneity determines pathogenicity in vitro. mSphere. 2020;5(3):e00371-20. doi: 10.1128/mSphere.00371-20
13. Ben-Ami R, Berman J, Novikov A, et al. Multidrug-Resistant *Candida haemulonii* and C. auris, Tel Aviv, Israel. Emerg. Infect. Dis. 2017, 23:195–203. doi: 10.3201/eid2302.161486
14. Fakhim H, Vaezi A, Dannaoui E, et al. Comparative virulence of *Candida auris* with *Candida haemulonii*, *Candida glabrata* and *Candida albicans* in a murine model. Mycoses. 2018; 61:377–382. doi:10.1111/myc.12754
15. Yue H, Bing J, Zheng Q, et al. Filamentation in *Candida auris*, an emerging fungal pathogen of humans: passage through the mammalian body induces a heritable

phenotypic switch. Emerg Microbes Infect. 2018;7(1). doi:10.1038/s41426-018-0187-x

16. Kean R, Brown J, Gulmez D, et al. *Candida auris*: A decade of understanding of an enigmatic pathogenic yeast. J Fungi. 2020;6(1):30. doi:10.3390/jof6010030
17. Torres SR, Pichowicz A, Torres-Velez F, et al. Impact of *Candida auris* infection in a neutropenic murine model. Antimicrob Agents Chemother. 2020;64:e01625-19. doi:10.1128/AAC.01625-19
18. Bruno M, Kersten S, Bain JM, et al. Transcriptional and functional insights into the host immune response against the emerging fungal pathogen *Candida auris*. Nat Microbiol. 2020;5(12):1516-1531. doi: 10.1038/s41564-020-0780-3
19. Kumar A, Baruah A, Tomioka M, et al. *Caenorhabditis elegans*: a model to understand host–microbe interactions. Cell Mol Life Sci. 2019. doi:10.1007/s00018-019-03319-7
20. Jemel S, Guillot J, Kallel K, et al. *Galleria mellonella* for the evaluation of antifungal efficacy against medically important fungi, a narrative review. Microorganisms. 2020;8(3):390. doi:10.3390/microorganisms8030390
21. Borman AM, Szekely A, Johnson EM. Comparative pathogenicity of United Kingdom isolates of the emerging pathogen *Candida auris* and other key pathogenic *Candida* species. mSphere. 2016;1(4):4-6. doi:10.1128/mSphere.00189-16
22. Sherry L, Ramage G, Kean R, et al. Biofilm-forming capability of highly virulent, multidrug-resistant *Candida auris*. Emerg Infect Dis. 2017;23(2):328-331. doi:10.3201/eid2302.161320
23. Eldesouky HE, Li X, Abutaleb NS, Mohammad H, Seleem MN. Synergistic interactions of sulfamethoxazole and azole antifungal drugs against emerging multidrug-resistant *Candida auris*. Int J Antimicrob Agents. 2018;52:754–761. https://doi.org/10.1016/j.ijantimicag.2018.08.016
24. Wang X, Bing J, Zheng Q, et al. The first isolate of *Candida auris* in China: Clinical and biological aspects article. Emerg Microbes Infect. 2018;7(1):0-8. doi:10.1038/s41426-018-0095-0

25. Wurster S, Bandi A, Beyda ND, et al. *Drosophila melanogaster* as a model to study virulence and azole treatment of the emerging pathogen *Candida auris*. J Antimicrob Chemother. 2019;74(7):1904-1910. doi:10.1093/jac/dkz100
26. Arias LS, Butcher MC, Short B, et al. Chitosan ameliorates *Candida auris* virulence in a *Galleria mellonella* infection model. Antimicrob Agents Chemother. 2020. https://doi.org/10.1128/AAC.00476-20
27. Romera D, Aguilera-Correa JJ, García-Coca M, et al. The *Galleria mellonella* infection model as a system to investigate the virulence of *Candida auris* strains. Pathog Dis. 2020;78(9):ftaa067. doi: 10.1093/femspd/ftaa067
28. Johnson CJ, Davis JM, Huttenlocher A, et al. Emerging fungal pathogen *Candida auris* evades neutrophil attack. MBio. 2018;9(4):1-9. doi:10.1128/mBio.01403-18
29. Lima SL, Rossato L, Salles de Azevedo Melo A. Evaluation of the potential virulence of *Candida haemulonii* species complex and *Candida auris* isolates in *Caenorhabditis elegans* as an *in vivo* model and correlation to their biofilm production capacity. Microb Pathog. 2020;22:148:104461. doi: 10.1016/j.micpath.2020.104461
30. Gago S, García-Rodas R, Cuesta I, et al. *Candida parapsilosis*, *Candida orthopsilosis*, and *Candida metapsilosis* virulence in the nonconventional host *Galleria mellonella*. Virulence. 2014;5(2):278-285. doi:10.4161/viru.26973
31. Ames L, Duxbury S, Pawlowska B, et al. *Galleria mellonella* as a host model to study *Candida glabrata* virulence and antifungal efficacy. Virulence. 2017;8(8): 1909-1917. doi:10.1080/21505594.2017.1347744
32. Ortega-Riveros M, De-la-Pinta I, Marcos-Arias C, et al. Usefulness of the nonconventional *Caenorhabditis elegans* model to assess *Candida* virulence. Mycopathologia. 2017;182:785–795. https://doi.org/10.1007/s11046-017-0142-8
33. Hernando-Ortiz A, Mateo E, Ortega-Riveros M, et al. *Caenorhabditis elegans* as a model system to assess *Candida glabrata*, *Candida nivariensis*, and *Candida bracarensis* virulence and antifungal efficacy. Antimicrob Agents Chemother. 2020;64:e00824-20. https://doi.org/10.1128/aac.00824-20

34. Oksuz S, Sahin I, Yildirim M, et al. Phospholipase and proteinase activities in different *Candida* species isolated from anatomically distinct sites of healthy adults. Jpn J Infect Dis. 2007;60(5):280-283
35. Júnior ADE, Silva AF, Rosa FC, et al. In vitro differential activity of phospholipases and acid proteinases of clinical isolates of *Candida*. Rev Soc Bras Med Trop. 2011;44:334–338. doi:10.1590/S0037-86822011005000036
36. L'Ollivier C, Labruère C, Jebrane A, et al. Using a Multi-Locus Microsatellite Typing method improved phylogenetic distribution of *Candida albicans* isolates but failed to demonstrate association of some genotype with the commensal or clinical origin of the isolates. Infect. Genet. Evol. 2012;12:1949–1957. https://doi.org/10.1016/j.meegid.2012.07.025
37. Atalay MA, Koc AN, Demir G, et al. Investigation of possible virulence factors in *Candida* strains isolated from blood cultures. Niger. J. Clin. Pract. 2015; 18(1):52-55
38. Peeters E, Nelis HJ, Coenye T. Comparison of multiple methods for quantification of microbial biofilms grown in microtiter plates. J Microbiol Methods. 2008;72(2):157-165. doi:10.1016/j.mimet.2007.11.010
39. Ramage G, VandeWalle K, Wickes BL, López-Ribot JL. Characteristics of biofilm formation by *Candida albicans*. Rev Iberoam Micol. 2001;18(4):163-170
40. Polak A. Virulence of *Candida albicans* mutants. Mycoses. 1992;35:9-16. https://doi.org/10.1111/j.1439-0507.1992.tb00813.x
41. Price MF, Wilkinson ID, Gentry LO. Plate method for detection of phospholipase activity in *Candida albicans*. Sabouraudia. 1982;20:7-14. https://doi.org/10.1080/00362178285380031
42. Cassone A, De Bernardis F, Mondello F, et al. 1987. Evidence for a correlation between proteinase secretion and vulvovaginal candidosis. J Infect Dis 156:777–83. https://doi.org/10.1093/infdis/156.5.777
43. Luo G, Samaranayake LP, Yau JY. 2001. *Candida* species exhibit differential in vitro haemolytic activities. J Clin Microbiol 39:2971-4. http://doi.org/10.1128/JCM.39.8.2971–2974.2001

44. Manns JM, Mosser DM, Buckley HR. 1994. Production of a haemolytic factor by *Candida albicans*. Infect Immun 62:5154-6
45. Breger J, Fuchs BB, Aperis G, et al. Antifungal chemical compounds identified using a *C. elegans* pathogenicity assay. PLoS Pathog. 2007. 3:e18. doi:10.1371/journal.ppat.0030018
46. Larkin E, Hager C, Chandra J, et al. The emerging pathogen *Candida auris*: growth phenotype, virulence factors, activity of antifungals, and effect of scy-078, a novel glucan synthesis inhibitor, on growth morphology and biofilm formation. Antimicrob Agents Chemother. 2017;61(5):1-13. doi: 10.1128/AAC.02396-16
47. Romera D, Aguilera-Correa JJ, Gadea I, et al. *Candida auris*: a comparison between planktonic and biofilm susceptibility to antifungal drugs. J Med Microbiol. 2019;68(9):1353-1358. doi:10.1099/jmm.0.001036
48. Short B, Brown J, Delaney C, et al. *Candida auris* exhibits resilient biofilm characteristics in vitro: implications for environmental persistence. J Hosp Infect. 2019;103(1):92-96. doi: 10.1016/j.jhin.2019.06.006
49. Tobouti PL, Casaroto AR, de Almeida RS, et al. Expression of secreted aspartyl proteinases in an experimental model of *Candida albicans*-associat ed denture stomatitis. J Prosthodont. 2016;25(2):127-134. doi:10.1111/jopr.12285
50. Chatterjee S, Alampalli SV, Nageshan RK, et al. Draft genome of a commonly misdiagnosed multidrug resistant pathogen *Candida auris*. BMC Genomics. 2015;16(1):1-16. doi:10.1186/s12864-015-1863-z
51. Muñoz JF, Gade L, Chow NA, et al. Genomic insights into multidrug-resistance, mating and virulence in *Candida auris* and related emerging species. Nat Commun. 2018;9(1):1-13. doi:10.1038/s41467-018-07779-6
52. Mesa-Arango AC, Forastiero A, Bernal-Martínez L, et al. The non-mammalian host *Galleria mellonella* can be used to study the virulence of the fungal pathogen *Candida tropicalis* and the efficacy of antifungal drugs during infection by this pathogenic yeast. Med Mycol. 2013;51(5):461-472. doi:10.3109/13693786.2012.737031

