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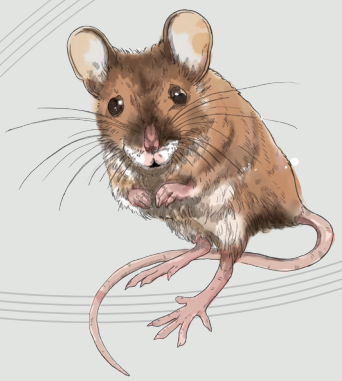
Contributing to the picture of northern Iberian Peninsula



Lucía Varela Castro

PhD Thesis - 2022

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Universidad del País Vasco

Euskal Herriko Unibertsitatea

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PhD Thesis

Submitted to Universidad del País Vasco/Euskal Herriko Unibertsitatea (UPV/EHU) in fulfilment of requirements for the degree of Doctor of Philosophy

Resulting from several researches conducted at the Animal Health Department, NEIKER-Basque Institute for Agricultural Research and Development, Basque Research and Technology Alliance (BRTA)

Author:

Lucía Varela Castro

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Supervisors:

Dr. Marta Barral Lahidalga

Dr. Iker Agirregomoskorta Sevilla

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Avui, Dolors, proposa un tema al cantant,
un que es rigui de tu i de mi i d'aquesta història que s'ha anat acabant.

Manel: *Ai, Dolors*. Els millors professors europeus (2008)

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LIST OF ABBREVIATIONS

AIC	Akaike Information Criteria
BCG	Bacillus Calmette–Guérin
CFU	Colony-forming units
CI	Confidence interval
CIT	Comparative intradermal tuberculin
CT	Cycle threshold
CTR	Camera trap
DIVA	Differentiating Infected from Vaccinated Animals
DNA	Deoxyribonucleic acid
EI	ELISA index
ELISA	Enzyme-linked immunosorbent assay
GLM	Generalized Linear Model
GLMM	Generalized Linear Mixed Model
HEYM	Herrold's Egg Yolk medium
***hsp*65**	Heat shock protein 65
IFN-γ	Interferon-gamma
ITS	Internal transcribed spacer
LN	Lymph node
Maa	*Mycobacterium avium* subspecies *avium*
MAC	*Mycobacterium avium* complex
Mah	*Mycobacterium avium* subspecies *hominissuis*
Map	*Mycobacterium avium* subspecies *paratuberculosis*
Mas	*Mycobacterium avium* subspecies *silvaticum*
MTC	*Mycobacterium tuberculosis* complex
MGIT	Mycobacteria growth indicator tube
mycoDB	Spanish Database of Animal Mycobacteriosis
NTM	Non-tuberculous mycobacteria
OADC	Oleic acid, albumin, dextrose and catalase
OC	Other carnivores
OD	Optical density
OR	Odds ratio
OTF	Officially Tuberculosis-Free

PCR	Polymerase Chain Reaction
PPD-A	*M. avium*-derived purified protein derivative
PPD-B	*M. bovis*-derived purified protein derivative
RD	Regions of difference
RNA	Ribonucleic acid
ROI	Republic of Ireland
rpoB	RNA polymerase β-subunit
SE	Standard error
SIT	Single intradermal tuberculin
TB	Tuberculosis
TST	Tuberculin skin test
TTD	Time to detection
UK	United Kingdom
USA	United States of America
UTM	Universal Transverse Mercator
WGS	Whole-genome sequencing
WHO	World Organization for Animal Health
16S rRNA	16S ribosomal ribonucleic acid

SUMMARY

The genus *Mycobacterium* encompasses more than 200 species of mycobacteria that are maintained and shared between the environment, domestic and wild animals, and humans. These microorganisms can cause medically and socio-economically significant diseases, and some of them are considered a One Health challenge because of their impact on public and animal health. Animal tuberculosis (TB) is a worldwide zoonotic infectious disease caused by *Mycobacterium bovis* and other members of the *M. tuberculosis* complex (MTC). This disease is a recognized public health problem, but also a source of significant economic losses in the livestock industry and a threat to the welfare of livestock, companion animals, farmed game animals and wild animals. Cattle (*Bos taurus*) are the main and most studied host of *M. bovis* and essentially the main target of bovine TB eradication programmes, which are mostly focussed on test and slaughter strategies. Nevertheless, the current control efforts are hampered by the limitations of diagnostic methods, which are partly attributable to the intricate development of the disease and to cross-reactions with other mycobacteria, as well as by the existence of other livestock and wild hosts contributing to the maintenance and transmission of MTC. TB host composition from the Iberian Peninsula might represent one of the most complexes worldwide. In addition to a wide spectrum of domestic hosts including cattle, goats (*Capra hircus*), sheep (*Ovis aries*) and pigs (*Sus scrofa domestica*), wild boar (*Sus scrofa*), red deer (*Cervus elaphus*) and fallow deer (*Dama dama*) are wild maintenance hosts of animal TB in south-central areas, while in the north the Eurasian badger (*Meles meles*) seems to be a potential TB reservoir in some Atlantic regions. Non-tuberculous mycobacteria (NTM) are colonizers of the environment that grow in a plethora of natural and human-made niches. In veterinary medicine, NTM imply a double concern: the potential to interfere with bovine TB diagnosis and to cause opportunistic or major infections that may lead to economic

losses and deprivation of animal welfare. Some NTM stand out among the rest in terms of veterinary significance, such as the members of the *M. avium* complex (MAC), *M. kansasii*, or *M. fortuitum*. Within the MAC, *M. avium* subspecies are the most clinically significant, particularly *M. avium* subsp. *paratuberculosis* (*Map*) and *M. avium* subsp. *avium* (*Maa*), the causative agents of paratuberculosis and avian TB. Despite the growing importance of NTM infections, limited information on their occurrence in animals is reported. Excluding *M. avium* subspecies, especially *Map*, published records are scarce and mainly emerge from secondary findings of MTC research.

As the prevalence of TB declines in livestock the role of wild hosts in its maintenance and transmission may become more relevant, and infections due to NTM become more readily recognized due to the increasing pressure to find remnant MTC infection.

The main objective of this doctoral thesis was to study the role of wildlife in the epidemiology of TB and other mycobacterial infections in the Basque Country, a low bovine TB prevalence region located in northern Iberian Peninsula, and to describe the potential pathways for mycobacteria transmission between cohabiting wild species and cattle in this area. This objective was addressed throughout five studies included in the present work. The first four studies focused on searching for mycobacterial infection and/or exposure in several wild species, and on comparing the situation with that of sympatric livestock. The last study was carried out to assess interactions between cattle and wildlife that could lead to interspecies transmission of mycobacteria.

While wild boar is considered one of the most important wild reservoirs of TB within Mediterranean habitats of south-central Iberian Peninsula, its role in northern regions is currently under debate. The first study was performed to evaluate the

exposure of wild boar populations of the Basque Country to MTC. For that purpose, a serological assay was conducted and risk factors associated with MTC seropositivity in this species were modelled. The seroprevalence found (17%) was higher than expected when compared with other areas with similar prevalence in cattle, and the models showed that the presence of livestock, the type of habitat and intrinsic characteristics of wild boar such as the age can influence on the exposure of this wild ungulate to MTC, even when bovine TB is almost eradicated. These findings might lead to reconsider the relevance of this species when developing control strategies in areas where bovine TB is close to eradication.

When comparing with the abundance of reports related to high prevalence or hot-spot areas, studies on wild mammals in low bovine TB prevalence scenarios are lacking. In the second study, a ten-year microbiological survey on the presence of MTC in wildlife from the Basque Country was performed. The spatial and temporal distribution of the spoligotypes obtained from these wild species was studied, also in relation to those identified during the Spanish National Bovine Tuberculosis Eradication Programme in cohabiting livestock. MTC were isolated from 1.12% of wild boar and 2.40% of red deer, but not from any other wild host. The strain diversity according to spoligotyping was remarkable. Five distinct spoligotypes belonging to *M. bovis* (SB0121, SB0134, SB0881, SB2354, SB1086) and one to *M. caprae* (SB0415) were detected in wildlife. Potential geographical and temporal links between the spoligotypes found in wild boar and cattle that are consistent with strain sharing were detected. This study has provided a wider picture of the understudied low TB prevalence areas of northern Iberian Peninsula.

Many studies related to mycobacterioses at the wildlife-livestock interface focus their attention on large and medium size mammals, but little is known about small

mammals. In the third study, we present an investigation on the detection of mycobacteria in small mammals trapped in cattle farms from the Basque Country with history of TB or NTM. Collected tissues from trapped animals were submitted to microbiological and molecular analyses for mycobacteria isolation and identification. Even though MTC members were not isolated from these animals, NTM that could be pathogenic or interfere with the diagnosis of TB such as *Map*, *M. celatum*, *M. gordonae*, *M. intracellulare* and *M. fortuitum* were detected in small rodents (mainly in *Apodemus sylvaticus*), entailing a prevalence of 6.5%. According to these findings, small mammals can carry mycobacteria in farm environments, a fact that should promote further research to deepen into the relevance of these animals in the epidemiology of mycobacterioses, in order to design more effective global control strategies.

Despite their ability to interfere with TB diagnosis and their potential to cause infections in their hosts, published records on NTM infections in animals are still scarce. The fourth study aimed at describing the diversity of NTM circulating among wild and domestic species from Spain (especially from the Basque Country), and to analyse their implications as potential pathogenic microorganisms or as sources of interferences in the diagnosis of bovine TB. For this purpose, 293 NTM isolates recovered from wild and domestic animals were analysed through a multigene approach for mycobacteria identification. Thirty-one species of mycobacteria were identified, being *Maa*, *M. avium* subsp. *hominissuis* (*Mah*), *M. bouchedurhonense*, *M. lentiflavum* and *M. nonchromogenicum* the most frequent ones. *Maa* and *M. lentiflavum* were isolated in several animals showing TB-like lesions, while *Maa*, *Mah* and *M. nonchromogenicum* were recovered from many cattle that had reacted to the tuberculin skin test (TST). Other NTM were also associated to these phenomena, but to a lesser extent. *Maa*, *Mah*, *M. nonchromogenicum* and *M. lentiflavum* were geographically

associated between wild boar and other hosts. The findings of the present study suggest that a high diversity of NTM circulates among wildlife and livestock. Wild boar and *M. avium* seem to play a relevant role in this epidemiological scenario.

Interactions between sympatric wildlife and livestock may contribute, among other phenomena, to interspecies transmission of MTC or NTM, leading to the spread of relevant mycobacterioses or to interferences with the diagnosis of TB. The fifth study aims at characterizing the interactions between several wild hosts and cattle through camera-trapping, providing a valuable insight into the dynamics of mycobacteria transmission opportunities within the Basque Country. Cross-species mycobacteria transmission, if occurring, would be mainly held through indirect interactions and most likely in pastures. Badger latrines might act as a source of exposure to mycobacteria for badger, wild boar, fox (*Vulpes vulpes*) and cattle, even though further studies will be needed to confirm this assumption. Wildlife visits were abundant but brief, and in contrast with the findings of previous studies, food and water sources did not act as aggregation points. The fox was the species that visited the farms more frequently and the one that showed more direct contacts with cattle, but the species that showed up in more numerous groups was the wild boar. Small rodents were the most frequent visitors inside farm buildings and the group that showed longest visits on average. The knowledge derived from this research could be useful to design effective control strategies when needed.

The results of this thesis work suggest that, overall, the risk of MTC transmission between wild animals and cattle in the Basque Country is currently low. Conversely, a risk of indirect NTM transmission could be more feasible. Four wild species might be most involved in the epidemiology of mycobacterial infections at the

wildlife-livestock interface of the Basque Country: wild boar, badger, fox and wood mouse, being wild boar probably the most relevant among them.

The outcomes of the five studies included in the present work greatly contribute to the general body of knowledge on animal MTC and NTM infection research from low bovine TB prevalence areas of northern Iberian Peninsula, and especially on the role of wildlife on the epidemiology of these mycobacterioses.

LABURPENA

Mycobacterium generoak 200 mikobakterio-espezie baino gehiago biltzen ditu, eta ingurumenean, etxe-abereetan, basa-animalietan eta gizakietan aurki daitezke. Mikroorganismo horietako batzuek medikuntzan eta albaitaritzan garrantzikoak eta sozioekonomikoki esanguratsuak diren gaixotasunak eragin ditzakete, eta horrek eragin handia du osasun publikoan zein animalien osasunean. Animalien tuberkulosia (TB) mundu osoan zehar hedatuta dagoen zoonosi infekziosoa da, *Mycobacterium bovis*-ek eta *M. tuberculosis* konplexuko (MTK) beste kide batzuek sortua. Gaixotasun hau osasun publikoaren arazo handietako bat izateaz gain, abeltzaintza-industrian galera ekonomikoen iturri garrantzitsua ere bada. Bestalde, etxe-abere eta basa-animalien osasunerako eta ongizaterako mehatxu handia da. Behia (*Bos taurus*) da *M. bovis*-en ostalari nagusi eta ikertuena, baita TB desagerrarazteko programen foku nagusia ere. Programa hauek, nagusiki, infektatutako animaliak detektatzeko eta ezabatzeko estrategiak dira. Hala ere, diagnostiko-metodoen mugek estrategia horiek oztopatzen dituzte, neurri batean gaixotasunaren garapen konplexuak eta beste mikobakterio batzuekin ematen diren erreakzio gurutzatuek eraginda, bai eta MTK mantentzen eta transmititzen laguntzen duten beste etxe- eta basa-ostalari batzuen presentziak ere. Iberiar Penintsulan, TBren ostalari espektroa munduko konplexuenetako bat da. Behi-, ahuntz- (*Capra hircus*), ardi- (*Ovis aries*) eta txerri-aziendaz (*Sus scrofa domestica*) gain, basurdeak (*Sus scrofa*), oreinak (*Cervus elaphus*) eta adarzabalak (*Dama dama*) basa-ostalari gisa jarduten dute, eta gaixotasuna mantentzen laguntzen dute Europako erdialde-hegoaldeko eremuetan. Iparraldean, berriz, azkonarra (*Meles meles*) basa-gordailu gisa aritu daiteke eskualde atlantiko batzuetan. Mikobakterio ez-tuberkulosoak (MET) ingurumenaren kolonizatzaileak dira, eta nitxo natural nahiz giza jatorriko nitxoetan hazteko gai dira. Albaitaritzan, METek arazo bikoitza dakarte: batetik, tuberkulosiaren diagnostikoan interferentziak sortzeko duten ahalmena dela eta, eta,

bestetik, galera ekonomikoak eragin ditzaketen infekzio oportunistak edo larriak sortarazi eta animalien osasunean eta ongizatean eragina izan dezaketelako. Mikobakterio ez-tuberkuloso espezie batzuk besteen gainetik nabarmentzen dira, albaitaritzan duten garrantziagatik, hala nola *M. avium* konplexuko kideak (MAK), *M. kansasii* edo *M. fortuitum*. MAKaren barruan, *M. avium*-en subespezieak dira klinikoki garrantzitsuenak, batez ere *M. avium* subsp. *paratuberculosis* (*Map*) eta *M. avium* subsp. *avium* (*Maa*), eta hauek dira, hurrenez hurren, paratuberkulosia eta hegaztien tuberkulosia sortzen duten eragileak. METek gero eta garrantzi handiagoa duten arren, oraindik oso informazio mugatua dugu animalietan duten presentziari buruz. *Mycobacterium avium*-en subespezieak izan ezik, *Map* batez ere, mikroorganismo horiekin lotutako argitalpenak urriak dira eta, oro har, MTKan ardaztutako ikerketen bigarren mailako aurkikuntzen ondorio dira.

Tuberkulosiaren prebalentziak abereetan behera egiten duenean, basa-ostalariek gaixotasun honen mantentzean eta transmisioan duten papera handitu daiteke, eta METek eragindako infekzioak errazago detektatzen dira, zaintza-presioa handitzen delako geratutako tuberkulosi-infekzioak aurkitzeko.

Doktorego-tesi honen helburu nagusia basa-animaliek Euskal Autonomia Erkidegoko tuberkulosiaren eta beste infekzio mikobakteriano batzuen epidemiologian duten eginkizuna aztertzea izan zen, Iberiar Penintsulako iparraldean dagoen eta behi-tuberkulosiaren prebalentzia txikia duen eskualdea baita. Horrez gain, eskualde horretako basa-espezieen eta behi-azienden artean mikobakterioak transmititzeko aukera posibleak deskribatzea ere izan zuen helburu. Helburu horiek tesi honetan bildutako bost ikerlanen bidez landu ziren. Lehenengo lau ikerketetan basa-espezietan mikobakterioek eragindako infekzioarekin eta/edo horien esposizioarekin lotutako aurkikuntza epidemiologikoak aztertu ziren, baita aurkikuntza horien eta ganaduan

hautemandakoen arteko lotura ikertu ere. Azken ikerlana behi-aziendaren eta basa-faunaren arteko interakzioak aztertzeko egin zen, mikobakterioen espezie arteko transmisioa erraztu dezaketelako.

Iberiar Penintsulako erdialde-hegoaldeko habitat mediterraneoetan, basurdea TBren basa-gordailu garrantzitsua da. Hala ere, penintsulako iparraldean duen garrantzia ez dago guztiz argi. Tesi honen lehen ikerlana Euskal Autonomia Erkidegoko basurde-populazioen MTKarekiko esposizioa ebaluatzean oinarritu zen. Horretarako, ELISA teknikaren bidez espezie horretatik jasotako serumak aztertu ziren, eta basurdearen seropositibotasunarekin zerikusia izan zezaketen arrisku-faktoreak modelizatu ziren. Hautemandako seroprebalentzia globala (% 17) uste baino handiagoa izan zen, behi-aziendan antzeko prebalentzia duten penintsulako iparraldeko beste eskualde batzuekin alderatzean. Eredu estatistikoek erakutsi zutenez, behi-aziendak, habitat-motak eta adinak eragina izan dezakete basurdeen MTKarekiko esposizioan, baita gaixotasuna aziendan ia erabat desagerrarazita dagoenean ere. Aurkikuntza horiei esker, kontrol-estrategiak garatzeko orduan, basurdearen garrantzia birplantearazi beharko litzake behi tuberkulosia desagerrarazteko zorian dagoen eremuetan.

Behi-tuberkulosiaren prebalentzia handiko eremuekin alderatuta, prebalentzia txikiko eremuetan ugaztun basatiekin egindako ikerketak urriak dira. Bigarren ikerlanean, 10 urtean bildutako Euskal Autonomia Erkidegoko basa-faunaren laginetan MTKaren detekzioa burutu zen, eta faunan aurkitutako espoligotipoen denbora- eta espazio-banaketa aztertu zen, Behien Tuberkulosia Desagerrarazteko Programa Nazionalean eskualde honetako abereetan detektatutakoekin alderatuz. Basurdeen % 1.12an eta oreinen % 2.40an MTK bakterioak isolatu ziren. Ez zen beste basa-espezietan aurkitu. Aurkitutako espoligotipoen aniztasuna handia izan zen. *M. bovis*-en bost espoligotipo (SB0121, SB0134, SB0881, SB2354, SB1086) eta *M. caprae*-ren

espoligotipo bat (SB0415) identifikatu ziren infektatutako basa-ungulatuetan. Denbora-erlazioak eta erlazio geografikoak hauteman ziren basurdeetan eta behi-aziendetan aurkitutako espoligotipoen artean, espoligotipo horien espezie arteko transmisioa adieraziz. Ikerlan honek, Iberiar Penintsulako iparraldeko behi-tuberkulosiaren prebalentzia txikiko eremuen egoera epidemiologikoa ulertzen lagundu du.

Basoko eta etxeko interfazean mikobakteriosiaren azterketarekin lotutako ikerketa gehienek tamaina ertaineko edo handiko ugaztunetan jarri dute arreta, baina mikrougaztunei buruz gutxi dakigu. Tesi honen hirugarren ikerlana TB edo MET historiala duten Euskal Herriko behi-ustiategietan harrapatutako mikrougaztunetan mikobakterioak detektatzea ardatz zuen lana izan zen. Harrapatutako animalietatik jasotako nodulu linfatikoak eta ehunak teknika mikrobiologiko eta molekularren bidez prozesatu eta aztertu ziren, mikobakterioak isolatu eta identifikatzeko. Animalia horietan ez zen MTKako kide den mikobakteriorik detektatu, baina detektatu ziren METak (gehienbat, basasaguan, *Apodemus sylvaticus*-en) patogenoak izan zitezkeenak edo TBren diagnostikoan eragin zezaketenak izan ziren: *Map*, *M. celatum*, *M. gordonae*, *M. intracellulare* eta *M. fortuitum*. Azterlan honetan hautemandako METen prebalentzia globala % 6.5ekoa izan zen. Gure emaitzen arabera, mikrougaztunek mikobakterioak garraia ditzakete abeltzaintza-ustiategietan, eta horrek etorkizunean ikerketak egitearen garrantzia azpimarratzen du, espezie horiek mikobakterioen epidemiologian duten garrantzian sakontzeko eta, horrela, kontrol-estrategia eraginkorragoak diseinatzeko.

Mikobakterio ez-tuberkulosoek TBren diagnostikoa oztopatzeko eta ostalarietan infekzioak eragiteko gaitasuna duten arren, animalietan burututako lanak urriak dira oraindik. Laugarren ikerlanaren helburua Espainiako basa- eta etxe-espezieen artean (batez ere Euskal Autonomia Erkidegoan) dabiltzan METen aniztasuna deskribatzea, eta organismo patogeno gisa edo behien tuberkulosiaren diagnostikoan interferentzia-iturri

gisa izan ditzaketen inplikazioak aztertzea izan zen. Horretarako, basa-animalietatik eta etxe-aberetatik lortutako METen 293 isolamendu, gen desberdinen anplifikazio edo sekuentziazio bidez identifikatu ziren. Guztira, 31 mikobakterio-espezie identifikatu ziren, eta ugarienak *Maa*, *M. avium* subsp. *hominissuis* (*Mah*), *M. bouchedurhonense*, *M. lentiflavum* eta *M. nonchromogenicum* izan ziren. *Maa* eta *M. lentiflavum* sarritan isolatu ziren tuberkulosiarekin bateragarriak ziren lesioak zituzten animalietan; *Maa*, *Mah* eta *M. nonchromogenicum*, berriz, intradermotuberkulinizazioaren proban erreakzionatzaile ziren behi gehienetan isolatu ziren. Beste MET espezie batzuk ere aurkikuntza horiekin lotu ziren, baina neurri txikiagoan. Gainera, aldi berean basurdean eta beste ostalari batzuetan isolatutako *Maa*, *Mah*, *M. nonchromogenicum* eta *M. lentiflavum*-en arteko harreman geografikoa hauteman zen. Ikerketa honen aurkikuntzek basa-faunaren eta abereen artean MET aniztasun handia dagoela erakutsi zuten. Eszenatoki epidemiologiko honetan, basurdeak eta *M. avium*-en subespezieek funtsezko eginkizuna dutela dirudi.

Basa-faunaren eta abereen artean gertatzen diren interakzioek MTKaren edo METen espezie arteko transmisioan lagun dezakete, mikobakterio garrantzitsuak zabalaraziz edo tuberkulosiaren diagnostikoan interferentziak sortuz. Bostgarren ikerlanaren helburua argazki-tranpa bidez basa-faunaren eta behi-aziendaren artean gertatzen diren interakzio-ereduak ezaugarritzea da, Euskal Autonomia Erkidegoan mikobakterioen transmisio-dinamika posibleei buruzko ezagutza sortuz. Gure emaitzen arabera, mikobakterioen espezie arteko transmisioa zeharkako interakzioengatik emango litzateke gehien bat, larreetan batez ere. Bestalde, azkonar-komunak mikobakterioekiko esposizio-puntu gisa jardun dezakete azkonarrentzako, basurdeentzako, azeriarentzako (*Vulpes vulpes*) eta behientzako, baina azterketa gehiago egitea beharrezkoa izango litzake hipotesi hori berresteko. Fauna basatiak ustiategietara

egindako bisitak ugariak baina laburrak izan ziren, eta beste ikerketa batzuetan aurkitutakoak ez bezala, ur- eta janari-baliabideek ez zuten animalien agregazioa eragin. Azeria izan zen behi-aziendarekin interakzio gehien eduki zuen espeziea, zuzenean zein zeharka, baina talde handiagoetan ustiategietara joaten zen espeziea basurdea izan zen. Saguek egin zituzten bisitarik luzeenak, eta, ustiategien barruan aurkitutako espezieen artean, haiek izan ziren bisitari ohikoenak. Ikerlan honetatik eratorritako ezagutza baliagarria izan liteke kontrol-estrategiak diseinatzeko, beharrezkoak izanez gero.

Doktorego-tesi honetan lortutako emaitzek iradokitzen dutenez, Euskadiko basa-faunaren eta behi-aziendaren artean MTK transmititzeko arriskua txikia da, MET transmititzeko arriskua handiagoa izan daiteken bitartean. Basurdea, azkonarra, azeria eta basasagua dira, antza, gure ikerketa-eremuan mikobakteriosien epidemiologian parte-hartze handiena duten basa-espezieak, eta, ziurrenik, basurdea da garrantzitsuena. Lan honek penintsula iparraldeko behi-tuberkulosiaren prebalentzia baxuko eremuetako animalietan infekzio tuberkuloso eta ez-tuberkulosoei buruzko ikerketan ekarpen lagungarria egin du, batez ere basa-faunak mikobakteriosi hauen epidemiologian duen eginkizunari dagokionez.

RESUMEN

El género *Mycobacterium* engloba más de 200 especies de micobacterias que se pueden encontrar tanto en el medio ambiente como en los animales domésticos, silvestres y en el ser humano. Algunos de estos microorganismos pueden causar enfermedades de relevancia médica-veterinaria y socioeconómica, provocando un fuerte impacto tanto en la salud pública como en la sanidad animal. La tuberculosis animal (TB) es una zoonosis infecciosa de distribución mundial causada por *Mycobacterium bovis* y otros miembros del complejo *M. tuberculosis* (CMT). Esta enfermedad es considerada uno de los grandes problemas para la salud pública, pero también constituye una fuente importante de pérdidas económicas en la industria ganadera y una gran amenaza para la salud y el bienestar de animales domésticos y silvestres. El ganado bovino (*Bos taurus*) es el hospedador principal y más estudiado de *M. bovis*, así como el principal objetivo sobre el que se centran los programas de erradicación de la TB, que mayoritariamente consisten en estrategias de detección y eliminación de animales infectados. Sin embargo, estas estrategias se ven obstaculizadas por las limitaciones de los métodos de diagnóstico, en parte debidas al complejo desarrollo de la enfermedad y a reacciones cruzadas con otras micobacterias, así como por la existencia de otros hospedadores domésticos y silvestres que contribuyen al mantenimiento y transmisión del CMT. En la Península Ibérica, el espectro de hospedadores de la TB representa uno de los más complejos a nivel mundial. Además del ganado bovino, caprino (*Capra hircus*), ovino (*Ovis aries*) y porcino (*Sus scrofa domestica*), el jabalí (*Sus scrofa*), el ciervo (*Cervus elaphus*) y el gamo (*Dama dama*) actúan como hospedadores silvestres que contribuyen al mantenimiento de la enfermedad en zonas del centro-sur, mientras que en el norte el tejón europeo (*Meles meles*) podría estar actuando como reservorio silvestre en algunas regiones Atlánticas. Las micobacterias no tuberculosas (MNT) son colonizadoras del medio ambiente capaces de crecer en una amplia variedad de nichos

tanto naturales como de origen humano. En veterinaria, las MNT implican una problemática doble: por un lado, su potencial para generar interferencias en el diagnóstico de la TB, y por otro lado su capacidad para causar infecciones oportunistas o graves que pueden derivar en pérdidas económicas y afectar a la salud y al bienestar de los animales. Algunas especies de MNT destacan sobre las demás debido a su relevancia veterinaria, como los miembros del complejo *M. avium* (CMA), *M. kansasii*, o *M. fortuitum*. Dentro del CMA, las subespecies de *M. avium* son las más importantes clínicamente, sobre todo *M. avium* subsp. *paratuberculosis* (*Map*) y *M. avium* subsp. *avium* (*Maa*), que son, respectivamente, los agentes causales de la paratuberculosis y de la TB aviar. A pesar de la creciente importancia de las MNT, todavía disponemos de información muy limitada sobre su presencia en animales. Exceptuando las subespecies de *M. avium*, sobre todo *Map*, las publicaciones relacionadas con estos microorganismos son escasas y generalmente se deben a hallazgos secundarios de investigaciones focalizadas en el CMT.

Cuando la prevalencia de TB disminuye en el ganado el papel de los hospedadores silvestres en su mantenimiento y transmisión puede volverse más relevante, y las infecciones provocadas por MNT se detectan más fácilmente debido al aumento de la presión de vigilancia para encontrar infecciones tuberculosas remanentes.

El principal objetivo de esta tesis doctoral fue estudiar el papel de la fauna silvestre en la epidemiología de la TB y otras infecciones micobacterianas en el País Vasco, una región de baja prevalencia de TB bovina situada en el norte de la Península Ibérica, así como describir posibles oportunidades de transmisión de micobacterias entre las especies silvestres y el ganado bovino en esta región. Este objetivo se abordó a través de cinco estudios incluidos en el presente trabajo. Los primeros cuatro estudios se centraron en la búsqueda de hallazgos epidemiológicos relacionados con la infección

por micobacterias y/o exposición a las mismas en diversas especies silvestres, así como en investigar estos hallazgos en relación con los detectados en el ganado. El último estudio se llevó a cabo para estudiar las interacciones entre el ganado bovino y la fauna silvestre que podrían favorecer la transmisión interespecífica de micobacterias.

En los hábitats Mediterráneos del centro-sur de la Península Ibérica, el jabalí es un importante reservorio silvestre de la TB. Sin embargo, su relevancia en el norte peninsular no está del todo clara. El primer estudio de esta tesis se centró en evaluar la exposición de las poblaciones de jabalí del País Vasco al CMT. Para ello, se analizaron sueros recogidos de esta especie mediante la técnica ELISA y se modelizaron factores de riesgo que podrían estar relacionados con la seropositividad del jabalí. La seroprevalencia global detectada (17%) fue mayor de lo esperado al compararla con otras regiones del norte peninsular con prevalencia similar en el ganado bovino. Los modelos mostraron que el ganado bovino, el tipo de hábitat y la edad pueden influir en la exposición del jabalí al CMT, incluso cuando la enfermedad en el ganado está prácticamente erradicada. Estos hallazgos nos pueden hacer reconsiderar la importancia del jabalí a la hora de desarrollar estrategias de control en zonas donde la TB bovina se encuentra en niveles próximos a la erradicación.

En comparación con la gran cantidad de investigaciones llevadas a cabo en mamíferos silvestres en zonas de alta prevalencia de TB bovina, los estudios en zonas de baja prevalencia son escasos. En el segundo estudio se llevó a cabo la detección del CMT en muestras de fauna silvestre del País Vasco recolectadas a lo largo de 10 años y se estudió la distribución espaciotemporal de los espoligotipos encontrados en fauna, comparándola con aquellos detectados en el ganado de esta misma región durante el Programa Nacional de Erradicación de la Tuberculosis Bovina. Se aislaron micobacterias del CMT en el 1,12% de los jabalíes y en el 2,40% de los ciervos, no

detectándose en ninguna otra especie silvestre. La diversidad de espoligotipos encontrada fue elevada. Se identificaron cinco espoligotipos de *M. bovis* (SB0121, SB0134, SB0881, SB2354, SB1086) y uno de *M. caprae* (SB0415) en los ungulados silvestres infectados. Se detectaron relaciones geográficas y temporales entre los espoligotipos encontrados en jabalí y ganado bovino que podrían estar indicando la transmisión interespecífica de los mismos. Este estudio ha contribuido al escenario epidemiológico de las zonas de baja prevalencia de TB bovina del norte de la Península Ibérica.

La mayor parte de los estudios relacionados con el estudio de las micobacteriosis en la interfaz silvestre-doméstico han centrado su atención en mamíferos de tamaño medio o grande, pero poco se sabe de los micromamíferos. El tercer estudio de esta tesis consistió en una investigación centrada en la detección de micobacterias en micromamíferos capturados en explotaciones de ganado bovino del País Vasco con historial de TB o MNT. Los linfonodos y tejidos recogidos de los animales capturados se procesaron y analizaron mediante técnicas microbiológicas y moleculares para el aislamiento e identificación de micobacterias. Aunque no se detectó ninguna micobacteria perteneciente al CMT en estos animales, sí se detectaron MNT (mayoritariamente en el ratón de campo, *Apodemus sylvaticus*) que podrían ser patógenas o interferir en el diagnóstico de la TB: *Map*, *M. celatum*, *M. gordonae*, *M. intracellulare* y *M. fortuitum*. La prevalencia global de MNT detectada en este estudio fue del 6.5%. Según nuestros resultados, los micromamíferos pueden transportar micobacterias por las explotaciones ganaderas, lo cual recalca la importancia de realizar futuras investigaciones para profundizar en la relevancia de este grupo de especies en la epidemiología de las micobacteriosis, y así diseñar estrategias de control más efectivas.

A pesar de la capacidad de las MNT para interferir en el diagnóstico de la TB y causar infecciones en sus hospedadores, los estudios en animales siguen siendo escasos. El objetivo del cuarto estudio fue describir la diversidad de MNT que circulan entre diferentes especies silvestres y domésticas de España (principalmente en el País Vasco), y analizar sus posibles implicaciones como organismos patógenos o como fuentes de interferencias en el diagnóstico de la TB bovina. Para ello, 293 aislados de MNT obtenidos de diferentes animales silvestres y domésticos fueron identificados mediante amplificación o secuenciación de distintos genes. En total, se identificaron 31 especies de micobacterias, siendo *Maa*, *M. avium* subsp. *hominissuis* (*Mah*), *M. bouchedurhonense*, *M. lentiflavum* y *M. nonchromogenicum* las más abundantes. *Maa* y *M. lentiflavum* se aislaron frecuentemente en animales que presentaban lesiones compatibles con TB, mientras que *Maa*, *Mah* y *M. nonchromogenicum* fueron aisladas en la mayoría de las vacas reaccionantes a la prueba de la intradermotuberculinización. Otras especies de MNT se asociaron también con estos hallazgos, aunque en menor medida. Además, se detectó una relación geográfica entre *Maa, Mah*, *M. nonchromogenicum* y *M. lentiflavum* aisladas simultáneamente en jabalí y en otros hospedadores. Los hallazgos de este estudio mostraron que existe una alta diversidad de MNT circulando entre la fauna silvestre y el ganado. El jabalí y las subespecies de *M. avium* parecen jugar un papel clave en este escenario epidemiológico.

Las interacciones que tienen lugar entre la fauna silvestre y el ganado pueden contribuir a la transmisión interespecífica del CMT o de MNT, provocando la propagación de micobacteriosis relevantes o la aparición de interferencias en el diagnóstico de la TB. El quinto estudio pretende caracterizar los patrones de interacción que ocurren entre las especies silvestres y el ganado bovino mediante fototrampeo, proporcionando conocimiento sobre las posibles dinámicas de transmisión de

micobacterias en el País Vasco. Según nuestros resultados, la transmisión interespecífica de micobacterias se daría fundamentalmente a partir de interacciones indirectas y principalmente en los pastos. Por otro lado, las letrinas de tejón podrían estar actuando como puntos de exposición a micobacterias para el tejón, el jabalí, el zorro (*Vulpes vulpes*) y las vacas, aunque será necesario realizar más estudios para confirmar esta hipótesis. Las visitas de la fauna silvestre a las explotaciones fueron abundantes pero cortas, y contrariamente a los hallazgos de otros estudios, los recursos de agua y comida no propiciaron la agregación de animales. El zorro fue la especie que interactuó más frecuentemente con el ganado bovino, tanto directa como indirectamente, pero la especie que visitó las granjas en grupos más numerosos fue el jabalí. Los ratones realizaron las visitas más largas y, entre las especies detectadas en el interior de las explotaciones, fueron los visitantes más frecuentes. El conocimiento derivado de este estudio podría resultar útil para diseñar estrategias de control, en el caso de que éstas fuesen necesarias.

Los resultados obtenidos en esta tesis doctoral sugieren que el riesgo de transmisión del CMT entre la fauna silvestre y el ganado bovino del País Vasco es bajo, mientras que el riesgo de transmisión de MNT podría ser mayor. El jabalí, el tejón, el zorro y el ratón de campo parecen ser las especies silvestres más involucradas en la epidemiología de las micobacteriosis en nuestra zona de estudio, siendo el jabalí probablemente la más relevante. El presente trabajo contribuye a la investigación sobre infecciones tuberculosas y no tuberculosas en animales procedentes de zonas de baja prevalencia de TB bovina del norte peninsular, especialmente en relación con el papel de la fauna silvestre en la epidemiología de estas micobacterioris.

GENERAL INTRODUCTION

1. Mycobacteria. A sip of taxonomy and main characteristics

At the time of writing this dissertation, more than 200 species/subspecies of mycobacteria have been described (Parte et al. 2020). These microorganisms belong to the single genus of the family *Mycobacteriaceae*, within the order *Actinomycetales*, phylum *Actinobacteria*, and kingdom *Bacteria*: the genus *Mycobacterium*. This genus was suggested for the first time by Karl Bernhard Lehmann and Rudolf Otto Neumann in 1896, in order to host organisms thought to be halfway between fungi and bacteria at that time. In fact, the prefix *myco-* (from greek μύκης (mykes)) means "fungus".

Mycobacteria are aerobic, Gram-positive, acid fast, non-motile, straight or slightly curved bacilli which do not form endospores. Their cell wall composition, extremely lipid-rich and with high mycolic acid content, is a distinctive phenotypic characteristic of these microorganisms that allows them to survive in the environment for long periods of time (Eppleston et al. 2014; Rodríguez-Hernández et al. 2016; Martínez et al. 2019). Their G + C content is distinctively high and their optimal growth temperature varies between species, ranging from temperatures well below ambient to over 45 ºC (Magee and Ward 2015). Mycobacteria are traditionally classified into two groups based on their growth rate: slow-growing mycobacteria need more than one week to develop colonies in solid media, while rapid-growing mycobacteria take shorter (Tortoli 2019). Colonies are mainly white- to cream-coloured, even though some strains produce yellow- to red-pigmented colonies (Magee and Ward 2015).

Phenotypic-based methods such as growth rate, morphology or pigmentation of colonies have been conventionally used for mycobacteria classification and identification, but later on molecular methods were proved to be quicker and more

accurate (Springer et al. 1996). The 16S ribosomal RNA gen (16S rRNA) has been the main pillar for many taxonomic and phylogenetic researches, being considered a reference identification tool for most mycobacteria (Turenne et al. 2001). However, because this gene is moderately variable among mycobacteria with high intraspecific similarity, the use of other genes, combined or not with 16S rRNA, such as the RNA polymerase β-subunit (*rpoB*), the hypervariable fragment of heat shock protein 65 (*hsp*65) or the internal transcribed spacer (ITS) region (16S-23S rRNA), among other targets, has also been implemented to better discriminate between species/subspecies and to study their phylogenetic relationships (Roth et al. 1998; Simmon et al. 2010; Higgins et al. 2011; Tortoli 2012). More recently, the advent of whole-genome sequencing (WGS) allowed for the revision of the *Mycobacterium* genus taxonomy and supported the global conclusions previously reported through the preceding molecular methods: An obvious distinction between slow and fast growers, with the *Mycobacterium terrae* complex occupying an intermediate position; fast growers occupying an ancestral position and members of the *M. chelonae-abscessus* complex identified as the most ancestral cluster (Figure 1) (Fedrizzi et al. 2017; Tortoli et al. 2017; Tortoli 2019).

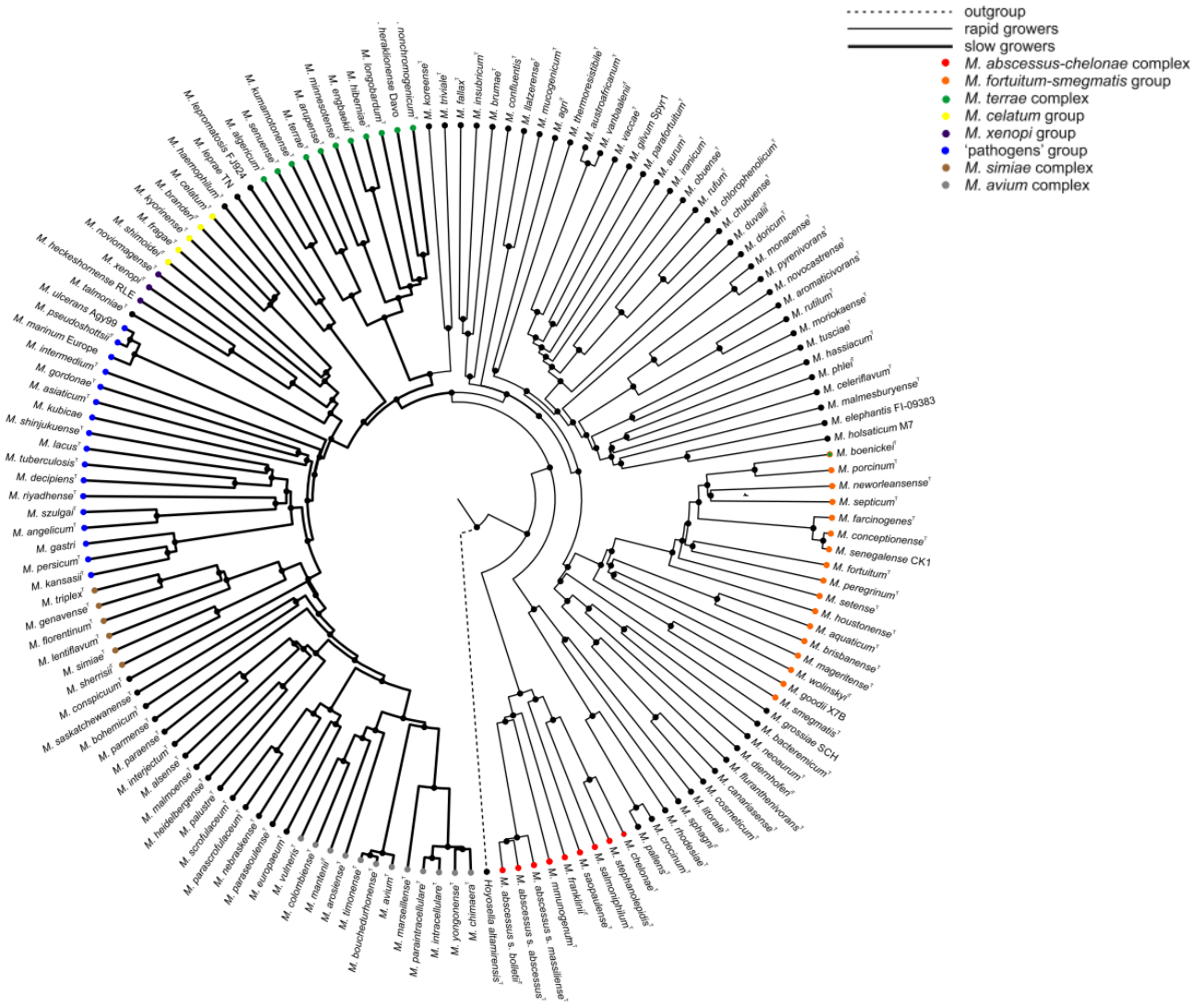

Figure 1. Phylogenetic tree of the genus *Mycobacterium*. Fast and slow growers are marked with different line width, while clusters of complexes/groups are shown with a colour code. Source: Tortoli et al. 2017.

In 2018, another research based on WGS technologies (Gupta et al. 2018) proposed to split the genus *Mycobacterium* into five genera: an emended genus *Mycobacterium* and four novel genera (*Mycolicibacterium* gen., *Mycolicibacter* gen., *Mycolicibacillus* gen. and *Mycobacteroides* gen.). Despite the overlap between the clades displayed in this study and those previously obtained through other phylogenomic approaches (Fedrizzi et al. 2017; Tortoli et al. 2017), the validation of the novel nomenclature has brought confusion and discrepancies among the scientific community (Tortoli et al. 2019). For this reason, the present dissertation adheres to the

classical nomenclature and the term “mycobacteria” is going to be used to refer to all the organisms comprised within these five genera.

Overall, mycobacteria include obligate pathogens, environmental opportunistic pathogens and saprophytes which contaminate or inhabit a broad spectrum of solid and aquatic substrates (Falkinham III et al. 2001; Hruska and Kaevska 2012; Biet and Boschiroli 2014). Obligate pathogens belong to slow-growing mycobacteria, while the opportunistic ones can be either fast or slow growers (see Figure 1). From a medical and veterinarian perspective, mycobacteria are usually grouped in: (1) the agents of tuberculosis (TB), (2) mycobacteria that cause leprosy, and (3) non-tuberculous mycobacteria (NTM), which is a wider group of species that include the causative agents of paratuberculosis, avian TB and a broad list of opportunistic mycobacterial infections in humans, domestic animals and wildlife (Biet and Boschiroli 2014; Malone and Gordon 2017; Martínez González et al. 2017; Claeys and Robinson 2018; Ploemacher et al. 2020).

2. Tuberculosis

2.1 Highlights

TB is a worldwide zoonotic infectious disease with a wide range of mammalian hosts. The causative agents of this disease are the members of the *M. tuberculosis* complex (MTC), maybe the most relevant group of mycobacteria: *M. tuberculosis*, *M. africanum*, *M. bovis*, *M. bovis* bacillus Calmette–Guérin (BCG), *M. caprae*, *M. microti* and *M. pinnipedii*. Recently it was proposed to also include *M. canettii, M. mungi, M. suricattae, M. orygis,* the Dassie bacillus and the Chimpazee bacillus, although they are not accepted by official bacterial taxonomical nomenclature yet (Malone and Gordon 2017; Riojas et al. 2018) (Figure 2). After the first isolation of *M. tuberculosis* by

Robert Koch from a TB patient in 1882, several tuberculous mycobacteria were isolated from different animal hosts, and isolates were designated based on the host from which they were originally or most commonly recovered (Malone and Gordon 2017). These findings led to the differentiation of human and animal TB, even though it has been demonstrated that MTC infection can jump from animals to humans and vice versa (Ocepek et al. 2005; Kock et al. 2021).

