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Mycoplasma bovis infection in Spanish cattle herds and evaluation of new control strategies

Infección por *Mycoplasma bovis* en rebaños bovinos españoles y evaluación de nuevas estrategias de control

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Infección por *Mycoplasma bovis* en rebaños bovinos españoles y evaluación de nuevas estrategias de control

Memoria presentada por la Graduada en Veterinaria

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Para optar al grado de Doctora con Mención Internacional

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A mis abuelos

A mi hermano

A mis padres

A Gonzalo

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1. General introduction

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1.1. Introduction

The present doctoral thesis consists of a compendium of five scientific publications and aspires to the mention “International doctoral research component”. The articles have been published in scientific journals indexed in the Journal of Citation Reports (JCR, 2020-2021), in the categories of Veterinary Sciences, Microbiology, and Biotechnology and Applied Microbiology. These publications constitute a scientific unit in the framework of *Mycoplasma bovis* research.

M. bovis is an important pathogen of cattle responsible for detrimental effects on economics and animal welfare on the cattle industry worldwide. The agent causes mastitis, pneumonia, arthritis, otitis media, keratoconjunctivitis and genital disorders (Maunsell et al., 2011; Nicholas & Ayling, 2003). *M. bovis* is involved in the bovine respiratory disease complex (BRD), a leading cause of economic losses in the global beef cattle industry, and that especially affects feedlot calves (Arcangioli et al., 2008; Pardon et al., 2013; Radaelli et al., 2008). In most countries, there are no effective vaccines commercially available and control strategies rely on good farming practices and antimicrobial treatment (Dudek et al., 2021; Perez-Casal et al., 2017). However, antimicrobial resistance has been reported by many countries over the past years (Ayling et al., 2014; Bokma et al., 2020; Gautier-Bouchardon et al., 2014; Gerchman et al., 2009; Hendrick et al., 2013; Heuvelink et al., 2016; Jelinski et al., 2020; Kawai et al., 2014; Kong et al., 2016; Soehnlen, Kunze, et al., 2011; Sulyok et al., 2014).

M. bovis circulates in Spain, as it has been detected in nasal swabs and lung tissue of young calves with clinical respiratory disease (Klein et al., 2017, 2019), and in pneumonic lungs of asymptomatic carriers (Fernández et al., 2020). Nevertheless, these studies provide little data about the distribution and features of circulating isolates, which is crucial to establishing effective preventive and control measures. There are also no studies that investigate the role of *M. bovis* in BRD affecting feedlot calves in Spain by combining histopathological analysis of pneumonic lesions with the identification of *M. bovis* antigens in the observed lesions. On the other hand, in France, a nearby country that exports a high number of animals to Spain, there are two main groups of isolates currently circulating. Based on a partial sequence of their *polC* gene, these are the subtypes (STs) 2 and 3 (Becker et al., 2015). Both STs are resistant to tilmicosin and tylosin (macrolides), and oxytetracycline (tetracycline) (Khalil et al., 2017). They differ in their capacity to acquire fluoroquinolone resistance *in vitro*. In this sense, ST3 easily

becomes resistant under selective pressure, whereas ST2 is somehow blocked in the development of resistance (Khalil et al., 2016). Notably, the first multiresistant ST3 isolate reported in France was isolated from a calf born in Spain and raised in a veal-calf herd in Southwest France (Becker et al., 2015). How widespread these two and other STs are in Spain and whether their antimicrobial susceptibility profile matches their *polC* typing is unknown.

Mycoplasma chromosomal transfer (MCT) is an unconventional mechanism of horizontal gene transfer (HGT) that was recently documented *in vitro* using *M. agalactiae*, a small ruminant pathogen phylogenetically close to *M. bovis* (Dordet-Frisoni et al., 2014). MCT enables the recipient cell to acquire small and large chromosomal fragments from any region of the donor genome, resulting in an endless variety of mosaic genomes. For MCT to occur, at least one partner must carry a functional integrative conjugative element (ICE) (Dordet-Frisoni et al., 2014, 2019). Although ICEs are widespread among *M. bovis* and other ruminant mycoplasmas (Tardy et al., 2015), the occurrence of MCT in the field remains to be addressed.

The introduction of *M. bovis*-infected cattle is thought to be the primary route of *M. bovis* entrance into a herd or area (Maunsell et al., 2011; Nicholas & Ayling, 2003). In herds or areas in which external animals are not introduced, the relative importance of diluted semen used for artificial insemination could be increased. Recently, semen was reported to be the source of *M. bovis* mastitis outbreaks in two closed dairy herds in Finland (Haapala et al., 2018). This reveals the need for a re-evaluation of the antimicrobials used for preparing seminal doses or contemplating other measures to prevent and/or control the transmission of *M. bovis* that might occur when using contaminated semen for artificial insemination.

The aforementioned questions were addressed among the five scientific articles that constitute the doctoral thesis, which are listed below:

1. *Mycoplasma bovis* in Spanish cattle herds: two groups of multiresistant isolates predominate, with one remaining susceptible to fluoroquinolones.
    - Authors: Ana García-Galán, Laurent-Xavier Nouvel, Eric Baranowski, Ángel Gómez-Martín, Antonio Sánchez, Christine Citti and Christian De la Fe.
    - Journal: Pathogens.
    - Date of publication: 7 July 2020.
    - Volume: 9.
    - Article number: 545.
    - Category of Journal Citation Reports (2020): Microbiology.
    - Impact factor: 3.492.
    - Position: 67/137 (Q2).
    - DOI: https://doi.org/10.3390/pathogens9070545

2. Importance and antimicrobial resistance of *Mycoplasma bovis* in clinical respiratory disease of feedlot calves.
    - Authors: Ana García-Galán, Juan Seva, Ángel Gómez-Martín, Joaquín Ortega, Francisco Rodríguez, Ángel García-Muñoz and Christian De la Fe.
    - Journal: Animals.
    - Date of publication: 20 May 2021.
    - Volume: 11.
    - Article number: 1470.
    - Category of Journal Citation Reports (2021): Veterinary Sciences.
    - Impact factor: 3.231.
    - Position: 16/144 (Q1).
    - DOI: https://doi.org/10.3390/ani11051470

3. Genome mosaicism in field strains of *Mycoplasma bovis* as footprints of in-host horizontal chromosomal transfer.
   - Authors: Ana García-Galán, Eric Baranowski, Marie-Claude Hygonenq, Mathilda Walch, Guillaume Croville, Christine Citti, Christian De la Fe and Laurent-Xavier Nouvel.
   - Journal: Applied and Environmental Microbiology.
   - Date of publication: 11 January 2022.
   - Volume: 88.
   - Article number: e0166121.
   - Category of Journal Citation Reports (2021): Biotechnology & Applied Microbiology.
   - Impact factor: 5.005.
   - Position: 46/158 (Q2).
   - DOI: https://doi.org/10.1128/AEM.01661-21

4. The addition of *Lactobacillus* spp., enrofloxacin or doxycycline negatively affects the viability of *Mycoplasma bovis* in diluted bovine semen.
   - Authors: Ana García-Galán, Ángel Gómez-Martín, Esther Bataller, Jesús Gomis, Antonio Sánchez, Joaquín Gadea, Luis Alberto Vieira, Empar García-Roselló and Christian De la Fe.
   - Journal: Animals.
   - Date of publication: 13 May 2020.
   - Volume: 10.
   - Article number: 837.
   - Category of Journal Citation Reports (2020): Veterinary Sciences.
   - Impact factor: 2.752.
   - Position: 19/146 (Q1).
   - DOI: https://doi.org/10.3390/ani10050837

5. The addition of *Lactobacillus* spp. negatively affects *Mycoplasma bovis* viability in bovine cervical mucus.
   - Authors: Ana García-Galán, Christian De la Fe, Jesús Gomis, Esther Bataller, Antonio Sánchez, Juan José Quereda, Empar García-Roselló and Ángel Gómez-Martín.
   - Journal: BMC Veterinary Research.
   - Date of publication: 20 July 2020.
   - Volume: 16.
   - Article number: 251.
   - Category of Journal Citation Reports (2020): Veterinary Sciences.
   - Impact factor: 2.741.
   - Position: 20/146 (Q1).
   - DOI: https://doi.org/10.1186/s12917-020-02454-9

1.2. Bibliographic review

1.2.1. The class *Mollicutes*

Mycoplasma bovis belongs to the class *Mollicutes* (trivial name “mycoplasma”), from the phylum Tenericutes. The term *Mollicutes* derives from the Latin words “molli” (soft) and “cutes” (skin). Since its first description, this class has been divided into five orders: *Mycoplasmatales, Entomoplasmatales, Haloplasmatales, Acholeplasmatales* and *Anaeroplasmatales*. According to this approach, *M. bovis* belongs to the order *Mycoplasmatales*, family *Mycoplasmataceae* and genus *Mycoplasma*. Recently, according to phylogenetic analysis of genomic data, several authors proposed sweeping changes to the nomenclature of members of the *Mollicutes* class, specifically involving the introduction of the order *Mycoplasmoidales* ord. nov (Gupta et al., 2018). In the new system, *M. bovis* belongs to the order *Mycoplasmoidales*, family *Metamycoplasmataceae* and genus *Mycoplasmopsis*, and so it is designated as *Mycoplasmopsis bovis* instead of *Mycoplasma bovis.* This change was validly proposed and published by the International Committee on Systematics of Prokaryotes (ICSP) (Oren & Garrity, 2018, 2019). However, the community of clinical mycoplasmologists and the International Committee on Systematics of Prokaryotes Subcommittee on the taxonomy of *Mollicutes* recommends the rejection of the new taxonomic approach (Balish et al., 2019; May & Brown, 2019). The present doctoral dissertation follows the recommendation of this collective.