53. Scorzoni L, de Lucas MP, Mesa-Arango AC, et al. Antifungal efficacy during *Candida krusei* infection in nonconventional models correlates with the yeast in vitro susceptibility profile. PLoS One. 2013;8(3):e60047. doi:10.1371/journal.pone.0060047
54. Kwon YJ, Shin JH, Byun SA, et al. *Candida auris* clinical isolates from South Korea: identification, antifungal susceptibility, and genotyping. J Clin Microbiol. 2019;57:e01624-18. doi.org/10.1128/JCM.01624-18
55. Muñoz JF, Welsh RM, Shea T, et al. Chromosomal rearrangements and loss of subtelomeric adhesins linked to clade-specific phenotypes in *Candida auris*. bioRxiv preprint. 2019: doi: 10.1101/ 754143
56. Szekely A, Borman AM, Johnson EM. *Candida auris* isolates of the Southern Asian and South African lineages exhibit different phenotypic and antifungal susceptibility profiles in vitro. J. Clin. Microbiol. 2019;57(5):e02055-18. doi: 10.1128/JCM.02055-18

VIRULENCE
2021, VOL. 12, NO. 1, 1063–1075
https://doi.org/10.1080/21505594.2021.1908765

Taylor & Francis
Taylor & Francis Group

RESEARCH PAPER

OPEN ACCESS Check for updates

Virulence of *Candida auris* from different clinical origins in *Caenorhabditis elegans* and *Galleria mellonella* host models

Ainara Hernando-Ortiz [a*], Estibaliz Mateo [a*], Aitzol Perez-Rodriguez [a], Piet W.J. de Groot [b], Guillermo Quindós [a], and Elena Eraso [a]

[a]Department of Immunology, Microbiology and Parasitology, Faculty of Medicine and Nursery, University of the Basque Country (UPV/EHU), Bilbao, Spain; [b]Regional Center for Biomedical Research, Castilla-La Mancha Science & Technology Park, University of Castilla-La Mancha, Albacete, Spain

ABSTRACT

Candida auris is an emerging multidrug-resistant fungal pathogen responsible for nosocomial outbreaks of invasive candidiasis. Although several studies on the pathogenicity of this species have been reported, the knowledge on *C. auris* virulence is still limited. This study aims to analyze the pathogenicity of *C. auris*, using one aggregating isolate and eleven non-aggregating isolates from different clinical origins (blood, urine and oropharyngeal specimens) in two alternative host models of candidiasis: *Caenorhabditis elegans* and *Galleria mellonella*. Furthermore, possible associations between virulence, aggregation, biofilm-forming capacity, and clinical origin were assessed. The aggregating phenotype isolate was less virulent in both *in vivo* invertebrate infection models than non-aggregating isolates but showed higher capacity to form biofilms. Blood isolates were significantly more virulent than those isolated from urine and respiratory specimens in the *G. mellonella* model of candidiasis. We conclude that both models of candidiasis present pros and cons but prove useful to evaluate the virulence of *C. auris in vivo*. Both models also evidence the heterogeneity in virulence that this species can develop, which may be influenced by the aggregative phenotype and clinical origin.

ARTICLE HISTORY
Received 9 November 2020
Revised 12 March 2021
Accepted 23 March 2021

KEYWORDS
Candida auris; emerging pathogen; candidiasis; virulence; invertebrate models

Introduction

Candida albicans is the predominant etiological agent of invasive candidiasis [1,2]. However, recently *Candida auris* is emerging as a major cause of invasive nosocomial infections with multidrug-resistance and high morbidity and mortality [3,4]. Since this species was first described in 2009, several clinical presentations, such as colonization, mucosal infection or bloodstream infection have been reported worldwide, mostly associated with outbreaks in surgical and medical intensive care units (ICU) [5,6]. Phenotypic and genotypic characterization studies of *C. auris* isolates from different countries have revealed the high diversity of this species identifying up to five possible different phylogeographical clades, four clearly distinct and a potential fifth clade, around the world [6,7].

A wide array of virulence mechanisms is encoded in the genome of *C. auris* [8]. Expression of phospholipase, proteinase, and hemolytic activities, adherence and biofilm formation, antifungal drug and environmental stress resistance are described among the most relevant virulence traits [9–11]. However, the knowledge on *C. auris* virulence remains scarce and further studies are required to understand its pathogenic behavior, which has been reported to be substantially variable among clades [12]. Currently, about 30 studies have incorporated *in vivo* models in the analysis of *C. auris* virulence, murine models being the most widely used [13–18]. However, ethical and economical issues for using these models of candidiasis are encouraging the use of alternative systems such as invertebrate models [19,20]. Several virulence studies of *C. auris* infections have been reported in invertebrate models using *Caenorhabditis elegans*, *Drosophila melanogaster*, *Danio rerio* or *Galleria mellonella* [11,21–29]. Moreover, non-mammalian hosts have been successfully implemented for studying host-pathogen interactions and invasive candidiasis caused by other *Candida* species, and to evaluate the effectiveness of new therapeutic approaches [11,30–33].

Advances in the knowledge of the virulence potential of *C. auris* will contribute to the control of infections by this emerging pathogen. Recently, two different

CONTACT Estibaliz Mateo estibaliz.mateo@ehu.eus

*These authors contributed equally to this paper.
Supplemental data for this article can be accessed here.

5. Eranskina/Anexo 5

Effectiveness of the combination Amphotericin B and Echinocandins against *C. auris* in vitro and in *Caenorhabditis elegans* host model

Ainara Hernando-Ortiz, Elena Eraso, Guillermo Quindós and Estibaliz Mateo

Department of Immunology, Microbiology and Parasitology, Faculty of Medicine and Nursery, University of the Basque Country (UPV/EHU), Bilbao, Spain

Manuscript in preparation

ABSTRACT

The multidrug-resistant fungal pathogen *Candida auris* has been declared a global health emergency, responsible for outbreaks of invasive candidiasis worldwide. Limited effective therapeutic choices make difficult the treatment against this emerging pathogen, and combinations of different antifungal drugs are considered a promising therapeutic alternative. Invertebrate animal models, such as the nematode *Caenorhabditis elegans*, bypass the ethical restrictions caused by murine models and provide interesting alternatives to assess the effectiveness of antifungal treatment against *Candida*. The aim of this work was to evaluate combinations of amphotericin B and echinocandins against five clinical blood isolates of *C. auris*, one of them with an aggregating phenotype, in vitro and in vivo during infection in the *C. elegans* model. In vitro results showed synergistic interactions between amphotericin B and echinocandins in most cases, although higher MIC values were observed in the aggregating isolate compared to non-aggregating counterparts. Caspofungin and amphotericin B combination exhibited the most potent protective effect during *C. elegans* infection with *C. auris* blood isolates, reaching *C. elegans* survival up to 99%; while micafungin and amphotericin B combination was the least effective. The findings of this study showed that amphotericin B and echinocandins combination is a good strategy for the treatment of candidiasis caused by blood isolates of *C. auris*. Moreover, the nematode *C. elegans* is a suitable alternative model in the screening of therapeutic drugs to achieve those capable of overcoming multidrug-resistant *C. auris* infection.

Keyboards: candidiasis, emerging pathogen, antifungal susceptibility, drugs combinations, invertebrate models

1. INTRODUCTION

Candida auris is an emerging multi-drug resistant pathogen that was described for the first time in 2009 (Borman et al., 2016; Lepak et al., 2017; Lockhart et al., 2017). This relevant pathogen in the medical field causes 30-50% mortality (Arastehfar et al., 2018; Lockhart et al., 2017). Its high capacity to develop resistance to commonly used antifungal drugs, its ability to form biofilms, to produce hydrolytic enzymes such as phospholipases, acidic proteases or hemolysins, to produce specific immune responses and to evade attack by neutrophils are some of virulence factors that contribute to persistence in the hospital environment and to promote candidemia (Welsh et al., 2017; Kenters et al., 2019; Johnson et al., 2018; Bruno et al., 2020; Rossata and Colombo, 2018).