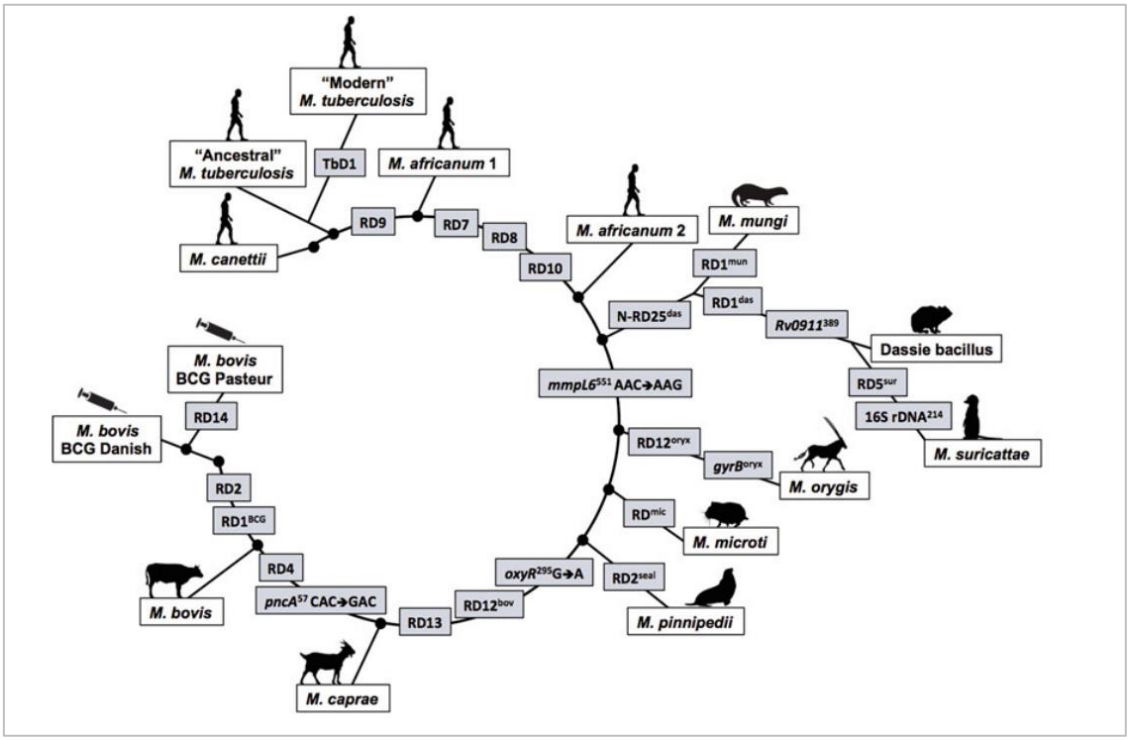

Figure 2. Members of the *Mycobacterium tuberculosis* complex and proposed evolutionary steps of its phylogeny. In grey boxes: molecular markers and regions of difference (RD) that differentiate the species. Source: Malone and Gordon 2017.

Human TB is mainly caused by *M. tuberculosis*, a bacterium that spreads through the air via droplets from human to human (Fogel 2015). The disease usually affects the lungs, even though extrapulmonary TB is not uncommon (Zaman 2010). It represents a leading cause of illness and one of the top causes of death globally, with an estimation of 1.3 million deaths in 2020 (World Health Organization 2021). For these reasons, human TB is still a major public health concern, especially in developing countries (Sarro et al. 2019). Unfortunately, and despite the "End TB" strategy

published and implemented by the World Organization for Animal Health (WHO) since 2014, estimations on global human TB mortality rate are not projected to get any better, partly due to the rising numbers of multiple, extensively and even totally drug-resistant strains (Nguyen 2017), and since collateral effects of COVID-19 pandemic emergency response are expected to increase the number of deaths by a 20% during the next five years (Hogan et al. 2020).

Animal TB is mainly caused by infection with *M. bovis*, even though other species of the MTC can be involved, like *M. caprae* (Kaneene et al. 2014). Because of its zoonotic potential, animal TB is a recognized public health problem, but also a source of significant economic losses in the livestock industry and a threat to the welfare of livestock, companion animals, farmed game animals and even wild mammals, which also turns this disease into a wildlife conservation issue (Zinsstag et al. 2006; Gortázar et al. 2012; Thapa et al. 2017; Pérez-Morote et al. 2020).

There are many pathways for animals to become infected with TB, but inhalation of infectious aerosols, either from an infected animal or dust particles, is considered to be the main one, at least in livestock (Menzies and Neill 2000; Serrano et al. 2018). When wildlife is also involved, the oral route may become more relevant and disease transmission is considered to be mainly indirect, for instance through shared water or food resources (Barasona et al. 2017). Infected animals can show variable disease states ranging from latent subclinical infection (no visible lesions or symptoms) to moderate or severe stages with generalized pathology. The typical lesions caused are firm and small granulomas (tubercles) that range in colour from white to yellowish and frequently show a central core of necrosis (Neill et al. 2005). Their development and distribution appears to be associated with the host, bacterial introduction route and stage of the disease, even though the respiratory system and related lymph nodes (LN) are the

most common target (Neill et al. 2005; Serrano et al. 2018; Thomas et al. 2021). Either if the infected animal is immunocompromised or if the immune system is not able to stop the infection from spreading, tissue damage can progress and the initial small granulomas located at the portal of entry become larger with time, resulting in chronic infection (Domingo et al. 2014). Generalized TB develops as a result of mycobacteria dissemination following the erosion of small blood or lymphatic vessels by growing tubercles (Domingo et al. 2014), being characterized by the spread of lesions to other organs (Neill et al. 2005; Domingo et al. 2014).

2.2 *M. tuberculosis* complex: multi-host pathogens

Although cattle has been historically considered the main host of *M. bovis* (Pesciaroli et al. 2014), this and other members of MTC are able to infect several wild and domestic mammals beyond this bovid, with just a few eventual exceptions (Mentaberre et al. 2010), and thus represent the perfect example of multi-host pathogens (see Table 1). In fact, a recent study has demonstrated that, in some regions of Europe, TB systems are dominated by non-bovine domestic and wild species (Santos et al. 2020). It is therefore obvious to assume that targeting all host species, not only cattle, is the only way to eradicate these multi-host microorganisms, which makes it particularly challenging. Interestingly, different scenarios can be observed all over the world, since host composition varies from one country to another or even between regions of the same country.

Table 1. Examples of animal hosts infected with MTC members.

MTC member	Reference Host*	Other Hosts	Reference
Dassie bacillus	Rock hyrax (*Procavia capensis*)	Meerkat (*Suricata suricattae*)	(Clarke et al. 2016)
Chimpanzee bacillus	Chimpanzee (*Pan troglodytes*)	Not described so far	(Coscolla et al. 2013)
M. africanum	Human (*Homo sapiens*)	Cattle (*Bos taurus*)	(Rahim et al. 2007)
		Non-human primates	(Thorel 1980)
M. bovis	Cattle	African buffalo (*Syncerus caffer*)	(Renwick et al. 2007)
		American bison (*Bison bison*)	(Miller and Sweeney 2013)
		Badger (*Meles meles*)	(Blanco Vázquez et al. 2021)
		Brushtail possum (*Trichosurus vulpecula*)	(Nugent et al. 2015)
		Camelids	(Álvarez et al. 2012)
		Common shrew (*Sorex araneus*)	(Skoric et al. 2007)
		Elephants	(Miller et al. 2021)
		Elk (*Cervus canadensis*)	(Miller and Sweeney 2013)
		Fallow deer (*Dama dama*)	(Amato et al. 2016)
		Field vole (*Microtus agrestis*)	(Skoric et al. 2007)
		Fox (*Vulpes vulpes*)	(Matos et al. 2014b)
		Goat (*Capra hircus*)	(Napp et al. 2013)
		Pig (*Sus scrofa domestica*)	(Parra et al. 2003)
		Red deer (*Cervus elpahus*)	(Gortázar et al. 2011b)
		Sheep (*Ovis aries*)	(Muñoz-Mendoza et al. 2012)
		White-tailed deer (*Odocoileus virginianus*)	(Miller and Sweeney 2013)
		Wild boar (*Sus scrofa*)	(Gortázar et al. 2011b)
		Wood mouse (*Apodemus sylvaticus*)	(Skoric et al. 2007)
M. canettii	Human	Not described so far	(Supply and Brosch 2017)
M. caprae	Goat	Cattle	(Ahmad et al. 2018)
		Domestic rabbit (*Oryctolagus cuniculus*)	(Sevilla et al. 2020)
		Fox	(Steinparzer et al. 2020)
		Pig	(Amato et al. 2017)
		Red deer	(Nigsch et al. 2019)
		Roe deer (*Capreolus capreolus*)	(Orłowska et al. 2020)
		Sheep	(Muñoz-Mendoza et al. 2012)
		Wild boar	(Csivincsik et al. 2016)
M. microti	Field vole	Badger	(Smith et al. 2009)
		Cat (*Catus felis*)	(Smith et al. 2009)
		Cattle	(Smith et al. 2009)
		Dog (*Canis lupus familiaris*)	(Michelet et al. 2015)
		Ferret (*Mustela putorius furo*)	(Smith et al. 2009)
		Goat	(Michelet et al. 2016)
		Llama (*Lama glama*)	(Smith et al. 2009)
		Otter (*Lutra lutra*)	(Michelet et al. 2015)
		Pig	(Michelet et al. 2015)
		Wild boar	(Pérez de Val et al. 2019)
		Wood mouse	(Cavanagh et al. 2002)
M. mungi	Banded mongoose (*Mungos mungo*)	Not described so far	(Alexander et al. 2010)

MTC member	Reference Host*	Other Hosts	Reference
M. orygis	East African oryx (*Oryx beisa*)	Antelope	(Smith et al. 2006)
		Cattle	(Refaya et al. 2019)
		Deer	(Smith et al. 2006)
		Gazelle	(Smith et al. 2006)
		Non-human primates	(Rahim et al. 2017)
		One-horned rhinoceros (*Rhinoceros unicornis*)	(Love et al. 2020)
		Waterbuck (*Kobus ellipsiprymnus*)	(Smith et al. 2006)
M. pinnipedii	Southern sea lion (*Otaria flavescens*)	Bactrian camel (*Camelus bactrianus bactrianus*)	(Jurczynski et al. 2011)
		Cattle	(Loeffler et al. 2014)
		Indian crested porcupine (*Hystrix cristata*)	(Jurczynski et al. 2011)
		Malayan tapir (*tapirus indicus*)	(Jurczynski et al. 2011)
		New Zealand fur seal *(Arctocephalus forsteri)*	(Roe et al. 2019)
		New Zealand sea lion *(Phocarctos hookeri)*	(Roe et al. 2019)
M. suricattae	Meerkat (*Suricata suricattae*)	Not described so far	(Parsons et al. 2013)
M. tuberculosis	Human	Cattle	(Hlokwe et al. 2017)
		Dog	(Parsons et al. 2012)
		Elephants	(Payeur et al. 2002)
		Non-human primates	(Shipley et al. 2008)

*The reference host is the species or group from which the MTC member was originally or most commonly recovered.

At global scale, there are five hot-spots considered as two-host or multi-host systems (Gortázar et al. 2015a): Within the two-host systems, host community is comprised by cattle and one wild species: In the British Islands, the Eurasian badger (*Meles meles*) (Corner et al. 2011); in New Zealand, the introduced Australian brushtail possum (*Trichosurus vulpecula*) (Nugent et al. 2015); and in Michigan, United States of America (USA), the white-tailed deer (*Odocoileus virginianus*) (VerCauteren et al. 2018). Multi-host systems are clearly recognized in sub-Saharan Africa and in Mediterranean habitats of south-central Iberian Peninsula. In southern Africa, animal TB is mainly maintained by the African buffalo (*Syncerus caffer*) or by the lechwe antelope (*Kobus leche*), depending on the setting, but MTC members have also been detected in a wide range of wild African mammals, which might add more complexity to the host community composition (Renwick et al. 2007). The host composition of the Iberian Peninsula might represent the most complex one worldwide. In south-central

areas, wild boar (*Sus scrofa*) and deer, mainly red deer (*Cervus elaphus*) but also fallow deer (*Dama dama*), are clearly wild maintenance hosts of animal TB (Gortázar et al. 2012), while in the north, the Eurasian badger seems to be a potential reservoir in some Atlantic regions (Acevedo et al. 2019; Blanco Vázquez et al. 2021) and the role of wild boar is still a matter of discussion (Muñoz-Mendoza et al. 2013; Mentaberre et al. 2014; Gortázar et al. 2017). Moreover, the disease has been detected to a lesser extent in other wild ungulates and carnivores, such as the roe deer (*Capreolus capreolus*), the fox (*Vulpes vulpes*), or the Iberian Lynx (*Lynx pardinus*) (Peña et al. 2006; Millán et al. 2008; Balseiro et al. 2009). The spectrum of domestic hosts is also remarkable in the Iberian Peninsula, since apart from cattle, goats (*Capra hircus*), sheep (*Ovis aries*) and pigs (*Sus scrofa domestica*) are also potential maintenance hosts (Samper et al. 1995; Parra et al. 2003; Napp et al. 2013; Muñoz-Mendoza et al. 2015). The recognition and investigation of these host communities, among others, has been an essential step to develop more effective control strategies.

2.3 TB control strategies in livestock and wildlife populations

In cattle (*Bos taurus*), the main and most-well studied host of *M. bovis*, the spread of the disease within herds has historically brought detrimental consequences for animal production, trade opportunities and human health (Cousins 2001). In light of these mayor threats, together with the severe economic losses that came along with them, the WHO adopted a resolution in 1983 calling for the eradication of *M. bovis* (Kaneene et al. 2014). Consequently, eradication programmes exclusively targeting cattle were implemented in Europe and other regions of the world through the last decades, being originally focussed on test and slaughter strategies. These programmes rarely include other domestic species from livestock industry (Cvetnić et al. 2007). However, in some countries such as Spain testing other domestic species (e.g., goats) is

compulsory when animals are epidemiologically related with cattle herds and some regions even have ongoing local eradication programmes directed to goat herds (Napp et al. 2013). Aside from searching for lesions compatible with TB during *post mortem* inspection of slaughtered animals, the tuberculin skin test (TST) is the standard diagnostic method for *in vivo* detection of infected individuals (Schiller et al. 2010). The European Communities Commission recognizes the single intradermal tuberculin (SIT) test and the comparative intradermal tuberculin (CIT) test as the official *in vivo* diagnostic assays for TB in the Member States, and the interferon-gamma (IFN-γ) assay as an alternative to TST since April 2021 (Commission delegated Regulation (EU) 2020/689 of 17 December 2019). In the SIT test only *M. bovis*-derived purified protein derivative (PPD-B) is injected in the mid-cervical region, while in the CIT test both avian (*M. avium*-derived) and bovine PPD (PPD-A and PPD-B) are injected at separate but close sites. These methods are based on cellular immune response. When an animal is infected or has been exposed to MTC bacteria, a delayed hypersensitivity reaction occurs at the injection site, which is related to T-memory lymphocyte activation. Lymphocytes can synthesize a variety of cytokines in response to this antigenic stimulation, including IFN-γ, which can be measured through the IFN-γ release assay (Biet and Boschiroli 2014). With the SIT test, the increase in skin-fold thickness caused by the immune reaction is measured. However, sensitization with NTM or vaccination against paratuberculosis, among other factors, can affect the specificity of the test (de la Rua-Domenech et al. 2006; Garrido et al. 2013), leading to the slaughter of non MTC-infected animals. With the CIT test, in which skin reactions to each PPD are compared, some of these issues can be solved but specificity and sensitivity can be also affected (Schiller et al. 2010). Despite their limitations, these procedures have successfully eradicated the disease in many countries from north-central Europe, which are declared

Officially Tuberculosis-Free (OTF) after more than 99.9% of their cattle herds were disease-free for more than six consecutive years. However, infection still prevails or re-emerge in some areas of the United Kingdom (UK) and the Republic of Ireland (ROI), in the Iberian Peninsula and in other several countries (Gortázar et al. 2012). Even though residual infections within herds seems to be the main cause of TB outbreaks among cattle, the disregard of wild and other domestic hosts when implementing disease control strategies has been one of the causes that has prevented the complete eradication of the disease (Guta et al. 2014).

Vaccination with BCG, a live attenuated strain of *M. bovis*, has been proved to reduce both progression and severity of TB in cattle, and thus, it is considered a measure that could complement the strategy of testing herds and culling reactor animals. However, it is currently prohibited by European Union legislation because of the variable protection that offers and the sensitization of vaccinated individuals to the tuberculin-based diagnostic tests (Chambers et al. 2014). In order to solve this last issue, studies have been performed for the development of DIVA tests, able to distinguish between infected and vaccinated animals. Instead of standard tuberculin, these tests use antigens present in *M. bovis* but lost in BCG during its attenuation (e.g., ESAT-6, CFP-10 or Rv3615c, this last being present in BCG genome but not secreted) (Vordermeier et al. 2011). Despite the progress made to improve the sensitivity and specificity of these tests, their implementation in field trials testing BCG vaccinated animals is required prior to any change in the legislation regarding the use of vaccines against TB in cattle (Vordermeier et al. 2016).

Even though it would be a fallacy to state that infection in cattle is solely attributable to a reservoir in wildlife, and despite TB in wildlife is believed to have its origin in cattle (Fitzgerald and Kaneene 2013), the increasing importance of wild hosts

has become a well-known reality over the time as the control of TB in cattle progresses (Richomme et al. 2013; Gortázar and Boadella 2014). Therefore, control strategies involving wildlife started to be developed in regions where wild hosts appeared to be a major obstacle for eradication. Nowadays these strategies, which should be ideally combined for an integrated control of the disease, range from no intervention, preventive actions or host population control to vaccination, and are considered on the basis of surveillance and monitoring schemes, cost-benefit ratio and stakeholders' willingness (Cowie et al. 2015; Gortázar et al. 2015b).

No intervention in wildlife can be an option when there is not a strong justification for action from a public or animal health point of view or when cost-efficient control tools are not available, provided that populations are monitored to allow to reconsider this strategy if the situation changes (Gortázar et al. 2015b). However, this decision can bring higher costs in the future (Woodroffe et al. 2006).

Preventive actions are those considering farm biosecurity, fencing, shared resources segregation and proper hunting-remains management, among others (Gortázar et al. 2015b). These tools have been developed with the aim of reducing wildlife-livestock interactions and therefore prevent disease transmission. For instance, the deployment of sheet metal gates, feed containers and electrified fences successfully kept badgers out of cattle farm facilities in the UK (Judge et al. 2011). In south-central Spain, the design of fencing strategies aimed at segregating wild ungulates and cattle from common water resources substantially reduced direct and indirect contacts between these species (Barasona et al. 2013), while in northern Spain the design of a calf-selective feeder hampered the access of wild boar to feed, reducing food-born indirect interspecies contacts (Balseiro et al. 2019). Carcasses and gut piles of wild ungulates represent a valuable food source for scavenger vertebrates, but they can also

contribute to the maintenance and spread of TB in wild reservoirs if carrion is infected (Vicente et al. 2011). Therefore, proper removal of big game waste (e.g. incineration or dropping the remains in feeding points for avian scavengers) is considered a complementary tool for the control of TB in wild populations (Royal Decree (Spain) 50/2018 of 12 February 2018), being its effectiveness already proved in wild boar from Mediterranean ecosystems (Cano-Terriza et al. 2018).

Host population control include random and selective culling strategies, which have been proved to achieve a certain reduction of TB prevalence in wild hosts and associated cattle herds (Corner et al. 2011; Mentaberre et al. 2014), but seems to be more suitable for isolated populations than for broad geographical scales (Gortázar et al. 2015b). Besides, a significant proportion of the society does not welcome using these tools, a fact which has triggered a strong debate among stakeholders and a search for more acceptable alternatives, such as immunocontraception or vaccination (Gortázar et al. 2015a, b).

Vaccination in wildlife is considered a promising measure that could be implemented for integrated control of TB in some contexts. Several laboratory trials have been carried out in cervids, wild boar, badger, brushtail possum, African buffalo and feral ferrets (*Mustela putorius furo*) (Cross et al. 2000; Palmer et al. 2007; de Klerk et al. 2010; Garrido et al. 2011; Nugent et al. 2016; Thomas et al. 2017; Balseiro et al. 2020). These experiments have tested different vaccine types, doses and administration routes, different challenge schemes and different methods of evaluation, with variable outcomes on vaccine efficacy. Nevertheless, it is likely that, for practical and economic reasons, any large-scale vaccination of wildlife populations against TB would be almost certainly based on oral delivery of the vaccine (Gormley and Corner 2011), which is indeed particularly challenging. Vaccine stability, environmental safety,

overconsumption and consumption by non-target species are some of the issues that need to be addressed before delivery in the field (Beltrán-Beck et al. 2014; Fischer et al. 2016). Currently, vaccination in wildlife is also forbidden, except in the UK, where an injectable BCG vaccine can be administered intramuscularly to badgers since 2010 (Gormley et al. 2017).

3. Non-tuberculous mycobacteria

3.1 Highlights

NTM are colonizers of the environment that grow in a plethora of natural and human-made niches, such as soil, water, plants, food products, dust, air or even extreme habitats (Pereira et al. 2020). This wide group of microorganisms encompasses fast and slow growers, being many of them clustered in complexes or groups based on phylogenetic relationships (Figure 1). Generally speaking, and strict saprophyte species put aside, NTM are mainly opportunistic pathogens of immunocompromised but also immunocompetent humans and animals, but within slow growers, *M. avium* complex (MAC) stands out among the rest of NTM in terms of medical and veterinary significance (Biet and Boschiroli 2014; Falkinham III 2016; Pereira et al. 2020). This complex consists of 12 validly published species: *M. avium*, *M. intracellulare*, *M. chimaera*, *M. colombiense*, *M. arosiense*, *M. vulneris*, *M. bouchedurhonense*, *M. timonense*, *M. marseillense*, *M. yongonense*, *M. paraintracellulare* and *M. lepraemurium* (Van Ingen et al. 2018). In turn, *M. avium* comprises three subspecies: *M. avium* subsp. *avium* (*Maa*) (including its variant *M. avium* subsp. *silvaticum* (*Mas*)), *M. avium* subsp. *hominissuis* (*Mah*) and *M. avium* subsp. *paratuberculosis (Map)* (Turenne et al. 2008). *Maa* and *Map* are of great relevance in veterinary medicine, since they are obligate pathogens and the causative agents of avian TB (Dhama et al. 2011) and paratuberculosis (Whittington et al. 2019), respectively.

In humans, the routes of entry of NTM mainly include inhalation, but also swallowing, aspiration and introduction through wounds (Pereira et al. 2020). Conversely to human TB, human-to-human transmission from patients with NTM respiratory disorders is considered unlikely (Koh 2017). Pulmonary infection with a clinical presentation similar to human TB is the most frequent manifestation among patients, even though skin, soft tissue and occasional osteoarticular infections are also described (Franco-Paredes et al. 2019; Ratnatunga et al. 2020). Disseminated infection is also possible, especially in patients with severely compromised immune systems (Wetzstein et al. 2021). Globally, the most clinically relevant species or groups are MAC, *M. abscessus*, *M. chelonae*, *M. fortuitum*, *M. kansasii*, *M. malmoense*, *M. marinum*, *M. ulcerans* and *M. xenopi* (Hoefsloot et al. 2013), being MAC members the most frequent NTM linked to lung disease around the world (Koh 2017).

Regarding animals, there are similarities in the transmission routes and clinical presentations in comparison with humans, but being the digestive route apparently more common than the respiratory one, and the infections usually more localized (Howard and Byrd 2000; Biet and Boschiroli 2014). In veterinary medicine, NTM infection implies a double concern: the potential to interfere with bovine TB diagnosis and to cause opportunistic or major infections, such as avian TB or paratuberculosis, that may lead to economic losses and deprivation of animal welfare (Biet and Boschiroli 2014).

As stated previously, PPD-B and PPD-A are used in the diagnostic methods for *in vivo* detection of MTC-infected animals. Because PPD contains antigens common to many mycobacteria (Infantes-Lorenzo et al. 2017), lymphocyte activation can also occur if animals were exposed to NTM harbouring antigens that are similar to those of *M. bovis* or other MTC members, sensitizing animals and leading to non-specific positive reactions. When the CIT test is used, individuals exposed to NTM are generally

differentiated from those infected with MTC because the reaction to PPD-A is usually bigger than the reaction to PPD-B. However, animals that are more immunoreactive to PPD-A than to PPD-B are often considered negative for TB, even though they might be actually coinfected with MTC, resulting in a decrease of the sensitivity of the test (Álvarez et al. 2009). Although some species of NTM have been isolated from domestic or farmed animals that reacted to tuberculin (e.g., *M. scrofulaceum*) (Bercovier and Vincent 2001), a few experimental studies on cross reactive immune responses between NTM and *M. bovis* have truly demonstrated the ability of some species to interfere with bovine TB diagnostic assays such as the TST or the IFN-γ assay: *Maa*, *Map*, *M. hiberniae*, *M. fortuitum*, *M. intracellulare* and *M. kansasii* represent some of these examples (Corner and Pearson 1978; de la Rua-Domenech et al. 2006; Biet and Boschiroli 2014). In addition to the interference with the *in vivo* diagnosis of bovine TB, NTM can cause lesions indistinguishable from those caused by MTC, which may also lead to TB misdiagnosis at *post mortem* inspections in slaughterhouses (Hernández-Jarguín et al. 2020). Therefore, many of these mycobacteria may hinder bovine TB eradication campaigns at *ante mortem* diagnosis and/or at *post mortem* examination of animals (Biet and Boschiroli 2014).

Records reporting NTM causing disease in wild and domestic animals include several species, such as MAC members, *M. abscessus*, *M. chelonae*, *M. farcinogenes*, *M. fortuitum*, *M. kansasii*, *M. marinum*, *M. phlei*, *M. porcinum*, *M. senegalense*, *M. smegmatis*, *M. scrofulaceum* and *M. xenopi*, among others (Pereira et al. 2020). However, MAC members are the mycobacteria mostly involved in animals' infections, which turns them into microorganisms of great relevance in veterinary medicine. Within MAC, *M. avium* subspecies are the most clinically significant.

3.2 *M. avium* subspecies

All subspecies of *M. avium* are recognized pathogenic mycobacteria responsible for infectious diseases in a wide spectrum of hosts and for causing interferences in the diagnosis of bovine TB (Biet and Boschiroli 2014; Scherrer et al. 2018), being *Map* and *Maa* obligate pathogens (Turenne et al. 2008). *Map* stands out among NTM for being the causative agent of paratuberculosis, a chronic wasting disease of ruminants that causes a huge impact on economy and animal welfare worldwide. It is also considered one of the most important causes of non-specific skin test reactions among cattle (Biet and Boschiroli 2014; Whittington et al. 2019). Besides, this mycobacterium arouses public health concerns due to its potential involvement in Crohn's disease in humans (Mendoza et al. 2009). Paratuberculosis, or Johne's disease, begins as a localised infection that can become systemic, resulting a in chronic granulomatous enteritis leading to emaciation and death (Whittington et al. 2019). Paratuberculosis is of great relevance in cattle, sheep and goat industry. However, this disease is also worrying in wild ruminants, which show clinical signs similar to those described in cattle (Carta et al. 2013), especially in cervids (Álvarez et al. 2005; Glawischnig et al. 2006; Kopecna et al. 2008; Robino et al. 2008; Pate et al. 2016; Matos et al. 2017; Galiero et al. 2018; Volpe et al. 2020). *Map* has also been detected in non-ruminant wildlife, even though the infection is usually subclinical (Carta et al. 2013).

Maa is the causative agent of avian TB and affects several species of domestic, captive and wild birds (Dhama et al. 2011). The disease primarily involves the digestive tract and the liver, with clinical manifestations including chronic and progressive emaciation and weakness (Dhama et al. 2011). This mycobacterium can infect and cause disease in other wild and domestic mammals, but infection does not usually lead to clinical disease and lesions are localized and less severe (Thorel et al. 2001). *Mas*

infects mainly wood pigeons (*Columba palumbus*) and is closely related to *Maa* (Uchiya et al. 2017), so close that some authors do not consider *Mas* a subspecies different from *Maa* in terms of taxonomy (Turenne et al. 2007). *Mah* is an environmental bacterium that mainly infects swine, being frequently isolated from pigs with subclinical infection showing lesions in LNs of the digestive tract and of the head (Domingos et al. 2009; Agdestein et al. 2014; Biet and Boschiroli 2014). Dissemination to other organs can also be detected at slaughter without preceding clinical symptoms (Hibiya et al. 2010). Clinical illness is rare in pigs, even though in the few cases reported the infection seems to produce reproductive disorders (Eisenberg et al. 2012). *Mah* is also an opportunistic pathogen for other mammals (Balseiro et al. 2011a; Pate et al. 2016; Ghielmetti et al. 2018, 2021), being able to cause, sporadically, severe infections with fever, diarrhoea and emaciation (Haist et al. 2008; Kriz et al. 2010; Klang et al. 2014).

Table 2 displays several examples of *M avium* infections in animals, highlighting the ability of these subspecies to infect many animal species.

Table 2. Examples of animal infections caused by *M. avium* subspecies.

***M. avium* subspecies**	**Host type**	**Species/groups**	**Reference**
Maa/Mas	Bird*	Columbiformes	(Dhama et al. 2011)
		Galliformes	
		Passerines	
		Psittacines	
		Raptors	
		Ratites	
		Waterfowl	
	Mammal	Badger (*Meles meles*)	(Balseiro et al. 2011a)
		Common shrew (*Sorex araneus*)	(Fischer et al. 2000)
		Cattle (*Bos taurus*)	(Dvorska et al. 2004)
		Goat (*Capra hircus*)	(Thorel et al. 2001)
		Pig (*Sus scrofa domestica*)	(Muwonge et al. 2012)
		Red deer (*Cervus elaphus*)	(Glawischnig et al. 2006)
		Sheep (*Ovis aries*)	(Thorel et al. 2001)
		Wild boar (*Sus scrofa*)	(Trcka et al. 2006)
		Yellow-necked mouse (*Apodemus flavicollis*)	(Fischer et al. 2000)
Mah	Swine	Pig	(Agdestein et al. 2014)
		Wild boar	(Ghielmetti et al. 2021)
	Others	Badger	(Balseiro et al. 2011a)
		Cattle	(Ghielmetti et al. 2018)
		Cat (*Felis catus*)	(Klang et al. 2014)
		Dog (*Canis lupus familiaris*)	(Haist et al. 2008)
		Horse (*Equus caballus*)	(Kriz et al. 2010)
		Red deer	(Pate et al. 2016)
Map	Ruminant	Cattle	(Fecteau 2018)
		Chamois (*Rupicapra rupicapra*)	(Kopecna et al. 2008)
		Fallow deer (*Dama dama*)	(Álvarez et al. 2005)
		Goat	(Windsor 2015)
		Mouflon (*Ovis musimon*)	(Kopecna et al. 2008)
		Red deer	(Glawischnig et al. 2006)
		Roe deer (*Capreolus capreolus*)	(Kopecna et al. 2008)
		Sheep	(Windsor 2015)
	Non-ruminant	Badger	(Beard et al. 2001)
		Black rat (*Rattus rattus*)	(Florou et al. 2008)
		Brown rat (*Rattus norvegicus*)	(Beard et al. 2001)
		Common vole (*Microtus arvalis*)	(Kopecna et al. 2008)
		Egyptian mongoose (*Herpestes ichneumon*)	(Cunha et al. 2020)
		European brown hare (*Lepus europaeus*)	(Beard et al. 2001)
		Fox	(Matos et al. 2014a)
		House mouse (*Mus musculus*)	(Florou et al. 2008)
		Otter (*Lutra lutra*)	(Matos et al. 2013)
		Pig	(Miranda et al. 2011)
		Stoat (*Mustela erminea*)	(Beard et al. 2001)
		Stone marten (*Martes foina*)	(Matos et al. 2014a)
		Weasel (*Mustela nivalis*)	(Beard et al. 2001)
		White-toothed shrew (*Crocidura suaveolens*)	(Kopecna et al. 2008)
		Wild rabbit (*Oryctolagus cuniculus*)	(Maio et al. 2011)
		Wild boar	(Zanetti et al. 2008)
		Wood mouse (*Apodemus sylvaticus*)	(Beard et al. 2001)

Maa/Mas= M. avium subsp. *avium*/*M. avium* subsp. *silvaticum*, *Mah= M. avium* subsp. *hominissuis*, *Map= M. avium* subsp. *paratuberculosis*. *Bird species are grouped in the main affected taxonomic groups.

3.3 NTM other than *M. avium*

Despite the growing importance of NTM infections, limited information on their occurrence in animals has been reported. Excluding *M. avium* subspecies, especially *Map*, published records are scarce and mainly emerge from secondary findings of MTC research. However, delving into the study of these microorganisms is necessary to understand their distribution, clinical significance and potential implications on TB eradication campaigns.

Table 3 summarizes the publications related to NTM other than *M. avium* isolated from free-ranging wild mammals in different countries of Europe, as well as the clinical findings detected. Despite the few published reports (n= 14), these researches are enough to reflect the high diversity of species that circulate among a broad variety of wild hosts from which ungulates, and mainly wild boar, are the most studied. Conversely, references related to carnivores or small mammals are negligible, with one report on small rodents, one on fox and one on badger. These studies are also useful to realise that, although not always, many of these microorganisms are able to cause lesions in the hosts they infect, at least in wild ungulates.

Table 3. Non-avium NTM species detected in free-ranging European wild mammals.

Host	Country	NTM species	Lesions found	References
Badger (*Meles meles*)	UK	*M. intracellulare*	/	(Hughes et al. 1993)*
Common shrew (*Sorex araneus*)	Czech Republic	*M. vaccae*	No	(Fischer et al. 2000)
Common vole (*Microtus arvalis*)	Czech Republic	*M. chelonae, M. fortuitum*	No	(Fischer et al. 2000)
Fallow deer (*Dama dama*)	Hungary	*M. nonchromogenicum, M. sinense*	**Yes**	(Rónai et al. 2016)
	Spain	*M. intracellulare, M. scrofulaceum, M. xenopi*	NIA	(Gortázar et al. 2011a)

Host	Country	NTM species	Lesions found	References
Fox (*Vulpes vulpes*)	Hungary	*M. thermoresistible*	No	(Rónai et al. 2016)
Red deer (*Cervus elaphus*)	Hungary	*M. arupense, M. fortuitum, M. intermedium, M. nonchromogenicum, M. palustre, M. parafortuitum, M. vaccae*	**Yes**	(Rónai et al. 2016)
	Slovenia	*M. confluentis, M. engbaekii, M. intracellulare, M. nonchromogenicum, M. peregrinum, M. vaccae*	No	(Pate et al. 2016)
	Spain	*M. interjectum, M. scrofulaceum*	NIA	(Gortázar et al. 2011a)
Roe deer (*Capreolus capreolus*)	Hungary	*M. fortuitum, M. kansasii*	No	(Rónai et al. 2016)
	Slovenia	*M. celatum*	No	(Pate et al. 2011)
		M. celatum, M. fortuitum, M. neoarum, M. terrae	No	(Pate et al. 2016)
White-toothed shrew (*Crocidura suaveolens*)	Czech Republic	*M. chelonae*	No	(Fischer et al. 2000)
Wild boar (*Sus scrofa*)	Croatia	*M. fortuitum, M. vaccae*	No	(Machackova et al. 2003)
		M. chelonae, M. fortuitum	No	(Cvetnić et al. 2011)
	Czech Republic	*M. chelonae, M. gordonae, M. terrae*	No	(Machackova et al. 2003)
		M. abscessus, M. chelonae, M. fortuitum, M. flavescens, M. phlei, M. smegmatis, M. scrofulaceum, M. terrae, M. triviale	No	(Trcka et al. 2006)[Ω]
	France	*M. peregrinum, M. setense, M. vaccae*	/	(Fellag et al. 2019)*
	Germany	*M. gordonae, M. terrae*	NIA	(Machackova et al. 2003)
	Hungary	*M. arosiense, M. bourgelatii, M. fortuitum, M. gordonae, M. intermedium, M. intracellulare, M. nonchromogenicum, M. scrofulaceum, M. sinense, M. vaccae*	**Yes**	(Rónai et al. 2016)
	Italy	***M. fortuitum***	**Yes**	(Serraino et al. 1999)
		M. interjectum, M. scrofulaceum	NIA	(Zanetti et al. 2008)
	Slovakia	***M. fortuitum*, *M. intracellulare***	**Yes**	(Machackova et al. 2003)

Host	Country	NTM species	Lesions found	References
	Slovenia	*M. intracellulare, M. peregrinum*	No	(Pate et al. 2016)
	Spain	*M. interjectum, M. intracellulare, M. scrofulaceum*	NIA	(Gortázar et al. 2011a)
		M. alvei, M. bohemicum, M. chelonae, M. chitae, M. colombiense, M. confluentis, M. elephantis, M. fortuitum, M. flavescens, M. intracellulare, M. kansasii, M. lentiflavum, M. nebraskense, M. palustre M. parmense, M. seoulense, M. thermoresistible	**Yes**	(García-Jiménez et al. 2015)
	Switzerland	*M. bourgelatii, M. celatum, M. colombiense, M. diernhoferi, M. engbaekii,* ***M. florentinum****, M. holsaticum, M. interjectum, M. intermedium, M. intracellulare* subsp. *chimaera,* ***M. lentiflavum****, M. monacense, M. nebraskense,* ***M. neoaurum****,* ***M. nonchromogenicum****, M. peregrinum, M. phlei, M. septicum, M. scrofulaceum, M. vaccae,* ***M. vulneris***	**Yes**	(Ghielmetti et al. 2021)

Unidentified and unclassified NTM are not included in this table. NTM associated with lesions are typed in bold if specified in the study. NIA= no information available. /= not applicable. *Faecal samples; $^{\Omega}$faecal and tissue samples.

Table 4 summarizes the publications related to NTM other than *M. avium* isolated from livestock in different countries of Europe, including the information on clinical and diagnosis findings. With a few more reports (n= 22), the spectrum of NTM species detected in livestock (mainly cattle and pigs) is also remarkable, as well as the number of species causing lesions or interferences in the diagnosis of bovine TB, which clearly indicates that these microorganisms deserve further attention.

Table 4. Non-avium NTM species detected in livestock from Europe.

Host	Country	NTM species	Lesions found	TST-reactor	References
Cattle (*Bos taurus*)	Czech Republic	***M. chelonae****,* ***M. fortuitum****,* ***M. intracellulare****,* ***M. phlei****,* ***M. terrae***	**Yes**	NIA	(Pavlik et al. 2002a)
		M. chelonae, ***M. terrae***	**Yes**	NIA	(Dvorska et al. 2004)
		M. chelonae*,* ***M. phlei****,* ***M. terrae***	**Yes**	Yes	(Pavlik et al. 2005)
	France	***M. terrae***	**Yes**	NIA	(Thorel et al. 1990)

Host	Country	NTM species	Lesions found	TST-reactor	References
		M. bourgelatii	NIA	NIA	(Guérin-Faublée et al. 2013)
		M. chimaera, M. confluentis, M. elephantis, M. flavescescens, M. fortuitum, M. gordonae, M. heckeshornense, M. holsaticum, M. intermedium, M. kansasii, M. komossense, M. monacense, M. neoaurum, M. peregrinum, M. phlei, M. pyrenivorans, M. rutilum, M. scrofulaceum, M. shimoidei, M. smegmatis, M. thermoresistible, M. xenopi, M. vaccae, M. nonchromogenicum, M. terrae	NIA	NIA	(Biet and Boschiroli 2014)
	Hungary	*M. arupense, M. bourgelatii, M. europaeum, M. fortuitum, M. intermedium, M. intracellulare, M. kansasii, M. malmoense, M. nebraskense, M. neoaurum, M. nonchromogenicum, M. palustre, M. peregrinum, M. phlei, M. scrofulaceum, M. shimoidei, M. smegmatis, M. sinense, M. thermoresistible*	**Yes**	Yes	(Rónai et al. 2016)
	Spain	*M. intracellulare*	NIA	NIA	(Gortázar et al. 2011a)
	Switzerland	*M. europaeum, M. hassiacum*,* ***M. kansasii****,* ***M. lymphaticum****,* ***M. persicum****, M. phlei*, M. vaccae**	**Yes**	No	(Ghielmetti et al. 2018)
	UK	*M. nonchromogenicum*	NIA	Yes	(Hughes et al. 1993)
		M. nonchromogenicum¥	/	No	(McCorry et al. 2004)
		M. bohemicum, M. holsaticum, M. kansasii, M. malmoense, M. nonchromogenicum, M. palustre	No	Yes	(Hughes et al. 2005)
		M. kansasii	**Yes**	Yes	(Houlihan 2010)
Goat (*Capra hircus*)	Spain	***M. kansasii***	**Yes**	Yes	(Acosta et al. 1998)
Pig (*Sus scrofa domestica*)	Croatia	*M. chelonae, M. fortuitum, M. peregrinum*	**Yes**	Yes	(Cvetnić et al. 2007)
	Czech Republic/ Slovakia	***M. intracellulare****,* ***M. scrofulaceum****,* ***M. terrae***	**Yes**	/	(Pavlas et al. 1985)
	Czech Republic	***M. chelonae****,* ***M. fortuitum****,* ***M. phlei****,* ***M. terrae***	**Yes**	/	(Pavlik et al. 2003)

Host	Country	NTM species	Lesions found	TST-reactor	References
		M. chelonae, M. fortuitum, M. phlei, M. terrae	**Yes**	Yes	(Pavlik et al. 2005)
		M. chelonae, *M. fortuitum, M. intracellulare,* ***M. scrofulaceum***, *M. terrae, M. smegmatis*	**Yes**	/	(Matlova et al. 2005)
	Finland	***M. palustre***	**Yes**	/	(Torkko et al. 2002)
	Hungary	***M. sinense***	**Yes**	/	(Rónai et al. 2016)
	Netherlands	*M. bohemicum, M. heckeshornense, M. malmoense, M. palustre*	No	/	(van Ingen et al. 2010)
	Norway	*M. bohemicum*$^{\Omega}$, *M. branderi**, *M. celatum*$^{\Omega}$, *M. malmoense*$^{\Omega}$, *M. palustre, M. triviale**	**Yes**	/	(Agdestein et al. 2014)
	Slovenia	***M. celatum***	**Yes**	/	(Pate et al. 2011)

Unidentified and unclassified NTM are not included in this table. TST= tuberculin skin test. NTM associated with lesions and/or reactor animals are typed in bold and/or underlined if specified in the study. NIA= no information available. /= not applicable. *Faecal samples; $^{\Omega}$faecal and tissue samples; $^{¥}$ mucus samples.

Interestingly, many of these reports belong to countries from Central Europe where cattle herd TB prevalence is currently low (0.00-0.05%), (EFSA and ECDC 2021), indicating that the emerging prevalence of these mycobacteria can become a matter of concern particularly in countries reporting low TB incidence.

4. The wildlife-livestock interface. From interspecies interactions to mycobacteria transmission

Interfaces between diverse host communities represent critical spots for cross-species transmission of diseases. This transmission is driven by direct and/or indirect interactions between individuals, which entail close contact between a susceptible individual and an infected one, or which occur when a susceptible individual comes into contact with a contaminated surface or an infected vector, respectively (Triguero-Ocaña et al. 2020a). Interactions enabling the exchange of pathogens at the wildlife-livestock interface have become a matter of study since several circumstances have promoted the

aggregation and space sharing of sympatric wild and domestic species. Livestock industry is the world's greatest land user, either through grazing or through the production of grains and fodder (Jori et al. 2021). This need for land has led to deforestation and habitat degradation, increasing areas of interaction between livestock and wildlife (Jori et al. 2021). In addition to this, consumer demand for improvement of animal welfare has triggered the shift of livestock industry from intensive to extensive farming systems in developed countries, allowing more contacts with wild animals (Jori et al. 2021). Management practices such as baiting or supplemental feeding are widespread among wildlife populations for different purposes, leading to aggregation of animals and potential intra or interspecific disease transmission (Sorensen et al. 2014). The studies dealing with this subject have been performed in different contexts, using diverse methodologies (camera trapping, telemetry, direct observations, questionnaires) and targeting several pathogens (Richomme et al. 2006; Payne et al. 2018; Triguero-Ocaña et al. 2019).

Among the many examples of pathogens exchanged between wildlife and livestock, the members of the MTC are microorganisms of great concern, particularly if TB is already eradicated or under control among the latter (Gortázar et al. 2007). Understanding the interactions taking place between wildlife and livestock is therefore a key for the identification of places, moments and circumstances that may entail highest risk for MTC interspecies transmission. Besides, when this information is combined with other epidemiological data, the assessment of this risk becomes more accurate and the design of control strategies can be better adapted to each context.

Several works have investigated how interaction patterns between wildlife and livestock may influence TB transmission all around the world, being most of them performed in TB infected areas and mainly focused in those wild species already

recognized as competent hosts of the disease (i.e., wild boar, deer and badger). Overall, indirect contacts occurring as a result of resource sharing are frequently described, while direct interactions are anecdotally reported. For instance, in south-central Spain, the transmission of MTC at this interface is thought to be influenced by the shared use of water and food sources between wild boar, red deer and cattle and pigs raised in extensive systems, which seems to depend on seasonal food availability and weather conditions (Kukielka et al. 2013; Barasona et al. 2014; Carrasco-Garcia et al. 2015; Triguero-Ocaña et al. 2019). More recently, another study in the same area evidenced the presence of cattle, pigs, sheep, goats, wild boar and red deer licking on mineral blocks, with rare overlap between wildlife and livestock (Martínez-Guijosa et al. 2021). However, with the record of 21 wildlife visits, the authors stated that this supplementation was less attractive to wildlife than the previously studied water points. In northern Spain, one study on GPS-collared badgers revealed the preference of this mustelid for apple orchards and paddocks used by TB-positive herds (Acevedo et al. 2019), while another one reported wild boar visits to farm facilities in order to feed from calf feeders (Balseiro et al. 2019). In a TB infected area of France, wild boar, red deer and badger visits to cattle farm facilities were analysed, showing that these were more frequent around water sources, salt licks and feed troughs placed in pastures, respectively (Payne et al. 2016). Later on, interactions between these three wild species were studied on baited places and waterholes in the same area, concluding that these resources could promote TB transmission through intra and interspecies interactions (Payne et al. 2017). The picture obtained from the interactions between badgers and cattle in the UK and ROI is more diverse. While some studies reported a clear avoidance of several types of farmyards by badgers, a low frequency of troughs use or a preference for pastures and for land more than 50 m apart from cattle (O ’mahony 2014;

Mullen et al. 2015; Woodroffe et al. 2016), others described a high frequency of badger incursions into farm buildings, being the animals recorded in feed stores and livestock housing and showing nose-to-nose contact with cattle (Tolhurst et al. 2009; Judge et al. 2011). In some cases, the risk of TB transmission was not linked to resource sharing between badgers and cattle, but to badgers latrines (Drewe et al. 2013). In the USA, interactions between white-tailed deer, raccoon (*Procyon lotor*) and Virginia opossum (*Didelphis virginiana*) were also associated with cattle-related resources such as stored feed or uneaten feed left in pastures (Berentsen et al. 2014; Lavelle et al. 2015, 2016).