Mollicutes derived from a common ancestor of *Clostridia* and *Bacilli* (phylum Firmicutes), Gram-positive bacteria with low GC content (Razin et al., 1998). This most likely occurred by a process called reductive evolution, which was marked by successive and drastic gene losses (Sirand-Pugnet, Citti, et al., 2007). As a result, bacteria of the class *Mollicutes* are characterized by their lack of a cell wall, the small cell diameter (0.15-0.45 µm) and genome size (0.5-2.0 Mb), and paucity of metabolic pathways (Razin et al., 1998). This last led them to adopt a parasitic or commensal lifestyle.

Based on their 16S rRNA gene sequence, *Mollicutes* can be classified into four phylogenetic groups: Spiroplasma*,* Pneumoniae*,* Hominis and Acholeplasma-Phytoplasma (Figure 1). Species of the Spiroplasma group infect plants and animals. Those from the Acholeplasma-Phytoplasma group are found in plants and their vector insects. Species from the Pneumoniae and Hominis groups infect humans and a wide range of animals. *M. bovis* belongs to the Hominis group and is closely related to the small ruminant pathogen *M. agalactiae.*

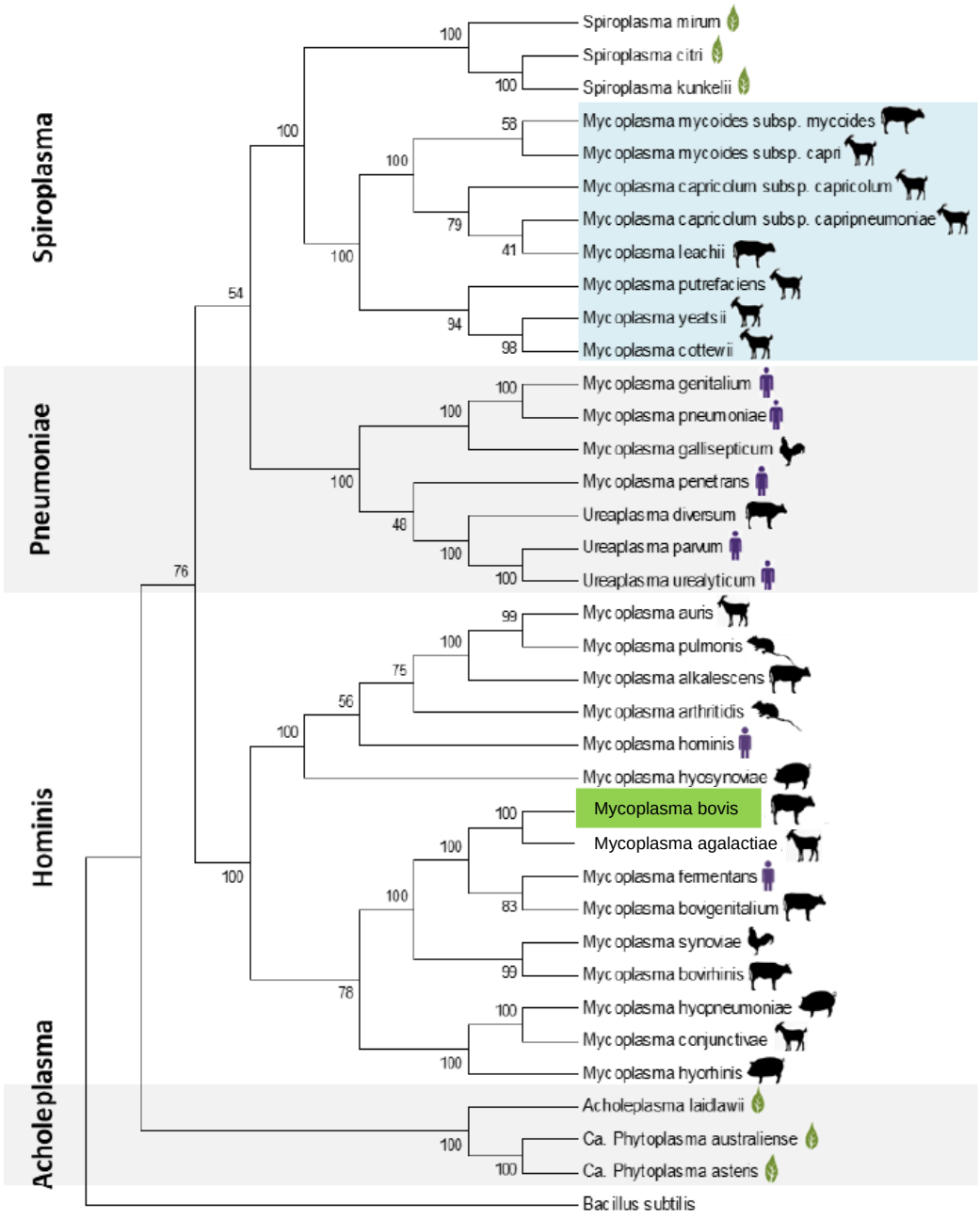

Figure 1. Phylogenetic tree of *Mollicutes*. Phylogenetic tree based on 16S ribosomal DNA sequences of 37 major representatives of *Mollicutes* aligned with clustal W (Mega 7), 1340 nucleotide positions, Neighbor-Joining method, and 500 bootstrap replicates (percentage indicated next to each branch). *Bacillus subtilis* is included as an outgroup. Phylogenetic groups are shown on the left. *M. bovis* is highlighted in green and the cluster mycoides in blue. The common host of each species is symbolized next to the name. Adapted from Faucher, 2018.

1.2.2. *Mycoplasma bovis*

M. bovis is an important pathogen of cattle responsible for detrimental effects on economics and animal welfare on the cattle industry worldwide. The agent causes mastitis, pneumonia, arthritis, otitis media, keratoconjunctivitis and genital disorders (Maunsell et al., 2011; Nicholas & Ayling, 2003). In addition to cattle, *M. bovis* is a primary pathogen of bison (Register et al., 2018). Sporadic infections in other animals, such as

buffaloes, sheep, pigs, white-tailed deer or chickens, have been reported (Dyer et al., 2004; Kumar et al., 2012; Marouf et al., 2011; Ongor et al., 2008; Spergser et al., 2013).

1.2.2.1. General features

The complete genome of *M. bovis* has an average size of 1.05 Mb, GC content of approximately 29.2% and 779 coding DNA sequences (CDSs) (*Genome List - Genome - NCBI*, n. d.). The genome is composed of one circular chromosome and there is no plasmid described for this mycoplasma species.

Given its small genome size, *M. bovis* has limited metabolic capacities and depends on external sources of amino acids, nucleic acid precursors and lipids (Calcutt et al., 2018). This species metabolizes neither glucose nor arginine but uses organic acids, such as lactate and pyruvate, as energy sources (Khan, Loria, et al., 2005). Some strains produce hydrogen peroxide as a product of metabolism, which could be a pathogenicity factor during infection (Khan, Miles, et al., 2005; Zhu et al., 2019).

M. bovis requires complex culture media and grows slowly at 37 °C and 5% of CO2 (Nicholas & Baker, 1998). On solid agar, colonies have a typical “fried egg” morphology, visible under a light microscope. This species can produce films and spots because of lipolytic activity (Thorns & Boughton, 1978) (Figure 2).

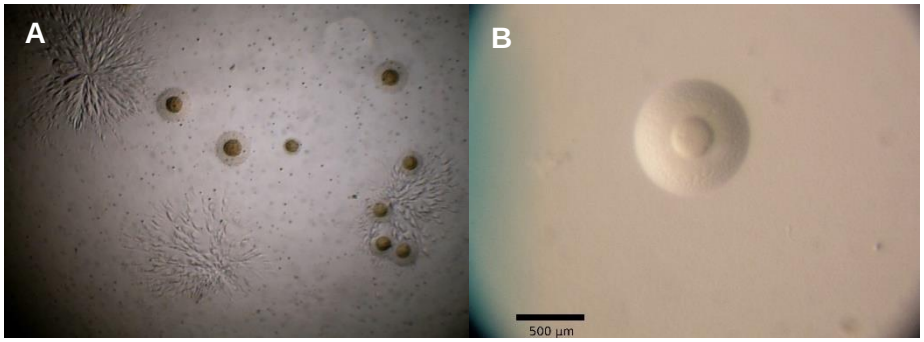

Figure 2. Colonies of *M. bovis* under a light microscope. (A) Colonies of *M. bovis* and films and spots. (B) Detail of a colony with the typical fried egg morphology.

The lack of a cell wall increases the susceptibility of mycoplasmas to environmental factors, such as pH variations, high temperatures, or osmotic stress, but renders them resistant to antimicrobials targeting the wall. The cell membrane contacts directly with the host, which can affect the host-pathogen interaction. For instance, *M. bovis* possesses a family of variable membrane surface lipoproteins (Vsps) that are major immunogenic antigens (Behrens et al., 1994; Lysnyansky et al., 1999). The Vsps can undergo high-frequency phase (ON-OFF) and size variation through complex mechanisms, which in turn provide the pathogen with the capacity to evade host immune defenses. Some Vsps have a role in adhesion to the host (Sachse et al., 1996), and

others in biofilm formation (McAuliffe et al., 2006). Comparative genome studies revealed differences in the number of *vsp* genes between strains. For instance, the reference strain PG45 has a 13-*vsp* gene cluster, whereas the strain HB0801 has a reduced set of 6-*vsp* genes (Qi et al., 2012).