The ability to grow in large cell aggregates is an interesting phenotypic characteristic of *C. auris* (Borman et al., 2016). Aggregating isolates are associated with lower pathogenicity than non-aggregating counterparts and with higher resistance to antifungal drugs (Borman et al., 2016; Sherry et al., 2017; Muñoz et al., 2020; Hernando-Ortiz et al., 2021). However, high heterogeneity has been observed within these different phenotypes of *C. auris* (Brown et al., 2020; Sherry et al., 2017). High genomic diversity of *C. auris* has also been reported among the five different clades based on distinct geographic location, and also high clonality of *C. auris* isolates within each clade, which supports the hypothesis of a simultaneous and independent outbreak of this species at different locations (Lockhart et al., 2017).

It is noteworthy that *C. auris* resistance to fluconazole in some cases can reach 100% of the studied isolates, the 43% to amphotericin B, and the 5% to echinocandins (Khan et al., 2018; Ahmad et al., 2020; Lockhart et al., 2017; Chowdhary et al., 2018). Therefore, and due to the low incidence of resistance against echinocandins, some authors have proposed these drugs as the most appropriate treatment against *C. auris* infection (Lockhart et al., 2017; Ruiz-Gaitán et al., 2017; Kenters et al., 2019). Moreover, several clinical cases have reported the effective use of antifungal drugs in combination, mainly echinocandins with amphotericin B or isavuconazole, for *C. auris* candidemia (Ruiz-Gaitán et al., 2017; Chamdramati et al., 2020; Mulet Bayona et al., 2020).

Most of the antifungal susceptibility studies of *C. auris* have been performed in vitro and fewer than 40 have been conducted in the in vivo models, being the murine model the most commonly used (Bruno et al., 2020; Lepak et al., 2017; Muñoz et al., 2020; Xin et al., 2019). However, to avoid ethical restrictions that cause mammalian models, the use of invertebrates animal models has been encouraged to study candidiasis (Borman et al., 2016; Hernando-Ortiz et al., 2020; Hernando-Ortiz et al., 2021; Muñoz et al., 2020; Wurster et al., 2019). The nematode *Caenorhabditis elegans* is an alternative host model of candidiasis widely used for its simplicity, for the possibility to use a very large number of individuals in each trail and the rapid results achievement. As such, it is a suitable non-mammalian host model to study the virulence of *Candida* species and the efficacy of antifungal drugs and new antifungal compounds as treatments for candidiasis (Desalermos et al., 2011; Segal and Frenkel, 2018; Hernando-Ortiz et al., 2020) including *C. auris* among these species (Eldesouky et al., 2018; Eldesouky et al., 2020[a]; Eldesouky et al., 2020[b]; Eldesouky et al., 2020[c]; Hernando-Ortiz et al., 2021; Lima et al., 2020).

The goal of this work was to analyze both in vitro and in vivo the susceptibility of five *C. auris* blood isolates, one of them with aggregating phenotype, against amphotericin B and echinocandins. In vitro antifungal susceptibility assays were performed using amphotericin B and anidulafungin, caspofungin or micafungin in monotherapy and in combination, amphotericin B with each echinocandin. The most effective treatment obtained in vitro were assayed in vivo using the *C. elegans* model, and the effectiveness of treatments in monotherapy were compared with those in combination against *C. auris* infection.

2. MATERIALS AND METHODS

2.1 *Candida auris* isolates and growth conditions

Five clinical *C. auris* blood isolates were used in this study (Table 1). Among them, four with non-aggregating phenotype, were obtained from the Hospital Universitario y Politécnico La Fe of Valencia, Spain (Dr. Alba Ruiz Gaitán and Dr. Javier Pemán), three of them being registered in the CBS-KNAW culture collection of Westerdijk Fungal

Biodiversity Institute. The remaining isolation, *C. auris* JMRC:NRZ 1101 from Jena Microbial Resource Collection was provided by the Institut für Hygiene und Mikrobiologie, Würzburg, Germany (Dr. Oliver Kurzai). This latter *C. auris* isolate present aggregating phenotype (Hernando-Ortiz et al., 2021).

The isolates were stored in vials containing sterile distilled water at room temperature. They were recovered on Sabouraud dextrose agar (Difco, Becton Dickinson, USA) and incubated at 37 °C for 24 h. For the assays with the *C. elegans* model, brain heart infusion (BHI) (Panreac, Spain) agar plates supplemented with kanamycin (90 µg/ml) were seeded with a cell suspension of 2 McFarland of each *C. auris* isolate and incubated at 37 °C for 24 h.

2.2 In vitro antifungal susceptibility of *C. auris*

The antifungal drugs assayed were amphotericin B (AmB) (Sigma-Aldrich Inc., USA), anidulafungin (AND) (Pfizer SA, Madrid, Spain), caspofungin (CAS) (Merk and Com Inc, NJ, USA) and micafungin (MCF) (Astellas Pharma Inc, Japan). The stock solutions of all of them were prepared in dimethyl sulphoxide (DMSO) for in vitro and in vivo studies, being 0.1% DMSO the concentration used in the *C. elegans* model.

The antifungal efficacy of each antifungal agent was performed by microdilution antifungal susceptibility testing as described in the protocol developed by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (Arendrup et al., 2020), using 96-well microplates. The concentrations of these antifungal drugs ranged from 0.03 to 16 µg/ml for AmB, and from 0.016 to 8 µg/ml for each drug of echinocandins. Briefly, *C. auris* inocula were prepared in distilled water and 200 µl of a 0.5 McFarland standard yeast suspension was transferred into 1800 µl of distilled water tube. Then, each well with 100 µl of antifungal drug was inoculated with 100 µl of the diluted inoculum. Microplates were incubated at 37 °C for 24 h and then, growth was measured at a wavelength of 450 nm with a spectrophotometer (Tecan, Switzerland). As appear in the protocol, the minimum inhibitory concentration (MIC) was considered as the lowest concentration which caused ≥ 50% (for echinocandins, MIC_1) and ≥ 90% (for AmB, MIC_0) inhibition of yeast growth compared to the growth without antifungal drug. The susceptibility of isolates was categorized according

to the provisional breakpoints proposed by the Centers for Disease Control and Prevention (CDC) (CDC, 2018): fluconazole (≥32 μg/ml), AmB (≥2 μg/ml), echinocandins (≥4 μg/ml for AND and MCF; ≥2 μg/ml for CAS).

The antifungal drug efficacy was also in vitro tested using combinations: AmB plus AND, AmB plus CAS, and AmB plus MCF. Antifungal drug combination assays were assessed by the broth microdilution checkerboard methodology using 96-well microplates (Mukherjee et al., 2005). In this case, the drugs concentrations assayed were ranged from 0.015 to 1 μg/ml for AmB and from 0.016 to 8 μg/ml for each echinocandins. Briefly, yeast cell suspensions of 0.5 McFarland for each *C. auris* isolate were prepared in distilled water and a ten-fold dilution was performed. Then, 100 μl of each yeast inoculum were mixed with 100 μl of antifungal drug in each well; after incubated at 37ºC for 24h, yeast growth was measured at 450nm. The interactions between antifungal drugs were performed by the fractional inhibitory concentration index (FICI) method. Values obtained of MIC of 90% of cell growth (MIC_0) were applied to the formula FICI= $MIC_{A/E}/MIC_A$ + $MIC_{E/A}/MIC_E$ ($MIC_{A/E}$= MIC for AmB in combination; MIC_A= MIC for AmB in monotherapy; $MIC_{E/A}$= MIC for echinocandins in combination with AmB; MIC_E= MIC for echinocandins in monotherapy). Interactions between antifungal compounds were classified as synergistic when FICI ≤0.5, additive when 0.5 < FICI ≤1; indifferent when 1< FICI ≤4 and antagonistic when FICI ≥4 (Te Dorsthorst et al., 2002; Mukherjee et al., 2005).

2.3 In vivo assays of antifungal combination in *Caenorhabditis elegans*

The *C. elegans* AU37 double mutant strain (*glp-4*(*bn2*); *sek-1*(*km4*)) obtained from the Caenorhabditis Genetics Center (University of Minnesota, USA) was used to perform the *in vivo* assays. These mutations make sterile nematodes at 25 °C (*glp-4*) and more susceptible to infection (*sek-1*), so ensure a constant number of individuals throughout the trials.

Survival studies were conducted with a synchronous population of *C. elegans* in the L4 larval stage as previously described (Breger et al., 2007; Ortega-Riveros et al., 2017). Nematodes were placed at 25 °C for 2 h onto BHI agar plates supplemented with kanamycin (90 μg/ml) seeded with lawns of clinical *C. auris* isolates to ingest them. After that,

nematodes were washed with M9 buffer (3 g of KH_2PO_4, 6 g of Na_2HPO_4, 5 g of NaCl, 1 ml of 1 M $MgSO_4$ and H_2O to 1 l) supplemented with kanamycin (90 µg/ml) and placed for 15 min on plates with nematode growth medium (NGM, 3 g of NaCl, 17 g of agar, 2.5 g of peptone, 1 ml of 1 M $CaCl_2$, 1 ml of 5 mg/ml cholesterol in ethanol, 1 ml of 1 M $MgSO_4$, 25 ml 1 M KPO_4, 975 ml H2O) to remove yeasts adhering to their cuticles. *C. elegans* infected with *C. auris* were then displaced in groups of 20 individuals in 24-well plates and antifungal compounds were added to the wells both in monotherapy and in combination. The antifungal drugs concentrations that best resulted in the in vitro antifungal susceptibility test were in vivo assayed. Plates were incubated at 25 °C for 120 h and nematode survival was observed every 24 h. Trials were performed in triplicate on different days. In each experiment, seven different treatments were evaluated for each *C. auris* isolate and 60 nematodes were included in each condition. Moreover, two control groups were utilized in each experiment, one control of uninfected nematodes and another one of infected nematodes without antifungal treatment. Uninfected nematodes were also assayed only in presence of antifungal drugs both in monotherapy and in combination to assess the toxicity of these compounds. Around 15.000 nematodes were assayed in this work.