Indirect contacts between wildlife and livestock may also favour interspecies spread and transmission of infections caused by NTM through environmental contamination (Daniels et al. 2003). However, NTM prevalence in wild and domestic animals populations should be investigated first.

5. The current scenario of northern Iberian Peninsula

Low bovine TB prevalence areas deserve specific attention at least for two reasons: On the one hand, when the disease is almost eradicated in livestock, the role of wild hosts in its maintenance and transmission may become more relevant (Richomme et al. 2013). On the other hand, as the prevalence in livestock falls, TB misdiagnosis due to NTM infections may increase, contributing to farmers' distrust in relation to *ante mortem* tests as described previously (Ciaravino et al. 2020).

In many regions from northern Iberian Peninsula, TB prevalence in cattle herds has been kept below one per cent over the last years (Direção Geral de Alimentação e Veterinária 2019; Ministerio de Agricultura Pesca y Alimentación 2021). However, eradication has not been achieved yet, and some of the regions even harbour hot-spot TB areas (Acevedo et al. 2019). Individuals infected with MAC have been detected also

in some northern areas in the course of the National Bovine Tuberculosis Eradication Programme among TST-reactor cattle (Balseiro et al. 2011a; Muñoz-Mendoza et al. 2013). Other livestock species or wild animals cohabiting with cattle and infected with mycobacteria could be contributing to the spread of these microorganisms and consequently to the emergence of new bovine TB outbreaks or reactor individuals among cattle infected with NTM.

In comparison to high TB prevalence areas from south-central Iberian Peninsula, where wild boar, red deer and fallow deer are considered wild reservoirs, research on MTC in wild mammals from northern Iberian Peninsula is scarce. Badger is considered a potential wild reservoir of TB in Asturias (north-western Atlantic Spain). There, this mustelid has been the focus of most of the research conducted on wild species in relation to TB, with reports on MTC isolation, gross pathology, histopathology, immunology and ecology (Balseiro et al. 2011b, 2013; Acevedo et al. 2019; Blanco Vázquez et al. 2021). However, we cannot assess if this mustelid could be relevant in other northern regions. In the most recent study, Blanco Vázquez and collaborators detected an overall prevalence and seroprevalence of 4.23% and 23.77% respectively after a 13-year survey. They confirmed that badger TB status was spatiotemporally associated with cattle TB status, indicating that both hosts may exert infection pressure on each other. In the same study, a trend towards increasing TB cases in cattle and badgers from eastern areas was observed and mostly associated with bovine TB hot-spots as well as with active badger TB surveillance. Regarding wild boar, there are records on TB infection in Atlantic (north-west) and Mediterranean (north-east) regions (Muñoz-Mendoza et al. 2013; Mentaberre et al. 2014). In accordance with the information reported so far, it is still not clear whether this wild ungulate may be acting as a TB spillover or reservoir. Most of the studies have reported low TB prevalence and

seroprevalence in this host (<5%), as well as a small proportion of individuals with localized macroscopic lesions (Boadella et al. 2011; Muñoz-Mendoza et al. 2013; Gortázar et al. 2017). However, one of those studies reported prevalences ranging from 6.5% to almost 8% at a local scale, or even higher in fenced hunting estates (Gortázar et al. 2017). In a serologic survey on wild boar from Portugal, authors did not detect any positive animal from the northern counties sampled (Santos et al. 2018). A different picture has been seen in a northern Mediterranean area, where higher prevalence (14%), seroprevalence (33.5%) and proportion of animals showing localized and generalized macroscopic lesions (24%) were detected and associated with the improper management of an infected cattle herd (Mentaberre et al. 2014; Pérez de Val et al. 2017). Oddly, a sympatric wild species, the Iberian wild goat (*Capra pyrenaica*), was not affected at all (Mentaberre et al. 2014). More recently, *M. microti*, the causative agent of TB in the field vole (*Microtus agrestis*) and other wild small rodents (Cavanagh et al. 2002; Kipar et al. 2014), has been detected in wild boar from the Catalan Pyrenees (north-eastern Iberian Peninsula) showing lesions compatible with TB (Pérez de Val et al. 2019). All these findings suggest that wild boar could eventually be a relevant species in the epidemiology of TB in northern Iberian Peninsula, at least under certain circumstances. Further studies should be performed to confirm this assumption.

NTM have also been isolated from badger in north-western Atlantic Spain. All the isolates belonged to *Maa* and *Mah* from individuals with or without visible lesions. (Balseiro et al. 2011a, b). Infections with *Maa* and *Mah* have also been detected in wild boar with histological lesions (Muñoz-Mendoza et al. 2013). Macroscopic lesions due to NTM have also been described in this host, but the identification at the species level was not performed (Mentaberre et al. 2014).

Badger and wild boar put aside, records of mycobacteria infections in other wild species from northern Iberian Peninsula are anecdotal and mostly focused on north-western habitats. These include a few reports of TB in red deer, TB or MAC infections in roe deer and fallow deer, and MAC infections in foxes and wild rabbits (*Oryctolagus cuniculus*) (Balseiro et al. 2008, 2009; Maio et al. 2011; Muñoz-Mendoza et al. 2013; Gortázar et al. 2017). Mycobacteria research targeting carnivores other than badger and fox is lacking. This is also the case for small rodents. To the best of our knowledge, the potential spectrum of NTM other than *M. avium* that could be infecting livestock and wildlife is currently unknown.

Regarding interactions at the wildlife-livestock interface, two studies have been published so far, both taking place in Asturias. In one study the authors described outcomes of TB in cattle and badger and focused on badger's spatial ecology in relation to TB transmission between these two species (Acevedo et al. 2019). The main findings indicated the preference of badger for apple orchards and paddocks used by TB-positive herds. In the other study, the effectiveness of a calf-selective feeder to prevent wild boar access was tested through camera trapping in a farm where wild boars approached to feed (Balseiro et al. 2019). The feeder was proved to hinder the access of wild boar and therefore to reduce the indirect contacts between this wild ungulate and cattle.

All this reveals a significant gap of knowledge on the role, if any, of wild mammals from northern Iberian Peninsula in the epidemiology of mycobacteria.

OBJECTIVES

This PhD thesis aims to contribute to drawing the epidemiological picture of mycobacterial infections in low bovine TB prevalence regions of northern Iberian Peninsula by investigating a broad community of wild species from a region not previously studied, the Basque Country. This will be addressed by focusing on two epidemiological aspects: On the one hand, the mycobacterial infection and/or exposure figures found in the wild hosts surveyed and the comparison of these findings with those related to livestock. On the other hand, the interactions between cattle and wildlife that could lead to interspecies transmission of mycobacteria.

The **main objective** of this work was to study the role of wildlife in the epidemiology of TB and other mycobacterial infections in the Basque Country, a low bovine TB prevalence region (<0.1%) located in northern Iberian Peninsula, and to describe the potential pathways for mycobacteria transmission between cohabiting wild species and cattle in this area.

This main objective is divided into three **specific objectives** that will be addressed throughout the five studies included in the "Studies" section:

1. To deep into the role of wildlife in the epidemiology of TB in the Basque Country (Studies I, II and III).
2. To characterize the diversity of NTM harboured by wild and domestic species, as well as to describe their potential implications on animal health and on the diagnosis of bovine TB (Studies III and IV).
3. To assess the interactions that take place between cattle and wildlife in relation to mycobacteria transmission (Study V).

STUDIES

Study I

Risk factors associated to a high *Mycobacterium tuberculosis* complex seroprevalence in wild boar (*Sus scrofa*) from a low bovine tuberculosis prevalence area

This study has been published as:

Varela-Castro L, Alvarez V, Sevilla IA, Barral M (2020). Risk factors associated to a high *Mycobacterium tuberculosis* complex seroprevalence in wild boar (*Sus scrofa*) from a low bovine tuberculosis prevalence area. PLOS ONE 15(4): e0231559.

Parts of this study have been presented at:

13th European Wildlife Disease Association Conference, Larissa, Greece, 27-31 August 2018: Varela-Castro L, Alvarez V, Martinez de Egidua M, Sevilla IA, Barral M. Temporal and spatial distribution of antibodies against *Mycobacterium bovis* in wild boar (*Sus scrofa*) in the Basque Country (Northern Spain). Oral presentation.

Workshop and Conferences for PhD candidates in Environmental Sustainability INGURU-DOK, Plentzia, Basque Country, 19 October 2018: Varela-Castro L, Alvarez V, Martinez de Egidua M, Sevilla IA, Barral M. The *Mycobacterium tuberculosis* complex at the wildlife-livestock interface in the Basque Country: Deepening into the epidemiology of tuberculosis. Oral presentation.

Abstract

Animal tuberculosis (TB) is a worldwide zoonotic disease caused principally by *Mycobacterium bovis,* a member of the *M. tuberculosis complex* (MTC). In southern Iberian Peninsula, wild reservoirs such as the wild boar, among other factors, have prevented the eradication of bovine TB. However, most of the studies have been focused on south-central Spain, where the prevalence of TB is high among wild ungulates and cattle herds. In northern regions, where wild boar density and bovine TB prevalence are lower, fewer studies have been carried out and the role of this species is still under debate. The aim of this study was to describe the temporal and spatial distribution of antibodies against MTC in wild boar from the Basque Country, northern Spain. Sera from 1902 animals were collected between 2010 and 2016. The seroprevalence was determined with an in house enzyme-linked immunosorbent assay and the search of risk factors was assessed by Generalized Linear Models. Overall, 17% of wild boars (326/1902; 95%CI, [15.5%–18.9%]) showed antibodies against MTC. Risk factors associated with seropositivity were the year and location of sampling, the number of MTC positive cattle, the distance to positive farms and the percentage of shrub cover. Younger age classes were associated with increased antibody titres among seropositive individuals. The seroprevalence detected was higher than those previously reported in neighbouring regions. Hence, further studies are needed to better understand the role of wild boar in the epidemiology of TB in low TB prevalence areas and consequently, its relevance when developing control strategies.

Keywords: *Mycobacterium bovis*, wild boar, ELISA, antibodies, epidemiology

1. Introduction

Animal TB is a worldwide zoonotic disease caused principally by *M. bovis*, a member of the MTC that infects a wide range of domestic and wildlife species (Santos et al. 2012). Because of its impact on public health and economic losses in livestock industry, eradication programmes in cattle have been implemented in Europe through the last decades (Gortázar et al. 2012). Meanwhile, the increase of wild ungulates populations reported in Europe results in biodiversity reduction and the increment of competent hosts for many diseases, including animal TB (Borowik et al. 2013; Massei et al. 2015; Lewis et al. 2017). This change comes partially from the absence of predators, which could potentially contribute to both wild ungulates populations and diseases control. The appearance of habitats suitable for wild ungulates due to increased food availability and rural abandonment may also favour this tendency (Massei et al. 2015; Lewis et al. 2017; Tanner et al. 2019). Thus, the implication of wild reservoirs, among other factors, has prevented the complete eradication of bovine TB in many countries (Schiller et al. 2010). Some recognized examples are the Eurasian wild boar and the red deer in the Iberian Peninsula (Gortázar et al. 2012). Moreover, other ungulates and carnivores seem to play a role in the epidemiology of bovine TB in this territory, either as spillovers, such as the red fox, the roe deer and the Iberian lynx; or as potential reservoirs, such as the fallow deer or the Eurasian badger (Peña et al. 2006; Millán et al. 2008; Balseiro et al. 2009, 2011b; Gortázar et al. 2011b). Together with the domestic hosts, including goats (Samper et al. 1995), sheep (Muñoz-Mendoza et al. 2015) and pigs (Parra et al. 2003), as well as the main and most well studied host, cattle (Cousins 2001), we are facing a multi-host pathogen system, where *M. bovis* persistence and transmission depends on several factors, such as the high resistance of this agent in the environment, the density of hosts and species interactions (Renwick et al. 2007), a

scenario most likely applicable to other members of the MTC like *M. caprae* and *M. microti*. Nevertheless, many evidences point to the wild boar as the most important wild reservoir within some Mediterranean epidemiological contexts (Naranjo et al. 2008), bearing in mind that domestic reservoirs (e.g. goats) might be even more relevant than this wild species (Napp et al. 2013). Besides, its opportunistic omnivorous diet and its capacity of living in a huge variety of habitats (Yamamoto 2007) turn this ungulate into an obstacle for bovine TB control strategies when its population is infected. However, the role of this host in the epidemiology of animal TB can vary from one country to another, or even between regions of the same country, since it will not only depend on the species characteristics, but also on the environment and the probability of interacting with other susceptible individuals (Nugent 2011). In the Iberian Peninsula, most of the studies performed on the epidemiology of animal TB in wild boar are focused on south-central Spain, where artificial management of game species has also increased their density and aggregation (Vicente et al. 2007). Moreover, the prevalence of TB is high among wild ungulates (Gortázar et al. 2011b) and cattle herds (Ministerio de Agricultura Pesca Alimentación y Medio Ambiente 2019) inhabiting this area. However, in northern Atlantic and Mediterranean regions, where wild boar density and aggregation are lower, as well as the TB prevalence among cattle herds (< 1%) (Ministerio de Agricultura Pesca Alimentación y Medio Ambiente 2019), fewer studies have been carried out and the research related to the role of wild boar is currently ongoing (Muñoz-Mendoza et al. 2013; Mentaberre et al. 2014; Gortázar et al. 2017). So far, whether this wild ungulate may act as a spillover or a reservoir is still under debate in northern Spain (Muñoz-Mendoza et al. 2013; Mentaberre et al. 2014). Hence, an increase of research is required in order to obtain a bigger picture of the understudied

low TB prevalence areas, since the relevance of wild boar may increase as the prevalence in livestock decreases (Richomme et al. 2013).

Therefore, this study aimed to increase the body of knowledge on animal TB epidemiology by describing the temporal and spatial distribution of antibodies against MTC in wild boar from a low bovine TB prevalence area, as well as to identify risk factors associated to the likelihood of having contact with the bacterium.

2. Materials and Methods

2.1 Ethics statement

Serum samples used in this study were obtained by competent local authorities from legally hunted wild boars or from wild boar carcasses found in the field, in complete agreement with Spanish and European regulations. No animals were killed specifically for this study. No ethical approval was deemed necessary.

2.2 Study area

This study was carried out in the Basque Country, northern Spain. This area covers 7234 km² and it is divided into three provinces (Araba, Bizkaia and Gipuzkoa), according to political and administrative criteria. In northern provinces an Atlantic climate predominates with mild winters and high precipitations. In the south, there is a Continental Mediterranean climate with hot summers and cold winters (Muñoz et al. 2010). Habitats also differ, being pine forests (mainly *Pinus radiata*) more common in the north and deciduous forests (dominated by *Fagus sylvatica* and *Quercus faginea*) alternated with pastures and crops in the south. Scrublands represent almost the ten per cent of the surface of the Basque Country, being distributed throughout the whole territory (Departamento de Desarrollo Económico e Infraestructuras 2018). The

prevalence of bovine TB among cattle herds from the Basque Country was less than 0.1 per cent in 2017, remaining close to official eradication (Ministerio de Agricultura Pesca Alimentación y Medio Ambiente 2019). On the other hand, the management of wild boar populations in this area does not imply artificial interventions such as fencing or feeding, but mostly relies in hunting activities within certain game preserves.

2.3 Wild boar sampling

Serum samples from 1902 wild boars belonging to 185 out of 247 hunting areas were collected during 2010-2016 in the context of a wildlife health serological surveillance programme in the Basque Country (Figure 1). Most of the animals (89.6%) were shot by authorized hunters during the regular hunting season (October to February) and sera were obtained in the field. Almost nine per cent of the serum samples were obtained from wild boar's population control programmes where animals were trapped and put down by competent authorities. A smaller proportion of sera were collected from animals with not recorded cause of death (1.5%) or from carcasses of run over animals (0.05%). Serum samples were mainly obtained by intracardiac puncture or intracavernous venipuncture, individually identified and stored at -20°C until processing.

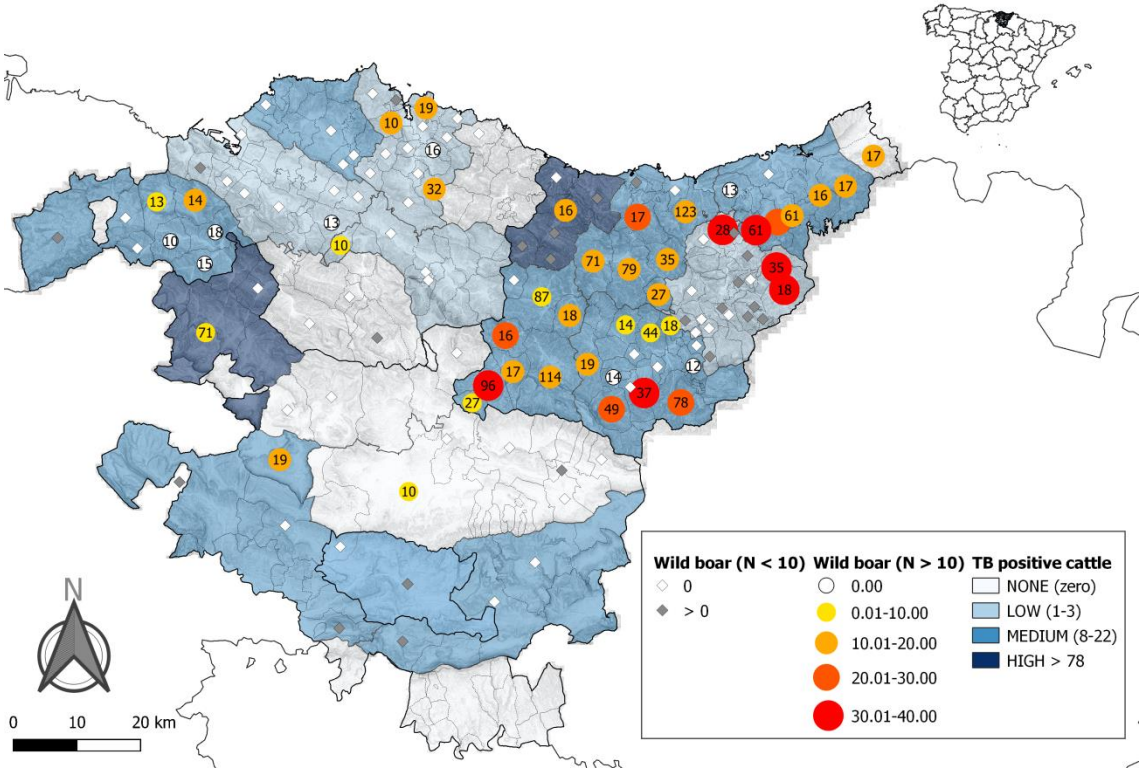

Figure 1. Spatial distribution of MTC seroprevalence (%) detected in wild boar and bovine TB positive cattle. Dot sizes and intensity of colour increase with the seroprevalence detected in municipalities where more than 10 wild boars were analysed. Labels inside these dots indicate the number (N) of animals analysed. Rhombuses indicate municipalities where less than 10 wild boars were analysed. Those in white mean they were negative and greys mean at least one animal was positive. Blue fill colour intensity increases with the number of TB positive cattle detected in each region: No (zero), low (1-3), medium (8-22) and high (> 78).

2.4 Serological assay

The presence of IgG antibodies against MTC was determined by using an in house enzyme-linked immunosorbent assay (ELISA) previously validated for wild boar, following the protocol previously described (Aurtenetxe et al. 2008). The control sera were the same used for the validation of this assay. All samples were analysed in duplicate. Optical densities (OD) were determined at 405 and 450 nm (MultiskanFC, ThermoScientific). $OD_{450\ nm}$ was subtracted from $OD_{405\ nm}$ and the results were expressed as an ELISA index (EI), calculating the ratio between the resulting mean

sample OD and the mean OD of the positive control. Samples with an EI ≥ 0.200 were considered positive.

2.5 Database

2.5.1 Wild boar data

Whenever it was possible, data of each wild boar such as sex (male; female), age (piglet < 1 year; yearling between 1 and 2 years; adult > 2 years), date and geographic location of collection (province, region and municipality) were recorded. Age of the animals was determined based on the sex, weight and tooth eruption patterns.

2.5.2 Livestock data

According to the last official 2009 census obtained from the Basque Statistics Institute (Eustat-Gobierno Vasco), there are about 136246 cattle in 5930 farms, 272167 sheep in 4539 farms and 21547 goats in 1605 farms in the study area. Attending to these data, variables based on livestock density (number of cattle-sheep-goats/Km^2) were calculated at region level. The whole livestock censuses of the Basque Country were taken into account for these estimations, because farms are not closed, biosafety measures are lacking and animals can remain in pastures regardless of the management system, allowing for potential direct or indirect contacts with animals outdoors, including wildlife.

2.5.3 MTC positive cattle

According to the information obtained from the Spanish Database of Animal Mycobacteriosis (mycoDB) (Rodriguez-Campos et al. 2012), 304 MTC-infected cattle were detected by official diagnostic methods and/or inspection at slaughter and confirmed by culture in the Basque Country between September 2009 and July 2017.

Herd prevalence and incidence of new positive herds during this period were highest in 2009 (0.57 and 0.55%, respectively) and lowest in 2017 (0.09 and 0.07%, respectively) according to the reports of the National Bovine TB Eradication Programme (Ministerio de Agricultura Pesca Alimentación y Medio Ambiente 2019). Taking advantage of these data, the amount of positive cattle per region was calculated and classified according to the number of positive cows detected (zero, low, medium, high).

In addition, the Euclidean distance from each wild boar to the nearest positive cattle herd was calculated. Because of the lack of information on the exact location of each wild boar, the centroid of every municipality of sampling was used. As for the positive cows, the finest scale available was also used, being the farm's UTM coordinates in 220 cases, the centroid of the village in 47 cases and the centroid of the municipality in 37 cases. The software QGIS Valmiera v2.2.0 (Quantum GIS Development Team 2014) was used for this spatial analysis.

2.5.4 Hunted wild boar

Counts of hunted wild boar within each hunting season and game preserve were obtained from the Provincial Councils. These counts were transformed into a measure of relative abundance (hunted wild boar per km²) (Acevedo et al. 2014; Keuling et al. 2018), only taking into account the habitable surface for this wild species within each game preserve, which was assessed with the software QGIS Valmiera v2.2.0 (Quantum GIS Development Team 2014).

2.5.5 Vegetation cover

The vegetation cover was obtained from the 2016 Forest inventory map of the Spatial Data Infrastructure of the Basque Country (GeoEuskadi-Gobierno Vasco) and

from the 2006 Spanish forestry map of the Nature Databank (BDN-MITECO). The vegetation cover of interest was reclassified into six categories: “pine forest”, “deciduous forest”, excluding the beech forests from this category due to their lack of undergrowth; “oak forest”, “beech forest”, “scrubland” and “pastures and crops” (Acevedo et al. 2006; Yamamoto 2007; Acevedo et al. 2014). An intersection between the surface of each municipality where every wild boar was hunted and the reclassified vegetation cover was created and the percentage of each vegetation category was calculated for every municipality. The software QGIS Valmiera v2.2.0 (Quantum GIS Development Team 2014) was used for this spatial analysis.

2.6 Statistical analysis

Two Generalized Linear Models (GLM) were implemented. The first model included 1811 wild boars and was adjusted to a binomial distribution and a logit link function, using the ELISA results (binomial variable: positive or negative) as the response variable. Then, a second model was built with a subset of positive wild boars (N = 168), using the antibody titres (continuous variable) as the response variable. This model was adjusted to a gamma distribution and a log link function. Before the implementation of these models, the normality of data was checked with the Kolgomorov-Smirnov test and several univariate analyses were performed between the response and the explanatory variables (N = 17) in order to identify potential risk factors. Non-parametric Mann-Whitney tests were used between continuous and categorical variables with two levels, while non-parametric Kruskal-Wallis tests were used when categorical variables had more than two levels; Chi-Square Tests were used between categorical variables and GLM adjusted to a gamma distribution and a log link function were used between continuous variables. In all tests, significance was set at $p < 0.05$. Explanatory variables for which $p < 0.25$ at the univariate analysis and that were

correlated by less than 0.7 were considered for inclusion in the models (Dormann et al. 2013). Finally, a manual bidirectional stepwise strategy was used to select the final models. First, the two models were built including all the selected predictors. Those predictors showing a non-significant association with the response variables were sequentially excluded from each model. Confounding variables were assessed by checking for changes in the regression coefficients when removing any variable. If changes were higher than 20%, the variable was included again in the model, otherwise it was definitely removed. The Akaike Information Criteria (AIC) and the percentage of explained deviance were taken into account when selecting the final models. All the statistical analyses were performed using the R Software 3.5.0 (R Development Core Team 2018). The data set employed for the statistical analyses is deposited in a public repository (Varela-Castro et al. 2020a).

3. Results

Overall, 17% of wild boars (326/1902; 95%CI, [15.5%–18.9%]) showed antibodies against MTC. In Figure 1, the spatial distribution of the seroprevalence detected in wild boar among the municipalities of the Basque Country is shown, as well as the spatial distribution of the MTC-positive cattle during the same period at a regional scale. The highest seroprevalences in wild boars were mainly observed in municipalities from the east of the study area, within the province of Gipuzkoa.

Results obtained after the univariate analysis are shown in Table 1 (categorical variables) and Table 2 (continuous variables) taking into account seroprevalence data (positive and negative wild boars). On the other hand, the distribution of EI values among positive wild boars according to the categorical and continuous variables are described in Table 3 and Figure 2, respectively. In these four images, p-values of each

univariate analysis are shown as a previous step for the selection of variables included in the binomial and gamma models.

Table 1. Seroprevalence of MTC detected in wild boars according to categorical variables.

Categorical variable	N. tested	% positives (95% CI)	p-value
Sex			0.513
Female	757	12.8 (10.6-15.4)	
Male	679	14.0 (11.6-16.8)	
Age			0.380
Piglet	217	15.7 (11.4-21.1)	
Yearling	438	12.1 (9.4-15.5)	
Adult	565	14.5 (11.8-17.7)	
Sampling year			**< 0.001***
2010	138	23.2 (16.9-30.9)	
2011	190	23.2 (17.7-29.7)	
2012	128	22.7 (16.3-30.6)	
2013	320	25.0 (20.6-30.0)	
2014	571	13.3 (10.8-16.3)	
2015	323	13.0 (9.8-17.1)	
2016	232	9.9 (6.7-14.4)	
Season			0.330
Spring	72	13.9 (15.0-19.4)	
Summer	108	23.1 (7.7-23.7)	
Autumn	1124	17.1 (16.2-31.9)	
Winter	598	16.6 (13.8-19.7)	
Positive cattle/region			**0.002***
Zero (0)	66	9.1 (4.2-18.4)	
Low (1-3)	392	23.2 (19.3-27.6)	
Medium (8-22)	1354	15.7 (13.8-17.7)	
High (> 78)	58	17.2 (9.6-28.9)	
Province			**< 0.001***
Araba	94	11.7 (6.7-19.8)	
Bizkaia	297	6.7 (4.4-10.2)	
Gipuzkoa	1511	19.5 (17.6-21.6)	

"*" indicates a significant association between the response and the explanatory variable at the univariate analysis ($p < 0.05$). P-value in bold type indicates variables included in the binomial model (after excluding correlated variables). The number of positive cattle per region was categorized as follows: zero (0), low (1-3), medium (8-22) and high (> 78).

Table 2. Descriptive statistics of the continuous variables' values among positive and negative wild boars.

Continuous variable	ELISA POSITIVE		ELISA NEGATIVE		
	N	**Median (IQR)**	**N**	**Median (IQR)**	**p-value**
Hunted wild boar/km^2	295	0.7 (0.5-1)	1503	0.8 (0.4-1)	**0.105**
Distance to positive cattle (km)	296	7.5 (3.3-9.4)	1515	6.1 (2.2-9.4)	**0.090**
Pine forest (%)	296	36.0 (27.8-45.7)	1515	36.0 (27.3-48.0)	0.597
Pastures & crops (%)	296	18.6 (14.6-22.2)	1515	18.6 (14.5-24.4)	0.686
Oak forest (%)	296	7.7 (2.9-12.2)	1515	5.6 (2.9-11.5)	0.789
Deciduous forest (%)	296	9.7 (3.8-16.5)	1515	7.6 (4.2-15.8)	0.676
Beech forest (%)	296	6.2 (2.5-16.9)	1515	5.0 (1.3-13.2)	**0.001***
Shrubs (%)	296	5.6 (4.7-8.9)	1515	4.8 (3.8-7.7)	**0.003***
Cattle/km^2	318	22.9 (16.0-32.1)	1545	18.0 (13.8-42.6)	0.551
Sheep/km^2	318	75.9 (56.8-90.8)	1545	64.3 (36.8-90.8)	**<0.001***
Goats/km^2	318	2.7 (2.1-2.7)	1545	2.7 (2.1-3.5)	**0.051**

"*" indicates a significant association between the response and the explanatory variable at the univariate analysis ($p < 0.05$). P-value in bold type indicates variables included in the binomial model (after excluding correlated variables). IQR = Interquartile Range.

Table 3. Descriptive statistics of the ELISA index of the positive wild boars according to categorical variables.

Categorical variable	ELISA index ≥ 0.200		p-value
	N	Median (IQR)	
Sex			0.897
Female	97	0.267 (0.234-0.389)	
Male	95	0.274 (0.229- 0.380)	
Age			**0.001***
Piglet	34	0.284 (0.249-0.523)	
Yearling	53	0.289 (0.241-0.500)	
Adult	82	0.244 (0.217-0.313)	
Sampling year			**0.241**
2010	32	0.257 (0.223-0.354)	
2011	44	0.274 (0.228-0.318)	
2012	29	0.279 (0.255-0.385)	
2013	80	0.271 (0.236-0.425)	
2014	76	0.283 (0.238-0.514)	
2015	42	0.272 (0.227-0.334)	
2016	23	0.261 (0.228-0.302)	
Season			0.505
Spring	10	0.274 (0.229-0.291)	
Summer	25	0.271 (0.237-0.369)	
Autumn	192	0.270 (0.228-0.379)	
Winter	99	0.284 (0.239-0.395)	
Positive cattle/region			**0.009***
Zero (0)	6	0.240 (0.230-0.254)	
Low (1-3)	91	0.376 (0.240-0.436)	
Medium (8-22)	212	0.274 (0.227-0.367)	
High (> 78)	10	0.229 (0.210- 0.253)	
Province			**0.009***
Araba	11	0.228 (0.218-0.239)	
Bizkaia	20	0.263 (0.226-0.297)	
Gipuzkoa	295	0.277 (0.234-0.400)	

"*" indicates a significant association between the response and the explanatory variable at the univariate analysis ($p < 0.05$). P-value in bold type indicates variables included in the gamma model (after excluding correlated variables). The number of positive cattle per region was categorized as follows: zero (0), low (1-3), medium (8-22) and high (> 78).

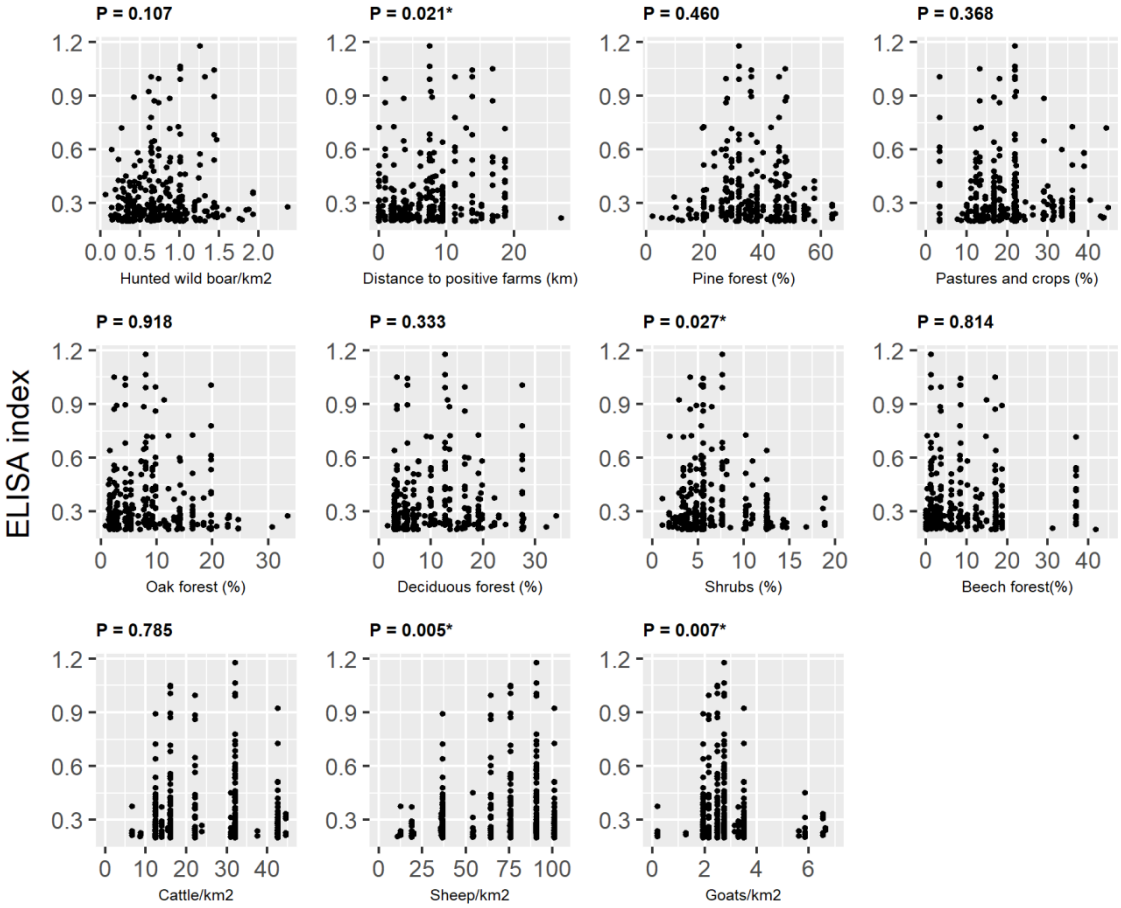

Figure 2. Descriptive statistics of the ELISA index of positive boars according to continuous variables. "*" indicates a significant association between the response and the explanatory variable at the univariate analysis ($p < 0.05$). Variables with a p-value lower than 0.25 were included in the gamma model.

The final (binomial) model explained 7% of the deviance (AIC = 1531.4). The results indicate that the probability of being positive for a wild boar changed over the sampling years, being significantly higher during the first years of the study period (2010-2013) when comparing with 2014 (Table 4). Hereafter, this probability began to decrease until the end of the study period, even though this change was not significant (2015-2016). As for the positive cattle, wild boars had a higher probability of being positive in regions where MTC-positive cattle were detected, compared to those where cattle were negative. Nevertheless, this increase was only significant in those regions with a low number of cattle outbreaks (Table 4). Moreover, a higher probability of

being seropositive was associated with the increase of the distance to MTC positive farms (Table 4). The sampling province was also associated with the probability of being positive. This probability was higher in Araba and Gipuzkoa, compared to Bizkaia (Table 4). Lastly, a higher probability of being positive was also observed with the increase of the percentage of shrub (Table 4). With regard to the analysis of the continuous variable, the gamma model (11% of explained deviance and AIC = -176.32) showed that piglets and yearlings were significantly associated with an increase of the EI, when comparing with adults (Table 4).

Table 4. Results of the Generalized Linear Models.

Response variable	Predictor	Level	OR (95%CI)	Estimate	P-value
ELISA results (binomial. N = 1811)	Intercept	-	**0.01 (0.00-0.03)**	-4.68	**<0.001**
	Positive cattle/region	Zero [a]	1	NA	NA
		Low	**3.28 (1.19-9.02)**	1.19	**0.021**
		Medium	1.44 (0.54-3.85)	0.36	0.468
		High	1.64 (0.51-5.26)	0.49	0.409
	Distance to TB positive farms (km)	-	**1.04 (1.02-1.07)**	0.04	**0.002**
	Percentage of shrubs	-	**1.05 (1.01-1.09)**	0.05	**0.011**
	Year of sampling	2010	**1.79 (1.08-2.98)**	0.58	**0.025**
		2011	**2.43 (1.54-3.85)**	0.89	**<0.001**
		2012	**2.41 (1.44-4.05)**	0.88	**<0.001**
		2013	**2.12 (1.44-3.13)**	0.75	**<0.001**
		2014 [a]	1	NA	NA
		2015	1.09 (0.71-1.67)	0.09	0.690
		2016	0.71 (0.42-1.19)	-0.34	0.196
	Provinces	Bizkaia [a]	1	NA	NA
		Araba	**4.30 (1.66-11.12)**	1.46	**0.003**
		Gipuzkoa	**5.70 (3.28-9.93)**	1.74	**<0.001**
ELISA index (gamma. N = 168)	Intercept	-	-	-1.21	**<0.001**
	Age	Adult [a]	-	NA	NA
		Yearling	-	0.33	**<0.001**
		Piglet	-	0.27	**0.012**

Significant values are written in bold letters. NA = Not applicable. "[a]" indicates the reference level selected for each categorical variable to run the model.

4. Discussion

The research on the epidemiology of animal TB in wild boar populations is quite scarce in the north of the Iberian Peninsula when comparing to the south. For this reason, this study was necessary to obtain a wider perspective of the epidemiology of TB in wild boars from low bovine TB prevalence Atlantic areas. The ELISA test is considered a useful tool when developing a first screening in wildlife, because of its speed, ease of use and relatively low cost (Che' Amat et al. 2015; Pérez de Val et al. 2017). The application of this method to the 1092 wild boar sera collected in this area revealed an overall seroprevalence (17%) unexpectedly higher than that detected in neighboring regions from northern Atlantic Spain (<5%) (Boadella et al. 2011; Muñoz-Mendoza et al. 2013). This suggests that the role of wild boar in the epidemiology of TB in northern Spain may be more relevant than it was expected. Despite this, the tendency observed throughout the study period points to a general drop of the seroprevalence, even though the lowest one detected in this survey (9.9% in 2016) is still high compared to data from the aforementioned studies. Several factors may have triggered this decreasing trend in TB seroprevalence in wild boar, but it could be related to the general drop of TB herd prevalence seen in cattle during the same period (from 0.37% in 2010 to 0.17% in 2016) (Ministerio de Agricultura Pesca Alimentación y Medio Ambiente 2019). On the other hand, considering that in a previous study from northern Spain MAC isolates were recovered from wild boar tissues in a higher proportion than MTC isolates (Muñoz-Mendoza et al. 2013) and being aware of the antigenic repertoire similarities found between different species of this genus, some cross-reactivity with other NTM cannot be completely excluded. Infection with members of the MTC other than *M. bovis* like *M. caprae* or *M. microti* is also detectable using PPD-B-based ELISAs (Beerli et al. 2015). For these reasons, further research

including not only serology, but also confirmatory microbiological culture and species identification are needed to better assess the significance of different mycobacterial infections in wild boar from this region. In any case, given the high specificity attributed to this ELISA test in its validation with field samples (Aurtenetxe et al. 2008), we think that the involvement of false positive results would minimally change these figures.

In the binomial model, a higher seroprevalence was found in regions where bovine outbreaks were detected, suggesting a potential risk of transmission at the wild-domestic interface. However, this increase was only significant when the amount of positive cattle was low. This could be due to the fact that interspecies interactions are not the only factor involved in the circulation and/or transmission of the bacterium. Actually, intraspecies interactions are often more common (Cowie et al. 2016; Payne et al. 2017), but this is influenced by each epidemiological scenario. In our study area, most of the seropositive animals were detected in Gipuzkoa, a province where wild boars showed also the highest antibody titres. This could be due to a higher dissemination of bacteria among wild boar. Therefore, despite a bacterial circulation between cattle and wild boars cannot be dismissed, wild boar intraspecies transmission might have a more relevant role in our study area and period. However, the seroprevalences observed in some municipalities suggest that wild populations could still represent a threat in terms of TB transmission and maintenance. Thus, more studies are needed to determine the mycobacteria species and spoligotypes circulating in wild boar from this area.

Another factor significantly related to the increase of the seroprevalence was the distance between wild boars and TB positive farms. However, this association showed just the opposite effect of what was expected, since the probability of wild boars being

positive increased with longer distances to the farms. Looking for a pattern at such a fine scale without the exact location of hunted animals could have led to an inaccuracy of the distance data and, consequently, to distort the statistics. Moreover, dichotomizing the EI into a binomial variable results in information loss, due to the inclusion of individuals displaying an index around 0.2 (probably exposure) with those displaying an index around 1 (probably infection) in the same level. This can result in a reduced precision of the odds ratio (OR) (Sroka and Nagaraja 2018). Despite this assumptions, a previous work found that exposure to MTC in wild boar was related to shorter distances between them and TB outbreaks in cattle, using the centroid of the commune of sampling as it was also the finest scale of spatial position available (Richomme et al. 2013). Nevertheless, the statistical approach was different, since it was carried out using a bootstrap method.

Lastly, the percentage of shrub was positively associated with the seroprevalence. Although wild boars can live in different kind of habitats, the shrub cover may be especially attractive from a survival perspective, because it can provide them a good shelter. In northern areas, unlike south-central areas in Spain (Vicente et al. 2007), spatial aggregation of wildlife seems less likely to occur, since wild boar densities are lower and humid habitats prevent wild species overcrowding (Muñoz-Mendoza et al. 2013). Thus, shrub cover may not produce a clear aggregation of wild boars, but it may hinder their movements, forcing them to use the same paths and limiting the excretion of and exposure to the bacterium to their own routes. Hence, it may not be about wild boar aggregation in northern bushy areas, but instead, we could think about a restricted movement capacity along this kind of vegetation as an enhancer of bacterial accumulation in their passages. In addition to this, shrub cover might also provide a moist microhabitat protected from the sun radiation that prompts

mycobacteria survival and persistence in the environment. In the Basque Country, the shrub cover has been gradually increasing through the years, ranging from six per cent of the surface in 1986 to almost 10% during our study period (Departamento de Desarrollo Económico e Infraestructuras 1986, 2018). This change in the vegetation cover seems to be linked with the abandonment of rural areas and thus, with an insufficient maintenance of forests and lands. If this rural abandonment phenomenon does not cease, other measures should be implemented to prevent the numerous problems that can derive from shrub progression, including the formation of potential hot-spots for bacterial persistence.

In the gamma model, it is remarkable that, among positive wild boars, increased antibody titres were mainly observed in yearlings and piglets, compared to adult individuals. It is generally considered that increased antibody titres are associated with more severe forms of TB in many wild species, including the wild boar (Garrido et al. 2011; Chambers 2013). Previous studies have found evidences of severe illness in young animals, rather than in adults, as animals with large lesions in more than one anatomical region were more frequently detected among juveniles (12 to 24 months) (Martín-Hernando et al. 2007), but these findings belong to a different epidemiological context (southern Spain). In another study, there was a decrease in the proportion of lesions from which mycobacteria could be isolated with increasing age (Corner et al. 1981). The social behaviour of this wild species might also explain this difference among age classes. Adult females and their young live in groups and maintain close contact, favouring exposure by different routes. Piglets may not only suckle from their own mother, if other sows have given birth at the same time (Yamamoto 2007), increasing their chances of exposure or even of acquiring an infection. Adult males, conversely, have a solitary lifestyle, reducing their chance of contact with other wild

boars out of the mating season (Yamamoto 2007) and, consequently, their risk of exposure to MTC. On the other hand, piglets and yearlings have had less time in their life to get in contact with the bacterium than the adults, and a detectable immune response needs time to develop after bacterial exposure (Pérez de Val et al. 2017). We expected to have higher antibody titers amongst adults than amongst younger boars, as it has been suggested that recent infections in younger age-class might cause lower antibody levels and lower ELISA sensitivity (Che' Amat et al. 2015). In spite of this, the same study reported a seroprevalence of 29.3% (95% CI 21.3–37.2) amongst 2–6 month-old piglets with or without visible lesions and, interestingly, the antibody levels detected by the PPD-B ELISAs did not correlate with the lesion score (Che' Amat et al. 2015). Based on the aforementioned studies, one hypothesis could be that part of adult individuals were exposed to the bacterium when they were younger but managed to control or even to clear the infection, and at the moment of hunting their immune response to an old contact or infection was less intense. Or it could be simply that reaching adulthood with progressive disease is less probable under the conditions of this area. Nevertheless, considering that the detection of higher antibody titres could be related to more extended lesions and, consequently, to higher excretion of mycobacteria (Chambers 2013), the dispersal behaviour of the yearlings (Sáez-Royuela and Tellerìa 1986) might be considered a factor that could easily contribute to the geographical spread of MTC.