Despite the cell wall-less characteristic, *M. bovis* can survive in the environment for long periods if protected from heat and desiccation. For example, the pathogen survives at 4 °C for nearly 2 months in sponges and milk, for over 2 weeks in water and wood, over 3 weeks in straw, and for years in frozen sperm, whereas at higher environmental temperatures (>20 °C), survival drops considerably (Pfützner & Sachse, 1996). Some strains can produce biofilms, which are bacterial cells attached to a surface, or each other, surrounded by a polysaccharide matrix (McAuliffe et al., 2006). McAuliffe et al (2006) observed that, at least *in vitro*, biofilm-forming cells were more resistant to heat and desiccation than planktonic cells and proposed that this capacity facilitates the survival of this species in the environment, and perhaps, contributes to the chronic persistence in the host.

1.2.2.2. Geographic distribution

M. bovis was first isolated in 1961 in the United States from a case of severe mastitis in cattle, reaching Europe in the 1970s (Dudek et al., 2020; Hale et al., 1962). International trade in cattle and cattle products has enabled its silent spread to all continents where cattle are kept (Dudek et al., 2020). Some studies report the circulation of *M. bovis* in Spain (Fernández et al., 2020; Klein et al., 2017, 2019), but provide little or no information regarding the origin of the isolates, and hence, do not allow to get a global picture of the pathogen distribution in our country.

1.2.2.3. Economic impact on cattle rearing-industry

Economic losses associated with *M. bovis* infections include (i) reduced milk and meat production; (ii) increased mortality, which mainly affects calves; (iii) premature culling of animals where welfare is compromised; (iv) treatment; (v) veterinary costs; and (vi) implementation of diagnostic, control and prevention measures (Maunsell et al., 2011). Across Europe, respiratory diseases caused by *M. bovis* were estimated to cause losses higher than 140 million euros per year (Nicholas & Ayling, 2003). In the United States, the costs of loss weight gain and carcass value were estimated at 32 million dollars per year, and losses due to *M. bovis*-mastitis at 108 million dollars per year (Rosengarten & Citti, 1999).

1.2.2.4. Transmission routes

The purchase of asymptomatic carriers is thought to be the primary route of *M. bovis* introduction into a herd (Maunsell et al., 2011). Once inside, infected animals may shed the agent in colostrum, milk, nasal, conjunctival or genital secretions (Figure 3) (Dudek et al., 2020), being respiratory tract and mammary gland shedding the major reservoirs of infection within a herd (Maunsell & Donovan, 2009). Stress may exacerbate disease and shedding (Calcutt et al., 2018). The Spanish beef cattle sector imports many animals from other countries, and the movement of animals between national farms is also a common practice (Figure 4). In the dairy sector, the movement of animals between farms is not so common. However, if the replacement rate of animals born at the farm is not sufficient to maintain the milk production, external animals may be introduced.

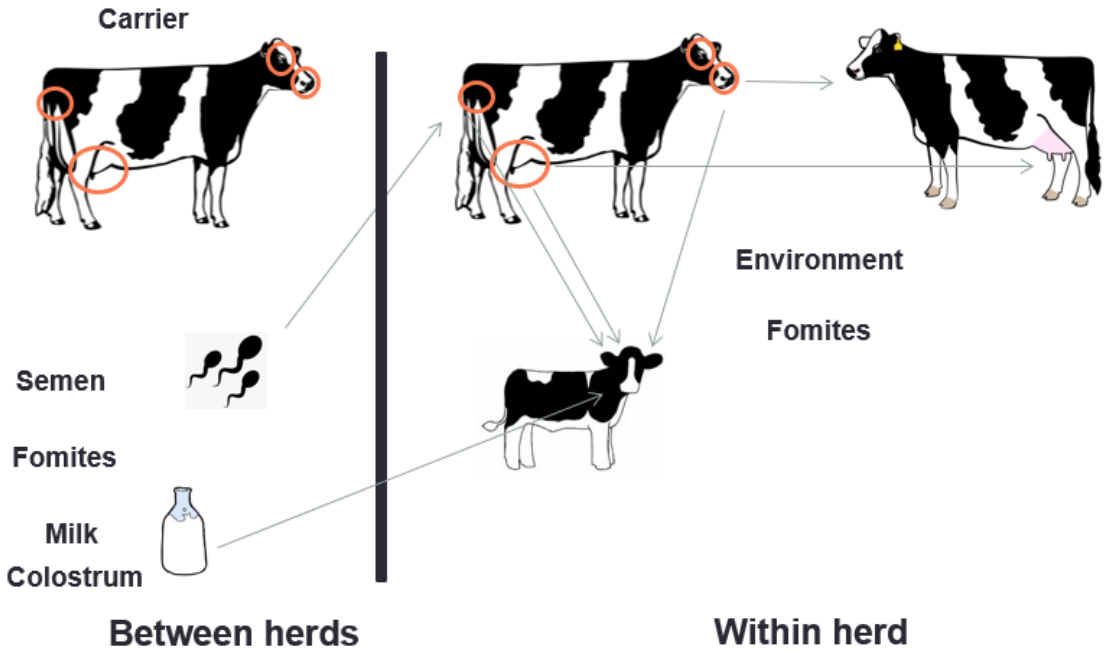

Figure 3. Possible pathways of *M. bovis* transmission. Orange circles represent the ways of excretion in the carrier.

Transmission can occur via aerosols, direct nose-to-nose contact and udder-to-udder during the milking process via fomites (e.g., milking unit liners, milker´s gloves, udder wash cloths) (Figure 3) (Fox & Gay, 1993; Maunsell et al., 2011). Other fomites, such as metal syringes, treatment materials, clothes of visitors and all equipment shared between farms and animals, could be a source of infection (Gonzalez et al., 1992; Maunsell et al., 2011). Calves can also become infected by ingesting infected milk or colostrum. Transmission from vaginal secretions of cows at calving, and via congenital during pregnancy is possible, although both events appear to occur infrequently (Maunsell & Donovan, 2009).

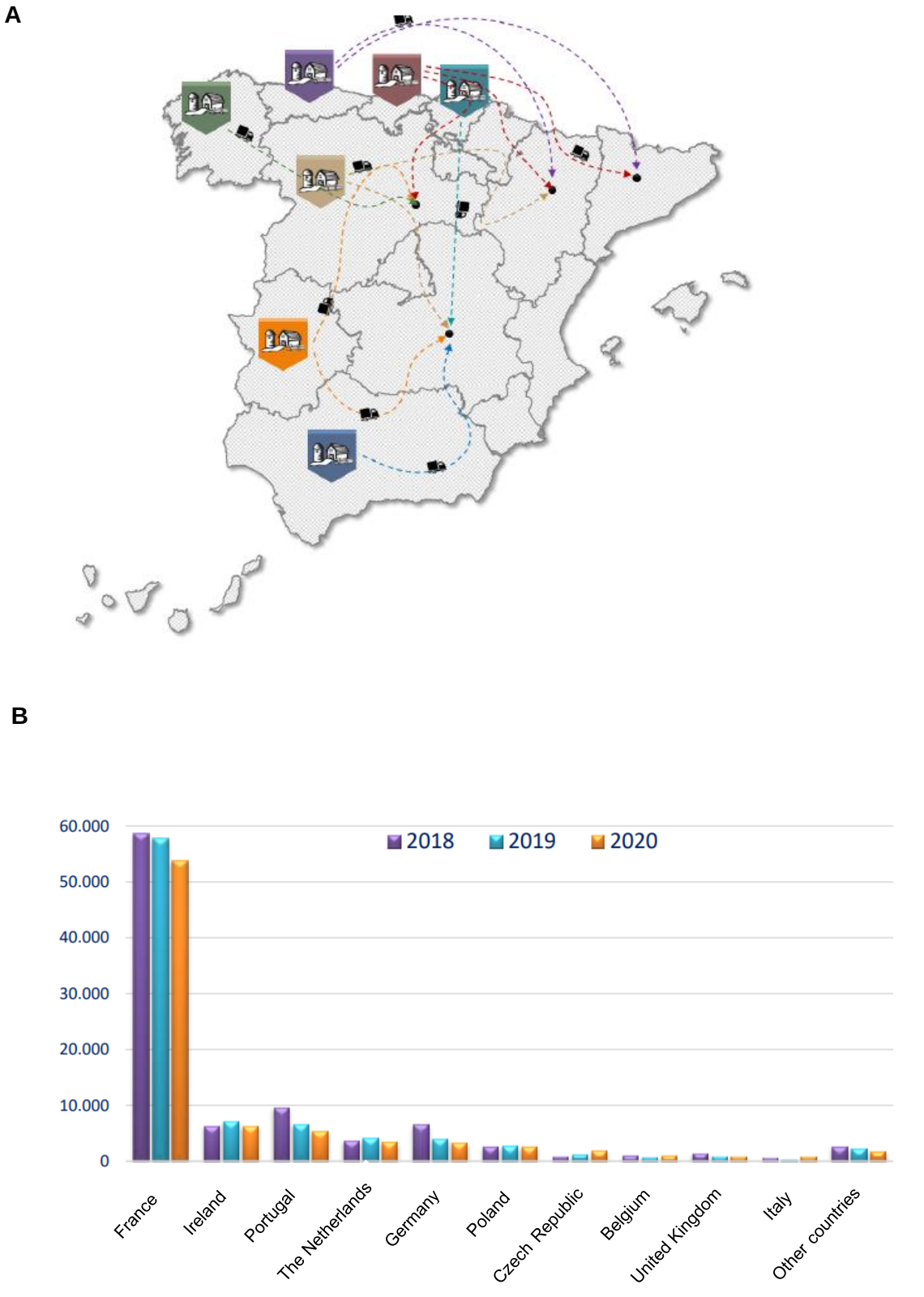

Figure 4. Main beef cattle movements in Spain. (A) Main animal movement to feedlots between Spanish autonomous communities (2017-2018). Adapted from *Estudio del sector español de vacuno de cebo: datos SITRAN*, 2019. (B) Main entries of beef cattle from other countries (tonnes) (2018-2020). Adapted from *Caracterización del sector vacuno de carne en España*, 2020.

