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On the trail of *Mycobacterium avium* subsp. *paratuberculosis* and *Mycobacterium bovis* in mainland Portugal: a microbiological and molecular survey in wildlife

Mestrado em Biologia Molecular e Genética

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Abstract

Paratuberculosis and bovine tuberculosis (bTB) are important infectious diseases of cattle caused by pathogenic mycobacteria, whose control present a challenge to livestock producers and veterinary authorities. These diseases represent a major problem for animal health and cause substantial economic losses associated with decreased productivity, limitations on animal trade and transactions of animal products, and slaughtering of infected animals in the case of bTB. Moreover, due to the zoonotic potential of these mycobacteria, and although the association of the paratuberculosis agent and Crohn's disease in humans is still ambiguous, it is necessary to strengthen the knowledge of the epidemiology of bTB and paratuberculosis, particularly the genetic signatures of the pathogens, their hosts, their routes of transmission and pathogenesis.

Mycobacterium avium subsp. *paratuberculosis* (*MAP*) is responsible for causing paratuberculosis or Johne's disease, a chronic infection of the gastrointestinal tract that affects ruminants worldwide. Ruminants appear to be the preferred or natural host, but *MAP* has already been isolated from other animal species, both domestic and wild.

Mycobacterium bovis (*M. bovis*) is the causative agent of bovine tuberculosis. Besides cattle, *M. bovis* can infect other domestic and wild species. In Iberian Peninsula, red deer and wild boar are known to act as maintenance hosts for *M. bovis* in hotspot areas.

In this work, the occurrence of *MAP* and *M. bovis* in wild carnivores, wild boar and red deer was assessed based on the molecular screening and microbiological culture of tissue samples opportunistically obtained from road-killed or hunted animals in mainland Portugal.

MAP detection was performed in feces and spleen samples from 225 animals, belonging to seven different species, namely Egyptian mongoose (n=149), red fox (n=40), common genet (n=5), stone marten (n=4), European badger (n=4), red deer (n=21) and wild boar (n=2), collected between 2005 and 2015. Samples were tested by culture in Herrold’s egg yolk solid medium, supplemented with and without mycobactin J, and by nested real-time PCR to amplify *IS900 MAP*-specific sequence.

The occurrence of *MAP*-positive animals, detected exclusively by PCR, was 8,4% (95%CI: 5,47-12,8%), which included nine Egyptian mongoose, four red fox, one stone marten, one common genet, one wild boar and three red deer, from several geographic locations. The percentages of *MAP*-positive Egyptian mongoose and red fox were 6,0% (95%CI: 3,2-11%) and 10% (95%CI: 4,0-23%), respectively.

The detection of *MAP*-positive red deer suggests an environmental source and indirect transmission, possibly indicating the presence of *MAP*-contamined water/pasture; while the identification of *MAP*-positive carnivores and wild boar also suggest contamination of the environment and/or ingestion of infected prey.

To our knowledge, *MAP* circulation in genets is reported for the first time. The presence of *MAP*-positive animals in several geographic locations, from north to the south of Portugal, also represents new data, since all the information published so far on wildlife is limited to animals from the center and northeast regions of mainland Portugal.

MAP DNA was detected in both biological matrices under study, being this survey the first report of *MAP* DNA in feces from wildlife. These results suggest contamination of the surrounding environment promoting the continuation of the infectious cycle.

The statistical analysis to identify risk factors for *MAP* exposure showed a significant association ($p<0,05$) between *MAP*-positive animals and their geographical location, being Faro, Aveiro and Viana do Castelo, the districts with increased risk. Other variables like species, gender, age class and type of organic matrix, were also analyzed, but no statistically significant differences were obtained.

Genotyping of *MAP*-positive samples was performed through MIRU-VNTR analysis, and, although several optimization approaches were attempted, it was not possible to obtain a complete allelic profile for any of the positive samples. Despite this fact, some alleles are reported for the first time, and multiple alleles were detected in VNTR X3, 7, 10, 25 and 292, which suggest co-infection with more than one *MAP* strain and/or mutation in these particular *loci*. Moreover, the occurrence of four alleles in VNTR X3 was registered for the first time.

The exposure to *M. bovis* was tested in feces and liver samples of 121 animals from five different species, namely Egyptian mongoose (n=93), red fox (n=4), stone marten (n=1), wild boar (n=2), and red deer (n=21) collected in 2012, 2013 and 2015, by culture in Lowenstein-Jensen pyruvate and Stonebrink solid media and using a semi-nested real-time PCR approach targeting *IS6110*.

M. bovis was isolated from one red deer from Castelo Branco, a geographic area enclosed in the epidemiological risk area for bovine tuberculosis in big game animals. None of the other samples under analysis was positive by culture or PCR.

The molecular characterization of *M. bovis* and *M. caprae* isolates from the bio-bank of INIAV was performed, by spoligotyping, disclosing the genetic diversity of 117 *M. bovis* and 10 *M. caprae* strains from domestic (cattle, sheep and pig) and wild animals (wild boar and red deer) isolated between 2011 and 2015. Spoligotyping exhibited a good discriminatory power ($D=0,91$) for the strains, revealing 27 different patterns. SB1174 was the most common spoligotype, accounting for 23% of the *M. bovis* isolates; and SB0157 was the only pattern obtained for *M. caprae*.

Four new spoligotypes (SB2354-SB2357) were identified and deposited in the international *M. bovis* database (http://www.Mbovis.org). The patterns SB1269, SB1265, SB1375, SB0948 and SB1060 were obtained for the first time in Portugal.

Eight patterns (SB1174, SB1264, SB0122, SB0265, SB1266, SB1195, SB0295 and SB1483) were common to wild boar and red deer and, with the exception of SB1483, the animals share the same geographical location, suggesting a common source of infection in those areas.

Despite the constraints related to our opportunistic sample, calculation of the diversity indices Shannon-Wiener, Simpson and Berger-Parker, combining information about species richness and abundance, and of the non-parametric estimators of species richness, chao 1 and chao 2, evidenced that, although sampling efforts could be reinforced, the panel of sampled species and number of specimens included was adequate for the purposes of this study, enabling inference of the epidemiological situation for the pathogens surveyed, particularly in the case of *MAP*.

Results from this work thus confirm that *MAP* and *M. bovis* circulate in livestock and widely distributed wildlife species from specific geographic regions of mainland Portugal and suggest the possibility of environmental contamination, reinforcing the need for increased surveillance and adjustment of control measures in order to enable successful eradication of these relevant diseases.

Key-words: *Mycobacterium avium* subsp. *paratuberculosis*, *Mycobacterium bovis*, wildlife, epidemiology, real-time PCR

Resumo

A paratuberculose e a tuberculose bovina são duas doenças infeciosas causadas por micobactérias patogénicas, com uma elevada importância ao nível da saúde e bem-estar animal e que provocam perdas económicas substanciais associadas à diminuição de produtividade, limitações ao comércio de animais e de produtos de origem animal e o abate sanitário de animais infetados, no caso da tuberculose. Acresce que, devido ao potencial zoonótico dos respetivos agentes etiológicos e apesar de estar ainda por demonstrar inequivocamente a associação do agente da paratuberculose à doença de Crohn em humanos, é necessário reforçar o conhecimento sobre a epidemiologia destas doenças, nomeadamente através de uma melhor caraterização dos agentes patogénicos, os seus hospedeiros, as vias de transmissão e a patogenia.

Mycobacterium avium subsp. *paratuberculosis* (*MAP*) é o microorganismo responsável pela paratuberculose ou doença de Johne, uma infeção crónica do trato gastrointestinal que afeta ruminantes em todo o mundo. Os ruminantes parecem ser o hospedeiro preferencial de *MAP*, contudo esta micobactéria já foi isolada a partir de amostras biológicas de um elevado número de outras espécies animais, domésticas e selvagens.

Mycobacterium bovis (*M. bovis*) é o agente etiológico da tuberculose bovina. À semelhança de *MAP*, também o isolamento de *M. bovis* já foi reportado num elevado número de hospedeiros, incluindo animais domésticos e selvagens. Na Península Ibérica, o veado e o javali estão identificados como reservatórios de *M. bovis* em determinadas áreas geográficas de elevada prevalência, tendo um importante papel no ciclo epidemiológico da doença.

Neste trabalho, pretendeu-se contribuir para o estudo da epidemiologia da paratuberculose e da tuberculose animal em Portugal, através da vigilância molecular e microbiológica de populações silvestres rastreadas de forma oportunística. Realizou-se a pesquisa de *MAP* e *M. bovis* em amostras biológicas de carnívoros, veados e javalis, doadas para fins científicos por várias entidades, e provenientes de atropelamento acidental, ações cinegéticas recreativas ou de correção de densidade de predadores devidamente autorizadas em Portugal continental.

Cento e trinta e quatro amostras de fezes e 149 amostras de baço, provenientes de 225 animais silvestres, foram rastreadas para a presença de *MAP*. Os espécimes testados pertenciam a sete espécies diferentes, nomeadamente, sacarrabos (n=149), raposa (n=40), geneta (n=5), fuinha (n=4), texugo (n=4), javali (n=2) e veado (n=21). As matrizes biológicas utilizadas foram processadas para realização de cultura em meio Herrold's com e sem micobactina J, e para extração de DNA. O DNA extraído foi testado para a presença da sequência de inserção *IS900*, específica de *MAP*, através de nested PCR em tempo real.

A presença da sequência *IS900* foi detetada em amostras provenientes de 19 dos animais testados (8,4%; 95%CI: 5,47-12,8%), evidenciando a sua exposição àquele agente. Os animais positivos incluem nove sacarrabos, quatro raposas, uma fuinha, uma geneta, um javali e três veados, originários de várias localizações geográficas. A percentagem de animais positivos das espécies sacarrabos e raposa foi de 6,0% (95%IC: 3,2-11%) e 10% (95%IC: 4,0-23%), respetivamente.

A deteção de *MAP* em veados sugere a existência de uma fonte ambiental e transmissão indireta, possivelmente através da existência de água e/ou vegetação contaminada com *MAP*, sendo que a identificação do microorganismo em amostras de carnívoros e javalis também sugere a possibilidade de contaminação do ambiente ou das presas, nas localizações geográficas de onde eram oriundos os animais positivos.

Tendo em consideração a informação disponível na literatura, este é o primeiro estudo onde é detetada a presença de DNA de *MAP* em genetas. A deteção de DNA de *MAP* em animais provenientes de norte a sul de Portugal, também representa uma nova informação, uma vez que todos os dados publicados até ao momento, se reportam a animais selvagens oriundos da região centro e nordeste do país.

Registou-se a deteção do DNA de *MAP* em ambas as matrizes biológicas, tratando-se este do primeiro estudo em que é reportada a deteção de *MAP* em fezes de animais selvagens. A deteção do microrganismo nas fezes sugere a sua excreção, que na forma viável poderá promover a continuação do ciclo infecioso.

A análise estatística dos resultados evidenciou uma associação significativa ($p<0,05$) entre os animais positivos para a presença de *MAP* e a sua localização geográfica, sendo Faro, Aveiro e Viana do Castelo, os distritos com maior risco. Por outro lado, para as variáveis espécie, género, classe etária e tipo de matriz biológica analisada, não foram obtidas diferenças estatisticamente significativas.

A caracterização molecular das amostras *MAP*-positivas foi realizada através da análise de MIRU-VNTR. Apesar de ter sido realizada uma otimização do protocolo aplicado, não foi possível obter um perfil alélico completo para nenhuma das amostras em análise.

Contudo, alguns alelos foram observados pela primeira vez, e alelos múltiplos foram detetados para o VNTR X3, 7, 10, 25 e 292, o que pode ser explicado pela presença de uma co-infeção e/ou ser devido a uma mutação nos referidos *loci*. Registou-se ainda pela primeira vez a ocorrência de quatro alelos para o VNTR X3.

Relativamente à pesquisa de *M. bovis*, foram analisadas 23 amostras de fezes e 98 amostras de fígado, provenientes de 121 animais. Os animais utilizados pertenciam a cinco espécies diferentes, especificamente sacarrabos (n=93), raposa (n=4), fuinha (n=1), javali (n=2) e veado (n=21). A metodologia seguida foi semelhante à aplicada para a pesquisa de *MAP*, contudo neste caso as matrizes biológicas utilizadas foram processadas para

realização de cultura em meio Lowenstein-Jensen com piruvato e Stonebrink, e o DNA extraído foi testado através de uma abordagem de PCR em tempo real semi-nested para a presença da sequência de inserção *IS6110*, a qual é específica para os membros do complexo *Mycobacterium tuberculosis*.

M. bovis foi isolada a partir de uma amostra de fezes de um veado, proveniente do distrito de Castelo Branco, uma área geográfica pertencente à zona epidemiológica de risco definida pelas autoridades Portuguesas para a tuberculose em caça maior. Nenhuma das restantes amostras em estudo demostrou ser positiva por cultura ou PCR.

O presente estudo vem também alargar o conhecimento sobre os genótipos de *M. bovis* e *M. caprae* que circulam em Portugal, e das relações epidemiológicas entre as diferentes espécies de hospedeiros, através da caraterização molecular de 127 isolados por *spoligotyping*. Os isolados utilizados (117 de *M. bovis* e 10 de *M. caprae*) encontravam-se depositados no biobanco do INIAV, e são provenientes de animais selvagens (veado e javali) e domésticos (bovinos, caprinos e suínos). A elevada diversidade de estirpes existente em Portugal é confirmada, tendo sido obtidos 27 perfis distintos e um bom poder de discriminação do método (D=0,91). O spoligotipo mais frequente entre os isolados de *M. bovis* foi SB1174 (23%), enquanto SB0157 foi o único perfil obtido para todos os isolados de *M. caprae*.

Quatro novos spoligotipos (SB2354-SB2357) foram identificados neste trabalho e adicionados à base de dados internacional (http://www.Mbovis.org). Os spoligotipos SB1269, SB1265, SB1375, SB0948 e SB1060 foram obtidos pela primeira vez em Portugal.

Oito dos spoligotipos identificados (SB1174, SB1264, SB0122, SB0265, SB1266, SB1195, SB0295 e SB1483) são comuns a javalis e veados, e à exceção do SB1483, os animais compartilham a mesma origem geográfica, sugerindo uma fonte comum de infeção nestas áreas.

Apesar das limitações inerentes a uma amostragem oportunística, o cálculo dos índices de diversidade Shannon-Wiener, Simpson e Berger-Parker, que combinam Informação sobre a ruiqueza e abundância de espécies, e dos estimadores não paramétricos de riqueza de espécies, chao 1 e chao 2, evidenciaram que, apesar do esforço de amostragem poder ser melhorado, o painel de espécies e número de espécimes incluídos neste estudo são adequados aos objetivos delineados, possibilitando inferências epidemiológicas acerca dos agentes patogénicos rastreados, particularmente no caso de *MAP*.

Concluindo, a paratuberculose e tuberculose bovina são doenças com elevado impacto ao nível da saúde animal e da economia. As micobactérias patogénicas responsáveis por estas doenças circulam em animais domésticos e selvagens em Portugal continental, reforçando assim a necessidade de uma maior vigilância e revisão das medidas de controlo, de modo a favorecer a erradicação das mesmas.