The seroprevalence observed in our survey was higher than that reported earlier in other northern areas, suggesting that the spillover role of wild boar in these regions might change at any time and become more relevant, if the appropriate factors are given (Mentaberre et al. 2014). Hence, in areas such as the Basque Country where TB prevalence among cattle herds is minimal, a possible spillback transmission from this

ungulate to cattle should not be neglected (Nugent 2011). We suggest a potential risk of transmission at the wildlife-livestock interface of the study area, even though it might not be as important as the risk of wild boar or cattle intraspecies transmission. Measures to reduce the surface of shrub cover should be considered, since in addition to other risks, such as bushfires, it could be related to the exposure of wild boars to MTC. Hunting strategies should keep in mind those individuals that can have an effect on bacterial circulation or spread, such as the piglets and yearlings. The role of other domestic animals should be deeply studied, in order to gather more information of this multi-host pathogen system. Considering that the general expansion of wild boar populations in Europe through the last decades is a widely recognized problem (Sáez-Royuela and Tellerìa 1986; Acevedo et al. 2006, 2014), we highlight the necessity of better understanding the relevance of wild boar in the epidemiology of animal TB in northern Spain, in order to develop appropriate surveillance and control strategies, if needed, able to prevent the dissemination of the disease within wild populations and transmission to livestock.

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Study II

A long-term survey on *Mycobacterium tuberculosis* complex in wild mammals from a bovine tuberculosis low prevalence area

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Abstract

The relevance of wild hosts in the maintenance and transmission of animal tuberculosis (TB) may increase as the prevalence in livestock decreases. However, studies on wild mammals in low bovine TB prevalence scenarios are scarce. The Basque Country is an understudied region from the Atlantic Iberian Peninsula with low bovine TB prevalence. In this ten-year survey we searched for *Mycobacterium tuberculosis* complex (MTC) infection in wildlife and studied the spatial and temporal distribution of the spoligotypes circulating among these wild species and cohabiting livestock. For these purposes, lymph nodes from 1472 wild mammals were cultured and isolates spoligotyped. Information on domestic TB cases was obtained from the Spanish Database of Animal Mycobacteriosis. Infection was confirmed in ten wild boar (1.12%; 95%CI 0.61-2.05) and four red deer (2.40%; 95%CI 0.94-6.00). MTC was not isolated from badgers or other wild species. The general spoligotype diversity in the region was high. Five distinct spoligotypes belonging to *M. bovis* (SB0121, SB0134, SB0881, SB2354, SB1086) and one to *M. caprae* (SB0415) were detected in wildlife. Wild ungulates harboured most of the *M. bovis* spoligotypes that were commonly found in cattle, being SB0121 and SB0134 geographically associated between wild boar and cattle. *M. caprae* SB0415 was also found in both wild species as well as in cattle and goats. Despite the absence of MTC-infected badgers and the overall low prevalence observed in wildlife, potential epidemiological links between cattle and wild boar have been revealed. No competent hosts should be ignored when developing global control strategies aimed at eradicating TB.

***Keywords*:** *Mycobacterium tuberculosis* complex, wildlife, wild boar, red deer, spoligotyping, bovine tuberculosis

1. Introduction

Infections caused by members of the MTC are commonly shared at the wildlife-livestock interface. The relevance of wild hosts in the maintenance and transmission of bovine TB may increase as the prevalence in livestock decreases (Richomme et al. 2013). However, according to the literature, studies on wild mammals in low bovine TB prevalence scenarios from northern Iberian Peninsula are still scarce (Balseiro et al. 2009, 2011b, 2013; Boadella et al. 2011; Muñoz-Mendoza et al. 2013; Mentaberre et al. 2014; Varela-Castro et al. 2020a) as well as in other European Atlantic regions (Gortázar et al. 2012) in comparison to the plentiful of reports related to high prevalence or hot-spot areas. In recent years outbreaks associated with high wildlife prevalence have been reported in some territories from the USA with low prevalence in cattle also (Kaneene and Pfeiffer 2006). In Atlantic habitats of northern Iberian Peninsula, prevalence of TB among cattle herds was less than 1% in 2018 according to the Spanish National Bovine TB Eradication Programme (Ministerio de Agricultura Pesca y Alimentación 2020). Unlike in Mediterranean habitats from southern Iberian Peninsula, where wild boar and deer, mainly red deer but also fallow deer, are considered wild maintenance hosts of TB (Gortázar et al. 2012), the European badger seems to be a potential reservoir in some Atlantic regions (Acevedo et al. 2019) where the role of wild boar is still under debate (Muñoz-Mendoza et al. 2013; Varela-Castro et al. 2020a). The Basque Country is a low bovine TB prevalence region from the Atlantic Iberian Peninsula where previous studies in wildlife have reported a MTC seroprevalence of 17% in wild boar and no MTC detection in small mammals (Varela-Castro et al. 2020a, b). The aims of this study were to identify MTC-infected wildlife in the Basque Country and to describe the spatial and temporal distribution of the spoligotypes circulating among these wild species and those identified in livestock.

2. Materials and Methods

Between 2010 and 2019, LNs from 1472 wild mammals (894 wild boar, 235 roe deer, 167 red deer, 175 carnivores and one lagomorph (*Lepus spp.*)) (Figure 1) were collected within the context of a wildlife health surveillance programme in the Basque Country. Since LNs were obtained from different sources (hunters and different administrative entities involved in wildlife surveillance), sample collection was not always systematic. For instance, when the whole carcass was available, either obtained from road-killed animals or from wildlife populations control activities (444 out of 1472 cases), a complete necropsy was performed and, whenever possible, mandibular, parotid, retropharyngeal, tracheobronchial, mediastinal and mesenteric LNs were collected. From 2017 onwards, the hepatic LN was also collected. However, when the collection of LNs was performed during hunting activities, those arriving to the laboratory often varied between individuals. All the LNs were submitted to *post mortem* examination for the detection of macroscopic TB-like lesions and subsequently stored at -20 °C until being processed for culture. If lesions compatible with TB were observed, LNs were individually processed. Otherwise, they were processed individually or in convenient pools. Samples (LN or pool) were homogenized in sterile distilled water (2 g in 10 ml or equivalently) (Serrano et al. 2018). These suspensions were decontaminated using the BD BBL™ MycoPrep™ kit and submitted to microbiological culture in BBL™ mycobacteria growth indicator tubes (MGIT™) (Becton Dickinson, Franklin Lakes, NJ, USA) following manufacturer's instructions. Inoculated MGITs were incubated at 37 °C for 42 days in an automated BACTEC MGIT 960 system (Becton, Dickinson and Company, Sparks, MD, USA). The pellets obtained from 1 ml of MGIT cultures with positive BACTEC time to detection (TTD) readouts were resuspended in 0.25 ml of distilled water and inactivated at 90 °C for 20 min. After centrifugation

pellets were discarded and the supernatants submitted to a previously described (Sevilla et al. 2017) tetraplex real-time polymerase chain reaction (PCR) for the detection of MTC members. A standard spoligotyping (Kamerbeek et al. 1997) was performed for the identification of confirmed MTC isolates. On the other hand, official information on TB cases detected in the course of the National Bovine TB Eradication Programme as well as on the spoligotypes involved was obtained from the website of the programme (https://www.mapa.gob.es/es/ganaderia/temas/sanidad-animal-higiene-ganadera/sanidad-animal/enfermedades/tuberculosis/Tuberculosis_bovina.aspx) and from mycoDB (Rodriguez-Campos et al. 2012), respectively. Spoligopatterns were used to calculate the Simpson's Index of Diversity as a measure of strain diversity according to the following formula:

Simpson's Index of Diversity=1-D, where $D=\Sigma(\text{number of isolates with a particular spoligotype/total number of isolates})^2$.

Figure 1. **Spatial distribution of the wild species sampled in the Basque Country between 2010 and 2019.** Numbers within the map refer to the number of animals analysed per area.

3. Results

Infection was confirmed in a total of 14 wild ungulates: ten wild boar (1.12%; 95%CI 0.61-2.05) and four red deer (2.40%; 95%CI 0.94-6.00) (Table 1). While MTC in red deer was only detected in two non-consecutive years, detection in wild boar was more constant throughout the study period (Table 2). Most of the isolates were obtained from LNs from the head (14/15), mainly mandibular LNs (see Table 1 for further details). Macroscopic lesions compatible with TB were observed in LNs of 32 animals. Out of these animals, MTC was isolated from two wild boars (from one mandibular and one parotid LN, respectively) and one red deer (from both mandibular LNs) that displayed only small visible lesions (≤ 3 mm). The remaining 29 animals were MTC-negative in culture and lesions were further investigated: in 52% of the cases, other

etiological agents such as NTM, *Streptococcus porcinus* or *Staphylococcus aureus* were detected. It was not possible to determine the cause of lesions in the rest of cases. Five spoligotypes of *M. bovis* were detected, being SB0881 found in one wild boar, SB0134 in two wild boars and two red deer, SB0121 in two wild boars, SB2354 in one wild boar and SB1086 in one red deer. The pattern SB0121 was isolated in two LNs of one individual (in mandibular and tracheobronchial LNs). One spoligotype of *M. caprae*, SB0415, was found in another two boars and one red deer. No spoligotypes were obtained for the remaining two isolates from two wild boars for which only old DNA from 2013 and 2014 was available. Regarding livestock, 301 cattle were MTC-infected during the study period. Isolates mainly belonged to *M. bovis* (27 spoligotypes) and SB1299 (N = 86), SB0881 (N = 62), SB0134 (N = 38) and SB0121 (N = 32) were the most frequent spoligotypes (Table 3). A few belonged to *M. caprae* (N = 2) and showed the spoligotype SB0415. Only four goats were reported to be MTC-infected, and all the isolates belonged to *M. caprae* spoligotypes SB0415 (N = 2) and SB0416 (N = 2). The *M. caprae* spoligopattern detected in wildlife was also detected in cattle and goats. Spoligotypes SB0121 and SB0134 were present in wild boar and cattle from the same or geographically close north-western areas of the Basque Country (Figure 2). The Simpson's Index of Diversity indicated high strain diversity among all the isolates identified (0.85), also considering separately those identified in livestock (0.85) and wildlife (0.82).

Table 1. Prevalence of *Mycobacterium tuberculosis* complex in wildlife from the Basque Country between 2010 and 2019.

Species	N (positive)	Prevalence (95%CI)	Spoligotype	N of animals	Isolation lymph node	TB-like lesion
Wild boar (*Sus scrofa*)	894 (10)	1.12% (0.61-2.05)	SB0121 (Mb)	1	Parotid	Yes
				1	Mandibular/ tracheobronchial	Yes (mandibular)
			SB0134 (Mb)	1	Tracheobronchial	No
				1	Mandibular	No
			SB0415 (Mc)	1	Mandibular	No
				1	LN from the head pool	No
			SB0881 (Mb)	1	Mandibular	No
			SB2354 (Mb)	1	LN from the head pool	No
			UD	1	Mandibular	No
				1	LN from the head pool	No
Roe deer (*Capreolus capreolus*)	235 (0)	0% (0.00-16.08)	–			
Red deer (*Cervus elaphus*)	167 (4)	2.40% (0.94-6.00)	SB0134 (Mb)	2	Mandibular	No
			SB0415 (Mc)	1	Mandibular	Yes*
			SB1086 (Mb)	1	Mandibular	No
Badger (*Meles meles)*	90 (0)	0.00% (0.00-4.10)	–			
Fox (*Vulpes vulpes*)	46 (0)	0.00% (0.00-7.71)	–			
Stone marten (*Martes foina*)	11 (0)	n.d.	–			
American mink (*Neovison vison*)	9 (0)	n.d.	–			
Wolf (*Canis lupus*)	4 (0)	n.d.	–			
Genet (*Genetta genetta*)	4 (0)	n.d.	–			
Marten (*Martes martes*)	3 (0)	n.d.	–			
European mink (*Mustela lutreola*)	2 (0)	n.d.	–			
Polecat (*Mustela putorius*)	2 (0)	n.d.	–			
Weasel (*Mustela nivalis*)	2 (0)	n.d.	–			
Wild cat (*Felis silvestris*)	2(0)	n.d.	–			
Hare (*Lepus spp.*)	1 (0)	n.d.	–			

n.d. = not determined. UD = undetermined. CI = confidence interval. LN = lymph node. Mb = *M. bovis*. Mc = *M. caprae.* *Lesion in both right and left mandibular LNs.

Table 2. Temporal trend (2010-2019) of *Mycobacterium tuberculosis* complex in wild boar, red deer and cattle from the Basque Country.

Year	Cattle herds (N)*	Herd prevalence*	Cattle (N)*	% positives*	Wild boar (N)	% positives	Red deer (N)	% positives
2010	6013	0.37	116,091	0.04	25	0.00	-	-
2011	5459	0.33	109,274	0.05	51	0.00	2	0.00
2012	5518	0.25	107,078	0.14	42	4.76	1	0.00
2013	5263	0.17	104,287	0.06	80	1.25	1	0.00
2014	5117	0.25	112,513	0.08	154	1.30	15	0.00
2015	4926	0.16	105,439	0.04	155	1.29	19	0.00
2016	4744	0.17	108,461	0.02	84	1.19	18	16.67
2017	5474	0.09	105,249	0.01	107	0.00	46	0.00
2018	5317	0.00	108,175	0	100	2.00	27	3.70
2019	3870	0.00	94,680	0	96	0.00	38	0.00

* Information on cattle from the Basque Country tested in the course of the National Bovine TB Eradication Programme has been extracted from its website (https://www.mapa.gob.es/es/ganaderia/temas/sanidad-animal-higiene-ganadera/sanidad-animal/enfermedades/tuberculosis/Tuberculosis_bovina.aspx). No official data is available for goats.

Table 3. Species identification and spoligotyping of the *Mycobacterium tuberculosis* complex isolates found in cattle, goats, wild boar and red deer in the Basque Country between 2010 and 2019.

Species	**Spoligotype**	**Cattle***	**Goat***	**Wild boar**	**Red deer**
		N isolates	**N isolates**	**N isolates**	**N isolates**
M. bovis	SB1299	86	-	-	-
	SB0881	62	-	1	-
	SB0134	38	-	2	2
	SB0121	32	-	2	-
	SB1141	11	-	-	-
	SB1312	9	-	-	-
	SB1366	9	-	-	-
	SB0963	8	-	-	-
	SB0265	7	-	-	-
	SB0890	5	-	-	-
	SB0875	3	-	-	-
	SB1350	3	-	-	-
	SB1662	3	-	-	-
	SB1995	3	-	-	-
	SB0119	3	-	-	-
	SB0130	2	-	-	-
	SB0152	2	-	-	-
	SB0920	2	-	-	-
	SB1074	2	-	-	-
	SB2003	2	-	-	-
	SB0120	1	-	-	-
	SB0296	1	-	-	-
	SB0339	1	-	-	-
	SB1275	1	-	-	-
	SB1315	1	-	-	-
	SB1333	1	-	-	-
	SB1340	1	-	-	-
	SB1086	-	-	-	1
	SB2354	-	-	1	-
M. caprae	SB0415	2	2	2	1
	SB0416	-	2	-	-
MTC	UD	-	-	2	-

MTC = *Mycobacterium tuberculosis* complex. UD = undetermined. *Data obtained from the Spanish Database of Animal Mycobacteriosis (mycoDB) (Rodriguez-Campos et al. 2012).

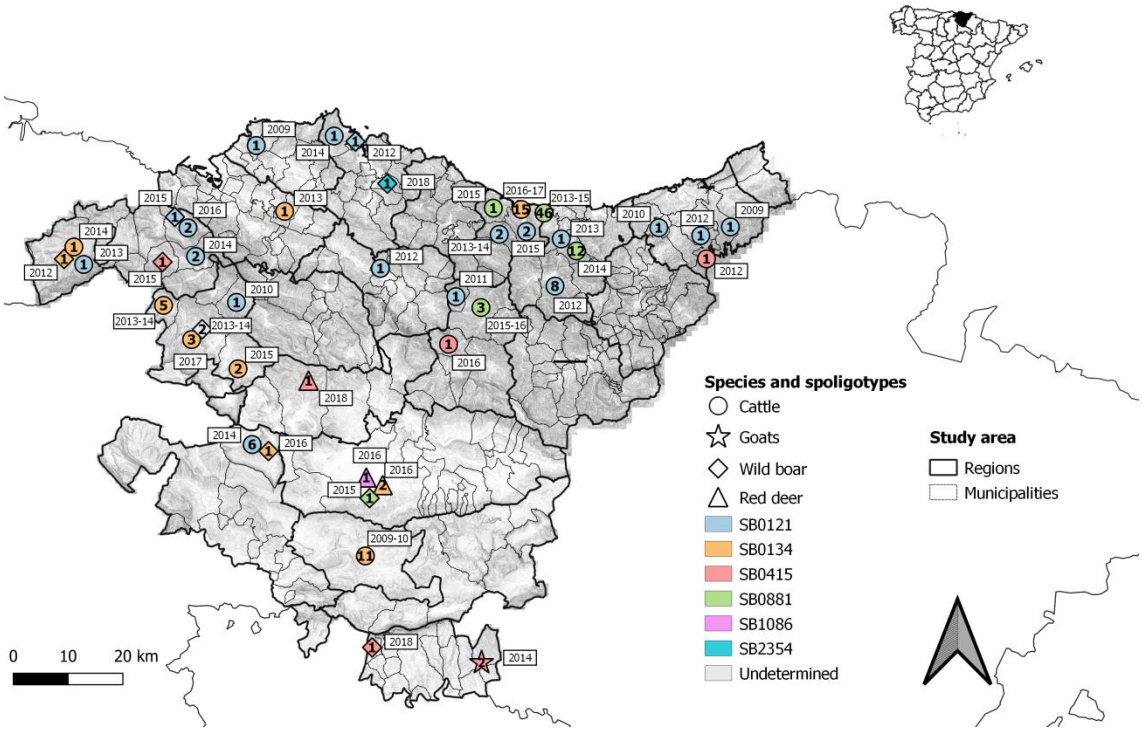

Figure 2. Spoligopatterns detected among cattle, goats, wild boar and red deer from the Basque Country. The map shows the spatial distribution of all the spoligotypes detected in wildlife and those simultaneously detected among the four species. Numbers inside the symbols (dots, stars, rhombuses and triangles) represent the number of positives. Numbers inside the rectangles represent the year(s) of sampling. Spoligotypes SB0121 and SB0134 are shared between cattle and wild boar in north-western areas.

4. Discussion

This study provides an insight into the epidemiology of TB in wild populations of the understudied low bovine TB prevalence areas of the Atlantic Iberian Peninsula. Our findings could contribute to represent the situation in similar regions (Gortázar et al. 2012). The low prevalence observed in wildlife was not unexpected because bovine TB prevalence among cattle herds was also very low during the study period and the number of TB cases detected in goats was negligible. Provided that a previous study reported a high seroprevalence in wild boar in the same area (Varela-Castro et al. 2020a) and in the absence of whole carcass analysis in most cases, the proportion of positive individuals among wild ungulates might represent an underestimation of the

true prevalence. It has to be pointed out that only a small proportion of the animals sampled for serum in the previous serological study (Varela-Castro et al. 2020a) had also tissue samples available for culture in the present study. Only a few of them belonged to north-eastern Basque Country (Gipuzkoa) where the seroprevalence was highest. In addition, the proportion of serum and tissue samples available that were included in each survey was not the same for the different areas/provinces in the Basque Country: north-western area (Bizkaia) was represented with 15.62% of sera and 48.66% of tissues, north-eastern area (Gipuzkoa) with 79.44% and 6.15% and southern area (Araba) with 4.94% and 45.19%, respectively. All spoligotypes were assigned to *M. bovis* and *M. caprae*, and no *M. microti* isolates were retrieved. The latter species has been recently detected in both domestic and wild animals in France and north-eastern Spain (Michelet et al. 2015, 2016; Pérez de Val et al. 2019), but it is difficult to culture (Boniotti et al. 2014). Antibodies against *M. microti* infection are also detectable on serum samples using the ELISA protocol employed in our previous serological survey, and thus it could have contributed in part to the high seroprevalence observed while the bacterium went undetected in culture (Varela-Castro et al. 2020a). A PCR with DNA extracted directly from the tissue samples might have identified some *M. microti* in our samples. However, this MTC member has not been detected to date in the Basque Country by tissue culture and/or tissue PCR, neither in a previous small mammal survey (Varela-Castro et al. 2020b) nor in the course of the National Bovine TB Eradication Programme, for which all MTC-culture-negative animals with positive skin test or with TB-like lesions are subsequently re-analysed including tissue PCR in search for the agent producing these interferences in the diagnosis of TB. Further research focused on this mycobacterium is ongoing.

The relatively high strain diversity and low prevalence in wild boar is in agreement with previous results from other Atlantic regions in northern Iberian Peninsula (Balseiro et al. 2013; Muñoz-Mendoza et al. 2013). This high diversity of isolates seems to indicate that there are different sources of MTC both for livestock and wild species. Local husbandry practices such us sharing or rotation of pastures, livestock trade or enclosures open to the field increase the likelihood of contacts with potential sources, even between distant areas. In contrast to previous results from Atlantic neighbouring regions, MTC infection was also detected in red deer and absent in all the carnivores, including badgers. Detection of MTC in red deer has been also described in other Atlantic habitats of Europe such as north-western France, a country deemed bovine TB-free since 2001 regardless of the appearance of several outbreaks among cattle herds in certain departments (Réveillaud et al. 2018). Access to carnivores' carcasses was always possible except for the four wolves included and for three badgers. Hence, badgers do not seem to be affected by TB nor entail a risk for cattle in the study area in comparison to the findings of previous studies from Atlantic Iberian Peninsula. However, methodological variations between studies might account for these differences. For instance, badgers' samples were only collected through passive surveillance, while in previous studies from neighbouring areas, badger trapping in cattle's TB positive areas was also implemented (Balseiro et al. 2011b, 2013). Besides, the histopathological examination performed in those studies identified evidences of TB in culture-negative badgers. Thus, this method could have revealed some positive individuals if used in our research. Few macroscopic lesions were observed in positive wild species, and all of them were located in LNs from the head, which means that MTC excretion could be limited. Since only LNs from the head region were received for these animals, we cannot confirm this hypothesis. In any case,

these wild ungulates harboured most of the *M. bovis* spoligotypes that are commonly found in cattle. Involvement of *M. caprae* in tuberculous infections in wild boar is not rare in central Spain (García-Jiménez et al. 2013). In our study *M. caprae* spoligotype SB0415 was also found in wild boar and deer as well as in cattle and goats, suggesting circulation and potential transmission of these *M. bovis* and *M. caprae* spoligotypes between wildlife and livestock in the study area. Hence, in regions such as the Basque Country where bovine TB prevalence is minimal, spillback transmission from these wild ungulates to domestic species should not be neglected. This risk may become even higher if effective biosafety measures are not implemented in the farms, this being the reality in the studied region, where most of the farm enclosures are open to the field even when managed under an intensive production system and no biosafety measures such as fencing are always implemented. On the other hand, spoligopatterns SB1086 and SB2354, detected in wild boar and red deer, have not been previously reported in Spain, neither in wildlife nor livestock. Both patterns seem to be relatively rare, even though a few isolates were detected in cattle (SB1086) and red deer (SB2354) in Algeria and Portugal, respectively (Sahraoui et al. 2009; Lopes dos Reis 2015). This could suggest the existence of a wild cycle in the study area where domestic animals are not involved, as other authors reported earlier in Mediterranean regions (Amato et al. 2016, 2018). Wild cervids are quite susceptible to MTC infection, being some species considered maintenance hosts (Gortázar et al. 2015b). However, this is not the case for roe deer, which does not appear to be a true reservoir (Balseiro et al. 2009; Lambert et al. 2017). The absence of positive individuals in our study supports this hypothesis. Although red deer showed a relatively higher prevalence than wild boar, it was also more sporadic throughout the years, and the number of analysed individuals was lower, which may have distorted these figures. Besides, wild boar shared more spoligotypes

with cattle, being SB0121 and SB0134 geographically associated between these two species. After reviewing the spoligotypes from neighbouring areas reported in mycoDB, a similar link has been found in municipalities adjacent to north-western Basque Country. These observations seem to indicate that there is a higher likelihood of bacterial transmission between wild boar and cattle than between deer and cattle in our area, maybe due to a more elusive behaviour of deer or simply to different geographical distributions. This, together with the high MTC seroprevalence previously observed in wild boar from the same region (Varela-Castro et al. 2020a), suggests that wild boar could represent a more relevant host in this area than it was expected. Considering that spoligotypes SB0121 and SB0134 are widely spread in the Iberian Peninsula, a finer isolate genotyping (e.g., WGS) should be implemented in the future in order to delve further into intra- and interspecies transmission events.

MTC-infected red deer of this study revealed that this species can be affected by TB in the Atlantic Iberian Peninsula, but this finding seems to be occasional and of low significance, even though the eventual existence of a bias due to the sampling approach should not be completely discarded. Although no MTC was isolated from badgers, in light of previous studies (Acevedo et al. 2019), further research including histopathological examination and active surveillance should be performed to determine the role of this host in the epidemiology of TB in the area.

The findings of this study are not sufficient to clearly establish epidemiological links between wildlife and livestock with respect to TB in this Atlantic region of the Iberian Peninsula with a bovine TB prevalence close to eradication, but it has contributed to increase the body of knowledge on the epidemiology of the disease. Collectively, in spite of the low MTC culture-positive wild boar prevalence observed, we have detected earlier a quite high MTC seroprevalence in this species and have

demonstrated now that it shares several spoligotypes with cattle. In the absence of more discriminatory genotyping of isolates, the potential epidemiological links revealed between cattle and wild boar in this region lead us to think that this wild ungulate could be more relevant than it was previously thought. Once TB establishes in wildlife populations, its control in those populations as well as in domestic ones becomes generally more difficult and needs a long-term dedication (Fitzgerald and Kaneene 2013). No competent hosts should be ignored when developing global TB control strategies aiming at reaching eradication.

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Study III

Detection of wood mice (*Apodemus sylvaticus*) carrying non-tuberculous mycobacteria able to infect cattle and interfere with the diagnosis of bovine tuberculosis

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XIV Congreso de la Sociedad Española para la Conservación y Estudio de los Mamíferos, Jaca, Spain, 5-8 December 2019: Varela-Castro L, Torrontegi O, Alvarez V, Martínez de Egidua M, Sevilla IA, Barral M. Detección de micobacterias no tuberculosas en ratones de campo (*Apodemus sylvaticus*) capturados en explotaciones de ganado vacuno del País Vasco. Oral presentation.

Workshop and Conferences for PhD candidates in Environmental Sustainability INGURU-DOK, Plentzia, Basque Country, 11 October 2019: Varela-Castro L, Torrontegi O, Alvarez V, Martínez de Egidua M, Sevilla IA, Barral M. Is the Wood mouse (*Apodemus sylvaticus*) relevant in the epidemiology of mycobacteriosis in cattle in the Basque Country? Oral presentation.

Abstract

Mycobacterial infections caused by the *Mycobacterium tuberculosis* complex (MTC) and non-tuberculous mycobacteria (NTM) are of great medical and veterinary relevance. The aim of this research was to study whether small mammals play a role in the epidemiology of mycobacterioses. Four samplings of 100 traps were performed in each of three cattle farms with previous history of tuberculosis (TB) or NTM between 2017 and 2018. A total of 108 animals belonging to seven species were trapped, classified, and necropsied, and tissues were submitted to microbiological and molecular methods for mycobacteria identification. The wood mouse (*Apodemus sylvaticus*) was the most abundant species (87%). No MTC was detected but six different NTM were identified (*M. intracellulare*, *M. avium* subsp. *paratuberculosis*, *M. gordonae*, *M. celatum*, *M. fortuitum*, and a not determined *Mycobacterium* sp.), showing a prevalence of 6.5%. No significant association was found between mycobacteria prevalence and the analysed factors. Although a role in the epidemiology of MTC could not be attributed to small mammals, *A. sylvaticus* carries NTM that could be pathogenic or interfere with the diagnosis of TB. According to our results, there is a risk of NTM transmission at the wildlife–livestock interface through potential indirect contacts between small mammals and cattle.

Keywords: Non-tuberculous mycobacteria, small mammals, *Apodemus sylvaticus*

1. Introduction

Aside from the agents responsible for leprosy, the genus *Mycobacterium* includes a large number of species that can be split into two main groups: the MTC and NTM. Several species of mycobacteria have been detected in wild and domestic animals (Bercovier and Vincent 2001; Pavlik et al. 2002b), in humans (Ashford et al. 2001), and also in the environment, which could represent an important reservoir due to the species' resistance to adverse factors and ubiquity (Hruska and Kaevska 2012). Those species belonging to MTC are the most studied, since they are the causative agents of human and animal TB. Human TB is a worldwide infectious disease mainly caused by *M. tuberculosis* with a 1.2 million death toll in 2018 according to the World Health Organization (World Health Organization 2019). Animal TB is a zoonotic disease that causes severe economic losses in the livestock industry of developed countries (Zinsstag et al. 2006). It is mainly caused by *M. bovis*, even though other species such as *M. caprae* can be involved (Kaneene et al. 2014). On the other hand, NTM are ubiquitous in a broad variety of soil and aquatic environments (Biet and Boschiroli 2014) and compose most of the species belonging to the genus *Mycobacterium*. However, because of an initial lack of knowledge on their clinical relevance, NTM were neglected for many years. Currently, conversely, they are associated with a wide range of infections in humans and animal species. Clinical manifestations caused by NTM range from skin and soft tissue infections to respiratory or digestive infections or diseases (Bercovier and Vincent 2001; Griffith et al. 2007; Biet and Boschiroli 2014). One meaningful example of veterinary relevance would be *Map*, a member of the MAC, which is the causative agent of paratuberculosis in ruminants. *Map* has also been related to Crohn's disease in humans, but this still remains controversial (Mendoza et al. 2009). Some species of NTM have been pointed out as a source of interference with bovine TB

diagnostic reagents, such as *Map* itself, *Maa*, and *M. fortuitum* (Bercovier and Vincent 2001; de la Rua-Domenech et al. 2006), or with the protection provided by the BCG vaccination (Buddle et al. 2002).

Soils shared between sympatric wildlife and livestock may become key zones for the indirect transmission of mycobacteria. Wild small mammals could have a role in the spread of these agents into those specific areas, since they are present in pastures and farm enclosures (sheds, straw, forage, etc.). Currently, rodent population control seems to be the most widespread measure to minimize the presence of small mammals within farm buildings, but the protection of forage, straw, and water to avoid small mammals feeding and excreting over these resources remains not completely solved (Fischer et al. 2000). The implementation of these measures may become even more complicated when feeders and troughs are also placed in the pastures. Pathogenic or opportunistic mycobacteria can colonize small mammals' tissues or simply pass through their digestive system and be shed intact in feces and body fluids, which could be further spread by the movements of these animals (Fischer et al. 2000). Previous studies have described the detection of mycobacteria in small mammals. Apart from *M. microti*, *M. bovis* was isolated from urban and wild rodents (Delahay et al. 2002; Mathews et al. 2006) and its ability to infect different species has been experimentally demonstrated (Clarke et al. 2007). Regarding NTM detection in small mammals, *Map*, *M. intracellulare*, *M. gordonae*, and *M. chelonae* have been isolated, among others (Kopecna et al. 2008; Durnez et al. 2011). Other studies have simultaneously detected the same species of NTM in livestock and cohabiting small mammals or even suggested a possible transmission of mycobacteria between them (Florou et al. 2008). Whether small mammals can act just as carriers or as true hosts or even reservoirs is not clear yet. Therefore, more in depth studies investigating the relevance of these mammals in the

epidemiology of mycobacterioses are needed if we want to design effective global control strategies. The goal of this research was to study the role of small mammals in cattle farms with a history of TB or NTM, using as reference three farms located in the Basque Country, Northern Spain. We also searched for factors associated with the detection of mycobacteria in these mammals.

2. Materials and Methods

2.1 Study area and small mammal sampling

Three cattle farms from the Basque Country with history of TB and/or NTM cases (Table 1) were selected, and permissions for small mammal trapping and euthanasia were obtained from the competent authorities (corresponding approval numbers and dates: 6387/2917 in December 2017, 1907 in March 2017 and 183 in February 2017). The selected farms had reactor cattle to the intradermal tuberculin test that were subsequently confirmed as *M. bovis*-infected or as false positives. From July 2017 to October 2018, 100 traps baited with chorizo (sausage-like cured meat product) were placed for small mammal live capture at the areas where cattle were located during the sampling. The selected bait is easy to insert and remove from the traps, does not rot rapidly, and can resist harsh weather conditions and feeding by invertebrates. Traps were placed overnight once every season, making a total of four samplings per farm. Sherman traps (7.6 cm by 8.9 cm by 22.9 cm; H.B.Sherman traps Inc., Tallahassee, FL, USA) were used indoors, while INRA traps (5 cm by 5 cm by 15 cm; BTS Mechanique, Besançon, France) were used along the edges between pastures and adjacent forests' shrubs.

Table 1. Mycobacteria detected in cattle and small mammals at farm level.

Farm locality	***Mycobacterium* species identified in cattle (2014–2017)**	**Small mammal species (N)**	**Total number trapped**	**Mycobacteria prevalence (%) in small mammals (95% CI)**	***Mycobacterium* species identified in small mammals**
Deba			34	11.8 (4.7–26.6)	
	M. bovis	*Apodemus sylvaticus* (29)			*M. intracellulare*
	Map	*Mus domesticus* (2)			*Map*
		Microtus agrestis (1)			*M. fortuitum*
		Microtus gerbei (1)			*M. gordonae*
		Apodemus sp. (1)			
Kortezubi			34	8.8 (3.0–23.0)	
	Maa	*A. sylvaticus* (32)			*Map*
		Crocidura russula (2)			*M.* sp.[¥]
					M. celatum
Kexaa			40	0.0 (0.0–8.7)	-
	M. bovis	*A. sylvaticus* (33)			
	Map	*Apodemus flavicollis* (3)			
	Maa	*Myodes glareolus* (1)			
	M. sp.*	*M. gerbei* (1)			
		M. domesticus (1)			
		C. russula (1)			

Maa = *M. avium* subsp. *avium*, Map = *M. avium* subsp. *paratuberculosis*, *M.* sp. = *Mycobacterium* sp. * Internal transcribed spacer (ITS) sequence showing 71%–75% base identities with the ITS sequence of different isolates of *M. insubricum* in BLAST analysis. [¥] The sequenced ITS amplicon showed a percentage of identity of 82.91% with *M. peregrinum* (BLAST). N = number of trapped animals.

2.2 Processing of small mammals and sample preparation

Captured individuals were transported to a Biosafety level 3 Laboratory and euthanized in a CO_2 chamber. Afterwards, weight and biometrics of each individual were recorded. At necropsy, sex and age (adult or juvenile) were determined and organs were inspected for the presence of macroscopic lesions. A pool of tissues was prepared for each animal including LNs from the head, the respiratory system, and the intestinal tract, lung, ileum, and muscle. All pools weighed less than 1 g. Finally, small mammal species were identified by dental alveoli patterns and skull and biometric features, following the indications of taxonomic keys and morphological studies (Panzironi et al. 1994; Blanco 1998). Prior to further processing, tissue pools were homogenized in 5 ml of sterile distilled water using a GentleMACS™ Dissociator (Miltenyi Biotec, Madrid, Spain) (RNA_02 program) and divided into two aliquots of 4.75 ml and 0.25 ml for culture and direct DNA extraction and real-time PCR analysis, respectively. A schematic representation of the laboratory methodology is given in Figure 1.

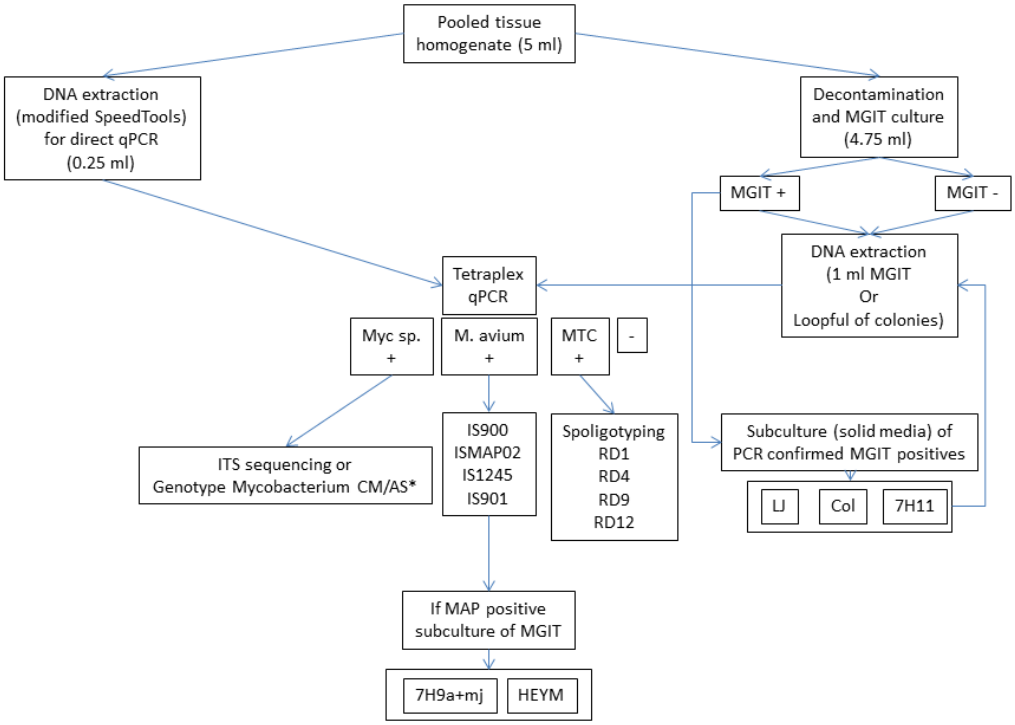

Figure 1. Schematic representation of the methodology. Culture media abbreviations: MGIT = Mycobacteria Growth Indicator Tube; LJ = Löwenstein–Jensen; Col = Coletsos; 7H11 = Middlebrook 7H11 supplemented with oleic acid-albumin-dextrose-catalase (OADC) enrichment; 7H9a+mj = agar-solidified 7H9 medium supplemented with OADC and mycobactin J; HEYM = in-house Herrold's Egg Yolk medium containing sodium pyruvate and mycobactin J. + = positive result; - = negative result. * Only with DNA from colonies obtained by subculture of positive MGITs.

2.3 Culture

Considering the small size of samples (<1 g), almost the whole volume of homogenized sample (4.75 ml) was destined to a single culture procedure. Homogenates were decontaminated using the BD BBL™ MycoPrep™ kit and processed for culture in BBL™ MGIT™ (Becton Dickinson, Franklin Lakes, NJ, USA) supplemented with BACTEC™ MGIT™ growth supplement and PANTA™ antibiotic mixture according to the manufacturer's instructions (Becton Dickinson, Franklin Lakes, NJ, USA). Inoculated MGITs were incubated in an automated BACTEC MGIT 960 system (Becton, Dickinson and Company, Sparks, MD, USA) at 37 °C for an

extended period of at least four months to enable isolation of slowly growing mycobacteria.

MGIT cultures confirmed as positive were subcultured in Difco Löwenstein–Jensen, Coletsos (Dismalab S.L., Madrid, Spain), and Middlebrook 7H11 supplemented with oleic acid-albumin-dextrose-catalase (OADC) enrichment (Becton Dickinson, Franklin Lakes, NJ, USA) in order to obtain isolated colonies for further molecular characterization. Since *Map* needs exogenous addition of mycobactin J for in vitro culture (Whittington et al. 2013), its growth requirements were not covered by the culture medium chosen in this study for primary isolation. To circumvent this methodological bias, if a DNA sample tested PCR-positive for *Map*, regardless of being DNA extracted from tissue homogenate or MGIT culture, its corresponding MGIT was subcultured in in-house prepared Herrold's Egg Yolk medium (HEYM) containing sodium pyruvate and mycobactin J (IDvet, Grabels, France) and in agar-solidified 7H9 medium supplemented with OADC and mycobactin J.

2.4 DNA extraction

DNA extraction from tissue homogenate aliquots (0.25 ml) was performed using a modified protocol of the Speedtools Tissue DNA extraction kit (BioTools, B&M Labs S. A., Madrid, Spain) as described previously (Sevilla et al. 2015, 2017). DNA was extracted from all MGIT cultures regardless of having positive or negative BACTEC TTD readouts. One milliliter of MGIT culture was centrifuged at 16,000× *g* for three minutes and the supernatant discarded. Pellets were resuspended in 0.25 ml of distilled water, inactivated at 90 °C for 20 min, and submitted to DNA extraction using the same modified protocol specified above for tissue homogenates.

2.5 Tetraplex real-time PCR for the screening of tissues and cultures

A previously described (Sevilla et al. 2015) and modified (Sevilla et al. 2017) tetraplex real-time PCR was performed for the screening of DNA extracted from MGIT cultures and homogenized tissue pools. This technique allows for the simultaneous detection of the *Mycobacterium* genus, all four *M. avium* subspecies, and MTC. The reaction was carried out in a total volume of 25 µl, containing 3 µl of extracted DNA and 22 µl of mastermix. Amplification was carried out in a 7500 real-time PCR thermal cycler (Applied Biosystems, Foster City, CA, USA) under previously described conditions (Sevilla et al. 2015, 2017). The estimation of valid cycle threshold (CT) and baseline was calculated automatically with the SDS software v. 1.5.1 (Applied Biosystems, Foster City, CA, USA), visually confirmed by checking amplification plots, and manually adjusted if needed.

2.6 Further molecular identification of mycobacteria detected by the tetraplex real-time PCR

2.6.1 Identification of Mycobacterium sp.-positive samples

Mycobacterium sp. detected by the tetraplex real-time PCR of DNA samples extracted from tissue homogenates were further identified by PCR and sequence analysis of the 16S-23S rRNA ITS. A previously described nested PCR was used for PCR amplification of the ITS region (Fyfe et al. 2008). After electrophoresis, PCR products were purified from agarose gels with the Genelute Gel Extraction kit (Sigma-Aldrich Co. Ltd., St. Louis, MO, USA) as recommended by the manufacturer. Purified amplicons and the same primers used for the second round of the nested PCR were adjusted to appropriate concentrations and shipped to EuroFins GATC Biotech GmbH (Konstanz, Germany) for sequencing. Inspection, edition, and alignment of sequences

was performed, assisted by Sequencing Analysis 5.2 software (Applied Biosystems, Foster City, CA, USA), and then compared with other published sequences using online BLAST analysis (NCBI, NLM, Bethesda, MD, USA).

Mycobacterium sp. isolates were identified using the Genotype Mycobacterium CM and AS kits (Hain Lifesciences GmbH, Nehren, Germany). For this purpose, a loopful of colonies growing in solid subcultures was resuspended in 100 µl of A-LYS/IC reagent of the GenoLyse kit (Hain Lifesciences GmbH, Nehren, Germany), and DNA was extracted following the protocol provided with the kit. Then, DNA was amplified and PCR amplicon identity revealed using the Genotype Mycobacterium CM and AS kits and the Twincubator hybridizer (Hain Lifesciences GmbH, Nehren, Germany) according to the indications of the manufacturer. These kits contain membrane strips coated with specific probes that are complementary to certain mycobacterial DNA sequences, allowing for the identification of MTC and 27 species of NTM in agreement with the hybridization pattern obtained. Isolates that could not be identified at the species level with this kit were further identified by the aforementioned ITS sequencing procedure.

2.6.2 Identification of M. avium subsp.-positive samples

For subspecies identification of samples yielding a positive result for *M. avium* in the tetraplex real-time PCR, DNA was analysed by different real-time or conventional PCR methods described earlier to amplify IS*900,* IS*Map02* (Sevilla et al. 2014), IS*1245,* and IS*901* (Slana et al. 2010). Identification was enabled by the interpretation of presence–absence signatures obtained for the genomic targets interrogated by PCR, which are subspecies-specific (Sevilla et al. 2017): *Map* is IS*900+*, IS*Map02+*, IS*1245−*, IS*901−*; *Maa* (and *Mas*) is IS*900−*, IS*Map02−*, IS*1245+*, IS*901+*; *Mah* is IS*900−*, IS*Map02−*, IS*1245+*, IS*901−*.

2.6.3 Identification of MTC-positive samples

The strategy outlined for the identification of MTC-positive samples included standard spoligotyping (Kamerbeek et al. 1997) as well as amplification of the regions of difference (RD) 1, 4, 9, and 12 of *M. tuberculosis* using previously described primers (Halse et al. 2011) in independent conventional singleplex PCR assays (Sevilla et al. 2017). The RD signature patterns for MTC species identification have been specified earlier (Halse et al. 2011).

2.7 Statistical analyses

Mycobacteria detection (positive/negative) and factors such as animal age, sex, season of capture, and sampling locality were analysed using Fisher's test. The combined results of direct PCR and culture were used as the dependent variable. Significance was set at $p < 0.05$. Statistical analyses were performed using the R Software 3.5.0 (R Development Core Team 2018).

3. Results

3.1 Identification and processing of small mammals

A total of 108 small mammals, 50 females (29 adults and 21 juveniles) and 58 males (28 adults, 25 juveniles and five undetermined), were trapped. Six species of rodents and one shrew species were identified, with *Apodemus sylvaticus* being the most frequently trapped species (87%; see Table 1 for further details). One rodent belonging to *Apodemus* genus could not be further identified due to massive teeth wear. Two individuals showed macroscopic lesions in the liver and in the kidney, respectively, but were not compatible with mycobacterial infections as assessed through histopathological and microbiological analyses.

3.2 Mycobacteria detection and identification

No members of the MTC were detected. As for NTM, the overall prevalence was 6.5% (7/108; 95% CI, (3.2%–12.8%)). More specifically, two species belonged to *M. avium* subspecies and five belonged to other NTM. Among them, one was detected in the unidentified *Apodemus* specimen and the other six were detected in *A. sylvaticus* individuals (Table 2). However, no animal tested positive for both direct PCR and culture.

Table 2. Mycobacteria detection and identification in positive small mammal specimens.

