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Epidemiology and Characterization of Mycoplasma Infections in Domestic Animals

Candidate
Dr. Mohamed Sh Mohamud Yusuf

Supervisor
Prof. Grazia Greco

Coordinator
Prof. Maria Tempesta

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"It is better to have less thunder in the mouth and more lightening in the hand"

Native American Proverb

I am dedicating this Doctoral dissertation to:

My lovely mother and father

Who made huge sacrifices to ensure that their children obtained the education they needed. Their unwavering dedication, persistence, and moral advice have shaped me into who I am today.

My Dear uncle, (Ismail Hussein)

Whose tremendous support throughout my degree provided the groundwork for my voyage. Your generosity, encouragement, and unfailing confidence in me have been a great blessing, and I will always be thankful for your help."

My beloved wife (Nimo Abdullahi)

Whose love, patience, and unwavering support have been my strength throughout this journey. Your encouragement has meant everything to me.

Preface

The studies herein described were designed at the 1) Department of Veterinary Medicine, University of Bari Aldo Moro, Italy with the cooperation of the 2) University of Córdoba, Faculty of Veterinary Sciences, Department of Genetics, Córdoba, Spain, and 3) the University of Animal Pathology Department, Zaragoza Veterinary Faculty, Zaragoza University, Miguel Servet 177, 50013, Zaragoza, Spain.

The following thesis is written in a "thesis by publication" format and features accepted and under-evaluation manuscripts of the selected topic.

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General Introduction

Mycoplasmas (class *Mollicutes*), derived from the Latin words mollis ("soft") and cutis ("skin"), are bacteria that exist as commensals or opportunistic pathogens in humans, animals, birds, reptiles, amphibians, fish, plants, and insects (Razin & Barile, 2013; Chalker, V. J. 2005; Bradbury, 2005; Greene, C. E., & Chalker, V. J. 2012; Brown 2010; Brown et al. 2010d; Fadiel et al. 2007; May et al. 2014). These organisms are characterized by the absence of a cell wall, instead possessing a thin tri-laminar membrane composed of approximately 60–70% proteins and 20–30% lipids (Bradbury, 2005, Razin and Hayflick, 2010). Mycoplasmas evolved from low G+C content, Gram-positive bacteria through reductive evolution, retaining the genes involved in replication and sacrificing the genes involved in biosynthesis and cell wall synthesis (Bradbury, 2005). Reductive evolution resulted in some interesting characteristics of mycoplasma, such as 1) a low G+C content of between 23 and 40%, 2) the requirement of sterols for growth and membrane function, as well as a host for many of their nutrients 3) very small genomes, with sizes ranging from 580 to 1350 kb, 4) a modification in the mycoplasma genetic code, where the UGA codon is translated to a tryptophan instead of a stop codon (Baseman and Tully, 1997; Bradbury, 2005; Armour, 2015), like *M. genitalium*, have a genome of about 600 kb, estimated to comprise less than 500 genes (Razin, 1985 a). Due to their limited metabolic capacity, which is a consequence of their small genome and lack of cell wall, they are fastidious and some of them are laborious to be cultured. As such, they can be both extracellular and intracellular pathogens whose lives depend on the largesse of their hosts (Morowitz and Tourtellotte, 1962). There is growing support for the hypothesis that Mollicutes evolved from gram positive bacteria by reductive evolution (Rogers et al., 1985).

Recent phylogenomic investigations have demonstrated that the conventional genus Mycoplasma is extensively polyphyletic and necessitates taxonomic revision. In a comprehensive study, analysed 140 genome sequenced species within the phylum Tenericutes using multiple methodologies, including phylogenetic reconstructions based on 63 conserved proteins, 45 ribosomal proteins, the three mains of RNA polymerase subunits, and 16S rRNA gene sequences. These analyses consistently resolved Tenericutes species into four distinct monophyletic clades: the "Acholeplasma", "Spiroplasma", "Pneumoniae", and "Hominis" clusters. As a result of these findings, a major taxonomic update was proposed (Gupta et al. 2018). Under the new taxonomies, mycoplasmas are included within the order Mycoplasmoidales, class Mollicutes, families Metamycoplasmataceae and

Mycoplasmoidaceae harbouring Eperythrozoon, Ureaplasma and five genera (Mycoplasmopsis, Mesomycoplasma, Metamycoplasma, Malacoplasma and Mycoplasmoides) (Gupta et al. 2018). Most members of the Mycoplasmoidales order act as opportunistic pathogens or primary disease agents in animals, affecting multiple organs, including the respiratory, urogenital, and hematopoietic systems (Gupta et al., 2018).

The great majority of human and animal mycoplasmas colonize the epithelial linings of the respiratory and urogenital tracts, mammary glands, eyes, alimentary canal, and joints (S. Razin and E. Jacobs, 1992).

Mycoplasmas have a strong propensity for causing chronic infections in humans and other vertebrates, effectively bypassing host immune responses (Krasteva et al. 2014; Razin et al. 1998). Several members have been implicated in the pathogenesis of severe respiratory and urogenital tract infections (May et al. 2014; Sha et al. 2005; Tully et al. 1983; Waites and Talkington 2004).

In equids, Mycoplasmopsis equigenitalium and Metamycoplasma subdolum have been associated with infertility, endometritis, vulvitis, abortions and balanoposthitis (Kirchhoff et al., 1973, 1980; Moorthy et al., 1977; Heitmann et al., 1979).

Mycoplasmopsis equigenitalium and M. subdolum are pleomorphic organisms forming coccoid to filamentous cells, with unassessed motility, both producing typical fried-egg colonies on solid medium, and growing optimally at 37°C in Hayflick, SP-4, or Frey's medium supplemented with glucose or arginine (Kirchhoff, 1978; Lemcke and Kirchhoff, 1979; Tully and Razin, 1983; Bergey, 1994; Tully, 1995). M. equigenitalium and M. subdolum have been reported with endometritis, vulvitis, balanoposthitis, impaired fecundity, and abortion in equids, though their virulence may vary among strains and both species are frequently detected in clinically normal animals (Spergser et al., 2002)

In dogs, many mycoplasmas are thought to form part of the normal bacterial flora of the upper airways (Koshimizu and Ogata 1974). Mycoplasmopsis canis, Mycoplasmopsis cynos, Mycoplasmopsis edwardii, and Mycoplasmopsis spumans all have been reported in dogs with upper/ lower respiratory tract diseases and reproductive system problems (Rosendal 1978; Armstrong et al. 1972; Jambhekar et al. 2019). Mycoplasmopsis canis and M. cynos are pleomorphic organisms capable of forming branched and filamentous structures, exhibiting a stable colony morphology with rough, granular surfaces, scalloped centers, and irregular margins that remain consistent upon subculturing, and they grow well in glucose-supplemented

Hayflick or SP-4 medium at 37°C (Edward, 1955; Røsendal, 1973; Damassa et al., 1994, Bergey, 1994).

Mycoplasmopsis canis is an opportunistic pathogen primarily associated with infertility, adverse pregnancy outcomes, endometritis, epididymitis, urethritis, cystitis, and pneumonia in domestic dogs, respiratory disease in cattle, and pneumonia in immunocompromised humans, showing a tendency toward upper respiratory tract commensalism and urogenital tract pathogenicity in dogs, and it has been isolated from a wide range of tissues including the reproductive, respiratory, and urinary tracts, as well as internal organs of dogs, cattle, humans, and non-human primates (Chalker, 2005). Mycoplasmopsis cynos is a pathogenic species that causes pneumonia, bronchitis, and occasionally cystitis in domestic dogs, transmitted via droplet aerosol as confirmed by sentinel dog studies, and has been isolated from the respiratory, urinary, and reproductive tracts, as well as conjunctivae (Chalker, 2005).

In cats, different haemotropic mycoplasmas including Mycoplasma haemofelis and M. haemominutum were reported (Tasker et al., 2003a; Peters et al., 2008; Zarea et al. 2022; Zarea et al. 2023). Haemoplasmas are widely distributed in wild and domestic animals worldwide (Assarasakorn et al., 2012; Pitchenin et al., 2019; Latrofa et al., 2020; Di Cataldo et al., 2021; Millán et al., 2021), causing a disease in mammalian animals ranging from mild to severe clinical diseases, characterized by anorexia, reduced appetite, dehydration, weakness, fever, weight loss, lethargy, haemolytic anemia, and even death (Messick, 2004; Sykes, 2010).

Transmission and Epidemiology

Mycoplasmas can be transmitted vertically or horizontally through direct contact with secretions, fomites, aggressive interactions, and blood transfusion (Alves et al., 2023; Willi et al., 2007b; Museux et al., 2009; Tasker, 2010; Cohen et al., 2018). Semi-intensive breeding systems facilitate greater spread due to close animal proximity (Fox, 2012; Mac.do et al., 2018). Kennel trading increases stress and pathogen exposure, and population changes further affect infection dynamics (Pesavento & Murphy, 2014).

Treatment and Resistance

Common anti-Mycoplasma agents include tetracyclines (McLellan, 2017), macrolides (Bauman, 2016), and fluoroquinolones (Emmerson & Jones, 2003), with doxycycline considered the most effective first-line treatment due to its broad-spectrum activity and favorable pharmacokinetics (Morley et al., 2005). Mycoplasmas are inherently resistant to

antimicrobials targeting folic acid metabolism and cell wall synthesis, such as sulfonamides, trimethoprim, and β-lactams (Maes et al., 2017; McCormack, 1993). The emergence of multidrug resistance (MDR) through antibiotic resistance gene transfer is an increasing concern (Forsberg et al., 2012; Faucher et al., 2019). Although vaccines exist for some Mycoplasma-related diseases, their efficacy remains limited, highlighting the urgent need for novel therapeutic and preventive strategies (Fair & Tor, 2014; Valentine-King et al., 2020).

Cultivation and Isolation

Mycoplasmas are slow-growing organisms requiring specialized media and conditions. Colonies typically appear after several days and exhibit a characteristic "fried egg" morphology (Citti & Blanchard, 2013; Razin & Hayflick, 2010). Media must include yeast extract, amino acid sources, sterols, energy substrates, selective agents, and pH indicators (Hannan, 2000; Caswell & Archambault, 2008). Despite high sensitivity, isolation is labor-intensive and influenced by factors such as prior antibiotic treatment, contamination by faster-growing organisms, and sample degradation, which may lead to false negatives (Caswell et al., 2010; Nicholas, 2011).

Diagnosis

Laboratory diagnosis relies on organism isolation and/or detection using molecular techniques (PCR), immunological assays (ELISA, immunohistochemistry), MALDI-TOF MS, and antimicrobial susceptibility testing (MIC assays) (Lauerman et al., 1995; Nicholas et al., 2016; Pereyre et al., 2013; Wiegand et al., 2008).

Despite their clinical significance, data on prevalence, transmission dynamics, and molecular characteristics remain limited in some animal species. Understanding these aspects is essential for improving animal health and assessing zoonotic risks under the One Health framework.

Objectives of the thesis

This thesis aims to investigate the molecular epidemiology and characterization of Mycoplasma species across diverse mammalian hosts through three studies:

1. Determine the prevalence and molecular identification of Mycoplasmopsis equigenitalium in donkey populations from Martina Franca (Italy) and Andalusia (Spain).

2. Assess the occurrence and risk factors associated with Mycoplasmopsis canis and Mycoplasmopsis cynos in dogs with reproductive disorders in Italy.
3. Explore the presence of vector-borne pathogens, including Mycoplasma spp., in clinically healthy stray cats in Zaragoza, Spain, and evaluate their potential role as zoonotic reservoirs.

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Thesis outline (Abstract)

The present thesis, that provides original data and novel information on epidemiological investigations and molecular analysis of *Mycoplasma* spp. infections in domestic animals, is structured into three chapters.

Chapter I. It is focused on the prevalence and molecular identification of *Mycoplasmopsis equigenitalium* infection in donkey populations from Martina Franca (Italy) and Andalusia (Spain). This study aimed at investigating the occurrence and risk factors of *Mycoplasma* spp. organisms in the genital tract of donkeys. Results out of 31/120 (25.83%, 95% CI: 18.00-33.67) for *M. equigenitalium* was recorded, with one jenny (0.833, 95% CI: 0.000-2.460) exhibiting a mixed infection of *Mycoplasmopsis equigenitalium* and *Metamycoplasma subdolum*. Biosecurity measures significantly influenced the infection frequence, with farm adhering to good management practices displaying significantly lower infection rates (p=0.0031, OR=0.173). No significant risk was detected for country, age, gender, or breed. The study reports *M. equigenitalium* and *M. subdolum* infections in healthy donkeys in Europe, identifying poor biosecurity as a risk factor and emphasizing the need for further research on their reproductive impact.

Chapter II. The aim of this study was to investigate the occurrence and the risk factors of *mycoplasmas* in canine populations affected with low reproductive efficiency. Out of 68 tested animals, *Mycoplasma* spp. DNA was detected in 26 dogs (38.24%, CI 95%: 26.68, 49.79) being 13 (52%, CI 95%: 32.42, 71.58) males and 13 (30.23%, CI 95%: 16.51, 43.96) females. When considering Mycoplasma species, M. cynos 15/68 (22%, CI 95%: 12.20, 31.91) was more frequently detected compared to M. canis 8/68 (11.76%, CI 95%: 4.11, 19.42), furthermore mixed infection was also detected 3/68 (4.4%, CI 95%: 0.00, 9.29), with males more at risk of M. cynos infection (OR: 5.07). Furthermore, animals affected with reproductive impairment

were three time to be more likelihood infected than healthy animals (OR: 3.38). This highlights their potential association with reproductive disorders and the need for adopting preventive measures.

Chapter III includes a Molecular survey on vector-borne pathogens in clinically healthy stray cats in Zaragoza (Spain). this study was to assess the prevalence of vector-borne pathogens (VBPs) in stray cats in Zaragoza, Spain, and to investigate potential risk factors for infection, including feline leukaemia virus (FeLV) and feline immunodeficiency virus (FIV). Results Nearly half of the cats (158/332, 47.6%) were positive for at least one VBP. Hepatozoon spp. were detected in 25.6%, haemotropic Mycoplasma spp. in 22.9%, B. henselae in 9.3% and L. infantum in 2.1% of the cats. Male sex had a statistically significant association with test results for haemotropic Mycoplasma spp. (odds ratio 1.38 [1.21;1.57]); regionality with Hepatozoon spp., B. henseale and FIV; and seasonality with Hepatozoon spp., haemotropic Mycoplasma spp., L. infantum and FeLV ($P \leq 0.05$ each). A strong positive correlation was reported for the amount of rainfall and the number of cats that tested positive for Hepatozoon spp. ($\rho = 753$, $P = 0.05$). None of the cats tested positive for A. phagocytophilum, A. platys, E. canis, Rickettsia spp., piroplasms, or microfilariae. Co-infections with multiple VBPs were detected in 56 out of 332 cats (16.9%). Thirty-one of the 332 cats included in the study (9.3%) tested positive for FeLV (6.9%) and for FIV (3.6%). In 20/31 cats (64.5%) that tested positive for FeLV/FIV, coinfections with VBP were detected ($P = 0.048$, OR 2.15 [0.99; 4.64]). VBPs were frequently detected in stray cats in Zaragoza. In particular, regionality and seasonality had a statistically significant association with PCR results for most VBPs included in the study.

CHAPTER 1

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Animal health

PREVALENCE OF *MYCOPLASMA EQUIGENITALIUM* IN MARTINA FRANCA (ITALY) AND ANDALUSIAN (SPAIN) DONKEY POPULATIONS

M.S.M. Yusuf[1], M. Burgio[1], G.G. Aiudi[1], J.V. Delgado Bermajo[2], C. Rivas López[2], M.S. Lucente[1], G. Lanave[1], M. Losito[1], M. Corrente[1], M. Tempesta[1], G. Greco[1]

[1]*Department of Veterinary Medicine - University of Bari Aldo Moro, Valenzano, Bari, Italy*
[2]*University of Córdoba, Faculty of Veterinary Sciences,Department of Genetics, Córdoba, Spain*

Introduction

Mycoplasma equigenitalium has been associated with infertility, endometritis in mares and with reduce fertility and balanoposthitis in stallions (1). Furthermore, *M. equigenitalium* was isolated from the genital tract of donkeys in Argentina (2).
Currently, there are no studies describing the presence and impact of *M. equigenitalium* on the reproductive sphere of donkeys in Italy and Spain. This study aimed at investigating the occurrence and risk factors for *Mycoplasma* spp. infection in genital tract of Martina Franca (Italy) and Andalusian (Spain) donkey populations.

Materials and Methods

From March 2023 to June 2024, a cross-sectional study was carried out on 120 healthy donkeys, with the majority being jennies (106, 88.3%), mainly reared under semi-extensive lifestyle in Italy (60.8%) and Spain (39.2%). Genital swab samples were collected, cultured in modified Hayflick media. The identification of the isolates was performed using different PCR assays targeting 16S, ITS (3) and rpoB (4) regions associated with sequence analysis.

Results

An overall prevalence of 25.83% (31/120, 95% CI: 18.00-33.67) of *M. equigenitalium* was recorded, with one donkey (0.833, 95% CI: 0.000-2.460) exhibiting a mixed infection of *M. equigenitalium* and *M. subdolum*. Biosecurity measures significantly influenced the infection frequence, with farm respecting good practice of biosecurity having significantly lower infection rates (p=0.0031, OR=0.173). Furthermore, no significant risk was detected for country, age, gender, or breed.

Discussion and Conclusion

This study represents the first report of *M. equigenitalium* and *M. subdolum* infections in healthy donkeys in Italy and Spain, and Europe in general, with low adoption of biosecurity measures identified as risk factors. However, more studies need to be performed to address the possible link to reproductive performances of donkeys.

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Prevalence of *Mycoplasmopsis equigenitalium* in Martina Franca (Italy) and Andalusian (Spain) donkey populations.

Yusuf M.SH M. [*1, 2], Burgio M. [1], Aiudi G.G. [1], Delgado Bermejo J.V.[3], Rivas López C.[3], Lucente MS [1], Lanave G. [1], Losito M. [1], Celentano F.G. [1], Corrente M. [1], Tempesta M. [1], Greco G. [1].

[1]Department of Veterinary Medicine - University of Bari Aldo Moro, Valenzano, Bari, Italy.

[2]Department of Veterinary Medicine and Animal Husbandry - Somali National University, Mogadishu, Somalia

[3]University of Córdoba, Faculty of Veterinary Sciences, Department of Genetics, Córdoba, Spain

ABSTRACT

Mycoplasmopsis (formerly, *Mycoplasma) equigenitalium* has been associated with infertility, endometritis in mares as well as reduced fertility and balanoposthitis in stallions. Furthermore, *M. equigenitalium* was isolated from the genital tract of donkeys in Argentina.
Currently, there are no studies describing the presence and impact of *M. equigenitalium* on the reproductive health of donkeys in Italy and Spain.

This study aimed at investigating the occurrence and risk factors of *Metamycoplasmatacea* organisms in the genital tract of Martina Franca (Italy) and Andalusian (Spain) donkey populations. From March 2023 to June 2024, a cross-sectional study was carried out on 120 healthy donkeys, with the majority being jennies (106, 88.3%), mainly reared under semi-extensive lifestyle in Italy (60.8%) and Spain (39.2%). Genital swab samples were collected, cultured in modified Hayflick media. The isolates were identified using different PCR assays targeting 16S rRNA, ITS and rpoB regions associated with sequence analysis.

A prevalence of 25.83% (31/120, 95% CI: 18.00-33.67) for *M. equigenitalium* was recorded, with one jenny (0.833, 95% CI: 0.000-2.460) exhibiting a mixed infection of *Mycoplasmopsis equigenitalium* and *Metamycoplasma subdolum*. Biosecurity measures significantly influenced the infection frequence, with farm adhering to good management practices displaying significantly lower infection rates (p=0.0031, OR=0.173). No significant risk was detected for country, age, gender, or breed.

This study represents the first report of *M. equigenitalium* and *M. subdolum* (first time in donkey) infections in healthy donkeys in Italy and Spain, and Europe in general, with low adoption of biosecurity measures identified as risk factors. However, more studies need to be performed to address the possible link to reproductive performances of donkeys.

Keywords: *Metamycoplasmatacea spp., Metamycoplasma spp., Mycoplasmopsis equigenitalium*, *Metamycoplasma subdolum,* reproductive tract, jennies, jacks, Martina Franca, Andalusia.

1.0. Introduction

Mycoplasmas are the smallest self-replicating bacteria, cell wall-lacking as a result of a reductive evolution from Gram-positive ancestors, with small genomes, limited metabolic capacities and a low G+C content in DNA (Waites et al., 1999).