Palavras-chave: *Mycobacterium avium* subsp. *avium*, *Mycobacterium bovis*, vida selvagem, epidemiologia, PCR em tempo real

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AFB: Acid Fast Bacillus

bTB: Bovine Tuberculosis

CE: *Cervus elaphus*

CD: Crohn's Disease

DGAV: Direção Geral de Alimentação e Veterinária

DR: Direct Repeat

EU: European Union

ELISA: Enzyme-Linked Immunosorbent Assay

GG: *Genetta genetta*

HEYM: Herrold's Egg Yolk Medium

HI: *Herpestes ichneumon*

HPC: Hexadecylpyridinium Chloride Monohydrate

IHQ: Immunohistochemistry

INIAV: Instituto Nacional de Investigação Agrária e Veterinária, IP

LJ: Lowenstein-Jensen

LPSN: List of prokaryotic names with standing in nomenclature

M. bovis: *Mycobacterium bovis*

MAC: *Mycobacterium avium* complex

MAP: *Mycobacterium avium* subspecies *paratuberculosis*

MF: *Martes foina*

MIRU: Mycobacterial Interspersed Repetitive Units

MM: *Meles meles*

MTBC: *Mycobacterium tuberculosis* complex

NaOH: Sodium hydroxide

NUTS: Statistical terrestrial units

OIE: World Organization for Animal Health

OMS: World Health Organization

OTF: Officially Tuberculosis Free

PCR: Polymerase Chain Reaction

PFGE: Pulsed-Field Gel Electrophoresis

REA: Restriction Endonuclease Analysis

RFLP: Restriction Fragment Length Polymorphism

RFU: Relative Fluorescence Units

SNP: Single Nucleotide Polymorphism

Spoligotyping: Spacer Oligonucleotide Typing

SS: *Sus scrofa*

SSR: Short Sequence Repeats

TR: Tandem Repeat

USA: United States of America

VNTR: Variable Number Tandem Repeats

VV: *Vulpes vulpes*

WAHID: Worldwide Animal Health Information Database

1. Introduction

1.1 The genus *Mycobacterium*

The genus *Mycobacterium* is part of the Mycobacteriaceae family, presently encompassing 170 species, as enlisted in LPSN (list of prokaryotic names with standing in nomenclature).

In general, it includes rod shaped, aerobic, alcohol-acid resistant (due to the waxy mycolic acid cell wall), non-motile organisms with an optimal growth temperature ranging from 25 to 45 °C, a high G+C content (61-71%) and several pathogens known to cause disease in numerous hosts, including man [1–3].

Mycobacteria are widespread microorganisms, and can be found in soil, water, vegetation and food; however, some species appear to be obligate parasites. According to growth in culture media, mycobacteria can be grouped as fast and slow growers, and the colonies formed can be non-pigmented or have a yellow to red color [1,2].

1.2 *Mycobacterium avium* subspecies *paratuberculosis*

Mycobacterium avium subspecies *paratuberculosis* (*MAP*) is a slow-growing intracellular obligate parasite and the causative agent of paratuberculosis or Johne's disease, a chronic infection of the gastrointestinal tract that affects ruminants worldwide.

MAP is a member of the *Mycobacterium avium* complex (MAC), a group of environmental slow-growing mycobacteria, also composed by *Mycobacterium avium* subspecies *avium, Mycobacterium avium* subspecies *silvaticum*, *Mycobacterium avium* subspecies *hominissuis*, *Mycobacterium intracellulare*, *Mycobacterium colombiense*, *Mycobacterium chimaera*, *Mycobacterium vulneris*, *Mycobacterium arosiense*, *Mycobacterium bouchedurhonense*, *Mycobacterium marseillense* and *Mycobacterium timonense* [4–11].

MAP genome is a circular chromosome with approximately 69% of G+C content, containing several insertion sequences like *IS900* (14 to 18 copies), *ISMav2* (three copies), *ISMap02* (six copies) and F57 element (one copy), which are *MAP* exclusive [12–15].

1.2.1 *MAP* strain types

In a general way, *MAP* can be divided into two major strain groups (or strain types) designated Type I/S/sheep and Type II/C/cattle (Table 1.1 and Table 1.2). Initially, strain types were named according to the species from which they were first isolated, however the designation of 'sheep' and 'cattle' is not entirely correct, since interspecies transmission of prototypical strains has been detected [16–18].

The Type III/intermediate as a subtype of Type I/S strains and Type B/Bison, as a subtype of Type II/C strains, were recently described [19–21].

Table 1.1 - Summary of the major phenotypic and epidemiological characteristics of *MAP* strain types.

Characteristic	Type I/Sheep	Type II/Cattle
Growth rate [17]	Very slow-growing isolates	Slow-growing isolates
Colony morphology[17]	Smooth and uniform colonies	Rough and non-uniform colonies
Pigmentation [17,19]	Mostly yellow colonies	Mostly non-pigmented colonies
Recommended solid medium [22]	LJ, Middlebrook 7H10 or 7H11 agar media with mycobactin J	HEYM with mycobactin J
Components of the medium [22]	Highly sensitive to certain antibiotics	Addition of sodium pyruvate enhances bacterial recovery
Typical incubation time for primary growth on solid media [23]	4-12 months	2-4 months
Decontamination [24]	Highly sensitive to decontamination	
Host range preference [16,25,26]	Preference for sheep and goats	Very broad host range, including ruminants and non-ruminants

Legend: LJ - Lowenstein Jensen, HEYM - Herrold's Egg Yolk Medium;

Using a polyphasic approach, the most common genotyping methods provide basic information on strain type, and sub-typing tools can be applied in order to further discriminate *MAP* isolates, and in that way, obtain a higher discrimination power [27] (Table 1.2).

Table 1.2 - Nomenclature of *MAP* strain types determined by different typing procedures and their relationship to the designated Type S and Type C strains.

Typing methods	Characteristics	Type S		Type C	
***IS900*-RFLP** [18,20]	*IS900*-based RFLP using *Pst*I, *Bst*EII and/or *Pvu*II as restriction endonucleases. Together with PFGE, these are the most widely used typing methods.	I/S	III	II/C	
PFGE [17,18]	Whole genome restriction using *Sna*BI and *Spe*I as restriction endonucleases, followed by electrophoresis with continuous changes in the electric field.	I/S	III	II/C	
***IS13111* PCR-REA** [21,28]	*IS1311* amplification followed by restriction with *Hinf*I and *Mse*I.	I/S		II/C	B
Sub-typing methods					
SSR analysis [29]	Analysis of G mononucleotide repeats and GGT trinucleotide repeats *loci.*				
VNTR analysis [30–32]	PCR method based on the polymorphism analysis of VNTR *loci*, namely the determination of the number of repetitions. MIRUs are a specific type of VNTR applicable to mycobacteria, and can be used in this analysis.				

Legend: RFLP (Restriction Fragment Length Polymorphism), PFGE (Pulsed-Field Gel Electrophoresis), PCR - Polymerase Chain Reaction, REA - Restriction Endonuclease Analysis, SSR - Short Sequence Repeats, VNTR (Variable Number Tandem Repeat) and MIRU - Mycobacterial Interspersed Repetitive Units.

1.2.2 Paratuberculosis or Johne's disease

1.2.2.1 *MAP* host species

Ruminants appear to be the preferred or natural host for *MAP*, primarily domestic ruminants as cattle, sheep and goat, but the disease in wild ruminants is also well documented including a large number of hosts, as red deer (*Cervus elaphus*), roe-deer (*Capreolus capreolus*), fallow deer (*Dama dama*), white-tailed deer (*Odocoileus virginianus*), alpine ibex (*Capra ibex*) and riverine buffalo (*Bubalus bubalis*) [33–38].

In non-ruminant animals, *MAP* was already detected in a very broad host range, that includes wild rabbit (*Oryctolagus cuniculus*), raccoon (*Procyon lotor*), coyote (*Canis latrans*),

stoat (*Mustela erminea*), weasel (*Mustela nivalis*), brown bear (*Ursus arctos*), and jackdaw (*Corvus monedula*) [34,39–45].

1.2.2.2 Paratuberculosis progression and *MAP* transmission

Disease progression and clinical signs are best characterized in domestic ruminants. *MAP* typically enters the host by the oral route and then, eventually, will cause a chronic inflammatory state with the presence of granulomatous lesions in the small and large intestine, leading to progressive weight lost, diarrhea and decreased milk production [46].

The animals can remain asymptomatic for two to five years, but during this period shedding at different levels may occur [47–49]. *MAP* excretion occurs, preferentially, in the feces but in an advanced-stage of infection, the pathogen may also be found in colostrum and milk, saliva, uterine fluid and semen, being also possible for fetal infections to occur *in utero* [46,50–52].

The main route of intra- and inter-species transmission is fecal-oral, by direct ingestion of *MAP*-contaminated feces, colustrum and milk or, indirectly, via *MAP*-fecal-contaminated water and/or feed and preys [53,54].

1.2.2.3 Potential sources of *MAP* infection

Despite the fact that *MAP* is only able to replicate inside host cell, it can be found in water, vegetation and soil, as well as in animal-derived products, which contributes to animal and/or human exposure to the pathogen. The human exposure raises public health concern, since the causal association between *MAP* and Crohn's disease in humans is still not clear.

In environment, *MAP* was cultured for up to 55 weeks from soil, for up to 24 weeks from the aerial parts of grasses and for up to 48 weeks from water, in a controlled environment in Australia [55,56].

In food, *MAP* has been detected by PCR and/or culture in raw and pasteurized milk from cows, sheep and goats; powdered infant milk; and cheeses [57–60]. Moreover, *MAP* has been detected by PCR in drinking water in the United States of America (USA) [61].

1.2.2.4 Paratuberculosis worldwide

Notification of paratuberculosis to OIE (World Organization for Animal Health) is mandatory and, according to the information on WAHID interface (Worldwide Animal Health Information Database), 54 out of 191 countries reported the presence of *MAP* infection and/or clinical disease during last year (Fig. 1.1). The worldwide herd prevalence of paratuberculosis is estimated to be 7 to 40%, based on serological monitoring tests [62].

In Europe, it is estimated that more than 50% of the dairy cattle is infected with *MAP*, however paratuberculosis was not included in the European Union (EU) programs of eradication, control and surveillance of animal diseases and zoonoses for 2015-2017 [63].

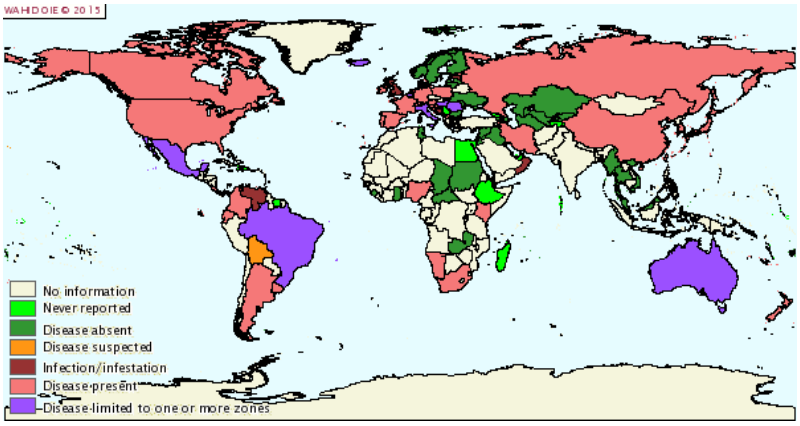

Fig. 1.1 - World map distribution of paratuberculosis, for both domestic and wild species, based on data related to last semester of 2014 reported by worldwide countries to OIE (accessed on October 2015).

1.2.2.5 Paratuberculosis in Portugal

So far, only a few geographically-limited studies have tried to assess the prevalence of paratuberculosis in Portugal, mainly in small ruminants [64–69], which suggest that probably this disease is under-diagnosed.

In the most recent data, the serological analysis by ELISA (Enzyme-Linked Immunosorbent Assay) of blood samples from 5370 sheep and goats and 2562 sheep has evidenced a prevalence of 27% and 9,1% in Lisboa and Serra da Estrela, respectively [66,67]. Moreover, a survey in sheep's from the Northeast of the country was performed, and 3,7% of the 3900 blood samples were seropositive by ELISA, and 18,7% of the pooled samples from a set of 1500 blood samples (in pools of five) were positive by *IS900* PCR approach [68,69].

Paratuberculosis cases have to be reported to the Portuguese authorities since 2009. However, in this work it was not possible to find official information regarding the numbers of cases. Furthermore, in the reports sent by Portugal to OIE, in the last five years, and available at WAHID, disease report is limited to the domestic population.

1.2.2.6 Diagnosis methods of paratuberculosis

Diagnostic methods include bacteriological culture of clinical samples, mainly from feces and intestine tissues, and serological/molecular tests (Fig. 1.2) [22,24,62,70–77].

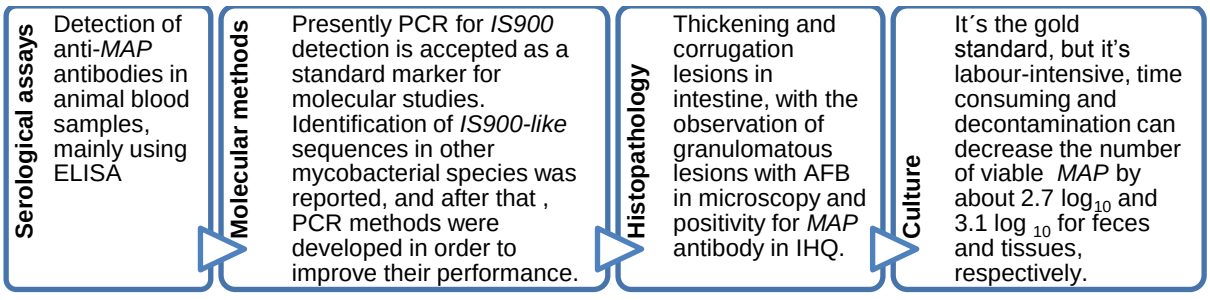

Fig. 1.2 - Summary of *MAP* diagnosis methods. AFB - Acid Fast Bacillus, IHQ - Immunohistochemistry.

Decontamination with oxalic acid and NaOH and culture in Lowenstein-Jensen (LJ) medium, or decontamination with HPC (hexadecylpyridinium chloride monohydrate) and culture in Herrold's Egg Yolk Medium (HEYM) or Middlebrook medium, are the common protocols followed for *MAP* culture. The culture media needs to be supplemented with an iron chelator, named mycobactin, being this dependence characteristic, but not *MAP* exclusive [78].

1.2.2.7 Control and eradication strategies for paratuberculosis

There are still no ideal, cost-effective, methods for the control of paratuberculosis. Control schemes based on a test-and-cull approach depend on the availability of suitable diagnostic assays to detect infected animals as early as possible, but the available serological methods are most reliable during the later stages of infection [23,71].

Vaccination has been used in some European countries, generally in small ruminants, and with good results (the number of clinical and subclinical animals was reduced), so is a possible control measure [23].

1.3 *Mycobacterium bovis*

Mycobacterium bovis (*M. bovis*), together with *Mycobacterium tuberculosis*, *Mycobacterium africanum*, *Mycobacterium microti*, *Mycobacterium canettii*, *Mycobacterium pinnipedii* and *Mycobacterium caprae*, constitute the *Mycobacterium tuberculosis* complex (MTBC) [79–82]. MTBC members, and the causative agents of tuberculosis in numerous species, can be best understood as a series of host-adapted ecotypes, with each ecotype having distinct host tropisms, phenotypes and pathogenicity, and being marked by molecular differences [83–86]. *M. bovis*, the causative agent of bovine TB (bTB), is an aerobic slow-growing bacteria with a genome formed by a circular chromosome with approximately 66% G+C content [87].