Rodent species	***Mycobacterium* isolation**	**MGIT PCR result**	**Direct PCR result (tissue homogenate)**	***Mycobacterium* identification method**	**Final identification**
A. sylvaticus	Yes	Positive (*M*. sp.)	Negative	Reverse hybridization and ITS sequencing	*M. fortuitum*
Apodemus sp.	Yes	Positive (*M*. sp.)	Negative	Reverse hybridization	*M. intracellulare*
A. sylvaticus	Yes	Positive (*M*. sp.)	Negative	Reverse hybridization	*M. gordonae*
A. sylvaticus	Yes	Positive (*M*. sp.)	Negative	Reverse hybridization	*M. celatum*
A. sylvaticus	No	Negative	Positive (*M. avium*)	IS*900*, IS*Map02*, IS*1245* and IS*901*	*Map*
A. sylvaticus	No	Negative	Positive (*M. avium*)	IS*900*, IS*Map02*, IS*1245* and IS*901*	*Map*
A. sylvaticus	No	Negative	Positive (*M.* sp.)	ITS sequencing	*M.* sp.*

Map = *M. avium* subsp. *paratuberculosis*. *M.* sp. = *Mycobacterium* sp. * ITS sequence with a percentage of identity of 82.91% with *M. peregrinum* IoA5 (BLAST).

Out of the 89 MGIT cultures displaying a positive TTD readout, only four were confirmed to contain mycobacteria with the tetraplex real-time PCR. The identification of these four isolates with the Genotype Mycobacterium CM and AS reverse hybridization kits was as follows: *M. fortuitum* complex (the ITS sequence obtained for this isolate displayed a percentage of identity of 98.74% with *Mycobacterium sp*. DL90, 96.68% with *M. fortuitum* sequevar Mfo D 16S-23S, and 96.68% with *M. fortuitum*

strain S358 in BLAST analysis), *M. intracellulare*, *M. gordonae*, and *M. celatum* (Table 2).

As for the homogenized tissue pools, two were positive to *M. avium* subspecies and one was positive to other NTM, according to the tetraplex real-time PCR. The sequence obtained for the sample positive to *Mycobacterium* sp. best matched with the ITS sequence available in GenBank for *M. peregrinum* isolate IoA5, displaying a percentage of identity of 82.91%, according to BLAST analysis. The two *M. avium* subspecies detected in the tissues of two animals by the tetraplex PCR were identified as *Map*, in agreement with the insertion sequence signature obtained (IS*900+*, IS*Map02+*, IS*1245–* and IS*901–*).

3.3 Statistics

Statistical analyses were performed considering only those individuals belonging to well-represented animal species, in this case, only *A. sylvaticus*. No statistically significant differences were detected in NTM distribution according to sex or age of small mammals, season, or farm (Table 3).

Table 3. Prevalence of non-tuberculous mycobacteria detected in *A. sylvaticus* according to the categorical variables.

Variable	Number tested	% Positives (95% CI)	p-value
Sex			1
Female	42	7.1 (2.5–19.0)	
Male	52	5.8 (2.0–15.6)	
Age			1
Juvenile	40	7.5 (2.6–19.9)	
Adult	50	6.0 (2.1–16.2)	
Season			0.3
Autumn	23	0.0 (0.0–14.3)	
Winter	36	11.1 (4.4–25.3)	
Spring	26	3.8 (0.7–18.9)	
Summer	9	11.1 (2.0–43.4)	
Farm Locality			0.1
Deba	29	10.3 (3.6–26.4)	
Kortezubi	32	9.4 (3.2–24.2)	
Kexaa	33	0.0 (0.0–10.4)	

4. Discussion

Studies researching the potential role of small mammals in the epidemiology of mycobacterial infections are lacking. To the best of our knowledge, this is the first reported survey searching for mycobacteria in small mammals present in Spanish cattle farms with a history of mycobacterioses. Most of the mycobacteria detected in this study were only found in *A. sylvaticus*. This species was also the most frequently trapped (Table 1) and it is the most abundant within the forests of the Iberian Peninsula, inhabiting a wide range of habitats (Torre et al. 2002). The type of traps and bait used in this study could have had a negative impact on targeting some species that are mainly herbivores (*Microtus gerbei*, *Microtus agrestis*, and *Myodes glareolus*).

Out of the 89 MGIT cultures displaying a positive TTD readout, 85 were not confirmed with the tetraplex real-time PCR. This high proportion of contaminated MGITs suggests that an improved decontamination and culture protocol for this type of samples might have yielded more mycobacterial isolates, since mycobacterial culture is generally problematic and very sample-matrix specific in terms of the procedure

adopted. With regard to the positive individuals, direct PCR and culture results were discrepant in all the cases. For those samples displaying a positive direct PCR but a negative culture, one explanation could be that while culture will only recover live mycobacteria, PCR can detect the DNA of both live and dead cells. Samples with positive direct PCR displayed high CTs (CTs > 35), which are normally related to a low bacterial load. In addition to this, the MGIT cultures of these samples were all contaminated, and thus, mycobacterial growth could have been prevented by the growth of contaminating flora. Regarding the two samples that were *Map*-positive by direct PCR, it is clear that MGIT without mycobactin J is not a suitable medium for *Map*. In spite of the attempt to recover *Map* cells by subculturing the MGIT broth of both samples in HEYM and M7H11, we were not able to isolate any *Map* colonies. On the other hand, those cases showing a positive culture but a negative direct PCR could be attributed to a higher sensitivity of culture over PCR. According to previous results, the minimum detectable concentration of *M. kansasii* (acting as a proxy for non-*M. avium* NTM) in artificially inoculated samples can be one colony-forming unit (CFU) log unit lower for MGIT-BACTEC than for the unmodified protocol of the tetraplex real-time PCR employed (Sevilla et al. 2015). It should also be mentioned that the volume of homogenized sample used for culture was almost 20 times the volume used for DNA extraction, from which only 3 out of 100 µl of the eluted DNA were loaded per PCR reaction.

Although we did not detect any species belonging to the MTC, we did find other mycobacteria of interest. For instance, two members of MAC were detected, *M. intracellulare* and *Map*, which have been demonstrated to sensitize cattle and interfere in the diagnosis of TB (de la Rua-Domenech et al. 2006; Biet and Boschiroli 2014). *M. intracellulare* is a NTM commonly found in patients with mycobacterial pulmonary

disease (Griffith et al. 2007). It has been recovered from water, soil, and biofilm samples (Falkinham III et al. 2001) and it is also implicated in infections of several wild and domestic animals, including cattle (Rastogi et al. 2001). As for small mammals, this bacterium was previously detected in the lungs of African rodents and insectivores (Durnez et al. 2011). *Map* is the causative agent of paratuberculosis, a chronic wasting disease that mainly affects ruminants, even though it has been isolated from many other wild and domestic species (Biet and Boschiroli 2014). Although some rodents seem to be resistant to *Map* infection (Koets et al. 2000), this bacterium has been previously detected in *A. sylvaticus* (Beard et al. 2001). On the other hand, *M. fortuitum*, *M. gordonae*, *M. celatum*, and a not determined *Mycobacterium* sp. with an ITS sequence similar to *M. peregrinum* (83% sequence identity) were also detected in this study. *M. fortuitum* is related to lung disease in humans and has been related to immune sensitization in cattle, leading to cross-reactive responses that can interfere with the diagnosis of TB (Michel 2008). This species has been described as naturally pathogenic for mice (Wolinsky 1979), and it has previously been detected in *Microtus arvalis* (Fischer et al. 2000). *M. gordonae* is the most commonly isolated mycobacterial species due to contamination when human respiratory specimens are cultured, even though it also can cause pulmonary or disseminated infection (Griffith et al. 2007). It has also been detected in cattle (Berg et al. 2009) and several species of small mammals (Durnez et al. 2011). This bacterium has been commonly found in water reservoirs (Honda et al. 2018). Besides, it has been described as an NTM species that could potentially express cross-reactive antigens and, consequently, affect the tuberculin test specificity (Vordermeier et al. 2007). *M. celatum* is an infrequently detected species of *Mycobacterium*, which is more common among immunocompromised patients (Rastogi et al. 2001). Its detection in animals is even less frequent and only two cases of *M.*

celatum infection in domestic ferret (*Mustela putorius furo*) and one in a white-tailed trogon (*Trogon viridis*) have been reported (Pate et al. 2011). Even though mice can be susceptible to *M. celatum* during experimental infection (Fattorini et al. 2000), this is the first report on the detection of this species in free-living rodents. It is worth mentioning that *M. celatum* can cause false *M. tuberculosis*-positive results in commercial molecular identification tests (Griffith et al. 2007). Lastly, *M. peregrinum* has been described as an opportunistic pathogen for humans and livestock (Bercovier and Vincent 2001), even though it has also been detected in wild animals (Pate et al. 2016).

Despite these findings, it was not possible to discern between true infection and passing-through microorganisms. Even though the small size of these animals may hinder the detection of macroscopic lesions, no visible lesions consistent with mycobacterial infection were observed in positive or negative animals. Pooling of tissues implies a loss of information about the body site where mycobacteria were located, and thus, possible entrance or excretion routes could not be further investigated. However, we have demonstrated that at least one small mammal species, *A. sylvaticus*, can act as a carrier of several NTM, among which *Map* is the only mycobacteria previously found in cattle from the same farm. Although *A. sylvaticus* does not seem to play a relevant role in the epidemiology of the MTC in our study area, we cannot reject the competence of this species to carry MTC if we take into account its ability to carry other mycobacteria, such as the NTM that have been isolated. The scarce information available in the literature on the epidemiology of natural *M. bovis* infection in small mammals does not suggest that *Apodemus* sp. or other small mammal species could maintain the infection in their population, and may be better considered as dead-end hosts (Delahay et al. 2002, 2007; Mathews et al. 2006). Besides, TB prevalence was

minimal during the study period among cattle and wildlife from the Basque Country, and, consequently, it is not striking that *M. bovis* was not found in small mammals. In contrast, the field vole is considered the maintenance host for *M. microti*, a role that similarly might be played by other small mammals (Smith et al. 2009; Kipar et al. 2014). This MTC member seems to be more widespread and infecting more species than previously thought, and has been recently identified in domestic and wild populations in France and northeastern Spain (Michelet et al. 2015, 2016; Pérez de Val et al. 2019). We did not detect *M. microti*, but it could also be that its prevalence is not high enough to favor the detection of infected animals, considering the limitations of our sample size and spatial scale. We cannot rule out the presence of mycobacteria in other small mammal species either, since the number of trapped individuals was very low.

Owing to the small representation of species other than *A. sylvaticus*, we focused the statistical analyses only towards this species. However, no statistically significant differences were detected in mycobacteria prevalence according to the analysed variables, albeit some tendencies were visualized. For instance, a higher prevalence was observed in females. Males normally have a bigger home range, particularly during the reproductive period (Torre et al. 2002), that could give them more chances to come in contact with mycobacteria. Nevertheless, females can be subjected to a higher reproductive stress and opportunistic infections could be more effective in immunocompromised individuals. On the other hand, juveniles showed a higher prevalence as compared to that of the adults. Accumulated risk of infection with increasing age has been previously described for *M. bovis* in other wild species, such as the European badger (Corner et al. 2011). The same pattern could have been expected with other mycobacteria but, conversely, we have found the opposite situation. As for

seasonality, both winter and summer showed a higher prevalence than autumn and spring. The small number of animals trapped during summer may have boosted the observed prevalence in this study, since only nine individuals were captured during this season. During winter, limited food sources could have promoted the entrance of small mammals into the farm buildings, increasing contact with cattle and, thus, mycobacteria circulation and exposure. When analyzing the effect of location, individuals trapped in one of the farms were all negative (Table 1). This could be related to the previous health status of this farm, which presented several outbreaks of paratuberculosis and TB, leading to a stronger implementation of biosecurity and sanitary measures that could have decreased the environmental load of mycobacteria. Despite the absence of a strong statistical relation between NTM prevalence and the explored variables in this study, if small mammals are attributed a vector role, their loitering behaviour could pose a risk of uncontrolled dissemination of mycobacteria or other agents of veterinary relevance among farms and their surroundings.

This is the first report on the detection of mycobacteria in small mammals captured in cattle farms from Spain and the first description of *M. celatum* detection in wild rodents. Conclusively, our results indicate that small mammals such as *A. sylvaticus* can carry potentially pathogenic NTM with the ability to cross-react with TB diagnosis in cattle, but do not seem to play a role in the epidemiology of TB in our study area and period. Due to the indirect interactions between small mammals and cattle that may take place in the environment of farms, a risk of mycobacteria transmission cannot be ruled out. Hence, further studies are required to determine the actual role of small mammals in the epidemiology of mycobacterial infections, as well as to assess if other species of small mammals are implicated. In line with this, active surveillance of NTM in cattle should be promoted in order to delve into the

epidemiology of these bacteria at the wildlife–livestock interface. In addition, novel biosecurity measures directed at minimizing the likelihood of contact between livestock and small mammals should be studied and implemented in agreement with the results obtained in further research.

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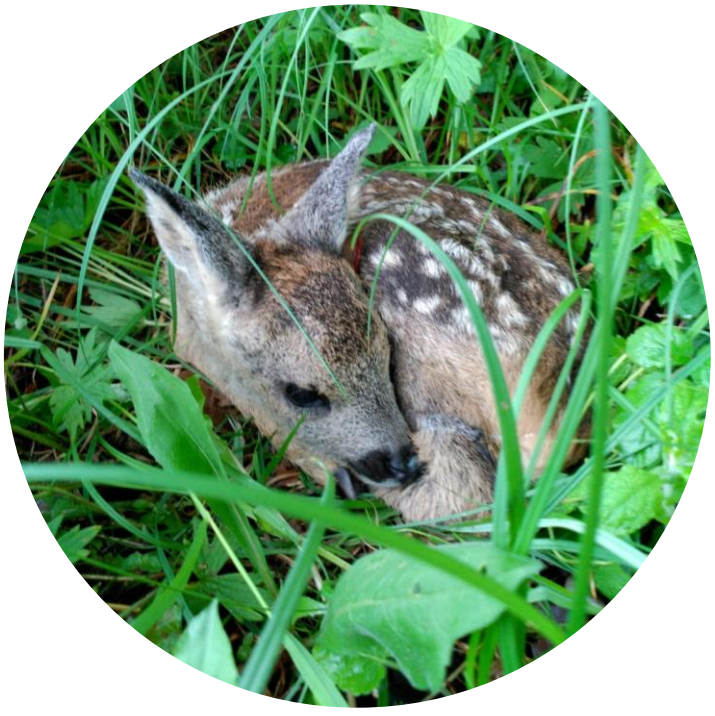
Study IV

Beyond Tuberculosis: Diversity and implications of non-tuberculous mycobacteria at the wildlife-livestock interface



This study is under review for publication:

Varela-Castro L, Barral M, Arnal MC, Fernández de Luco D, Gortázar C, Garrido JM, Sevilla IA (2022). Beyond Tuberculosis: Diversity and implications of non-tuberculous mycobacteria at the wildlife-livestock interface. Transboundary and Emerging Diseases (under review).

Parts of this study have been presented at:

XV Congreso de la Sociedad Española para la Conservación y Estudio de los Mamíferos, Córdoba, Spain, 4-7 December, 2021: Varela-Castro L, Gerrikagoitia X, Alvarez V, Tello M, Molina E, Barral M, Sevilla IA. Más allá de la tuberculosis: Micobacterias no tuberculosas en la interfaz silvestre-doméstica del País Vasco. Poster.

Abstract

Non-tuberculous mycobacteria (NTM) circulate between the environment, animals and humans entailing a double concern: their ability to interfere with tuberculosis (TB) diagnosis and their potential to cause infections in their hosts. However, published records on NTM infections in animals are still scarce. The aims of the present study were to describe the diversity of NTM circulating among wild and domestic species from Spain, and to analyse their implications as potential pathogenic microorganisms or as sources of interferences in the diagnosis of bovine TB. Overall, 293 NTM isolates of 277 animals were obtained from tissue samples collected between 2012 and 2019, and analysed through a multigene approach for mycobacteria identification. Thirty-one species were identified, being *M. avium* subsp. *avium* (*Maa*) and *M. avium* subsp. *hominissuis* (*Mah*), but also *M. bouchedurhonense*, *M. nonchromogenicum* and *M. lentiflavum*, the most abundant ones. *Maa* and *M. lentiflavum* were isolated in several animals showing TB-like lesions. *Maa*, *Mah* and *M. nonchromogenicum* were recovered from many cattle that had reacted to the TST. Other NTM were also associated to these phenomena. These four mycobacterial species were geographically associated between wild boar and other hosts. The findings of the present study suggest that a high diversity of NTM circulates among wildlife and livestock. Wild boar and *M. avium* seem to play a relevant role in this epidemiological scenario.

Keywords: Non-tuberculous mycobacteria, wild boar, *M. avium* complex, *M. nonchromogenicum*, *M. lentiflavum*

1. Introduction

According to the List of Prokaryotic names with Standing in Nomenclature (www.bacterio.net) while writing this manuscript, the genus *Mycobacterium* contained 259 species and 24 validly published subspecies that range from innocuous saprophytes to relevant pathogens (Parte et al. 2020). Some well-known members of the MTC, such as *M. tuberculosis* or *M. bovis*, have historically stood out because of their medical and veterinary relevance, but nowadays so do several species of NTM (Biet and Boschiroli 2014; Saxena et al. 2021).

NTM are widely distributed in the environment, being isolated from a broad variety of sources including water, feed, soil and dust (Falkinham III 2016). Their cell wall composition and their adaptability allow them to survive in different habitats for long periods of time (Hruska and Kaevska 2012) even under adverse conditions. In veterinary medicine, NTM entail a twofold problem: the interference in the diagnosis of bovine TB and the potential to cause infections that may lead to economic losses and deprivation of animal welfare (Bercovier and Vincent 2001; de la Rua-Domenech et al. 2006; Michel 2008; Jaroso et al. 2010; Biet and Boschiroli 2014). However, excluding *Map*, published records on NTM infections in animals are scarce and mainly report secondary findings of MTC research, since active surveillance for the detection of NTM is not contemplated nor is far less mandatory in livestock health control campaigns and wildlife surveillance programmes in general. Even though many of those publications have contributed with substantial information on the implications of a wide range of NTM detected in terrestrial or aquatic, domestic, farmed and/or wild animals (Bercovier and Vincent 2001; Zanetti et al. 2008; Balseiro et al. 2011a; Gcebe et al. 2013, 2018; Pate et al. 2016; Rónai et al. 2016; Gcebe and Hlokwe 2017; Ghielmetti et al. 2018,

2021), there is still a long way to go before being able to understand the relevance of many of these microorganisms. In the Iberian Peninsula, few studies have reported the isolation of NTM in wildlife: Among *M. avium* subspecies, *Maa* and *Mah* have been detected in badger and wild boar (Domingos et al. 2009; Balseiro et al. 2011a; Muñoz-Mendoza et al. 2013), while *Map* has been found in more species: fallow deer, otter (*Lutra lutra*), wild boar and wild rabbit (Álvarez et al. 2005; Balseiro et al. 2008; Maio et al. 2011; Matos et al. 2013, 2016a). Besides, MAC-infected roe deer and foxes have been also reported (Muñoz-Mendoza et al. 2013). Apart from *M. avium*, other NTM have been isolated from wildlife. For instance, *M. intracellulare* was found in red deer, wild boar and wood mouse; *M. interjectum* in red deer and wild boar, and *M. scrofulaceum* in fallow deer, red deer and wild boar (Gortázar et al. 2011a; García-Jiménez et al. 2015; Varela-Castro et al. 2020b). Some species have been detected in one host only, such as *M. xenopi* in fallow deer (Gortázar et al. 2011a), *M. chelonae*, *M. nebraskense* or *M. triplex*, reported in wild boar (García-Jiménez et al. 2015); and *M. fortuitum*, *M. gordonae* or *M. celatum*, found in wood mice (Varela-Castro et al. 2020b). Regarding livestock from the Iberian Peninsula, *M. avium* subspecies such as *Map* have been reported in cattle, goat and sheep, while *Mah* being detected in pigs (Sevilla et al. 2005; de Juan et al. 2006; Domingos et al. 2009; Álvarez et al. 2011), but records of NTM other than *M. avium* have been anecdotally described only: For instance, *M. kansasii* was isolated from a tuberculin-positive goat, while *M. intracellulare* has been described in cattle (Acosta et al. 1998; Gortázar et al. 2011a).

In the last decade, NTM have been isolated from several wild and domestic species during wildlife TB surveillance programmes, bovine TB eradication campaigns or TB-related research. Taking advantage of the material obtained from several regions of Spain, the aims of the present study were 1) to describe the diversity of NTM

circulating in wild and domestic species and 2) to assess the possible implications of these mycobacteria as potential pathogens or as a source of interferences in the diagnosis of bovine TB.

2. Materials and Methods

2.1 Mycobacterial isolates: Study sites and sample collection

The 293 cultures used in this study were recovered from tissue samples belonging to wild and domestic animals from seven different sites in Spain (AR, BA, BC, BU, CR, GR and RI, see Figure 1) that were received in the laboratory between 2012 and 2019. Information on the total number of wild animals analysed per host species and region was only available for the Basque Country (BC): NTM from this region were isolated from 123 animals in the course of a wildlife health surveillance programme for which samples from 818 wild boars, 208 roe deer, 165 red deer, 84 badgers, 41 foxes, 37 carnivores other than badger and fox, one lagomorph (*Lepus* spp.) and one common buzzard (*Buteo buteo*) were cultured. Wildlife samples were collected from road-killed animals or through wildlife population management and hunting activities following different collection protocols according to the entities involved and to the reasons for which they were collected (surveillance/control programmes or confirmation of cases compatible with TB). Although not always possible, routine sampling consisted in collection of mandibular, parotid, retropharyngeal, tracheobronchial, mediastinal, hepatic and mesenteric LNs, as well as any tissue with TB-compatible lesions. Samples collected from livestock belonged to animals slaughtered at abattoirs from the Basque Country for being suspected of TB through *in vivo* official diagnostic methods and/or inspection at slaughter. In agreement with the Spanish National Bovine TB Eradication Programme, in the presence of TB-compatible

lesions, at least the lesioned site including adjacent tissue was analysed in the laboratory. If lesions were not visible, at least one of the LNs from each of the following sites were analysed: head (retropharingeal or mandibular), thoracic cavity (mediastinic or bronquial), thoracic body (cervical or prescapular), abdominal cavity (mesenteric or hepathic) and mammary gland (supramammary). These animals were subsequently ruled out as MTC-infected or confirmed as coinfected with *M. bovis* and NTM. No animal was slaughtered or hunted specifically for this study.

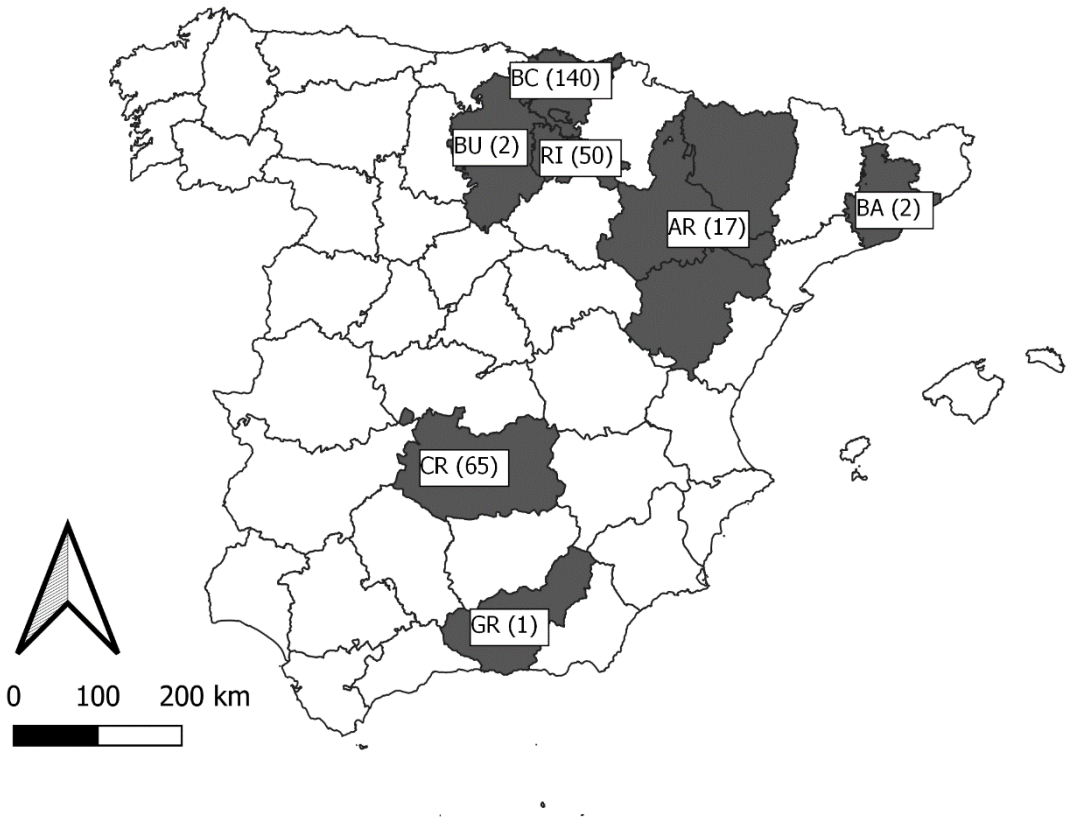


Figure 1. Map showing the sampling sites. The number of animals analysed per site is displayed in brackets.

2.2 Pathological examination

A *post mortem* examination of carcasses (if available) or samples received was performed for the detection of gross pathological changes. Histological analysis was also performed in samples from livestock and in some samples with lesions from

wildlife. Samples were fixed in 10% buffered formalin, dehydrated and embedded in paraffin wax. Sections (3-5 µm thick) were stained with Carazzi's hematoxylin and eosin for histopahological analysis, and Ziehl-Neelsen for acid-fast bacilli detection.

2.3 Culture of samples

Samples were processed for culture at the Biosafety level 3 Laboratory of NEIKER (Bizkaia, Basque Country). Samples were individually processed if macroscopic lesions were observed, provided that they were not pooled originally. Otherwise, they were processed individually or in convenient pools. Samples (LN or pool) were homogenized in sterile distilled water (2 g in 10 ml or equivalently) (Serrano et al. 2018) and subsequently decontaminated using the BD BBL™ MycoPrep™ kit, following manufacturer's instructions. BBL™ MGIT™ (Becton Dickinson, Franklin Lakes, NJ, USA) supplemented with BACTEC™ MGIT™ growth supplement (OADC) and PANTA™ antibiotic mixture were inoculated with the resulting decontaminated samples and incubated in an automated BACTEC MGIT 960 system (Becton, Dickinson and Company, Sparks, MD, USA) at 37ºC for 42 days (Varela-Castro et al. 2021a). *Map*-specific media were not used.

2.4 Tetraplex real-time PCR screening of positive cultures

Pelleted material obtained from 1 ml of MGIT cultures with positive BACTEC TTD readouts was resuspended in 0.25 ml of distilled water, inactivated at 90ºC for 20 min and disrupted with zirconia/silica beads (0.1 mm) for 10 min in a TissueLyser II (Qiagen, Hilden, NRW, Germany). Hereafter, it was centrifuged and the supernanant used in a previously described (Sevilla et al. 2015) and improved (Sevilla et al. 2017) tetraplex real-time PCR for the simultaneous detection of the *Mycobacterium* genus, the subspecies of *M. avium* and the species of the MTC. Finally, extracted DNA from 293

NTM isolates coming from 277 animals (255 wild and 22 domestic) was preserved at -20 °C for further species or *M. avium* subspecies identification: 202 belonged to 192 wild boar, 23 to 21 badgers, 21 to 19 cattle, 17 to 17 red deer, 17 to 16 roe deer, 3 to 3 foxes, 2 to 2 stone martens (*Martes foina*), 2 to 2 American minks (*Neovison vison*), 2 to 2 domestic rabbits, 2 to 1 common buzzard, 1 to 1 goat and 1 to 1 Iberian wild goat.

2.5 Species Identification of NTM isolates

2.5.1 Identification of Mycobacterium sp.-positive and M. avium-negative cultures

PCR amplification and sequence analysis of a portion of the 16S rRNA and the *rpoB* genes was performed for the identification of *Mycobacterium* sp. isolates using three previously described protocols: two for 16S rRNA gen (Wilton and Cousins 1992; Harmsen et al. 2003) and one for *rpoB* gen (Adékambi et al. 2003). PCR mixtures of 50 µl contained 5 µl of 10X *Taq* buffer, 2.5 µl of 50 mM MgCl2, 0.4 µl of 25 mM of each deoxynucleotide triphosphate (dATP, dCTP, dGTP, and dTTP), 0.2 µl of 5 U/µl *Taq* DNA polymerase (Invitrogen S.A., Barcelona, Spain), 1 µl of 10 µM of each forward and reverse primers, 38 µl of RNase/DNase-free water and 2 µl of DNA. PCR amplification conditions and primer sequences are shown in Table 1. A proportion of samples with weak or no amplification were reanalysed under the same PCR conditions but using the CERTAMP Kit for Complex Amplifications (Biotools, Madrid, Spain) following manufacturer's instructions.

Table 1. Primers used for conventional PCR and sequencing.

Target gen	PCR protocol	Sequence (5'-3')	Reference
16S rRNA	A	16S27F: AGAGTTTGATCMTGGCTCAG 16S907R: CCGTCAATTCMTTTRAGTTT	Harmsen et al. 2003
16S rRNA	B	MycGen_F: AGAGTTTGATCCTGGCTCAG MycGen_R: TGCACACAGGCCACAAGGGA	Wilton et al. 1992
rpoB	C	Myco-F: GGCAAGGTCACCCCGAAGGG Myco-R: AGCGGCTGCTGGGTGATCATC	Adekambi et al. 2003

A: 28 cycles of denaturation at 94°C for 45 s, primer annealing at 53°C for 1 min and DNA elongation at 72°C for 90 s. Initial denaturation step of 80°C for 5 min and final elongation step of 72°C for 10 min.

B: 40 cycles of denaturation at 94°C for 30 s, primer annealing at 62°C for 30 s and DNA elongation at 75°C for 90 s. Initial denaturation step of 95°C for 5 min and final elongation step of 72°C for 10 min.

C: 35 cycles of denaturation at 94°C for 30 s, primer annealing at 64°C for 30 s and DNA elongation at 72°C for 90 s. Initial denaturation step of 95°C for 5 min and final elongation step of 72°C for 10 min.

PCR products were treated with the ExoSAP-IT™ kit (Thermo Fisher scientific Inc., Waltham, MA, USA) or purified from agarose gels with the Genelute Gel Extraction kit (Sigma-Aldrich Co. Ltd., St. Louis, MO, USA), as recommended by the manufacturers. Purified amplicon and appropriate primer were mixed at requested concentrations and sent to EuroFins GATC Biotech GmbH (Konstanz, Germany) for sequencing. Sequencing primers were the same used for amplification in both cases. Inspection, edition and alignment of sequences was performed using the Unipro UGENE 40.1 free software (Unipro, Novosibirsk, Russia) and then compared with other published sequences using online BLAST analysis (NCBI, NLM, Bethesda, MD, USA). Because the 5' end sequence of 16S rRNA gene is considered sufficient for species identification of most mycobacteria (Harmsen et al. 2003), the 5' end region flanked by 16S27F/MycGen_F and BKL1-R primer was retained for BLAST and phylogenetic analysis (≈ 477 bp) (Monteserin et al. 2016). To assign species, ≥ 99.7% similarity to reference sequences was required for 16S rRNA and ≥ 97% for *rpoB* (Monteserin et al. 2016). Afterwards, a consensus for species identification was performed as follows:

When species assignation derived from 16S rRNA and *rpoB* gene sequences was concordant and achieved the percentage of similarity for both of them, the species identification consensus was recorded. When genes were not in complete agreement in assigning species and both achieved the percentage of similarity, both results, excluding *Mycobacterium* sp. hits, were recorded for species identification. When the percentage of similarity was not accomplished for one of the genes, species assignation was conducted with the other gene only. If the percentage of similarity was not accomplished for any of the genes, isolates remained unidentified at species level and they were recorded as *Mycobacterium* sp., but the hit with highest percentage of similarity of the BLAST search was recorded to state to which NTM species was the sequence closest to. If the final consensus was an unclassified mycobacterium (e.g., *Mycobacterium* sp. GN-9680) this result was recorded, but the next BLAST hit with species designation displaying the highest percentage of similarity was also recorded to indicate to which species could be most related to.

Some isolates could not be identified through the aforementioned methods due to failing of PCR amplification or to poor-quality sequences (mycobacteria with no sequencing result). To discard non-specific results in the screening PCR and confirm that these isolates belonged to the genus *Mycobacterium*, the Genotype Mycobacterium CM kit (Hain Lifesciences GmbH, Nehren, Germany) was used following the indications of the manufacturer. The presence-absence of a band in the Genus Control position of the membrane strips of the kit was used for this purpose.

2.5.2 Identification of M. avium subsp.-positive cultures

For *M. avium* subspecies identification, DNA was analysed by two real-time PCR methods described earlier to amplify IS*1245* and IS*901* insertion sequences (Slana et al. 2010). Identification was performed on the basis of presence–absence signatures obtained for these genomic targets (Bartos et al. 2006): *Maa*, including the variant called *Mas* is IS*1245*+, IS*901*+, while *Mah* is IS*1245*+, IS*901*−.

2.6 Phylogenetic analyses

For phylogenetic analyses, incomplete sequences missing some informative base positions at 5' and/or 3' ends were removed to avoid loosing information of complete sequences. Two phylogenetic trees, one per gen, were constructed with MEGA-X 10.2.0 software, using the neighbour-joining method (Saitou and Nei 1987). The evolutionary distances were computed using the Kimura 2-parameter method (Kimura 1980) and all positions containing gaps and missing data were eliminated (complete deletion option). Bootstrap tests were calculated on 1000 replicates and trees were rooted using *Nocardia farcinica* as the outgroup. The style of the trees was edited using the online tool iTOL 6.4 (Letunic and Bork 2021).

3. Results

Out of the 293 isolates, 83 belonged to *M. avium* subspecies and 210 to other NTM. For NTM other than *M. avium* we obtained valid sequences of both genes in 50.50% of the isolates (106). We obtained mycobacteria sequences of 16S rRNA gene alone in 25/210 isolates and of *rpoB* gene alone in 28/210 isolates, either because genes were not amplified or were weakly amplified despite several PCR attempts, because sequences did not display sufficient quality or because other microorganisms

contaminating the cultures were co-amplified (mainly with the 16S rRNA PCR protocol described by Harmsen et al. 2003). Altogether, isolate identification was possible to conduct in 76% of the NTM analysed through sequencing (159/210) and in 83% of all the isolates (242/293) (see Supplementary Table 1). Still, the Genotype Mycobacterium CM kit confirmed the presence of mycobacterial DNA in all the cases with no sequencing result.

Out of 242 isolates, 166 belonged to 31 known species, 37 displayed homology with more than one species (in 28 cases 16S rRNA and *rpoB* species assignation was not in full agreement and in the other 9 cases *rpoB* was lacking and 16S rRNA sequence was identical for more than one NTM species), 27 belonged to 7 unclassified mycobacteria strains (e.g., *Mycobacterium* sp. GN-9680 or *Mycobacterium* sp. 34028-3) and 12 did not meet the established similarity criteria with any deposited sequence (#39, #56, 219#, #225, #226, #227, #328, #367, #376, #386, #449 and #454 (unidentified mycobacteria) (See Supplementary Table 1 for further detail).

Regarding the single region with information on the number of wild animals analysed per host species (BC region), an overall NTM prevalence of 9.10% was detected. At host species level, this prevalence was 21.42% for badger, 10.64% for wild boar, 5.29% for roe deer, 4.88% for fox, 3.66% for red deer and 2.70% for carnivores other than badger and fox. The NTM found in the different hosts considering all study sites is shown in Table 2. NTM belonging or related to MAC were the most abundant, particularly *Maa* (n=48) and *Mah* (n=35), but also *Mycobacterium* sp. GN-9680 (n=15) and *M. bouchedurhonense* (n=12). The predominance of MAC was also true for the hosts with more isolates (badger, cattle, red deer, roe deer and wild boar), being *Maa* or *Mah* again the most abundant except for red deer, for which strain *Mycobacterium sp.* 8115-1 was the most frequent. Out of the MAC, the species most frequently isolated

were *M. nonchromogenicum* (n=12) and *M. lentiflavum* (n=10). Two cases of coinfection with different NTM were detected, both of them in the Basque Country: one badger was coinfected with *M. kumamotonense* and *M. septicum* and one roe deer with *Maa* and *Mah*. Besides, coinfection with *M. bovis* and NTM was identified in 14 wild boar from CR infected with *M. alvei*, *M. bouchedurhonense* (n=3), *M. paraffinicum*, *M. porcinum*, *M. vulneris/colombiense/intracellulare/bouchedurhonense* (n=2), *Mycobacterium* sp. GN-9680 (n=2), *Mycobacterium* sp. IEC1808 (related to *M. interjectum*), *Mycobacterium* sp. 2 (n=2) and a NTM with no sequencing result, and in two cattle from the Basque Country infected with *Maa* and *Mah*.

Table 2. NTM identified per host species. The proportion of isolates out of the total isolates obtained from each host species and their geographic origin is indicated.

NTM species (N of isolates)	Host sp. (N of isolates/total isolates)	(%)	Region
Maa (48)	Badger (2/23)	8.70%	BC, RI
	Cattle (6/21)	**28.60%**	BC
	Buzzard (2/2)	100%	BC
	Rabbit (2/2)	100%	BA
	Fox (1/3)	33.33%	BC
	Wild goat (1/1)	100%	AR
	American mink (2/2)	100%	RI
	Red deer (2/17)	11.76%	BC, RI
	Roe deer (9/17)	**52.94%**	BC, RI
	Stone marten (1/2)	50%	RI
	Wild boar (20/202)	**9.90%**	AR, BC, CR, RI
Mah (35)	Badger (7/23)	**30.40%**	BC
	Cattle (4/21)	19.05%	BC
	Red deer (1/17)	5.88%	BC
	Roe deer (3/17)	17.64%	BC
	Stone marten (1/2)	50%	BC
	Wild boar (19/202)	9.41%	BC, RI, CR, AR
M. alvei (1)	Wild boar (1/202)	0.50%	CR
M. arosiense (1)	Wild boar (1/202)	0.50%	BC
M. arosiense/colombiense/intracellulare/bouchedurhonense (1)	Wild boar (1/202)	0.50%	CR
M. bohemicum (2)	Wild boar (2/202)	0.99%	BC, RI
M. bouchedurnonense (12)	Badger (2/23)	8.70%	BC
	Cattle (1/21)	4.76%	BC
	Red deer (2/17)	11.76%	BC
	Roe deer (1/17)	5.88%	BC
	Wild boar (6/202)	2.97%	CR, RI
M. bouchedurhonense/intracellulare (1)	Wild boar (1/202)	0.50%	RI
M. colombiense/intracellulare/bouchedurhonense (14)	Badger (1/23)	4.34%	RI
	Red deer (1/17)	5.88%	RI
	Wild boar (12/202)	5.94%	CR, RI
M. colombiense/intracellulare/intracellulare subsp. *yongonense/bouchedurhonense* (2)	Wild boar (2/202)	0.99%	CR, RI
M. confluentis (2)	Wild boar (2/202)	0.99%	BC, RI
M. diernhoferi (1)	Wild boar (1/202)	0.50%	RI
M. elephantis (1)	Wild boar (1/202)	0.50%	AR
M. elephantis/holsaticum (1)	Red deer (1/17)	5.88%	RI
M. engbaekii (2)	Wild boar (2/202)	0.99%	BC
M. europaeum (1)	Wild boar (1/202)	0.50%	BC
M. florentinum (1)	Badger (1/23)	4.34%	BC
M. fortuitum (3)	Roe deer (1/17)	5.88%	BC
	Wild boar (2/202)	0.99%	BC
M. hiberniae (1)	Wild boar (1/202)	0.50%	BC
M. interjectum (6)	Badger (3/23)	13.00%	BC, RI
	Wild boar (3/202)	1.49%	BC, CI, RI
M. intermedium (2)	Wild boar (2/202)	0.99%	BC, RI
M. intracellulare (3)	Wild boar (3/202)	1.49%	BC, CR
M. intracellulare subsp. *chimaera*/subsp. *yongonense* (1)	Roe deer (1/17)	5.88%	BC
M. kansasii (1)	Roe deer (1/17)	5.88%	BC
M. kumamotonense (1)	Badger (1/23)	4.34%	BC
M. lentiflavum (10)	Red deer (2/17)	11.76%	AR
	Roe deer (1/17)	5.88%	AR
	Wild boar (7/202)	3.47%	AR, CR, RI
M. nebraskense (1)	Wild boar (1/202)	0.50%	BC
M. nonchromogenicum (12)	Cattle (3/21)	14.29%	BC, GR

NTM species (N of isolates)	Host sp. (N of isolates/total isolates)	(%)	Region
	Wild boar (9/202)	4.46%	BC, RI
M. paraense (2)	Wild boar (2/202)	0.99%	CR
M. paraffinicum (1)	Wild boar (1/202)	0.50%	CR
M. parascrofulaceum (6)	Wild boar (6/202)	2.97%	BC
M. peregrinum/arcueilense/montmartrense/lutetiense/septicum (1)	Wild boar (1/202)	0.50%	BC
M. porcinum (1)	Wild boar (1/202)	0.50%	CR
M. scrofulaceum (2)	Cattle (1/21)	4.76%	BC
	Wild boar (1/202)	0.50%	BC
M. senegalense/farcinogenes/houstonense/fortuitum/conceptionense (1)	Fox (1/3)	33.33%	BC
M. senuense (1)	Goat (1/1)	100%	BC
M. seoulense (2)	Wild boar (2/202)	0.99%	BC, CR
M. septicum (3)	Badger (1/23)	4.34%	BC
	Wild boar (2/202)	0.99%	BC, RI
M. triplex (1)	Cattle (1/21)	4.76%	BC
M. vulneris/colombiense/intracellulare/bouchedurhonense (11)	Badger (1/23)	4.34%	BC
	Red deer (1/17)	5.88%	RI
	Wild boar (9/202)	4.46%	CR, RI
Mycobacterium sp. 34028-3* (5)	Wild boar (5/202)	2.48%	BC, CR, RI
Mycobacterium sp. 3582* (2)	Wild boar (2/202)	0.99%	BC
Mycobacterium sp. 3582/J16* (4)	Red deer (1/17)	5.88%	BC
	Wild boar (3/202)	1.49%	BC
Mycobacterium sp. 8115-1* (3)	Red deer (3/17)	**17.64%**	RI
Mycobacterium sp. GN-9680* (15)	Wild boar (15/202)	7.43%	CR
Mycobacterium sp. IEC1808* (1)	Wild boar (1/202)	0.50%	CR
Mycobacterium sp. TY59* (1)	Wild boar (1/202)	0.50%	BC
Mycobacterium sp. 1 (2)	Wild boar (2/202)	0.99%	CR
Mycobacterium sp. 2 (7)	Wild boar (7/202)	3.47%	CR
Mycobacterium sp. 3 (1)	Wild boar (1/202)	0.50%	BC
Mycobacterium sp. 4 (1)	Wild boar (1/202)	0.50%	BC
Mycobacterium sp. 5 (1)	Cattle (1/21)	4.76%	BU
Mycobacteria with no sequencing result (51)	Badger (4/23)	17.40%	BC
	Cattle (4/21)	19.00%	BC, BU
	Fox (1/3)	33.33%	CR
	Red deer (3/17)	17.65%	BC, RI
	Wild boar (39/202)	19.31%	AR, BC, CR, RI

n.d.=not determined, %=proportion of the specified NTM out of the total number of isolates retrieved from each host species. *Maa=M. avium* subsp. *avium*, *Mah=M. avium* subsp. *hominissuis*, *Mycobacterium* sp. 1= *Mycobacterium* sp. close to species belonging to *M. avium* complex, *Mycobacterium* sp. 2= *Mycobacterium* sp. close to *M. palustre*/*lentiflavum/paraense*, *Mycobacterium* sp. 3= *Mycobacterium* sp. close to *M. scrofulaceum*, *Mycobacterium* sp. 4= *Mycobacterium* sp. close to *M. wolinskyi*. *Mycobacterium* sp. 5= *Mycobacterium* sp. close to *M. chitae*. *: *Mycobacterium* sp. GN-9680 and TY59 are related to *M. avium* complex, *Mycobacterium* sp. 34028-3 to *M. triplex/stomatepiae*/*montefiorense*, *Mycobacterium* sp. IEC1808 to *M. interjectum*, *Mycobacterium* sp. 3582 to *M. nebraskense*, *Mycobacterium* sp. J16 to *M. scrofulaceum* and *Mycobacterium* sp. 8115-1 to *M. duvalii*. See Supplementary Table 1 for more detailed information. The proportion of the NTM species detected more frequently in each animal species is marked in bold letters for those hosts with more than 3 isolates.

Maa, *Mah*, *M. nonchromogenicum* and *M. lentiflavum* were isolated from different hosts within the same municipality, being wild boar always one of the implicated hosts (see Table 3).

Table 3. NTM species isolated from different hosts in the same municipality.

Region	Municipality	NTM species	Hosts
BC	Aiara	*Maa*	Wild boar and cattle
	Gueñes	*Mah*	Wild boar and cattle
	Kortezubi	*Maa*	Wild boar and roe deer
	Kuartango	*M. nonchromogenicum*	Wild boar and cattle
	Vitoria-Gasteiz	*Maa*	Wild boar, badger and roe deer
	Zuia	*Maa*	Wild boar and red deer
		Mah	Wild boar and red deer
AR	Jaca	*M. lentiflavum*	Wild boar and red deer

Maa=M. avium subsp. *avium, Mah=M. avium* subsp. *hominissuis*

Records on lesions compatible with TB and on mycobacteria isolated from the animals showing those lesions are summarized in Table 4. Lesions were detected in a total of 47 individuals (mainly wild boar). Macroscopic findings consisted in encapsulated or diffuse lesions such as single or multiple caseous, necrotic or calcified nodules ranging in size from 1 to 30 mm. Histological findings were mainly observed as necrotic granulomas. Among animals for which pathological analysis was performed at both macroscopic and microscopic levels, gross and histological lesions were found in 18 individuals, while other six only had microscopic (n=3) or macroscopic (n=3) lesions. The remaining animals with lesions (n=23) were only macroscopically examined. In 37 out of the 47 individuals showing lesions (78.72%), NTM were isolated from the sample where lesions were detected, being *Maa* (in 11 animals) and *M. lentiflavum* (in 8 animals) the most frequent species. Among the remaining 10 individuals, only *M. bovis* was isolated from the lesioned tissue (4 wild boar), no mycobacterium was isolated from those tissues in 5 animals and the lesioned tissue was not available for culture in one animal.