Despite their reductive evolution, mycoplasmas own attaching virulent factors, such as adhesins, lipoproteins, and variable surface proteins, therefore their primary habitats are the mucosa surfaces of the respiratory and urogenital tracts, as well as eyes, joints and mammary glands of a wide range of vertebrate hosts, including livestock, wild animal species, and humans (Razin et al., 1998). However, due to the high culturing requirements of mycoplasmas, information regarding the occurrence, prevalence and their pathogenic role in some animal species is underestimated or still unknown (Razin et al., 1998; Rosengarten et al., 2001; Citti and Blanchard, 2013; Arfi et al., 2021).

According to the recently accepted taxonomy, mycoplasmas are microorganisms within the genus *Mycoplasmopsis*, class *Mollicutes*, family *Metamycoplasmataceae* (Gupta et al. 2018). Most members within the *Mycoplasmopsis* genus act as primary or opportunistic pathogens for animals (Gupta et al., 2018).

Horses (*Equus caballus*) and donkeys (*Equus asinus*) are mammals belonging to genus Equus, which emerged approximately 4.5 million years ago (Jónsson et al., 2014).

According to Food and Agriculture organization of the United Nations (FAO, 2018), the global donkey populations were estimated at approximately 50.4 million head (FAOSTAT, 2018) with the majority being in Africa (60.6%) followed by Asia (26.2) and Central/South America (12%) (FAOSTAT, 2021). Currently, in the context of the green economy, donkey breeding has been garnering renewed attention in western countries due to their suitability for new forms of

production, including meat and milk, hides, cosmetics, onotherapy and tourism thereby allowing for the growth of small rural businesses (F. Camillo et al., 2018; Colombo E, et al., 2020; Tully, P. A., & Carr, N., 2022; Papademas et al., 2022).

M. equigenitalium and *M. subdolum,* have been frequently associated with infertility, endometritis, vulvitis and abortions in mares, with isolation rate of 5-34% from the genital tract and 7% from aborted equine foetuses and in stallions balanoposthitis and reduced fertility (Moorthy et al., 1977; Heitmann et al., 1979; Kirchhoff et al., 1979; Spergser et al., 2002; Nehra et al., 2015, Khurana et al., 2015, gorniák-Nowesiélka et al., 1984).

In donkeys (*Equus asinus*), information on mycoplasma infections is scant compared to o horses and limited to only one study in Argentina (Tamiozzo et al., 2022). In general, donkeys are often treated as "small horses"; however, there are several differences that must be taken into consideration when analysing the impact of infectious diseases on donkeys' health (Barrandeguy et al., 2018). First of all, donkeys may have different susceptibilities to certain infectious agents, and they usually have mild clinical manifestations compared to horses (Câmara et al., 2020).

The present study aimed at investigating the occurrence and prevalence of organisms of the *Metamycoplasmaceae* family in the genital tract of donkeys and identifying possible risk factors.

2. Materials and methods

2.1 Ethical statement

All procedures were conducted in agreement with institutional guidelines on animal welfare and use, with the informed consent of the owner and the approval of the ethics committee of the University of Bari 'Aldo Moro' (protocol no. 14/2023).

2.2 Animal and study area

The study was conducted in the southern regions of two European countries: Italy and Spain. In Italy, it focused on the regions of Apulia and Basilicata, where Martina Franca donkeys are predominantly bred. In Spain, the study focused on Andalusia, particularly on the Andalusian donkey breed, in the provinces of Córdoba and Seville. Shape files of study locations were obtained from GADM (Global Administrative Area Maps) (version 2.8) online tools, then

imported into Quantum Geographic Information System (QGIS) software, version 3.36.0. for visualization (Figure 1).

2.3 Sampling design and collection

A sampling plan was designed to collect genital swabs from donkeys randomly distributed throughout the regions of Italy and Spain. Considering the current consistency of 2100 units of two populations, to determine the sample size, the animals were considered as sampling units, with an assumed a priori prevalence of 50%, a confidence level of 95% and an absolute precision of 10% (Thrusfield, 2018). Based on these parameters, the minimum required number of primary units was 92 animals. Between March 2023 and June 2024, 120 donkeys were investigated. Among these, 73 (60.8%) were Martina Franca donkeys from Italy, and 47 (39.2%) were Andalusian donkeys from Spain. All the animals were reared in a semi-extensive system. For each animal, identification data (including sex, age, breed and location) and clinical information were collected during the clinical examination. Information was also collected on the application of an internal control system for biosecurity procedures. During the clinical examination, two different genital swabs were taken from each animal for analysis. Before sampling, the perineal and genital areas were thoroughly cleaned with warm water. All swabs were collected by the same employee using clean gloves for each animal, and by opening the vulvar labia or foreskin to avoid contact of the swab with the skin. To detect the carrier status of mycoplasmas, a swab was rotated over the vaginal walls or glands and the inner part of the foreskin and immediately placed in a sterile tube containing PPLO broth (Becton Dickinson, Le Pont de Claix, France) with 20% equine serum without any inhibitor (antimicrobial or thallium acetate). The second swab was placed in a tube containing Dulbecco's Modification of Eagle's Medium (D-MEM) (Corning, Mediathec Inc. Manassas, VA-USA) for DNA extraction.

The sample swabs were delivered to the laboratory under refrigerated conditions. The Veterinary Medicine Department of the University of Bari in Valenzano (Italy) performed testing.

2.4 Data management and Statistical analysis

The recorded data were entered in Excel® spreadsheets (Microsoft), and the prevalence was calculated at both the animal and farm levels, with a 95% confidence interval determined using exact binomial confidence intervals. Pearson's chi-square or Fisher's exact tests were used, with a 95% confidence interval (CIs) to compare the differences between the proportions and the risk factors in donkeys. All statistics were performed using the Epitools AUSVET calculator (https://epitools.ausvet.com.au/).

2.5. Culturing

For *Mycoplasma* spp. detection, 500 µL of each swab was inoculated in 4.5 ml Hayflick broth media (Tully and Razin, 1983) and incubated under 5% CO_2 conditions (Oxoid^{Tm}) at 37ºC for 4 to 7 days (in case of negative at 4$^{th\ day}$). Cultures were observed daily for colour changes, indicating the presence of live mycoplasmas. Positive liquid cultures (50 µl) were inoculated on *Mycoplasm*a Hayflick solid media (Tully and Razin, 1983) and incubated for a further 4 to 7 days at 37ºC under microaerophilic conditions and observed daily. Five colonies per plate were cloned three times to ensure purity (Rosendal, 1973). The cloned cultures were stored at -80°C for subsequent classification studies.

2.6. Molecular investigation

2.6.1. DNA extraction and purification of the samples

Whole genomic DNA for Mycoplasma spp. PCR was extracted from 200 µL of the genital swab samples, as well as from Hayflick agar when visible colonies were present. The IndiSpin ® Pathogen Kit (Indical Bioscience GmbH, Leipzig, Germany) was used according to the manufacturer's instructions. DNA was eluted in 100 µL of AE buffer and stored at −40°C until testing.

2.6.2. Polymerase chain reaction (PCR) for detection of mycoplasmas

All swab samples were screened for *Mycoplasma* spp. using the generic primers (forward primer GPO-3; reverse primer MGSO) directed against 16S rRNA gene as described previously (Table 1) (van Kuppeveld et al., 1992, 1994).

PCR master mix was composed of 0.2 µL of Taq Gold DNA polymerase, 0.25 µL of each primer, 2.5 µL of dNTP's, 3 µL MgCl2, 2.5 µL of 10X PCR Buffer II, 2.5 µL of dNTPs, 14.3 µL of H2O. Finally, 2 µL of the DNA template was added, and the terminal PCR reaction

volume was 25 µL. The PCR amplification was performed by applying the following parameters: initial denaturation at 94 °C for 10 min, followed by 40 cycles of denaturation at 94 °C for 1min, primer annealing at 52 °C for 1 min, elongation at 72 °C for 1 min, and final elongation step at 72 °C for 10 min. Every PCR run contained a negative control (a sample, containing all reagents except DNA template), and reference strains including *M. bovigenitalium* ATCC 19852 and *M. californicum* (laboratory collection) used as positive controls. The products were analysed by electrophoresis in 1.5% agarose gel, followed by UV visualization after ethidium bromide staining and the approximate length of amplicons were assessed with a molecular-weight size marker (100 bp DNA ladder) as a reference. All PCR products of the expected size were submitted for sequencing for species identification.

Furthermore, for species identification and genotyping, all the isolates on Hayflick plates were submitted to MLST analyses using additional conventional PCR (cPCR) assays that amplify RNA polymerase beta subunit-encoding gene *(rpoB),* DNA gyrase subunit B gene (*gyrB*) (Volokhof et al., 2007), 16S rRNA, 16S–23S rRNA intergenic space (ITS) and 23S rRNA (Volokhof et al., 2019) (Table 1).

2.6.3. Purification of PCR Products and Phylogeny analysis

All cPCR amplicons of expected sizes were purified using the NEB Exo-SAP PCR purification kit (New England Biolabs, Inc., Ipswich, MA, USA) and subsequently sequenced in both directions with the same primer for species identification and phylogenetic analysis by Eurofins Genomics (Ebersberg I Germany). The obtained nucleotide sequences were compared with GenBank database entries by Basic Local Alignment Search Tool (BLAST) (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Chromatogram evaluation, primer trimming and sequence alignment were conducted using the Geneious® 10.3.1 software (Biomatters Ltd., Auckland, New Zealand). To align each sequence and assess homologous genes or targets for genetic variation, the Clustal W algorithm was used. For phylogenetic analysis, newly obtained 16S, ITS and 23S, *rpoB* and *gyrB* sequences and those from representative known *Mycoplasma* isolates were analysed with Mega-X v. 10.0.5 software (Kumar et al., 2018). Each gene was first analysed individually to assess its phylogenetic signal. Subsequently, the sequences were concatenated to increase phylogenetic resolution..Phylogeny relationships were calculated using the 'find best DNA/protein model' tool from MEGA-X. The maximum likelihood approach, with the Hasegawa-Kishino-Yano (HKY) substitution model, a proportion of

invariable sites and a gamma distribution of rate variation across sites, was applied. Statistical confidence was ensured through bootstrap resampling with 1,000 replicates.

2.7 Phenotypic antimicrobial susceptibility testing by MIC

All isolates were tested for susceptibility to a panel of 10 antimicrobials belonging to 7 different classes, selected because they represent antimicrobial families commonly used for the treatment of mycoplasmas. Antimicrobial susceptibility was determined using the broth microdilution method following the Hannan protocol for mycoplasmas (Hannan, 1997, 2000**)**, with SENSITITRE custom MIC plates (TREK Diagnostic Systems/Thermo Fisher Scientific, West Sussex, UK). As quality control strains, *Mycoplasma bovis* PG45 and *Mycoplasma* bovigenitalium were used. Briefly, pure cultures obtained from logarithmic-phase cultures on Hayflick broth medium were suspended in sterile diluent (Hayflick broth without inhibitors) to achieve a colour-changing unit (CCU) equivalent to approximately 10^3–10^5 CFU/mL, as recommended in Hannan's method. Of this standardized inoculum, 100 µL were dispensed into each well of the MIC plate containing serial two-fold dilutions of each antimicrobial agent. Plates were incubated at 36 ± 1°C for 72–96 h, or until visible colour change was detected in the positive controls, indicating mycoplasma growth. MIC values were defined as the lowest concentration of antimicrobial that completely inhibited the colour change. A growth control without antibiotics and a sterility control were included in each plate. Because of the absence of CLSI-approved and standardized breakpoints for many veterinary *Mycoplasma* species, the isolates were not classified as susceptible, intermediate or resistant to the different antimicrobials.

3. RESULTS

3.1. Description of animal population

he characteristics of all enrolled donkeys, expressed as median/mode (range) or percentage (%), where appropriate, are reported in Table 2 and Supplementary Table 1 (TS1). Of the 120 donkeys, 14 (11.7%) were jacks and 106 (88.3%) were jennies, with ages ranging from 1 to 30 years (median: 6 years; mode: 5). The majority of donkeys (73; 60.8%) were from Italy, and 47 (39.2%) were from Spain. All donkeys were reported to have a semi-intensive lifestyle.

3.2. Culture and Molecular Methods

Results of Mycoplasma isolation are presented in Table 3 and Supplementary Table 1 (TS1). Out of 120 swab samples, 15 (12.50%; 95% CI: 6.58–18.42) successfully grew in culture, all originating from Italian animals.

Based on both the 16S rRNA cPCR assay and sequence analyses of the obtained products, *M. equigenitalium* DNA was detected in 31 (25.83%; 95% CI: 18.00–33.67) of the 120 swab samples (Table 3). One sample (0.83%; 95% CI: 0.00–2.46) from animal #109.23-1 (TS1) was co-infected with *M. subdolum* and *M. equigenitalium* (Table 3).

Among the 31 PCR-positive samples, 11, 11, and 10 were confirmed as *M. equigenitalium* through cPCR assays targeting the 16S rRNA, ITS, 23S rRNA, gyrB, and rpoB loci, respectively (Table 4 and Figure 2). Additionally, one sample was confirmed as *M. subdolum* through cPCR targeting the ITS and gyrB loci, while the same sample was identified as *M. equigenitalium* through rpoB analysis, confirming a co-infection case (Table 4).

The rpoB sequence of *M. equigenitalium* from Italian donkey farms showed that the sample from Farm 1 (F1, #318.24-1) was 99.71% similar to *M. equigenitalium* isolated from the reproductive tract of a horse in Germany (CP101808). Samples from Farm 2 (F2, #120.24-10), Farm 3 (F3, #109.23-1), and Farm 4 (F4, #348.24-116) were 99.6% similar to the same reference. The gyrB, 16S rRNA, ITS, and 23S rRNA sequences from Italian donkeys were 99.87% and 99.5% similar to *M. equigenitalium* T37 (CP101808). Additionally, one donkey from Farm 3 showed a co-infection: the rpoB gene sequence was 99.6% identical to *M. equigenitalium* (CP101808), while the gyrB and ITS sequences showed 93.61% and 100% identity, respectively, to *M. subdolum* isolated from a horse (CP137846).

3.3. Multiple Sequence Alignment Analysis

The sequences of the rpoB, gyrB, and 16S rRNA, ITS, and 23S rRNA genes/regions of *M. equigenitalium* and *M. subdolum* obtained in this study were compared to the closest reference sequences from GenBank (CP101808 for ITS, gyrB, and rpoB; CP137846 for ITS and gyrB) using multiple sequence alignment analysis. Data on base substitutions are shown in Table 5. Single-nucleotide polymorphisms (SNPs) were detected at eight nucleotide positions of the rpoB gene. Transitions (point mutation that changes a purine nucleotide to another purine (A ↔ G) were found at various positions along the rpoB gene fragment sequences, depending on

the farm. The sample from Farm 1 (F1, #318.24-1) exhibited five SNP variations from the reference strain, while samples from Farms 2, 3, and 4 displayed seven SNP variations each. These findings indicate the presence of two distinct rpoB genotypes of *M. equigenitalium* among the farms.

In the *M. equigenitalium* gyrB gene fragment sequences, transitions were observed at two nucleotide positions. Additionally, *M. subdolum* gyrB gene fragment sequences showed 101 SNP differences from the reference strain (CP137846), including 66 transitions and 35 transversions (substitution of a purine (A or G) for a pyrimidine (C or T) or vice versa). Among the transversions, 14 led to non-synonymous mutations, resulting in amino acid changes.

In the *M. equigenitalium* 16S rRNA, ITS, and 23S rRNA regions, transitions were observed at twelve nucleotide positions compared to the reference strain (CP101808). Transitions at positions 295 and 2170 were observed in the 23S rRNA and 16S rRNA regions, respectively, while the remaining ten transitions were observed in the ITS region. In the *M. subdolum*, 16S rRNA, ITS, and 23S rRNA regions showed 100% identity with the sequences of the reference strain CP137846.

3.4. Nucleotide Sequence Accession Numbers

Any unique sequences (according to pathogen, locus, and animal host) were submitted to the GenBank database under the following accession numbers:

PV987805 and PV987806 for *M. equigenitalium* 16S rRNA, ITS, 23S rRNA (donkeys #318.24-1, #120.24-10);

PV987807 for *M. subdolum* 16S rRNA, ITS, 23S rRNA (donkey #109.23-1);

PX071974 and PX071975 for *M. equigenitalium gyr*B (donkeys #318.24-1, #120.24-10);

PX071976 for *M. subdolum* gyrB (donkey #109.23-1).

PX071977, PX071978 for *M. equigenitalium rpo*B (donkeys #318.24-1, #120.24-10).

3.5. Antimicrobial Susceptibility Findings

MIC values for the tested antimicrobial are presented in Table 6. Among the macrolides, tylosin and tilmicosin demonstrated strong in vitro activity, with consistently low MIC_{50} values (0.12–0.5 µg/mL), indicating high susceptibility across isolates. Spectinomycin also showed uniform efficacy (MIC_{90} = 4 µg/mL), reinforcing its reliable performance. Florfenicol exhibited generally favourable activity (MIC_{50} = 1 µg/mL), although a slightly elevated MIC_{90} (4 µg/mL)

may suggest reduced susceptibility in some strains. Oxytetracycline remained effective for most isolates (MIC_{50} = 0.25 µg/mL), but low susceptibility was detected in a subset (MIC >32 µg/mL). Tiamulin, a pleuromutilin, showed a wide range of MIC values (≤0.008 to >16 µg/mL), indicating high eterogeneity among strains. Similarly, lincomycin displayed a bimodal distribution, with some isolates fully susceptible (MIC ≤0.5 µg/mL) and others exhibiting high-level resistance (MIC >32 µg/mL). Enrofloxacin presented a dichotomous profile, with several isolates showing low MICs (0.25 µg/mL) and others resistant (MIC >16 µg/mL), suggesting possible acquired resistance mechanisms. In contrast, erythromycin and spiramycin showed limited efficacy, with elevated MIC_{90} values (32 µg/mL and >16 µg/mL, respectively). Overall, tylosin, tilmicosin, spectinomycin, and florfenicol were the most active agents. The observed variability in susceptibility to erythromycin, spiramycin, lincomycin, and enrofloxacin highlights the importance of ongoing surveillance and susceptibility profiling to guide effective treatment strategies.

3.6. Risk Factor Analysis

Among the donkey populations, country, age, gender, and breed were not significant risk factors. Notably, poor adherence to biosecurity measures (P = 0.0031; OR = 0.173; 95% CI: 0.05–0.55) had a significant effect on *M. equigenitalium* occurrence (Table 5).

4.0. Discussion

This study reports a 25.83% prevalence of mycoplasmas in two donkey populations from Italy and Spain, with all positive cases detected in females. Of the 120 donkeys tested, 15 (12.50%) were culture-positive, with *M. equigenitalium* and *M. subdolum* successfully isolated. To our knowledge, this is the first report of *M. equigenitalium* and *M. subdolum* infection in donkeys from these regions using both culture and PCR methods.

These findings align with previous studies performed in horses. Nehra et al. (2015) reported *M. equigenitalium* in mares and stallions from northern India, with 22.13% PCR-positive and 9.01% culture-positive cases. Similarly, Tamiozzo *et al.* (2022) found a 33.3% prevalence in apparently healthy donkeys in Argentina, closely matching our results. However, information

on the role of *M. equigenitalium* and *M. subdolum* in equine reproductive disorders remains scarce (Spergser et al., 2002).

A notable finding of this study is the difference in culture success between Italian and Spanish samples. Fresh Italian samples yielded *M. equigenitalium* in 15 of 18 PCR-positive cases, resulting in 16 isolates, hosting one animal from an Italian farm both *M. equigenitalium* and *M. subdolum.* In contrast, none of the 13 cPCR 16S-positive Spanish samples, which were frozen prior to processing, grew in culture, likely due to the detrimental effects of freezing and thawing on bacterial viability.

In stallions, mixed genital *Mycoplasma* infections have been associated with reproductive disorders (Moorthy et al., 1977; Zgorniák-Nowesiélka et al., 1984). Although *M. equigenitalium* has been detected in both fertile and infertile horses (Moorthy et al., 1977; Bermudez et al., 1987), our study represents only the first investigation of *Mycoplasma* in the reproductive tract of healthy donkeys in Europe, the previous being Tamiozzo et al. (2022) in Argentina. This limitation hinders a comprehensive understanding of the epidemiology, pathogenicity, transmission, and clinical relevance of *Mycoplasma* infections in donkeys.