1.3.1 *M. bovis* genotyping

The molecular typing techniques most commonly used for *M. bovis* genotyping can also be applicable to all members of MTBC and are described in Fig. 1.3 [88–92].

Spoligotyping

- PCR and reverse hybridization method based on the polymorphisms of DR locus, which is comprised of conserved direct repeats of 36 bp interspersed with unique spacer sequences. The simplicity of the method, that detects the presence/absence of these spacer sequences, allowed the establishment of international, open-source, spoligotype databases, such as *M.bovis.org.*

VNTR analysis

- Several VNTR *loci*, including MIRUs, were already used for genotyping *M. bovis*. The common approach determines the number of repetitions of 12, 15 or 24 locus per analysis and the final result can be introduced in international databases, such as *MIRU-VNTR plus* database.

***IS6110*-RFLP**

- *IS6110* is a multi-copy sequence (1-25 copies) found within MTBC, although *M. bovis* normally contains only one copy. *IS6110*-RFLP uses *Pvu*II as restriction endonuclease. This method has low discriminatory power, especially for strains with low-copy number of *IS6110.*

Fig. 1.3 - Summary of the most common typing techniques used for *M. bovis*. Spoligotyping - Spacer Oligonucleotide Typing, DR - Direct Repeat.

1.3.2 Animal Tuberculosis

M. bovis is the main etiological agent of bTB but occasionally *M. caprae* is also isolated from suspected tuberculosis lesions. The disease caused by both ecotypes is not substantially different and the same diagnostic methods can be applied [77].

1.3.2.1 *M. bovis* host species

Besides cattle, *M. bovis* is also able to infect other animal species, including domestic animals as sheep, goat, pig, dogs and cats [93,94], but also several wildlife species worldwide, causing animal TB. Some of the wildlife species are known to act as maintenance hosts and source of infection to other animals, namely the African buffalo (*Syncerus caffer*) in South Africa, lechwe antelope (*Kobus leche*) in Zambia, brushtail possums (*Trichosurus vulpecula*) in New Zealand, white-tailed deer (*Odocoileus virginianus*) in EUA, Canadian bison (*Bison bison*) in Canada, badger (*Meles meles*) in United Kingdom and Republic of Ireland, and red deer (*Cervus elaphus*) and wild boar (*Sus scrofa*) in Iberian Peninsula [95–98].

Surveys in other wildlife species were already performed and the presence of *M. bovis* was detected in a very broad host range that includes coyote (*Canis latrans*), red fox (*Vulpes vulpes*), black bear (*Ursus americanus*), raccoon (*Procyon lotor*), fallow deer (*Dama dama*) and Iberian lynx (*Lynx pardinus*) [99–101].

1.3.2.2 Tuberculosis progression and transmission

Bovine TB is an infectious, chronic, but progressive, disease characterized by the formation of typical granulomatous lesions in several different organs. Clinical signs include weakness, anorexia, emaciation, dyspnoea, and cough, particularly in the advanced stages [77].

The excretion of virulent and viable bacilli is dependent on the localization of the infection. Thus, the excretion of *M. bovis* occurs, essentially, through aerosols and respiratory secretions, feces and urine. In advanced stages of the disease, the pathogen can also be found in milk, saliva, vaginal and uterine discharges, and purulent material of cutaneous abscesses or open lesions of peripheral lymph nodes [97,102–104].

The anatomical localization of the lesions provides some information about the route of transmission: animals with lesions restricted to the thoracic cavity are presumed to have been infected by the inhalation of aerosols through sharing of the same habitat (food/water spots); while those with lesions in abdominal organs are thought to have acquired the infection by ingestion of infected animals or contaminated pastures/water [102–104].

In a controlled environment in the USA, *M. bovis* persisted up to 88 days in soil and 58 days in water, an evidence that shows that this pathogen can represent a risk of exposure for

cattle and/or wildlife [105]. Moreover, in Portugal, the presence of *M. bovis/ caprae* was already detected in the environment in areas where bTB is highly prevalent in wildlife [106].

1.3.2.3 Animal tuberculosis worldwide

Bovine TB is notifiable to OIE and, according to the information on WAHID interface, 71 out of the 191 countries reported the presence of *M. bovis* infection and/or clinical disease during the last year (Fig. 1.4).

Concerning Europe, several countries and specific regions, including the Algarve in Portugal, achieved the OTF (Officially Tuberculosis Free) status in 2012, in accordance with EU legislation (Decision 2012/204/EU) [107]. In the EU non-OTF regions, infection with *M. bovis* was reported in 18,256 (1,33%) cattle herds in 2013 [107]. Bovine TB is one of the diseases included in the EU programs of eradication, control and surveillance of animal diseases and zoonoses for 2015-2017 [63].

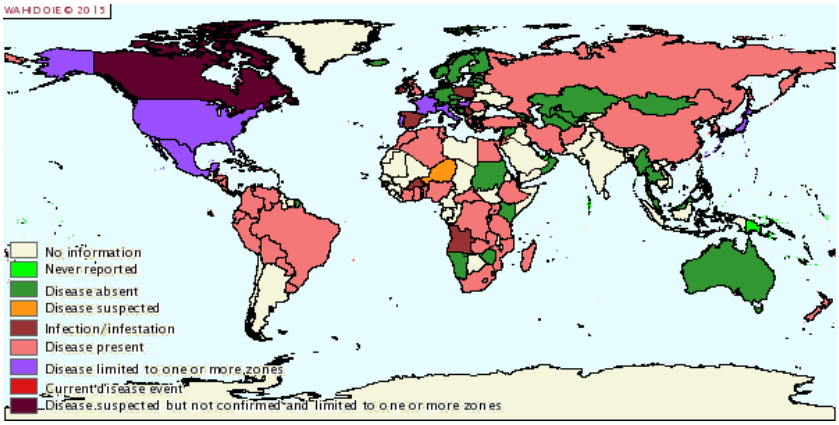

Fig. 1.4-World map distribution of animal tuberculosis, for both domestic and wild species, based on data related to last semester of 2014 reported by worldwide countries to OIE (accessed on October 2015).

1.3.2.4 Animal tuberculosis in Portugal

All bTB cases detected in Portugal have to be reported to the Portuguese authorities since 1953, and also to the European authorities (EU Comission) and to OIE.

The prevalence of *M.bovis* infected cattle in mainland Portugal has remained low and has been decreasing, with animal and herd prevalence being 0,03% and 0,11%, respectively, in 2008 (Fig. 1.5 A) [108]. However, in 2009 the number of animals almost doubled, reaching in 2010 the values of 0,26% prevalence in animals and 0,9% prevalence in herds [108].

This increase of bTB indicators in Portugal may be a combination of genuine disease increase with higher vigilance efficacy [109]. The reorganization of the veterinary services that was performed in 2009, and the reorganization of the existent farms might also have contributed to the escalation of epidemiological indicators [108].

Since 2012, the values have remained constant and low (0,06% and 0,37% of animal and herd prevalence, respectively, in 2014).

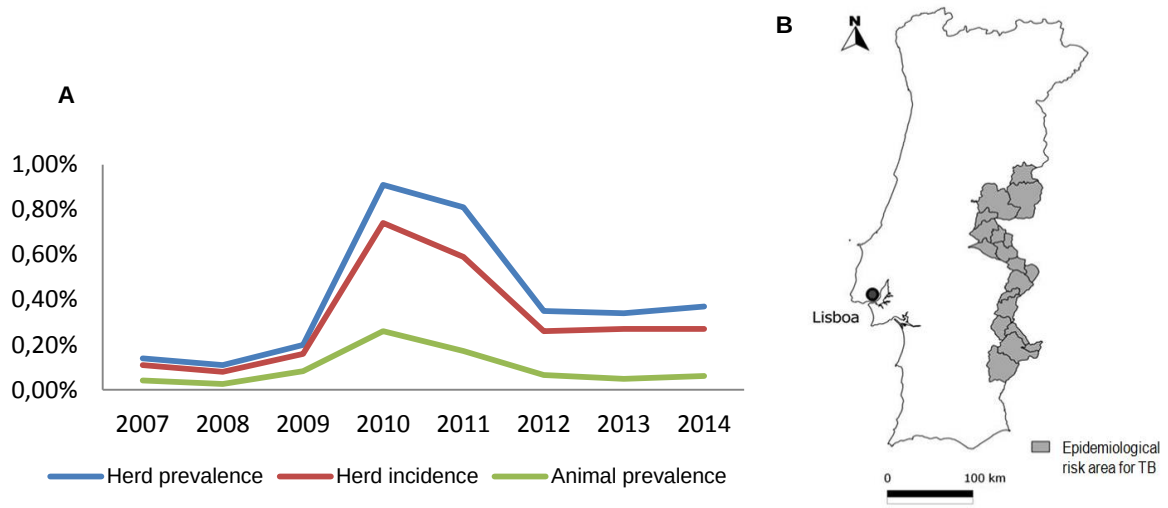

Fig. 1.5 - (A) Trend of bovine tuberculosis epidemiological indicators in mainland Portugal for the period 2007-2014. Adapted from DGAV (2014) [108]**. (B) Epidemiological risk area for big game species. Adapted from DGAV (2014)** [110]**.**

The eradication program of bTB in Portugal is based on the detection of positive live animals by the single intradermal comparative tuberculin test and interferon-γ test; routine surveillance at slaughterhouse; compulsory slaughter of positive animals; compensation to owners of slaughtered animals; and restriction of movements and pre-movement testing [108]. Only the isolation of *M. bovis* during culture or the observation of typical TB lesions during histopathological analysis confirms the animal as being TB infected, being INIAV the reference laboratory for diagnosis [109].

Overpopulation of big game animals, namely red deer and wild boar, occurs in certain regions of mainland Portugal, increasing intra and interspecific transmission. After the publication of several studies evidencing the circulation of *M. bovis* in wild populations [111–113], the Portuguese authorities defined in April 2011 an epidemiological risk area for TB in big game (Fig. 1.5 B). This implies that big game hunting activities may be accompanied by a credentialed veterinarian to perform a *post-mortem* examination of hunted animals and eventually collect samples from suspected tuberculosis lesions for laboratorial confirmation; and to ensure proper disposal of hunting by-products [110].

In mainland Portugal, the isolation of *M. bovis* from red deer and wild boar is well documented and genotyping studies of circulating isolates obtained from domestic and wild animals, from different geographic locations have been performed over the years [111–115].

1.3.2.5 Diagnosis methods

Bovine tuberculosis infection in cattle is usually diagnosed in living animals on the basis of delayed hypersensitivity reactions and, after death, with the use of histopathological, bacteriological and molecular techniques (Fig. 1.6) [88,90,91,116]

For the microbiological culture, decontamination with detergent (0,375-0,75% HPC), alkali (2-4% NaOH) or acid (5% oxalic acid), and incubation in LJ pyruvate, Stonebrink and Coletsos base are the common approaches. Moreover, the culture media needs to be supplemented with pyruvate, since *M. bovis* has an alteration in genes involved in the pyruvate metabolism [117].

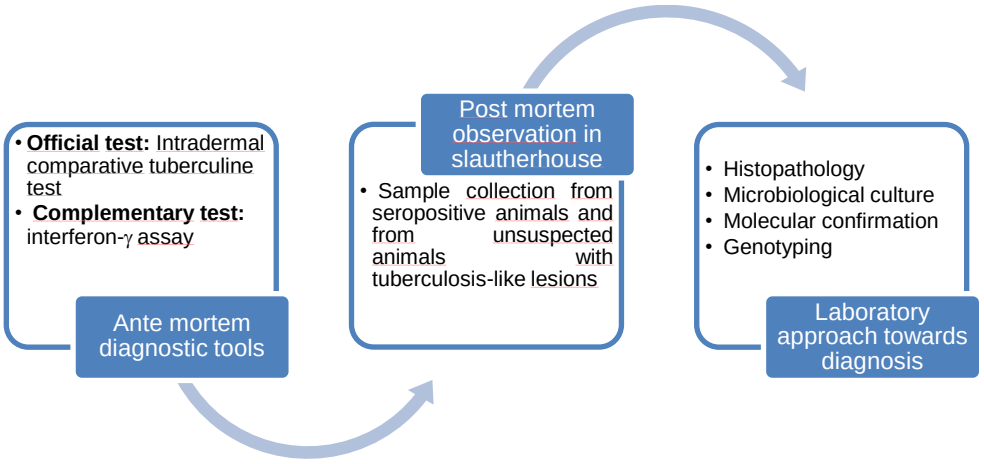

Fig. 1.6 - General laboratory algorithm used for bovine tuberculosis diagnosis.

1.3.3 Impact of *M. bovis* in human health

In countries with programs of control and eradication of bTB, *M. bovis* can especially affect people that work directly with infected animals by airborne transmission, such as veterinarians, zoo workers, hunters, slaughterhouse workers and farmers. In countries without surveillance, the contact might happen through the consume of unpasteurized dairy products or raw/undercooked meat from infected animals [118–121]. There are evidences of human-to-human transmission through the airborne via, especially among immunossupressed people [122,123].

1.4. Objectives of the present work

MAP and *M. bovis* are pathogenic mycobacteria that can infect livestock and wildlife, causing paratuberculosis and animal TB, respectively. These diseases may have a huge impact in economy and animal health throughout the world, and, due to their zoonotic potential, raising concerns for public health. In that way, their control and eradication can only be achieved through the understanding of the transmission routes of the pathogens and the ascertainment of the importance of wildlife species in the epidemiological cycle.

As such, the main objective of this work was to contribute to the disclosure of wild hosts that may potentially be exposed to *MAP* or *M. bovis* in mainland Portugal. For that purpose, geographically widely distributed animal specimens from the Mustelidae, Herpestidae, Viverridae, Canidae, Suidae and Cervidae families were surveyed for the presence of these bacteria using a polyphasic diagnostic approach based on microbiological and molecular

methods. The clarification of epidemiological links between wildlife and livestock animals was also envisaged based on genotyping techniques applied to isolates of both origins and using the limited epidemiological information available.

Samples for *M. bovis* survey were selected based on previous knowledge, meaning that the majority of the samples used are from animals killed in the epidemiological risk area.

2. Methods

All the experimental work described below was developed at Instituto Nacional de Investigação Agrária e Veterinária (INIAV, IP; UEISPSA strategic unit) in Lisboa, Portugal.

2.1 Animal samples used in this study

The biological matrices used in this study resulted from animals found dead on the road and from legal hunting actions, and were donated for scientific purposes, by the hunting associations and under a protocol established by Dr. Mónica Cunha with CE3C, FCUL, University of Aveiro and Fencaça. All tissue and fecal samples were collected during necropsy, and conserved at -20ºC until being processed in agreement with OIE and OMS (World Health Organization) guidelines.

2.2 Samples used for *Mycobacterium avium* subsp. *paratuberculosis* survey

The circulation of *MAP* in wildlife was investigated in biological specimens of wild carnivores, wild ruminants (red deer) and wild boar, collected between 2005 and 2015, in mainland Portugal (Supplementary Table 1).

Feces (n=134) and spleen (n=149) samples from 225 animals, 105 males and 101 females, representing seven different species, were the biological matrices screened for *MAP* (Table 2.1).

Table 2.1 - Family, species, number and type of animal specimens processed for *MAP* detection.