Table 4. NTM isolated from hosts showing microscopic/gross lesions.

NTM identified (N of animals)	Host	N of animals	Location of lesion	Tissue from which NTM were isolated	*M. bovis* isolation (tissue)	Region
Maa (13)	Buzzard	**1**	Liver/intestines	Liver/intestines	No	BC
	Cattle	1	RPh LN$^{\mu}$	TBr LN	No	BC
		1	RPh/Med LNs	Med LN	No	BC
		1	RPh$^{\mu}$/TBr*/Med* LNs	Med LN	Yes (TBr LNs)	BC
	Wild goat	**1**	Lung*	Lung	No	AR
	Rabbit	**2**	Caecal appendix*	Caecal appendix	No	BA
	Wild boar	**4**	Mand LN*	Mand LN	No	AR
		1	Tonsil	Med+TBr LNs	No	CR
		1	Mand LN	Mand LN	No	BC
Mah (3)	Cattle	1	Med LN$^{\mu}$	RPh LN	Yes (Med and TBr LNs)	BC
	Wild boar	**1**	Mand LN	Mand LN	No	BC
	Wild boar	**1**	Mand LN*	Mand LN	No	AR
M. bouchedurhonense (3)	Cattle	**1**	Tonsil	Tonsil	No	BC
	Wild boar	1	Med LN	Mand LN+Tonsil	Yes (Med+TBr LNs)	CR
		1	Mand LN	Med+TBr LNs	Yes (Mand LN+Tonsil)	CR
M. col/int/bou (3)	Wild boar	**1**	Mand LN	Mand LN+Tonsil/Med+TBr LNs	No	CR
		1	Mand LN	Mand LN+Tonsil	No	CR
		1	Lung $^{\Omega}$	Mand LN+Tonsil	No	CR
M. elephantis (1)	Wild boar	**1**	Mand LN*	Mand LN	No	AR
M. intracellulare (1)	Wild boar	**1**	Mand LN	Mand+Par LNs pool	No	BC
M. lentiflavum (9)	Red deer	**2**	RPh LN*	RPh LN	No	AR
	Roe deer	**1**	Prescp LN*	Prescp LN	No	AR
	Wild boar	**5**	Mand LN*	Mand LN	No	AR
		1	Mand LN	Med+TBr LNs	No	CR
M. nonchromogenicum (2)	Cattle	**1**	Med LN$^{\mu}$/Lung	Lung	No	BC
	Wild boar	**1**	Head LNs	Head LNs	No	BC
M. paraense (1)	Wild boar	**1**	Mand LN	Mand LN+Tonsil	No	CR
M. paraffinicum (1)	Wild boar	1	Mand LN	Med+TBr LNs	Yes (Mand LN+Tonsil)	CR
M. porcinum (1)	Wild boar	**1**	Mand/TBr/Med/Lung $^{\Omega}$ /Mes $^{\Omega}$ LNs	Mand LN+Tonsil	Yes (Med+TBr LNs)	CR
M. parascrofulaceum (1)	Wild boar	**1**	Mand LN	Mand LN	No	BC
M. seoulense (1)	Wild boar	1	Mand LN	Med+TBr LNs	No	CR
M. triplex (1)	Cattle	**1**	RPh LN	RPh LN	No	BC
M. sp. IEC1808$^{¥}$ (1)	Wild boar	**1**	Mand /TBr LNs	Mand LN+Tonsil	Yes (Med+TBr LNs)	CR
Mycobacteria with no sequencing result (5)	Wild boar	**3**	Mand LN	Mand LN	No	AR, BC
		1	Mand LN	Mand LN+Tonsil	No	CR
		1	Mand LN	Med+TBr LNs	No	CR

LN=lymph node. *Maa= M. avium* subsp. *avium*, *Mah=M. avium* subsp. *hominissuis*, *M. col/int/bou=M. colombiense/intracellulare/bouchedurhonense*. Med=mediastinal, Mand=Mandibular, Mes= Mesenteric, Par=Parotid, Prescp=Prescapular,

TBr=Tracheobronquial, RPh=Retropharyngeal. ¥: *Mycobacterium* sp. IEC1808 is related to *M. interjectum*. *: Lesions were both macro and microscopic. µ: Lesions were only microscopic. Ω: Not available for culture. The number of animals from which NTM were isolated from the sample with lesions are highlighted in bold and underlined.

Different NTM were isolated from 20 domestic ruminants that were analysed under TB surveillance procedures. The causes for having been submitted to TB microbiological diagnosis (TST reactivity, TB-compatible lesions detected at slaughter or follow-up or depopulation operation of herds where TB-positive animals were detected) are summarized in Table 5, together with the mycobacteria isolated.

Table 5. Causes for slaughter or for laboratory TB diagnosis in NTM-infected livestock.

NTM isolated	Livestock (N)	TST reactor	TB-compatible lesions at slaughter	Cause for slaughter	TB confirmation
M. senuense	Goat (1)	Yes	No	TST-reactor	No
Maa	Cattle (2)	Yes	No	TST-reactor	No
	Cattle (1)	Yes	Yes	TST-reactor	No
	Cattle (1)	Yes	No	TST-reactor	Yes
Mah	Cattle (1)	Yes	No	TST-reactor	Yes
	Cattle (1)	No	No	Depopulation	No
	Cattle (2)	Yes	No	TST-reactor	No
M. nonchromogenicum	Cattle (3)	Yes	No	TST-reactor	No
M. scrofulaceum	Cattle (1)	Yes	No	TST-reactor	No
M. triplex	Cattle (1)	Yes	No	TST-reactor	No
M. bouchedurhonense	Cattle (1)	No	Yes	Depopulation	No
Mycobacterium sp.5	Cattle (1)	No	No	Follow-up	No
Mycobacteria with no sequencing result	Cattle (3)	Yes	No	TST-reactor	No
	Cattle (1)	No	No	Follow-up	No

TST=Tuberculin skin test. *Mycobacterium* sp. 5 is close to *M. chitae*.

Maa and *M. nonchromogenicum* were the species most frequently isolated from TB-negative but TST-reactor cattle, followed by *Mah*. *M. scrofulaceum* and *M. triplex* were also isolated from reactor cattle, as well as *M. senuense* from one reactor goat.

The phylogenetic relatedness between isolates based on 16S rRNA and *rpoB* sequences is graphically represented by the phylogenetic trees shown in Figure 2 (16S rRNA) and Figure 3 (*rpoB*). As illustrated in these figures, both trees separated slow-growing mycobacteria from rapid-growing mycobacteria. Even though members of the

MAC are clearly the most represented mycobacteria in this study and therefore within the trees (even more taking into account that *M. avium* subspecies are not included in the trees), NTM belonging to *M. terrae* complex, *M. simiae* complex and *M. fortuitum* group were also detected, as shown in these figures. However, we have not detected any NTM from *M. abscessus-chelonae* complex, *M. celatum* group or *M. xenopi* group. When outputs of both genes were available, results were mostly concordant. *rpoB* tree was more ramified showing a higher power of discrimination between isolates than the tree for 16S rRNA sequences (e.g., # 116, # 139 or # 353; See Supplementary Table 1).

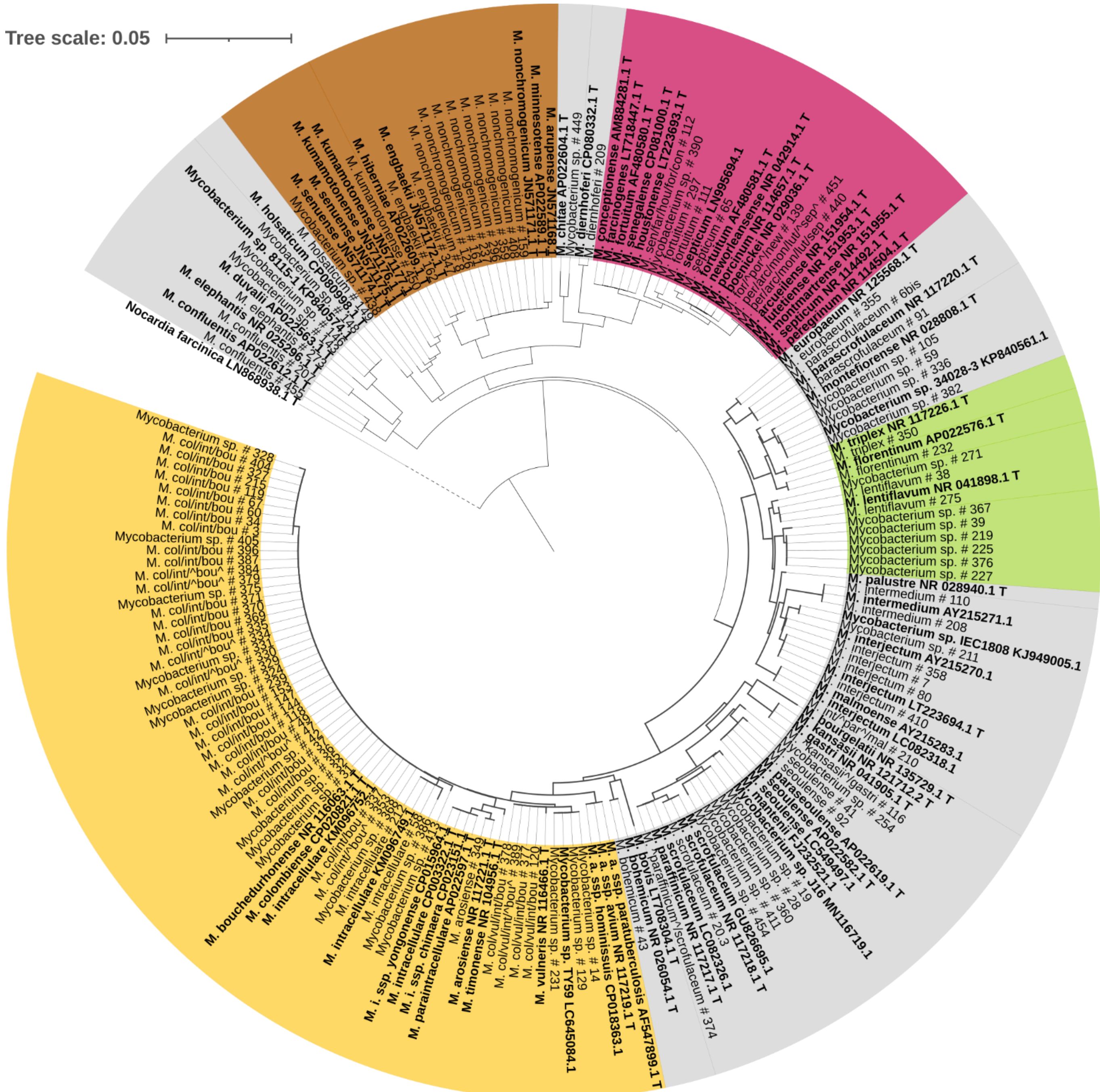


Figure 2. Phylogenetic tree based on 16S rRNA sequences obtained from NTM isolates other than *M. avium*. It was constructed using the neighbour-joining method, the Kimura 2-parameter method and the complete deletion option in MEGA-X 10.2.0 software. Bootstrap test was calculated on 1000 replicates. *Nocardia farcinica* was used as the outgroup. Reference sequences are shown in bold letters. *M. terrae* complex, *M. fortuitum* group, *M. simiae* complex, *M. avium* complex and their related *Mycobacterium* spp. are shown in brown, fuschia, green and yellow respectively (complex/group designation according to Tortoli et al. 2017). Branches of slow growers are drawn thicker than those of rapid growers. M. a. ssp=*M. avium* subspecies, M. i. ssp=*M. intracellulare* subspecies. Isolates displaying more than one species (because all options had identical 16S rRNA sequence) were abbreviated with a three-letter code: M. col/int/bouch=*M. colombiense/intracellulare/bouchedurhonense*, M. col/vul/int/bouch=*M. colombiense/vulneris/intracellulare/bouchedurhonense*, M. int/par/mal=*M. interjectum/paraense/malmoense*, M. sen/far/hou/for/con= *M. senegalense/farcinogenes/houstonense/fortuitum/conceptionense*. M for/por/new=*M. fortuitum, porcinum/neworleansense*, M. per/arc/mon/lut/sep= *M. peregrinum/arcueilense/montmartrense/lutetiense/septicum*. When the 16S rRNA sequence was identical for more than one NTM, the species assigend by *rpoB* sequencing (if available) was marked by including its designation between ^ symbols.

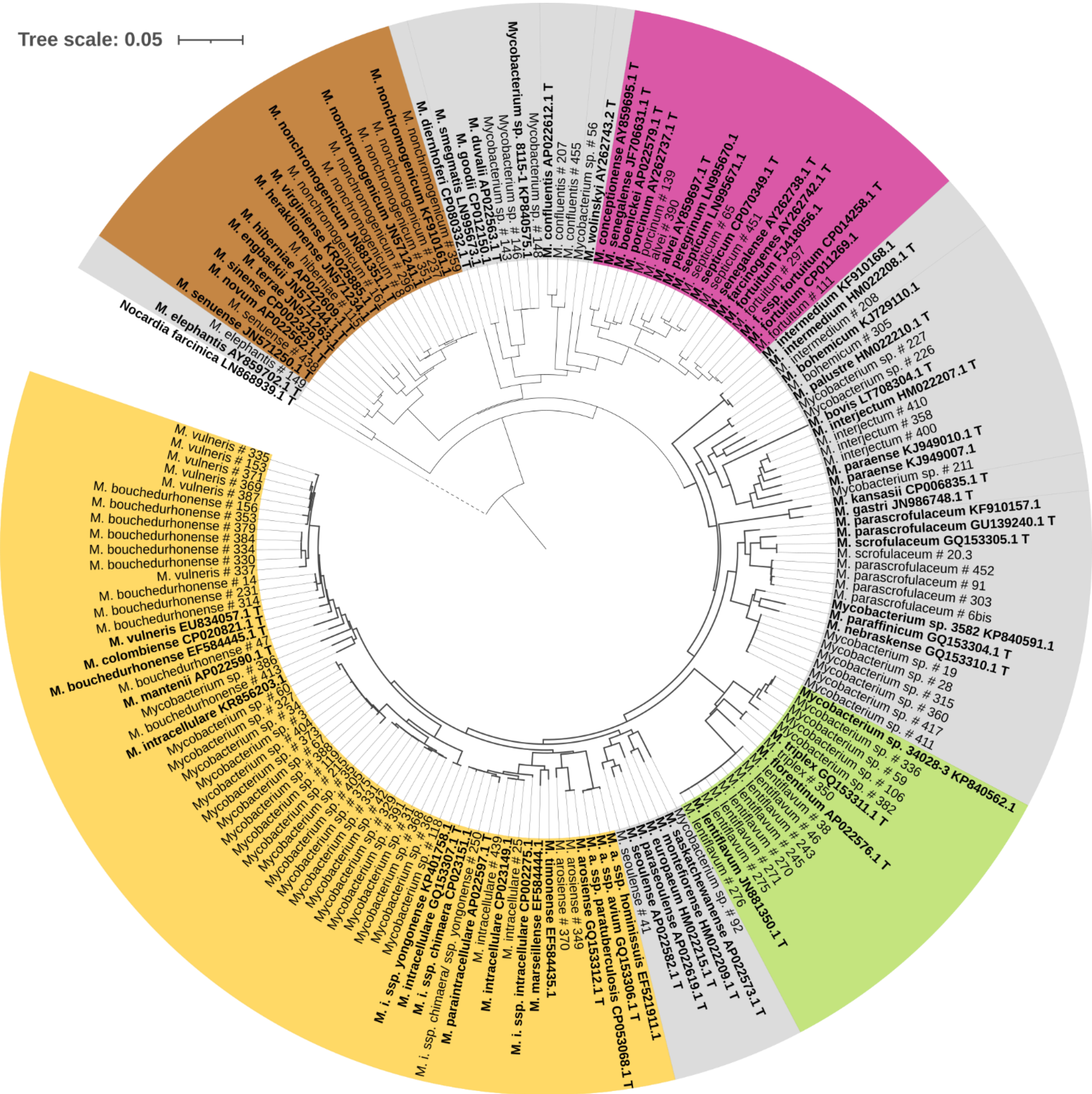


Figure 3. Phylogenetic tree based on *rpoB* sequences obtained from NTM isolates other than *M. avium*. It was constructed using the neighbour-joining method, the Kimura 2-parameter method and the complete deletion option in MEGA-X 10.2.0 software. Bootstrap test was calculated on 1000 replicates. *Nocardia farcinica* was used as the outgroup. Reference sequences are shown in bold letters. *M. terrae* complex is shown in brown, *M. fortuitum* group is shown in fuchsia, *M. simiae* complex and related *Mycobacterium* spp. are shown in green and *M. avium* complex and related *Mycobacterium* spp. are shown in yellow (complex/group designation according to Tortoli et al. 2017). Branches of slow growers are drawn thicker than those of rapid growers. M. a. ssp=*M. avium* subspecies, M. i. ssp=*M. intracellulare* subspecies, M. f. ssp=*M. fortuitum* subspecies.

4. Discussion

The information gained in this study greatly contributes to the body of knowledge on NTM epidemiology, especially to that concerning the Iberian Peninsula (Álvarez et al. 2005; Balseiro et al. 2008, 2011b; Domingos et al. 2009; Gortázar et al. 2011a; Maio et al. 2011; Matos et al. 2013, 2016a; Muñoz-Mendoza et al. 2013; García-Jiménez et al. 2015; Varela-Castro et al. 2020b). *Maa*, *Mah* and *M. bouchedurhonense* from the MAC, *M. nonchromogenicum* from the *M. terrae* complex and *M. lentiflavum* from the *M. simiae* complex were the species most frequently isolated. *Maa* causes avian TB in several species of birds (Dhama et al. 2011) and *Mah*, even though considered more environmental and ubiquitous (Turenne et al. 2008; Biet and Boschiroli 2014), infects mainly swine (Agdestein et al. 2014), but they can also infect other wild and domestic hosts (Dvorska et al. 2004; Glawischnig et al. 2006; Balseiro et al. 2011a; Muñoz-Mendoza et al. 2013). We have isolated both or at least one of these subspecies from almost all the hosts. *M. nonchromogenicum* is an environmental mycobacterium that has been frequently isolated in wildlife and cattle (Biet and Boschiroli 2014; Rónai et al. 2016). In our study, *M. nonchromogenicum* was one of the most prevalent species, being isolated from cattle and wild boar. *M. lentiflavum* is considered an emerging pathogen for humans causing cervical lymphadenitis (Miqueleiz-Zapatero et al. 2018). It has been previously detected in wild boar, warthog, buffalo, gazelle and cattle, among other hosts (Katale et al. 2014; García-Jiménez et al. 2015), a list of host species extended to the red deer and the roe deer according to our results. Information on *M. bouchedurhonense* is scarce in the literature. It has been reported in an antelope (eland) and a leopard from South Africa (Gcebe and Hlokwe 2017) as well as from human patients from France and Zambia (Salah et al. 2009; Mwikuma et al. 2015). In our study, its presence in several hosts was noteworthy,

including cattle, wild boar, badger, red deer and roe deer. Some of the unclassified mycobacteria detected (*Mycobacterium* sp. GN-9680, TY59 and IEC1808) were previously isolated from human patients (Gitti et al. 2011; Fusco Da Costa et al. 2015), while others were found in cattle and red deer (*Mycobacterium* sp. 8115-1, related to *M. duvalii*) or in wild boar (*Mycobacterium* sp. 34028-3 and 3582, related to *M. triplex*/*stomatepiae*/*montefiorense* and to *M. nebraskense*) (Rónai et al. 2016). Most of our isolates were retrieved from wild boar from specific sites, except for *Mycobacterium* sp. 8115-1 and *Mycobacterium* sp. 3582/J16 (the latter related to *M. scrofulaceum*), which were detected only in red deer or in both red deer and wild boar from different sites, respectively. Finally, 12 isolates (4% of the total) did not meet the similarity criteria established for species designation and could belong to novel NTM species or variants not reported yet.

Although most of the NTM species detected throughout this study have been already reported in wild or domestic species (Biet and Boschiroli 2014; García-Jiménez et al. 2015; Rónai et al. 2016; Gcebe and Hlokwe 2017), we have greatly contributed to swell the list of potential hosts for different NTM. For instance, we report several NTM not detected before in badger (e.g., *M. florentinum*, *M. interjectum* or *M. septicum*), a host species for which very little is known in Europe with regard to NTM apart from reports on MAC infections (Balseiro et al. 2011a). This is also the first report on *M. bouchedurhonense* and *M. nonchromogenicum* detection in cattle and wild boar from Spain. Some NTM were only detected in wildlife (e.g., *M. hiberniae*, *M. kansasii*, *Mycobacterium* sp. 34028-3) or in livestock (e.g., *M. triplex*, *M. senuense*), which could mean they would be exclusively circulating in those animal populations for reasons not assessed in this study. However, *Maa*, *Mah* or *M. nonchromogenicum* were detected in both wild and domestic species, indicating that wildlife and livestock can be infected

with the same species and contribute to the environmental spread of such NTM. In addition, these three species together with *M. lentiflavum* were found in different host species from the same geographic location (municipality level). This geographical association was remarkable for wild boar, which was always involved. Despite being the best represented host species in this work, these findings, together with the high NTM prevalence observed in the Basque Country (>10%) make of this wild ungulate a good candidate host for NTM spreading in this region.

Even though the occurrence of TST-reactor cattle due to NTM is not very frequent, in low TB prevalence settings its impact can be considered more relevant, causing great concern to both farmers and authorities. Excluding those cases where coinfection with MTC was confirmed, *Maa*, *Mah* and *M. nonchromogenicum* were the NTM most commonly isolated from TST-reactor cattle. All *Maa*, two out of the three *Mah* and all *M. nonchromogenicum* isolates from livestock were retrieved from cows reacting to TST. This is in agreement with the literature in that *Maa* and *Mah* have been pointed out as a cause of interference in the diagnosis of bovine TB (de la Rua-Domenech et al. 2006; Biet and Boschiroli 2014; Scherrer et al. 2018). This cross-reacting role has also been suggested for *M. nonchromogenicum* (Hughes et al. 1993). *M. scrofulaceum*, *M. triplex* and *M. senuense* were also sporadically isolated from TST-reactor animals not coinfected with MTC. *M. scrofulaceum* is an opportunistic pathogen for animals that has been isolated from farmed and domestic species (e.g., buffaloes, deer, swine and cattle) that reacted to TST, specially to PPD-A (Bercovier and Vincent 2001). As far as we know from the literature, *M. triplex* and *M. senuense* are also opportunistic pathogens, but despite of having been isolated from wild boar (*M. triplex*) and from pigs and sheep (*M. senuense*) (Muwonge et al. 2012; Zeng et al. 2013; García-Jiménez et al. 2015), detection in reactor animals has not been previously reported. To

the best of our knowledge, interferences with the diagnosis of bovine TB have not been experimentally described for any of these two NTM, but in the light of our results their ability to cross react with TST official reagents should not be ruled out. In spite of not having been detected infecting livestock in this study, some NTM species considered to be able to interfere with bovine TB diagnosis (Biet and Boschiroli 2014) were detected in wildlife. Specifically, *M. fortuitum* was isolated from wild boar and roe deer, *M. hiberniae* from wild boar and *M. kansasii* from roe deer. Although we did not isolate *M. kansasii* from cattle, this species is of great significance in terms of TB diagnosis because it seems to be cross-reactive not only with PPD, but also with defined antigens based on ESAT-6 or CFP-10 (Scherrer et al. 2019). Regarding the isolates belonging to unclassified mycobacteria strains (e.g., *Mycobacterium* sp. GN-9680 or *Mycobacterium* sp. 34028-3) or to unidentified mycobacteria, none of them were related to cross reactions in TST.

Isolating NTM from animal tissues does not necessarily entail active infection or disease in those animals, especially taking into account that whole carcass inspection was not possible in most cases. But most of the species detected in this work could be pathogenic if conditions for infection development are favourable (e.g., immunocompromised animals, high-dose exposure etc.). In fact, in addition to a few strict pathogens (*Maa* and *Map*) (Turenne et al. 2007, 2008) many of these NTM have already been described as opportunistic pathogens of animals and humans (Pereira et al. 2020). *Maa* and *M. lentiflavum* were the species most frequently isolated from animals showing lesions compatible with TB, including cattle, common buzzard, Iberian wild goat, rabbit, red deer, roe deer and wild boar. *Mah* is frequently isolated from pigs with subclinical infection showing lesions in LNs (Agdestein et al. 2014). In the current study we have recovered *Mah* from lesioned tissues of wild boar. *M. bouchedurhonense*

has been previously pointed out as pathogenic for an African antelope after its isolation from an individual with lesions compatible with TB (Gcebe and Hlokwe 2017). In our study one cow from which *M. bouchedurhonense* was isolated displayed visible lesions. Although not being usually associated with lesions, *M. nonchromogenicum* was isolated from lesioned tissues from wild boar as well as from a cow. Despite having been detected less frequently, other NTM species from this study seemed to be involved in the lesions observed in some animals: *M. elephantis*, *M. intracellulare*, *M. parascrofulaceum*, *M. paraense*, *M. porcinum*, *M. triplex* and MAC members other than *M. avium* subspecies. The remaining NTM species of this study were apparently not related with the presence of lesions in their hosts. But some of those NTM have been previously associated with lesions or clinical signs in the same or other hosts (*M. alvei*, *M. confluentis*, *M. engbaekii*, *M. fortuitum*, *M. florentinum*, *M. interjectum*, *M. intermedium*, *M. kansasii*, *M. kumamotonense*, *M. nebraskense*, *M. scrofulaceum*, *M. senuense* and *M. septicum*) (Bercovier and Vincent 2001; Rastogi et al. 2001; Zanoni et al. 2008; Kik et al. 2010; Zeng et al. 2013; Biet and Boschiroli 2014; Katale et al. 2014; Gcebe and Hlokwe 2017; Gcebe et al. 2018; Ghielmetti et al. 2018, 2021; Timm et al. 2019; Krajewska-Wędzina et al. 2019; Hernández-Jarguín et al. 2020). With regard to the isolates belonging to unclassified mycobacteria strains, only one identified as IEC1808 was cultured from lesioned tissues belonging to a wild boar from CR. None of the isolates belonging to unidentified mycobacteria were cultured from lesioned tissues. On the other hand, culture of some samples displaying lesions did not yield any mycobacterial isolate. This could indicate that other microorganisms might have caused these lesions or that culture failed to grow living mycobacteria from the sample, especially for tissues with necrotic and calcified lesions.

In the present study, the combined use of 16S rRNA and *rpoB* genes was the selected identification approach for NTM isolates other than *M. avium*. 16S rRNA sequencing is considered a standard and reference identification tool for most NTM (Turenne et al. 2001; Tortoli 2012) and its combined use with other genetic targets like *rpoB* or *hsp65*, among others, is recommended for a more precise identification at the species or subspecies level (Adékambi and Drancourt 2004; Devulder et al. 2005; Gomila et al. 2007; Simmon et al. 2010). However, we obtained good quality mycobacteria sequences of both genes only for the half of the isolates. Besides, for a quarter of them no sequencing result was obtained for any of the two genes studied. One of the reasons that contributed to these negative results was the difficulty or impossibility to amplify the targeted genes, maybe due to differences between primer sequences and targeted NTM sequences, the formation of secondary structures or other problems related to PCR amplification. We believe that it is important to report these negative results and thus, to share relevant information on the methodological difficulties that entail working with mycobacteria with other researchers. Approaches based on WGS can avoid these issues and be the ultimate tool for identification purposes of unknown mycobacteria isolates. In some cases, we obtained bad or poor-quality sequences that could not be assessed. In others, some electropherograms showed mixed NTM sequences or sequences belonging to microorganisms other than mycobacteria. The liquid culture medium selected (MGIT) may have accounted for the latter two issues. MGIT displays higher sensitivities of detection and offers faster results compared to solid media cultures (Idigoras et al. 2000; Griffith et al. 2007). However, MGIT does not produce isolated colonies and it is related to higher contamination rates, issues that hinder identification procedures. Using solid media to obtain isolated

colonies for DNA extraction would have helped to solve these issues, but unfortunately most of the preserved material was DNA extracted from MGIT cultures.

In conclusion, our findings support that a wide diversity of NTM circulates among domestic and wild hosts in the studied areas, including species potentially pathogenic and causative of interferences in the diagnosis of TB in cattle, being *Maa*, *Mah*, *M. nonchromogenicum* and *M. lentiflavum* above all. Further studies are needed to evaluate the ability of all these NTM to infect different hosts as well as to cross react with the reagents of *in vivo* diagnosis of animal TB. Controlling NTM spread and infection in livestock and wildlife seems difficult, to say the least. Exploring new alternative or confirmatory diagnostic reagents and tests as well as monitoring the presence of NTM in livestock and wildlife populations would greatly contribute to improving the efficiency of TB eradication programmes.

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Study V

Interaction patterns between wildlife and cattle reveal opportunities for mycobacteria transmission in farms from north-eastern Atlantic Iberian Peninsula

This study has been published as:

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14th European Wildlife Disease Association Conference, Virtual Conference, 31 August-2 September 2021: Varela-Castro L, Sevilla IA, Payne A, Gilot-Fromont E, Barral M. Interaction patterns between wildlife and cattle assessed by camera traps reveal chances for mycobacteria transmission in Atlantic habitats. Oral presentation.

Abstract

Interactions taking place between sympatric wildlife and livestock may contribute to interspecies transmission of the *Mycobacterium tuberculosis* complex (MTC) or non-tuberculous mycobacteria (NTM), leading to the spread of relevant mycobacterioses or to interferences with the diagnosis of tuberculosis (TB). The aim of this study was to characterize the spatiotemporal patterns of interactions between wildlife and cattle in a low bovine TB prevalence Atlantic region. Camera traps were set during a one-year period in cattle farms with a history of TB and/or non-tuberculous mycobacterioses. The frequency and duration of wildlife visits, and the number of individuals per visit, were analysed through Generalized Linear Mixed Models. The seasons, type of place, type of point, and period of the day were the explanatory variables. A total of 1293 visits were recorded during 2741 days of camera observation. Only 23 visits showed direct contacts with cattle, suggesting that mycobacteria transmission at the wildlife–livestock interface would occur mainly through indirect interactions. Cattle pastures represented the most appropriate habitat for interspecies transmission of mycobacteria, and badgers' latrines appear to be a potential hot-spot for mycobacteria circulation between badgers, wild boars, foxes, and cattle. According to both previous epidemiological information and the interaction patterns observed, wild boars, badgers, foxes, and small rodents are the species or group most often in contact with livestock, and thus may be the most involved in the epidemiology of mycobacterioses in the wildlife–livestock interface in this area.

Keywords: Camera-traps; interactions; wildlife-livestock interface; tuberculosis; non-tuberculous mycobacteria

1. Introduction

Multi-host pathogens are often of wide concern because of the complexity that entails their control (Roche et al. 2013). This control may become harder to manage when wild species are involved in their maintenance and transmission and even more difficult when poor or lacking farm biosecurity measures enable the occurrence of interactions between livestock and wildlife. In order to improve biosecurity, it is necessary to identify the places, moments, and circumstances that entail highest risk. Generally speaking, the rate of interactions between species tends to increase when scarce water or food sources are shared by domestic and wild species, such as in Mediterranean ecosystems, due to a high spatial and/or temporal overlap between them (Kukielka et al. 2013; Carrasco-Garcia et al. 2015) and so does the probability of pathogen spread and transmission. Indirect transmission, more likely than direct transmission, tends to be involved across a community of host species (Triguero-Ocaña et al. 2019). However, when several host species are involved in the transmission of the same pathogen, it is crucial to identify the most important epidemiological connections between species and where/when these connections occur. Understanding interactions that can potentially lead to pathogen transmission at the wildlife-livestock interface is therefore a key for the implementation of appropriate disease control strategies in a multi-host system. However, this is often difficult to assess.

Animal TB is a worldwide zoonotic disease caused mainly by *M. bovis* and other mycobacteria belonging to the MTC. Although cattle are considered its main and most well-studied host, *M. bovis* represents the perfect example of a multi-host pathogen with a complex and diverse spectrum of both domestic and wild hosts. In fact, a recent study has demonstrated that TB systems in some regions of Europe are

dominated by non-bovine domestic and wild species (Santos et al. 2020). *M. bovis* survival in the environment is highly variable according to environmental conditions but may last for several months (Rodríguez-Hernández et al. 2016), enhancing the likelihood of interspecies transmission within shared habitats (mainly through indirect contacts) (Kukielka et al. 2013; Carrasco-Garcia et al. 2015). On the other hand, the emerging prevalence of NTM has become a matter of concern (Baldwin et al. 2019), even in countries reporting a low TB incidence (Hoefsloot et al. 2013). Some of these NTM are associated with opportunistic or major mycobacterioses affecting humans and several domestic and wild species, as well as with interferences in the diagnosis of bovine TB (Biet and Boschiroli 2014). NTM are widely distributed in a broad variety of aquatic and terrestrial environments (Hruska and Kaevska 2012). Some species of veterinary relevance, such as *Map*, are able to persist in the environment for long periods (Eppleston et al. 2014).

In the Iberian Peninsula, multiple domestic and wild hosts are implicated in the epidemiology of animal TB. Among domestic species, cattle is still considered the main reservoir (Gortázar and Boadella 2014), despite the fact that other livestock can also play this epidemiological role (e.g., goats (Napp et al. 2013), sheep (Muñoz-Mendoza et al. 2015), and pigs (Parra et al. 2003)). Although Spain is far from being considered officially TB free, the herd-level prevalence in cattle has been greatly reduced since the introduction of the National Eradication Programme in 1987. In Atlantic regions in particular, this prevalence has been kept below one per cent (Ministerio de Agricultura Pesca y Alimentación 2020) for the last twelve years. However, eradication has not been accomplished yet. In spite of the absence of a mandatory NTM surveillance programme, NTM-infected cattle have also been detected in these regions during the national TB eradication campaigns among cattle showing false positive reactions to the

TST (Muñoz-Mendoza et al. 2013; Varela-Castro et al. 2020b). The interactions between cattle and competent cohabiting wild hosts could contribute to this epidemiological picture of Atlantic Iberian Peninsula. There, the European badger has been described as a potential wild reservoir of TB (Acevedo et al. 2019; Blanco Vázquez et al. 2021). Furthermore, occasional TB cases have been detected in red deer, and wild boar seems to be implicated in the epidemiology of the disease (its role still being under debate) (Muñoz-Mendoza et al. 2013; Varela-Castro et al. 2020a, 2021a). Besides, several species of NTM have been detected in these three wild species as well as in roe deer, wood mice, fox, and other carnivores such as the stone marten and the mink (Varela-Castro, unpublished data) (Muñoz-Mendoza et al. 2013; Varela-Castro et al. 2020b, 2021a). Before designing and implementing strategies aimed at reducing pathogens transmission between wild and domestic animals, deepening our current understanding of wild-domestic interaction dynamics is necessary. Among the current tools available for this purpose, camera trapping is a non-invasive technique useful for the assessment of a broad variety of ecological phenomena (Rowcliffe et al. 2008; Niedballa et al. 2016, 2019; Triguero-Ocaña et al. 2020b), which can be helpful to delve into disease transmission mechanisms.

The aims of the present research were (1) to study through camera trapping the nature of interactions (direct or indirect, frequency, duration and number of animals per wildlife visit, and observed behaviours) between cattle and wild mammal species from the Basque Country, a low bovine TB prevalence Atlantic region, and (2) to investigate whether these interactions may vary according to season, period of the day, places and points sampled. The results will provide useful information for assessing the risk of transmission of mycobacteria that could help in designing potential control strategies adapted to this specific scenario.

2. Materials and Methods

2.1 Study area

This study was carried out in the Basque Country, northern Iberian Peninsula, where the annual prevalence of TB among cattle herds has been less than one per cent for the last 17 years and kept below 0.1% since 2017 (Ministerio de Agricultura Pesca y Alimentación 2020). According to official censuses from 2018 (Gobierno Vasco), there are 134,611 cattle in 4703 farms. These animals graze in the pastures regardless of the management system. Even when managed under an intensive production system, enclosures are open to the field and no biosafety measures such as fencing are always implemented. Therefore, cattle may share the pastures with cohabiting wildlife. Traditional husbandry practices are still maintained by some farmers in the Basque Country, being communal pastures shared by cattle and other domestic species such as horses and sheep, during the summer. MTC infection among wild mammals from this region has been detected in wild boar (1.12%) and red deer (2.40%) (Varela-Castro et al. 2021a). Several species of NTM able to infect cattle and interfere with the diagnosis of bovine TB have been also detected in wood mice (Varela-Castro et al. 2020b) and other wild species from the study area (Varela-Castro, unpublished data: wild boar, red deer, roe deer, badger, fox and stone marten).

Three cattle farms located in the municipalities of Kexaa, Kortezubi and Deba (named A, B and C, respectively (see Figure 1)) were selected to represent farms where TB and other mycobacterioses, mainly provoked by *Map* and/or *Maa*, have been recently diagnosed. These farms hosted TST-reactor cattle that were subsequently confirmed as *M. bovis*-infected or as false positive cases. Almost half of the MTC-positive cattle of the last ten years in the Basque Country were detected in farms A and

C and NTM were also detected in these farms, while in farm B only *Maa* was detected (Varela-Castro et al. 2020b). Farms A and B are dairy farms and farm C is a fighting bull farm. All three farms follow a free-range system. Cattle from farm A can either graze in the pastures or stay indoors, since facilities are open all year long. Facilities from farm B are completely closed and cattle are kept indoors during autumn and winter. Bulls of farm C are always kept outdoors. There are no other domestic species in these farms that could be important in terms of MTC transmission.

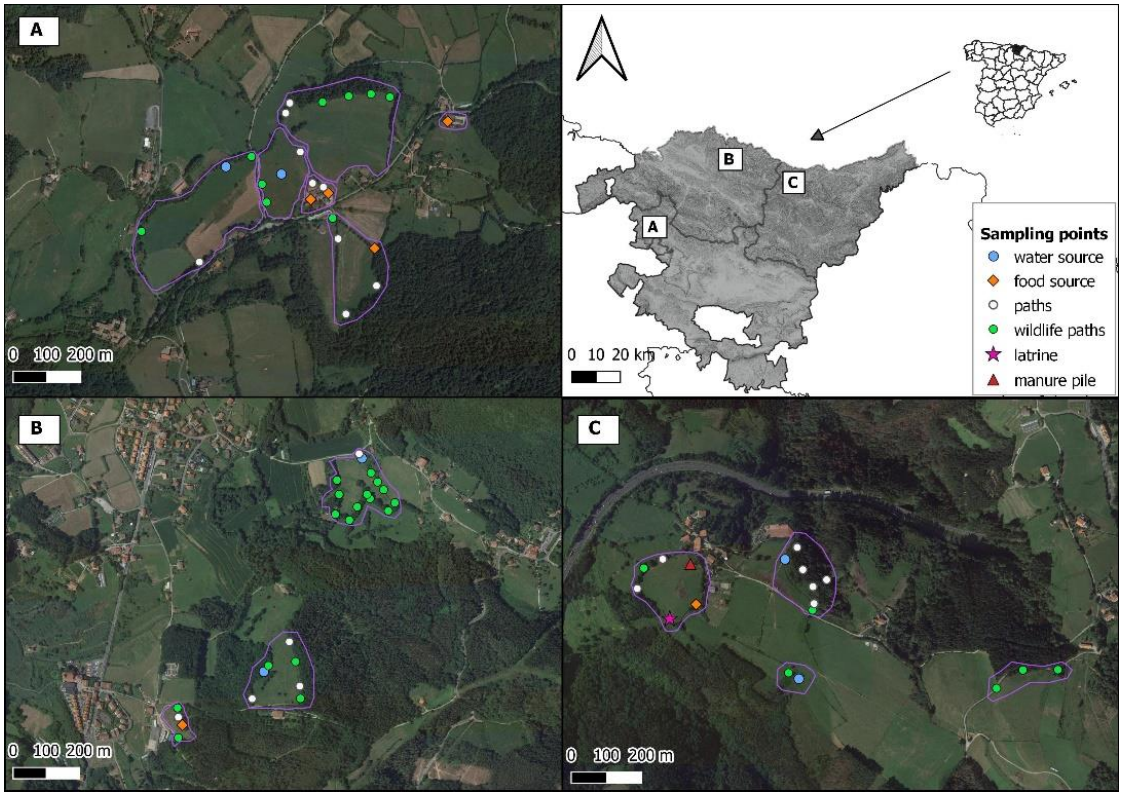

Figure 1. Field sampling design. The map shows the location of the studied farms A, B and C within the Basque Country (northern Iberian Peninsula). The spatial distribution of the sampling points recorded within farms A, B and C is displayed on the satellite photographs A, B and C, respectively (see legend for sampling point description). Purple lines surround the sites (1 to 13) included in the models as a random factor.

2.2 Camera trap survey

During a one-year period (January to November 2017), a total of twenty-three infrared motion-triggered camera traps (CTR) (Trophy Cam HD Aggressor, Bushnell, Overland Park, KS, USA) were used for the detection of wild mammal visits in different places of the farms while cattle were present (either in the field or inside facilities connected with outdoors). The field design comprised a two-week sampling period per farm and season except for farm B, where cattle are kept in closed facilities with no contact with outdoors during autumn and winter; thus, no sampling periods were recorded for those seasons. Overall, 10 sampling periods were recorded. CTRs owned movement detection up to 25 m and a response time of 0.2 s. They were programmed to work day and night, recording 10 s videos each time a movement was detected with a triggered interval of 5 s. Date and time were displayed for each video. CTRs were tied on trees, spikes, fences, or walls at ≈50 cm above the ground or up to 150–200 cm with a downward inclination, depending on the sampling point. When needed, branches that fell in the field of vision were removed. There was no overlap between the CTRs' field of view.

The sampled places were cattle pastures, bushy edges between pastures, farm buildings, and a pine forest. In each place CTRs were set in one to several points that could be *a priori* attractive for some wild species, such as water or food sources, a badger latrine and a manure pile, as well as in points that could potentially indicate the presence of wildlife, such as wildlife paths or paths that could be used by both cattle and wild species (see Figure 1). Water sources were all located outdoors and could be either a stream, a pond or cattle troughs settled with a certain height but surrounded by a flooded ground. Food sources were located indoors and outdoors. Those located indoors could be cattle feeders settled on the ground or feed-storages with some spillage of

grain, while those located outdoors were piles of straw or hay delivered on the ground or a hazelnut trees plantation. Because of husbandry practices such as the rotation of herds among different pastures, the number of cameras varied between sampling periods and some points were not recorded during all the sampling periods of each farm. Overall, we sampled 67 points located in 17 places (Table 1). Due to the unbalanced availability among sampling places and points in the study area, pastures and wildlife paths were the type of place and point of the survey with longer surveillance time (Table 1). All videos were checked for species identification. If a wild mammal was detected, the number of animals, their behaviour and the duration of the visit was also registered.

Table 1. Number of visits, surveillance recording hours and sessions grouped by type of place and point.

Type of place	Number surveyed	Type of point	Number surveyed	Number of surveillance hours	Number of Sessions
Pasture (1070)	11	Water source (114)	6	4514.83	17
		Food source (10)	3	939.60	4
		Manure (12)	1	1343.82	4
		Latrine (54)	1	511.27	2
		Wildlife path (703)	30	20,115.23	77
		Path (177)	13	8590.40	30
Farm building (22)	2	Food source (8)	3	2564.35	11
		Path (16)	2	1144.52	7
Forest (61)	1	Wildlife path (12)	1	360.23	1
		Path (49)	2	2756.07	8
Edge (140)	3	Wildlife path (17)	1	1455.08	4
		Path (109)	3	3689.23	12
		Water source (14)	1	1027.97	3
Total	17		67	49,012.60	180

Numbers in brackets indicate the number of wild mammal visits.

2.3 Variables definition

Since some CTRs were located relatively close to each other, their observations could be non-independent. In each farm, we thus defined three to six sites, a "site" being a spatial unit corresponding to either a farm building or a pasture, including its bushy edges and the forest when present (see Figure 1). Each site (n = 13) was thus considered an independent area from other sites from the same farm, while the non-independence of observations within a site was accounted for in the analysis (see below). Distances between sampling points situated within a site varied among sites (range = 16 to 393 m).

We defined a "session" as a continuous period of monitoring on the same sampling point with the same camera. Although sessions were planned to last two weeks, some of them terminated earlier due to cattle moving CTRs, thefts and unexpected battery depletion. For this reason, the duration (in hours) of each session was taken into account for subsequent analyses. We defined independent visits as (1) consecutive videos of individuals of different species; (2) consecutive videos of individuals of the same species more than 30 min apart; or (3) non-consecutive videos of a different or same species (Payne et al. 2017). The number of visits per wild species, their duration (interval between the time displayed at the beginning of the first video and at the end of the last video included in the same visit, in minutes), and the number of animals per visit were the dependent variables. Animals could not always be individually identified, so the maximum number of individuals seen simultaneously in any of the videos of each visit was recorded. A direct interaction was defined as the simultaneous presence of cattle and at least one wild mammal on the same video. All other visits were considered as indirect interactions with cattle, since all points included areas used by cattle. Explanatory variables were the season (spring: April–June,

summer: July–September, autumn: October–November, and winter: January–March), the period of the day (dawn, day, night and sunset, being the time slots determined according to the season where visits were observed), the place (pasture, farm building, forest, and edge) and the sampling point (water source, food source, manure, latrine, path, and wildlife path).