Our analysis revealed that country, age, and breed had no significant impact on *Mycoplasma* prevalence, but gender did, with all positive cases found in females. This mirrors findings from Denmark, where all riding stallions tested negative for *Mycoplasma* spp. (Baczynska et al., 2007). Another key finding is the significantly reduced infection risk ($P = 0.0031$, $OR = 0.173$) in farms with stringent biosecurity protocols, particularly those involved in food production.

This study provides new insights into the genetic variability of *Mycoplasma equigenitalium* and *Mycoplasma subdolum* by analyzing key gene regions, including *rpo*B, *gyr*B, 16S rRNA, ITS, and 23S rRNA. The detection of single-nucleotide polymorphisms (SNPs) across these loci reveals notable intra- and inter-species genetic diversity, which may have implications for diagnostics, epidemiology, and understanding evolutionary relationships.
The identification of two distinct **rpoB** genotypes of *M. equigenitalium* across different farms suggests possible geographic or host-associated genetic divergence. The presence of five SNPs in the sample from Farm 1 and seven SNPs in samples from Farms 2–4 indicates a level of genetic heterogeneity that could reflect either independent evolutionary events or selective pressures in different environments. These findings underscore the importance of monitoring

genetic variation in more field isolates, particularly in genes like **rpoB**, which are often used in molecular typing and antimicrobial resistance studies.

In the **gyrB** gene, the limited variation observed in *M. equigenitalium* (two transitions) contrasts sharply with the extensive polymorphism found in *M. subdolum*, which exhibited 101 SNPs, including 14 non-synonymous mutations. Since *gyr*B encodes a subunit of DNA gyrase (essential for DNA replication), these amino acid-altering changes may affect protein functions such enzyme activity or susceptibility to antibiotics and could be explored further to assess their impact on bacterial physiology or pathogenicity. The high level of divergence in *M. subdolum* suggests that this species may be undergoing more rapid genetic evolution or may represent a more genetically diverse population.

The analysis of ribosomal RNA regions revealed that *M. equigenitalium* exhibits moderate variability, with twelve transitions across the 16S rRNA, ITS, and 23S rRNA regions. Notably, the ITS region accounted for the majority of these changes, which is consistent with its known role as a hypervariable spacer region. In contrast, *M. subdolum* showed complete identity with the reference strain in these regions, suggesting a more conserved ribosomal profile or possibly a clonal population structure.

Overall, these findings contribute to the growing body of knowledge on *Mycoplasma* species diversity and highlight the utility of multi-locus sequence analysis in differentiating strains and understanding their evolutionary dynamics. Future studies should investigate the functional consequences of the observed SNPs, particularly those leading to amino acid substitutions, and explore their potential role in virulence, host adaptation, or antimicrobial resistance. Additionally, expanding the sample size and geographic scope could help clarify the population structure and transmission patterns of these organisms.

Finally, the study assessed the **antimicrobial susceptibility** of *M. equigenitalium* isolated strains. The antimicrobial susceptibility profiles presented in this study offer valuable insights into the efficacy of commonly used agents against *Mycoplasma* isolates. The consistently low MIC_{50} values for **tylosin** and **tilmicosin** (0.12–0.5 µg/mL) confirm their strong in vitro activity and suggest that macrolides remain a reliable therapeutic option for treating *Mycoplasma* infections in equid populations. Their effectiveness across all tested isolates supports their continued use as first-line agents, particularly in settings where resistance surveillance is limited. **Spectinomycin** also demonstrated uniform efficacy (MIC_{90} = 4 µg/mL), reinforcing its role as a dependable alternative, especially in cases where macrolide resistance may emerge. The performance of **florfenicol** was generally favourable, with a MIC_{50} of 1 µg/mL. However,

the slightly elevated MIC_{90} (4 µg/mL) may indicate the presence of strains with reduced susceptibility, warranting further monitoring to detect early signs of resistance development. These results are consistent with previous studies on *M. hyopneumoniae* in pigs (Felde et al., 2018) and *M. bovis* in cattle (Barberio et al., 2016).

Oxytetracycline showed good activity overall (MIC_{50} = 0.25 µg/mL), but the detection of isolates with MIC values >32 µg/mL is concerning. This suggests the emergence of resistant subpopulations, which could compromise treatment efficacy if not addressed through targeted therapy or rotation of antimicrobials.

The wide range of MIC values observed for **tiamulin** (≤0.008 to >16 µg/mL) highlights significant heterogeneity among isolates. This variability may reflect genetic differences in drug target sites or efflux mechanisms and underscores the need for individualized susceptibility testing prior to treatment. Similarly, **lincomycin** exhibited a bimodal distribution, with some isolates fully susceptible and others highly resistant (MIC >32 µg/mL), suggesting the coexistence of distinct resistance phenotypes within the population, resembling the **bimodal susceptibility** seen in *M. bovis* (Barberio et al., 2016).

Enrofloxacin presented a dichotomous profile, with several isolates showing low MICs (0.25 µg/mL) and others exhibiting high-level resistance (MIC >16 µg/mL). This pattern is indicative of acquired resistance mechanisms, such as mutations in DNA gyrase or efflux pump activation, which have been documented in other *Mycoplasma* species. The presence of resistant strains calls for cautious use of fluoroquinolones and emphasizes the importance of resistance screening.

In contrast, erythromycin and spiramycin showed limited efficacy, with elevated MIC_{90} values (32 µg/mL and >16 µg/mL, respectively). These findings suggest that these macrolides may no longer be suitable for empirical treatment and should be reserved for cases with confirmed susceptibility (Paterna et al., 2016, Gautier-Bouchardon, 2018). Overall, tylosin, tilmicosin, spectinomycin, and florfenicol emerged as the most active agents against the tested *Mycoplasma* isolates. However, the observed variability in susceptibility to erythromycin, spiramycin, lincomycin, and enrofloxacin underscores the importance of ongoing antimicrobial surveillance and susceptibility profiling. These measures are essential to guide effective treatment strategies, prevent therapeutic failures, and mitigate the spread of resistance.

5.0. Conclusion

In summary, *Mycoplasma* spp. is an emerging and often underrecognized bacterium in donkeys, capable of causing a range of clinical diseases from respiratory infections to systemic and reproductive issues. The observed prevalence, genetic variability, and association with suboptimal biosecurity practices highlight the need for increased awareness and targeted interventions. Further research is essential to clarify its clinical impact, transmission routes, and potential reproductive consequences in donkey populations. Continued surveillance, advanced molecular diagnostics, and improved management practices are key to protecting donkey health and preventing the spread of *Mycoplasma* spp. infections.

Furthermore, evaluating MIC data is pivotal for monitoring AMR. The authors provided for the first-time evidence for the AMR risk in mycoplasmas of the genital tract of donkeys. Therefore, risk awareness when administering antimicrobials to animals and to take the necessary precautions when handling animals and their products are recommended.

CRediT authorship contribution statement

M.SH M Yusuf: Visualization, Investigation, Data curation, Writing – original draft, Formal analysis. **M. Burgio:** Sampling collection, Data curation. **Aiudi G.G:** Data curation. **Bermejo J.V. Delgado:** Data curation. **López C. Rivas:** Data curation. **MS Lucente:** Investigation. **F. Pellegrini:** Formal analysis. **M. Losito** Data curation. **M. Corrente**: Formal analysis. **M. Tempesta**: review & editing. **G. Greco:** Study design, Investigation, Writing – original draft, Formal analysis, Writing – review & editing, Supervision.

Conflict of interest

The authors declare that they have no conflict of interest or personal relationships that could have appeared to influence the work reported in this paper.

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Fig. 1. Shape files of study locations were obtained from the QGIS online map tools and imported for visualization into QGIS version 3.36 Maidenhead.

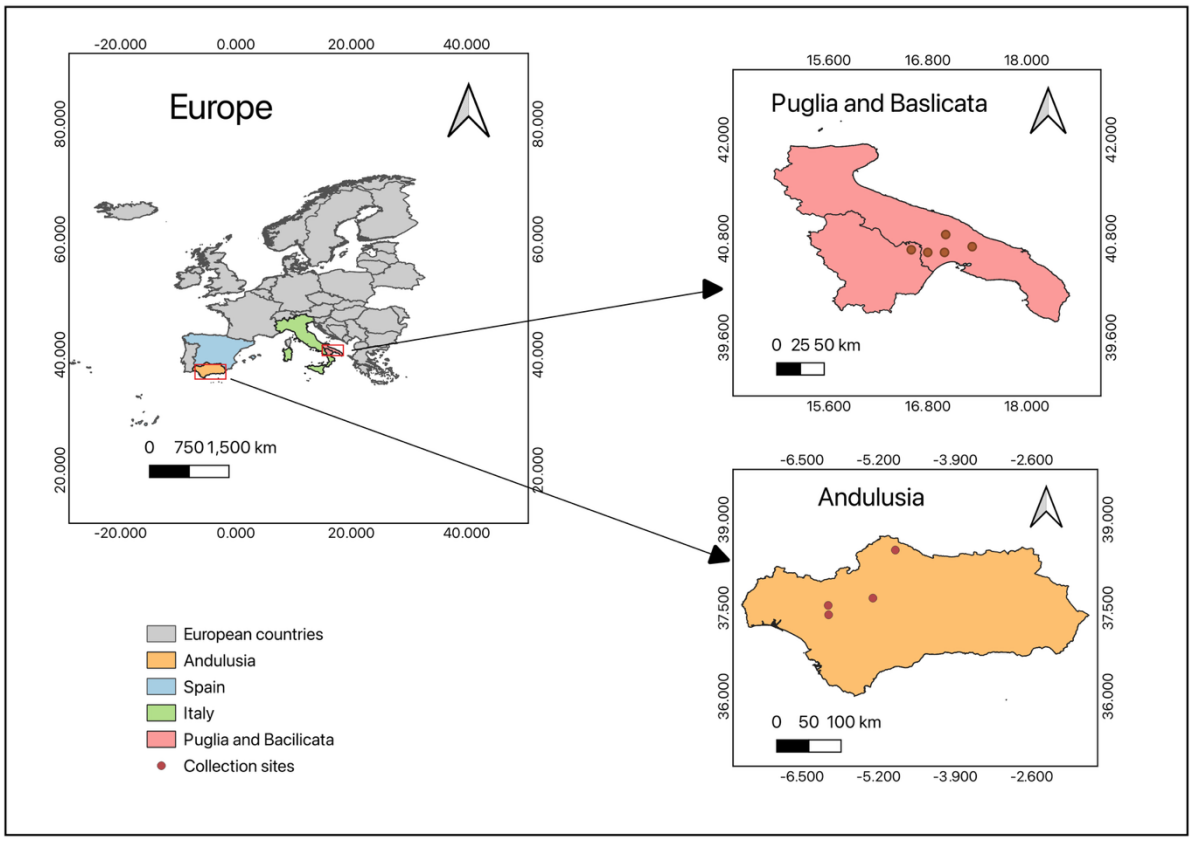

TABLE 1: Primers and amplification conditions used for the detection of *Mycoplasma* spp. from genital mucosal surfaces of donkeys.

Target gene	Primer name	Primer sequence	Amplification conditions (x40)	Product size (bp)	Reference
16S	GPO-3-F	5'GGGAGCAAACAG GATTAGATACCC T 3'	94 °C 10 min, 94 °C 1min, 52 °C 1 min, 72 °C 1 min, 72 °C 10 min	280 bp	Van Kuppeveld et al. 1994
	MGSO-R	5'TGC ACC ATC TGT CAC TCT GTT AAC CTC 3"			Van Kuppeveld et al. 1992
16S + ITS + 23S	27 F	AGAGTTTGATCCTG GCTCAG	94 °C 10 min, 94 °C 1min, 50 °C 1 min, 72 °C 1 min, 72 °C 10 min	2300	Volokhof et al., 2019
	23s R	TNCTTTTCACCTTTC CCTCACGGTAC			
Universal *rpo*B	rpoB-FMYC	AGTTATCACAATTTA TGGATCAAA	94 °C 10 min, 94 °C 1min, 52 °C 1 min, 72 °C 1 min, 72 °C 10 min	1700-2000	Volokhof et al., 2007
	rpoB-R-MYC	GCTCAHACTTCCATT TCHCCAAA			
*gyr*B	gyrB-FMYC	AAAACGWCCAGGK ATGTATATTGG	94 °C 10 min, 94 °C 1min, 52 °C 1 min, 72 °C 1 min, 72 °C 10 min	1700-2000	Volokhof et al., 2007
	gyrB-R-MY	GGATCCATTGTTGTT TCTCATAATTG			

Table 2: Characteristics of Martina Franca (Italy) and Andalusian (Spain) donkeys (n.120) enrolled in the study

Locality	Donkeys from Italy n (%)	Donkeys from Spain n (%)
Donkeys	73 (60.8%)	47 (39.2%)
Age		
Median/mode age (range) Years	5/5 (1-30)	7/8 (1-20)
≤4 (Young)	27 (37)	13(27.7)
>4 (Adult)	46 (63)	34 (72.3)
Gender		
Female	65 (89)	41 (87.2)
Male	8 (11)	6 (12.8)

Table 3: Prevalence of *M. equigenitalium* and *M. subdolum* in donkeys.

Pathogen	Total	PCR Positive (%)	95% CI	Culture Positive (%)	95% CI
M. equigenitalium	120	31(25.83)	18.00, 33.67	15 (12.50)	6.58, 18.42
Mixed infectious	120	1(0.833)	0.000, 2.460	1(0.833)	0.000, 2.460

Table 4: Sequencing results of *Mycoplasma* spp. positive Donkeys, including the closest relative sequence (query cover 93.61-100) and identity results (ID %) for ITS, *gyr*B and *rpoB* loci, number (n.) and original of findings.

No of samples and farms		Sample ID	Origin	Closest relative DNA sequences (Accession No)					
				16S rRNA, ITS, 23S rRNA	ID %	***gyr*B**	ID %	***rpo*B**	ID %
1	F1	318-24-1	Lamacarvotta (TA), ITA	*M. equigenitalium* (CP101808)	99.5%	*M. equigenitalium* (CP101808)	99.87%	M. equigenitalium (DQ219494)	99.71%
2	F2	120-24-8	Ferradina (MT), ITA	M. equigenitalium (CP101808)	99.5%	*M. equigenitalium* (CP101808)	99.87%	*M. equigenitalium* (CP101808)	99.6%
3	F2	120-24-10	Ferradina (MT), ITA	*M. equigenitalium* (CP101808)	99.5%	*M. equigenitalium* (CP101808)	99.87%	*M. equigenitalium* (CP101808)	99.6%
4	F2	120-24-13	Ferradina (MT), ITA	*M. equigenitalium* (CP101808)	99.5%	*M. equigenitalium* (CP101808)	99.87%	*M. equigenitalium* (CP101808)	99.6%
5	F2	120-24-16	Ferradina (MT), ITA	*M. equigenitalium* (CP101808)	99.5%	*M. equigenitalium* (CP101808)	99.87%	*M. equigenitalium* (DQ219494)	99.6%
6	F2	120-24-17	Ferradina (MT), ITA	*M. equigenitalium* (CP101808)	99.5%	*M. equigenitalium* (CP101808)	99.87%	*M. equigenitalium* (CP101808)	99.6%
7	F2	120-24-18	Ferradina (MT), ITA	*M. equigenitalium* (CP101808)	99.5%	*M. equigenitalium* (CP101808)	99.87%	*M. equigenitalium* (DQ219494)	99.6%
8	F3	109-23-1A	La Longhiera (BA), ITA	*M. equigenitalium* (CP101808)	99.5%	*M. equigenitalium* (CP101808)	99.87%	*M. equigenitalium* (DQ219494)	99.6%
9	F3	109-23-1B	La Longhiera (BA), ITA	*M. subdolum* (CP137846)	100%	*M. subdolum* (CP137846)	93.61%		
10	F4	348-24-110	Mottola (TA), ITA	*M. equigenitalium* (CP101808)		*M. equigenitalium* (CP101808)	99.87%	*M. equigenitalium* (CP101808)	99.6%
11	F4	348-24-116	Mottola (TA), ITA	*M. equigenitalium* (CP101808)	99.5%	*M. equigenitalium* (CP101808)	99.87%	*M. equigenitalium* (DQ219494)	99.6%

Table 5: Single Nucleotide Polymorphisms (Nr. of SNPs) for each *locus* based on the sequence analysis of donkey samples compared to the reference strains

Target gene and farms	Strain	GenBank Accession Number	Homology (%)	Nucleotide Position								Total SNPs
16S rRNA, ITS, 23S rRNA				295	555	645	710	714-718	750-751	2170		
F1, F2, F4	MEG	PV987805	99.15%	C	T	C	T	TTTGA	AA	G		12
	Reference	CP101808		T	C	T	G	GG--G	--	A		
F3	MSD	PV987807	100%									
	Reference	CP137846										
				Nucleotide Position								
gyrB				447	978							
F1, F2, F4	MEG		99.87%	A	T							2
	Reference	CP101808		G	C							
F3	MSD		93.61%									
	Reference	CP137846										
				Nucleotide at Position								
rpoB				27	120	159	228	312	1002	1311	1449	
F1	MEG		99.71%	T	A	A	C	C	T	C	C	8
F2, F3, F4	MEG		99.6%	C	G	A	C	C	T	T	T	
	Reference	DQ219494		T	A	G	T	T	C	T	C	
Total												22

Legend: MEG: *Mycoplasmopsis equigenitalium*, MSD: *Metamycoplasma subdolum*

Figure 2. Phylogenetic tree based on concatenated partial sequences of the 16S rRNA, ITS, 23S rRNA, gyr*B*, and rpo*B* genes from *Mycoplasma* species. The tree was constructed using the UPGMA method with the Hasegawa-Kishino-Yano (HKY) substitution model in Geneious® 10.3.1 software. Bootstrap values (1,000 replicates) are shown at the nodes. GenBank accession numbers are provided for reference samples, with the sequence from *Mycoplasma arginini* used as an outgroup. The sequences generated in the present study are marked in bold.