Family	Species	Total of animals	Total samples	Animals with both matrices analyzed
Canidae (*n*=40)	Red fox (*Vulpes vulpes*)	40	*n*= 26 F; *n*=29 S	16
Mustelidae (*n*=8)	Stone marten (*Martes foina*)	4	*n*=3 F; *n*=3 S	2
	European badger (*Meles meles*)	4	*n*=2 F; *n*=4 S	2
Viverridae (*n*=5)	Common genet (*Genetta genetta*)	5	*n*=2 F; *n*=4 S	1
Herpestidae (*n*=149)	Egyptian mongoose (*Herpestes ichneumon*)	149	*n*=78 F; *n*=109 S	37
Suidae (*n*=2)	Wild boar (*Sus scrofa*)	2	*n*=2 F	
Cervidae (*n*=21)	Red deer (*Cervus elaphus*)	21	*n*=21 F	
Total		225	*n*=134 F; *n*=149 S	58

Legend: F - feces, S - spleen

For bacterial culture purposes, 1 g of feces and 2 g of spleen samples were macerated and decontaminated with 0,9% (v/v) and 0,7% (v/v) of HPC (*Sigma-Aldrich*), respectively, following the methodology described in the OIE guidelines (2008). After decontamination, the macerated suspensions were divided into two equal parts. One part was maintained at 4ºC until further processing for DNA extraction and the other one was centrifuged and the sediment inoculated (100μL) in HEYM with and without mycobactin J. After inoculation, the cultures were maintained at 37ºC and observed for bacteriological growth every week.

Mycrobiological culture in HEYM with and without mycobactin J was attempted for all the feces samples. Subsequently, based on the positive results from nested real time *IS900* PCR, nine other spleen samples were processed for culture.

2.2.1 DNA extraction from feces and tissue suspension

Direct DNA extraction from 5 mL of feces and 5 mL of spleen suspensions was performed using the commercial system Invisorb® Spin Tissue Mini Kit (*Stratec Molecular*) and High Pure PCR Template Preparation Kit (*Roche*), respectively, following the manufacturer's instructions. A step of mechanical disruption, before applying Proteinase K, was added using a bead-beating protocol consisting of 6.5 ms^{-1} cycles for 45 sec, repeated twice, in the FastPrep FP120 Bio101 (*Savant Instruments*). Genomic DNA suspensions were stored at -20 °C until further use.

2.2.2 Nested real-time PCR for the detection of *IS900* insertion sequence

The detection of *MAP* in the genomic DNA extracted from biological samples was performed by a nested real-time PCR targeting *IS900* (Leão et al., unpublished results).

This PCR assay consists of two amplification steps: (i) a first standard PCR targeting a 224 bp sequence; and (ii) a second real-time PCR, using the previous amplification product as template, that amplifies a 66 bp sequence (Supplementary Table 2).

In addition to samples, positive control (DNA extracted from *MAP* ATCC 19698 and DNA from positive bovine tissue sample) and a negative control (water) were included in all PCR batches. All samples were tested at least twice.

The amplified products from both PCR reactions were analyzed in a 2% agarose gel.

2.2.3 Genotyping of *MAP*-positive samples by MIRU-VNTR technique

The *MAP*-positive samples detected by nested real-time PCR targeting I*S900* were tentatively characterized by MIRU-VNTR (Mycobacterial Interspersed Repetitive Units - Variable Number Tandem Repeat), based on eight *loci*, following the method described by Thibault and collaborators [30].

PCR reactions were performed for all *MAP*-positive samples using a conventional protocol, however, and in order to try to improve the signal, in a subset of samples was also attempted

a real-time PCR (Supplementary Table 2 and 3). Optimization of the method was attempted by using different PCR cycles, different annealing temperatures and different DNA quantities from the samples, namely 2μL, 5μL, 10 μL and 1:10 dilution. A positive (DNA extracted from *MAP* ATCC 19698 strain) and a negative control (water) were included in all PCR assays.
The PCR products were analyzed in 2% agarose gel. Data available in the open-source MAC INMV database (http://mac-inmv.tours.inra.fr/) were used to assign the hypothetical size of each amplicon to their correspondent tandem repeat copy number.

2.2.4 Strain type differentiation of *MAP*- positive samples

The DNA from *MAP*-positive samples, as inferred by nested real time *IS900* PCR, was also tested to attempt strain type differentiation, using an approach based on SNP (Single Nucleotide Polymorphism) detection (Leão et al., unpublished results).
In this methodological approach, a genomic region harboring a SNP that allows the differentiation between S and C strains is amplified by PCR, followed by restriction with endonucleases or direct Sanger sequencing (Supplementary table 2). Positive control (DNA extracted from *MAP* strain K-10) and negative control (water) were included in all reaction batches, and the PCR product was observed in a 2% agarose gel.

2.3 Samples used for *Mycobacterium bovis* survey

The circulation of *M. bovis* in wildlife was investigated in biological specimens of wild carnivores, wild ruminants (red deer) and wild boar, collected in 2012, 2013 and 2015, in mainland Portugal (Supplementary Table 1).
Ninety eight liver samples and 23 feces from 121 animals, 60 males and 61 females, representing five different species, were used in this survey (Table 2.2).

Table 2.2 - Family, species, number and type of animal specimens processed for *M. bovis* detection.

Family	Species	Total of animals	Total of samples
Canidae (*n*=4)	Red fox (*Vulpes vulpes*)	4	*n*=4 L
Mustelidae (*n*=1)	Stone marten (*Martes foina*)	1	*n*=1 L
Herpestidae (*n*=93)	Egyptian mongoose (*Herpestes ichneumon*)	93	*n*=93 L
Suidae (*n*=2)	Wild boar (*Sus scrofa*)	2	*n*=2 F
Cervidae (*n*=21)	Red deer (*Cervus elaphus*)	21	*n*=21 F
Total		121	*n*=98 L; *n*=23 F

Legend: L - liver; F - feces

For bacteriological culture purposes, 1 g of feces and 2 g of liver were macerated and decontaminated with 0,9% (v/v) and 0,7% (v/v) of HPC (*Sigma-Aldrich*), respectively, following the methodology described in the OIE guidelines (2008). After decontamination, one part of the macerated suspension was maintained at 4ºC until further processing for DNA extraction and the other part was centrifuged and the sediment (100 μL) inoculated into

Lowenstein Jensen (LJ) pyruvate and Stonebrink (*Biogerm*). After inoculation, the cultures were maintained at 37ºC and observed for bacteriological growth every week.

2.3.1 DNA extraction from feces and tissue suspension

Direct DNA extraction from feces and liver suspensions was performed, following the procedures already referred in the *MAP* survey methods (section 2.2.1).

2.3.2 DNA extraction from culture

Colonies on solid media were suspended in TE 1 M pH 8.0 and heated at 99 °C for 30 min. After centrifugation (1500 g, for 5 min), the suspension was stored at -20ºC.

2.3.3 Semi-Nested real-time PCR for the detection of *IS6110* insertion sequence

The detection of *IS6110* followed the methodology and reactional conditions developed by Costa and collaborators [124].

This PCR assay follows the same principle of the methodology previously described for the detection of *IS900*. However, in this case, one of the primers used (*IS6110*-RV) is common to both PCR reactions (Supplementary Table 4). The first standard PCR amplifies a 199 bp fragment and the second real-time PCR a 63 bp sub-fragment.

Two positive (DNA extracted from *M. tuberculosis* H37RV and DNA from a positive tissue sample) and one negative control (water) were used in every PCR batch. All samples were tested, at least, twice.

Analysis in 2% agarose gel was performed for the PCR products of both steps.

2.3.4 Species identification of bacterial isolates by *gyrB* PCR-REA

Species confirmation of bacterial isolates growing in Stonebrink and LJ pyruvate medium was performed by *gyrB* PCR-REA (Supplementary Table 4).

In every PCR batch were included two positive (DNA extracted from *M. tuberculosis* H37RV and *M. bovis BCG*) and one negative control (water); and the resulting amplified products were visualized in a 1,5 % agarose gel.

The enzymatic restriction reactions with *Rsa*I and *Sac*I were carried out for a final volume of 10 μl, following the manufacturer's instructions (*New England Biolabs*), and using 3 μl of PCR reaction product. The restriction products were visualized in a 2% agarose gel.

2.4 Spoligotyping of *M. bovis* and *M. caprae* isolates

One hundred and seventeen *M. bovis* and 10 *M. caprae* isolates, from five animal species, preliminarily identified by *gyrB* PCR-REA methodology, were characterized at the molecular level by spoligotyping (Table 2.3). This analysis included *M. bovis* isolates from 2014 and 2015 and *M. caprae* isolates from 2011 onwards.

Table 2.3 - Number of bacterial isolates, per year and animal species, spoligotyped.

Year	Red Dear	Wild Boar	Cattle	Sheep	Pig
2011			1		
2012				2	
2013			3	2	
2014	17	16		1	
2015	34	42	7		2
Total	51	58	11	5	2

DNA extracted from *M. tuberculosis* H37RV and *M. bovis BCG* were used as positive controls while water was included as negative control in every PCR assay for the amplification of the DR (Direct Repeat) region (Supplementary Table 4). The amplified products were analyzed in a 2% agarose gel before initiating the spoligotyping-associated hybridization procedure.

The membranes used in the spoligotyping technique were prepared in INIAV, using 43 oligonucleotide sequences corresponding to the 43 spacer regions of *locus* DR.

The PCR targeting the DR region and hybridization of PCR products was performed according to the procedure described by Kamerbeek and collaborators [88], with the following changes: hybridization time was increased to 90 min and membranes were re-used after three washes at 85ºC in 1% SDS, followed by two washes at room temperature with 20 mM EDTA.

The spoligotyping pattern obtained for each isolate was converted into a binary code of 43 digits and it was inserted in the international data base Mbovis.org (http://www.mbovis.org/index.php). The new spoligotypes were re-tested for the confirmation of the pattern, submitted to the international database and assigned a new spoligotype designation.

The discriminatory capacity of spoligotyping technique was evaluated with the use of discriminatory power (D), which was calculated through an online application tool available on the website: http://insilico.ehu.es.

2.5 Statistical analyses of the results

Chi-square test and Mann-Whitney test (α=0,05) were performed in IBM SPSS Statistics 22.0; and graphics were produced in Excel and GraphPad Prism 6.0. The confidence intervals (95%) were calculated through an online application tool available on the website: http://vassarstats.net/.

2.6 Diversity analyses of the samples under study

Diversity analyses was performed through the establishment of the relationship between abundance and distribution; the calculation of diversity indices (Shannon-Wiener, Simpson and Berger-Parker); and the determination of non-parametric estimators (chao 1 and chao 2) of species richness (Supplementary Table 5) [125,126].

3.1 Detection of *MAP* in feces and spleen of wild species

3.1.1 Demographic characteristics of the samples under study

Animals were sexed and aged during necropsy, however for a few specimens, gender (n=19) and age class (n=9) was not registered. The 225 animals screened belong to seven different species, being 45% females, 47% males and 8% unknown (Supplementary Fig. 1).

Each specimen was assigned to one of four age classes: adults, sub-adults, juveniles and cubs, according to body length and dental growth, being the majority of surveyed specimens adult individuals (n=177; 78,7%) (Supplementary Fig. 2).

Regarding geographical distribution, the districts of mainland Portugal were considered the sampling unit. Most individuals originated from Beja region (n=68), while Vila Real and Castelo Branco were the districts with more species analysed (n=4) (Fig. 3.1 A and B). The districts of Braga, Porto, Bragança and Setúbal were not represented in this survey, since there were no samples originating from these geographic locations.

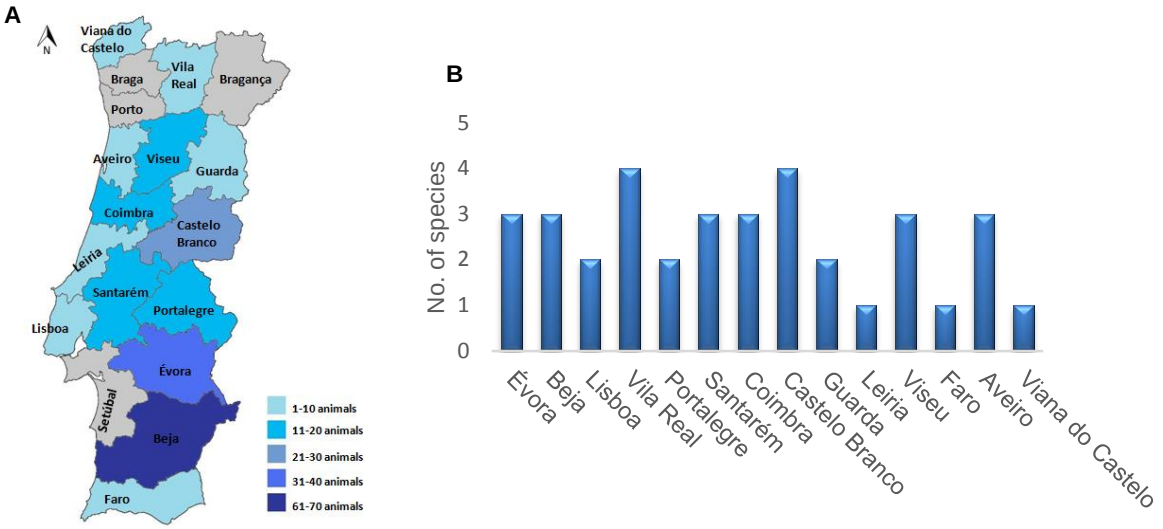

Fig. 3.1 - (A) Geographic distribution of the 225 animals under analysis across the districts of mainland Portugal. (B) Number of species analyzed per sampled district.

3.1.2 Diversity analyses of the samples under study

Despite the constraints related to our opportunistic sample, a diversity analyses was performed to assess if our sample would enable proper inferences of the epidemiological situation of the pathogenic agents surveyed. A positive relationship between the abundance of animals from a particular species and their distribution across districts was registered, meaning that the two species from which more individuals were sampled are indeed the species that are distributed in a higher number of geographic locations (Fig. 3.2). Considering the R^2 value, results suggest reinforcement of sampling, including more individuals from a larger number of districts.

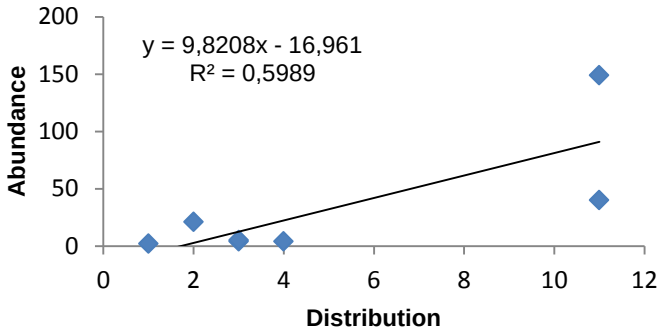

Fig. 3.2 - Relationship between abundance of animals and their distribution across sampled districts.

The diversity indices Shannon-Wiener, Simpson and Berger-Parker, allow the appraisal of the balance between the number of individuals and the number of species under analysis in a given district (community). These indices were chosen since they represent a coherent system for diversity estimates, and they are also the most common indices used in diversity analysis in ecological studies [127].

Since only one animal species was sampled from the districts of Leiria, Faro and Viana do Castelo, this analysis was not carry out for those regions (Table 3.1).

Table 3.1 - Diversity indices values by sampled district and NUTS (Statistical terrestrial units).