2.4 Statistical analysis

The observed behaviours were classified focusing on those that could represent a risk of mycobacteria acquisition or excretion (Table 2). When none of these behaviours was observed, animals were considered as “moving through”. If more than one behaviour were detected during a visit, either by one or more individuals, they were all recorded, except for “moving through” (Payne et al. 2016). The percentage of occurrence of the different behaviours was calculated for each species. Then, the frequencies of wildlife visits were described, for each species, in terms of means and standard errors (SEs) by computing the number of visits per month, based on the observations obtained for each session. The means and SEs were also computed for the duration of the visits and the number of individuals per visit. Afterwards, a description of the direct interactions between each species and cattle was performed.

Table 2. Description of behaviours observed among wild species.

Behaviour	Description
Grazing (for roe deer)	Feeding from grass, plants or fruits from a surface
Foraging (for badger and wild boar)	Searching for food by digging the ground with the snout
Sniffing (for all species)	Smelling the ground to search for food or to explore a surface/object
Excreting (for all species)/Scent marking (for all carnivores)	Urinating or defecating. For carnivores, lifting the tail and approaching the pelvis to the ground
Grooming/Scratching/Wallowing (for all species)	Applying tongue or paws to parts of the body in repeated motions, shaking the body, scraping against a surface, rolling in a water point
Drinking (for all species)	Drinking from water sources
Moving through (for all species)	Passing through a sampling point without performing any of the aforementioned behaviours

Then, generalized linear mixed models (GLMMs) were used to analyse how the number of visits per each species, their duration and the number of individuals varied among seasons, periods of the day, type of place and type of point, using the farms (A, B and C) and the sites (1–13) within each farm as random effects in order to take into account the likely dependence of wildlife visits within farms and each site of every farm. For the number of visits, a model adjusted to a Poisson distribution was used and, in order to consider the sampling effort of the sessions, the logarithm of the number of surveillance hours per session was included in the model as an offset. For the number of animals and the duration of visits, models adjusted to Poisson and Gamma distributions were used respectively. A total of 18 models were initially fit, one per response variable and species. For each one, a maximal model including all the variables was first created. Hereafter, the dredge function of R software was used to generate a selection table of models with combinations of the fixed variables originally included in the maximal model. The selection of the best combination was made following the parsimony

principle (Burnham and Anderson 2002): among models that had similar AIC values (delta < 2), the one with fewest parameters was selected.

Finally, we used the overdisp.glmer function of R in order to check whether overdispersion was still present in the residuals of the selected Poisson models (Zuur et al. 2009). Nakagawa and Schielzeth R-squared were used to determine the variability explained by the fixed and random parts of the selected models (using the r.squaredGLMM function of R software). All of the statistical analyses were performed using the R 4.0.0 software (R Development Core Team 2020). The data sets employed for the statistical analyses are submitted as Supplementary Material: Tables S1 and S2 (Varela-Castro et al. 2021b).

3. Results

3.1 Data collected from the field sampling

Data were recorded during 2741 camera days (i.e., data obtained from a given camera over a given day) distributed into 180 sessions (mean duration ± SE: 271.76 h ± 7.73). A total of 127,091 videos were recorded. Among them, 48,976 involved only cattle, 1329 other domestic species (cats, dogs, and horses), 4942 birds, 2320 wild mammals, and 4 reptiles. In 71 videos, it was not possible to identify the species. Wild mammal videos involved wild boar, roe deer, badger, fox, other carnivores (hereafter OC group, which includes genet (*Genetta genetta*), stone martens and pine martens (*Martes martes*)), small rodents (mouse-like), hedgehogs (*Erinaceus europaeus*), squirrels (*Sciurus vulgaris*), and bats. After excluding those species without previous epidemiological data on mycobacterial infection in the study area (hedgehogs, squirrels and bats), 2182 videos of wild mammals were retained for the analyses.

A total of 1293 visits by wild species of interest were registered, each visit being recorded by 1 to 33 videos. All species visited the farms during all seasons. Pastures and wildlife paths received the highest number of visits (Table 1). Since the observed species were mainly nocturnal, most of the visits (85%), including direct contacts with cattle, took place at night. Visits occurred in 64 out of the 67 sampling points. The three points that did not receive any visits were food sources located inside a farm building (2 points) and in a pasture (1 point). Wild boar, fox and small rodents were the only visitors of farm buildings (Table 3).

Table 3. Description of wild mammal visits. Mean ± SE and range are shown for frequency of visits per month, visit duration, and number of individuals per visit.

	Badger (*n* = 315)	**Wild boar (*n* = 304)**	**Roe deer (*n* = 175)**	**Fox (*n* = 376)**	**Other carnivores (*n* = 38)**	**Small rodents (*n* = 85)**
Frequency of visits (all visits, number per month)	4.73 ± 0.61 0–46.67	4.41 ± 0.58 0–46.33	2.76 ± 0.64 0–83.72	5.69 ± 0.66 0–59.20	0.54 ± 0.16 0–19.66	1.85 ± 0.74 0–117.2
Frequency of visits in buildings only (number per month)	0	1.07 ± 0.76 0–12	0	1.30 ± 0.76 0–12.12	0	2.04 ± 2.04 0–36.76
Visit duration (min)	0.89 ± 0.19 0.17–31	1.64 ± 0.26 0.17–38	2.33 ± 0.60 0.17–56	1.31 ± 0.31 0.17–86	0.47 ± 0.13 0.17–4	5.12 ± 1.68 0.17–95
Number of individuals per visit	1.05 ± 0.01 1–4	2.57 ± 0.10 1–10	1.16 ± 0.03 1–3	1.04 ± 0.01 1–3	1 ± 0 1–1	1.08 ± 0.04 1–3

Numbers in brackets indicate the total number of visits.

3.2 Frequency and characterization of visits per species

Figure 2 shows the proportion of occurrence of the behaviours exhibited per species. The most frequent behaviour was moving through (60% of the visits), followed by sniffing (31%), being both behaviours displayed by all species. Table 3 describes wild mammal visits in terms of frequency, number of individuals per visit, and duration of visits. The frequency of visits was highest for foxes, followed by badgers, wild boar, roe deer, small rodents, and the OC group. Small rodents were the group that showed longest visits on average (5.12 min ± 1.68), while the OC group showed the shortest on average (0.47 min ± 0.13). The species that showed up in more numerous groups was

the wild boar (2.57 individuals ± 0.10, up to 10 individuals), while the rest of the species showed mainly solitary incursions (82% of visits performed by a single individual) or appeared, punctually, in small groups (up to four badgers, up to three roe deer, foxes, and small rodents). Even though visits longer than half an hour occurred sporadically (1% of the visits) except for the OC group, short visits (less than 5 min) were predominant (93% of the visits). Twenty-three direct contacts with cattle were recorded (see Table 4). Thus, the other 1270 visits were considered as indirect interactions. The fox was the species which showed most direct contacts with cattle (eight), followed by small rodents (six) and wild boar (six), badger (two) and roe deer (one). No direct interaction was recorded for the OC group. More than half of these direct contacts took place during autumn (13/23) and within pastures (17/23), being more frequent in wildlife paths (9/23). Those that took place in farm buildings were mostly between small rodents and cattle (4/5). Even though the most common behaviour recorded during the visits was moving through, when a direct interaction took place, wild animals showed other behaviours such as sniffing, scent marking or foraging, except for small rodents (Table 4).

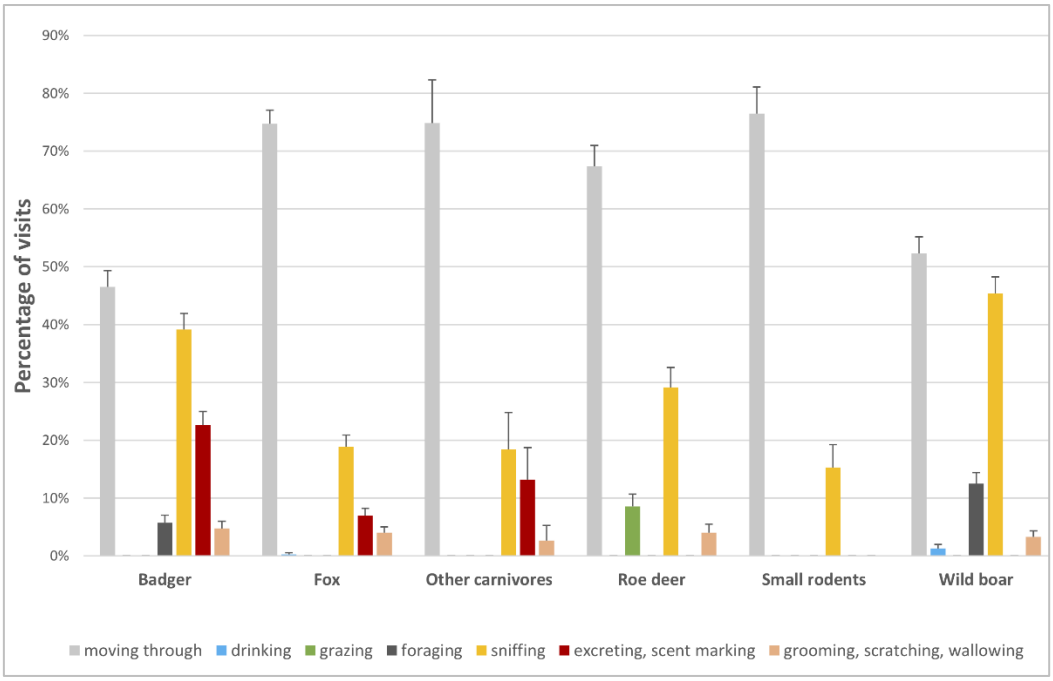

Figure 2. Percentage of the occurrence of each behaviour exhibited per species.

Table 4. Description of direct contacts between wild mammals and cattle.

	Badger	Wild boar	Roe deer	Fox	Small rodents
Number of direct interactions	2	6	1	8	6
Most frequent season	Autumn (2)	Autumn (5)	Summer	Summer (5)	Autumn (4)
Most frequent place	Pasture (2)	Pasture (5)	Pasture	Pasture (7)	Farm building (4)
Most frequent point	Latrine (2)	Manure (2)/Wildlife path (2)	Wildlife path	Wildlife path (6)	Path (4)
Behaviours observed	Sniffing/ scent marking	Sniffing/foraging/moving through	Sniffing	Moving through/ sniffing	Moving through

Numbers in brackets indicate the number of direct contacts.

Table 5 shows the outputs of the models selected to explain the frequency of visits, the number of individuals per visit, and the duration of visits for each wild mammal. Models related to the number of individuals could only be fit for wild boar data, due to the quasi-absence of variability for other species. The overdispersion of residuals was limited for all Poisson models, ranging from 0.6 to 3.03. The random effects (farms and sites within farms) accounted for 0 to 91.5% of the variations.

Table 5. Models selected per wild species and response variable. For each model, the table gives the percentage of variation explained by fixed and random parts of the model, and the OR, estimate and p-value of Wald test for each contrast between the reference level and the given level. The number of individuals was analysed for wild boar only due to the quasi-absence of variability for other species.

Species	Response variable	V.E by fixed part	V.E by random part	Fixed effect	Level	OR (95% CI)	Estimate	*p*-value
Badger	Frequency of visits	28.35%	40.94%	Season	Autumn	0.87 (0.59–1.26)	−0.14	0.454
				(ref: summer)	Winter	**2.49 (1.75–3.54)**	0.91	**<0.001**
					Spring	1.16 (0.86–1.58)	0.15	0.325
				Place	Edge	**0.56 (0.34–0.91)**	−0.59	**0.020**
				(ref: pasture)	Forest	1.06 (0.53–2.11)	0.06	0.875
				Point	Latrine	**3.81 (1.97–7.35)**	1.34	**<0.001**
				(ref: wildlife path)	Manure	**0.04 (0.01–0.31)**	−3.17	**0.002**
					Path	**0.46 (0.31–0.68)**	−0.78	**<0.001**
					Water source	0.81 (0.54–1.20)	−0.21	0.287
	Duration of visits	1.03%	9.09%	Place	Edge	**0.43 (0.20–0.89)**	−0.85	**0.024**
				(ref: pasture)	Forest	**0.35 (0.15–0.80)**	−1.06	**0.013**
				Point	Latrine	0.43 (0.18–1.03)	−0.84	0.059
				(ref: wildlife path)	Manure	0.27 (0.02–3.04)	−1.29	0.292
					Path	**0.41 (0.25–0.70)**	−0.88	**<0.001**
					Water source	1.60 (0.95–2.70)	0.47	0.079
Wild boar	Frequency of visits	29.08%	19.64%	Season	Autumn	**1.57 (1.13–2.18)**	0.45	**0.007**
				(ref: summer)	Winter	**0.39 (0.23–0.67)**	−0.94	**<0.001**
					Spring	**1.38 (1.02–1.88)**	0.32	**0.038**
				Point	Food source	**0.34 (0.13–0.94)**	−1.07	**0.037**
				(ref: wildlife path)	Latrine	**9.07 (4.47–18.42)**	2.20	**<0.001**
					Manure	1.30 (0.49–3.41)	0.26	0.599
					Path	0.77 (0.55–1.08)	−0.26	0.128
					Water source	1.23 (0.86–1.76)	0.21	0.260
	Number of animals	10.85%	0.60%	Season	Autumn	**1.52 (1.20–1.94)**	0.42	**<0.001**
				(ref: summer)	Winter	0.70 (0.46–1.07)	−0.35	0.103
					Spring	1.04 (0.83–1.30)	0.04	0.746
				Period of the day	Dawn	0.70 (0.34–1.43)	−0.35	0.332
				(ref: night)	Sunset	**0.59 (0.38–0.92)**	−0.53	**0.020**
	Duration of visits	0.32%	10.88%	Season	Autumn	1.12 (0.60–2.07)	0.11	0.730
				(ref: summer)	Winter	**3.41 (1.54–7.57)**	1.23	**0.003**
					Spring	1.21 (0.71–2.06)	0.19	0.480
Roe deer	Frequency of visits	4.87%	91.50%	Season	Autumn	1.57 (0.83–2.97)	0.45	0.162
				(ref: summer)	Winter	0.54 (0.25–1.18)	−0.61	0.123
					Spring	**0.30 (0.21–0.44)**	−1.19	**<0.001**
	Duration of visits	6.21%	20.20%	Season	Autumn	**0.28 (0.10–0.81)**	−1.26	**0.020**
				(ref: summer)	Winter	0.95 (0.33–2.72)	−0.05	0.925
					Spring	**0.10 (0.06–0.16)**	−2.32	**<0.001**
				Point	Food source	**22.15 (4.46–110.05)**	3.10	**<0.001**
				(ref: wildlife path)	Path	1.43 (0.59–3.46)	0.35	0.434
					Water source	1.85 (0.42–8.11)	0.62	0.413
Fox	Frequency of visits	28.42%	6.43%	Point	Food source	**0.15 (0.05–0.42)**	−1.89	**<0.001**
				(ref: wildlife path)	Latrine	**2.79 (1.42–5.49)**	1.03	**0.003**
					Manure	0.48 (0.19–1.23)	−0.73	0.127
					Path	1.02 (0.79–1.32)	0.02	0.863
					Water source	**0.62 (0.42–0.93)**	−0.47	**0.020**
	Duration of visits	3.88%	3.89%	Season	Autumn	**0.44 (0.20–0.97)**	−0.83	**0.042**
				(ref: summer)	Winter	0.86 (0.43–1.73)	−0.15	0.670
					Spring	**2.02 (1.10–3.68)**	0.70	**0.022**
				Period of the day	Dawn	0.89 (0.25–3.19)	−0.12	0.859
				(ref: night)	Day	**3.76 (1.61–8.78)**	1.33	**0.002**
					Sunset	0.60 (0.25–1.44)	−0.51	0.254
				Place	Edge	**0.38 (0.15–0.97)**	−0.97	**0.042**
				(ref: pasture)	Farm building	1.48 (0.13–16.54)	0.39	0.750
					Forest	0.45 (0.16–1.25)	−0.80	0.125
				Point	Food source	2.22 (0.15–33.41)	0.80	0.563
				(ref: wildlife path)	Latrine	0.23 (0.04–1.34)	−1.47	0.103
					Manure	0.12 (0.01–1.03)	−2.13	0.053
					Path	0.68 (0.33–1.41)	−0.39	0.301

Species	Response variable	V.E by fixed part	V.E by random part	Fixed effect	Level	OR (95% CI)	Estimate	*p*-value
					Water source	**0.32 (0.13–0.83)**	−1.13	**0.019**
Other carnivores	Frequency of visits	2.68%	3.58%	Place (ref: edge)	Pasture	**0.19 (0.06–0.60)**	−1.68	**0.005**
				Point (ref: path)	Water source	**0.20 (0.06–0.67)**	−1.61	**0.009**
					Wildlife path	0.34 (0.11–1.02)	−1.08	0.054
	Duration of visits	6.9%	0.00%	Place (ref: edge)	Pasture	**0.36 (0.20–0.66)**	−1.02	**<0.001**
Small rodents	Frequency of visits	23.92%	18.23%	Season (ref: autumn)	Summer	**0.03 (0.01–0.09)**	−3.64	**<0.001**
					Winter	**0.11 (0.05–0.23)**	−2.19	**<0.001**
					Spring	**0.15 (0.08–0.29)**	−1.91	**<0.001**
				Place (ref: pasture)	Edge	**0.33 (0.15–0.72)**	−1.11	**0.005**
					Farm building	1.86 (0.16–21.88)	0.62	0.623
				Point (ref: wildlife path)	Food source	0.20 (0.03–1.13)	−1.63	0.068
					Path	**0.53 (0.31–0.92)**	−0.63	**0.023**
					Water source	0.42 (0.17–1.04)	−0.86	0.060
	Duration of visits	11.08%	0.00%	Season (ref: autumn)	Summer	**0.02 (0.00–0.14)**	−3.74	**<0.001**
					Winter	**0.04 (0.01–0.11)**	−3.31	**<0.001**
					Spring	**0.02 (0.01–0.06)**	−3.74	**<0.001**

Significant values are written in bold letters. V.E = Variation explained. ref = reference level of the fixed effect.

3.2.1 Badger

No badger visit was recorded during the day, inside farm buildings, or in food sources. The two direct interactions with cattle were both observed in the latrine (Table 4). Pastures were the place where badger visits were most frequent and longest. The frequency of visits was also significantly higher in winter than in summer (OR = 2.49, 95% CI: 1.75–3.54). Moreover, visits were significantly more frequent in the badger latrine than in wildlife paths (OR = 3.81, 95% CI: 1.97–7.35), but significantly less frequent in paths (OR = 0.46, 95% CI: 0.31–0.68) or in the manure pile (OR = 0.04, 95% CI: 0.01–0.31) than in wildlife paths. The duration of visits was also significantly shorter in paths (OR = 0.41, 95% CI: 0.25–0.70) compared to wildlife paths (Table 5).

3.2.2 Wild boar

No diurnal visit was recorded. Apart from one case that was recorded in a farm building, direct interactions with cattle took place in pastures (5/6) (Table 4), including one in the latrine. The frequency of visits was significantly higher in autumn (OR = 1.57, 95% CI: 1.13–2.18) and spring (OR = 1.38, 95% CI: 1.02–1.88) compared to

summer, while it was significantly lower during winter (OR = 0.39, 95% CI: 0.23–0.67). The same tendency was also observed for the number of animals per visit, being significantly higher in autumn compared to summer (OR = 1.52, 95% CI: 1.20–1.94). However, the visits were significantly longer in winter (OR = 3.41, 95% CI: 1.54–7.57). Wild boar visits were significantly more frequent in the badger's latrine (OR = 9.07, 95% CI: 4.47–18.42) and significantly less frequent in food sources (OR = 0.34, 95% CI: 0.13–0.94). Lastly, wild boars were significantly less numerous during the dawn than at night (OR = 0.59, 95% CI: 0.38–0.92) (Table 5).

3.2.3 Roe deer

No visits were recorded in farm buildings, in the manure pile or in the latrine. The only direct interaction with cattle recorded took place in summer, during the day and in a wildlife path located in pastures (Table 4). Visits were significantly less frequent during spring (OR = 0.30, 95% CI: 0.21–0.44) and shorter during spring (OR = 0.10, 95% CI: 0.06–0.16) and autumn (OR = 0.28, 95% CI: 0.10–0.81), if compared with summer. Visits were significantly longer in food sources than in wildlife paths (OR = 22.15, 95% CI: 4.46–110.05) (Table 5).

3.2.4 Fox

Foxes were seen at all periods of the day, type of places and type of points. All direct interactions with cattle but two that were recorded in the latrine and in a path took place in wildlife paths (6/8) (Table 4). Compared to wildlife paths, visits were significantly more frequent in badger's latrine (OR = 2.79, 95% CI: 1.42–5.49) and less frequent in food (OR = 0.15, 95% CI: 0.05–0.42) or water sources (OR = 0.62, 95% CI: 0.42–0.93). Water sources received shorter visits (OR = 0.32, 95% CI: 0.13–0.83) than wildlife paths did. In comparison to summer, these were significantly longer in spring

(OR = 2.02, 95% CI: 1.10–3.68) and shorter in autumn (OR = 0.44, 95% CI: 0.20–0.97). Moreover, they were significantly longer during the day than during the night (OR = 3.76, 95% CI: 1.61–8.78), and shorter in edges than in pastures (OR = 0.38, 95% CI: 0.15–0.97) (Table 5).

3.2.5 Other carnivores

Most of these visits were performed by genets (33/38), even though stone martens (3/38) and martens (2/38) could be sporadically observed. All visits by these species were recorded in the pastures and their edges. No visit was recorded in food sources, in the latrine, or in the manure pile. Visits were significantly less frequent (OR = 0.19, 95% CI: 0.06–0.60) and shorter (OR = 0.36, 95% CI: 0.20–0.66) in pastures than in edges, and less frequent in water sources than in paths (OR = 0.20, 95% CI: 0.06–0.67) (Table 5).

3.2.6 Small rodents

No visit was recorded during sunset, in the forest, in the manure pile, or in the latrine. Unlike the rest of the species, direct interactions between cattle and small rodents were more frequent inside farm buildings (4/6) (Table 4). The selected models showed that frequency and duration of visits were significantly lower in summer (OR = 0.03, 95% CI: 0.01–0.09), winter (OR = 0.11, 95% CI: 0.05–0.23) and spring (OR = 0.15, 95% CI: 0.08–0.29) compared to autumn. Their frequency was also lower in edges compared to pastures (OR = 0.33, 95% CI: 0.15–0.72) and in paths compared to wildlife paths (OR = 0.53, 95% CI: 0.31–0.92) (Table 5).

4. Discussion

4.1 Methodology

Camera trapping has proved to be a useful tool for studying interactions with a minimal disturbance to animals. However, failures in the detection due to intrinsic characteristics of the CTRs (distance detection, response time), the position angle when hanging the devices, camera malfunctioning, and loss of battery power or adverse weather conditions may have led to an underestimation of the number and duration of visits and the number of individuals. Hence, our observations correspond to a minimum of what is actually occurring in these farms. On the other hand, due to husbandry practices and organization issues, there was an imbalance in the field sampling, since all points were not sampled at all seasons. This could have limited our ability to detect seasonal variations. Besides, the small sample size of some types of places and points (e.g., forest, latrine; see Table 1) has narrowed the information obtained from them and may be underrepresented.

4.2 Spatiotemporal patterns of wildlife-cattle interactions

Our observations confirmed that as well as in other regions (Drewe et al. 2013; Kukielka et al. 2013; Carrasco-Garcia et al. 2015; Payne et al. 2016), indirect interactions between wildlife and cattle can be considered more frequent than direct interactions. Different spatial and temporal patterns were observed depending on the species surveyed.

Winter was the most favourable season for badger visits to occur. This season is a period of food scarcity for this species, whose diet in the Basque Country is mainly based on earthworms and garden fruits (Zabala et al. 2002). However, badgers did not approach farm buildings or food sources, probably due to the fact that cattle resources

(e.g., silage or hay) lack attractiveness for them. These findings are consistent with those previously reported in a medium density population area, where badgers clearly avoided farmyards (Mullen et al. 2015) but differ with some British (Tolhurst et al. 2009; Judge et al. 2011) and French studies (Payne et al. 2016), where high rates of building use were described. Resource availability and badger population density might account for these differences. In agreement with previous reports (Woodroffe et al. 2016), pastures were the preferred place for badgers in our study area. Earthworm intake might explain the attractiveness of pastures, since their soft ground is suitable for foraging. The most attractive point for this mustelid was the badger latrine. Moreover, the few direct interactions recorded between cattle and badgers always took place in the latrine. For these reasons, this point might be considered as a potential hot-spot for both indirect or direct interactions between these two species. However, in the absence of other latrines, it is not clear whether this high frequency of visits was due to the latrine itself or to another specific feature of this particular point.

The seasonal differences observed in the frequency and duration of wild boar visits, as well as in the number of individuals per visit, may be due to different factors. In winter, wild boar density is at its lowest and the duration of incursions may be longer when searching for food. However, farm food sources were the less attractive point for this species. This could be related to a higher availability of natural resources in the study area, at least compared to areas from southern Spain, where baited points turned out to be very attractive to wild boar (Kukielka et al. 2013). As well as with badgers, wild boar visits were more frequent in the badger latrine, possibly due to an attraction effect of its characteristic scent. Thus, the latrine could also represent a significant point for wild boar to interact with other species.

Roe deer visits were most frequent and longest in summer, coinciding with the mating period of this species. Some of the activities during this period, such as the defence of the territory, the avoidance of dangerous fights and the chasing of females by bucks (Hoem et al. 2007) might make them more visible. Actually, the only direct contact recorded between roe deer and cattle took place in pastures during summer and by day. Longer visits were observed in food sources than in other sampling points. Roe deer is mainly a browser, not a grazer (Duncan et al. 1998), so they are not expected to use the same food resources as cattle. Indeed, all roe deer visits to food sources were recorded in the hazelnut trees plantation, which represents the only resource not interesting for cattle.

As for badgers and wild boar, the badger latrine was the most visited point by foxes and also the scene of a direct interaction with cattle, which supports considering the badger latrine a potential hot-spot for intra and interspecies interactions in our study area. Despite visiting all types of points, foxes showed less interest for food and water sources. This may reflect their interest in other food supplies, such as small mammals to prey on. Conversely to other species, fox visits were longer during the day because they spent a long time resting on the pastures. Lastly, visits were shorter in autumn and longer in spring compared to the summer. These findings could be also related to their rest times, which were longer during the warmest seasons.

OC group species turned out to be less often seen and always alone. Visits were most frequent and longest in the edges, which is consistent with their search for protection from predators and unfavourable weather conditions (Palomares and Delibes 1994). The majority of visits were performed by genets, and almost half of them were recorded in one specific path within the edge of one pasture. Genets spend most of their

time resting in the same place (Palomares and Delibes 1994). Thus, the potential existence of a resting site close to this path might explain the output of the models.

Although small rodent species could not be determined, in a previous study conducted in the same farms the wood mouse was the species most frequently captured (Varela-Castro et al. 2020b). Small rodents visits occurred more often and were longer in autumn than during other seasons, probably since this is a period when most rodent populations, such as the widely distributed wood mouse population, are at their maximal abundance (Torre et al. 2002). These wild rodents typically move along field margins of farmlands and are known to be common in hedgerows (Montgomery and Dowie 1993), which might explain their preferences for pastures and wildlife paths from the study area.

4.3 Opportunities of mycobacteria transmission

Since indirect interactions were much more common than direct interactions, mycobacteria transmission at the wildlife-livestock interface, if occurring, would be mainly held through indirect interactions. In general, pastures represent the most appropriate place for interspecies transmission of mycobacteria in the study area. Our results suggest that badger latrines can be suitable places for both indirect and direct contacts at least between badgers, wild boar, foxes and cattle. During the visits to the latrine, individuals of these three wild species showed behaviours related to possible excretion of or exposure to pathogens such as sniffing or scent marking, where cattle was also seen sniffing or grazing. Consequently, these points could be considered potential hot-spots for mycobacteria circulation in this habitat. However, a single latrine was found and recorded, and therefore, further studies are needed to confirm this hypothesis.

Some remarkable differences have been identified between this study and previous reports. In our study, whatever the wild species considered, the average duration of the visits was shorter (<5 min) than in studies from France (Payne et al. 2016, 2017). For instance, the average duration of wild boar visits was significantly shorter in our study area (1.64 min) than in a bovine TB-infected area in France (14.5 min) (Payne et al. 2016). The most common behaviour in our study area was "moving through". Wild species mainly move around shared habitats with cattle, but resources such as water or food supplies do not act as aggregation points, conversely to the results of previous studies (Kukielka et al. 2013; Barasona et al. 2014; Payne et al. 2016, 2017). A higher availability of natural resources throughout the whole year may account for these differences. These findings, together with the absence of MTC-infected individuals among wildlife, except for wild boar, and the low TB prevalence reported for this wild ungulate and cattle from the Basque Country (Varela-Castro et al. 2021a), suggest that the risk of MTC transmission between wild animals and cattle would be, overall, low. On the contrary, we suspect that a risk of indirect NTM transmission could be more feasible in the study area, since this group of mycobacteria have been detected in all species and the prevalence observed in some of them was significant.

Since interaction patterns and infection figures differed among wild species, some of them might be more involved than others in the epidemiology of mycobacterioses in the Basque Country. Thus, depending on the species and the situation, different control strategies could be implemented to maximize effectiveness. Foxes, badgers and wild boars were the species observed most frequently. Even though not considered as a TB reservoir in the Atlantic Iberian Peninsula, wild boar is the only species observed in this study and found to be infected with MTC in the study area, since red deer distribution is limited to a few settings that do not encompass these

farms. Wild boar has shown an unexpectedly high MTC seroprevalence of 17% in this region (Varela-Castro et al. 2020a) and, despite the low prevalence detected by culture (<2%) and the absence of animals with disseminated lesions/infection, a potential geographical link was found between spoligotypes identified in cattle and wild boar (Varela-Castro et al. 2021a). Furthermore, culture methods revealed a 9% prevalence of NTM in this species. Considering this information, the high frequency of visits and the high proportion of individuals per visit, wild boar could contribute to the dynamics of mycobacteria transmission in the Basque Country. Conversely, the badger is already considered a potential reservoir of TB in neighbouring Atlantic regions (Acevedo et al. 2019; Blanco Vázquez et al. 2021) but no infected individual was found in our study area (Varela-Castro et al. 2021a). However, a high prevalence of NTM infection (17%) was detected in this mustelid, as well as in other regions of northern Iberian Peninsula (Balseiro et al. 2011a). Since the ability of badger to transmit MTC is already confirmed, either as a TB maintenance host or as a bridge between other species through its latrines (Caron et al. 2015), the potential role of this carnivore in the epidemiology of mycobacterioses in the Basque Country should not be ruled out. MTC-infected foxes have been sporadically found in Spain (Millán et al. 2008) but not in the Basque Country, where 46 individuals were analysed throughout a 10-year survey (Varela-Castro et al. 2021a). The fox is currently considered a spillover host of TB in Europe (Michelet et al. 2018) (i.e., populations cannot maintain infection on the long-term, but may transmit it to other species), even though the prevalence reported in foxes ranged from 9% in four TB endemic areas of France (Richomme et al. 2020) to 26% in Portugal (Matos et al. 2014b, 2016b). In addition, the prevalence of NTM in foxes from the Basque Country (4.3%) was lower compared to badgers and wild boar. Nevertheless, the fox was the species most often observed and for which most direct

contacts with cattle were recorded, so its behaviour could counteract its apparent irrelevance in the epidemiology of mycobacterioses. A study carried out earlier in the same three farms proved that small rodents such as *A. sylvaticus* can carry potentially pathogenic NTM with the ability to cross-react with TB diagnosis in cattle, reporting an overall prevalence of 6.5% (Varela-Castro et al. 2020b). However, no species belonging to the MTC were detected. The scarce literature available on the epidemiology of natural *M. bovis* infection in small rodents suggests that these animals could be dead-end hosts (i.e., not able to transmit infection to other species (Delahay et al. 2002, 2007; Mathews et al. 2006)). However, the field vole (*Microtus agrestis*) is considered as a natural maintenance host for *M. microti*, a role that other small rodents like the wood mouse might play, maintaining the infection and spreading the bacteria through wounds inflicted to their predators or by indirect transmission through sputum, saliva or skin crusts (Smith et al. 2009; Kipar et al. 2014). These routes should not be ruled out for NTM transmission. Although most of the visits in our study were recorded in pastures and wildlife paths, small rodents were also observed inside the farm buildings and, conversely to the rest of the studied species, most of their direct contacts with cattle took place inside the enclosures. Roe deer visits were on average more frequent than those of small rodents and longer compared to badgers, wild boars and foxes. In the Basque Country, no cases of TB were detected (Varela-Castro et al. 2021a) and the prevalence of NTM in roe deer was 4.70%. Like the fox, the roe deer has been considered a spillover host, particularly in endemic areas (Lambert et al. 2017). However, TB cases in roe deer are reported even more sporadically (Balseiro et al. 2009). The behaviour of this species during this study (mostly solitary, not observed close to farm buildings, preference for food sources disregarded by other species and almost no direct contacts with cattle) suggests that roe deer is unlikely to play any role

in the epidemiology of TB in this low prevalence area. Accordingly, its relevance in the epidemiology of other mycobacterial infections seems to be limited. Finally, OC group could be considered the least threatening in terms of mycobacteria transmission risk in the study area. If we focus on the species observed in this study, *M. avium* was detected in one stone marten out of 18 specimens analysed in the Basque Country. Besides, to the best of our knowledge the only cases of MTC infection reported in Europe belonged to one stone marten and two genets from Portugal (Matos et al. 2016b). These epidemiological features, as well as the behaviour observed (lowest frequency and duration of visits that are always performed by one individual, no direct interaction with cattle and preference of edges over pastures) support our statement.

The findings of this study together with previous results in wildlife from the Basque Country and the low TB infection prevalence observed in cattle do not show a strong justification for intervention to reduce the risk of mycobacteria transmission at the wildlife–livestock interface. However, monitoring has proved to be an essential tool for defining the most appropriate measures if the situation changes. To reduce wild visits to farms, combined strategies rather than a single one would be more effective (Gortázar et al. 2015b). This study suggests that biosafety should particularly concern pastures, for example by activating electric fencing at night and at different heights, taking into account the anatomy of the species of interest. Furthermore, according to our results the presence of badger latrines inside pastures should be at least identified and hereafter reduced or cattle access to them should be avoided. These measures may reduce indirectly the presence of wild boar and foxes in these particular points as well. Because measures directed at minimizing contacts between cattle and small rodents are difficult to implement, rodent population control strategies may indirectly help to reduce these interactions. Notwithstanding, MTC and *M. leprae* put aside and in spite of

a few recognized pathogens such as *Map* and *Maa* (Turenne et al. 2007, 2008), the vast majority of NTM are generally regarded as environmental and ubiquitous or opportunistic pathogens at the most (Kasperbauer and Huitt 2013; Claeys and Robinson 2018). This fact makes NTM control even more challenging. Strategies to avoid exposure to these mycobacteria may rely more strongly on hygiene than on preventing contact between animals. Possible measures include avoidance of animal-driven farm environmental contamination, water contamination, biofilm formation, and surface spreading (Claeys and Robinson 2018).

The results of the present study combined with the information derived from our previous epidemiological surveys suggest that four wild species or groups might be most involved in the epidemiology of mycobacterioses in the Basque Country: wild boar, badgers, foxes, and small rodents. Cattle pastures were the most frequently visited habitat and indirect interactions represent the most likely route for the potential transmission of mycobacteria between cattle and wild species. Conversely to previous studies, food and water sources did not attract wild species in this region, while badger latrines could have acted as aggregation points and as a source of mycobacteria exposure for badgers, wild boars, foxes and cattle. Further studies are first needed to confirm that interactions between wild species and cattle in pastures occur preferentially on the latrines. If so, their identification and management would be a key to avoid interspecies transmission on pastures in this area. Moreover, analysing interactions among these three wild species around latrines and in other interfaces (elsewhere than in farm environment) will be needed to completely understand mycobacteria transmission dynamics. In agreement with the low TB prevalence of the study area, the risk of MTC transmission at the wildlife-livestock interface is expected to be low. However, the risk of NTM interspecies transmission in the Basque Country is more likely than that of

MTC, which could result in the maintenance and spread of potentially pathogenic mycobacteria that could also affect the tuberculin test specificity in cattle. The current quantification and qualification of connections among several hosts provides a valuable insight into the dynamics of transmission within the wildlife-livestock interface.

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GENERAL DISCUSSION

Mycobacteria are maintained and shared between the environment, domestic and wild animals, and humans. These microorganisms can cause medically and socio-economically significant diseases, including TB and several non-tuberculous infections, and some of them are considered a One Health challenge because of their impact on public and animal health.

Despite decades of efforts and measures to control bovine TB, the disease continues to be a substantial source of health and socioeconomic concern (Caminiti et al. 2016; Olea-Popelka et al. 2017). As the prevalence of the disease declines in livestock, the role of wild hosts in its maintenance and transmission may become more relevant (Richomme et al. 2013). However, studies on wild mammals in low bovine TB prevalence scenarios are scarce compared with high prevalence or hot-spot areas (Gortázar et al. 2012; Mentaberre et al. 2014). In those countries with ongoing bovine TB eradication programmes, infections due to NTM are more frequently detected as the incidence of the disease falls and the pressure to find remnant infection increases (Biet and Boschiroli 2014). However, and despite their growing importance, the information on animal NTM infection is scarce (with the exception of *M. avium* subspecies) in comparison with animal MTC infection and insufficient to understand the clinical relevance and implications on TB eradication programmes of this broad group of mycobacteria.

The studies included in this work provide valuable insights into the role of several wild mammals in the epidemiology of animal mycobacterioses in a low bovine TB prevalence area of northern Iberian Peninsula. This is the first time that a global study of these characteristics is carried out in these areas.

Conclusions derived from this work indicate that the contribution of wildlife to the maintenance of animal TB in the Basque Country would be overall low, especially compared to south-central Iberian Peninsula (Vicente et al. 2013; Matos et al. 2016b) (Studies II and III). This finding would be in agreement with the few studies previously carried out in other northern regions (Muñoz-Mendoza et al. 2013; Gortázar et al. 2017). MTC members were only detected in a small proportion of wild boar (1.12%) and red deer (2.40%), and absent in the remaining wild mammals studied, including badger, entailing a global TB prevalence of 0.89% among wildlife (14/1580; including 14/1472 wild mammals from Study II and 0/108 small rodents from Study III). Nevertheless, we detected a high seroprevalence in wild boar populations (17%), which suggests that this wild ungulate might deserve further attention (Study I). Conversely, NTM able to cause infection or to interfere with bovine TB diagnosis were isolated from a wide range of wild animals from the Basque Country and other regions of Spain, as well as from livestock (mainly cattle) suspected of TB through official diagnostic methods and/or inspection at slaughter (Studies III and IV). The overall NTM prevalence observed among wildlife from the Basque Country, 8.89% (123/1463; including 7/108 small rodents from Study III and 123/1355 wild animals from Study IV), was remarkable compared to that of MTC. In some particular species such as badger and wild boar, it was even more striking (21.42% and 10.64%, respectively). The overall prevalence was similar to those detected in previous NTM researches from other countries (Pate et al. 2016; Rónai et al. 2016). Even though *Map* was excluded from the general focus of this work, *M. avium* subspecies (*Maa* and *Mah*) were isolated most frequently. Other mycobacteria such as *M. nonchromogenicum* or *M. bouchedurhonense* were also found recurrently.

Findings from the second and fourth studies suggest that circulation and transmission of mycobacteria between wildlife and livestock could take place in the study area. For instance, wild boar and red deer harboured most of the main *M. bovis* and *M. caprae* spoligotypes found in cattle and goats (Study II). Besides, *Maa*, *Mah*, *Map*, *M. bouchedurhonense* and *M. nonchromogenicum* were found both in wildlife and livestock (Studies III and IV). Nevertheless, some of the *M. bovis* spoligotypes (SB1086 and SB2354), as well as *M. hiberniae*, *M. kansasii* or *M. parascrofulaceum*, were only detected among wild hosts, which could suggest the existence of a wild cycle in the study area where domestic animals are not involved. Likewise, the detection of some spoligotypes (e.g., SB1299 and SB0416) and NTM species (*M. triplex* and *M. senuense*) only in livestock might indicate that they have been exclusively circulating between domestic animals so far.

The camera-trap survey allowed us to analyse the interactions between cattle and sympatric wild mammal species from which mycobacteria were isolated in the Basque Country, except for the red deer, whose distribution in this region does not cover the farms studied during this survey. Thanks to this last study, we identified opportunities for potential mycobacteria transmission at the wildlife-livestock interface. Wildlife visits to cattle farms were abundant and frequent but with differing spatiotemporal patterns depending on the species surveyed. According to our results, and in agreement with the literature, indirect interactions were more frequent than direct ones (Drewe et al. 2013; Carrasco-Garcia et al. 2015; Payne et al. 2016), and thus represent the most likely route for the potential transmission of mycobacteria between cattle and wild species. Cattle pastures represented the most appropiate habitat for interspecies transmision of mycobacteria and badger latrines could be acting as a source of mycobacteria exposure for badgers, wild boars, foxes and cattle. Nevertheless, it is

noteworthy that the average duration of wildlife visits was remarkably short whatever the species considered (<5 min). The most common behaviour observed throughout the visits was "moving through" and, in contrast with previous research (Kukielka et al. 2013; Barasona et al. 2014), water or food supplies did not act as aggregation points.

Because interaction patterns and infection figures differed among the wild species studied, it seems that the likelihood of being involved in the epidemiology of mycobacterioses in the Basque Country would be higher for some of them:

Many evidences indicate that wild boar is the most important TB wild reservoir in some Mediterranean epidemiological contexts (Naranjo et al. 2008). However, with the information reported so far, the role of this wild ungulate in northern Iberian Peninsula is still a matter of debate (Muñoz-Mendoza et al. 2013; Mentaberre et al. 2014; Gortázar et al. 2017; Santos et al. 2018). Wild boar was one of the two wild species found to be infected with MTC in the Basque Country (Study II). Despite the absence of individuals with disseminated lesions and the low TB prevalence observed in comparison with south-central areas (Gortázar et al. 2011b), a potential epidemiological link was found between spoligotypes identified in cattle and this wild ungulate. In addition, we detected a seroprevalence unexpectedly higher (Study I) than that observed in neighbouring locations (Boadella et al. 2011; Muñoz-Mendoza et al. 2013) and demonstrated that the presence of livestock, the type of habitat and some wild boar's intrinsic characteristics (e.g., age) can influence on its likelihood of being exposed to MTC, even in a region where bovine TB is almost eradicated. These findings suggest that wild boar could be more relevant in the epidemiology of animal TB in northern Atlantic Iberian Peninsula than it was thought, although not as remarkable as in Mediterranean ecosystems. Furthermore, wild boar displayed one of the highest NTM prevalences among wildlife from the Basque Country (Study IV) and three species

(*Maa*, *Mah* and *M. nonchromogenicum*) were geographically associated as they were isolated from this and other host species, including cattle, in the same area. All these evidences, together with the high frequency of visits to cattle farms and the high number of individuals per visit (Study V), and considering the general expansion of wild boar populations in Europe through the last decades (Massei et al. 2015), suggest that wild boar is the wild mammal species which more likely could play a role in mycobacteria maintenance and spread in the Basque Country.

Some species of wild cervids such as red deer, white-tailed deer and fallow deer are quite susceptible to MTC infection, and are actually considered TB maintenance hosts in some specific areas (Gortázar et al. 2012; VerCauteren et al. 2018). In the Basque Country, red deer showed a relatively higher TB prevalence than wild boar, but the number of red deer analysed was more than five times smaller (Study II). Besides, MTC was detected more sporadically and no spoligotype was geographically linked between this and other species. This could be explained because of a more elusive behaviour of red deer or simply because its distribution is restricted to a few specific settings in the study area. These observations seem to indicate that in our study area, MTC transmission is more likely to occur between wild boar and cattle than between deer and cattle. Red deer was also found to be carrying NTM such as *Maa* and *Mah*, but the prevalence detected in the Basque Country was one of the lowest (3.66%) (Study IV). Because of the distribution of this wild cervid in the Basque Country, interaction patterns between cattle and this species could not be analysed in the Study V. As a consequence, further conclusions with regard to the relevance of red deer on the epidemiology of mycobacterial infections in the Basque Country cannot be drawn.

In contrast with other wild cervids, roe deer appears to be a TB spillover host (Balseiro et al. 2009; Lambert et al. 2017). In fact, the absence of MTC-infected

individuals in the Basque Country supports this assumption (Study II). Conversely, we did find a proportion of NTM-infected roe deer in the Basque Country (5.29%), being some of the detected species (*Maa*, *M. fortuitum* or *M. kansasii*) of veterinary interest (Biet and Boschiroli 2014; Scherrer et al. 2019) (Study IV). Roe deer were solitary, did not visit sites close to farm buildings, preferred food sources ignored by other animals and barely interacted directly with cattle (Study V). Therefore, we consider unlikely that this wild species plays any role in the epidemiology of TB in this region. Accordingly, its relevance in the epidemiology of NTM infections seems to be limited.

Overall, carnivores are considered spillover hosts of TB, except for the badger, whose relevance in the epidemiology of this disease has been confirmed in some areas of Europe, including Asturias (Atlantic northern Spain) (Blanco Vázquez et al. 2021). MTC infection was absent in all the carnivores analysed from the Basque Country, including badgers (Study II). Thus, in comparison to previous research from neighbouring regions, badgers from the Basque Country do not seem to be affected by TB nor pose a risk for cattle, although methodological differences might account for the lack of agreement between studies (e.g., passive surveillance vs passive surveillance + active surveillance in TB hot-spot areas and culture vs culture + histopathology) (Balseiro et al. 2011b, 2013). The findings with regard to NTM are completely different. The highest prevalence of mycobacteria other than MTC was detected in this mustelid, being *Mah* but also *M. interjectum* the NTM most frequently detected (Study IV). Additionally, badger was one of the mammals that visited cattle farms more frequently, and despite more research is necessary to confirm this hypothesis, badger's latrines might act as potential hot-spots for interactions between this species, wild boar, foxes and cattle, and consequently, for mycobacteria circulation (Study V). In light of these results, and taking into account the ability of badgers to transmit MTC either as a

TB maintenance host or as a bridge between other species through its latrines (Caron et al. 2015), the ability of this carnivore to maintain and transmit mycobacterial infections in the Basque Country should not be ruled out.