Venereal transmission is not excluded. In cows, the genital infection can occur by artificial insemination with infected semen (Figure 3), and in males, by the licking of shedder animals (Haapala et al., 2018; Pfützner & Sachse, 1996). *M. bovis*-infected embryos might be another source of infection (Bielanski et al., 2000).

The capacity of *M. bovis* to survive in bedding materials, such as feed, water, straw, manure or recycled bedding sand, has led to the hypothesis that the environment could be a source of transmission (Figure 3) (Justice-Allen et al., 2010; Pfützner & Sachse, 1996; Piccinini et al., 2015). Some authors reported that bedding sand positive for *M. bovis* showed no evidence of being a source of infection to naïve calves despite exposure for several weeks along with extensive diagnostic testing for the agent in the animals (Wilson et al., 2011). However, according to Piccinini et al (2015), the housing and transport conditions should be considered to evaluate the environmental risk of *M. bovis* transmission.

Other animals – bison, buffaloes, sheep, pigs, white-tailed deer and chickens – can become infected, and they should be taken into account as possible sources of infection (Dyer et al., 2004; Kumar et al., 2012; Marouf et al., 2011; Ongor et al., 2008; Register et al., 2018; Spergser et al., 2013).

Although most *M. bovis* organisms reside on mucosal surfaces or extracellularly within lesions, the agent can also invade and survive in a variety of host cell types (e.g. alveolar macrophages, lymphocytes, erythrocytes, epithelial cells), which could enhance evasion of the host immune response and dissemination by the hematogenous route (Maunsell & Chase, 2019).

Once in the herd, *M. bovis* is very difficult to eradicate. Infected cattle can develop clinical symptoms or become asymptomatic carriers. In both cases, these animals constitute a source of infection for other animals.

1.2.2.5. Clinical manifestations

M. bovis is a major cause of mastitis, pneumonia and arthritis in cattle. In addition, the pathogen can cause otitis media, genital disorders, keratoconjunctivitis and other manifestations (Maunsell et al., 2011; Nicholas & Ayling, 2003; Pfützner & Sachse, 1996).

1.2.2.5.1. Mastitis

M. bovis-mastitis is highly contagious and affects cows of any age or stage of lactation. The herd presentation varies from endemic subclinical disease to severe clinical outbreaks (Maunsell et al., 2011). Clinical mastitis is characterized by inflammation of

more than one quarter, a great alteration of milk consistency (from watery to purulent) and a drastic drop in milk production (Pfützner & Sachse, 1996). In subclinical cases, cows can have an increased somatic cell count, decreased milk yield and lower milk quality (Timonen et al., 2017). *M. bovis*-mastitis can be accompanied by arthritis or respiratory disease in mastitic cows or other animals of the herd (Maunsell et al., 2011).

1.2.2.5.2. Pneumonia

M. bovis-pneumonia can occur in any age cattle. It is part of the bovine respiratory disease complex (BRD), a leading cause of economic losses in the global beef cattle industry, and that especially affects feedlot calves (Arcangioli et al., 2008; Pardon et al., 2013; Radaelli et al., 2008). Other infectious agents involved in BRD are bovine herpesvirus type 1 (BHV-1), bovine viral diarrhea virus (BVDV), bovine respiratory syncytial virus (BRSV), bovine coronavirus (BCOV), parainfluenza-3 (PI-3) virus, *Mannheimia haemolytica*, *Pasteurella multocida*, *Histophilus somni* and *Trueperella pyogenes* (Arcangioli et al., 2008; Cirone et al., 2019; Radaelli et al., 2008).

Clinical signs of *M. bovis*-pneumonia are usually indistinguishable from those caused by the other respiratory pathogens and include fever, dyspnea, coughing, nasal discharge, depression and decreased or no appetite (Caswell & Archambault, 2007; Maunsell et al., 2011). In subclinical cases, production variables such as carcass weight are decreased and days of fattening are increased (Fernández et al., 2020). Features that specifically suggest *M. bovis* pneumonia are concurrence with arthritis, otitis media, or both, in the same animal or other animals in the herd and chronicity (Caswell & Archambault, 2007; Maunsell et al., 2011).

In the lungs, the pathogen can cause caseonecrotic bronchopneumonia with multiple foci of caseous necrosis (Figure 5) (Caswell & Archambault, 2007; Gagea et al., 2006). Some authors consider bronchiolitis as another *M. bovis*-distinctive lesion (Oliveira et al., 2020; Rodríguez, Castro, et al., 2015; Rodríguez, González, et al., 2015). In addition, the pathogen may contribute to necrosis of the bronchiolar epithelium, bronchus-associated lymphoid tissue (BALT) hyperplasia, and bronchiolar fibrosis (Hermeyer et al., 2011; Rodríguez, Castro, et al., 2015; Rodríguez, González, et al., 2015). The agent can contribute to bronchopneumonia with foci of coagulative necrosis or abscesses, but usually in co-infection with other bacteria (Caswell et al., 2010).

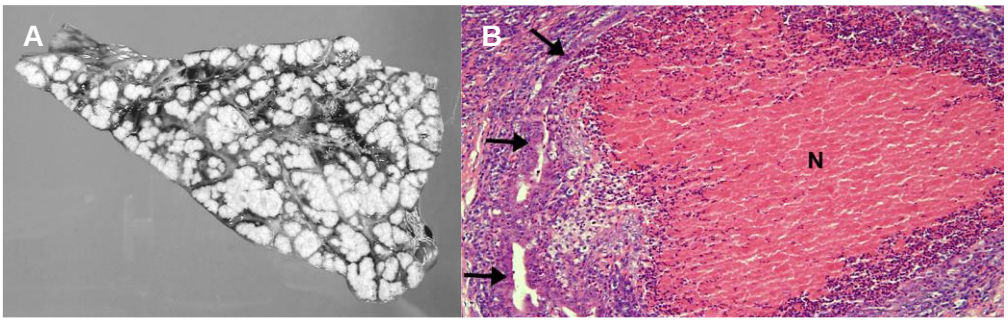

Figure 5. Caseonecotric bronchopneumonia caused by *M. bovis*. (A) Gross pathology. Cut surface of lung section with multiple foci of caseous necrosis. Adapted from Gagea et al., 2006. (B) Histopathology. Extended caseonecrotic focus with eosinophilic center (N) demarcated by inflammatory cells and remnants of the necrotic bronchiolar epithelium (arrows). Adapted from Hermeyer et al., 2012.

1.2.2.5.3. Arthritis

Cattle of any age can be affected by *M. bovis*-arthritis, which usually concurs with mastitis and/or pneumonia. Clinical signs include lameness, joint swelling, pain, heat on palpation, poor appetite and fever. Large rotator joints (e.g. shoulder, elbow, carpal, hip, stifle, hock) are the most commonly affected (Maunsell et al., 2011).

1.2.2.5.4. Otitis media

M. bovis-otitis media occurs in calves and sporadically in adult cattle, and often concurs with pneumonia, arthritis or both. Clinical signs can be unilateral or bilateral and include ear pain (evidenced by head shaking and scratching ears), ear droop, ptosis, epiphora, fever and poor appetite. Purulent discharge may appear if the tympanic membrane has broken (Maunsell et al., 2011; Walz et al., 1997).

1.2.2.5.5. Genital disorders

Genital disorders due to *M. bovis* are normally found only in a few individuals. In bulls, the pathogen may cause orchitis, vesiculitis and decrease sperm quality (Pfützner & Sachse, 1996), whereas in cows, it may cause endometritis, salpingitis, oophoritis, infertility and abortion (Hirth et al., 1966).

1.2.2.5.6. Other manifestations

M. bovis can be isolated from the conjunctiva of clinically healthy animals or cattle suffering from keratoconjunctivitis, and meningitis can appear as a complication of otitis media. In addition, the pathogen may cause endocarditis or decubital abscesses (Kanda et al., 2019; Maunsell et al., 2011).

1.2.2.6. Diagnosis

Clinical and pathological signs can guide the diagnosis, but they are not pathognomonic. Laboratory confirmation is thus especially important for the accurate diagnosis of *M. bovis*. Culture, PCR (polymerase chain reaction), immunohistochemistry and indirect

ELISA (enzyme-linked immunoabsorbent assay) are among the techniques more widely used for *M. bovis* diagnosis. For extended review on diagnostic techniques see (Calcutt et al., 2018; Caswell et al., 2010; Dudek et al., 2020; Parker et al., 2018).

1.2.2.6.1. Direct diagnosis

In clinically affected cattle, samples of choice are milk (mastitis), bronchoalveolar lavage or nasal swabs (pneumonia), synovial fluid (arthritis), ear swabs (otitis media), eye swabs (keratoconjunctivitis), and semen, genital discharge or preputial washings (genital disorders) (Nicholas & Ayling, 2003). In post-mortem analysis, tissue samples or tissue swabs can be collected from affected organs. In the absence of symptoms, bulk tank milk (BTM) can be an easy and informative sample for dairy herds (Nicholas et al., 2016). At the individual level, a milk sample or a nasal swab sample may help to detect carriers.

1.2.2.6.1.1. Culture

Culture enables creating a collection of isolates that can be used, for instance, for molecular epidemiological studies or antimicrobial resistance surveys. However, it requires specific conditions, as mycoplasmas demand high nutritional requirements. Media should contain at least three components: (i) a broth base suitable for mycoplasmas (e.g. PPLO broth base without crystal violet), (ii) yeast extract and (iii) serum (generally horse or bovine serum). The addition of one or more antimicrobials active against cell-wall bacteria enables to make the medium partially selective (Freundt, 1983; Tully, 1995). The culture of mycoplasmas is slow, and that of *M. bovis* requires 2 to 5 days of incubation. On solid agar, it is possible to visualize the colonies with the typical “fried egg” morphology, as well as films and spots (Figure 2).