2.4 Statistics

Kaplan-Meier curves were generated with GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA) to assess the survival of *C. elegans* during the five clinical *C. auris* isolates infection with and without exposed to the different antifungal drugs treatments. Differences in *C. elegans* survival were analyzed by long-rank test with the statistical program SPSS v24.0 (IBM, Chicago, IL, USA). Values of $p < 0.05$ were considered as statistically significant.

3. RESULTS

3.1. In vitro susceptibility of *C. auris* blood isolates to AmB and echinocandins

The antifungal susceptibility of the five *C. auris* blood isolates, with aggregating and non-aggregating phenotype, was evaluated for AmB and echinocandins (Table 1). All isolates were susceptible to AmB, with MIC_0 between 0.5 and 1 μg/ml. Among the five *C. auris* isolates, four with non-aggregating phenotype were susceptible to echinocandins, MIC_1 for CAS was 0.25 μg/ml, and for AND and MCF ranging from 0.12 to 0.25 μg/ml. Strikingly, the *C. auris* JMRC:NRZ 1101 isolate, with aggregating phenotype, was resistant to the three echinocandins, with MIC_1 values ≥4 μg/ml.

Antifungal drugs activity was also performed by the combination of AmB with each of the echinocandins (Table 2). The three combinations showed a synergistic interaction against *C. auris* isolates, except the MCF and AmB combination that showed an additive interaction against the aggregating *C. auris* isolate (JMRC:NRZ 1101).

Interestingly, echinocandins and AmB combination showed MIC value lower than in monotherapy. These values were lower against non-aggregating *C. auris* isolates compared to the aggregating counterpart; ranged from 0.03 to 0.06 μg/ml versus from 0.25 to 0.5 μg/ml, respectively. Regarding to echinocandins, the combination with AmB showed also a reduction of MIC values against the five *C. auris* blood isolates. It is noteworthy that MIC_0 for echinocandins in monotherapy was in all cases ≥8 μg/ml. However, MIC values were not higher than 2 μg/ml when AND (0.5-2 μg/ml) and MCF (0.25-2 μg/ml) were combined with AmB against all *C. auris* isolates, and also when CAS and AmB combination (0.5-2 μg/ml) was assayed against *C. auris* isolates with non-aggregating phenotype. The drugs CAS and AmB combination showed a MIC of 4 μg/ml for the aggregating *C. auris* isolate. The MCF and AmB combination was the most active against the *C. auris* blood isolates, as MIC values of MCF were equal or less than to those of the other two echinocandins. The geometric mean (GM) obtained for AmB combined with AND and CAS was 1.148 μg/ml, while for AmB and MCF combination the GM was 0.574 μg/ml.

Table 1. In vitro antifungal activity of echinocandins (anidulafungin (AND), caspofungin (CAS) and micafungin (MCF)) and amphotericin B (AmB) against five *C. auris* blood isolates.

***C. auris* isolate**	**Source**	**Phenotype**	**MIC_0(µg/ml)[a]**	**MIC_1(µg/ml)[b]**		
			AmB	**AND**	**CAS**	**MCF**
CJ94	Hospital La Fe (Spain)	Non-aggregating	0.5	0.25	0.25	0.12
CBS 15605	Hospital La Fe (Spain) Westerdijk Fungal Biodiversity Institute	Non-aggregating	0.5	0.12	0.25	0.12
CBS 15606	Hospital La Fe (Spain) Westerdijk Fungal Biodiversity Institute	Non-aggregating	0.5	0.12	0.25	0.12
CBS 15607	Hospital La Fe (Spain) Westerdijk Fungal Biodiversity Institute	Non-aggregating	0.5	0.12	0.25	0.12
JMRC:NRZ 1101	Institut für Hygiene und Mikrobiologie (Germany) Jena Microbial Resource Collection	Aggregating	1	4	>8	4

[a]MIC_0 (Minimal inhibitory concentration of 90% of cell growth)
[b]MIC_1 (Minimal inhibitory concentration of 50% of cell growth)

Table 2. In vitro antifungal activity of echinocandins (anidulafungin (AND), caspofungin (CAS) and micafungin (MCF)) in combination with amphotericin B (AmB) against five *C. auris* blood isolates.

C. auris isolate	MIC_0 (µg/ml)					MIC_0 (µg/ml)					MIC_0 (µg/ml)				
	AmB	AND	AmB/AND	FICI	Effect	AmB	CAS	AmB/CAS	FICI	Effect	AmB	MCF	AmB/MCF	FICI	Effect
CJ94	0.5	>8	0.03/1	0.122	S	0.5	>8	0.03/1	0.122	S	0.5	>8	0.03/0.25	0.076	S
CBS 15605	0.5	>8	0.03/0.5	0.091	S	0.5	>8	0.03/0.5	0.091	S	0.5	>8	0.03/0.5	0.091	S
CBS 15606	0.5	>8	0.06/2	0.245	S	0.5	>8	0.06/1	0.128	S	0.5	>8	0.06/2	0.245	S
CBS 15607	0.5	>8	0.03/1	0.122	S	0.5	>8	0.03/1	0.122	S	0.5	>8	0.06/0.5	0.151	S
JMRC:NRZ 1101	1	>8	0.25/2	0.375	S	1	>8	0.25/4	0.5	S	1	>8	0.5/0.5	0.562	Ad
GM	0.574	>8	0.054/1.148	-	-	0.574	>8	0.054/1.148	-	-	0.574	>8	0.071/0.574	-	-
Range	0.5-1	>8	0.03-0.25/ 0.5-2	-	-	0.5-1	>8	0.03-0.25/ 0.5-4	-	-	0.5-1	>8	0.03-0.5/ 0.25-2	-	-

FICI (fractional inhibitory concentration index); Effect of the interaction: S= Synergistic interaction; Ad= Additive interaction.
GM (geometric mean)
MIC_0 (Minimal inhibitory concentration of 90% of cell growth)

3.2. Antifungal therapy efficacy during *Caenorhabditis elegans* infection with *Candida auris*

Therapeutic drugs of AmB and echinocandins were assessed in the *C. elegans* in vivo model of candidiasis. The result showed that these drugs were not toxic to nematodes (100% survival) neither in monotherapy nor in combination. The five *C. auris* blood isolates were able to kill the nematode (7.6-52.3% survival), while uninfected nematodes used as controls remained viable (100% survival) during the 120 h post-infection. Efficacy of the antifungal drugs during *C. elegans* infection with *C. auris* isolates was detailed in Figure 1 and Table S1.

Treatment with AmB in monotherapy no significantly increased the survival of infected nematodes over infected and untreated ones. However, when nematodes were treated with echinocandins (AND and CAS) both in monotherapy, and combined with AmB, protection against *C. auris* infection was observed.

Treatment of CAS and AmB combination was the most effective, reaching a *C. elegans* survival between 56.8% and 99% and a mean mortality reduction of 53.48%, compared to infected and untreated nematodes. The drug CAS used in monotherapy also has a protective effect during *C. elegans* infection, being the nematode mortality reduction between 9.4% and 56.9%. The combination CAS and AmB significantly reduced the *C. elegans* mortality compared to monotherapy ($p \leq 0.02$); except for the infection by *C. auris* CJ94 isolate, that against this infection CAS monotherapy was as effective as combined (survival of 39.7% versus 33.4%, respectively) (Figure 1a).

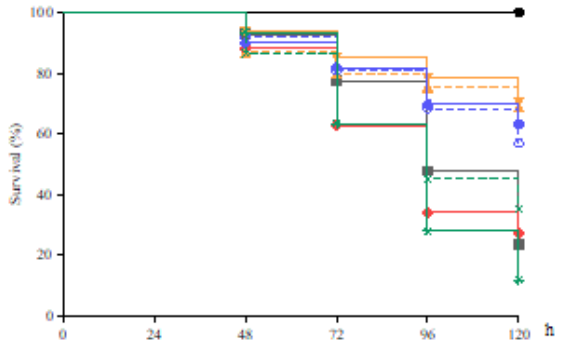
The antifungal drug of AND was also effective against *C. auris* infection compared to infected and untreated nematodes. Treatment of AND and AmB combination achieved better protection than AND in monotherapy during *C. elegans* infection with the *C. auris* CBS 15605 and CBS 15607 isolates ($p \leq 0.032$) (Table S1). Whereas, this combined treatment against the remaining three *C. auris* isolates presented similar effect than AND in monotherapy regimen. The AND and AmB combination reached a survival of *C. elegans* ranging from 39.9% to 98.8% and the mean of nematode mortality reduction was 48.78%,

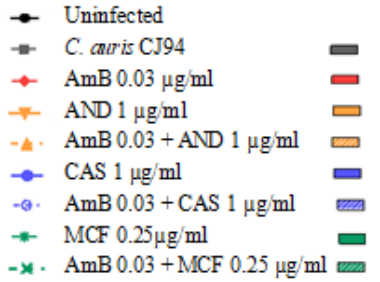
compared to infected and untreated nematodes. This combination was the best against the *C. auris* CJ94 isolate (45.6% mortality reduction), although there was no significant difference with AND in monotherapy (47.1% mortality reduction). Moreover, AND and AmB combination was as effective as CAS and AmB combination against the aggregating *C. auris* JMRC:NRZ 1101 isolate. However, the CAS and AmB combination was better compared to AND and AmB combination for *C. auris* CBS 15605 and CBS 15607 infection ($p \leq 0.022$). Furthermore, an equal or lower concentration of CAS than AND, combined with AmB, was more effective or equal against the *C. elegans* infection with non-aggregating *C. auris* isolates, respectively (Figure 1).