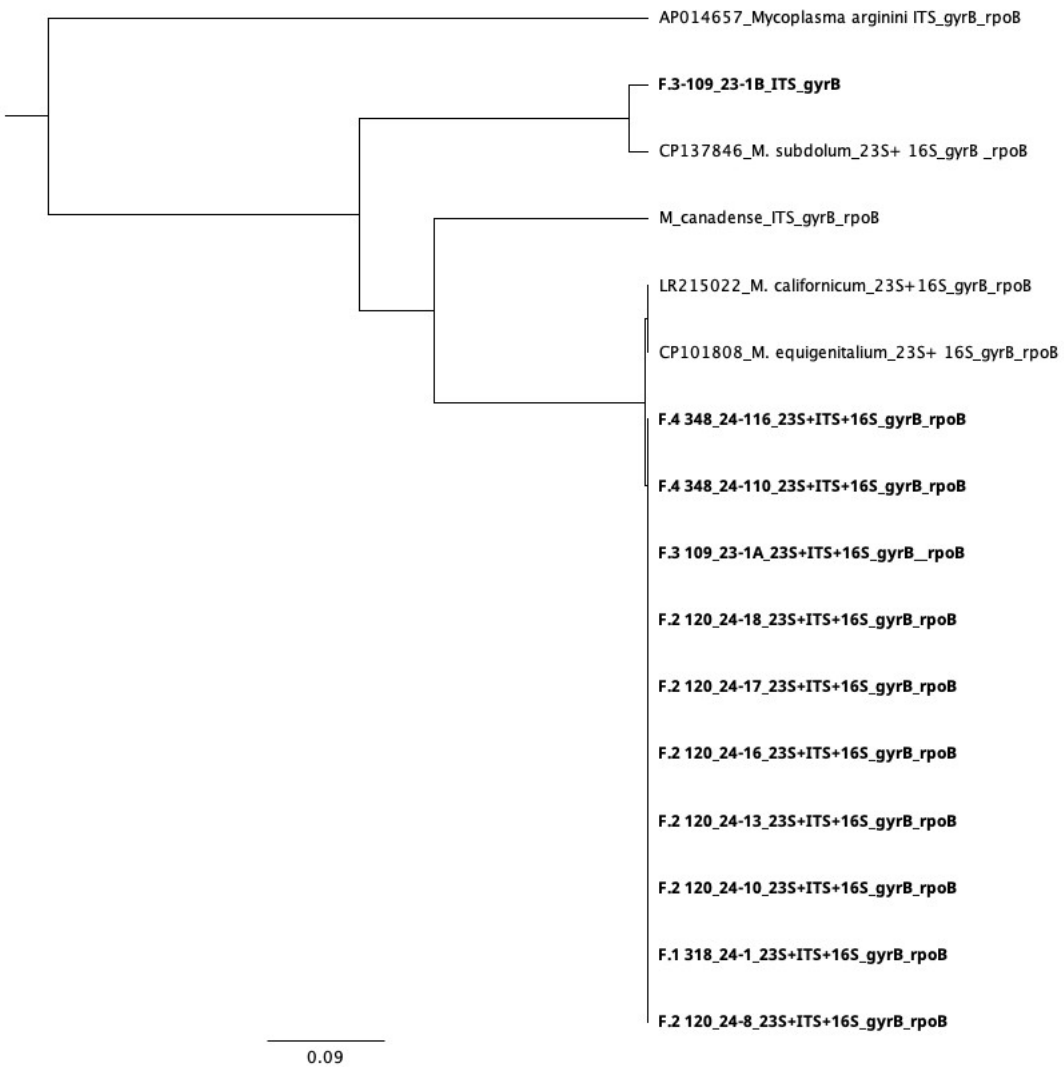

Table 6: Phenotypic antimicrobial susceptibility testing by MIC of *Mycoplasmopsis equigenitalium* isolates detected from reproductive tract swabs collected from healthy donkeys.

alco	**Range**	***M. equigenitalium* strains**							**MIC50**	**MIC90**
		20.24-11	**120.24-3**	**120.24-6**	**120.24-16**	**120.24-17**	**348.24-112**	**348.24-116**		
Macrolides										
Tilmicosin	0.03-64	0.5	0.25	0.25	2	1	0.5	0.12	0.5	2
Erythromycine	0.12-32	16	16	16	32	32	32	16	16	32
Spiramycin	0.5-16	>16	<=0.5	>16	>16	<=0.5	<=0.5	>16	>16	>16
Tylosin tatrate	0.015-64	0.12	0.12	0.06	0.25	0.25	0.25	0.12	0.12	0.25
Pleuromutilins										
Tiamulin	0.008-16	>16	<=0.008	>16	0.03	0.015	0.015	>16	0.03	>16
Lincosamide										
Lincomycin	0.05-32	>32	<=0.5	>32	>32	<=0.5	<=0.5	>32	>32	>32
Tetracyclines										
Oxytetracycline	0.12-32	>32	<=0.12	>32	0.25	0.25	0.25	0.25	0.25	>32
Aminocyclitol										

Spectinomycine	0.25-256	2	1	2	2	4	2	0.5	2	4
Fluoroquinolones										
Enrofloxacin	0.12-16	>16	0.25	>16	0.25	0.25	0.25	0.25	4	>16
Amphenicols										
Flortenicol	0.25-16	1	1	1	4	2	2	1	1	4

Table 7: Association between variables and *Mycoplasma* spp. status with corresponding chi square ($\chi 2$), *P*-value, odds ratio (OR) and 95% confidence interval (CI). Significant values are in bold.

Variable	Category	Total	Positive n (%)	95% CI	X2	*P*-value	OR	95% CI
Mycoplasma Spp.								
Country								
	Italy	73	18 (24.7)	14.77,34.55	0.134	0.7138		
	Spain	47	13 (27.7)	14.87,40.45				
Age								
	≤4	40	9 (22.5)	9.56, 35.44	0.348	0.5553		
	>4	80	22 (27.5)	17.72,37.28				
Gender								
	Male	14	0	0				
	Female	106	31(25.83)	18.00,33.67				
Breed								
	Martina franca	73	18 (24.7)	14.77,34.55	0.134	0.7138		
	Andalusian	47	13 (27.7)	14.87,40.45				
Biosecurity measure								
	Adopted	37	3(8.11)	0.00, 16.90	8.772	**0.0031**	**0.173**	0.0541, 0.5555
	Not adopted	83	23(33.73)	23.56,43.91	Ref			

Supplementary Table 1: Characteristics of the *Mycoplasma* species detected on the genital mucosa of donkeys through multiple diagnostic methods.

Number	ID	Country ESP(Spain) ITA(Italy)	Gender M (male) F (female)	Age	Culture (Hayflick)	c-PCR for 16S	c-PCR for ITS	c-PCR for *rpo*B	c- PCR for *gyr*B	Phenotypical antibiotics resistance
1	1-109/23	ITA	F	4	grow	pos	pos	pos	pos	
2	2-109/23	ITA	F	2	--	--	--	--	--	
3	1-227/23	ITA	F	3	--	--	--	--	--	
4	2-227/23	ITA	M	4	--	--	--	--	--	
5	1-292/23	ITA	F	15	--	pos	--	--	--	
6	2-292/23	ITA	F	3	--	--	--	--	--	
7	3-292/23	ITA	F	12	--	--	--	--	--	
8	4-292/23	ITA	F	3	--	--	--	--	--	
9	5-292/23	ITA	F	5	--	--	--	--	--	
10	6-292/23	ITA	F	10	--	--	--	--	--	
11	7-292/23	ITA	F	8	--	--	--	--	--	
12	8-292/23	ITA	F	8	--	--	--	--	--	
13	9-292/23	ITA	F	15	--	--	--	--	--	
14	10-292/23	ITA	M	1	--	--	--	--	--	
15	11-292/23	ITA	F	4	--	--	--	--	--	
16	12-292/23	ITA	F	15	--	--	--	--	--	
17	13-292/23	ITA	F	1	--	--	--	--	--	
18	14-292/23	ITA	M	20	--	--	--	--	--	
19	15-292/23	ITA	F	4	--	--	--	--	--	
20	16-292/23	ITA	F	9	--	--	--	--	--	
21	17-292/23	ITA	M	1	--	--	--	--	--	
22	18-292/23	ITA	F	30	--	--	--	--	--	
23	19-292/23	ITA	F	2	--	--	--	--	--	
24	20-292/23	ITA	F	12	--	--	--	--	--	
25	21-292/23	ITA	F	15	--	--	--	--	--	
26	22-292/23	ITA	F	8	--	--	--	--	--	
27	1-322/23	ITA	F	5	--	--	--	--	--	
28	2-322/23	ITA	F	6	--	--	--	--	--	
29	3-322/23	ITA	F	8	--	--	--	--	--	
30	4-322/23	ITA	F	7	--	--	--	--	--	
31	5-322/23	ITA	F	8	--	--	--	--	--	
32	6-322/23	ITA	F	5	--	--	--	--	--	
33	7-322/23	ITA	F	6	--	--	--	--	--	
34	8-322/23	ITA	F	9	--	--	--	--	--	
35	9-322/23	ITA	F	8	--	--	--	--	--	
36	10-322/23	ITA	F	7	--	--	--	--	--	
37	11-322/23	ITA	F	5	--	--	--	--	--	
38	1-563/23	ESP	F	3	--	--	--	--	--	
39	2-563/23	ESP	F	9	--	pos	--	--	--	
40	3-563/23	ESP	F	10	--	pos	--	--	--	
41	4-563/23	ESP	F	14	--	--	--	--	--	
42	5-563/23	ESP	F	8	--	--	--	--	--	
43	6-563/23	ESP	M	2	--	--	--	--	--	
44	7-563/23	ESP	F	17	--	pos	--	--	--	
45	8-563/23	ESP	M	8	--	--	--	--	--	
46	9-563/23	ESP	M	1	--	--	--	--	--	
47	10-563/23	ESP	F	2	--	--	--	--	--	
48	11-563/23	ESP	F	14	--	pos	--	--	--	
49	12-563/23	ESP	F	8	--	--	--	--	--	
50	13-563/23	ESP	F	7	--	pos	--	--	--	

51	14-563/23	ESP	F	8	--	pos	--	--	--	
52	15-563/23	ESP	F	4	--	--	--	--	--	
53	16-563/23	ESP	F	6	--	--	--	--	--	
54	17-563/23	ESP	F	5	--	--	--	--	--	
55	18-563/23	ESP	F	4	--	--	--	--	--	
56	19-563/23	ESP	F	1	--	--	--	--	--	
57	20-563/23	ESP	F	12	--	--	--	--	--	
58	21-563/23	ESP	F	10	--	--	--	--	--	
59	22-563/23	ESP	F	20	--	--	--	--	--	
60	23-563/23	ESP	F	6	--	--	--	--	--	
61	24-563/23	ESP	F	7	--	--	--	--	--	
62	25-563/23	ESP	F	8	--	pos	--	--	--	
63	26-563/23	ESP	F	5	--	--	--	--	--	
64	27-563/23	ESP	M	8	--	--	--	--	--	
65	28-563/23	ESP	F	8	--	--	--	--	--	
66	29-563/23	ESP	F	8	--	--	--	--	--	
67	30-563/23	ESP	F	6	--	--	--	--	--	
68	31-563/23	ESP	F	9	--	--	--	--	--	
69	32-563/23	ESP	F	11	--	pos	--	--	--	
70	33-563/23	ESP	F	5	--	pos	--	--	--	
71	34-563/23	ESP	F	5	--	--	--	--	--	
72	35-563/23	ESP	F	1	--	pos	--	--	--	
73	36-563/23	ESP	F	5	--	--	--	--	--	
74	37-563/23	ESP	M	5	--	pos	--	--	--	
75	38-563/23	ESP	F	6	--	pos	--	--	--	
76	39-563/23	ESP	F	15	--	pos	--	--	--	
77	40-563/23	ESP	F	6	--	--	--	--	--	
78	41-563/23	ESP	F	10	--	--	--	--	--	
79	42-563/23	ESP	M	10	--	--	--	--	--	
80	43-563/23	ESP	F	1	--	--	--	--	--	
81	44-563/23	ESP	F	1	--	--	--	--	--	
82	45-563/23	ESP	F	1	--	--	--	--	--	
83	46-563/23	ESP	F	1	--	--	--	--	--	
84	47-563/23	ESP	F	2	--	--	--	--	--	
85	1-120/24	ITA	F	2	grow	pos	--	--	--	
86	2-120/24	ITA	F	3	--	--	--	--	--	
87	3-120/24	ITA	F	3	grow	pos	--	--	--	
88	4-120/24	ITA	M	1	--	--	--	--	--	
89	5-120/24	ITA	M	1	--	--	--	--	--	
90	6-120/24	ITA	F	1	grow	pos	--	--	--	
91	7-120/24	ITA	F	10	--	--	--	--	--	
92	8-120/24	ITA	F	6	grow	pos	pos	pos	pos	
93	9-120/24	ITA	F	11	--	--	--	--	--	
94	10-120/24	ITA	F	11	grow	pos	pos	pos	pos	
95	11-120/24	ITA	M	2	--	--	--	--	--	
96	12-120/24	ITA	F	1	--	--	--	--	--	
97	13-120/24	ITA	F	1	grow	pos	pos	pos	pos	
98	14-120/24	ITA	F	2	--	--	--	--	--	
99	15-120/24	ITA	F	5	grow	pos	--	--	--	
100	16-120/24	ITA	F	2	grow	pos	pos	pos	pos	
101	17-120/24	ITA	F	7	grow	pos	pos	pos	pos	
102	18-120/24	ITA	F	3	grow	pos	pos	pos	pos	
103	19-120/24	ITA	F	8	--	--	--	--	--	
104	1-318/24	ITA	F	5	--	pos	pos	pos	pos	
105	2-318/24	ITA	F	5	--	pos	--	--	--	
106	3-318/24	ITA	F	5	--	--	--	--	--	
107	4-318/24	ITA	F	5	--	--	--	--	--	

108	1-348/24	ITA	F	14	--	--	--	--	--	
109	2-348/24	ITA	F	17	--	--	--	--	--	
110	3-348/24	ITA	F	16	grow	pos	--	--	--	
111	4-348/24	ITA	F	12	--	--	--	--	--	
112	5-348/24	ITA	F	3	grow	pos	pos	pos	pos	
113	6-348/24	ITA	F	17	--	--	--	--	--	
114	7-348/24	ITA	F	5	--	--	--	--	--	
115	8-348/24	ITA	M	5	--	--	--	--	--	
116	9-348/24	ITA	F	16	grow	pos	pos	pos	pos	
117	10-348/24	ITA	F	15	grow	pos	--	--	--	
118	11-348/24	ITA	F	12	--	--	--	--	--	
119	12-348/24	ITA	F	1	--	--	--	--	--	
120	13-348/24	ITA	F	1	--	--	--	--	--	

CHAPTER 2

Occurrence and risk factors of *Mycoplasmopsis* spp. in canine reproductive tracts in Italy

PREVALENCE OF MYCOPLASMA CANIS AND CYNOS IN DOGS AFFECTED WITH REPRODUCTIVE FAILURES, ITALY

Yusuf M.SH M. (1), Burgio M. (1), Cicirelli V. (1), Mrenoshki D. (1), Tempesta M. (1), Buonavoglia D. (1), Aiudi G.G. (1), Greco G. (1)

(1) Department Of Veterinary Medicine - University of Bari

Corresponding Author: **Giulio Guido Aiudi** - giulioguido.aiudi@uniba.it

The genus Mycoplasma includes cell wall less bacteria that affect different animal species including carnivores (Razin et al., 1983, Chalker 2005). In dogs, Mycoplasma spp. infections are associated, either exclusively or in co-infection, with viral or bacterial agents, with multiform clinical signs such as complex respiratory disease (CIRD), anaemia, arthritis. (Chalker 2005). Previous studies described the presence of Mycoplasma spp. in the genital tract of both male and female dogs including healthy or unhealthy dogs (Doig et al., 1981). In particular, several Mycoplasma species have been isolated from the prostate, epididymis and bladder in dogs with urogenital diseases and/or hypofertility (LAbee-Lund et al., 2003). Currently, in Italy there are no studies describing the presence and impact of Mycoplasma species on the reproductive sphere. Our study aims to investigate the occurrence and the risk factor analyses of Mycoplasma spp. in canine populations affected with low reproductive efficiency.

Sixty-eight dogs, 25 males and 43 females, were included in the analyses. For each animal genital samples were screened for Mycoplasma canis and Mycoplasma cynos by using molecular methods (cPCR). Samples were submitted for the DNA extraction and analysed by using two specific cPCR assays. Out of 68 tested animals, Mycoplasma spp. DNA was detected in 29 dogs (42.65%, CI 95%: 30.89-54.40) being 13 (19.12%, CI 95%: 9.77-28.46) males and 16 (23.53%, CI 95%: 13.45-33.61) females. When considering Mycoplasma species, M. cynos (18/68, 26.47%, CI 95%: 8.59-26.71) was more frequently detected compared to M. canis (11/68, 16.18%, CI 95%: 7.42-24.93) with males more at risk of M. cynos infection (OR: 2.916). Furthermore, animals affected with reproductive impairment were four times more likelihood infected than healthy animals (OR: 4.34).

The present study describes for the first time M. canis and M. cynos in dogs in Italy describing a possible link between the presence of Mycoplasma spp. infection and the impairment of canine reproductive efficiency, thus supporting the need to adopt appropriate prevention measures

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CHAPTER 2

Occurrence and risk factors of *Mycoplasmopsis* spp. in canine reproductive tracts in Italy

Yusuf M. Sh.M.[1],[2], Aiudi G.G.[1], Burgio M. [1], Sasso R.[1], Cicirelli V. [1], Buonavoglia D. 1, Decaro N.[1], Tempesta M. [1], Martella V [1], Greco G.[1].

[1]Department Of Veterinary Medicine - University of Bari "Aldo Moro", Bari

[2] Department of Veterinary Medicine and Animal Husbandry - Somali National University, Mogadishu, Somalia.

ABSTRACT

The genus *Mycoplasmopsis* includes cell-wall-less bacteria that affect a wide range of animal species, including carnivores (Razin et al., 1983). In dogs, mycoplasmas, either as primary pathogens or in conjunction with viral or bacterial agents, have been associated with various clinical conditions, such as canine infectious respiratory disease (CIRD), anaemia, and arthritis (Chalker, 2005). These microorganisms have been previously detected in the genital tracts of both clinically healthy and diseased male and female dogs (Doig et al., 1981). Moreover, several species have been isolated from the prostate, epididymis, and bladder of dogs presenting with urogenital disorders and/or subfertility (L'Abee-Lund et al., 2003). To date, no studies have documented the presence or pathological significance of *Mycoplasma* spp. in the canine reproductive system within Italy. The present study aimed to assess the occurrence of *Mycoplasma* spp. and evaluate associated risk factors, with particular emphasis on their potential impact on reproductive performance. Genital samples were collected from 68 dogs (25 males and 43 females) and analyzed using conventional polymerase chain reaction (cPCR). *Mycoplasma* spp. DNA was detected in 26 dogs (38.24%; 95% CI: 26.68–49.79), comprising 13 males (52%; 95% CI: 32.42–71.58) and 13 females (30.23%; 95% CI: 16.51–43.96). *M. cynos* was the most frequently detected species (22%; 95% CI: 12.20–31.91), followed by *M. canis* (11.76%; 95% CI: 4.11–19.42). Mixed infections were observed in 4.4% of cases. Statistical analysis revealed that male dogs were significantly more likely to be infected with *M. cynos* ($p = 0.006$; OR: 5.07). Furthermore, dogs exhibiting reproductive impairments were three times more likely to test positive for *Mycoplasma* spp. compared to clinically healthy individuals ($p = 0.04$; OR: 3.38).

This study represents the first report of *M. canis* and *M. cynos* in the canine population in Italy and suggests a potential association between *Mycoplasma* spp. infection and compromised reproductive efficiency. These findings underscore the importance of implementing targeted diagnostic and preventive strategies in breeding and clinical settings.

Keywords: *Metamycoplasmatacea spp., Mycoplasmopsis canis*, *Mycoplasmopsi cynosis,* reproductive tract.

1. Introduction

Mycoplasmas are the smallest known prokaryotic organisms, characterized by the absence of a cell wall and a typically strict host and tissue specificity (Razin, Yogev, and Naot, 1998). In dogs, various *Mycoplasma* species can colonize the mucosal surfaces of the respiratory and urogenital tracts, as well as the auditory canal (Chalker, 2005). To date, 14 *Mycoplasma* species have been isolated from dogs (May and Brown, 2009; Spergser et al., 2011; Chalker, 2005). *Mycoplasmopsis cynos*, first identified over 40 years ago (Rosendal, 1973), is considered the most pathogenic among canine *Mycoplasma* spp. (Chalker, 2005; Zeugswetter et al., 2007). While *Mycoplasmopsis canis* can infect multiple mammalian hosts including humans and calves, *M. cynos* appears to be host-specific to dogs (Chalker, 2005).

M. canis is frequently associated with reproductive disorders, including infertility, mucopurulent discharges, cystitis, epididymitis, and prostatitis in males, as well as uterine pathologies in females (Chalker, 2005; Doig et al., 1981; Jambhekar et al., 2019; L'Abee-Lund et al., 2003; Ülgen et al., 2006; Barreto et al., 2022).

Among the *Mycoplasma* species affecting dogs, *M. cynos* is primarily linked to respiratory disease. It often acts synergistically with other bacterial and viral pathogens within the Canine Infectious Respiratory Disease (CIRD) complex, exacerbating clinical symptoms (Decaro et al., 2016; Jambhekar et al., 2019).

Transmission of *Mycoplasma* spp. can occur both vertically and horizontally, primarily through direct contact with secretions or contaminated fomites (Alves et al., 2023). Semi-intensive breeding environments facilitate the spread of infection due to close animal proximity (Fox,

2012; Macedo et al., 2018). Additionally, commercial dog trade in kennels increases direct and indirect contact, elevates stress levels, and contributes to the circulation of diverse pathogens. These factors, along with fluctuations in host population size, influence colonization dynamics and infection risk (Pesavento and Murphy, 2014).

Accordingly, the aim of this study was to determine the prevalence of *M. canis* and *M. cynos*, and to identify associated risk factors in dogs presenting with reproductive disorders.

2. Material and methods

2.1. Animal and study area

This study was conducted in the Puglia (40.7928° N, 17.1012° E) and Basilicata (40.6431° N, 15.9700° E) regions of southern Italy, both characterized by a Mediterranean climate with hot, dry summers and mild, wet winters. Geospatial data for the study sites were obtained from GADM online tools and visualized using Quantum Geographic Information System (QGIS) software, version 3.36.0. A cross-sectional convenience sampling approach was employed, including 48 dogs presenting with reproductive failures at veterinary clinics and 20 kennel-housed, mixed-breed stray dogs without reproductive issues, serving as controls. The minimum sample size (n = 68) was calculated based on a 95% confidence level, 10% margin of error, and an assumed infection prevalence of 50% (Thrusfield, 2007). Between January 2017 and May 2023, clinical and demographic data (gender, age, breed, and locality) were recorded for each animal. Genital swab samples were collected for diagnostic purposes, placed in sterile tubes containing DMEM transport medium, and transported under cold chain conditions to the Infectious Diseases Unit, Department of Veterinary Medicine, University of Bari. Samples were stored at −40 °C until further analysis.