	Shannon-Wiener	Simpson	Berger-Parker
Districts			
Évora	1,081	0,655	2,5
Beja	0,153	0,058	1,030
Lisboa	0,349	0,198	1,125
Vila Real	1,168	0,640	2
Portalegre	0,655	0,463	1,571
Santarém	0,947	0,570	1,778
Coimbra	0,537	0,277	1,188
Castelo Branco	1,102	0,627	2,364
Guarda	0,562	0,375	1,333
Viseu	0,600	0,314	1,222
Aveiro	1,099	0,667	3
NUTS			
North	1,424	0,722	2,400
Center	1,176	0,577	1,633
Alentejo	0,783	0,427	1,368
Lisboa	0,349	0,198	1,125

Legend: North - Viana do Castelo, Braga, Porto, Vila Real and Bragança; Center – Aveiro, Viseu, Guarda, Castelo Branco, Leiria, Santarém and Coimbra; Alentejo – Portalegre, Évora, Beja and Setúbal.

Regarding the calculated diversity indices, the higher the value, the high is the diversity of the community in analysis. So, the districts of Vila Real, Castelo Branco, Évora and Aveiro yielded the highest values, which thus can be considered the most balanced communities; while in contrast, Beja and Lisboa were the most unbalanced. The same diversity indices were obtained for clustered geographical areas - NUTS (statistical terrestrial units), and the

North region, which includes Vila Real and Viana do Castelo, is the most balanced community.

Non-parametric estimators of species richness chao 1, which analyses species abundance data, and chao 2, that considers species incidence data, were also calculated, evidencing a completeness of the sampling method of 93% and 82%, respectively.

All parameters considered, we conclude that the panel of sampled species and number of specimens is adequate for the purposes of this study. However, and since chao 2 estimator exhibits inferior values, supplementary sampling efforts should however be carried out in the future in order to include more specimens from the less represented species and to reach more balanced communities across the mainland territory.

3.1.3 Detection of *MAP*-positive animals

An animal was classified as *MAP*-positive if the bacterium was isolated from at least one biological matrix in selective media and/or if *MAP* DNA was detected by *IS900* nested real time PCR. According to these criteria, 19 of the 225 animals tested were considered *MAP*-positive (8,4%; 95%CI: 5,47-12,8%).

MAP bacteria were not isolated in none of the selective media used. In contrast, *MAP* DNA was detected by nested real-time PCR in six out of seven species, in both feces and spleen samples (Fig. 3.3 A and B).

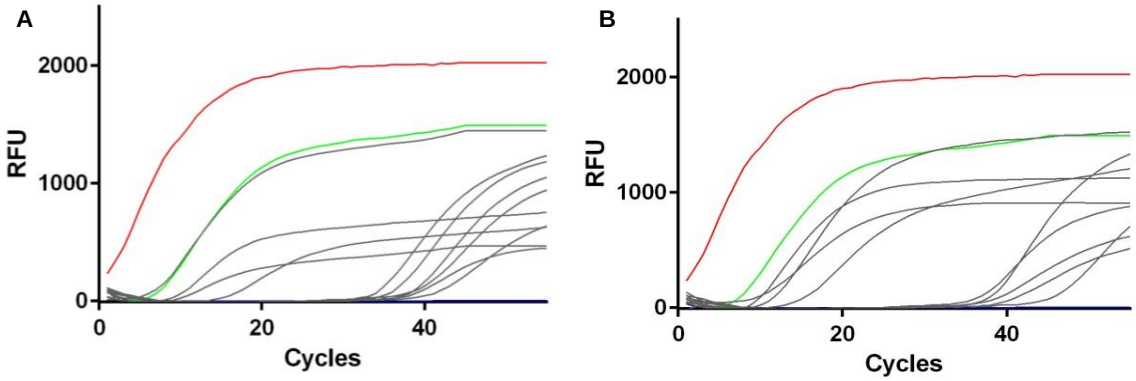

Fig. 3.3 - Real-time PCR amplification curves targeting *IS900* in fecal (A) and spleen (B) samples. Positive controls (DNA extracted from *MAP* ATCC19698 (red line) and from a previously positive fecal sample (green line)). Positive samples analyzed in this study are depicted in grey lines and the negative control (water instead of DNA) is represented by the blue line. RFU - Relative Fluorescence Units.

In fact, nine Egyptian mongoose (*Herpestes ichneumon*); four red fox (*Vulpes vulpes*), one stone marten (*Martes foina*), one common genet (*Genetta genetta*), one wild boar (*Sus scrofa*) and three red deer (*Cervus elaphus*) were positive by molecular methods. The four European badger (*Meles meles*) tested negative for *MAP* DNA (Table 3.2). The percentage of *MAP*-positive animals was 6,0% (95%CI: 3,2-11%) in Egyptian mongoose and 10% (95%CI: 4,0-23%) in red fox (Fig. 3.4 A).

Table 3.2 - Characteristics of *MAP* DNA positive animals.

Species	Gender	Age class	Year	Geographic location	DNA-positive matrix
Red fox (*Vulpes vulpes*)	F	Adult	2010	Vila Real	Feces
n=40	M	Adult	2010	Portalegre	Feces
	F	Adult	NK	Évora	Feces[a]
	F	Adult	2011	Aveiro	Spleen
Egyptian mongoose (*Herpestes*	M	Adult	2011	Lisboa	Feces[a]
ichneumon)	M	Adult	2011	Faro	Spleen
n=149	M	Adult	2011	Beja	Spleen
	F	Adult	2011	Beja	Spleen
	M	Adult	2011	Viseu	Spleen
	F	Adult	2011	Beja	Spleen
	F	Adult	2011	Beja	Spleen
	F	Adult	2011	Beja	Spleen
	M	Adult	2011	Faro	Spleen
Common genet (*Genetta genetta*) *n*=5	F	Juvenile	2009	Viseu	Feces
Stone marten (*Martes foina*) *n*=4	M	Adult	2005	Aveiro	Feces
Red deer (*Cervus elaphus*)	M	Juvenile	2015	Évora	Feces
n=21	M	Adult	2015	Castelo Branco	Feces
	F	Adult	2015	Castelo Branco	Feces
Wild boar (*Sus Scrofa*) *n*=2	M	Adult	2015	Viana do Castelo	Feces

Legend: a. Spleen sample from that animal was also tested, NK - unknown

Among all *MAP*-positive animals, 10,5% were juveniles and 89,5% were adults; 47,4% were females and 52,6% were males.

The Chi-square test (α=0,05) was used to compare the results across species, gender and age class, indicating no significant differences (p>0,05), meaning that the probability of testing positive for *MAP* is independent of species, gender and age.

As for geographic location *MAP*-positive animals were detected in 10 out of the 14 districts analyzed, the majority being located in the border with Spain (Fig. 3.4 B). The district from where more *MAP*-positive animals were detected was Beja (n=5; 7,35%). In contrast, none of the animals sampled from Santarém, Coimbra, Guarda and Leiria tested positive for *MAP*.

Analysis of the proportion of *MAP*-positive animals per NUTS, by the Chi-square test (α=0,05), indicates no significant statistical differences (*p*>0,05). However, when the proportion of *MAP*-positive animals is compared by district, using the Chi-square test (α=0,05), and considering only those regions where *MAP*-positive animals were detected, results indicate significant statistical differences (*p*<0,05). Moreover, Faro, Aveiro and Viana do Castelo are the districts that are contributing for the dependence between the variable "geographic location" and "*MAP*-positive test" (value of adjusted residuals=2,1; 3,0 and 2,0 respectively). This result is in agreement with the fact that those were the districts with the highest proportion of *MAP*-positive animals. However, it also needs to be considered the fact that the number of sampled animals was low, specifically five specimens from Faro, three from Aveiro and two from Viana do Castelo. So, a larger number of animals from these locations would have to be analyzed to verify if this association still remains.

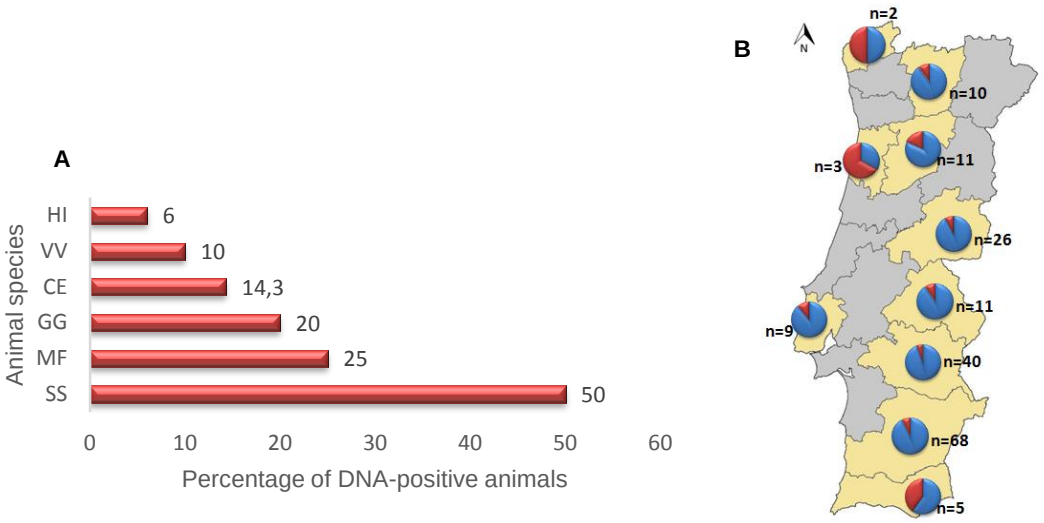

Fig. 3.4 - (A) Percentage of *DNA*-positive animals within each species (HI - *Herpestes ichneumon*; VV - *Vulpes vulpes*; MF - *Martes foina*; GG - *Genetta genetta*; CE - *Cervus elaphus*; and SS - *Sus scrofa*). (B) Geographical distribution of *MAP*-positive animals. The pie charts show the proportion of *MAP*-positive animals within each district; red=*MAP*-positive animals, blue= *MAP*-negative animals.

MAP DNA was detected in feces (n=10) and spleen (n=9), corresponding to 7,5% and 6%, respectively, of the biological matrices under analyses (Fig. 3.3A and B). Statistical analysis by the Chi-square test (α=0,05) across biological matrices types evidenced no significant difference (p>0,05).

The distribution of Cq values among fecal and spleen samples ranged from 7,74 to 41 and from 10,28 to 44,98; being 27,2 and 27,3 the mean values, respectively (Fig. 3.5A). The mean Cq values of the two matrix types under analysis was compared by the Mann-Whitney test (α=0,05) and no significant differences were obtained (p>0,05).

Lower Cq values, detected in both matrices, possibly reflect one of several aspects when compared to higher Cq value samples: higher *MAP* burden in the sample, better DNA extraction efficiency or better preservation of the sample.

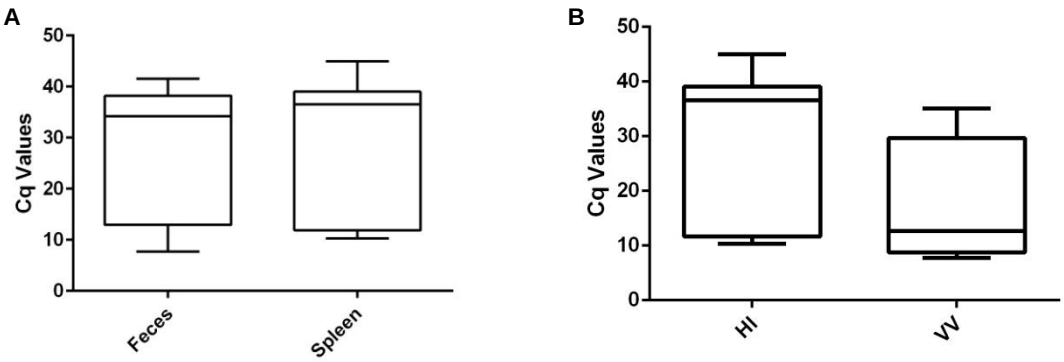

Fig. 3.5 - Distribution of the Cq values obtained by semi-nested real-time *IS900* PCR for feces and spleen samples (A); and for Egyptian mongoose and red foxes samples (B). HI - *Herpestes ichneumon* and VV - *Vulpes vulpes*.

Considering that red fox and Egyptian mongoose were the most represented species, the mean Cq values obtained for their biological samples were compared by the Mann-Whitney test (α=0,05) and no significant differences were obtained (Fig. 3.5B).

Two of the *MAP*-positive animals were screened both in feces and spleen, but only feces were positive. This may be the result of an early state of infection and low burden of the pathogen that could only be present in the lower gastrointestinal tract, or else *MAP* could be present but below the limit of detection of the PCR assay.

Detection of *MAP*-positive animals exclusively by molecular methods and absence of microbiological isolation in selective media might be explained by the fact that conventional culture methods require aggressive chemical decontamination that possibly impairs microbial growth of bacilli, which in addition with the required amount of bacilli that should accumulate in solid media to perceive bacterial growth, imply a very high limit of *MAP* detection [22,24,27,76]. Since PCR detection methods do not require any of the above mentioned steps, the loss of viability associated with decontamination is not an issue. Moreover, the samples used belong to animals killed on the road and in hunting actions, so by the time of necropsy the organs already show signs of autolysis, which then again complicates bacteriological isolation; at last, the conditions related to the management of the samples, namely repeated freeze–thawing cycles, may also have influenced these results.

There is presently no reliable data in Portugal about *MAP* shedding in feces, as this matrix was not tested in any of the previous surveys in wildlife or small ruminants, with the exception of a study with bovines from the North of the Portugal that isolated *MAP* from feces [128]. Therefore, our survey is the first report of *MAP* DNA in feces from wildlife. The detection of *MAP* DNA in feces of a subset of the sampled population might indicate shedding of the pathogen, allowing the cycle of infection to continue; in contrast, the detection in spleen might be an indication of systemic infection.

The detection of *MAP*-positive red deer (n=3) was, somehow, predictable, since this animal species is a ruminant, which is described as *MAP* natural host. The detection of *MAP* in deer is well documented, not only in *Cervus elaphus*, but also in other species (roe deer, fallow deer, white-tailed deer) [33–36]. Moreover, it was recently reported the isolation of *MAP* from kidney samples of red deer from the Center of Portugal [129], and two of the positive animals detected in our study were from the same geographic location of those animals, while the other one was from Alentejo. The detection of *MAP*-positive red deer suggests an environmental source and indirect transmission, possibly indicating the presence of *MAP*-contamined water/pasture in those regions of the country.

The identification of *MAP*-positive carnivores and wild boar also suggest contamination of the environment and/or ingestion of infected prey. Previous reports in Portugal described *MAP* isolation from wild boar lymph nodes and from several organs of carnivores (red foxes,

beech martens, Eurasian otters, Egyptian mongooses, and European badger), from the center region of Portugal [130,131]. However, to our knowledge, we report the detection of *MAP* in a common genet for the first time.

Finally, a recent survey performed in rabbits from the Northeast of the country detected positive animals, by PCR and ELISA, so this prey species could act as a source of *MAP* infection for carnivores [132]. And in fact, a *MAP*-positive red fox from a close geographic location was detected in the present study.

3.1.4 *MAP*-positive samples genotyping by MIRU-VNTR

Genotyping of *MAP*-positive samples was performed by MIRU-VNTR enabled by the detection of eight TR (Tandem Repeat) sequences: X3, 3, 7, 10, 25, 32, 47 and 292 [30]. VNTR analysis starts to be commonly used in genotyping studies since is simpler, requires less template DNA and can be applicable to non-cultured strains, when compared with the most widely used methods. The approach followed in this work has a high discriminatory power and an online database associated - MAC INMV database (http://mac-inmv.tours.inra.fr/), allowing the worldwide comparison of *MAP* profiles.