1.2.2.6.1.2. Molecular-based identification

Currently, both conventional and real-time PCR remain the molecular methods most widely used for *M. bovis*-specific detection. Identification can be performed following culture or directly from the clinical sample, although culture enrichment of the samples improves detection when DNA is present at low concentrations. The chosen targets for diagnosis must be both interspecies-specific and intraspecies-conserved. Several targets can be used, such as the 16S rRNA gene (Cai et al., 2005; Chávez González et al., 1995), the *uvrC* gene (Clothier et al., 2010), the *oppD* gene (Sachse et al., 2010), the *fusA* gen (Boonyayatra et al., 2012), the *polC* gene (Marenda et al., 2005), or the membrane protein 81 gene (Foddai et al., 2005).

1.2.2.6.1.3. Immunohistochemistry

The detection of antigen-coding sequences by PCR does not necessarily mean that the identified agents are linked to a specific lesion or disease. Immunohistochemistry reveals the location of the agent antigen within a lesion in formalin-fixed paraffined embedded tissue sections by using monoclonal or polyclonal antibodies (Magaki et al., 2019). This technique can thus be used to establish a causal role of the agent in the development of lesions or disease.

1.2.2.6.2. Indirect diagnosis

1.2.2.6.2.1. Serology

Serology identifies antibodies in plasma, serum and milk. This method is usually employed for herd status screening because, on an individual animal level, titers may sometimes be poorly correlated with infection or disease (Maunsell et al., 2011). Currently, indirect diagnosis is mainly performed by commercially produced ELISAs, based on recombinant proteins (Andersson et al., 2019).

1.2.2.7. Molecular typing

Molecular typing of *M. bovis* isolates provides information about their genetic diversity and phylogenetic relationships, which could be of interest to trace their history and route of dissemination, as well as to assess the efficiency of sanitary measures. A very often used system is multilocus sequence typing (MLST). This system differentiates among strains based on the comparison of partial DNA sequences from housekeeping genes. There are two MLST systems commonly used to type *M. bovis*. One is based on partial sequences of the genes *dnaA*, *metS*, *recA*, *tufA*, *atpA*, *rpoD and tkt* (Rosales et al., 2015) and the other on partial sequences of the genes *dnaA*, *gltX*, *gpsA*, *gyrB*, *pta-2*, *tdk and tkt* (Register et al., 2020). Only the second scheme is publicly available and hosted at https://pubmlst.org/organisms/mycoplasma-bovis. On the other hand, the analysis of whole-genome sequence - single nucleotide polymorphism (WGS-SNP) is becoming popular because of its higher degree of discriminatory power (Yair et al., 2020).

In France, typing by analysis of a partial sequence of the housekeeping gene, *polC,* distinguished between old and recent *M. bovis* strains, collected before and after 2000, respectively (Becker et al., 2015). Currently, more than 80% of the isolates circulating in that country belong to subtype (ST) 2, followed by ST3, which represents almost 20% of the isolates, and ST5, which has only been detected in one animal (Becker et al., 2020). Another study observed a difference between ST2 and ST3 in their capacity to achieve fluoroquinolone resistance under selective pressure *in vitro*. In this sense, ST3 rapidly

acquires mutations in the quinolone resistance-determining regions (QRDR) and becomes resistant, whereas the genetic context of ST2 seems less prone to acquire this resistance (Khalil et al., 2016). On the other hand, field isolates of both STs were resistant to tilmicosin and tylosin (macrolides), and oxytetracycline (tetracycline), whatever the associated clinical condition (respiratory disease, arthritis, otitis, mastitis) (Khalil et al., 2017). The first description of a ST3 isolate was reported in France in 2011. Curiously, this isolate was obtained from a calf born in Spain and raised in Southwest France (Becker et al., 2015). However, how ST2, ST3 and other STs are distributed in Spain and whether their antimicrobial susceptibility profiles are congruent with *polC* typing remain unknown.

1.2.2.8. Prevention and control

1.2.2.8.1. Vaccination

In most countries, including Spain, there are no effective vaccines commercially available. Some inactivated and live-attenuated vaccines showed some efficacy in experimental studies, but they have not been reproduced commercially (Dudek et al., 2021). Autogenous vaccination might be a small-scale solution in closed herds but will have limited success in herds with frequent animal movements such as feedlots, as the highly antigenic variation of *M. bovis* implies that a vaccine produced from one isolate may not fully protect animals exposed to other isolates (Perez-Casal et al., 2017). In France, a live attenuated vaccine was recently approved for temporary use (*Protivity lyophilisat et solvant pour suspension injectable pour bovins* | *Anses - Agence nationale de sécurité sanitaire de l'alimentation, de l'environnement et du travail*, 2021), but its efficacy under field conditions remains to be proved. Two inactivated vaccines are commercially available in the United States, although their effectiveness is not fully satisfactory (Soehnlen, Aydin, et al., 2011). Improvements are therefore needed in this area, and reverse vaccinology is under research (Perez-Casal et al., 2017). By using this approach, all possible protein antigens conserved in all the isolates can be identified through bioinformatic tools, which can be then produced synthetically and tested in model animals (Rappuoli, 2000). Ideally, vaccines should be safe, effective against all disease manifestations and all *M. bovis* isolates, usable at all stages of animal production, stable, single shot, part of BRD vaccines involving other respiratory pathogens and provide long-term effective protective immunity (Calcutt et al., 2018).

1.2.2.8.2. Herd management and treatment

A closed herd policy is the best way of preventing the entrance of *M. bovis* into the herd. If that is not possible, quarantine, and laboratory testing can be effective to ensure that purchased animals are free of infection before the entrance. Combining testing of prior exposure (e.g., Indirect ELISA for detecting antibodies) and of current infection (e.g., PCR for detecting DNA) would offer great control against new infections and possibly latent infections (Calcutt et al., 2018).

If an infection is detected, isolation of the infected animals and early treatment with effective antimicrobials offers a chance to control the disease (Calcutt et al., 2018). It should be noted that antibiotherapy, if effective, may reduce the shedding and/or the clinical signs, but may not provide a bacteriological cure. On the other hand, there is often a poor response to treatment in cases of mastitis and chronic respiratory disease. In those cases, treatment is discouraged and culling is recommended (if not of all the affected animals, at least where welfare is compromised) (Nicholas et al., 2016; Calcutt et al., 2018). Metaphylactic treatment has been justified when high levels of morbidity and mortality because of *M. bovis*-associated disease could be expected in high-risk cattle (Maunsell et al., 2011). According to the Regulation EU 2019/6 (2019), "antimicrobial medicinal products should be used for metaphylaxis only when the risk of spread of an infection or of an infectious disease in a group of animals is high and where no appropriate alternatives are available" (p. 48).

Above all, it is important to maintain regular screening of the animals and/or the BTM to recognize asymptomatic carriers (Dudek et al., 2020).

Cleaning and disinfection can be applied to prevent a possible infection from the environment, or any equipment shared between farms and animals. This species is susceptible to commonly used chlorine-, chlorhexidine- acid-, or iodine-based disinfectants (Maunsell et al., 2011).

Other good farming practices are maintaining adequate ventilation, providing proper nutrition, vaccinating against other respiratory pathogens, avoiding sources of stress, such as overcrowding or thermal stress (heat, cold), and avoiding the mixing of animals from different age groups and lots. Biological materials, such as milk, colostrum, semen, or embryos, must be free of *M. bovi*s. Pasteurization of milk (at 71.7 °C for 15 s) and colostrum (at 60 °C for 30 min) should be enough to inactivate the pathogen (Godden et al., 2006; Stabel et al., 2004).

1.2.3. Antimicrobial susceptibility and mechanisms of antimicrobial resistance

Until effective vaccines are universally commercialized, good farming practices and antimicrobial treatment are the only approaches that can be used in attempt to control *M. bovis* infections. Antimicrobial agents active against *M. bovis* include fluoroquinolones, macrolides, lincosamides, phenicols, pleuromutilins, tetracyclines and aminoglycosides. However, many countries have reported a drastic reduction of the *in vitro* antimicrobial susceptibility of *M. bovis* isolates over the past years (Ayling et al., 2014; Bokma et al., 2020; Gautier-Bouchardon et al., 2014; Gerchman et al., 2009; Hendrick et al., 2013; Heuvelink et al., 2016; Jelinski et al., 2020; Kawai et al., 2014; Kong et al., 2016; Soehnlen, Kunze, et al., 2011; Sulyok et al., 2014).

The level of resistance/susceptibility of a bacterium to a given antimicrobial can be estimated by calculating the minimum inhibitory concentration (MIC). MIC is the minimum concentration of an antimicrobial that completely inhibits the growth *in vitro*. The microbroth dilution method is one of the most used techniques to determine the MIC value (Figure 6). There are general guidelines for testing the susceptibility of veterinary mycoplasmas to antimicrobials (Hannan, 2000), but no standard breakpoints are available. Therefore, MIC data are often compared to standard breakpoints given for other bacteria of veterinary interest (e.g. *Pasteurellaceae*) (Gautier-Bouchardon et al., 2014), breakpoints proposed for mycoplasma species by other authors (Hannan, 2000), or by documentation of genetic mutations conferring resistance (Lysnyansky & Ayling, 2016).