2.2. Ethical Statement

All procedures involving animal care were conducted in accordance with relevant authority guidelines. Genital samples were collected fat the time of clinical visit from sick and from apparently healthy breeding animals with the prior consent of owners and under the ethical

approval of the Animal Ethics Committee of the Department of Veterinary Medicine, University of Bari (Protocol No. 1609-III/13; Approval No. 14/2023).

2.3. Mycoplasma spp. Cultivation and Isolation

Cultivation was performed directly on Hayflick agar plates enriched with equine serum, as described by Tully and Razin (1983), and incubated at 37 °C under microaerophilic conditions for 7 days. Colonies exhibiting the characteristic "fried egg" morphology, indicative of *Mycoplasma* spp., were subsequently subjected to molecular analysis for species identification.

2.4. Molecular Investigation

2.4.1. DNA Extraction

Genomic DNA for *Mycoplasma* spp. PCR was extracted from 200 µL of genital swab samples and from Hayflick agar plates when visible colonies were present. The IndiSpin® Pathogen Kit (Indical Bioscience GmbH, Leipzig, Germany) was used according to the manufacturer's instructions. DNA was eluted in 100 µL of AE buffer and stored at −40 °C until use.

2.4.2. Polymerase Chain Reaction (PCR) for Detection of Mycoplasma spp.

A set of generic primers—forward primer GPO and reverse primer MGSO—was used to amplify a fragment of *Mycoplasma* spp. DNA (Van Kuppeveld et al., 1992, 1994). PCR was performed in a 25 µL reaction mixture containing 14.3 µL of nuclease-free water, 3 µL of $MgCl_2$, 2.5 µL of 10× PCR Buffer II, 2.5 µL of dNTPs, 0.2 µL of Taq Gold DNA polymerase, 0.25 µL (50 pmol) of each primer, and 2 µL of DNA template (sample, positive control, or negative control using 2 µL of water) (Table 1).

To identify *Mycoplasma canis* and *Mycoplasma cynos* species, two species-specific PCR assays targeting the ITS regions were performed as described by Chalker et al. (2004). Clinical isolates were compared with reference strains of *M. canis* and *M. cynos*. Briefly, the forward primer Myc1, common to both species, was used in combination with species-specific reverse primers (Table 1).

2.5 Data management and Statistical analysis

3. Statistical Analysis

The recorded data were entered into Microsoft Excel® spreadsheets, and the prevalence of *Mycoplasma* spp. was calculated. Pearson's chi-square test or Fisher's exact test was applied, as appropriate, to compare differences in proportions and assess associations between potential risk factors and reproductive disorders in dogs. A 95% confidence interval (CI) was used for all statistical analyses, which were performed using WinEpi software (http://winepi.net/).

3.1. Description of the Animal Population

The characteristics of the study population are summarized in Table 2. Among the 48 breeding dogs, 35 (72.9%) were female and 13 (27.1%) were male. Ages ranged from 12 to 96 months (median: 28 months; mode: 12 months). All breeding dogs were purebred, including German Shepherd (n = 4), Australian Shepherd (n = 3), Boston Terrier (n = 4), Rhodesian Ridgeback (n = 5), English Bulldog (n = 1), Golden Retriever (n = 12), Bracco Italiano (n = 12), Maltese (n = 1), Miniature Pinscher (n = 1), and Belgian Shepherd (n = 5). Most dogs were from Puglia (n = 43, 89.6%), with a smaller number from Basilicata (n = 5, 10.4%).

The breeding dogs included in this study had experienced reproductive problems within the previous year. Specifically, bitches that failed to conceive after at least two matings with proven fertile males, and males that had ineffective matings with at least two proven fertile bitches, were enrolled. Each dog underwent a comprehensive clinical evaluation, including physical examination and ultrasonographic examination of the reproductive tract. All breeding dogs were confirmed to be clinically healthy.

Among the 20 rescued stray dogs, 12 (60%) were male and 8 (40%) were female. Their ages ranged from 12 to 156 months (median: 90 months; mode: 120 months). All were mixed-breed dogs from Puglia. No reproductive abnormalities were detected during the veterinary examinations conducted upon their admission to the shelter (Table 2).

3.2. *Mycoplasma* spp. Detection

Out of the 68 dogs tested, 26 (38.24%; 95% CI: 26.68–49.79) were positive for *Mycoplasma* spp. (Table 3). Among the identified species, *Mycoplasma cynos* was the most prevalent, detected in 15 dogs (22.06%; 95% CI: 12.20–31.91), followed by *Mycoplasma canis* in 8 dogs (11.76%; 95% CI: 4.11–19.42). Co-infection with both *M. canis* and *M. cynos* was observed in 3 dogs (4.41%; 95% CI: 0.00–9.29) (Table 3).

3.3. Risk Factor Analysis

Purebred dogs were significantly more likely to be infected with *Mycoplasma* spp. compared to mixed-breed dogs ($P = 0.04$; OR = 3.38; 95% CI: 0.91–5.80) (Table 4). Lifestyle and clinical condition were also significantly associated with *Mycoplasma* spp. infection ($P < 0.05$), as was the geographical origin of the dogs ($P < 0.05$).

Regarding *M. cynos* infection specifically, male dogs were at significantly higher risk than females ($P = 0.006$; OR = 5.07; 95% CI: 1.33–8.93) (Table 4).

4. Discussion

This study is the first to report on the detection of *M. canis* and *M. cynos* in dogs from southern Italy and to explore their potential association with reproductive disorders. The overall prevalence of *Mycoplasma* spp. was 38.24%, indicating a relatively high occurrence in the studied population. *M. cynos* was the most frequently detected species (22.06%), followed by *M. canis* (11.76%), with co-infections observed in 4.41% of cases.

These findings contrast with those of Alves et al. (2023), who reported a higher prevalence of *M. canis* (29.7%; 33/111) and no detection of *M. cynos*. Similarly, other studies have reported lower detection rates of *M. cynos*. For instance, Hong and Kim (2012) identified *M. cynos* in only 5% (1/20) of Beagle dogs with respiratory disease, while Canonne et al. (2018) detected it in 6.67% (4/60) of dogs with eosinophilic bronchopneumopathy or chronic bronchitis and in

3.33% (2/60) of healthy dogs. Barreto et al. (2020) found *M. cynos* in only one of 15 dogs with respiratory symptoms, with *M. canis* and *M. edwardii* being more prevalent.

In our study, 45.83% (22/48) of dogs with reproductive disorders tested positive for *Mycoplasma* spp., compared to 20% (4/20) of healthy dogs. This difference was statistically significant ($P < 0.05$), with an odds ratio of 3.98, supporting a potential link between *Mycoplasma* infection and reproductive impairment. Similarly, Zheng et al. (2023) reported significant differences in the endometrial microbiome of dogs with pyometra compared to healthy controls ($P < 0.05$), with *Mycoplasma* among the most prevalent genera in diseased animals.

Gender also appeared to influence infection rates. In our study, 52% (13/25) of infected dogs were male, compared to 30.23% (13/43) of females. Specifically, *M. cynos* infection was significantly more common in males (40%) than in females (11.63%), with an odds ratio of 5.07 ($P = 0.0065$), suggesting that males may be more susceptible to *Mycoplasma* spp., particularly *M. cynos*.

The association between *Mycoplasma* infection and reproductive disorders is further supported by our finding that dogs with reproductive issues had a 3.38 times higher risk of infection compared to healthy controls. This highlights the potential role of *Mycoplasma* spp. in canine infertility.

Although the urogenital tract is a common site for *Mycoplasma* isolation, its role in disease remains unclear due to its presence in both healthy and affected animals (L'Abee-Lund et al., 2003; Maksimović et al., 2018; Ülgen et al., 2006). *M. canis* and *M. cynos* are among the most clinically relevant species, associated with urogenital infections, infertility, respiratory disease, and anemia. Currently, no vaccines are available for canine *Mycoplasma* spp., and inappropriate treatment may worsen infections. Moreover, the isolation of unidentified *Mycoplasma* species (Kirchner et al., 1999; Chandler and Lappin, 2002; Chalker and Brownlie, 2004; Chalker, 2005) suggests that novel species remain to be described, underscoring the need for further research into these understudied microorganisms.

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3. Conclusion

This study investigates the occurrence and potential impact of Mycoplasma spp. on reproductive efficiency in dogs in Italy. Genital samples from 68 dogs (25 males, 43 females) were analyzed using conventional PCR. Mycoplasma spp. DNA was detected in 38.24% of the animals, with M. cynos being the most prevalent species. Males were more likely to be infected with M. cynos (OR: 5.07), and dogs with reproductive impairments were three times more likely to be infected (OR: 3.38). This is the first report of M. canis and M. cynos in the reproductive tracts of dogs in Italy, suggesting a potential link to reduced reproductive efficiency.

CRediT authorship contribution statement

- **Yusuf M.SH.M** Visualization, Investigation, Data curation, Writing – original draft, Formal analysis. **Aiudi G.G:** study design and Data curation. **Burgio M.:** Data curation. **Sasso R.** Investigation. **Cicirelli V., Buonavoglia D., Martella V., Decaro N.: Buonavoglia D. and M. Tempesta:** review & editing. review & editing. **G. Greco:** Study design, Investigation, Writing – original draft, Formal analysis, Writing – review & editing, Supervision.

Conflict of interest

The authors declare that they have no conflict of interest or personal relationships that could have appeared to influence the work reported in this paper.

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and the research has been developed in the framework of the activities of the “Centro Interdipartimentale di Ricerca per la Cooperazione allo Sviluppo” (CPS) of the University of Bari, Italy.

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Fig. 1. Shape files of study locations were obtained from the QGIS online map tools and imported for visualization into QGIS version 3.36 Maidenhead.

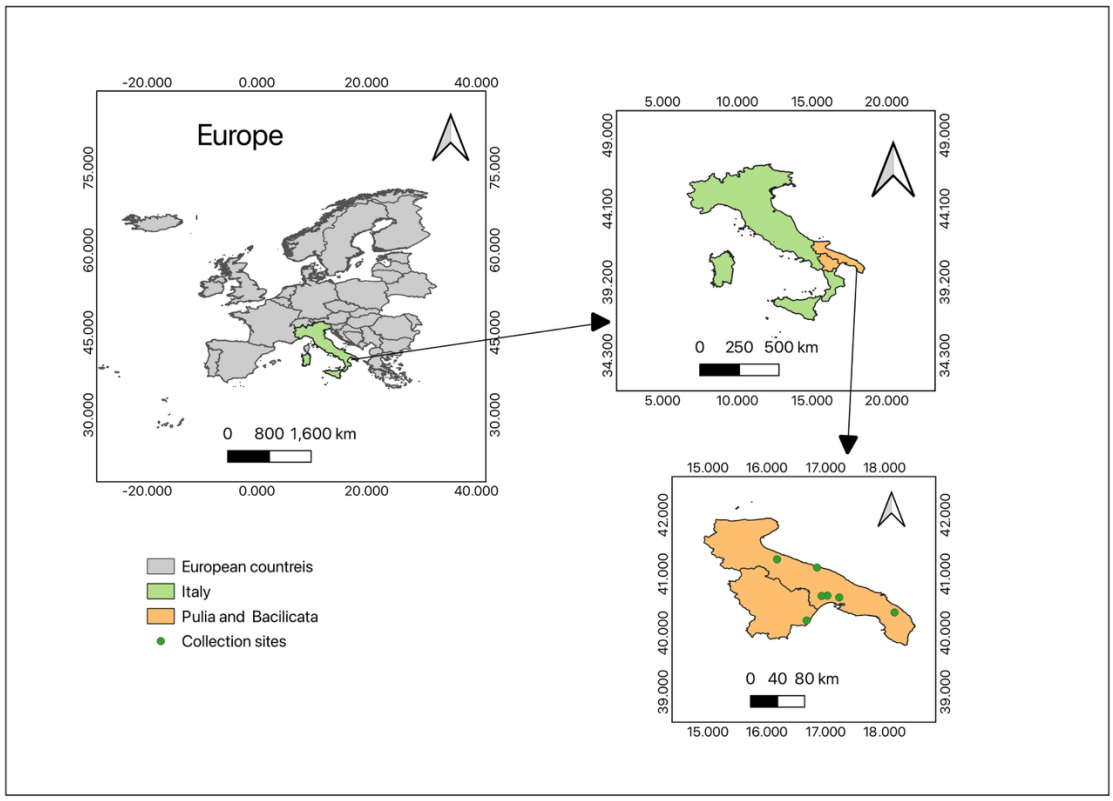

TABLE 1: Primers and amplification conditions for the detection of *M. canis* and *M. cynos,* from swabs of genital tract of dogs.

Target gene	Primer name	Primer sequence	Amplification conditions (x40)	Product size (bp)	Reference
16S	GPO-3-F	5'GGGAGCAAACAG GATTAGATACCC T 3'	94 °C 10 min, 94 °C 1min, 52 °C 1 min, 72 °C 1 min, 72 °C 10 min	1013	Van Kuppeveld et al. 1994
	MGSO-R	5'TGC ACC ATC TGT CAC TCT GTT AAC CTC 3"	94 °C 10 min, 94 °C 1min, 52 °C 1 min, 72 °C 1 min, 72 °C 10 min	1013	Van Kuppeveld et al. 1992
ITS	Myc1-F	5'CACCGCCCGTCACA CCA3'	94 °C 10 min, 94 °C 1min, 52 °C 1 min, 72 °C 1 min, 72 °C 10 min		Chalker et al. 2004
	M. canis-R	5'CTGTCGGGGTTATCT CGAC3'	94 °C 10 min, 94 °C 1min, 52 °C 1 min, 72 °C 1 min, 72 °C 10 min	247	
	M. *cynos*-R	5'GATACATAAACACAA CATTATAATATTG3'	94 °C 10 min, 94 °C 1min, 52 °C 1 min, 72 °C 1 min, 72 °C 10 min	227	

Table 2: Characteristics of breeding and rescued stray dogs investigated for *Mycoplasma* spp. Infection.

	Breeding dogs n (%)	Rescued stray dogs n (%)
Dogs	48(80)	20(20)
Age		
Median/mode age (months) (range)	28/24 (12-96)	90/120 (12-156)
Gender		
Female	35(72.9)	8(40)
Male	13(27.1)	12(60)
Breed		
Mixed	0	20(100)
Pure	48(100)	0
Shepherd dog	12(25)	
Golden retriever	12(25)	
Bracco Italian	12(25)	
Rhodesian ridgeback	5(10.4)	
Boston terrier	4(8.3)	
Bulldog	1(2.1)	
Maltese	1(2.1)	
Pinscher	1(2.1)	
Location		
Puglia	43(89.6)	20(100)
Basilicata	5 (10.4)	0
Clinical condition		
Healthy	0	20(100)
Unhealthy	48(100)	0

Table 3: Prevalence of *Mycoplasma* spp.in dogs.

Pathogen	Total	Positive (%)	95% CL
Mycoplasma spp	68	26 (38.24)	26.68-49.79
M. canis	68	8 (11.76)	4.11-19.42
M. cynos	68	15 (22)	12.20-31.91
Both *M. canis* and *M. cynos*	68	3 (4.4)	0.00-9.29

Table 4: Association between variables and *Mycoplasma* spp. status with corresponding chi square (χ2), *P*-value, odds ratio (OR) and 95% confidence interval (CI). Significant values are in bold.

Variable	Category	Total	Positive n (%)	95% CI	X2	*P*-value	OR	95% CI
Mycoplasma spp.								
Age								
	12-31	26	11 (42.3)	23.32, 61.30	0.659	0.7		
	32-51	18	9 (50)	26.90, 73.10				
	>52	24	9 (37.50)	18.13, 56.87	Ref.			
Gender								
	Male	25	13 (52)	32.42, 71.58	3.2	0.07		
	Female	43	13 (30.23)	16.51, 43.96	Ref.			
Breed								
	Pure	48	22 (45.83)	31.74, 59.93	**3.98**	**0.04**	3.38	**0.9052, 5.8020**
	Mixed	20	4 (20)	2.47, 37.53	Ref.			
Pure breed								
	shepherd dog	12	4 (28.57)	6.66, 60.01				
	Golden retriever	12	4 (28.57)	6.66, 60.01				
	Bracco Italian	12	4 (28.57)	6.66, 60.01	Ref.			
	Rhodesian ridgeback	5	4 (80)	44.94, 100.00	6.2	0.18		

	Boston terrier	4	4 (100)					
	Bulldog, Maltese, Pinscher	3	0					
Locality								
	Bari	29	15 (51.72)	33.54-69.91				
	Taranto (Crispiano and Mottola, Castellaneta)	32	7 (21.88)	7.55, 36.20				
	Other Provence (BAT and Lecce)	2	0	0				
	Matera (Policoro)	5	4 (80)	44.94, 100.00				
Lifestyle								
	Breeding farm	48	22 (45.83)	31.74, 59.93	**3.98**	**0.04**	3.38	**0.9052, 5.8020**
	Rescue stray dogs	20	4 (20)	2.47, 37.53	Ref.			
Clinical condition								
	Unhealth	48	22 (45.83)	31.74, 59.93	**3.98**	**0.04**	3.38	**0.9052, 5.8020**
	Health	20	4 (20)	2.47, 37.53	Ref.			
M. canis								
	Male	25	3 (12)	0.00, 24.74	Ref.			
	Female	43	5 (11.63)	2.05, 21.21	0.002	0.96		
Clinical condition								
	Un health	48	12 (25)	12.75, 37.25	3.65	0.056		
	Health	20	1 (5)	0.00, 14.55	Ref.			
Lifestyle								
	Breeding farm	48	12 (25)	12.75, 37.25	3.65	0.056		

	Rescue stray dogs	20	1 (5)	0.00, 14.55	Ref.			
M. cynos								
	Male	25	10 (40)	20.80, 59.20	7.4	**0.0065**	5.07	**1.3255, 8.9275**
	Female	43	5 (11.63)	2.05, 21.21	Ref.			
Clinical condition								
	Un health	48	10 (20.8)	9.34, 32.32	0.3	0.58		
	Health	20	3 (15)	0.00, 30.65	Ref.			
Lifestyle								
	Breeding farm	48	10 (20.8)	9.34, 32.32	0.3	0.58		
	Rescued stray dogs	20	3 (15)	0.00, 30.65	Ref.			
Both *M. canis* and *M. cynos*								
	Female	43	3(6.98)	0.00, 14.59				
	Male	25	0	0				

CHAPTER 3

Vector_borne pathogens including haemoplasmas in stray cat

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Parasites & Vectors

RESEARCH Open Access

Molecular survey on vector-borne pathogens in clinically healthy stray cats in Zaragoza (Spain)

Sergio Villanueva-Saz[1,2,3*], Marivi Martínez[1,2], Ard M. Nijhof[4,5], Bastian Gerst[4,5], Michaela Gentil[6], Elisabeth Müller[6], Antonio Fernández[1,2], Ana González[7], Mohamed Sh. Mohamud Yusuf[8], Grazia Greco[8], Maite Verde[1,2,3], Giovanni Sgroi[9], Delia Lacasta[1,2,3], Diana Marteles[1,2,3], Michele Trotta[1] and Ingo Schäfer[6*]

Abstract

Background In Europe, feline vector-borne infections are gaining importance because of the changing climate, expanding habitats of potential vectors and expanding pathogen reservoirs. The main objective of this study was to assess the prevalence of vector-borne pathogens (VBPs) in stray cats in Zaragoza, Spain, and to investigate potential risk factors for infection, including feline leukaemia virus (FeLV) and feline immunodeficiency virus (FIV).

Methods Blood samples from stray cats presented to the veterinary faculty in Zaragoza between February 2020 and 2022 were tested by polymerase chain reaction (PCR) for the presence of *Anaplasma phagocytophilum*, *Anaplasma platys*, *Bartonella henselae*, *Ehrlichia canis*, *Rickettsia* spp., haemotropic *Mycoplasma* spp., *Hepatozoon* spp., *Leishmania infantum*, piroplasms and microfilariae at the LABOKLIN laboratory. The cats were also tested for FeLV and FIV by PCR.