Due to the fact that bacterial growth on selective media was not observed, this analysis had to be adapted to the existing material and performed with total DNA extracted from specimen matrices. Optimization approaches were carried out, namely by changing the annealing temperature, the number of cycles of the PCR assay and the quantity of template DNA in the reaction, so that it would be possible to obtain a complete profile. However, only partial profiles were obtained, as evidenced in Table 3.3 and in Fig. 3.6 and Fig. 3.7.

Table 3.3 - MIRU-VNTR profile for the positive samples under analysis.

Sp	SC	GL	X3	3	7	10	25	32	47	292
VV	F7	VR		5	2	1	1			
	F20	PG	4	9	11+ >12	1			1	2
	F111	ÉV					1+7	3		
	S131	AV					1			
HI	F16	LS		0	9	1+3			0	
	S77	FR								
	S79	BJ								
	S88	BJ				0α	2+4+6	1		4
	S98	VS				2 β	1+2+6	0		3 + >12
	S102	BJ				2 β	1+2+6	0		1
	S104	BJ		0		2 β	1+2	1		3
	S105	BJ		0		2 β	1+2	1		2
	S112	FR	3				1+2			0
GG	F101	VS	3			1				
MF	F102	AV								
CE	FV9	ÉV				0	2			0
	FV16	CB	3+4+8+ >12			0	3+5+11	4+12	> 12	0+ 7
	FV18	CB				0		4		7 + >12
SS	FJ2	VC	1+6							

Legend: Sp - animal species, SC - sample code, GL - geographic location, F- Fecal samples and S - spleen samples; blank space refers to absence of amplification; α -existence of one band inferior to allele 0; 2β - existence of the same pattern of two bands (both inferior to the allele 0); VR-Vila Real, PG-Portalegre, EV-Évora, AV-Aveiro, LS-Lisboa, FR-Faro, BJ-Beja, VS-Viseu, CB-Castelo Branco and VC-Viana do Castelo.

For the sample F20 was obtained the most complete profile, with the characterization of six out of eight TR, while samples S77, S79 and F102 were not typeable by this method as we were not able to detect any TR. Difficulties in obtaining a complete profile have been previously reported by others, even when using fresh DNA directly extracted from bacterial culture [133–135].

Related to the number of repetitions, and to avoid confusion in the evaluation of the results, repetitions higher than 12 were noted as >12. For some of the TR, namely 3, 7, 10, 25 and 47, some repetition numbers were observed for the first time.

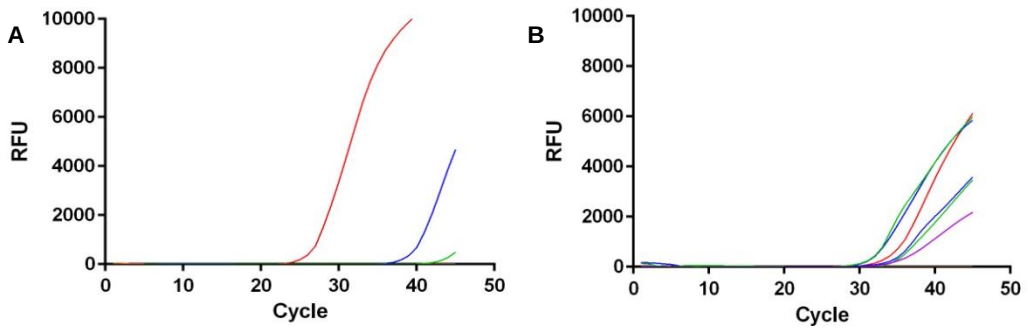

Fig. 3.6 - Real time PCR amplification curves of TR 47 (A) and TR 10 (B) *loci*. Positive control (DNA extracted from *MAP* ATCC 19698) is represented by red line, sample F16 by green line, sample F20 by blue line, sample F7 (only TR 10) by purple line and negative control by orange line.

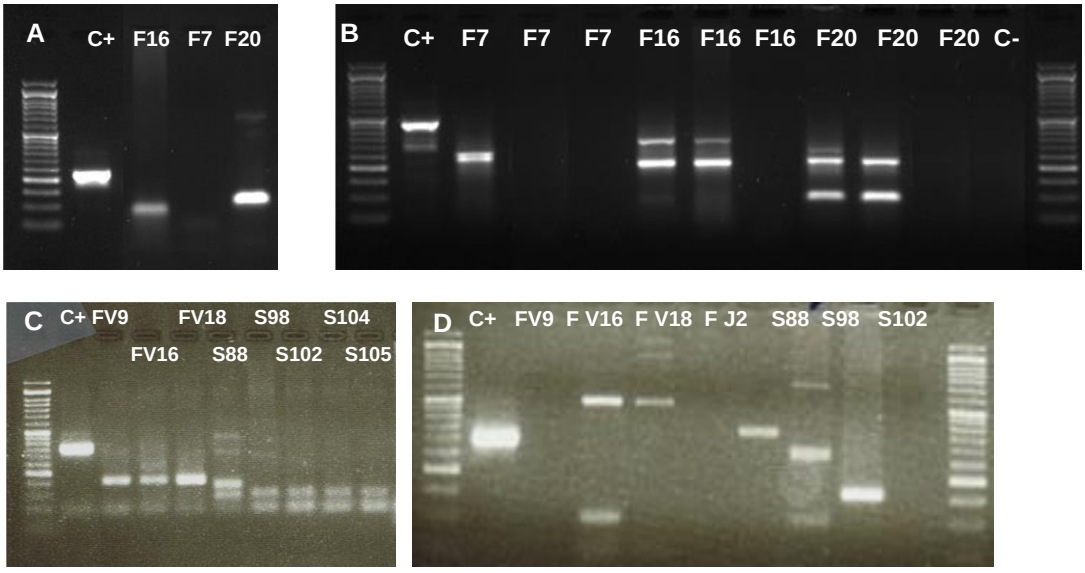

Fig. 3.7 - Agarose gel (2%) electrophoresis of the amplification products of the PCR targeting TR 47 (A), TR 10 (B, C) and TR 292 (D) *loci*. (B) Results of *MAP*-positive samples in different dilutions. Results for positive culture control (*MAP* ATCC 19698, C+) and *MAP*-positive samples are depicted in the image (molecular size marker – Nzytech ladder VI).

All TR showed variability and in tandem repeats X3, 7, 10, 25 and 292, multiple allele was observed, indicated by the consistent presence of two, three or four clear bands (Fig. 3.7). Multiple alleles for all of the TR are already described in the literature, but this analysis is the first evidence of the occurrence of 4 alleles in TR X3. The presence of more than one allele

might be due to co-infection of multiple strains, a possibility previously described for MAC members including MAP; and/or due to mutation at these particular *loci* [26,32,133,136–139]. In TR 10, two bands located at an inferior position of the location of allele 0 were observed (Fig. 3.7). This situation might be due to stutter peaks and be related to strand slippage of the polymerase during PCR amplification.

Samples S98, S102, S104 and S105 have the same profile for TR 10, and similar one for TRs 25 and 32 (Fig. 3.7). All of the samples belong to animals of the same species (Egyptian mongoose), being three of them from Beja district.

Not all of the published data about VNTR analysis uses the same TR, which complicates the analysis and the comparison of the results. The genotyping of 12 bovine isolates from the North of Portugal is the only Portuguese information available. For all the 12 isolates it was obtained the same profile – INMV2 [128]. Despite the fact that only a partial profile was obtained for S112 and F101, these are the only samples that show concordance with bovine isolates, namely in the number of repetitions of TRX3.

3.1.5 Strain differentiation of *MAP*-positive samples

The attempt to differentiate *MAP*-positive samples in S and C type strains was performed by PCR, but none of the positive samples showed amplification of the target regions.

As it happened with the molecular characterization of samples by MIRU-VNTR, total DNA extracted from specimen matrices is a less easy template for PCR when compared to DNA from culture, since it is rich in eukaryotic DNA and possibly includes many short genomic fragments of the bacteria due to autolysis, impairing amplification of larger fragments as compared to the short fragments underlying the real-time PCR technique.

3.2 Detection of *Mycobacterium bovis* in liver and feces of wild species

3.2.1 Demographic characteristics of the samples under study

Animals were sexed and aged during necropsy. The 121 animals used in this screening survey belong to five different species, 50,5% being females and 49,5% males. Each specimen was assigned to one of four age classes, previously mentioned, and the majority of the individuals surveyed were adults (n=80; 66%) (Supplementary Fig. 3 A and B).

Concerning geographical distribution, the districts of mainland Portugal were considered the sampling unit. Most individuals originated from Beja region (n=44), while Évora, Castelo Branco e Coimbra were the districts with more species analysed (n=2) (Fig. 3.8 A and B). Several districts, namely Setúbal, Portalegre, Leiria, Guarda, Aveiro, Braga, Porto, Bragança and Vila Real were not represented in this survey, since there were no samples originating from these geographic locations.

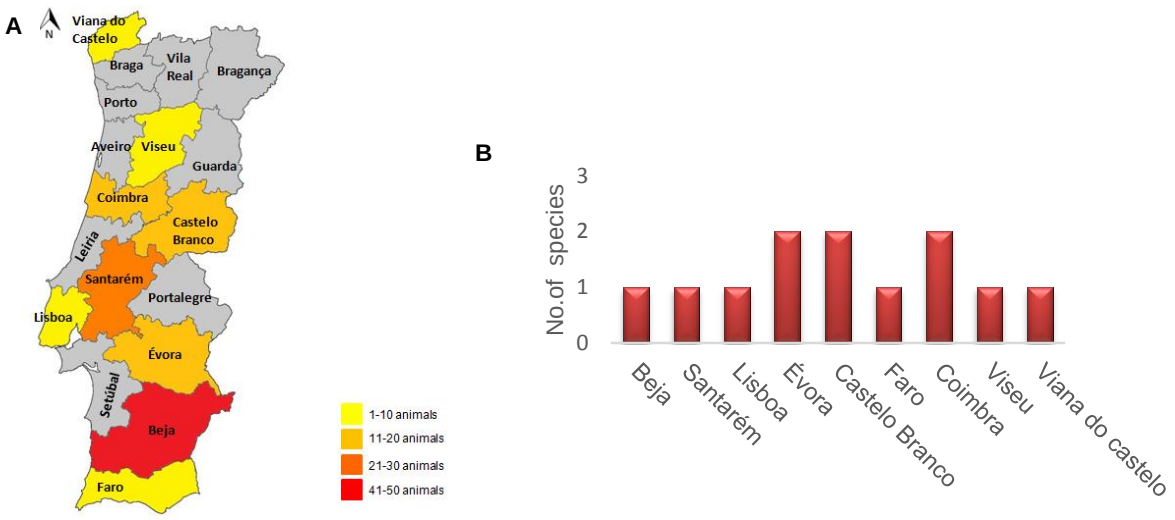

Fig. 3.8 - (A) Geographic distribution of the 121 animals under analysis across the districts of mainland Portugal. (B) Number of species analyzed per sampled district.

As for the diversity analysis, performed as indicated previously, it was registered a positive relationship between the abundance of animals of a particular species and its distribution across districts, and in the case of this survey the Egyptian mongoose is the most representative species of the survey (77%) and was distributed in six out of nine of the districts under analysis (Fig. 3.9).

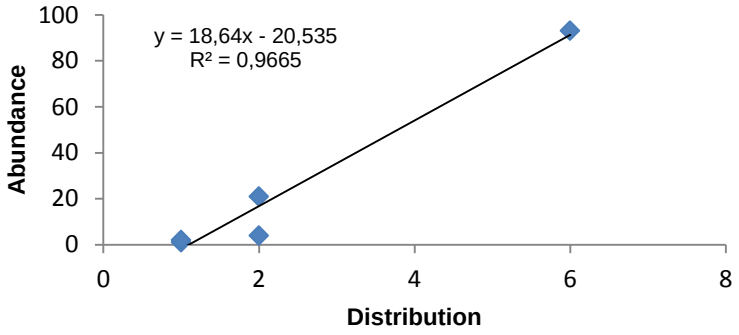

Fig. 3.9 - Relationship between abundance of animals and their distribution across sampled districts.

Comparing these results with the ones obtained for *MAP* survey, in this case is clear that an effort in sampling more individuals and in a bigger number of districts is necessary.

The diversity indices Shannon-Wiener, Simpson and Berger-Parker were obtained for Évora, Castelo Branco and Coimbra, since only these districts had more than one sampled species (Table 3. 4). Due to the fact that the majority of the sampled districts only had animals from one species, this diversity analyses is not very informative. However, combining estimates from all these diversity indices suggest that sampling balance in Évora and Castelo Branco are very similar. When the analysis is based in a wider geographic area, the Center region, which includes Coimbra and Castelo Branco, is the most balanced community.

Table 3. 4 - Diversity index values by sampled districts and NUTS.

District	Shannon-Wiener	Simpson	Berger-Parker
Évora	0,540	0,355	1,300
Coimbra	0,271	0,142	1,083
Castelo Branco	0,227	0,391	1,364
NUTS			
Center	3,983	0,518	1,750
Alentejo	0,540	0,355	1,300

Legend: Center - Aveiro, Viseu, Guarda, Castelo Branco, Leiria, Santarém and Coimbra; Alentejo - Portalegre, Évora, Beja and Setúbal.

Non-parametric estimators of species richness, chao 1 and chao 2, were also calculated, evidencing a completeness of the sampling method of 91% and 45% respectively.

All parameters considered, we conclude that our sampling method has an imperfection regarding to communities with more than one species sampled, so supplementary sampling efforts should be carried out in the future, in order to include more specimens from the less represented species in order to reach a more balanced communites.

3.2.2 Detection of *M. bovis*-positive animals

An animal was classified as *M. bovis*-positive if the bacterium was isolated from the biological matrix in selective media and/or if *M. bovis* DNA was detected by *IS6110* semi-nested real-time PCR and the species confirmed by *gyrB*-REA. According to these criteria, only one animal was considered *M. bovis*-positive.

This *M. bovis* was isolated from a red deer fecal sample in both selective media used. Yellow colonies were visible in LJ pyruvate and ST after 6 weeks of incubation. DNA extracted from these colonies was positive for *IS6110* in the semi-nested real-time PCR and *gyrB*-REA methodology confirmed it to be *M. bovis* (Fig. 3.10 A and B).

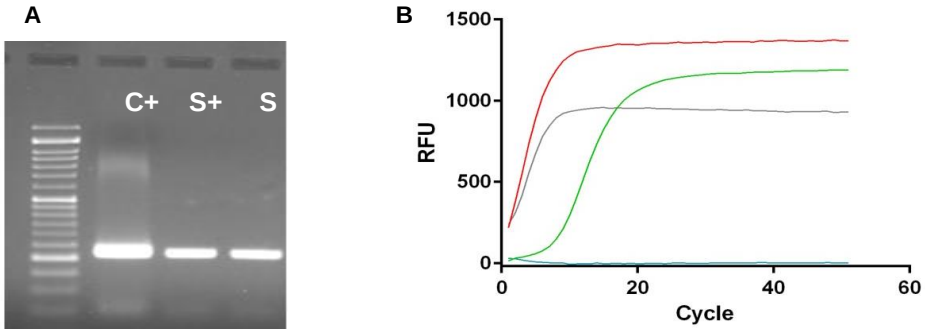

Fig. 3.10 - (A) Agarose gel (2%) electrophoresis of *IS6110* PCR for positive controls (*M. tuberculosis* H37RV (C+) and positive sample (S+)) and DNA from fecal samples under analysis (S) (molecular size marker – Nzytech ladder VI). (B) *IS6110* targeted real-time PCR amplification curve obtained from DNA of the red deer positive fecal sample culture. Positive controls (DNA extracted from *M. tuberculosis* H37RV culture (red line) and from positive spleen sample (green line)). Positive sample is depicted in grey and negative control (water instead of DNA) is represented by the blue line.