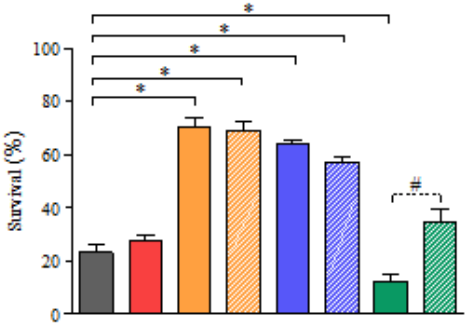
The echinocandin MCF was the least effective against *C. auris* infection in vivo. The drug MCF, both in monotherapy and combined with AmB, only significantly protected during *C. elegans* infection with the *C. auris* CBS 15606 ($p \leq 0.0001$) and CBS 15607 ($p \leq 0.0001$) isolates, compared to the infected and untreated *C. elegans* (Table S1). *C. elegans* survival increased up to 68.7% with this drug combination, however, the mean of mortality reduction was 18.02% due to the effect of these treatments was not as effective against the aggregating isolate (JMRC:NRZ 1101) and three non-aggregating isolates (CJ94, CBS 15605, CBS 15607); it was significantly less effective than the other two combinations of echinocandins - AmB ($p \leq 0.0001$) (Figure 1).

Survival curves of *C. elegans* infected with	Legend of graphics	Survival percentages of *C. elegans* at 120 hours post-infection

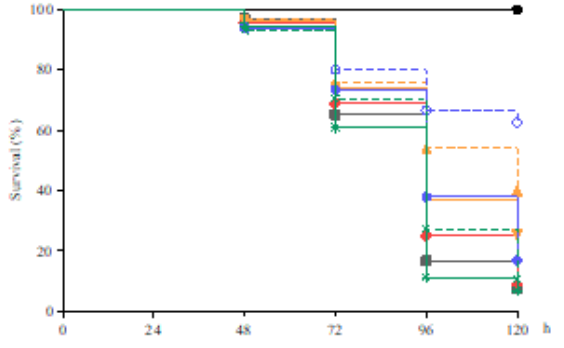
a) CJ94

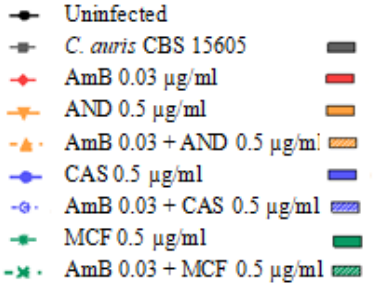


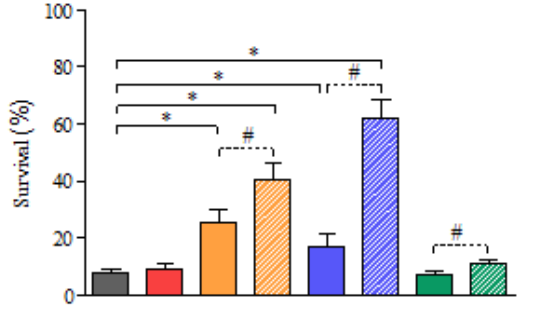




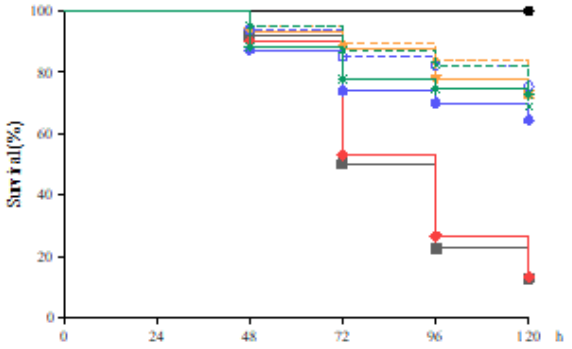
b) CBS 15605

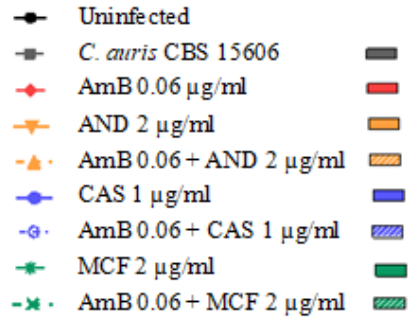


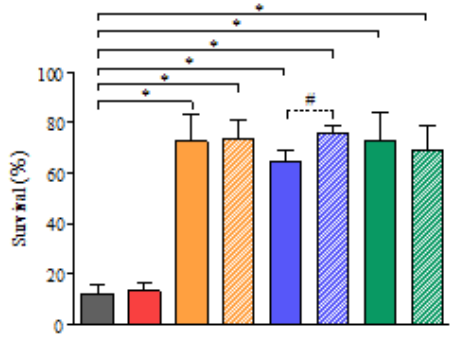




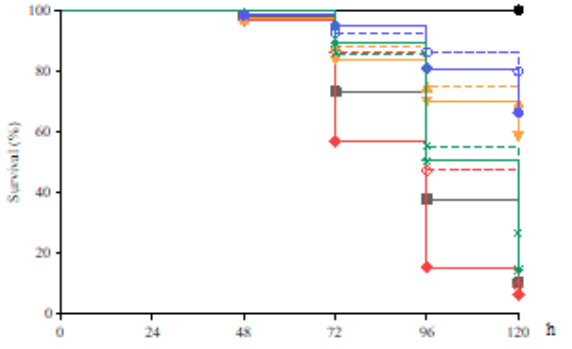
c) CBS15606

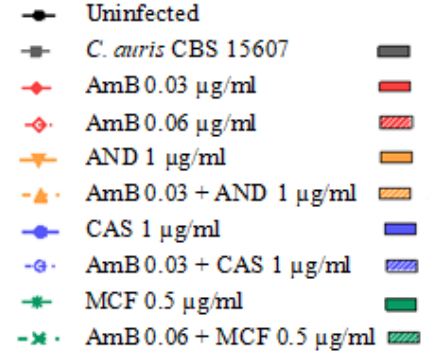


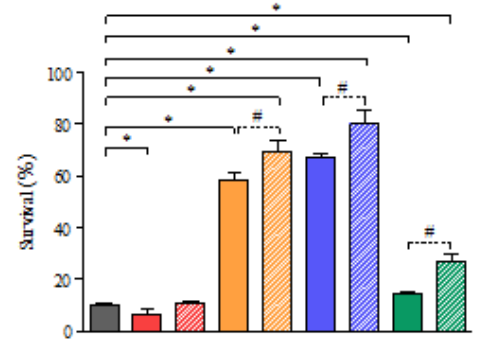




d) CBS15607







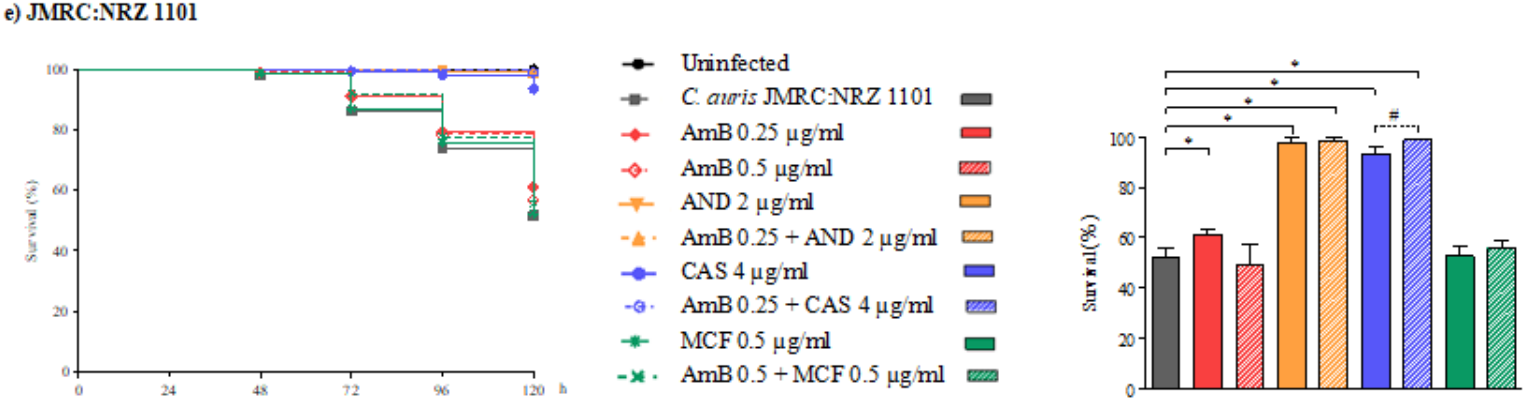


Figure 1: Survival curves and survival percentages at 120 hours post-infection of *Caenorhabditis elegans* infected with *C. auris* blood isolates CJ94 (a), CBS 15605 (b), CBS 15606 (c), CBS 15607 (d) and JMRC:NRZ 1101 (e), and, treated with the combinations AmB plus AND, AmB plus CAS, and AmB plus MCF. Statistically significant differences were indicated compared to nematodes of *C. elegans* infected and untreated (*), and to nematodes infected and treated with its corresponding echinocandins in monotherapy (#).