Results Nearly half of the cats (158/332, 47.6%) were positive for at least one VBP. *Hepatozoon* spp. were detected in 25.6%, haemotropic *Mycoplasma* spp. in 22.9%, *B. henselae* in 9.3% and *L. infantum* in 2.1% of the cats. Male sex had a statistically significant association with test results for haemotropic *Mycoplasma* spp. (odds ratio 1.38 [1.21;1.57]); regionality with *Hepatozoon* spp., *B. henseale* and FIV; and seasonality with *Hepatozoon* spp., haemotropic *Mycoplasma* spp., *L. infantum* and FeLV ($P \leq 0.05$ each). A strong positive correlation was reported for the amount of rainfall and the number of cats that tested positive for *Hepatozoon* spp. ($\rho = 753$, $P = 0.05$). None of the cats tested positive for *A. phagocytophilum*, *A. platys*, *E. canis*, *Rickettsia* spp., piroplasms, or microfilariae. Co-infections with multiple VBPs were detected in 56 out of 332 cats (16.9%). Thirty-one of the 332 cats included in the study (9.3%) tested positive for FeLV (6.9%) and for FIV (3.6%). In 20/31 cats (64.5%) that tested positive for FeLV/FIV, coinfections with VBP were detected ($P = 0.048$, OR 2.15 [0.99; 4.64]).

Conclusions VBPs were frequently detected in stray cats in Zaragoza. In particular, regionality and seasonality had a statistically significant association with PCR results for most VBPs included in the study.

*Correspondence:
Sergio Villanueva-Saz
svs@unizar.es
Ingo Schäfer
i.schaefer@laboklin.com
Full list of author information is available at the end of the article

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Methods Blood samples from stray cats presented to the veterinary faculty in Zaragoza between February 2020 and 2022 were tested by polymerase chain reaction (PCR) for the presence of *Anaplasma phagocytophilum, Anaplasma platys, Bartonella henselae, Ehrlichia canis, Rickettsia* spp*., haemotropic Mycoplasma* spp., Hepatozoon spp., *Leishmania infantum, piroplasms* and microfilariae at the LABOKLIN laboratory. The cats were also tested for FeLV and FIV by PCR.

Results Nearly half of the cats (158/332, 47.6%) were positive for at least one VBP. *Hepatozoon* spp. were detected in 25.6%, haemotropic mycoplasma spp. in 22.9%, *B. henselae* in 9.3% and *L. infantum* in 2.1% of the cats. Male sex had a statistically significant association with test results for haemotropic mycoplasma spp. (odds ratio 1.38 [1.21;1.57]);
regionality with *Hepatozoon* spp., *B. henseale* and FIV; and seasonality with *Hepatozoon* spp., haemotropic mycoplasma spp., *L. infantum* and FeLV ($P \leq 0.05$ each). A strong positive correlation was reported for the amount of rainfall and the number of cats that tested positive for Hepatozoon spp. ($\rho = 753$, $P = 0.05$). None of the cats tested positive for A. phagocytophilum, A. platys, E. canis, Rickettsia spp., piroplasms, or microfilariae. Co-infections with multiple VBPs were detected in 56 out of 332 cats (16.9%). Thirty-one of the 332 cats included in the study (9.3%) tested positive for FeLV (6.9%) and for FIV (3.6%). In 20/31 cats (64.5%) that tested positive for FeLV/FIV, coinfections with VBP were detected ($P = 0.048$, OR 2.15 [0.99; 4.64]).

Conclusions VBPs were frequently detected in stray cats in Zaragoza. In particular, regionality and seasonality had a statistically significant association with PCR results for most VBPs included in the study.

Highlights

• Nearly half of the cats tested positive for at least one vector-borne pathogen by PCR

• Hepatozoon felis, haemotropic Mycoplasma, Bartonella and Leishmania were detected

• Stray cats should be monitored to prevent and manage potential zoonotic diseases

Keywords Arthropod-transmitted infections, Feline vector-borne infections, Laboratory diagnostics, PCR, Tick transmitted Infections.

Introduction

The importance of feline vector-borne pathogens (VBPs), transmitted by blood-feeding arthropods, is increasingly being recognized in Europe. Cats are exposed to arthropods, especially when living outdoors or as stray cats without ectoparasite prophylaxis and veterinary care [1–3]. Ectoparasites can transmit various parasitic, bacterial, or viral pathogens to animal hosts such as cats. Cats with immunosuppression as a result of feline leukaemia virus (FeLV) and feline immunodeficiency virus (FIV) infections are predisposed to vector-borne infections including leishmaniosis [4], hepatozoonosis [5–7] and cytauxzoonosis [8]. Screening for VBPs may be pivotal for animal and environmental risk assessment.

The distribution of feline VBPs is mainly dependent on the habitats of the transmitting vectors [2]. Consequently, *Anaplasma phagocytophilum* infections have most often been recognized in northern, central, or eastern Europe [9]. The occurrence of Leishmania infantum infections has been linked to habitats of phlebotomine sand flies in the Mediterranean and Southeastern Europe [9]. Infections with *Hepatozoon felis* and less frequently with *H. canis* and *H. silvestris* were detected in the Mediterranean and Southeastern Europe [10–14]. Feline infections with Dirofilaria immitis are mainly reported within the Mediterranean and Southeastern Europe [15, 16]. Finally, infections with Dirofilaria repens are rare in cats [17]. Other documented VBPs affecting cats in Europe

include helminths (e.g. *Thelazia callipaeda*, *Dipylidium caninum*), bacteria (e.g. *Borrelia burgdorferi* sensu lato, *Bartonella* spp., *Francisella tularensis*), protozoa (e.g. *Babesia* spp., *Theileria* spp., *Cytauxzoon* sp.) and viruses (*Flaviviridae*) [2]. For many of these feline VBPs, infections in humans have been reported as well. This, for example, includes B. henselae and

B. clarridgeiae as the causative agents of cat scratch disease in humans [18], *Leishmania infantum* causing human leishmaniosis [19], *A. phagocytophilum* causing human granulocytic anaplasmosis [20], *D. repens* causing subcutaneous dirofilariosis [21] and *D.immitis* causing heartworm infections [21]. Haemotropic mycoplasmas are bacteria that are classified within the Mycoplasma genus but cannot be cultivated [22]. *Mycoplasma haemofelis*, Candidatus *Mycoplasma haemominutum* and *Candidatus* Mycoplasma turicensis are the most commonly detected species in cats [23].

The aims of this study were to investigate the prevalence of several VBPs in stray cats in the city of Zaragoza, Spain, and to identify potential risk factors for infection.

Materials and methods

Study areas, cat population and sampling

The study was carried out in the city of Zaragozam (41°38′58.8948″N, 0°53′15.7632″W, the Aragon region of Spain). The study population included stray European shorthair cats captured in different areas of Zaragoza from February 2020 to October 2022 as part of a trap, neuter and release sterilization programme that ran locally to control stray populations. Captured stray cats were anaesthetized with a combination of dexmedetomidine (Dexdomitor®; 15 µg/kg, subcutaneous injection), ketamine (Anaestamine®; 5 mg/kg, subcutaneous injection) and methadone (Semfortan®; 0.3 mg/kg, subcutaneous injection). Data on the breed, sex, blood collection date and colony of origin (postal code) of each cat were recorded. A complete physical examination was carried out before sampling. Only cats older than 1 year and classified as apparently healthy based on the general examination were included. Prior to blood collection, the fur of the cats was trimmed around the jugular region. Sampling consisted of collecting 1 ml of blood aseptically by jugular venepuncture, with the collected volume placed in a tube containing ethylenediaminetetraacetic acid (EDTA) anticoagulant for PCR analysis. Blood was stored at − 20 °C until processing.

This survey was included under Project Licence PI75/20 approved by the Ethics Committee for Animal Experiments for the University of Zaragoza. The care and use of animals were performed according to the Spanish Policy for Animal Protection RD 53/2013, which meets the European Union Directive 2010/63 on the protection of animals used for experimental and other scientific purposes.

Molecular analysis

PCR testing of EDTA-blood was performed to detect A. phagocytophilum, A. platys, B. henselae, E. canis, Rickettsia spp., haemotropic Mycoplasma spp., Hepatozoon spp., L. infantum, piroplasms and microfilariae in the LABOKLIN laboratory (Bad Kissingen, Germany). Additionally, PCR testing was performed for FeLV and FIV. The molecular methods applied for investigation with descriptions of the gene targets at the LABOKLIN laboratory are summarized in Table 1. Furthermore, additional PCR protocols were used for sequence analysis of Bartonella spp. and Hepatozoon spp. (Table 1).

Bartonella and Hepatozoon species identification and sequence analysis For Bartonella sp. identification, samples that tested positive with Bartonella sp. qPCR were submitted to a conventional PCR (cPCR) assay amplifying an ~ 603 bp fragment of the 16S-23S intergenic transcribed spacer (ITS) region (Table 1) [24]. The cPCR cycling conditions consisted of an initial denaturation step at 94 °C for 2 min, followed by 40 cycles of 94 °C for 15 s, 60 °C for 15 s and 72 °C for 45 s. The ITS cPCR products were sequenced for further identification and characterization. Furthermore, the partial 16S rRNA fragment from all ITS cPCR positive samples was amplified for genotype characterization by using the type-specific primers Bh1_R (5′- CCG ATA AAT CTT TCT CCC TAA -3′) and Bh2_R (5′- CCG ATA AAT CTT TCT CCA AAT -3′) in combination with the broad-host-range primer 16SF [5′-AGA GTT TGA TCC TGG(CT)TCAG-3′] as described previously (51) (Table 1). The cPCR cycling conditions consisted of an initial denaturation step at 94 °C for 2 min, followed by 40 cycles of 94 °C for 15 s, 57 °C for 15 s and 72 °C for 15 s. All cPCR assays were performed in a final volume of 25 µl that contained 2 µl of DNA extract, 2.5 µl of 10 Å~ TaKaRa LA Taq buffer, 0.25 µl of TaKaRa LA Taq enzyme (Takara Bio Europe S.A.S.Saint-Germain-en-Laye, France), 2.5 µl of MgCl2 (25 mM), 1 µl each of forward and reverse primers (50 µM), 4 µl of dNTPs and PCR grade water up to 25 µl.

For species and genotype characterization, the obtained ITS cPCR products were sequenced in both directions using BigDye 3.1 Ready Reaction Mix (Applied Biosystems) according to the manufacturer's instructions. For Hepatozoon species identification, a 779-bp fragment of the 18S rRNA gene was amplified using the primers Hep18S-F74 (5'- CAG TAA AAC TGC AAA TGG CTCAT-3') and Hep18S-R853 (5'-CCA ATA ATG TAG AAC CAA AAT CCT -3'). The 25 µl PCR mixture contained the following: 15.25 µl PCR-grade water, 5 µl 5 Å~ reaction buffer (Biozym, Oldendorf, Germany), 1 µl of each primer (10 pmol), 0.25 µl of Biozym HS Taq DNA Polymerase and 2.5 µl template DNA. The cPCR cycling conditions consisted of an initial denaturation step at 98 °C for 2 min followed by 40 cycles of 98 °C for 15 s, 55.5 °C for

15 s and 72 °C for 15 s. Hepatozoon 18S rRNA amplicons were Sanger sequenced by LGC Genomics, Berlin, Germany.

The genomic sequences obtained in this study were edited using Geneious 10.1.3 2020 version (Biomatters Ltd., Auckland, New Zealand) and submitted to a BLAST search (BLASTn; https:// blast. ncbi. nlm. nih. gov/ Blast. cgi? PROGR AM= blast n& PAGE_ TYPE= Blast Searc h& LINK_ LOC= blast home) using default values to find homologous hits. For the phylogenetic analyses, sequences were aligned using the L-INS-I algorithm of the Geneious MAFFT plugin with default settings. A maximum likelihood (ML) phylogenetic tree with 1000 bootstrap resamplings using the GTR-GAMMA nucleotide model was performed using the Geneious RAxML plugin.

Statistical analysis

Statistical analysis was carried out using SPSS for Windows (version 28.0; IBM). $P \leq 0.05$ was regarded as statistically significant. The dataset was tested for adherence to a normal distribution in cats that tested positive for VBPs by Shapiro-Wilk testing. For normally distributed data, the chi-square test and Fisher's exact test were used to calculate statistical significance. For data on seasonality, which were not normally distributed, Kruskal-Wallis testing was used to calculate statistical significance. The different seasons were defined as follows: spring from 21 March to 20 June, summer from 21 June to 22 September, autumn from 23 September to 31 December and winter from 1 January to 20 March. Binomial logistic regression was performed to determine the effect of sex, regional distribution and seasonality on the PCR test results. Spearman's rank correlation coefficient (ρ) was calculated to correlate the percentage of cats testing positive for the individual VBPs with the amounts of rainfall and average temperatures in the individual months of the study (https:// www. aemet. es/ es/ servi ciosc limat icos/ datos clima tolog icos). $\rho < 0.2$ was classified as a very low correlation, $\rho = 0.2–0.5$ as a low correlation, $\rho > 0.5–0.7$ as a medium correlation, $\rho > 0.7–0.9$ as a strong correlation and $\rho > 0.9$ as a very strong correlation.

Results

Out of the 332 cats included in this study, 158 (47.6%) tested positive for at least one VBP (Table 2). Hepatozoon spp. (85 out of 332 cats, 25.6%), haemotropic Mycoplasma spp. (76 out of 332 cats, 22.9%), B. henselae (31 out of 332 cats, 9.3%) and L. infantum (7 out of 332 cats, 2.1%) were detected. None of the cats tested positive for A. phagocytophilum, A. platys, E.

canis or Rickettsia spp. Twenty-three of 332 cats (6.9%) tested PCR positive for FeLV, and 12 of 332 cats (3.6%) tested PCR positive for FIV. In 21 of the 31 cats that tested positive for FeLV and/ or FIV, coinfections with VBPs were detected (P = 0.048, OR = 2.15 [95% CI 0.99–4.64]).

In 56/158 cats (35.4%) that tested positive, co-infections with more than one vector-borne pathogen were recognized. Most often, haemotropic Mycoplasma spp. (46 out of 56 cats, 82.1%) were involved, followed by Hepatozoon spp. (29 out of 56 cats, 51.8%), B. henselae (17 out of 56 cats, 30.4%) and L. infantum (5 out of 56 cats, 8.9%). In 43 out of 56 cats (76.8%), co-infections with two pathogens were recognized; in 12 cats (21.4%), co-infections with three pathogens; in one cat (1.8%), coinfections were with four pathogens.

Of 31 samples that yielded amplicons of the expected size when using cPCR targeting the 16S-23S ITS region of Bartonella spp., 28 (96.6%) were characterized as 16S B. henselae type II and one as B. henselae type I based on the 16S rRNA cPCR assay (Table 2). The identity of the two remaining samples (cats #48 and #122) could not be determined because of the low quantity of the available DNA. Of the ITS-positive samples, 11/29 (37.5%) were successfully sequenced. Ten of the sequences were identical, so only two sequences were deposited in GenBank® under accession numbers: OQ918678 and OQ918679. According to the sequence data, B. henselae was the only species present with two different ITS sequence types detected that matched with the partial ITS sequences of the reference strains Houston I (BAO16RB) (catalogue #284–OQ918679) and Marseille II (CP072904) (catalogue #196–OQ918678), as stated in Additional file 1: Fig. S1.

A partial fragment of the 18S rRNA gene from Hepatozoon species was successfully amplified from 70 samples that tested positive in TaqMan real-time PCR for Hepatozoon spp. Nineteen H. felis sequence variants (variants A–S, OR263276-OR263294) were detected that showed 99.1–100% identity with the 18S rRNA gene of a Spanish H. felis isolate (AY620232). In one cat (catalogue #98), an H. canis sequence (OR263295) that was 100% identical to another H. canis isolate from Spain (AY150067) was detected (Additional file 1: Fig. S2). Considering regional distribution, sequence variants were different between areas: Central (variants A, D, G, O, Q and S), North (variants A, E, M and Q), South (variants A, E, F, I, O, P and Q), East (variants A, H, Q, R and S) and West (variants A, J, K and S).

Of 332 cats, 179 (53.9%) were female, and 153 (46.1%) were male. A statistically significant impact of sex on the test results was detected only for haemotropic Mycoplasma spp. (21/179 female [11.7%] and 55/153 male cats [35.9%] tested positive, P < 0.001, OR = 1.38; in 21 of

the 31 cats testing positive for FeLV and/or FIV, coinfections with VBPs were detected (P = 0.048, OR = 2.15 [95% CI 1.21 -1.57]). There was no statistically significant impact of sex on Hepatozoon spp. (47/179 female [26.3%] and 38/153 male cats [24.8%] tested positive, P = 0.802), L. infantum (2/179 female [1.1%] and 5/153 male cats [3.3%] tested positive, P = 0.255), B. henselae (16/179 female [8.9%] and 15/153 male cats [9.8%] tested positive, P = 0.851), FeLV (8/179 female [4.5%] and 15/153 male cats [9.8%] tested positive, P = 0.081) or FIV (3/179 female [1.7%] and 9/153 male cats [5.9%] tested positive, P = 0.073).

The postal code of the area in which the cats were caught in Zaragoza was known for 322/332 cats (97.0%). There were statistically significant differences between regions and PCR positivity for Hepatozoon spp. ($\chi2 = 17.396$, df = 4, P = 0.002), B. henselae ($\chi2 = 14.544$, df = 4, P = 0.006) and FIV ($\chi2 = 11.961$, df = 4, P = 0.018), which could not be demonstrated in haemotropic Mycoplasma spp. ($\chi2 = 4.395$, df = 4, P = 0.355), L. infantum ($\chi2 = 7.523$, df = 4, P = 0.111) and FeLV ($\chi2 = 3.060$, df = 4, P = 0.548) (Table 3).

Positive test results were not normally distributed for seasonality in cats testing positive for VBPs, FeLV and/ or FIV (P < 0.001 each). Significant differences between seasons and PCR test results were observed for Hepatozoon spp. (P < 0.001), haemotropic Mycoplasma spp. (P = 0.048), L. infantum (P = 0.007) and FeLV (P = 0.045) when using the Kruskal-Wallis test but not for B. henselae (P = 0.111) and FIV (P = 0.674) (Table 4). Statistically significant differences in PCR results were observed when autumn and winter were compared for Hepatozoon spp. (P = 0.030, r = 0.2), haemotropic Mycoplasma spp. (P = 0.049, r = 0.2), L. infantum (P = 0.019, r = 0.3) and FeLV (P = 0.036, r = 0.2). Additionally, this was observed for spring and winter in Hepatozoon spp. (P < 0.001, r = 0.4) and for summer and winter in L. infantum (P = 0.010, r = 0.3) with weak to moderate effect sizes. In binominal logistic regression analysis including 322 cats, seasonality with spring/autumn compared to summer/ winter (B = 0.823, Wald = 8.867, P = 0.003, OR = 2.28 [95% CI 1.33–3.92]) and regionality with southern parts of Zaragoza compared to all other areas (B = 1.195, Wald = 7.48, P = 0.006, OR = 3.30 [95% CI 1.40–7.78]) contributed significantly to positive test results for Hepatozoon spp. but not male sex compared to female sex (P = 0.945). The binomial logistic regression model was statistically significant ($\chi2(3) = 15.233$, P = 0.002). Goodness of fit was assessed using the Hosmer-Lemeshow test, indicating a good model fit ($\chi2(3) = 0.731$, P > 0.05). Correlations between predictor variables were low (r < 0.70), indicating that multicollinearity was not a confounding factor in the analysis. For haemotropic Mycoplasma spp., the binomial

logistic regression model was statistically significant ($\chi2(3) = 30.557$, $P < 0.001$). Goodness of fit was assessed using the Hosmer-Lemeshow test, indicating a good model fit ($\chi2(3) = 1.200$, $P > 0.05$). Correlations between predictor variables were low ($r < 0.70$), indicating that multicollinearity was not a confounding factor in the analysis. Male sex had a statistically significant impact on the PCR results (B = 1.477, Wald = 23.747, $P < 0.001$, OR = 4.38 [95% CI 2.42–7.94]). Regionality with southern parts compared to all others ($P = 0.104$) and seasonality with winter compared to all other seasons ($P = 0.246$) did not show statistical significance. For B. henseale, the binomial logistic regression model did not show any statistically significant impact of regionality (southern areas compared to all others, $P = 0.989$), seasonality (winter compared to all other seasons, $P = 0.146$) or male sex ($P = 0.624$) on the results of PCR-testing.