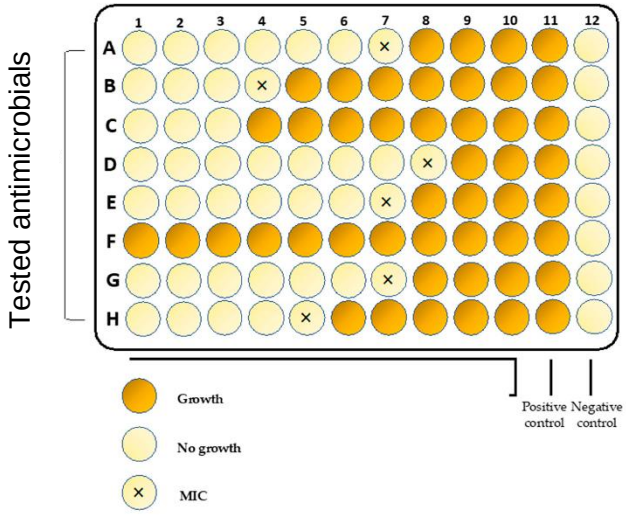

Figure 6. Scheme of the microbroth dilution method in a 96 well plate for MIC determination of eight antimicrobials (rows A-H). Each well contains a fixed volume of broth culture with a pH indicator. In columns 1 to 10, each well contains a variable concentration of the corresponding antimicrobial (column 1, the higher concentration; column 10, the lower concentration) and a fixed volume of bacterial inoculum. In column 11 (positive control) each well contains broth culture and bacterial inoculum and in column 12 (negative control) each well contains broth culture. The bacterial growth is indicated by a change of color from light yellow (no growth) to orange (growth). MIC is defined as the minimum antimicrobial concentration at which no growth (no change of color) is observed. Adapted from Jaśkiewicz et al., 2019.

1.2.3.1. Intrinsic resistance

Lacking the cell wall, *Mollicutes* are intrinsically resistant to antimicrobials that inhibit cell-wall synthesis (e.g., β-lactams, glycopeptides, cycloserines, or fosfomycin). The lack of membrane lipopolysaccharides and enzymes for the synthesis of folic acid results, respectively, in resistance to polymyxins and sulfonamide/trimethoprim (McCormack, 1993; Olaitan et al., 2014). A conservative mutation in the *rpoB* gene results in intrinsic resistance to rifampicin (Gadeau et al., 1986). *M. bovis* and other members of the Hominis and Spiroplasma phylogenetic groups are intrinsically resistant to the macrolide, erythromycin (Faucher, 2018; Waites et al., 2014).

1.2.3.2. Molecular mechanisms of acquired resistance

The three main molecular mechanisms of acquired antimicrobial resistance are (Figure 7):

A) The enzyme-catalyzed antimicrobial deactivation, which consists in degrading the antimicrobial or modifying it by the addition of chemical groups to its key active center.

B) The modification of the target through mutations of the coding target gene, or its protection by the addition of chemical groups that prevent the fixation of the antimicrobial.

C) The prevention of intracellular accumulation by reducing the membrane permeability, which decreases the uptake of the antimicrobial, or by enhancing the activity of efflux pumps, which increases the excretion outside of the cell.

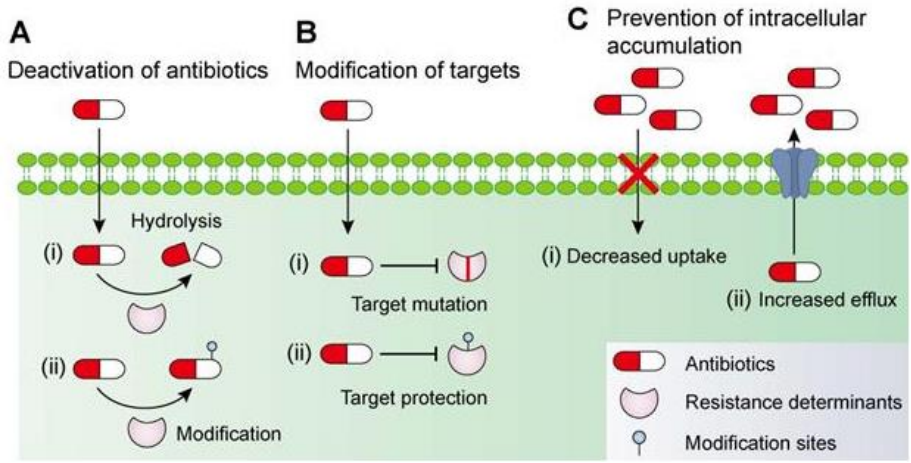

Figure 7. Scheme of the main molecular mechanisms of acquired antimicrobial resistance. (Liu et al., 2021).

The modification of the target through mutations of the coding target gene is the main molecular mechanism of acquired antimicrobial resistance in *Mollicutes*, and the only reported so far in *M. bovis.* In the literature, the mutations are systematically indicated according to positions in *Escherichia coli* (unless stated otherwise).

1.2.3.2.1. Target modification by mutation of the corresponding coding gene

Below, the prime mechanism of action of antimicrobials active against *M. bovis* and the main mutations linked to resistance in this species are briefly described.

Fluoroquinolones inhibit the activity of DNA gyrase and topoisomerase IV. These enzymes are involved in the regulation of DNA supercoiling, which is essential for replication, transcription, and cell division (Correia et al., 2017). The DNA gyrase is composed of the subunits GyrA and GyrB, encoded by the *gyrA* and *gyrB* genes, respectively. The topoisomerase IV is composed of the subunits ParC and ParE, encoded, respectively, by the *parC* and *parE* genes. The main resistant mechanism to fluoroquinolones is due to non-synonymous mutations in the QRDR of those genes. The mutations result in amino acid substitutions that alter the target protein structure and hence, the fluoroquinolone binding affinity to the enzyme, leading to drug resistance (Redgrave et al., 2014). In fluoroquinolone-resistant *M. bovis,* these hotspots are often found at codon 83 of *gyrA* and codons 80, 81 and 84 of *parC*. There is also a cumulative effect, as a single non-synonymous mutation at *gyrA* (codon 83) is enough to acquire an intermediate level of susceptibility, but additional non-synonymous mutations in *parC* are needed to achieve resistance (Gautier-Bouchardon, 2018; Hata et al., 2019; Khalil et al.,

2016; Lysnyansky, Mikula, et al., 2009; Mustafa et al., 2013; Sato et al., 2013; Sulyok et al., 2017).

Macrolides, lincosamides, phenicols and pleuromutilins are protein synthesis inhibitors that bind to the 50S ribosomal subunit and interact with the 23S rRNA. In addition, macrolides and lincosamides interact with the ribosomal proteins L4 and L22, and pleuromutilins with the ribosomal protein L3 (Leclercq, 2002; Schwarz et al., 2016). The 23S rRNA is encoded by one or more copies of the *rrl* genes and the ribosomal proteins L4, L22 and L3 are encoded, respectively, by the *rplD*, *rplV*, and *rplC* genes. In macrolide-resistance *M. bovis*, mutations are often found at positions 748, 752, 2058 and 2059 of the *rrl* genes*, rrl3* and *rrl4*, whereas mutations at positions 2059 and 2060 are key to achieving lincomycin resistance (Gautier-Bouchardon, 2018; Hata et al., 2019; Khalil et al., 2017; Kong et al., 2016; Lerner et al., 2014; Sulyok et al., 2017). Resistance to florfenicol was associated with mutations at positions 2062, 2063 and 2506, whereas resistance to pleuromutilins was linked with mutations at positions 2035, 2060, 2062, 2448, 2500, and 2611 (Sulyok et al., 2017). Additional non-synonymous mutations in L4 and L22 proteins have been evidenced but only the substitution Q93K/H in L22 (*M. bovis* PG45 numbering) was linked to macrolide resistance (Khalil et al., 2017). No mutation in protein L3 has been evidenced in *M. bovis*. Of note, *M. bovis* and other members of the Hominis and Spiroplasma phylogenetic groups are intrinsically resistant to the macrolide, erythromycin, due to a point mutation (G→A) at position 2057 (Faucher, 2018; Waites et al., 2014).

Tetracyclines and aminoglycosides are protein synthesis inhibitors that bind to the 30S ribosomal subunit and interact with the 16S rRNA (Brodersen et al., 2000; Kotra et al., 2000). The 16S rRNA is encoded by one or more copies of the *rrs* genes. In *M. bovis*, resistance to tetracyclines is often linked to mutations at positions 965 and 967 of the *rrs* genes, *rrs3* and *rrs4*, whereas mutations at position 1192 are linked to spectinomycin resistance (Amram et al., 2015; Gautier-Bouchardon, 2018; Hata et al., 2019; Khalil et al., 2017; Sulyok et al., 2017).

1.2.3.2.2. Other mechanisms of acquired resistance

Additional mechanisms have been described in other mycoplasma species. For instance, an active efflux that prevents the intracellular accumulation of fluoroquinolones has been reported in *M. hominis* and *M. mycoides* subsp. *capri* (Antunes et al., 2015; Raherison et al., 2002, 2005). A transposon carrying a protein named Tet(M) that confers protection of the ribosome from the action of tetracyclines was described in *M. hominis* and *Ureaplasma urealyticum* (Roberts et al., 1985; Roberts & Kenny, 1986). Another

example is a prophage carrying genes conferring resistance to aminoglycosides identified in *M. bovirhinis* (Lysnyansky & Borovok, 2021).