The DNA from the cultured *M. bovis* was genotyped by spoligotyping and confirmed as SB1195. This spoligotype pattern was previously detected in red deer from the same

geographic region as this positive animal (Castelo Branco) [114]. During the necropsy of this animal, tuberculosis-like lesions in lymph nodes were macroscopically visible.

M. bovis was not isolated from any of the other samples under analysis and *IS6110* DNA was not detected by semi-nested real time PCR. Despite the fact that none of the wild carnivores screened in this survey was positive for *M. bovis,* this microorganism has already been isolated in terrestrial carnivores from Portugal (Cunha et al., unpublished results), and was also very recently reported in red foxes, Egyptian mongoose, beech marten, Eurasian otter and common genet, from the center of Portugal [140].

3.3 Spoligotyping

One hundred and seventeen *M. bovis* and 10 *M. caprae* isolates from wild boar, red deer, sheep, cattle and pig were genotyped by spoligotyping, in order to improve knowledge about the molecular characterization of the *M. bovis*/*caprae* strains circulating in Portugal. The technique showed a good discriminatory power (D=0,91), revealing 27 different patterns, 26 for *M. bovis* and 1 for *M. caprae* (Table 3.5). Four new patterns were identified for the first time in the world (SB2354-SB2357).

Table 3.5 - Spoligotyping patterns, hosts and geographic location of *M. bovis* and *M. caprae* isolates.

Spoligotypes[a]		No. isolates					Geographic location
		W	R	S	C	P	
SB1174		14	13				BI, AL
SB1264		8	8				BI, AL
SB0122		6	9				BI, AL
SB0157		1		5	4		TM, EDM, BI, AL, SP
SB0121		8			1		BI, AL, ER
SB0265		3	5		1		BI, AL
SB1266		3	4				BI
SB1195		2	2		1		BI, AL
SB0295		1	1			1	BI, IP
SB1483		2	2				BI, AL
SB1060		2					BI
SB0119			1				BI
SB1190			1		1		AL, ER
SB0856		2					BI
SB0124					2		AL, TM
SB0120		2					BI
SB1090					1		AL
SB1269		1					BI
SB1608						1	IP
SB0948			1				BI
SB1191		1					BI
SB1265		1					BI
SB1375			1				BI
SB2354			1				BI
SB2355			1				AL
SB2356		1					BI
SB2357			1				AL

Legend: a. Allocated by database http://www.mbovis.org. W-wild boar, R-red deer, C-cattle, S-sheep, P pig, AL-Alentejo, BI-Beira Interior, EDM – Entre Douro e Minho, ER- Extremadura e Ribatejo, IP - Iberian Peninsula and TM – Trás-os-Montes

Spoligotypes SB1174 (23%), SB1264 (14%) and SB0122 (13%) were the most common among *M. bovis* isolates. Gathering the data obtained in this study together with the publish

genotyping surveys in Portugal, SB1174 is the most common pattern for wild boar and SB0122 for red deer [109,115].

All isolates from *M. caprae*, obtained from livestock and wild boar, are SB0157, which is in agreement with previous indications for other *M. caprae* isolates [112,115].

3.3.1 Spoligotype distribution by geographical location

From the 127 isolates examined in this study, 125 were obtained from infected animals from mainland Portugal and 2 from Iberian Peninsula. Beira Interior with 78 isolates was the most represented region and also the geographic location where the majority of the profiles (n=21) were isolated.

The patterns SB1269, SB1265, SB1375, SB0948 and SB1060 were obtained for the first time in Portugal, all in Beira Interior, however, with the exception of SB1060, they have already been identified in Spain [141].

Twelve patterns were geographically limited to Beira Interior; and three to Alentejo, being SB0157 the spoligotype with wider geographical distribution.

3.3.2 Spoligotype distribution by species

Eight out of the 26 *M. bovis* spoligotypes detected were common to wild boar and red deer, which included the most frequent patterns. In contrast, the isolation of seven patterns was limited to wild boar, six to red deer, two to cattle and one to the Iberian swine.

Spoligotypes SB1174, SB0265, SB0121, SB1195, SB0119 and SB0120 were identified in wild boar and/or red deer from Beira Interior and Alentejo, which is in agreement with previously published results [111,114,115].

The patterns SB0856 and SB1483 were detected for the first time in wild boar from Beira Interior, since the data available only refers to wildlife and livestock from other geographic locations of mainland Portugal, being Alentejo one of those [114,115]. So, the animals with this spoligotype might have been infected when in contact with others in Alentejo and then moved up north to Beira Interior. A similar situation might have happened with the red deer with pattern SB1264, which was for the first time reported in Alentejo, and the information available so far concerns wildlife and livestock from Beira Interior.

The pattern SB1190 was detected in a red deer from Alentejo, and it has been previously reported from wild boar and livestock from the same geographic region, which might be an evidence of the existence of a common source of infection in that region. The same is valid for patterns SB0122 and SB0295 that were detected in wild boar and red deer, respectively, from Beira Interior, and the available information only concerns other wildlife and livestock in that region, and in the regions surrounded namely Alentejo [111,114,115].

For pattern SB1191, the epidemiological data available reports its detection in domestic animals, so this study is the first evidence of the presence of this pattern in wildlife from the same region (Beira Interior), again reinforcing the idea of interspecies transmission.

SB1195 was detected for the first time in livestock from Alentejo and the data available only reports its detection in wildlife from Beira Interior. Livestock in Alentejo might have been the source of infection, for the animals in Beira Interior; or the opposite situation might have happened.

Finally, two patterns were obtained for the Iberian swine: SB0295 that was already detected in wildlife and livestock in Portugal and Spain, while SB1608 is reported for the first time in Iberian Peninsula [112,115,142].

4. Final Discussion and Perspectives

Paratuberculosis and animal TB have a huge impact in animal health, being associated with important economic losses and presenting a zoonotic potential. *M. bovis* can cause TB in man, while the causal relation between *MAP* and Crohn's disease is not yet clear. Understanding the epidemiology of these two diseases is thus crucial in order to control their occurrence in domestic animals and spill-over to wildlife, in which the disease may be established.

The main objective of this work was to improve the knowledge of the host range of both *MAP* and *M. bovis* in the Portuguese mainland territory, and to accomplish that goal, 225 biological samples were screened for *MAP* presence and 121 for *M. bovis*.

In *MAP* survey, 19 (8,4%; 95%CI: 5,47-12,8%) of the tested animals were considered to be positive, being this study, to our knowledge, the first evidence of *MAP* detection in a common genet.

Statistical analysis was performed in order to identify possible risk factors, and geographic location was the only variable that is statistically related to the detection of *MAP*-positive animals in this work, being Viana do Castelo, Faro and Aveiro, the districts that influenced that association.

Considering the data obtained and the diversity analysis performed, sampling efforts should be reinforced in order to include animals from the less represented species and from the districts that were not (sufficiently) sampled (especially in the case of *M. bovis*). Moreover, the districts of Viana do Castelo, Faro and Aveiro should be analyzed carefully to see if the statistical relationship established in this survey, is consistent when a larger number of animals is considered.

This survey is also the first report on the detection of *MAP* DNA in feces of wildlife species. This result suggests the excretion of the pathogen, which might allow the continuation of the infectious cycle. Since the pathogen can survive for a long period of time in water/soil, environmental contamination is a problem not only for the animals living in that geographical location, but also for others that may travel large distances in search for food and become indirectly exposed.

Genotyping by MIRU-VNTR of *MAP*-positive samples, revealed the presence of multiple alleles for TR X3, 7, 10, 25 and 292, which might be explained by co-infection with different *MAP* strains and/or mutation in that particular *loci*. The information provided by genotyping techniques is very important, especially, when trying to study the transmission routes and persistence of a given genotype on a given host or geographic location. In the case of *MAP* characterization, there is still little information available, and VNTR analysis does not always include the same TR. So, in this concrete subject, it would be helpful to establish an international consensus and to refine protocols for MIRU-VNTR typing, especially when genotyping is performed directly with the DNA extracted from a tissue/fecal sample, thus enabling uniformity of procedures and profile interpretation that would favor the comparison of the different outbreaks occurring worldwide.

For *M. bovis* survey, only the feces from one red deer, hunted in the district of Castelo Branco, were positive. The positive red deer was hunted in a geographic location included in the bTB risk area for big game animals defined by the Portuguese authorities and, in the necropsy, tuberculosis-like lesions were identified, which constitute a clinical sign of infection.

The genetic diversity of 117 *M. bovis* and 10 *M. caprae* strains from domestic and wild animals was investigated by spoligotyping. The majority of the isolates were originated from free-ranging animals hunted in two of Portugal's most important hunting areas that are also included in the risk area of bTB: Beira Interior and Alentejo.

The diversity of sub-types found (n=27) confirms that spoligotyping is a valuable tool for epidemiological purposes (D=0,91). SB1174 was the most common pattern, accounting for 23% of *M. bovis* isolates, and SB0157 pattern obtained for *M. caprae* isolates. Some of the patterns obtained were common to wild boar and red deer co-existing in the same geographic area, which suggest inter-species transmission. Further characterization of these isolates by MIRU-VNTR would be helpful in order to gain more insights on *M. bovis* transmission, and also for *M. caprae,* since spoligotyping did not differentiate the strains within this ecotype and only a single pattern was obtained.

Wild animals are susceptible to infection with many of the same pathogenic agents that affect domestic animals, and the epidemiological data gathered so far show that transmission between livestock and wildlife can occur in both directions. Considering these evidences, in order to break the infection cycle of *MAP* and *M. bovis*, and apart from biosecurity control

measures in livestock, there are also some actions that might be used to reduce the contact between animals, such as imposing physical barriers between cattle and wildlife and separate feed and water for different livestock species; proper management of animal left-overs and by-products; lower density of animals; limiting the artificial feeding to wildlife species; and compulsory and systematic sanitary inspection in big game species [109].

In the specific case of the paratuberculosis scenario in Portugal, since there are evidences of active disease in domestic animals, and considering that *MAP* has already been isolated from several wildlife species, it would be important to implement a control and eradication program of the disease. Presently, there is not enough information to conclude if some of the *MAP*-positive wildlife species could act as a maintenance hosts, so more research in this field would, also, be very important.

Relatively to the bTB scenario in Portugal, the epidemiological situation is different, since more knowledge has been accumulated and reported, and an eradication program is implemented in cattle, while a risk area has been defined for wild boar and red deer. Since *M. caprae* is the main causative agent of caprine TB, and also poses a health risk for other domestic animals and wildlife, increasing the information available about TB in goats would be an important step, so it could be considered an extension of the bTb eradication program to include goats, at least in the cases where small ruminants co-exist with bovines in the same herd.

Recognition that red deer and wild boar act as maintenance hosts for *M. bovis* in Iberian Peninsula, and data accumulated from spoligotyping and MIRU-VNTR techniques, evidencing common patterns between livestock and wildlife from the same geographic location, which supports interspecies transmission, suggest that the efficacy of the eradication program should be reevaluated, since wild species may reintroduce the pathogen in herds already free of tuberculosis and jeopardize the efforts completed so far.

The establishment of a common cooperation protocol between Portugal and Spain for the eradication of bovine tuberculosis could be considered, not only because the same big game species act as maintenance hosts in both countries, but also because the defined TB risk area for big game animals in Portugal is adjacent with the Spanish border. Finally, vaccination of wildlife species in hotspot areas in the near future could also be a strategy envisaged by national authorities, as the vaccination experience in wild boars from neighboring Spain is achieving promising results [143].

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6. Supplementary Tables

Supplementary Table 1 - Characteristics of the animal population under study, namely species, gender, cause and year of death, age and geographical location.

Sp.	Gender	Cause of death	Age	Samples analysed	Year of death	Geogra-phic location	Sample Code
HI	M	Hunting	Juvenile	F and S	2010	EV	HI62
				S	2011	BJ	HI141, HI 199, HI 178
						ST	HI 238
				L	2012	BJ	HI512
						FA	HI525
						ST	HI489
					2013	BJ	HI629, HI628
						CO	HI603
				F and L	2013	CO	HI602
			Sub-adult	L	2012	BJ	HI470, HI472
					2013	ST	HI597
						BJ	HI642
			Adult	F	2010	BJ	HI64
					2013	BJ	HI556, HI562, HI573, HI563, HI560, HI550, HI580, HI574,HI566
				S	2010	CB	HI142, HI145, HI173
						BJ	HI169
						ST	HI143
					2011	BJ	HI240, HI223, HI149, HI16 HI172, HI175,HI176, HI202
						PG	HI 225, HI 204, HI 222
						VS	HI 219, HI144, HI 220
						FA	HI 243
						CB	HI 246
						CO	HI 247
				L	2012	ST	HI531, HI496 HI532, HI537 HI539, HI535 HI534
						BJ	HI477, HI516,HI509, HI500, HI510 HI503, HI518,HI514, HI502, HI513
						CB	HI523, HI522
					2013	ST	HI598, HI596 HI590, HI589
						BJ	HI637, HI616 HI634, HI633
						CO	HI544, HI546, HI540
				Fand S	2010	BJ	HI66
						CB	HI96
					2011	CO	HI139
						FA	HI133
						ST	HI135
						FA	HI131
						ST	HI136
				F and L	2013	CO	HI601
		RK	Sub-adult	L	2013	CB	HI611
			Adult	F	2011	EV	HI92
				S		FA	HI174
				L	2012	CB	HI520
					2013	CO	HI612
		NK	Juvenile	F	2009	CB	HI18
				S	2010	CO	HI50
				F and S	2010	EV	HI67
						PG	HI75
						BJ	HI61
			Adult	F	2008	CO	HI29
						PG	HI31
					2009	GD	HI16

						CB	HI20
					2010	EV	HI 70, HI60 HI24, HI22
						ST	HI57
				S	2010	VS	HI49
						BJ	HI52
					2011	LS	HI73
						ST	HI53, HI55
						BJ	HI56
				F and S	2010	PG	HI76
						BJ	HI59
						EV	HI58, HI71
					2011	LS	HI86, HI80 HI72
						LE	HI74
HI	F	Hunting	Cub	L	2013	BJ	HI623, HI625, HI624
			Juvenile	F and S	2011	BJ	HI93
				S	2011	BJ	HI 181
				L	2012	ST	HI488
					2013	BJ	HI613, HI620, HI614
			Sub-adult	L	2012	BJ	HI506, HI473, HI529
					2013	ST	HI587
						BJ	HI641
			Adult	F and S	2010	ÉV	HI63
						BJ	HI65
						CB	HI137, HI134
					2011	CO	HI94, HI138, HI132
						BJ	HI97
						ST	HI95
						VS	HI130
				F and L	2012	VS	HI497
					2013	VS	HI610
				F	2013	CO	HI605
						BJ	HI577, HI554, HI576, HI572, HI565, HI570, HI569
				S	2011	ST	HI 245
						FA	HI 237
						CO	HI146, HI148
						BJ	HI140, HI147,HI200, HI166,HI167, HI168,HI170, HI171, HI177,HI179, HI180, HI182,HI201, HI203 HI205, HI206,HI207, HI208,HI209, HI218 HI224, HI226,HI227, HI239,HI241, HI242,HI244
				L	2012	BJ	HI475, HI517,HI504,HI501,HI505, HI507,HI511,HI515,HI508
						ST	HI487, HI491,HI486, HI536,HI533, HI530, HI538
						FA	HI526, HI528, HI527, HI524
					2013	BJ	HI635, HI617, HI636, HI618,HI631
						ST	HI608, HI593, HI609
						CO	HI542, HI541, HI545, HI600,HI543
		RK	Adult	F and S	2011	EV	HI90, HI89
				L	2012	VS	HI499
		NK	Juvenile	F	2009	GD	HI17
						LS	HI27
					2010	CO	HI41
			Adult	F	2008	CO	HI15
					2009	CB	HI21
					2010	EV	HI23
					2011	LS	HI69
				S	2010	BJ	HI51
					2011	VS	HI54
						LS	HI81
				F and S	2010	PG	HI77