A strong positive correlation was reported for the amounts of rainfall and the percentages of cats testing positive for Hepatozoon spp. ($\rho = 753$, $P = 0.05$, Table 5). For haemotropic Mycoplasma spp. and L. infantum, no statistically significant correlations were detected with the amounts of rainfall (Additional file 1: Fig. S3) and the average temperature (Additional file 1: Fig. S4) in the individual months of the study ($P > 0.05$ each) (Table 5).

Discussion

Almost half of the cats in our study (47.6%) tested positive for at least one VBP by molecular analysis. This is highly indicative of infection, in contrast to positive serological antibody detection demonstrating pathogen contact in the past. This study shows that clinicians should consider vector-borne diseases as potential differential diagnoses in cats that originate from or have travelled to endemic regions. In general, the occurrence of VBPs is associated with the distribution of their vectors, which are influenced by factors such as climate, land use and human density. Spain has a highly variable climate, ranging from a continental humid climate to a Mediterranean climate. In Zaragoza, the climate is Mediterranean, with a marked continental influence, characterized by low rainfall (320 mm per year) and moderate average temperatures (15.3 °C). However, the confluence in the study area of several rivers may favour the existence of differentiated environments. This particularity could explain the results obtained in the central area (Table 3), with a higher number of animals testing positive for the pathogens evaluated in this study.

Seasonal distribution has also been analysed in the present study. We detected seasonal differences mainly in Hepatozoon spp., haemotropic Mycoplasma spp. and L. infantum. For

Hepatozoon spp., more positive samples were detected in spring, followed by autumn and finally summer. Although little is known about the epidemiology of Hepatozoon in cats, arthropod activity in these seasons in Spain could pose a risk to free-roaming cats. For haemotropic Mycoplasma spp., a higher number of positive animals were detected in the winter, and a possible justification could be that during this season the animals are more likely to live in groups, favouring the transmission of these pathogens. Finally, L. infantum has been detected more frequently in animals in winter, which might be explained by the variable and long incubation time after sandfly infection during the warmer seasons.

We observed differences between the regional distributions of selected VBPs in Zaragoza (Table 3). The comparison of results between this study and other studies published in Spain and other European Mediterranean countries is difficult and not always possible. The highly statistically significant impact of rainfall on Hepatozoon spp. with a strong positive correlation ($P = 0.005$, $P = 0.753$) is remarkable. To the authors' knowledge, this type of association has never published before.

Co-infections were recognized in 35.4% (56/158) of the cats that tested positive. One cat (1.8%) had a quadruple infection, 12 cats (21.4%) had a triple infection, and 43 cats (76.8%) had a double infection. Co-infections with multiple VBPs can complicate diagnosis and treatment in dogs. A higher percentage of co-infections with other VBPs may lead to more marked laboratory abnormalities and severity in the case of canine leishmaniosis [25]. However, limited information is available in cats, although it is plausible that co-infections could predispose cats to immune system exhaustion.

Hepatozoon sp. DNA was detected in 25.6% (85/332) of the stray cats in this study. The diagnosis of Hepatozoon spp. in cats from Zaragoza reported herein supports that this protozoan is widespread on the Iberian Peninsula, considering the descriptions recently reported in cat populations from Portugal and different regions of Spain [7, 26–29]. The Hepatozoon infection prevalence described in previous Spanish studies varied from 0.6% [28] to 4% in cats from the Barcelona area [29] and was as high as 16% in a cat colony from Barcelona [7], in all cases lower than the prevalence reported in our study. These differences in the number of cats that tested positive could be due to the vector distribution, characteristics of the cat populations and differences in the molecular detection methods used. In the present study, the stray status of cats and possible major exposure to ectoparasites may explain the higher prevalence detected. Hepatozoon spp. are transmitted by ingestion of the final host containing mature oocysts by the intermediate host [30]. Although the vectors of feline

hepatozoonosis are still unknown, it is expected that H. felis is transmitted by a haematophagous arthropod, as demonstrated for other Hepatozoon spp. transmitted by fleas, ticks, mites, lice, mosquitoes and sand flies [11]. Variations in the possible vector distribution can explain, at least partially, the differences in

Hepatozoon sp. infection rates described in studies performed in different regions. Other transmission modes have been described for some Hepatozoon spp., including intrauterine transmission and carnivorism [12, 31–33].

Hepatozoon canis was detected in one of the cats (catalogue #98). Although H. canis is usually found in dogs and wild carnivores, it was previously also detected in cats from other countries, including France, Italy and Israel [12, 13, 34].

In the present study, a high haemotropic Mycoplasma sp. infection percentage (107/332, 32.2%) was recorded, with Candidatus M. haemominutum most frequently detected (61/332, 18.4%), followed by M. haemofelis (35/332, 10.5%) and Candidatus M. turicensis (11/332, 3.1%). These data are quite similar to those from previous studies in Spain. In the Barcelona region, the previously reported haemotropic Mycoplasma sp. prevalence ranged from 7.8% to 11.9% [35, 36], and it was 10.6% in Madrid [37]. In Italy, similar prevalence estimates (11.6% to 18.3%) were reported [1, 3, 38, 39]. However, higher haemotropic Mycoplasma spp. prevalence has been reported in Cyprus (26.4%) [40] and Portugal (27.1%) [41]. Haemotropic Mycoplasma spp. is frequent in European cats, with slight differences within the countries. A higher risk of testing positive for haemotropic Mycoplasma spp. in

male cats than in female cats was also demonstrated in astudy of cats in Bangkok, Thailand [42], which is consistent with our results.

The occurrence of Bartonella sp. in cats from different areas of Spain is reported with frequencies ranging from 0.3 to 38.3% [29, 35, 43–45]. The detection of Bartonella sp. infection (9.3%) in stray cats from Zaragoza reported herein confirms that these bacteria are widespread in Spain, similar to previous observations in cats from other areas (11.9% in Barcelona) of the country [36]. Furthermore, our results are in line with those of previous studies investigating free-roaming cats in Germany (16.3%) [46] and Italy (18%) [47]. In contrast, the Bartonella sp. prevalence estimate reported herein is higher than that recorded in studies investigating pet cats with indoor lifestyles in the USA (< 5.0%) [48] or Egypt (3.0%) [49], highlighting the different risk of cats being exposed to risk factors (e.g. ectoparasite infestation) based on their lifestyle.

Based on the heterogeneity of the ITS and 16SrRNA region sequences, the species B. henselae includes genotype I (Houston I) and genotype II (Marseille) and different subtypes [24, 50]. The two genotypes display geographical heterogeneity, wherein genotype II is prevalent in European countries and the USA [1, 3, 46, 51], while genotype I is mainly reported in Asia and North Africa [49, 52, 53]. In the present study, B. henselae was
the unique species detected, with 16S rRNA/ITS type II (8.4%) being found more frequently than type I (0.3%), in line with what has already been documented in Western
countries.

The cat is the main animal host reservoir of Bartonella henselae, B. clarridgeiae and B. koehlerae [18, 54]. Bartonella clarridgeiae and B. koehlerae were not detected in stray cats from the Zaragoza region in the present study, although their presence cannot be confidently excluded in the area, as two of the samples that tested positive by generic qPCR could not be re-amplified by conventional PCR because of the low DNA quantity available, so the identity of these two samples could not be determined.

Of interest, Bartonella sp. and haemotropic Mycoplasma spp. co-infection (7/332, 2.1%) was observed herein, similar to in previous studies conducted in the Barcelona region (4.4%) [36] and in Italy (0.1% to 3%) [1, 38, 39].

In the present study, the Leishmania sp. infection percentage was 2.1% (7/332). In Spain, PCR positive percentages of 5.6% and 6% were recorded in blood samples from cats in Zaragoza [55] and Murcia [56], respectively. A study performed by Ortuno et al. (2023) revealed a higher detection percentage of the pathogen by PCR in the skin and lymphoid tissue than in the blood of cats and in the skin of healthy cats than in the skin of cats with clinical signs [56]. Another study suggested that PCR testing of conjunctival swabs is more sensitive test than that of peripheral blood [57]; however, this is highly dependent on the cellularity of the swabs. The relatively low infection percentage observed in the present study may thus in part be explained by the type of sample used, as demonstrated for canine leishmaniosis as well [58], and the fact that only clinically healthy stray cats were included
in the study.

Anaplasma phagocytophilum, A. platys and E. canis DNA was not detected in this study. The prevalence of these two tick-transmitted genera described in previous Spanish studies varied from 0 to 1% [36]. In contrast, a high molecular prevalence of E. canis (9.9%) and A. phagocytophilum (8.4%) was described in cats from Madrid, Spain [43]. Anaplasma and Ehrlichia infections in cats are generally rare in southern European countries [36, 38, 59],

which is in accordance with the results of the present study. For A. phagocytophilum, this is probably due to the distribution of ticks of the Ixodes persulcatus complex as competent vectors, which are only rarely detected in Mediterranean countries and more frequently found in Central or Northern Europe [60].

Rickettsia spp., Piroplasmida (Babesia spp., Theileria spp., Cytauxzoon sp.) and microfilariae (D. immitis, D. repens, Acanthocheilonema reconditum) DNA was not detected in the present study. Feline piroplasmid infection is quite common in Europe. Piroplasmid infection is caused by Cytauxzoon sp., with a prevalence in Spain of 1.2% [8]. In Italy, a few reports of Cytauxzoon sp. Infection have been published from the northern and central regions [61, 62]. Other piroplasms usually associated with dogs, such as Babesia vogeli [63] and Babesia canis [27], have also been described in European cats. Microfilariae infections in cats appear to be rare and have only been sporadically documented in cats from Mediterranean regions, although a seropositivity of 24.4% was recently reported in stray cats from Zaragoza [64], and infections of two cats by D. immitis and D. repens were recently reported in Italy [65]. However, limitations in cats associated with PCR testing should be considered, as cats most often do not show prominent numbers of microfilariae in the peripheral blood, resulting in false-negative PCR results [66].

Limitations of this study

The limitations of this study are mainly its retrospective design, predominantly due to missing anamnesis and information regarding clinical signs consistent with vector-borne infections. Probably, no ectoparasite prophylaxis was applied in the cats included in the study, but this could not be ruled out with certainty. We were not able to include the haematological and biochemistry results of the tested cats.

Conclusions

Almost half of the cats (47.6%) tested positive for at least one vector-borne pathogen. The cats in the present study were apparently healthy, underlining the need for epidemiological studies of stray cats in Europe to identify zoonotic and non-zoonotic pathogens and the relevance of screening domestic cats for VBPs, especially in endemic areas. Immunosuppression (FeLV-/FIV infections) and male sex may contribute to positive results in molecular tests for vector-borne infectious agents.

Abbreviations

DNA Deoxyribonucleic acid

EDTA Ethylenediaminetetraacetic acid

FeLV Feline leukaemia virus

FIV Feline immunodeficiency virus

OR Odds ratio

PCR Polymerase chain reaction

rRNA Ribosomal ribonucleic acid

VBP Vector-borne pathogen

Supplementary Information

The online version contains supplementary material available at https:// doi.
org/ 10. 1186/ s13071-023-06046-y.

Additional file 1: Fig. S1. Maximum likelihood (ML) phylogenetic tree genepercentaged using the RAxML plugin in Geneious 10.1.3 software, calculated from the partial internal transcribed spacer (ITS) sequences of Bartonella species. Sequence data genepercentaged in the present study are highlighted in bold. Brucella melitensis was used as outgroup. Bootstrap values > 50% are shown at the nodes.

Additional file 2: Fig. S2. Maximum likelihood (ML) phylogenetic tree genepercentaged using the RAxML plugin in Geneious 10.1.3 software, calculated from the partial 18S rRNA gene sequences of selected Hepatozoon species. Sequence data genepercentaged in the present study are highlighted in bold. Adelina dimidiata was used as outgroup. Bootstrap values > 50% are shown at the nodes.

Additional file 3: Fig. S3: Average amount of rainfall sorted by months in the time frame of the study (blue line) and historically (red line) in Zaragoza (Spain)

Additional file 4: Fig. S4: Average temperature sorted by months in the time frame of the study (blue line) and historically (red line) in Zaragoza (Spain).

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Declarations

Ethics approval and consent to participate

This survey was included under Project Licence PI75/20 approved by the Ethic Committee for Animal Experiments for the University of Zaragoza. The care and use of animals were performed according with the Spanish Policy for Animal Protection RD 53/2013, which meets the European Union Directive 2010/63 on the protection of animals used for experimental and other scientific purposes.

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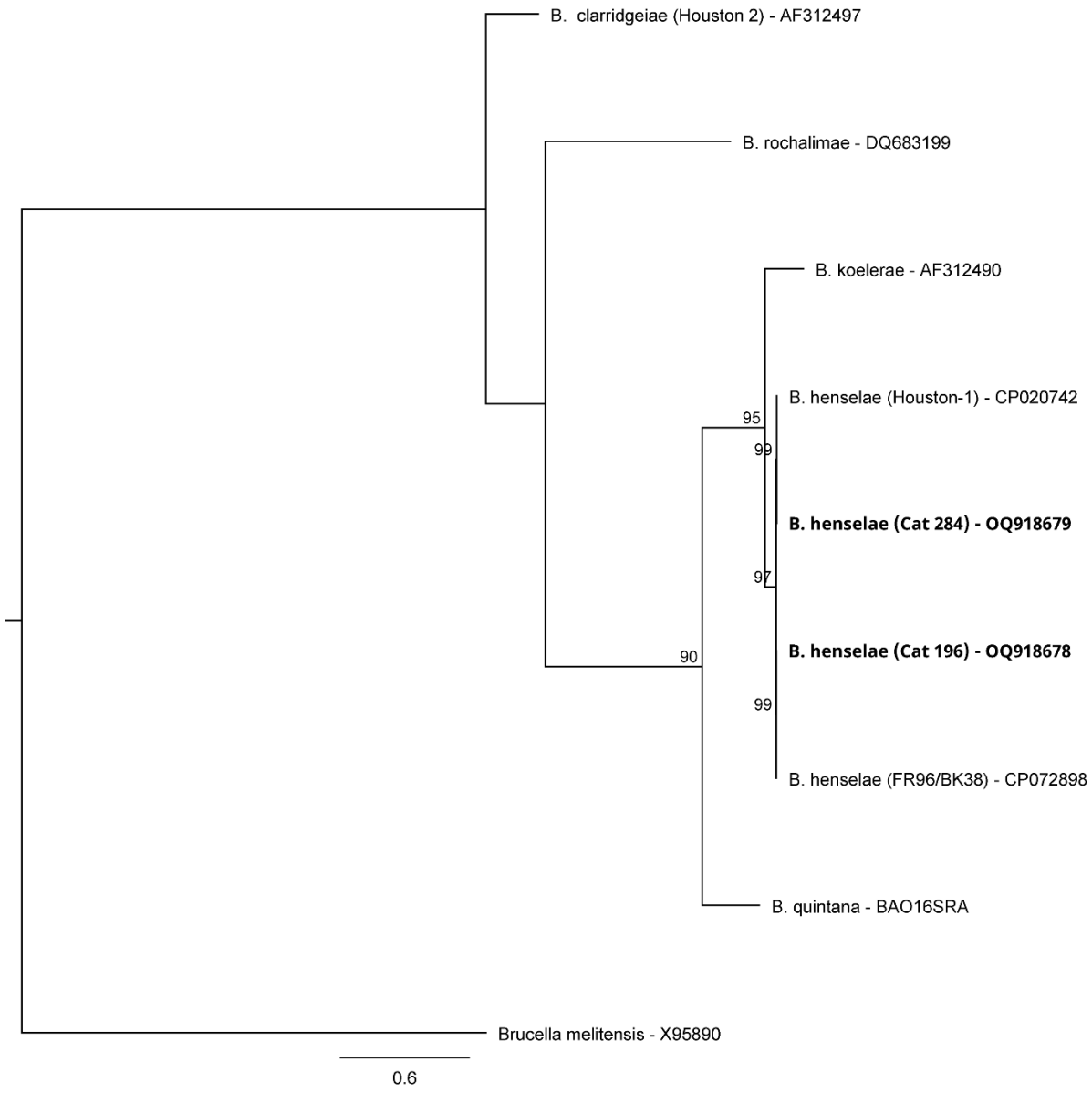
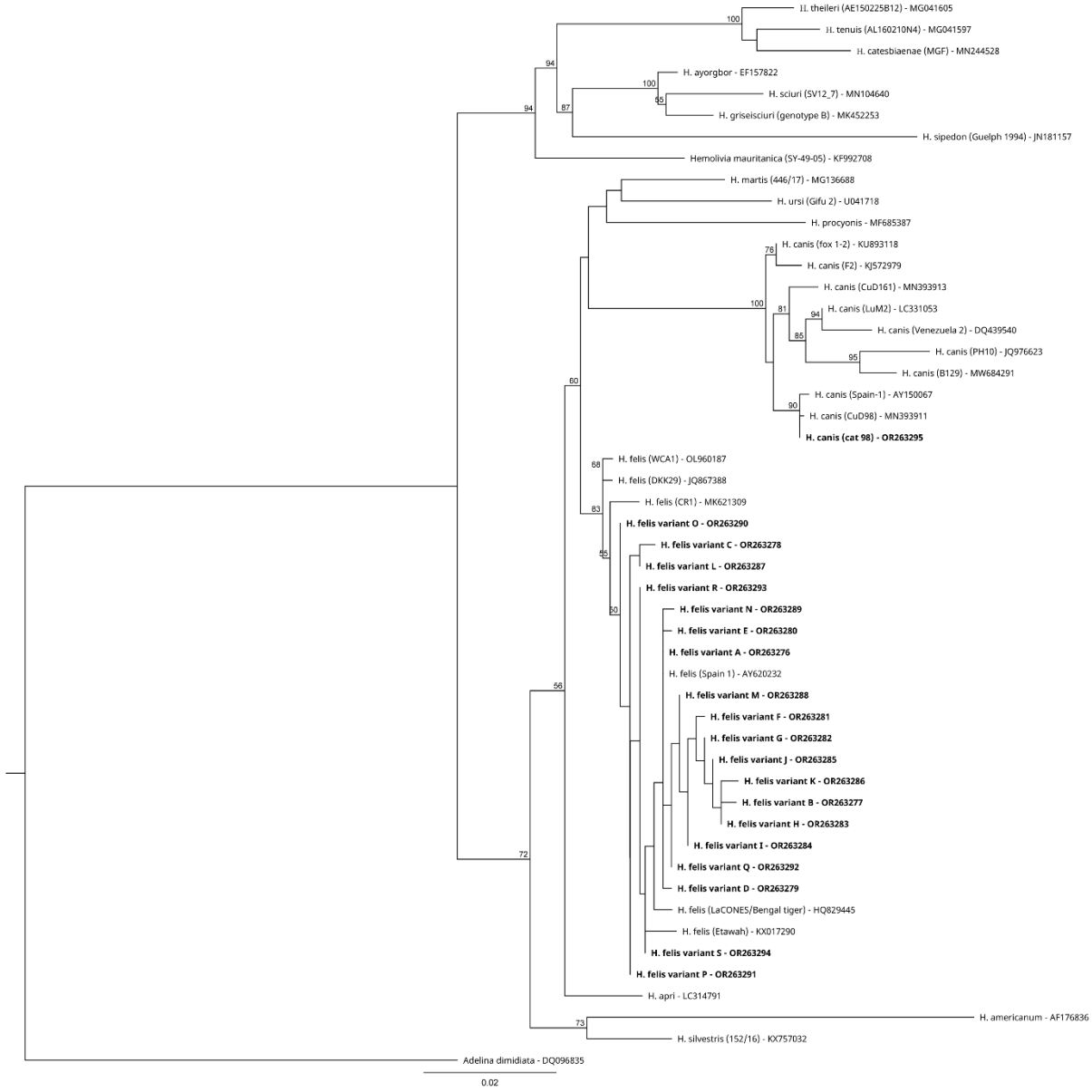
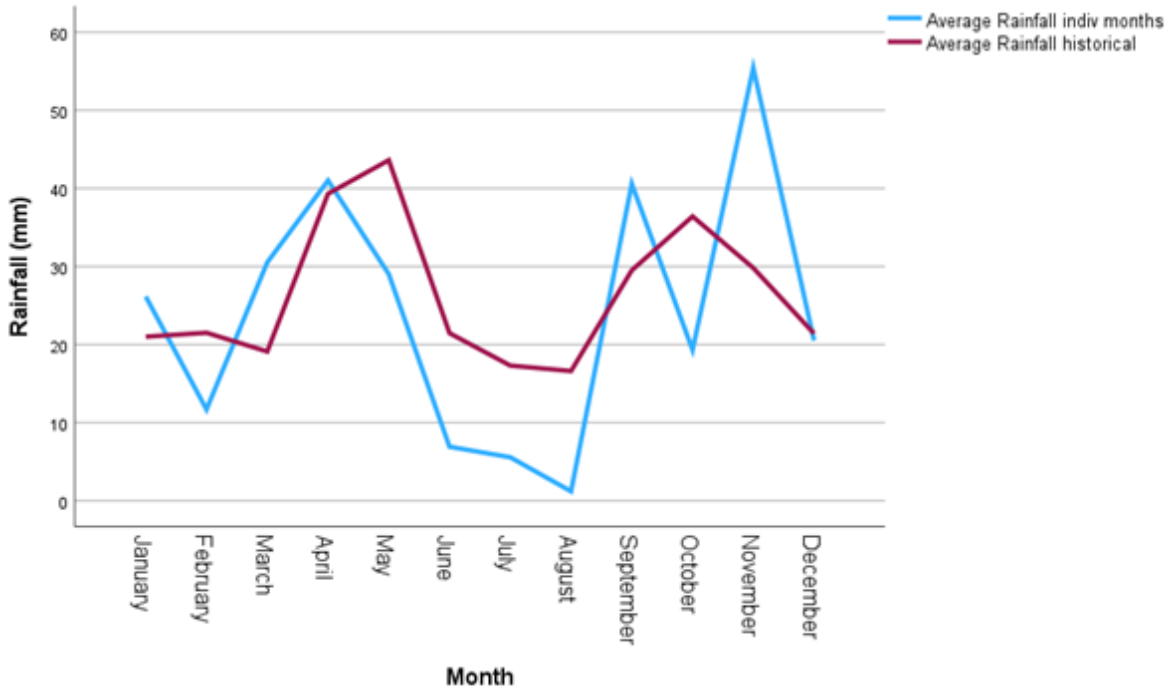
Additional file 1: Fig. S1. Maximum likelihood (ML) phylogenetic tree genepercentaged using the RAxML plugin in Geneious 10.1.3 software, calculated from the partial internal transcribed spacer (ITS) sequences of *Bartonella* species. Sequence data genepercentaged in the present study are highlighted in bold. *Brucella melitensis* was used as outgroup. Bootstrap values > 50% are shown at the nodes.