1.2.4. Horizontal gene transfer

Horizontal gene transfer (HGT) is the movement of genetic material between organisms that are not in a parent-offspring relationship. HGT enables the genetic mixing with transfers of large or small portions of genetic material, called mobile genetic elements (MGE), from a donor bacterium to a recipient bacterium. The acquisition of new genes may enable the recipient cell to eliminate deleterious mutations or to acquire new phenotypic features, such as resistance to antimicrobials, new metabolic properties, or new host specificity (Ochman et al., 2000). The three main mechanisms of HGT are (Figure 8):

A) Transduction: foreign DNA is introduced into a donor cell by a virus, called phage or bacteriophage. During viral encapsulation in the host/donor bacterium, fragments of bacterial DNA may be packaged into the viral capsid. The phages released by lysis will then infect other susceptible bacteria (Chiang et al., 2019).
B) Conjugation: the transfer of DNA occurs through a pore that connects the membranes of the donor and the recipient bacteria. This is the mechanism by which plasmids and integrative conjugative elements (ICEs) are transferred and requires direct contact to occur (Llosa et al., 2002; Wozniak & Waldor, 2010).
C) Transformation: this process occurs when a bacterium enters a “competence state" that allows the uptake of free DNA from its surroundings, which often originates from the lysis of other bacteria (Chen & Dubnau, 2004).

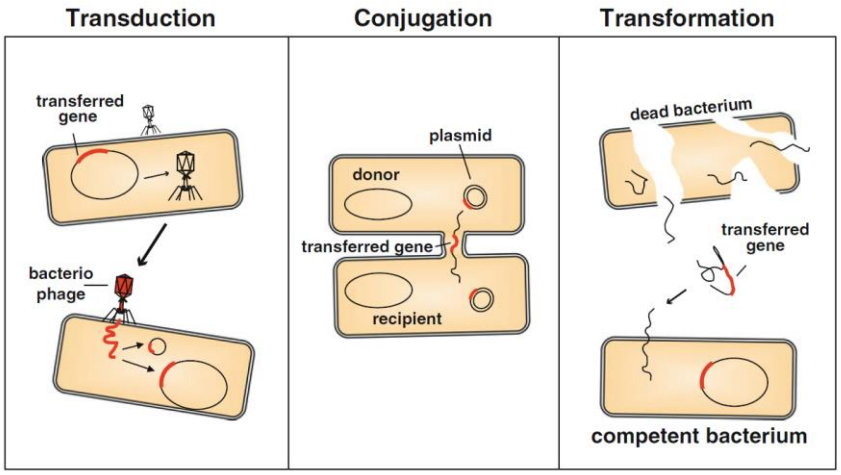

Figure 8. Major mechanisms of HGT in bacteria: transduction, conjugation and transformation. Red fragments represent transferred genes from the donor to the recipient strain (Blokesch, 2016).

Traditionally, HGT was assumed to be marginal in *Mollicutes*, but recent *in silico* comparative genomic studies revealed past events of HGT between phylogenetically distant species sharing the ecological niche. Important exchanges took place between *M. agalactiae* and mycoplasmas of the cluster mycoides, all pathogens of ruminants (Sirand-Pugnet, Lartigue, et al., 2007). Other exchanges took place between the human pathogens *M. hominis* and *U. parvum* (Pereyre et al., 2009), and between the avian pathogens *M. synoviae* and *M. gallisepticum* (Sirand-Pugnet, Lartigue, et al., 2007; Vasconcelos et al., 2005). Finally, the genomic comparison between the insect pathogen *Spiroplasma atrichopogonis*, and the crustacean pathogen *S. eriocheiris*, suggests that the transfer occurred in their common ancestor (Lo et al., 2015).

1.2.4.1. Mobile genetic elements

MGEs, in particular, phages, ICEs, and plasmids are key players in HGT. These MGEs are present in *Mollicutes*, although their distribution varies among genera and species (Breton et al., 2012; Citti et al., 2018; Marenda, 2014). Other MGEs such as insertion sequences (ISs) are found in many bacteria, including *Mollicutes*. Although not directly involved in HGT, ISs contribute to genome plasticity, as they can relocate themselves, promote genome re-arrangements or disrupt genes (Marenda, 2014). Of these MGEs, *M. bovis* carries ICEs, prophages and ISs, briefly described below.

1.2.4.1.1. Insertion sequences

ISs are short DNA segments (0.7-2.5 kb) capable of moving from place to place in a genome. They are composed of a gene encoding a transposase and two inverted repeats located upstream and downstream of the IS. When they are inserted into the genome, a sequence of the host genome is found duplicated on both sides. These are the direct repeats (Siguier et al., 2014) (Figure 9).

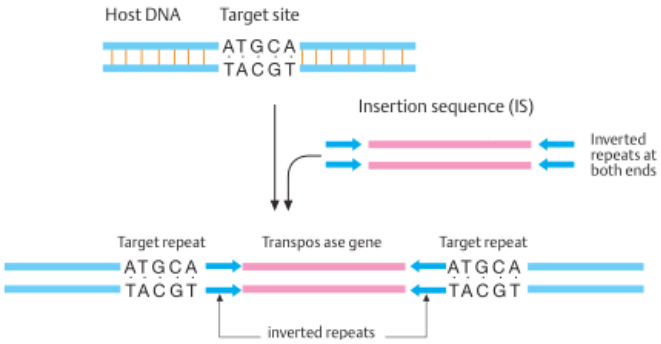

Figure 9. Structure of and IS. Blue arrows indicate the orientation of inverted repeats. Adapted from *Transposition*, 2017.

ISs are present in many species of the four phylogenetic groups: Spiroplasma, Pneumoniae, Hominis, Acholeplasma-Phytoplasma. Eight different ISs have been identified in *M. bovis*. They belong to the families IS*3*, (IS*Mbov4*), IS*30* (IS*Mbov1*, IS*Mbov6*) and IS*1634* (IS*Mbov2*, IS*Mbov3*, IS*Mbov5*, IS*Mbov8*, IS*Mbov9*) and represent up to 6.4% of the genome (Li et al., 2011; Lysnyansky, Calcutt, et al., 2009; Qi et al., 2012; Siguier et al., 2006; Thomas et al., 2005). Apart from gene disruptions, their position suggests that they are involved in genome re-arrangements. For instance, there is a large chromosomal inversion (540kb) between *M. bovis* strains PG45 and Hubei-1, which is located between two IS*Mbov3* in PG45 (Li et al., 2011).

1.2.4.1.2. Prophages

Phages are viruses that infect bacteria and can follow a lytic or lysogenic cycle. During the lysogenic cycle, the DNA of the virus integrates into the host chromosome or plasmid, a state in which the phage is called “prophage” and replicates with them. During cell damage, the prophage can be excised and enter the lytic cycle. The viral capsids are released, which will allow the infection of other bacteria and the horizontal transfer of genetic material by transduction (Chiang et al., 2019).

The mechanism of transduction has never been demonstrated in *Mollicutes*, but prophages have been identified in the phylogenetic groups Acholeplasma, Spiroplasma and Hominis (Citti et al., 2020; Marenda, 2014). The *M. bovis* strain RM16 carries the prophage MAgV1-like (31.7 kb) (Figure 10). The prophage MAgV1 was first described in the genome of an unusual *M. agalactiae* strain linked to an episode of mortality affecting Alpine ibexes in France (Tardy et al., 2012). Another prophage was observed in the genome of the *M. bovis* strain 3308MB (Citti et al., 2020). It was found to harbor sequences having some similarity to virus P1 (11.6 kb) of *M. pulmonis*, a rodent pathogen (Tu et al., 2001). Whether these viruses provide particular virulence or biological properties to *M. bovis* is unknown.

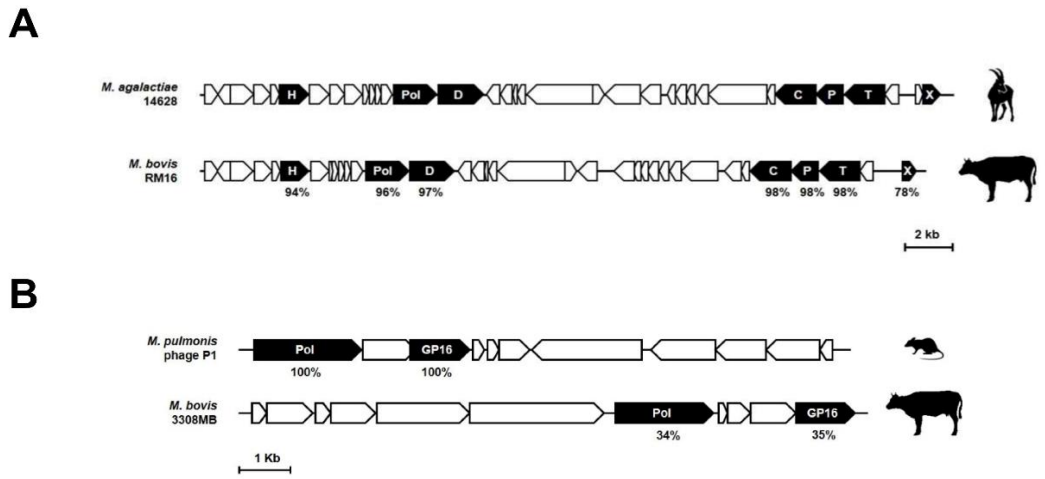

Figure 10. Genomic organization of phages MAgV1-like and P1-like. (A) Genomic organization of *M. agalactiae* phage MAgV1 of strain 14628 and comparison with MAgV1-like sequences identified in *M. bovis* RM16. The letter code in black arrows refers to CDS products: H, helicase; Pol, DNA polymerase; D, DNA primase; C, prohead protein; P, portal; T, terminase; X, Xer. For each black CDS the percentage of the global similarity with MAgV1 is indicated. (B) Genomic organization of *M. pulmonis* phage P1 and P1-like in *M. bovis* 3308MB. Black arrows indicate CDSs in phage P1 displaying some similarity with sequences of 3308MB genome. The letter code in black arrows refers to CDS products: Pol, DNA polymerase; GP16, DNA encapsidation protein. In A and B, animal shadows are used to illustrate the host tropism of each strain. Adapted from Citti et al., 2020.