					2011	LS	HI68
						VS	HI84
	NK	Hunting	NK	F	2014	CO	HI662, HI664, HI663
		RK	Adult	S	2012	CB	Hi-TB006
VV	M	Hunting	Cub	F and S	NK	EV	VV15
			Juvenile	F, S, L	NK	EV	VV09
			Sub-adult	F, S, L	NK	EV	VV11
			Adult	F	2010	PG	C3-II, C3-IV, C3-III
					2011	ST	Vv-TB044
				S	2011	ST	Vv-TB044
				F and S	2011	ST	Vv-TB046
					NK	EV	VV16
		RK	Adult	S	2011	GD	Vv-TB055
		NK	Adult	S	NK	ST	Vv-TB042
				F	2010	VS	C3-IX
	F	Hunting	Cub	F and S	NK	EV	VV18, VV21
			Sub-adult	F, S, L	NK	EV	VV10
			Adult	F	2010	PG, CO, BJ	C3-V, C3-VI, C3-XXIV
				S	2006	CO	VV 01
					2011	EV	VV 03
							VV 04
							12-35539
				F and S	2011	ST	Vv-TB045
					NK	EV	VV17, VV20
				F, S, L	NK	LS	VV08
		RK	Adult	S	2011	GD, AV, EV	Vv-TB041, Vv-TB043, Vv-TB054
		NK	Adult	F	2010	VR	C3-XIII
	NK	RK	Juvenile	S	NK	VR	Vv-TB049
			Adult	F and S	2011	VR	Vv-TB050
				S	2011	CB	Vv-TB051, Vv-TB052
					NK	VR	Vv-TB047, Vv-TB048
		NK	NK	F and S	NK	EV	VV22, VV23, VV24
MM	M	RK	Adult	F and S	NK	CB	MM05
	F	RK	Cub	S	2011	ST	Mm-TB003
	NK	Hunting	Adult	F and S	2011	VR	Mm-TB005
		RK	Adult	S	2011	ST	Mm-TB004
MF	M	RK	Adult	F	2010	BJ	Mf11-TB003
				F and S	2005	AV	MF 01
				F, S, L	NK	CO	MF02
	NK	RK	Adult	S	2011	VR	Mf-TB011
GG	F	RK	Adult	F and S	NK	AV	GG01
		NK	Juvenile	F	2009	VS	C3-Gg-TB001
	NK	NK	NK	S	NK	VR	Gg-TB004,Gg-TB003,Gg-TB005
CE	F	Hunting	Juvenile	F	2015	EV	RD2, RD4
						CB	RD17
			Sub-adult	F	2015	EV	RD1
						CB	RD12, RD20
			Adult	F	2015	EV	RD3, RD5
						CB	RD18, RD21
	M	Hunting	Juvenile	F	2015	EV	RD7, RD8, RD9, RD10
						CB	RD11
			Sub-adult	F	2015	CB	RD13, RD19
			Adult	F	2015	CB	RD14,RD15, RD16
						EV	RD6
SS	F	Hunting	Sub-adult	F	2015	VC	WB1
	M	Hunting	Adult	F	2015	VC	WB2

Legend: Species: HI - *Herpestes ichneumon*; VV - *Vulpes vulpes*; MM - *Meles meles*; MF - *Martes foina*; GG - *Genetta genetta*; CE - *Cervus elaphus* and SS - *Sus scrofa*;
Gender: F - female, M – male and NK – unknown

Cause of death: NK – unknown and RK – road killed
Samples analyzed: F - feces, S – spleen, and L - liver
Geographic location: EV - Évora, BJ - Beja, LS- Lisboa, VC - Viana do Castelo, AV - Aveiro, VR - Vila Real, PG - Portalegre, CO -Coimbra, CB - Castelo Branco, ST - Santarém, FA - Faro, VS – Viseu, GD - Guarda and LE - Leiria

Supplementary Table 2 - Primers and probes used in the nested real-time PCR for detection of *IS900* and for the MIRU-VNTR technique.

		Primer/Probe sequence (5'→3')	Fragment size (pb)	Target region	PCR conditions
MAP Detection	*IS900*-EXT-FW	a	224	*IS900*	1. 95 °C-3 min, 2. 35 cycles at 95 °C for 45 sec, 55 °C for 30 sec, and 72 °C for 90 sec 3. 72 °C-10 min
	IS900-EXT-RV	a			
	IS900-INT-FW	CCGGTAAGGCCGACCATT	66	*IS900*	1. 95°C-2 min 2. 45 cycles at 95ºC for 5 sec and 60ºC for 10 sec
	IS900-INT-RV	ACCCGCTGCGAGAGCA			
	IS900 Tqn	TET-CATGGTTATTAACGACGACGCG CAGC-BHQ1			
MAP Typing	32	CCACAGGGTTTTTGGTGAAG		TR 32 *MAP*	Conventional PCR: 1.95°C-10min, 2. 38 or 45 cycles at 98 °C for 10 sec, proper annealing temperature for each TR for 30 sec, and 72 °C for 90 sec 3. 72ºC -10 min Real time PCR: 1.98°C-5min, 2. 38 or 45 cycles at 98 °C for 10 sec and at proper annealing temperature for each TR for 30 sec
		GGAAATCCAACAGCAAGGAC			
	292	CTTGAGCAGCTCGTAAAGCGT		TR 292 *MAP*	
		GCTGTATGAGGAAGTCTATTCATGG			
	X3	AACGAGAGGAAGAACTAAGCCG		TR X3 *MAP*	
		TTACGGAGCAGGAAGGCCAGCGGG			
	25	GTCAAGGGATCGGCGAGG		TR 25 *MAP*	
		TGGACTTGAGCACGGTCAT			
	3	CATATCTGGCATGGCTCCAG		TR 3 *MAP*	
		ATCGTGTTGACCCCAAAGAAAT			
	7	GACAACGAAACCTACCTCGTC		TR 7 *MAP*	
		GTGAGCTGGCGGCCTAAC			
	10	GACGAGCAGCTGTCCGAG		TR 10 *MAP*	
		GAGAGCGTGGCCATCGAG			
	47	CGTTGCGATTTCTGCGTAGC		TR 47 *MAP*	
		GGTGATGGTCGTGGTCATCC			
	S/C strain F	a	528	a	1. 98 °C-3 min, 2. 35 cycles at 98 °C for 10 sec, 63 °C for 30 sec, and 72 °C for 30 sec 3. 72 °C-10 min
	S/C strain R	a			

Legend: a - confidential information (Leão et al., unpublished results), b - PCR conditions are described in section 2.2.4, TET – Tetrachloro-Fluorescein; BHQ1 – Black Hole Quencher 1

Supplementary Table 3 - PCR conditions tested in the MIRU-VNTR analyses.

	Conventional PCR		Real time PCR	
MIRU-VNTR *loci*	**Number of cycles**	**Annealing temperature tested**	**Number of cycles**	**Annealing temperature tested**
X3/47/292	38 and 45	64ºC, 62ºC	38 and 45	64ºC
3	45	58ºC, 56ºC	45	58ºC
7	38	68ºC, 64ºC	38	64ºC
25/10	45	58ºC, 56ºC, 60ºC	45	58ºC
32	38 and 45	54ºC	45	54ºC

Supplementary Table 4 - Primers and probes used in the semi-nested real-time PCR for detection of *IS6110* and for *M. bovis* identification and characterization.

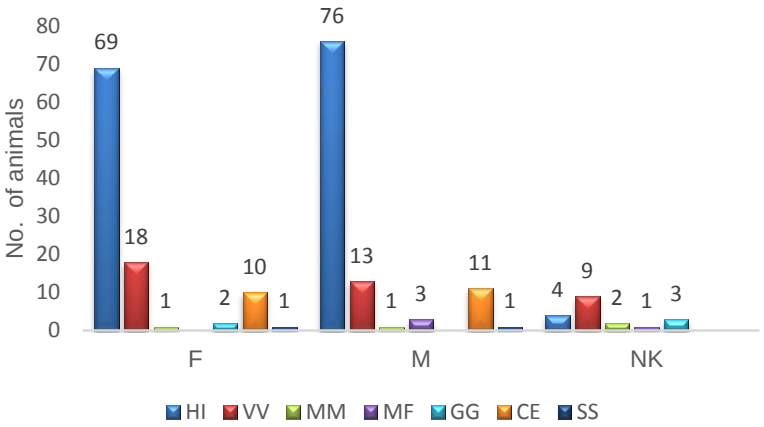
		Primer/Probe sequence (5'→3')	Fragment size (pb)	Target region	PCR conditions
MTBC Detection	IS6110-F	GGGTCGCTTCCACGATG	199	*IS6110*	**1. 95 °C-10 min 2. 45 cycles at 95 °C for 30 sec, 65 °C for 30 sec, and 72 °C for 30 sec 3. 72ºC-10 min**
	IS6110-RV	GGGTCCAGATGGCTTGC			
	IS6110-FN	CTCGTCCAGCGCCGCTTCGG	66	*IS6110*	1. 95 °C-2 min 2. 45 cycles at 95 °C for 5 s and 60 °C for 10 s.
	IS6110-RV	GGGTCCAGATGGCTTGC			
	IS6110 Tqn	FAM-CGCGTCGAGGACCATGGAG GT-BHQ1			
M. bovis characterization	MTUB F	TCGGACGCGTATGCGATATC	1020	*gyrB*	1. 95 °C-3 min 2. 35 cycles at 95 °C for 60 sec, 65 °C for 90 sec, and 72 °C for 90 sec **3. 72ºC-5 min**
	MTUB R	ACATACAGTTCGGACTTGCG			
	DRa	GGTTTTGGGTCTGACGAC	DR of 36	DR region	1. 96 °C-3 min 2. 35 cycles at 96 °C for 60 sec, 55 °C for 60 sec and 72 °C for 30 sec **3. 72ºC-5 min**
	DRb	CCGAGAGGGGACGGAAAC-			

Legend: FAM - 6-fluorescein amidite; BHQ1 – Black Hole Quencher 1

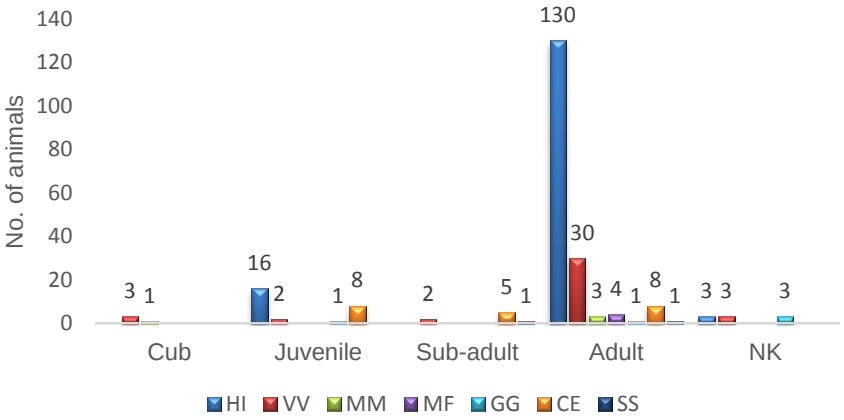
Supplementary Table 5 - Mathematical formula for the calculation of diversity indices and non-parametric estimators.

	Formula
Shannon-Wiener	$H' = -\sum_{i=1}^{n}(pi)ln(pi),\ \ pi = \frac{ni}{Nt}$ proportional abundance of species i in the sample
Simpson	$D = 1 - \sum_{i=1}^{n}(pi^2)$
Berger-Parker	$N\infty = \frac{1}{p1}$ $p1$= proportional abundance of the most common species in the sample
Chao 1	$S_{tot} = S_{obs} + (a^2 / 2b)$, a=singleton, b=doubleton
Chao 2	$S_{tot} = S_{obs} + (a^2 / 2b)$, a=unique, b=duplicate

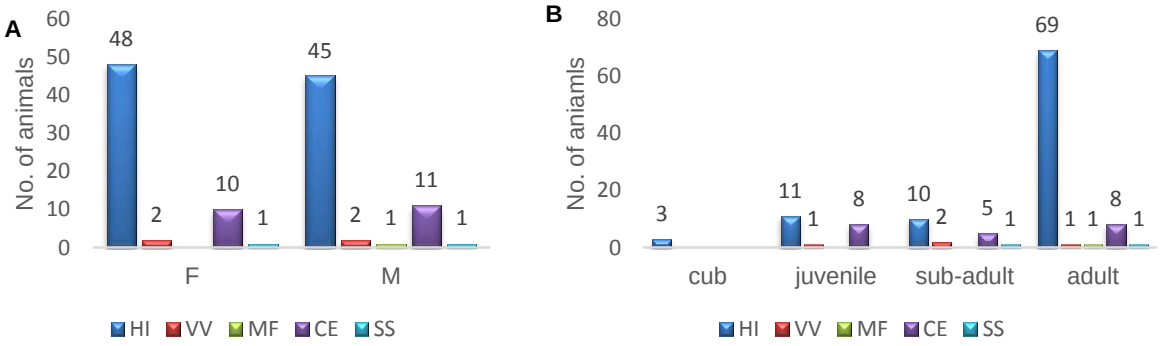
Legend: Sobs – total of observed species in our sample; singleton and doubleton, refers to abundance data, being the singletons the communities with one individual and doubleton the communities with two individuals; and unique and duplicate, refers to incidence data, being unique the communities with one species and duplicates the communities with two species.



Supplementary Fig. 1 - Gender distribution across species of the specimens under analysis (HI - *Herpestes ichneumon*; VV - *Vulpes vulpes*; MM - *Meles meles*; MF - *Martes foina*; GG - *Genetta genetta*; CE - *Cervus elaphus*; SS - *Sus scrofa*; F - female; M - male and NK - unknown).



Supplementary Fig. 2 - Age distribution across species of the specimens under analysis (HI - *Herpestes ichneumon*; VV - *Vulpes vulpes*; MM - *Meles meles*; MF - *Martes foina*; GG - *Genetta genetta*; CE - *Cervus elaphus*; SS - *Sus scrofa*; and NK - unknown).



Supplementary Fig. 3 - (A) Gender distribution across species of the specimens under analysis. (B) Age distribution across species of the specimens under analysis. (HI - *Herpestes ichneumon*; VV - *Vulpes vulpes*; MF - *Martes foina*; CE - *Cervus elaphus*; SS - *Sus scrofa*; F - female and M - male).