4. DISCUSSION

C. auris has become a dangerous emerging pathogen worldwide for its ability to generate biofilms in medical devices, remain in the hospital environment for weeks or even months, evade neutrophil attack, and develop multi-resistance to commonly used antifungal (Lockhart et al., 2017; Muñoz et al., 2018; Brown et al., 2020; Bruno et al., 2020). The limited antifungal therapeutic options against *C. auris* infection are an unresolved challenge that requires attention (Bruno et al., 2020).

C. auris is highly resistant to fluconazole, exceeding the 90% of resistant isolates in most cases, and some ranging from 99 to 100% (Lockhart et al., 2017; Chowdhary et al., 2018; Khan et al., 2018; Ahmad et al., 2020; Zhu et al., 2020). A reduced susceptibility to AmB was also reported for *C. auris*, with resistance variability higher than that to fluconazole: Chowdhary et al. (2018) observed AmB resistance in only 8% of *C. auris* isolates, while Tsay et al. (2017) did so in 43%; however, most authors described values between 8 and 43% of AmB-resistant *C. auris* isolates (Lockhart et al., 2017; Tsay et al., 2017; Chowdhary et al., 2018; Khan et al., 2018; Ninan et al., 2020; Pfaller et al., 2021). Echinocandins, the main treatment currently recommended, have a low incidence in most studies, between 2% and 3% of *C. auris* isolates (Tsay et al., 2017; Chowdhary et al., 2018); or even only 1% of the *C. auris* isolates in a New York outbreak from 2016 to 2018 (Zhu et al., 2020). In the present study only five *C. auris* blood isolates were analyzed, but in vitro results showed the efficacy of AmB and echinocandins, except against *C. auris* isolate with aggregating phenotype that showed the MIC highest value of AmB and was resistant to the three echinocandins (MIC $\geq$ 4 μg/ml) in monotherapy regimens. High MIC values for echinocandins in *C. auris* isolates were reported in an outbreak from the UK (Rhodes et al., 2018), in different clinical studies from EEUU (Biagi et al., 2019; Woodworth et al., 2019), and even in a previous study from India, in which high MIC values for CAS were detected for 37% of *C. auris* isolates, 24% of them being resistant ($\geq$2 μg/ml) (Kathuria et al., 2015). Interestingly, reversible induction of the aggregating phenotype was reported in isolates of the Southern Asian lineage exposed to

triazoles or echinocandins, which may pose an additional problem in the treatment against *C. auris* infection (Szekely et al., 2019).

Combination therapies are a promising strategy against candidiasis by multidrug-resistant *Candida* species. However, scarce evidence exists about antifungal combinations against *C. auris* infection. Few clinical cases of patients affected by *C. auris* candidemia have been treated with combinations of echinocandins - AmB (Ruiz-Gaitán et al., 2017; Chamdramati et al., 2020; Ostrowsky et al., 2020) and of echinocandins - isavuconazole (Mulet Bayona et al., 2020). In addition, fewer than ten in vitro studies have analyzed the effectiveness of antifungal compounds combinations against *C. auris* (Bidaud et al., 2019; Bidaud et al., 2020; Caballero et al., 2021; Fakhim et al., 2017; O´Brien et al., 2020; Pfaller et al., 2021; Schwarz et al., 2020; Wu et al. 2020). Among them, synergistic interaction of the MCF and voriconazole combination was observed against *C. auris* isolates, and indifferent interaction of CAS plus voriconazole (Fakhim et al., 2017). Synergistic effect was also obtained with the combination of AND plus voriconazole and AND plus isavuconazole against 14% and 31% of the *C. auris* isolates analyzed, respectively (Pfaller et al., 2021). Specifically, the combination of isavuconazole with echinocandins analyzed with different in vitro approaches has also achieved synergism and fungistatic activity against *C. auris* (Caballero et al., 2021). In addition, the interaction of echinocandins (AND and CAS) with flucytosin or with the non-antifungal drug colistine presented synergistic effect, while these two compounds with MCF showed indifferent effect against *C. auris* (Bidaud et al., 2019; Bidaud et al., 2020; O´Brien et al., 2020). Our in vitro results showed much better results in combination than in monotherapy, although we also observed different interaction with AmB plus MCF (additive effect), and with AmB plus AND and AmB plus CAS (synergistic effect) against the *C auris* aggregating isolate (JMRC:NRZ 1101) isolate. However, the AmB-echinocandins combination in vitro resulted in a synergistic interaction against all *C auris* non-aggregating isolates, and the combination AmB plus MCF showed a higher potency since the lowest MIC values of MCF were obtained. These finding highlight the differences, probably in which the phenotype influences, within the species *C. auris*; fact

that has already been noted both in antifungal drug interactions (Caballero et al., 2021), as well as in virulence (Hernando-Ortiz et al., 2021) and in the genomic diversity of this species (Brown et al., 2020).

This synergistic effect observed in vitro for echinocandins - AmB was assessed in vivo, using the *C. elegans* host model, to establish a possible correlation. The nematode *C. elegans* as host model of *Candida* infection has been useful to increase knowledge about the virulence and the effectiveness of treatments for candidiasis caused by *Candida* species (Ortega-Riveros et al., 2017; Elkabti et al., 2018; Hernando-Ortiz et al., 2020) including *C. auris* (Eldesouky et al., 2018; Eldesouky et al., 2020[a]; Eldesouky et al., 2020[b]; Eldesouky et al., 2020[c]; Hernando-Ortiz et al., 2021; Lima et al., 2020). Concretely, in vivo studies in *C. elegans* model on antifungal combination therapy in *C. auris* infections have been conducted to analyze the combination of azole drugs with other antimicrobial agent such as the sulfa drug sulfamethoxazole (Eldesouky et al., 2018), the HIV protease inhibitor lopinavir (Eldesouky et al., 2020[a]), the antiemetic agent aprepitant (Eldesouky et al., 2020[b]), and the stilbene compound ospemifene (Eldesouky et al., 2020[c]). All of them reported that these combination treatments enhanced survival of *C. elegans* compared to those treated with the respective monotherapy.

However, combinations of AmB and echinocandins have not been previously evaluated in vivo using experimental animals infected by *C. auris*, then, for the first time the efficacy of these antifungal combination therapies have been confirmed both in vitro and in vivo in a *C. elegans* model of *C. auris* infection. Our results identified the combination of CAS plus AmB as the most effective; it achieved higher nematode survival during infection by four of the five *C. auris* blood clinical isolates. Although the effect of CAS-AmB was similarly good to AND-AmB against the infection by *C. auris* aggregating isolate (JMRC:NRZ 1101) in *C. elegans*, enhancing nematode survival by nearly 100% (99% and 98.8%, respectively); moreover, these drugs in monotherapy also showed protective effect during *C. elegans* infection. Strikingly, the combination of AmB and MCF, and also these drugs in monotherapy, were the least effective even ineffective in some cases during *C. elegans* infection with *C. auris*. However, in a retrospective cohort

study of neonates who suffered *C. auris* sepsis the treatment of AmB and MCF combination was effective, as patient survival increased to 83% (Chandramati et al., 2019). The differences on antifungal drugs therapies found between in vitro and in vivo assays highlight the importance of confirming the in vitro results using in vivo systems due to its complexity, such as the non-mammalian host *C. elegans*, which has once again proven its usefulness as model of candidiasis.

To conclude, this study demonstrates the synergistic effect in vitro and in vivo of AmB and echinocandins combinations against *C. auris*. Variations on antifungal drugs effect were observed, likely associated to the capacity of *C. auris* to form cell aggregates; the aggregating phenotype showed in vitro higher MIC values both in monotherapy and in combination assays compared to their non-aggregating counterparts. Overall, the combination of AmB and CAS was the most effective to reduce mortality of *C. elegans* for *C. auris* infection, while the AmB and MCF combination was the least effective. Effectiveness of the combination AmB and echinocandins against *C. auris* in vitro and in *C. elegans* host model promotes the combination therapy as a promising tool against *C. auris* infection and warrants further studies in this regard.