Additional file 2: Fig. S2. Maximum likelihood (ML) phylogenetic tree genepercentaged using the RAxML plugin in Geneious 10.1.3 software, calculated from the partial 18S rRNA gene sequences of selected *Hepatozoon* species. Sequence data genepercentaged in the present study are highlighted in bold. *Adelina dimidiata* was used as outgroup. Bootstrap values > 50% are shown at the nodes.



Additional file 3: Fig. S3: Average amount of rainfall sorted by months in the time frame of the study (blue line) and historically (red line) in Zaragoza (Spain)



Additional file 4: Fig. S4: Average temperature sorted by months in the time frame of the study (blue line) and historically (red line) in Zaragoza (Spain).

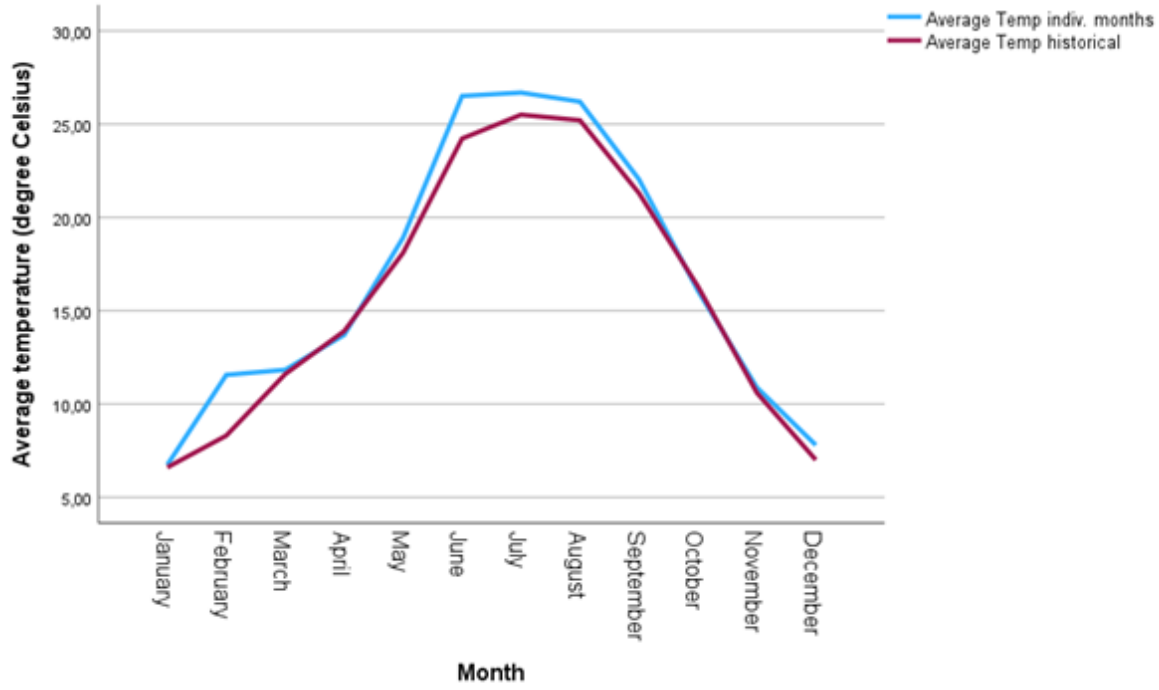


Table 1. Methodology of PCR testing applied for the detection of vector-borne pathogens performed in the present study

Vector-borne pathogen	Molecular method	Gene target	Primer sequence (5'-3 ')
Anaplasma phagocytophilum	TaqMan® real-time PCR	Heat shock protein 60	F: CTCTGAGCACGCTTGTACT
			R: GCCTTTACAGCAGCAACT TGAAG
Anaplasma platys	TaqMan® real-time PCR	groEL	F: CCGATCCTTGAAAACGTT GCT
			R: TCCTTCTACATCCTCAGC GATGAT
Ehrlichia canis	TaqMan® real-time PCR	Disulphide-oxidoreductase	F: CCCTCAAAAAGTGATAGA TCTGCTCA
			R: TCCAATTCAGATTTGTAT TTTTTTATGTTTTGACTCA
Rickettsia spp.	TaqMan® real-time PCR	23S rRNA	F: AGCTTGCTTTTGGATCAT TTGG
			R: TTCCTTGCCTTTTCATAC ATCTAGT
Mycoplasma haemofelis	TaqMan® real-time PCR	16S rDNA	F: GTGCTACAATGGCGAAC ACA
			R: TCCTATCCGAACTGAGAC GAA
Mycoplasma haemominutum	TaqMan® real-time PCR	16S rDNA	F: TGATCTATTGTKAAAGGC ACTTGCT
			R: TTAGCCTCYGGTGTTCCT CAA
Mycoplasma turicensis	TaqMan® real-time PCR	16S rDNA	F: AGAGGCGAAGGCGAAAA CT
			R: CTACAACGCCGAAACAC AAA
Bartonella henselae (for detection)	TaqMan® real-time PCR	Alr-gcvP IGS	F: GAGGGAAATGACTCTCTC AGTAAAA
			R: TGAACAGGATGTGGAAG AAGG
Bartonella spp. (for typing)	cPCR	16S—23S ITS	F: CTTCAGATGATGATCCCA AGCCTTYTGGCG
			R: GAACCGACGACCCCCTGC TTGCAAAGCA
Bartonella henselae (for typing, I or II)	cPCR	16S rRNA	Bh_F: AGAGTTTGATCCTGG (CT)TCAG
			BhI_R: CCGATAAATCTTTCT CCCTAA
			BhII_R: CCGATAAATCTTTC TCCAAAT
Leishmania infantum	TaqMan® real-time PCR	Kinetoplast minicircle DNA	F: AACTTTTCTGGTCCTCCG GGTAG
			R: ACCCCCAGTTTCCCGCC
Hepatozoon spp. (for detection)	TaqMan® real-time PCR	18S rRNA	F: AACACGGGAAAACTCAC CAG

			R: CCTCAAACTTCCTCGCGTTA
Hepatozoon spp. (for typing)	cPCR and sequencing	18S rRNA	F: CAGTAAAACTGCAAATGGCTCAT
			R: CCAATAATGTAGAACCAAAATCCT
Piroplasms	Conventional PCR	Small subunit ribosomal	F: AATACCCAATCCTGACACAGGG
			R: TTAAATACGAATGCCCCCAAC
Microfilariae	TaqMan® real-time PCR	5.8S rDNA	F: AGTGCGAATTGCAGACGC
			R: ATTGACCCTCAACCAGACG
FeLV	TaqMan® real-time PCR	U3 long terminal repeat	F: TCAAGTATGTTCCCATGAGATACAA
			R: GAAGGTCGAACTCTGGTCAACT
FIV	TaqMan® real-time PCR	Gag	F: GGCATATCCTATTCAAACAG
			R: AAGAGTTGCATTTTATATCC

Table 2. Proportions of the molecularly detected pathogens in stray cats from Zaragoza, Spain

Pathogen	***n* tested positive/*N* total**	**Percentage (%)**
Bacterial pathogens		
Haemotropic *Mycoplasma* spp.	76/332	22.9
Candidatus M. hemominutum	61/332	18.4
M. hemofeli	35/332	10.5
Candidatus M. turicensis	11/332	3.3
Bartonella spp.	31/332	9.3
B. henselae type I	1/332	0.30
B. henselae type II	28/332	8.73
Bartonella spp.	2/332	0.6
Anaplasma phagocytophilum	0/332	0
Anaplasma platys	0/332	0
Ehrlichia canis	0/332	0

Rickettsia spp.	0/332	0
Protozoal pathogens		
Hepatozoon spp.	85/332	25.6
Leishmania infantum	7/332	2.1
Piroplasma	0/332	0
Helminths		
Microfilariae	0/332	0
Viral pathogens		
Feline leukaemia virus (FeLV)	23/332	6.9
Feline immunodeficiency virus (FIV)	12/332	3.6

Table 3. Regional distribution of positive molecular test results for selected vector-borne pathogens and FeLV/FIV in stray cats in Zaragoza, Spain, according to postal codes (*n* tested positive/*N* total [%])

Pathogen	Central	North	South	East	West	Total	*P*
Hepatozoon spp.	27/131 (20.6%)	9/60 (15%)	12/25 (48%)	22/56 (39.3%)	15/50 (30%)	85/322 (26.4%)	0.002
Haemotropic *Mycoplasma spp.*	24/131 (18.3%)	17/60 (28.3%)	8/25 (32%)	12/56 (21.4%)	9/50 (18%)	70/322 (21.7%)	0.355
Bartonella henseale	5/131 (3.8%)	9/60 (15%)	0/25 (0%)	6/56 (10.7%)	9/50 (18%)	29/322 (9.0%)	0.006
Leishmania infantum	1/131 (0.8%)	4/60 (6.7%)	0/25 (0%)	1/56 (1.8%)	1/50 (2.0%)	7/322 (2.2%)	0.111
FeLV	13/131 (9.9%)	3/60 (5%)	1/25 (4%)	4/56 (7.1%)	2/50 (4%)	23/322 (7.1%)	0.548
FIV	8/131 (6.1%)	0/60 (0%)	0/25 (0%)	0/56 (0%)	0/50 (0%)	8/322 (2.5%)	0.018
Total	131/322 (40.7%)	60/322 (18.6%)	25/322 (7.8%)	56/322 (17.4%)	50/322 (15.5%)	322/322 (100%)	-

Central: 50,001, 50,003, 50,004, 50,005, 50,009, 50,010, 50,012, 50,014, 50,017, 50,018, 50,190, 50,191, 50,720

North: 50,015, 50,019, 50,020, 50,193, 50,820
South: 50,007, 50,021
East: 50,002, 50,013, 50,016, 50,057, 50,194
West: 50,011, 50,197, 50,620
FeLV Feline leukaemia virus, FIV feline immunodeficiency virus

Table 4. Seasonal distribution of positive molecular test results for selected vector-borne pathogens and FeLV/FIV in stray cats in Zaragoza, Spain (*n* tested positive/*N* total [%])

Pathogen	**Spring**	**Summer**	**Autumn**	**Winter**	**Total**	***P*[1]**
Hepatozoon spp.	28/71 (39.4)	20/83 (24.1)	31/111 (27.9)	6/67 (9.0)	85/332 (25.6)	< 0.001
Haemotropic *Mycoplasma spp.*	18/71 (25.4)	16/83 (19.3)	19/111 (17.1)	23/67 (34.3)	76/332 (22.9)	0.048
Bartonella henselae	6/71 (8.5)	3/83 (3.6)	12/111 (10.8)	10/67 (14.9)	31/332 (9.3)	0.111
Leishmania infantum	1/71 (1.4)	0/83 (0)	1/111 (0.9)	5/67 (7.5)	7/332 (2.1)	0.007
Total	39/71 (54.9)	33/83 (39.8)	51/111 (45.9)	35/67 (52.2)	158/332 (47.6)	0.233
FeLV	4/71 (5.6)	7/83 (8.4)	12/111 (10.8)	0/67 (0)	23/332 (6.9)	0.045

Spring: 21 March–20 June; summer: 21 June–22 September; autumn: 23 September–31 December; winter = 1 January–20 March
FeLV feline leukaemia virus; *FIV* feline immunodeficiency virus
[1]Kruskal-Wallis test.

Table 5. Correlation between percentages of cats tested positive for haemotropic *Mycoplasma* spp., *Hepatozoon* spp. and *Leishmania infantum* in the individual months of the study with amounts of rainfall (mm) and temperature (°C) using Spearman's rank correlation coefficient

		***Mycoplasma* spp.**	***Hepatozoon* spp.**	***Leishmania* spp.**	**Rainfall (mm)**	**Temperature (°C)**
Haemotropic *Mycoplasma* spp.	CC	1.000	-0.512	0.484	-0.081	0.385
	Sig	–	0.089	0.111	0.803	0.216
	N	12	12	12	12	12
Hepatozoon spp.	CC	− 0.512	1.000	− 0.465	0.753	0.238
	Sig	0.089	–	0.128	0.005	0.456
	N	12	12	12	12	12
Leishmania infantum	CC	0.484	− 0.465	1.000	− 0.078	− 0.382
	Sig	0.111	0.128	–	0.810	0.220
	N	12	12	12	12	12
Rainfall (mm)	CC	− 0.081	0.753	− 0.078	1.000	− 0.154
	Sig	0.803	0.005	0.810	–	0.633
	N	12	12	12	12	12
Temperature (°C)	CC	− 0.385	0.238	− 0.382	− 0.154	1.000
	Sig	0.261	0.456	0.220	0.633	–
	N	12	12	12	12	12

General discussion and conclusions

This doctoral Thesis investigated the molecular epidemiology and characterization of *Mycoplasma* infections across different animal species, namely dogs, donkeys, and stray cats. The combined findings provide new insights into the prevalence, risk factors, and potential impact of mycoplasmas and other associated pathogens in both domestic and free-ranging populations.

In Chapter 1 the study represents the first report of *M. equigenitalium* and *M. subdolum* infections in donkeys in Italy and Spain, and more broadly in Europe. A prevalence of 25.8% was detected by PCR, while culture identified fewer cases (12.5%). The disparity between culture and PCR is consistent with previous observations in equids and other species, where the fastidious growth requirements of mycoplasmas and sample handling conditions (e.g., freezing of Spanish samples) reduce isolation success compared to molecular methods (Caswell et al., 2010; Citti & Blanchard, 2013).

Interestingly, infections were detected in clinically healthy donkeys, mirroring earlier reports in horses where *M. equigenitalium* and *M. subdolum* were found in both symptomatic and asymptomatic animals (Spergser et al., 2002). This suggests that these organisms can act as commensals or opportunistic pathogens depending on host factors or co-infections. Although no reproductive disorders were documented in the sampled donkeys, their known association with infertility, endometritis, and abortion in horses (Moorthy et al., 1977; Heitmann et al., 1979; Nehra et al., 2015) raises concerns about potential subclinical impacts on donkey fertility.

Risk factor analysis showed that poor biosecurity was significantly associated with higher prevalence, while age, sex, breed, and country were not. This finding reinforces the importance of husbandry and management practices in controlling transmission, aligning with earlier studies showing management as a critical determinant in mycoplasma epidemiology (Fox, 2012; Macêdo et al., 2018).

The antimicrobial susceptibility test of *M. equigenitalium* isolates showed good activity of tylosin, tilmicosin, florfenicol, and spectinomycin, while variable resistance was observed for enrofloxacin, lincomycin, and pleuromutilins. These findings are consistent with previous reports on *Mycoplasma* spp., where macrolides and tetracyclines remain the most effective first-line agents (Hannan et al., 1997; Morley et al., 2005; McLellan, 2017). Resistance to β-

lactams and sulphonamides was expected given the absence of a bacterial cell wall (McCormack, 1993; Maes et al., 2018). The detection of heterogeneous MIC patterns for enrofloxacin and lincomycin is in line with the emergence of multidrug resistance mechanisms in veterinary mycoplasmas (Forsberg et al., 2012).

In Chapter 2 the study provides evidence of a notable prevalence (38.2%) of *Mycoplasma* spp. in dogs with reproductive problems, with *M. cynos* (22%) detected more frequently than *M. canis* (11.7%). These results corroborate previous reports identifying *M. canis* and *M. cynos* as important canine pathogens affecting reproductive and respiratory systems (Chalker, 2005; Jambhekar et al., 2019). The detection of mixed infections further emphasizes the complex microbial ecology of the canine reproductive tract.

A key finding was the higher risk of *M. cynos* infection in males (OR: 5.07), consistent with the notion that sex-related behavioral or anatomical differences may influence exposure and colonization. Additionally, dogs with reproductive impairment were more likely to be positive for *Mycoplasma* spp. than healthy controls (OR: 3.38), suggesting a possible causal association between infection and reduced fertility. While causality cannot be confirmed in this cross-sectional design, the findings align with earlier studies implicating *Mycoplasma* spp. in infertility, endometritis, and epididymitis (Edward, 1955; Damassa et al., 1994). From a clinical perspective, these findings underline the importance of including *Mycoplasma* testing in the diagnostic work-up of canine infertility, particularly in breeding contexts where reproductive efficiency is paramount. Preventive strategies, such as regular screening of breeding dogs and improved kennel hygiene, may help reduce transmission.

In Chapter 3 the molecular survey of stray cats in Zaragoza revealed a high burden of vector-borne pathogens (VBPs), with 47.6% of animals positive for at least one agent. Haemotropic *Mycoplasma* spp. was found in 22.9% of cats, confirming their widespread distribution in feline populations, as reported globally (Tasker, 2010; Millán et al., 2021). The presence of *Bartonella henselae*, *Hepatozoon spp.*, and *Leishmania infantum* in co-infections highlights the complex pathogen ecology of stray cats at the human–animal–vector interface.

Risk factor analysis revealed that male cats were more likely to be infected with haemoplasmas, which may reflect sex-related behavioral differences such as fighting and roaming, leading to increased exposure (Willi et al., 2007; Cohen et al., 2018). Seasonal and geographical variations also influenced prevalence, supporting the role of vector dynamics and environmental conditions in transmission. The positive correlation between rainfall and *Hepatozoon* prevalence emphasizes the influence of ecological factors.

The co-occurrence of VBPs with retroviral infections (FeLV and FIV) is particularly concerning. Co-infections were detected in 64.5% of retrovirus-positive cats, suggesting immunosuppression may facilitate pathogen persistence. This finding is consistent with prior studies reporting synergistic interactions between retroviruses and vector-borne infections in cats (Assarasakorn et al., 2012; Latrofa et al., 2020).

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About the author

Mohamed Sh Mohamud Yusuf was born in Garbaharey, Gedo, Somalia on 10th of January 1993. Following the high School education, he started his under graduation in Veterinary Medicine in Somali National University, Somalia, and obtained his bachelor's degree of Veterinary Medicine in 2019. He employed as assistant lecture in faculty of Veterinary medicine at Somali National University. In 2020-2021 he obtained her master's degree in veterinary medicine. In 2022, he started his PhD study in "Animal Health and Zoonosis" at the University of Bari Aldo Moro, under the supervision of Prof. Grazia Greco.

Dr. Yusuf's research focuses on emerging infectious diseases and the One Health approach, with a particular emphasis on the epidemiology and distribution of pathogens in domestic animals. He has contributed to multiple collaborative research projects, especially those addressing vector-borne diseases and other infectious threats affecting animal and public health.

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