Significant TB prevalences have been reported in foxes from Europe (Matos et al. 2014b, 2016b; Richomme et al. 2020). However, in Spain MTC-infected individuals have been only sporadically detected (Millán et al. 2008). We did not find any MTC-infected fox (Study II) and NTM-infection was confirmed only in two out of 41 individuals (Study IV). However, considering that fox was the species with more farm visits and direct contacts with cattle (Study V), these behaviours could eventually counteract its apparent low relevance in the epidemiology of mycobacterioses in this region.

To the best of our knowledge, records on MTC infection in carnivores other than badger and fox are anecdotal in Europe (Matos et al. 2016b). If we focus on those species analysed in the Basque Country we only obtained one NTM isolate (*Mah*), from a stone marten (Studies II and IV). In addition, we observed that genets, martens and stone martens showed the lowest frequency and duration of visits, which were always performed by one individual, did not interact directly with cattle and prefered edges over pastures (Study V). These carnivores may be therefore considered the least threatening wild group in terms of mycobacteria transmission risk.

According to the scarce research on the epidemiology of natural *M. bovis* infection in wild small rodents, these animals could be considered dead-end hosts (Delahay et al. 2002, 2007; Mathews et al. 2006). However, the field vole is thought to be a natural maintenance host for *M. microti*, a MTC member that infects more species than previously thought (Kipar et al. 2014; Michelet et al. 2015; Pérez de Val et al.

2019). This wild rodent spreads the bacteria through wounds inflicted to their predators or by indirect transmission via sputum, saliva or skin crusts (Smith et al. 2009; Kipar et al. 2014). This role could be eventually played by other small rodents, such as the bank vole (*Clethrionomys glareolus*) or the wood mouse (Cavanagh et al. 2002), and could also be feasible for NTM transmission. Although we did not detect any species belonging to the MTC in small rodents captured in the environment of cattle farms, we did find a NTM prevalence of 6.5% and demonstrated that at least one species, the wood mouse, can act as a carrier of several potentially pathogenic mycobacteria with the ability to cross-react with TB diagnosis in cattle (Study III). On the other hand, we observed that small rodents showed the longest visits on average, were the most frequent visitors inside the farm buildings and, in contrast to the rest of the species, most of direct contacts with cattle took place inside the enclosures (Study V). Although species differentiation was not possible to conduct through the cameras, the wood mouse was the most frequently captured small rodent in the studied farms (Study III). Considering all these findings, we believe there could be a risk of mycobacteria transmission between small rodents (at least wood mouse) and cattle that should not be dismissed.

To sum up, the combined results of all the studies included in this work suggest that four wild species might be most involved in the epidemiology of mycobacterial infections at the wildlife-livestock interface of the Basque Country: wild boar, badger, fox and wood mouse, being wild boar probably the most relevant among them. The findings of the camera trap survey, together with the lack of MTC-infected individuals among wildlife, except for wild boar and red deer, as well as the low TB prevalence reported for these wild ungulates and cattle, suggest that the risk of MTC transmission between wild animals and cattle would be, overall, low. Nevertheless, in regions where

bovine TB prevalence is minimal, spillback transmission from these wild ungulates to domestic species should not be neglected. On the contrary, and in the absence of a standarised monitoring of NTM in cattle, the risk of NTM spreading into the environment and thus, the likelihood of indirect transmission seem to be more important in the study area, provided that this group of mycobacteria have been isolated from many wild species and that the prevalence detected among some of them was really significant. Even though the results obtained from these studies do not show a strong justification for intervention to reduce the risk of mycobacteria transmission between livestock and wildlife, monitoring has been useful to define the most appropriate control strategies, if ever needed, adapted to the epidemiological context of the Basque Country. Biosafety measures should particularly concern fencing of pastures, with special attention to wild boar, and rodent population control in order to reduce wildlife visits to the farms and, consequently, interspecies interactions. If further studies confirm that interactions between wild species and cattle in pastures occur preferentially on badger latrines, the location of these points should be detected and cattle access to them avoided. Strategies relying also in hygiene within the farms should be implemented to avoid spread and exposure to NTM. Further monitoring and characterization of NTM in livestock would help improving current bovine TB control programmes based mainly on tuberculin skin testing.

Collectively, the outcomes and conclusions of the five studies included in the present work greatly contribute to the general body of knowledge on animal MTC and NTM infection research from low bovine TB prevalence areas of northern Iberian Peninsula, and especially on the role of wildlife on the epidemiology of these mycobacterioses. As TB in livestock gets close to eradication, wildlife can still

contribute to MTC maintenance and NTM emerge as the subsequent challenge to deal with in the context of TB control strategies.

CONCLUSIONS

1. The global prevalence of TB in wildlife from the Basque Country, including all the animals analysed between 2010 and 2019, was low (0.89%). MTC (*M. bovis* and *M. caprae*) was isolated only from wild boar (1.12%) and red deer (2.40%). MTC was not detected in the remaining wild mammal species studied, including badger.
2. Wild boar and red deer harboured most of the main *M. bovis* and *M. caprae* spoligotypes found in cattle and goats in the study area, suggesting circulation and potential cross-species transmission of MTC.
3. Wild boar showed a high seroprevalence (17%) and shared spoligotypes SB0121 and SB0134 with geographically related cattle. Therefore, this wild species might be more involved in the epidemiology of animal TB in northern Iberian Peninsula than previously considered, although not as remarkably as in Mediterranean ecosystems.
4. Red deer seems to be less relevant than wild boar in the epidemiology of animal TB in this region because, apart of having a distribution restricted to few specific settings, MTC was detected more sporadically and no spoligotype was geographically linked between this and other species.
5. NTM were isolated from a wide range of wild animal species (badger, wild boar, red deer, roe deer, fox, stone marten and wood mouse) as well as from livestock (mainly cattle) suspected of TB through official diagnostic methods or inspection at slaughter.
6. The overall NTM prevalence observed among wildlife from the Basque Country between 2012 and 2019 was remarkable (8.89%). The highest figures were obtained for badgers (21.42%), wild boar (10.64%) and small rodents (mainly

wood mice) (6.5%), indicating that these would be the wild species mostly contributing to the spread of NTM.

7. *M. avium* subspecies (*Maa* and *Mah*) were the NTM species isolated most frequently. Other mycobacteria such as *M. nonchromogenicum* or *M. bouchedurhonense* were also found recurrently. These four species can interfere with the diagnosis of bovine TB and were found both in wildlife and livestock, suggesting these hosts can share mycobacteria or the sources for mycobacteria acquisition.
8. The high NTM prevalence detected in wild boar, and the fact that three species (*Maa*, *Mah* and *M. nonchromogenicum*) were geographically associated between this and other host species in the Basque Country, suggest that this wild ungulate also plays a relevant role in the maintenance and spread of NTM in the study area.
9. Indirect interactions represent the most likely route for the potential transmission of mycobacteria between cattle and wild mammals. Wildlife visits to cattle farms were abundant and frequent, but remarkably short, and their spatiotemporal patterns differed depending on the species surveyed.
10. Cattle pastures represented the most appropiate habitat for interspecies transmision of mycobacteria. Food and water sources did not act as aggregation points. Badger latrines could be acting as a source of mycobacteria exposure for badgers, wild boars, foxes and cattle.
11. Taking all the studies into account, four wild species might be most involved in the epidemiology of mycobacterial infections at the wildlife-livestock interface of the Basque Country: wild boar, badger, fox and wood mouse, being wild boar probably the most relevant among them.

SUPPLEMENTARY MATERIAL

Supplementary Table 1. Identification results algorithm (tetraplex real-time PCR, IS*1245* real-time PCR, IS*901* real-time PCR, 16S rRNA and *rpo*B PCR, sequencing and BLAST analysis) obtained for each NTM isolate, including final identification consensus.

Isolate ID	Host ID	Host species	Tetraplex real-time PCR	*IS1245/IS901* real-time PCR	16S rRNA PCR	16S rRNA BLAST query coverage (%)	16S rRNA BLAST base identities (%)	*rpoB* PCR	*rpoB* BLAST query coverage (%)	*rpoB* BLAST base identities (%)	Consensus
4	1	Wild boar	*M. avium*	*Maa*							*Maa*
9	2	Wild boar	*M. avium*	*Mah*							*Mah*
10	3	Wild boar	*M. avium*	*Mah*							*Mah*
11	4	Wild boar	*M. avium*	*Mah*							*Mah*
12	5	Wild boar	*M. avium*	*Mah*							*Mah*
13	6	Cow	*M. avium*	*Maa*							*Maa*
16	**7**	Buzzard	*M. avium*	*Maa*							*Maa*
21	8	Mink	*M. avium*	*Maa*							*Maa*
22	9	Wild boar	*M. avium*	*Maa*							*Maa*
23	10	Wild boar	*M. avium*	*Mah*							*Mah*
24	11	Roe deer	*M. avium*	*Maa*							*Maa*
26	12	Wild boar	*M. avium*	*Mah*							*Mah*
27	13	Wild boar	*M. avium*	*Mah*							*Mah*
45	14	Wild boar	*M. avium*	*Maa*							*Maa*
62	15	Wild boar	*M. avium*	*Mah*							*Mah*
64	16	Cow	*M. avium*	*Mah*							*Mah*
69	17	Red deer	*M. avium*	*Mah*							*Mah*
70	18	Wild boar	*M. avium*	*Mah*							*Mah*
82	19	Roe deer	*M. avium*	*Maa*							*Maa*
109	20	Wild boar	*M. avium*	*Maa*							*Maa*
127	7	Buzzard	*M. avium*	*Maa*							*Maa*
165	21	Red deer	*M. avium*	*Maa*							*Maa*
166	22	Roe deer	*M. avium*	*Maa*							*Maa*
167	23	Roe deer	*M. avium*	*Maa*							*Maa*
168	24	Roe deer	*M. avium*	*Maa*							*Maa*
169	25	Roe deer	*M. avium*	*Maa*							*Maa*
170	26	Mink	*M. avium*	*Maa*							*Maa*
172	27	Badger	*M. avium*	*Maa*							*Maa*
173	28	Stone marten	*M. avium*	*Maa*							*Maa*
175	29	Wild boar	*M. avium*	*Maa*							*Maa*
187	30	Wild boar	*M. avium*	*Maa*							*Maa*
198	31	Wild boar	*M. avium*	*Maa*							*Maa*
214	32	Wild boar	*M. avium*	*Maa*							*Maa*
229	33	Roe deer	*M. avium*	*Mah*							*Mah*
230	34	Roe deer	*M. avium*	*Mah*							*Mah*
238	35	Badger	*M. avium*	*Mah*							*Mah*
246	**36**	Cow	*M. avium*	*Maa*							*Maa*
247	36	Cow	*M. avium*	*Maa*							*Maa*

Isolate ID	**Host ID**	**Host species**	**Tetraplex real-time PCR**	***IS1245/IS901* real-time PCR**	**16S rRNA PCR**	**16S rRNA BLAST query coverage (%)**	**16S rRNA BLAST base identities (%)**	***rpoB* PCR**	***rpoB* BLAST query coverage (%)**	***rpoB* BLAST base identities (%)**	**Consensus**
248	36	Cow	*M. avium*	*Maa*							*Maa*
255	**37**	Wild boar	*M. avium*	*Maa*							*Maa*
256	37	Wild boar	*M. avium*	*Maa*							*Maa*
278	38	Wild boar	*M. avium*	*Mah*							*Mah*
279	39	Wild boar	*M. avium*	*Mah*							*Mah*
280	40	Fox	*M. avium*	*Maa*							*Maa*
281	41	Wild boar	*M. avium*	*Mah*							*Mah*
282	42	Badger	*M. avium*	*Mah*							*Mah*
283	43	Badger	*M. avium*	*Maa*							*Maa*
285	44	Wild boar	*M. avium*	*Maa*							*Maa*
287	45	Cow	*M. avium*	*Mah*							*Mah*
289	46	Wild boar	*M. avium*	*Maa*							*Maa*
291	47	Wild boar	*M. avium*	*Mah*							*Mah*
292	48	Wild boar	*M. avium*	*Maa*							*Maa*
295	49	Wild boar	*M. avium*	*Mah*							*Mah*
316	50	Badger	*M. avium*	*Mah*							*Mah*
323	51	Wild boar	*M. avium*	*Maa*							*Maa*
339	52	Roe deer	*M. avium*	*Maa*							*Maa*
341	53	Roe deer	*M. avium*	*Maa*							*Maa*
342	**54**	Roe deer	*M. avium*	*Maa*							*Maa*
343	54	Roe deer	*M. avium*	*Mah*							*Mah*
344	55	Wild boar	*M. avium*	*Mah*							*Mah*
345	56	Badger	*M. avium*	*Mah*							*Mah*
346	57	Wild boar	*M. avium*	*Maa*							*Maa*
356	58	Red deer	*M. avium*	*Maa*							*Maa*
361	59	Wild boar	*M. avium*	*Maa*							*Maa*
362	60	Wild boar	*M. avium*	*Mah*							*Mah*
364	61	Wild boar	*M. avium*	*Mah*							*Mah*
402	62	Wild boar	*M. avium*	*Maa*							*Maa*
414	63	Cow	*M. avium*	*Mah*							*Mah*
415	64	Wild boar	*M. avium*	*Mah*							*Mah*
437	65	Cow	*M. avium*	*Maa*							*Maa*
442	66	Wild boar	*M. avium*	*Mah*							*Mah*
445	**67**	Badger	*M. avium*	*Mah*							*Mah*
446	67	Badger	*M. avium*	*Mah*							*Mah*
447	68	Wild boar	*M. avium*	*Maa*							*Maa*
458	69	Wild goat	*M. avium*	*Maa*							*Maa*
459bis	70	Rabbit	*M. avium*	*Maa*							*Maa*
460bis	71	Rabbit	*M. avium*	*Maa*							*Maa*
G1	72	Stone marten	*M. avium*	*Mah*							*Mah*
G2	73	Wild boar	*M. avium*	*Maa*							*Maa*

Isolate ID	**Host ID**	**Host species**	**Tetraplex real-time PCR**	***IS1245/IS901* real-time PCR**	**16S rRNA PCR**	**16S rRNA BLAST query coverage (%)**	**16S rRNA BLAST base identities (%)**	***rpoB* PCR**	***rpoB* BLAST query coverage (%)**	***rpoB* BLAST base identities (%)**	**Consensus**
G3	74	Cow	*M. avium*	*Mah*							*Mah*
G4	75	Cow	*M. avium*	*Maa*							*Maa*
G5	76	Wild boar	*M. avium*	*Maa*							*Maa*
G6	77	Badger	*M. avium*	*Mah*							*Mah*
3	78	Wild boar	*M.* sp.		*M. col/int/bou*	100%	99.78%	*M.* sp. GN-9680	96%	99.85%	*M. col/int/bou*
7	79	Badger	*M.* sp.		*M. interjectum*	100%	100%	*M. interjectum*	100%	100%	*M. interjectum*
8	80	Wild boar	*M.* sp.		*M. nonchromogenicum*	100%	100%	*M. nonchromogenicum*	100%	99.86%	*M. nonchromogenicum*
14	81	Badger	*M.* sp.		*M.* sp. TY59	100%	100%	*M. bouchedurhonense*	99%	99.30%	*M. bouchedurhonense*
19	82	Red deer	*M.* sp.		*M.* sp. J16	100%	99.79%	*M.* sp. 3582	99%	98.44%	*M.* sp. 3582/J16
20.3	83	Cow	*M.* sp.		*M. scrofulaceum*	100%	100%	*M. scrofulaceum*	100%	100%	*M. scrofulaceum*
25	84	Wild boar	*M.* sp.		not valid			*M. intracellulare*	100%	100%	*M. intracellulare*
28	85	Wild boar	*M.* sp.		*M.* sp. J16	100%	99.79%	*M.* sp. 3582	96%	98.46%	*M.* sp. 3582/J16
29	86	Wild boar	*M.* sp.		*M. paraense*	100%	99.75%	not valid			*M. paraense*
30	87	Wild boar	*M.* sp.		not valid			not valid			no sequencing result
31	**88**	Wild boar	*M.* sp.		*M. col/int/bou*	100%	99.58%	*M.* sp. GN-9680	96%	98.10%	*M.* sp. GN-9680
32	88	Wild boar	*M.* sp.		*M. col/int/bou*	100%	99.58%	*M.* sp. GN-9680	96%	98.33%	*M.* sp. GN-9680
33	89	Wild boar	*M.* sp.		not valid			not valid			no sequencing result
34	**90**	Wild boar	*M.* sp.		*M. col/int/bou*	100%	99.79%	*M.* sp. GN-9680	96%	99.71%	*M. col/int/bou*
35	**91**	Wild boar	*M.* sp.		*M. col/int/bou*	100%	99.58%	*M.* sp. GN-9680	96%	98.10%	*M.* sp. GN-9680
36	92	Wild boar	*M.* sp.		*M. col/int/bou*	100%	100%	*M.* sp. GN-9680	96%	97.37%	*M. col/int/bou*
37	93	Wild boar	*M.* sp.		*M. col/int/bou*	100%	100%	*M.* sp. GN-9680	96%	97.52%	*M. col/int/bou*
38	94	Wild boar	*M.* sp.		*M. lentiflavum*	100%	100%	*M. lentiflavum*	100%	100%	*M. lentiflavum*
39	**95**	Wild boar	*M.* sp.		*M. lent/par*	100%	99.35%	*M. palustre*	100%	95.17%	*M.* sp. 2
41	96	Wild boar	*M.* sp.		*M. seoulense*	100%	99.79%	*M. seoulense*	100%	98.31%	*M. seoulense*
42	97	Wild boar	*M.* sp.		*M. col/int/bou*	100%	99.58%	*M.* sp. GN-9680	96%	98.25%	*M.* sp. GN-9680
43	98	Wild boar	*M.* sp.		*M. bohemicum*	100%	100%	not valid			*M. bohemicum*
44	99	Cow	*M.* sp.		not valid			*M. nonchromogenicum*	100%	100%	*M. nonchromogenicum*
46	100	Wild boar	*M.* sp.		*M. lentiflavum*	100%	100%	*M. lentiflavum*	100%	100%	*M. lentiflavum*
47	101	Red deer	*M.* sp.		*M. col/int/bou*	100%	100%	*M. bouchedurhonense*	100%	97.33%	*M. bouchedurhonense*
49	102	Wild boar	*M.* sp.		*M. col/int/bou*	100%	100%	not valid			*M. col/int/bou*
53	103	Wild boar	*M.* sp.		not valid			not valid			no sequencing result
56	104	Wild boar	*M.* sp.		not valid			*M. wolinskyi*	100%	92.64%	*M.* sp. 4
58	105	Wild boar	*M.* sp.		*M. intracellulare*	100%	100%	*M. bouchedurhonense*	100%	97.50%	*M. bou/int*
59	106	Wild boar	*M.* sp.		*M.* sp. 34028-3	100%	100%	*M.* sp. 34028-3	99%	99.72%	*M.* sp. 34028-3
60	107	Badger	*M.* sp.		*M. col/int/bou*	100%	99.79%	*M.* sp. GN-9680	96%	99.42%	*M. col/int/bou*
61	108	Red deer	*M.* sp.		not valid			not valid			no sequencing result
65	109	Wild boar	*M.* sp.		*M. septicum*	100%	100%	*M. septicum*	99%	99.58%	*M. septicum*
66	110	Fox	*M.* sp.		not valid			not valid			no sequencing result
67	91	Wild boar	*M.* sp.		*M. col/int/bou*	100%	99.79%	*M.* sp. GN-9680	96%	99.71%	*M. col/int/bou*
80	111	Badger	*M.* sp.		*M. interjectum*	100%	100%	not valid			*M. interjectum*
91	112	Wild boar	*M.* sp.		*M. parascrofulaceum*	100%	100%	*M. parascrofulaceum*	100%	97.19%	*M. parascrofulaceum*

Isolate ID	Host ID	Host species	Tetraplex real-time PCR	*IS1245/IS901* real-time PCR	16S rRNA PCR	16S rRNA BLAST query coverage (%)	16S rRNA BLAST base identities (%)	*rpoB* PCR	*rpoB* BLAST query coverage (%)	*rpoB* BLAST base identities (%)	Consensus
92	113	Wild boar	*M.* sp.		*M. seoulense*	100%	100%	*M. sask/mont*	100%	96.06%	*M. seoulense*
94	114	Wild boar	*M.* sp.		not valid			*M. parascrofulaceum*	100%	97%	*M. parascrofulaceum*
101	115	Wild boar	*M.* sp.		not valid			*M. septicum*	99%	99.56%	*M. septicum*
103	116	Wild boar	*M.* sp.		not valid			not valid			no sequencing result
105	117	Wild boar	*M.* sp.		*M.* sp. 34028-3	100%	99.77%	*M.* sp. 34028-3	100%	99.12%	*M.* sp. 34028-3
106	117	Wild boar	*M.* sp.		not valid			*M.* sp. 34028-3	99%	99.29%	*M.* sp. 34028-3
110	118	Wild boar	*M.* sp.		*M. intermedium*	100%	100%	*M. intermedium*	100%	99.85%	*M. intermedium*
111	119	Wild boar	*M.* sp.		*M. fortuitum*	100%	100%	*M. fortuitum*	100%	99.86%	*M. fortuitum*
112	120	Fox	*M.* sp.		*M. sen/far/hou/for/con*	100%	100%	*M. porcinum*	100%	96.90%	*M. sen/far/hou/for/con*
115	121	Wild boar	*M.* sp.		not valid			*M. hiberniae*	100%	97.92%	*M. hiberniae*
116	122	Roe deer	*M.* sp.		*M. kansasii/gastri*	100%	100%	*M. kansasii*	100%	100%	*M. kansasii*
117	123	Badger	*M.* sp.		not valid			not valid			no sequencing result
118	124	Wild boar	*M.* sp.		*M. col/int/bou*	100%	100%	*M.* sp. GN-9680	96%	97.37%	*M. col/int/bou*
119	125	Wild boar	*M.* sp.		*M. col/int/bou*	100%	99.79%	*M.* sp. GN-9680	96%	99.42%	*M. col/int/bou*
120	126	Wild boar	*M.* sp.		*M. col/vul/int/bou*	100%	99.79%	not valid			*M. vul/col/int/bou*
126	127	Wild boar	*M.* sp.		*M. nonchromogenicum*	100%	100%	not valid			*M. nonchromogenicum*
129	128	Wild boar	*M.* sp.		*M.* sp. TY59	100%	100%	not valid			*M.* sp. TY59
133	129	Red deer	*M.* sp.		not valid			not valid			no sequencing result
134	130	Cow	*M.* sp.		not valid			not valid			no sequencing result
138	131	Wild boar	*M.* sp.		not valid			not valid			no sequencing result
139	132	Wild boar	*M.* sp.		*M. for/por/new*	100%	100%	*M. porcinum*	100%	99.31%	*M. porcinum*
140	133	Wild boar	*M.* sp.		*M. col/Miy/int/tim/bou*	100%	99.51%	*M.* sp. GN-9680	99%	98.07%	*M.* sp. GN-9680
141	134	Wild boar	*M.* sp.		not valid			not valid			no sequencing result
143	135	Red deer	*M.* sp.		*M.* sp. 8115-1	100%	100%	*M.* sp. 8115-1	99%	100%	*M.* sp. 8115-1
144	136	Red deer	*M.* sp.		*M. col/int/bou*	100%	100%	not valid			*M. col/int/bou*
146	137	Red deer	*M.* sp.		*M.* sp. 8115-1	100%	100%	*M.* sp. 8115-1	99%	100%	*M.* sp. 8115-1
147	138	Red deer	*M.* sp.		*M. col/int/bou*	100%	100%	*M. vulneris*	99%	98.33%	*M. vul/col/int/bou*
148	139	Red deer	*M.* sp.		*M.* sp. 8115-1	100%	100%	*M.* sp. 8115-1	99%	100%	*M.* sp. 8115-1
149	140	Red deer	*M.* sp.		*M. holsaticum*	99%	100%	*M. elephantis*	100%	97.33%	*M. ele/M. hol*
152	141	Wild boar	*M.* sp.		*M. col/int/Miy/bou*	100%	100%	not valid			*M. col/int/Miy/bou*
153	142	Wild boar	*M.* sp.		*M. col/int/bou*	100%	100%	*M. vulneris*	98%	98.43%	*M. vul/col/int/bou*
154	143	Wild boar	*M.* sp.		not valid			not valid			no sequencing result
156	144	Wild boar	*M.* sp.		not valid			*M. bouchedurhonense*	99%	97.89%	*M. bouchedurhonense*
159	145	Wild boar	*M.* sp.		*M. nonchromogenicum*	100%	100%	*M. nonchromogenicum*	100%	100%	*M. nonchromogenicum*
161	146	Cow	*M.* sp.		*M. nonchromogenicum*	100%	100%	*M. nonchromogenicum*	100%	99.03%	*M. nonchromogenicum*
162	147	Wild boar	*M.* sp.		*M. engbaekii*	100%	100%	*M. engbaekii*	99%	96.80%	*M. engbaekii*
163	148	Wild boar	*M.* sp.		not valid			not valid			no sequencing result
179	149	Wild boar	*M.* sp.		*M. nonchromogenicum*	100%	100%	*M. nonchromogenicum*	100%	100%	*M. nonchromogenicum*
185	150	Wild boar	*M.* sp.		not valid			not valid			no sequencing result
186	151	Wild boar	*M.* sp.		not valid			not valid			no sequencing result
188	152	Wild boar	*M.* sp.		not valid			not valid			no sequencing result

Isolate ID	Host ID	Host species	Tetraplex real-time PCR	*IS1245/IS901* real-time PCR	16S rRNA PCR	16S rRNA BLAST query coverage (%)	16S rRNA BLAST base identities (%)	*rpoB* PCR	*rpoB* BLAST query coverage (%)	*rpoB* BLAST base identities (%)	Consensus
189	153	Red deer	*M.* sp.		not valid			not valid			no sequencing result
191	154	Wild boar	*M.* sp.		not valid			not valid			no sequencing result
197	155	Wild boar	*M.* sp.		not valid			not valid			no sequencing result
205	156	Wild boar	*M.* sp.		not valid			not valid			no sequencing result
206	157	Wild boar	*M.* sp.		not valid			not valid			no sequencing result
207	158	Wild boar	*M.* sp.		*M. confluentis*	100%	99.78%	*M. confluentis*	99%	99.44%	*M. confluentis*
208	159	Wild boar	*M.* sp.		*M. intermedium*	100%	100%	*M. intermedium*	100%	100%	*M. intermedium*
209	160	Wild boar	*M.* sp.		*M. diernhoferi*	100%	100%	*M. diernhoferi*	100%	100%	*M. diernhoferi*
210	161	Wild boar	*M.* sp.		*M. inter/par/mal*	100%	100%	*M. paraense*	99%	97.84%	*M. paraense*
211	162	Wild boar	*M.* sp.		*M.* sp. IEC1808	100%	100%	*M.* sp. FI-13041	98%	95.77%	*M.* sp. IEC1808
212	**163**	Wild boar	*M.* sp.		*M. intracellulare*	100%	100%	*M.* sp. GN-9680	96%	98.78%	*M. intracellulare*
215	164	Wild boar	*M.* sp.		*M. col/int/bou*	100%	99.79%	*M.* sp. GN-9680	96%	98.98%	*M. col/int/bou*
216	165	Wild boar	*M.* sp.		not valid			*M. interjectum*	100%	98.06%	*M. interjectum*
219	166	Wild boar	*M.* sp.		*M. lent/par*	100%	99.35%	*M. palustre*	100%	94.05%	*M.* sp. 2
220	163	Wild boar	*M.* sp.		not valid			*M.* sp. GN-9680	99%	99.11%	*M.* sp. GN-9680
224	167	Wild boar	*M.* sp.		not valid			not valid			no sequencing result
225	168	Wild boar	*M.* sp.		*M. lent/par*	100%	99.35%	not valid			*M.* sp. 2
226	169	Wild boar	*M.* sp.		not valid			*M. palustre*	100%	94.31%	*M.* sp. 2
227	95	Wild boar	*M.* sp.		*M. lent/par*	100%	99.35%	*M. palustre*	100%	95%	*M.* sp. 2
231	170	Roe deer	*M.* sp.		*M.* sp. TY59	100%	100%	*M. bouchedurhonense*	99%	99.15%	*M. bouchedurhonense*
232	171	Badger	*M.* sp.		*M. florentinum*	100%	100%	*M. florentinum*	100%	99.85%	*M. florentinum*
235	172	Wild boar	*M.* sp.		not valid			not valid			no sequencing result
236	173	Wild boar	*M.* sp.		not valid			not valid			no sequencing result
237	174	Wild boar	*M.* sp.		*M. nonchromogenicum*	100%	100%	*M. nonchromogenicum*	100%	100%	*M. nonchromogenicum*
239	175	Badger	*M.* sp.		*M. col/int/bou*	100%	100%	*M. vulneris*	98%	98.63%	*M. vul/col/int/bou*
240	176	Wild boar	*M.* sp.		not valid			not valid			no sequencing result
243	177	Wild boar	*M.* sp.		not valid			*M. lentiflavum*	100%	100%	*M. lentiflavum*
244	178	Wild boar	*M.* sp.		not valid			not valid			no sequencing result
245	179	Red deer	*M.* sp.		not valid			*M. lentiflavum*	100%	100%	*M. lentiflavum*
249	180	Cow	*M.* sp.		not valid			not valid			no sequecing result
250	181	Roe deer	*M.* sp.		not valid			*Mich/Miy*	100%	99.86%	*Mich/Miy*
251	182	Wild boar	*M.* sp.		not valid			not valid			no sequencing result
252	183	Wild boar	*M.* sp.		*M. scrofulaceum*	100%	100%	not valid			*M. scrofulaceum*
254	184	Wild boar	*M.* sp.		*M.* sp. L2008	100%	99.37%	*M. nebraskense*	100%	97.16%	*M. nebraskense*
270	185	Wild boar	*M.* sp.		*M. lentiflavum*	100%	100%	*M. lentiflavum*	100%	100%	*M. lentiflavum*
271	186	Wild boar	*M.* sp.		*M. lentiflavum*	100%	99.57%	*M. lentiflavum*	100%	100%	*M. lentiflavum*
272	187	Wild boar	*M.* sp.		not valid			*M. lentiflavum*	100%	100%	*M. lentiflavum*
273	188	Wild boar	*M.* sp.		*M. elephantis*	100%	100%	*M. elephantis*	100%	98.95%	*M. elephantis*
274	189	Roe deer	*M.* sp.		not valid			*M. lentiflavum*	100%	99.85%	*M. lentiflavum*
275	190	Red deer	*M.* sp.		*M. lentiflavum*	100%	100%	*M. lentiflavum*	100%	100%	*M. lentiflavum*
276	191	Wild boar	*M.* sp.		not valid			*M. lentiflavum*	100%	100%	*M. lentiflavum*

Isolate ID	Host ID	Host species	Tetraplex real-time PCR	*IS1245/IS901* real-time PCR	16S rRNA PCR	16S rRNA BLAST query coverage (%)	16S rRNA BLAST base identities (%)	*rpoB* PCR	*rpoB* BLAST query coverage (%)	*rpoB* BLAST base identities (%)	Consensus
277	192	Cow	*M.* sp.		not valid			not valid			no sequencing result
290	193	Badger	*M.* sp.		not valid			not valid			no sequencing result
296	194	Cow	*M.* sp.		*M. nonchromogenicum*	100%	100%	*M. nonchromogenicum*	99%	100%	*M. nonchromogenicum*
297	195	Roe deer	*M.* sp.		*M. fortuitum*	100%	100%	*M. fortuitum*	99%	100%	*M. fortuitum*
303	196	Wild boar	*M.* sp.		not valid			*M. parascrofulaceum*	100%	97.05%	*M. parascrofulaceum*
304	197	Wild boar	*M.* sp.		not valid			not valid			no sequencing result
305	198	Wild boar	*M.* sp.		not valid			*M. bohemicum*	100%	99.44%	*M. bohemicum*
307	199	Wild boar	*M.* sp.		not valid			not valid			no sequencing result
308	**200**	Wild boar	*M.* sp.		not valid			not valid			no sequencing result
309	200	Wild boar	*M.* sp.		not valid			not valid			no sequencing result
310	201	Wild boar	*M.* sp.		not valid			*M. parascrofulaceum*	100%	97.16%	*M. parascrofulaceum*
312	202	Badger	*M.* sp.		not valid			not valid			no sequencing result
313	203	Wild boar	*M.* sp.		not valid			not valid			no sequencing result
314	204	Red deer	*M.* sp.		not valid			*M. bouchedurhonense*	99%	99.15%	*M. bouchedurhonense*
315	205	Wild boar	*M.* sp.		not valid			*M.* sp. 3582	99%	98.44%	*M.* sp. 3582
317	206	Wild boar	*M.* sp.		not valid			not valid			no sequencing result
318	207	Wild boar	*M.* sp.		not valid			not valid			no sequencing result
324	208	Wild boar	*M.* sp.		*M. col/int/bou*	100%	99.58%	*M.* sp. GN-9680	99%	98%	*M.* sp. GN-9680
326	209	Wild boar	*M.* sp.		*M. col/int/Miy/bou*	100%	99.76%	not valid			*M. col/int/Miy/bou*
327	210	Wild boar	*M.* sp.		*M. col/int/bou*	100%	99.79%	*M.* sp. GN-9680	96%	99.42%	*M. col/int/bou*
328	211	Wild boar	*M.* sp.		*M. col/int/bou*	100%	99.58%	not valid			*M.* sp. 1
329	212	Wild boar	*M.* sp.		*M. col/int/bou*	100%	99.58%	*M.* sp. GN-9680	96%	98.24%	*M.* sp. GN-9680
330	213	Wild boar	*M.* sp.		*M. col/int/bou*	100%	100%	*M. bouchedurhonense*	99%	97.75%	*M. bouchedurhonense*
331	214	Wild boar	*M.* sp.		*M. col/int/bou*	100%	99.58%	*M.* sp. GN-9680	96%	98.25%	*M.* sp. GN-9680
332	215	Wild boar	*M.* sp.		not valid			not valid			no sequencing result
333	216	Wild boar	*M.* sp.		not valid			not valid			no sequencing result
334	217	Wild boar	*M.* sp.		*M. col/int/bou*	100%	100%	*M. bouchedurhonense*	99%	97.75%	*M. bouchedurhonense*
335	218	Wild boar	*M.* sp.		*M. col/int/bou*	100%	100%	*M. vulneris*	98%	98.43%	*M. vul/col/int/bou*
336	219	Wild boar	*M.* sp.		*M.* sp. 34028-3	100%	100%	*M.* sp. 34028-3	99%	100%	*M.* sp. 34028-3
337	220	Wild boar	*M.* sp.		*M. col/int/bou*	100%	100%	*M. vulneris*	98%	97.72%	*M. vul/col/int/bou*
338	221	Wild boar	*M.* sp.		not valid			not valid			no sequencing result
347	222	Wild boar	*M.* sp.		*M. engbaekii*	100%	100%	not valid			*M. engbaekii*
349	223	Wild boar	*M.* sp.		*M. arosiense*	100%	100%	*M. arosiense*	100%	99.86%	*M. arosiense*
350	224	Cow	*M.* sp.		*M. triplex*	100%	100%	*M. triplex*	100%	99.86%	*M. triplex*
351	225	Wild boar	*M.* sp.		*M. nonchromogenicum*	100%	100%	*M. nonchromogenicum*	99%	100%	*M. nonchromogenicum*
352	226	Cow	*M.* sp.		not valid			not valid			no sequencing result
353	227	Cow	*M.* sp.		*M. col/int/bou*	100%	100%	*M. bouchedurhonense*	99%	98.31%	*M. bouchedurhonense*
354	228	Wild boar	*M.* sp.		*M. nonchromogenicum*	100%	100%	*M. nonchromogenicum*	100%	99.86%	*M. nonchromogenicum*
355	229	Wild boar	*M.* sp.		*M. europaeum*	100%	100%	not valid			*M. europaeum*
358	230	Wild boar	*M.* sp.		*M. interjectum*	100%	100%	*M. interjectum*	100%	98.31%	*M. interjectum*
359	231	Wild boar	*M.* sp.		*M. nonchromogenicum*	100%	100%	*M. nonchromogenicum*	100%	100%	*M. nonchromogenicum*

Isolate ID	Host ID	Host species	Tetraplex real-time PCR	*IS1245/IS901* real-time PCR	16S rRNA PCR	16S rRNA BLAST query coverage (%)	16S rRNA BLAST base identities (%)	*rpoB* PCR	*rpoB* BLAST query coverage (%)	*rpoB* BLAST base identities (%)	Consensus
360	232	Wild boar	*M.* sp.		*M.* sp. J16	100%	99.79%	*M.* sp. 3582	99%	98.44%	*M.* sp. 3582/J16
366	233	Wild boar	*M.* sp.		not valid			not valid			no sequencing result
367	234	Wild boar	*M.* sp.		*M. paraense*	100%	99.35%	*M. palustre*	100%	94.82%	*M.* sp. 2
368	235	Wild boar	*M.* sp.		*M. col/int/bou*	100%	99.58%	*M.* sp. GN-9680	96%	98.10%	*M.* sp. GN-9680
369	236	Wild boar	*M.* sp.		*M. col/int/bou*	100%	100%	*M. vulneris*	98%	98.29%	*M. vul/col/int/bou*
370	237	Wild boar	*M.* sp.		*M. col/int/bou*	100%	100%	*M. arosiense*	100%	97.61%	*M. aro/col/int/bou*
371	238	Wild boar	*M.* sp.		*M. col/int/bou*	100%	100%	*M. vulneris*	98%	98.43%	*M. vul/col/int/bou*
373	239	Wild boar	*M.* sp.		not valid			*M.* sp. GN-9680	96%	99.71%	*M.* sp. GN-9680
374	240	Wild boar	*M.* sp.		*M. paraf/scrof*	100%	100%	*M. paraffinicum*	100%	100%	*M. paraffinicum*
375	241	Wild boar	*M.* sp.		*M. col/int/bou*	100%	99.58%	*M.* sp. GN-9680	96%	98.25%	*M.* sp. GN-9680
376	242	Wild boar	*M.* sp.		*M. lent/par*	100%	99.35%	not valid			*M.* sp. 2
377	243	Wild boar	*M.* sp.		*M. col/vul/int/bou*	100%	99.79%	not valid			*M. vul/col/int/bou*
378	244	Wild boar	*M.* sp.		*M. col/vul/int/bou*	100%	99.79%	not valid			*M. vul/col/int/bou*
379	245	Wild boar	*M.* sp.		*M. col/int/bou*	100%	100%	*M. bouchedurhonense*	99%	97.75%	*M. bouchedurhonense*
380	**246**	Wild boar	*M.* sp.		not valid			not valid			no sequencing result
381	246	Wild boar	*M.* sp.		not valid			not valid			no sequencing result
382	247	Wild boar	*M.* sp.		*M.* sp. 34028-3	100%	100%	*M.* sp. 34028-3	99%	98.59%	*M.* sp. 34028-3
384	248	Wild boar	*M.* sp.		*M. col/int/bou*	100%	100%	*M. bouchedurhonense*	99%	97.75%	*M. bouchedurhonense*
385	249	Wild boar	*M.* sp.		not valid			not valid			no sequencing result
386	250	Wild boar	*M.* sp.		*M. intracellulare*	100%	99.58%	*M. colombiense*	100%	96.48%	*M.* sp. 1
387	**251**	Wild boar	*M.* sp.		*M. col/int/bou*	100%	100%	*M. vulneris*	98%	98.57%	*M_vul/col/int/bou*
388	251	Wild boar	*M.* sp.		not valid			M. sp. GN-9680	96%	99.56%	M. sp. GN-9680
389	252	Wild boar	*M.* sp.		*M. col/vul/int/bou*	100%	99.79%	*M. bouchedurhonense*	99%	98.53%	*M. bouchedurhonense*
390	253	Wild boar	*M.* sp.		*M. for/por/new*	100%	99.13%	*M. alvei*	100%	97.22%	*M. alvei*
391	254	Wild boar	*M.* sp.		not valid			*M.* sp. GN-9680	96%	98.10%	*M.* sp. GN-9680
396	255	Wild boar	*M.* sp.		*M. col/int/bou*	100%	100%	not valid			*M. col/int/bou*
400	256	Wild boar	*M.* sp.		not valid			*M. interjectum*	100%	97.78%	*M. interjectum*
404	90	Wild boar	*M.* sp.		*M. col/int/bou*	100%	99.79%	*M.* sp. GN-9680	96%	99.71%	*M. col/int/bou*
405	257	Wild boar	*M.* sp.		*M. col/int/bou*	100%	99.58%	*M.* sp. GN-9680	96%	98.10%	*M.* sp. GN-9680
407	258	Wild boar	*M.* sp.		not valid			not valid			no sequencing result
408	259	Wild boar	*M.* sp.		*M. nonchromogenicum*	100%	99.79%	not valid			*M. nonchromogenicum*
410	260	Badger	*M.* sp.		*M. interjectum*	100%	100%	*M. interjectum*	100%	100%	*M. interjectum*
411	261	Wild boar	*M.* sp.		*M.* sp. J16	100%	99.79%	*M.* sp. 3582	99%	98.30%	*M.* sp. 3582/J16
413	262	Badger	*M.* sp.		*M. col/int/bou*	100%	99.58%	*M. bouchedurhonense*	99%	97.18%	*M. bouchedurhonense*
416	263	Wild boar	*M.* sp.		not valid			not valid			no sequencing result
417	264	Wild boar	*M.* sp.		not valid			*M.* sp. 3582	99%	99.48%	*M.* sp. 3582
438	265	Goat	*M.* sp.		*M. senuense*	100%	99.58%	*M. senuense*	99%	99.58%	*M. senuense*
439	266	Wild boar	*M.* sp.		*M. int/Mich/Miy/paraint*	100%	100%	*M. intracellulare*	100%	100%	*M. intracellulare*
440	267	Wild boar	*M.* sp.		*M. per/arc/montm/lut/sep*	100%	100%	not valid			*M. per/arc/montm/lut/sep*
443	268	Wild boar	*M.* sp.		not valid			not valid			no sequencing result
448	269	Badger	*M.* sp.		not valid			not valid			no sequencing result
449	270	Cow	*M.* sp.		*M. chitae*	100%	98.26%	not valid			*M.* sp. 5
450	**271**	Badger	*M.* sp.		*M. kumamotonense*	100%	100%	not valid			*M. kumamotonense*

Isolate ID	**Host ID**	**Host species**	**Tetraplex real-time PCR**	***IS1245/IS901* real-time PCR**	**16S rRNA PCR**	**16S rRNA BLAST query coverage (%)**	**16S rRNA BLAST base identities (%)**	***rpoB* PCR**	***rpoB* BLAST query coverage (%)**	***rpoB* BLAST base identities (%)**	**Consensus**
451	271	Badger	*M.* sp.		*M. per/arc/montm/lut/sep*	100%	100%	*M. septicum*	100%	99.72%	*M. septicum*
452	272	Wild boar	*M.* sp.		not valid			*M. parascrofulaceum*	100%	97.33%	*M. parascrofulaceum*
454	273	Wild boar	*M.* sp.		*M.* sp. J16	100%	99.36%	not valid			*M.* sp. 3
455	274	Wild boar	*M.* sp.		*M. confluentis*	100%	100%	*M. confluentis*	100%	98.33%	*M. confluentis*
456	275	Wild boar	*M.* sp.		*M. fortuitum*	100%	100%	not valid			*M. fortuitum*
457	276	Wild boar	*M.* sp.		not valid			not valid			no sequencing result
6bis	277	Wild boar	*M.* sp.		*M. parascrofulaceum*	100%	100%	*M. parascrofulaceum*	100%	97.19%	*M. parascrofulaceum*

Not valid= targeted gene not amplified or weakly amplified, poor-quality sequences or sequences belonging to non-mycobacterial microorganisms. *Maa= M. avium* subsp. *avium*, *Mah= M. avium* subsp. *hominissuis*, *M. arc= M. arcueilense*, *M. aro= M. arosiense*, *M. bou= M. bouchedurhonense*, *M. col= M. colombiense*, *M. con= M. conceptionense*, *M. ele= M. elephantis*, *M. far= M. farcinogenes*, *M. for = M. fortuitum*, *M. hol= M. holsaticum*, *M. hous= M. houstonense*, *Mich= M. intracellulare* subsp. *chimaera*, *M. int= M. intracellulare*, *M. inter= M. interjectum*, *Miy= M. intracellulare* subsp. *yongonense*, *M. lent= M. lentiflavum*, *M. lut= M. lutetiense*, *M. mal= M. malmoense*, *M. mont=M. montefiorense*, *M. montm=M. montmartrense*, *M. new= M. neworleansense*, *M. par= M. paraense*, *M. paraf= M. paraffinicum*, *M. paraint= M. paraintracellulare*, *M. per= M. peregrinum*, *M. por= M. porcinum*, *M. sask= M. saskatchewanense*, *M. scrof= M. scrofulaceum*, *M. sen= M. senegalense*, *M. sep= M. septicum*, *M. tim= M. timonense*, *M. vul= M. vulneris*. *M.* sp. 1= *Mycobacterium* sp. close to species belonging to *M. avium* complex, *M.* sp. 2= *Mycobacterium* sp. close to *M. palustre/lentiflavum/paraense*. *M.* sp. 3= *Mycobacterium* sp. close to *M. scrofulaceum*, *M.* sp. 4= *Mycobacterium* sp. close to *M. wolinskyi*, *M.* sp. 5= *Mycobacterium* sp. close to *M. chitae*. *Mycobacterium* sp. GN-9680 and TY59 are related to *M. avium* complex, *Mycobacterium* sp. 34028-3 to *M. triplex/stomatepiae/montefiorense*, *Mycobacterium* sp. IEC1808 to *M. interjectum*, *Mycobacterium* sp. 3582 to *M. nebraskense*, *Mycobacterium* sp. J16 to *M. scrofulaceum* and *Mycobacterium* sp. 8115-1 to *M. duvalii*. Host identities (ID) of animals with more than one isolate are marked in bold letters.

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Instituto Nacional de Investigación
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