5. BIBLIOGRAPHY

1. Aigner M, Erbeznik T, Gschwentner M, Lass-Flörl C. 2017. Etest and Sensititre YeastOne susceptibility testing of echinocandins against *Candida* species from a single center in Austria. Antimicrob Agents Chemother **61**(8):1-8. doi:10.1128/AAC.00512-17
2. Arendrup MC, Meletiadis J, Mouton JW, et al. EUCAST DEFINITIVE DOCUMENT E.DEF 7.3.2. Method for the determination of broth dilution minimum inhibitory concentrations of antifungal agents for yeasts. 2020. https://www.eucast.org/astoffungi/methodsinantifungalsusceptibilitytesting/susceptibility_testing_of_yeasts/

3. Barchiesi F, Spreghini E, Tomassetti S, Giannini D, Scalise G. 2007. Caspofungin in combination with amphotericin B against *Candida parapsilosis*. Antimicrob Agents Chemother **51**(3):941-945. doi:10.1128/AAC.00880-06
4. Biagi MJ, Wiederhold NP, Gibas C, et al. 2019. Development of high-level echinocandin resistance in a patient with recurrent *Candida auris* candidemia secondary to chronic candiduria. Open Forum Infect Dis **6**(7):1-5. doi:10.1093/ofid/ofz262
5. Bidaud AL, Botterel F, Chowdhary A, Dannaouia E. 2019. In vitro antifungal combination of flucytosine with amphotericin B, voriconazole, or micafungin against *Candida auris* shows no antagonism. Antimicrob. Agents Chemother. **63**:1–7. https://doi.org/10.1128/AAC.01393-19
6. Bidaud AL, Djenontin E, Botterel F, Chowdhary A, Dannaoui E. 2020. Colistin interacts synergistically with echinocandins against *Candida auris*. Int J Antimicrob Agents **55**(3):105901. doi:10.1016/j.ijantimicag.2020.105901
7. Borman AM, Szekely A, Johnson EM. 2016. Comparative pathogenicity of United Kingdom isolates of the emerging *Candida auris* and other key pathogenic *Candida* species. mSphere **1**(4):4-6. doi:10.1128/mSphere.00189-16.Editor
8. Breger J, Fuchs BB, Aperis G, Moy TI, Ausubel FM, Mylonakis E. 2007. Antifungal chemical compounds identified using a *C. elegans* pathogenicity assay. PLoS Pathog **3**(2):0168-0178. doi:10.1371/journal.ppat.0030018
9. Bruno M, Kersten S, Bain JM, et al. 2020. Transcriptional and functional insights into the host immune response against the emerging fungal pathogen *Candida auris*. Nat Microbiol **5**(12):1516-1531. doi: 10.1038/s41564-020-0780-3
10. Caballero U, Kim S, Eraso E, Quindós G, Vozmediano V, Schmidt S, Jauregizar N. 2021. In Vitro Synergistic Interactions of Isavuconazole and Echinocandins against *Candida auris*. Antibiotics. **10**(4):355. doi: 10.3390/antibiotics10040355
11. Chakrabarti A, Singh S. 2020. Multidrug-resistant *Candida auris*: an epidemiological review. Expert Rev Anti Infect Ther **18**(6):551-562. doi:10.1080/14787210.2020.1750368

12. Center for Disease Control and Prevention (CDC). *Candida auris* Identification. Atlanta, GA. 2018. https ://www.cdc.gov/fungal/candida-auris/recommendations.html
13. Chandramati J, Sadanandan L, Kumar A, Ponthenkandath S. 2020. Neonatal *Candida auris* infection: Management and prevention strategies. A single centre experience. J Paediatr Child Health. doi:10.1111/jpc.15019
14. Chowdhary A, Prakash A, Sharma C, et al. 2018. A multicentre study of antifungal susceptibility patterns among 350 *Candida auris* isolates (2009-17) in India: Role of the ERG11 and FKS1 genes in azole and echinocandin resistance. J Antimicrob Chemother **73**(4):891-899. doi:10.1093/jac/dkx480
15. Denardi LB, Keller JT, Oliveira V, Mario DAN, Santurio JM, Alves SH. 2017. Activity of combined antifungal agents against multidrug-resistant *Candida glabrata* strains. Mycopathologia **182**(9-10):819-828. doi:10.1007/s11046-017-0141-9
16. Desalermos A, Muhammed M, Glavis-Bloom J, Mylonakis E. 2011. Using *Caenorhabditis elegans* for antimicrobial drug discovery. Expert Opin Drug Discov **6**(6):645-652. https://doi.org/10.1517/17460441.2011.573781
17. Eldesouky HE, Li X, Abutaleb NS, Mohammad H, Seleem MN. 2018. Synergistic interactions of sulfamethoxazole and azole antifungal drugs against emerging multidrug-resistant *Candida auris*. Int J Antimicrob Agents. doi:10.1016/j.ijantimicag.2018.08.016
18. [a] Eldesouky HE, Salama EA, Lanman NA, Hazbun TR, Seleem MN. 2020. Potent Synergistic Interactions between Lopinavir and Azole Antifungal Drugs against Emerging Multidrug-Resistant *Candida auris*. Antimicrob Agents Chemother **65**(1):e00684-20. doi: 10.1128/AAC.00684-20
19. [b]Eldesouky HE, Lanman NA, Hazbun TR, Seleem MN. 2020. Aprepitant, an antiemetic agent, interferes with metal ion homeostasis of *Candida auris* and displays potent synergistic interactions with azole drugs. Virulence **11**(1):1466-1481. doi: 10.1080/21505594.2020.1838741

20. [c]Eldesouky HE, Salama EA, Hazbun TR, Mayhoub AS, Seleem MN. 2020. Ospemifene displays broad-spectrum synergistic interactions with itraconazole through potent interference with fungal efflux activities. Sci Rep **10**(1):6089. doi: 10.1038/s41598-020-62976-y
21. Elkabti A.B, Issi L, Rao RP. 2018. *Caenorhabditis elegans* as a model host to monitor the *Candida* infection processes. J Fungi **4**:123. https://doi.org/10.3390/jof4040123
22. Fan S, Yue H, Zheng Q, Bing J, Tian S, Chen J, Ennis CL, Nobile CJ, Huang G, Du H. 2021. Filamentous growth is a general feature of *Candida auris* clinical isolates. Med Mycol **23**:myaa116. doi: 10.1093/mmy/myaa116.
23. Fakhim H, Chowdhary A, Prakash A, et al. 2017. *In Vitro* Interactions of echinocandins with triazoles against multidrug-resistant *Candida auris*. Antimicrob Agents Chemother **61**(11):e01056-17. doi:10.1128/AAC.01056-17
24. Hernando-Ortiz A, Mateo E, Ortega-Riveros M, et al. 2020. *Caenorhabditis elegans* as a model system to assess *Candida glabrata, Candida nivariensis*, and *Candida bracarensis* virulence and antifungal efficacy. Antimicrob Agents Chemother. **64**(10):e00824-20. doi: 10.1128/AAC.00824-20
25. Hernando-Ortiz A, Mateo E, Perez-Rodriguez A, de Groot PWJ, Quindós G and Eraso E. 2021. Virulence of *Candida auris* from different clinical origins in *Caenorhabditis elegans* and *Galleria mellonella* host models. Virulence. doi.org/10.1080/21505594.2021.1908765
26. Johnson CJ, Davis JM, Huttenlocher A, Kernien JF, Nett JE. 2018. Emerging fungal pathogen *Candida auris* evades neutrophil attack. mBio **9**(4):e01403-18. doi: 10.1128/mBio.01403-18
27. Kathuria S, Singh PK, Sharma C, Prakash A, Masih A, Kumar A, Meis JF, Chowdhary A. 2015. Multidrug-Resistant *Candida auris* Misidentified as *Candida haemulonii*: Characterization by Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry and DNA Sequencing and Its Antifungal Susceptibility Profile Variability by Vitek 2, CLSI Broth

Microdilution, and Etest Method. J Clin Microbiol. **53**(6):1823-30. doi: 10.1128/JCM.00367-15.

28. Kenters N, Kiernan M, Chowdhary A, et al. 2019. Control of *Candida auris* in healthcare institutions: Outcome of an International Society for Antimicrobial Chemotherapy expert meeting. Int J Antimicrob Agents **54**(4):400-406. doi:10.1016/j.ijantimicag.2019.08.013
29. Lepak AJ, Zhao M, Berkow EL, Lockhart SR, Andes DR. 2017. Pharmacodynamic optimization for treatment of invasive *Candida auris* infection. Antimicrob Agents Chemother **61:**1–5. https://doi.org/10.1128/AAC.00791-17
30. Lima SL, Rossato L, Salles de Azevedo Melo A. 2020. Evaluation of the potential virulence of *Candida haemulonii* species complex and *Candida auris* isolates in *Caenorhabditis elegans* as an in vivo model and correlation to their biofilm production capacity. Microb Pathog **148**:104461. doi: 10.1016/j.micpath.2020.104461.
31. Lockhart SR, Etienne KA, Vallabhaneni S, et al. 2017. Simultaneous emergence of multidrug-resistant *Candida auris* on 3 continents confirmed by whole-genome sequencing and epidemiological analyses. Clin Infect Dis **64**:134-140. https://doi.org/10.1093/cid/ciw691
32. Lockhart SR. 2019. *Candida auris* and multidrug resistance: Defining the new normal. Fungal Genet Biol **131**:103243. doi: 10.1016/j.fgb.2019.103243
33. Montagna MT, Lovero G, Coretti C, et al. 2015. Susceptibility to echinocandins of *Candida* spp. strains isolated in Italy assessed by European Committee for Antimicrobial Susceptibility Testing and Clinical Laboratory Standards Institute broth microdilution methods. BMC Microbiol **15**(1):1-6. doi:10.1186/s12866-015-0442-4
34. Mukherjee PK, Sheehan DJ, Hitchcock CA, Ghannoum MA. 2005. Combination treatment of invasive fungal infections. Clin Microbiol Rev **18(**1):163-194. doi:10.1128/CMR.18.1.163

35. Mulet Bayona JV, Tormo Palop N, Salvador García C, et al. 2020. Characteristics and management of candidaemia episodes in an established *Candida auris* outbreak. Antibiotics (Basel) **9**(9):E558. doi:10.3390/antibiotics9090558
36. Muñoz J, Ramirez L, Dias L dos S, et al. 2020. Pathogenicity Levels of Colombian strains of *Candida auris* and Brazilian strains of *Candida haemulonii* species complex in both murine and *Galleria mellonella* experimental models. J Fungi **6**(3):104. doi:10.3390/jof6030104
37. Ninan MM, Sahni RD, Chacko B, Balaji V, Michael JS. 2020. *Candida auris:* Clinical profile, diagnostic challenge and susceptibility pattern: Experience from a tertiary-care centre in South India. J Glob Antimicrob Resist **21**:181-185. doi:10.1016/j.jgar.2019.10.018
38. O'Brien B, Chaturvedi S, Chaturvedi V. 2020. In vitro evaluation of antifungal drug combinations against multidrug-resistant *Candida auris* isolates from New York outbreak. *Antimicrob Agents Chemother* **64**(4):1-14. doi:10.1128/AAC.02195-19
39. Ortega-Riveros M, De-la-Pinta I, Marcos-Arias C, Ezpeleta G, Quindós G, Eraso E. 2017. Usefulness of the non-conventional *Caenorhabditis elegans* model to assess *Candida* virulence. Mycopathologia **182**(9-10):785-95. https://doi.org/10.1007/s11046-017-0142-8
40. Ostrowsky B, et al. 2020. *Candida auris* Isolates resistant to three classes of antifungal medications — New York, 2019. MMWR Morb Mortal Wkly Rep. **69**(1):6-9. doi:10.15585/mmwr.mm6901a2
41. Pfaller MA, Messer SA, Deshpande LM, Rhomberg PR, Utt EA, Castanheira M. 2021. Evaluation of synergistic activity of isavuconazole or voriconazole plus anidulafungin and the occurrence and genetic characterisation of *Candida auris* detected in a surveillance program. Antimicrob Agents Chemother AAC.02031-20. doi: 10.1128/AAC.02031-20
42. Rhodes J, Abdolrasouli A, Farrer RA, et al. 2018. Genomic epidemiology of the UK outbreak of the emerging human fungal pathogen *Candida auris*. Emerg Microbes Infect **7**(1). doi:10.1038/s41426-018-0045-x

43. Rossato L, Colombo AL. 2018. *Candida auris*: What have we learned about its mechanisms of pathogenicity?. Front Microbiol. **9**:308.1 doi:10.3389/fmicb.2018.03081

44. Romera D, Aguilera-Correa JJ, García-Coca M, Mahillo-Fernández I, Viñuela-Sandoval L, García-Rodríguez J, Esteban J. 2020. The *Galleria mellonella* infection model as a system to investigate the virulence of *Candida auris* strains. Pathog Dis **78**(9):ftaa067. doi: 10.1093/femspd/ftaa067

45. Ruiz Gaitán AC, Moret A, López Hontangas JL, Molina JM, Aleixandre López AI, Cabezas AH, Mollar Maseres J, Arcas RC, Gómez Ruiz, MD, Chiveli MÁ, Cantón E, Pemán J. 2017. Nosocomial fungemia by *Candida auris*: First four reported cases in continental Europe. Rev. Iberoam Micol **34:**23–27. https://doi.org/10.1016/j.riam.2016.11.002

46. Sakagami T, Kawano T, Yamashita K, et al. 2019. Antifungal susceptibility trend and analysis of resistance mechanism for *Candida* species isolated from bloodstream at a Japanese university hospital. J Infect Chemother **25**(1):34-40. doi:10.1016/j.jiac.2018.10.007

47. Schwarz P, Bidaud AL, Dannaoui E. 2020. In vitro synergy of isavuconazole in combination with colistin against *Candida auris*. *Sci Rep* **10,** 21448. https://doi.org/10.1038/s41598-020-78588-5

48. Scorzoni L, de Lucas MP, Mesa-Arango AC, Fusco-Almeida AM, Lozano E, Cuenca-Estrella M, Mendes-Giannini MJ, Zaragoza O. 2013. Antifungal efficacy during *Candida krusei* infection in non-conventional models correlates with the yeast in vitro susceptibility profile. PLoS One **8**(3): e60047. https://doi.org/10.1371/journal.pone.0060047

49. Segal E, Frenkel M. 2018. Experimental in vivo models of candidiasis. J Fungi **4**:21. https://doi.org/10.3390/jof4010021

50. Sharma C, Wankhede S, Muralidhar S, et al. 2013. *Candida nivariensis* as an etiologic agent of vulvovaginal candidiasis in a tertiary care hospital of New Delhi, India. *Diagn* Microbiol Infect Dis **76**(1):46-50. doi:10.1016/j.diagmicrobio.2013.02.023

51. Sherry L, Ramage G, Kean R, et al. 2017. Biofilm-forming capability of highly virulent, multidrug-resistant *Candida auris*. Emerg Infect Dis **23**(2):328-331. doi:10.3201/eid2302.161320
52. Souza ACR, Fuchs BB, Alves V, Jayamani E, Colombo AL, Mylonakis E. 2018. Pathogenesis of the *Candida parapsilosis* complex in the model host *Caenorhabditis elegans*. Genes **9**(8):401. https://doi.org/10.3390/genes9080401
53. Szekely A, Borman AM, Johnson EM. 2019. *Candida auris* isolates of the Southern Asian and South African lineages exhibit different phenotypic and antifungal susceptibility profiles *in vitro*. J Clin Microbiol **57**:e02055-18. https://doi.org/10.1128/JCM.02055-18
54. Te Dorsthorst DTA, Verweij PE, Meletiadis J, Bergervoet M, Punt NC, Meis JFGM, Mouton JW. 2002. In vitro interaction of flucytosine combined with amphotericin B or fluconazole against thirty-five yeast isolates determined by both the fractional inhibitory concentration index and the response surface approach. Antimicrob Agents Chemother **46**(9):2982-2989. doi:10.1128/AAC.46.9.2982-2989.2002
55. Tóth Z, Forgács L, Locke JB, et al. 2019. In vitro activity of rezafungin against common and rare *Candida* species and *Saccharomyces cerevisiae*. J Antimicrob Chemother **74**(12):3505-3510. doi:10.1093/jac/dkz390
56. Valentín A, Cantón E, Pemán J, Fernandez-Rivero ME, Tormo-Mas MA, Martínez JP. 2016. In vitro activity of anidulafungin in combination with amphotericin B or voriconazole against biofilms of five *Candida* species. J Antimicrob Chemother **71**(12):3449-3452. doi:10.1093/jac/dkw316
57. Vatanshenassan M, Boekhout T, Meis JF, et al. 2019. *Candida auris* identification and rapid antifungal susceptibility testing against echinocandins by MALDI-TOF MS. Front Cell Infect Microbiol **9**(February):1-9. doi:10.3389/fcimb.2019.00020
58. Welsh RM, Bentz ML, Shams A, Houston H, Lyons A, Rose LJ, Litvintseva P. 2017. Survival, persistence, and isolation of the emerging multidrug-resistant

pathogenic yeast *Candida auris* on a plastic health care surface. J Clin Microbiol **55**:2996 –3005. https://doi.org/10.1128/JCM.00921-17

59. Woodworth MH, Dynerman D, Crawford ED, et al. 2019. Sentinel case of *Candida auris* in the Western United States following prolonged occult colonization in a returned traveler from India. Microb Drug Resist **25**(5):677-680. doi:10.1089/mdr.2018.0408
60. Wu Y, Totten M, Memon W, Ying C, Zhang SX. 2020. In vitro antifungal susceptibility of the emerging multidrug- resistant pathogen *Candida auris* to miltefosine alone and in combination with amphotericin B. Antimicrob Agents Chemother **64**(2):2019-2021. doi:10.1128/AAC.02063-19
61. Wurster S, Bandi A, Beyda ND, et al. 2019. *Drosophila melanogaster* as a model to study virulence and azole treatment of the emerging pathogen *Candida auris*. J Antimicrob Chemother **74**: 1904–1910. doi:10.1093/jac/dkz100
62. Xin H, Mohiuddin F, Tran J, Adams A, Eberle K. 2019. Experimental mouse models of disseminated *Candida auris* infection. mSphere **4**(5):1-9. doi:10.1128/msphere.00339-19
63. Yamada SM, Tomita Y, Yamaguchi T, Matsuki T. 2016. Micafungin versus caspofungin in the treatment of *Candida glabrata* infection: a case report. J Med Case Rep **10**(1):1-5. doi:10.1186/s13256-016-1096-z
64. Zhu YC, O'Brien B, Leach L, et al. 2020. Laboratory analysis of an outbreak of *Candida auris* in New York from 2016 to 2018: Impact and lessons learned. J Clin Microbiol **58**(4):1-16. doi:10.1128/JCM.01503-19