1.2.4.1.3. Mycoplasma ICEs

Mycoplasma ICEs are large chromosomal regions (20-30kb) that can excise, circularize, transfer through conjugation and integrate randomly into the genome of the recipient cell, where they replicate as part of the host chromosome. They belong to a new family of ICEs that appear to be specific to the *Mollicutes* class and have been identified in species of the phylogenetic groups Hominis, Pneumoniae and Spiroplasma (Citti et al., 2018; Marenda, 2014).

These self-transmissible elements are composed of about 20 structural genes similarly orientated and flanked by two inverted repeats, which are juxtaposed in the free circular form (Figure 11). A minimal ICE backbone consisting of CDS1, CDS3, CDS5, CDS14, CDS16, CDS17, CDS19 and CDS22 is present across most documented ICEs. ICEs can be present as single or multiple copies into the host genome. Incomplete versions (shorter or with pseudogenes), called vestiges of ICE (vICE), are found in some species. *M. bovis* PG45 carries two ICE copies, ICEB-1 (21.9 kb) and ICEB-2 (37.1 kb), the latter being a vestigial form with 3 pseudogenes (CDS16, CDS17 and CDS19). ICEB-2 harbors the complete backbone, whereas ICEB-1 lacks CDS1 (Figure 11).

ICEs are widespread among *M. bovis* and other ruminant mycoplasmas (Tardy et al., 2015). Based on their gene organization and sequences they are divided into the Spiroplasma or the Hominis type. The two types especially differ at the CDS22, which is present in all the ICEs but is homologous to one or another type. A single strain can carry

both types. For instance, in *M. bovis* PG45, the ICEB-1 belongs to the Hominis type and the ICEB-2 to the Spiroplasma type.

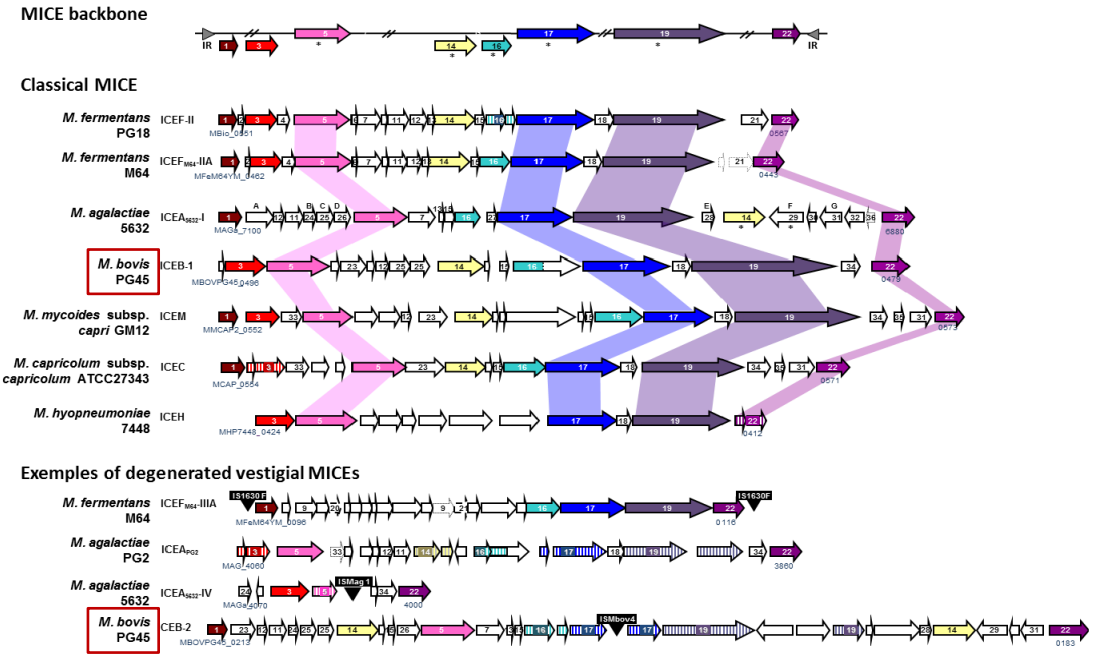

Figure 11. Mycoplasma ICE (MICE) backbone and genomic organization of major MICE representatives. *M. bovis* PG45 is highlighted in red. The MICE backbone is represented in the first line. CDSs conserved across MICEs are positioned on the solid line, whereas CDSs that are absent or truncated in particular MICEs are below. Inverted repeats (IR) are represented by grey triangles. CDSs containing transmembrane domains are indicated with an asterisk. Pseudogenes are represented by arrows filled with hatched color. ISs are represented as black boxes. Adapted from Citti et al., 2018.

The mechanism of ICE transfer from ICE-positive to ICE-negative cells was demonstrated using *M. agalactiae* as a model organism (Dordet Frisoni et al., 2013). The process was illustrated into 5 steps that represent the current proposed model for ICE-transfer in mycoplasmas (Figure 12) (Baranowski et al., 2018). Once in the recipient strain, the ICE integrates into the host chromosome interrupting genes and/or conferring conjugative properties to the recipient strain (Dordet-Frisoni et al., 2014, 2019; Faucher et al., 2019). Indeed, ICEs are key players in mycoplasma chromosomal transfer (MCT) (See below).

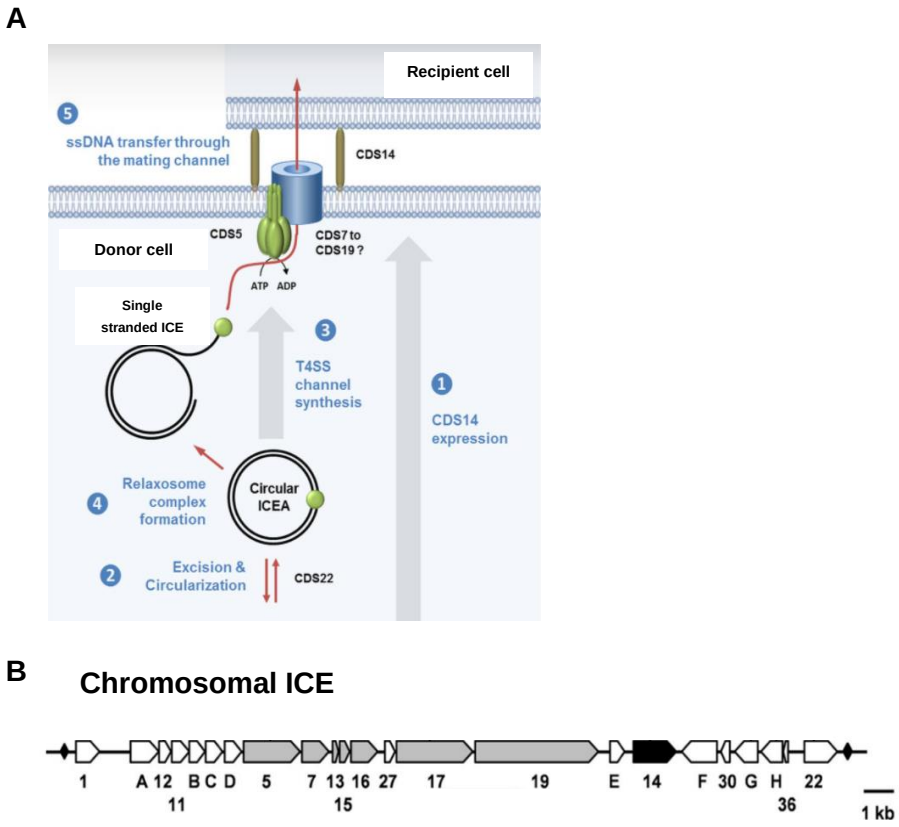

Figure 12. Mechanism of transfer and organization of ICEs of *M. agalactiae* 5632. (A) Illustration of the 5 steps of ICE transfer in *M. agalactiae*: 1) A surface lipoprotein (CDS14 product) would offer the initial contact between the donor and the recipient cell, required for bacterial conjugation; 2) Specific cellular conditions (e.g. cold stress) or random events would induce the ICE excision and circularization into a circular double-stranded DNA. Both events would be mediated by a DDE recombinase (CDS22 product); 3) The transmembrane conjugative channel would be formed as a result of the expression and assembly of the conjugative module (products of CDS5, CDS7, CDS13, CDS15, CDS16, CDS17, CDS19); 4) A protein complex known as relaxome would recognize the origin of transfer (oriT) on the circular ICE, and a relaxase would generate a linear single-stranded DNA by nicking the ICE DNA; 5) The transfer of the single-stranded DNA through the conjugative channel would be facilitated by TraG/VirD4 energetic component (CDS5), found at the inner side of the membrane. Once in the recipient strain, the ICE would re-circularize, become double-stranded and integrate randomly into the host chromosome. (B) Structure of a *M. agalactiae* 5632 ICE. The CDS14 lipoprotein is colored in black and the CDSs containing transmembrane domains in grey. Adapted from Baranowski et al., 2018.

1.2.4.2. Mycoplasma chromosomal transfer and genome mosaicism

MCT is a novel conjugative mechanism of HGT that was discovered in *M. agalactiae* under *in vitro* conditions. MCT involves the horizontal transmission of small or large chromosomal regions from any part of the donor genome that are incorporated into the recipient genome by homologous recombination (Dordet-Frisoni et al., 2014, 2019; Faucher et al., 2019). This distributive process can generate an infinitive variety of mosaic genomes, which offers the recipient genome a remarkably adaptative potential (Figure 13). Faucher et al., (2019) observed that MCT could accelerate fluoroquinolone resistance *in vitro* under enrofloxacin selective pressure, providing susceptible bacteria with the capacity to rapidly acquire several chromosomal loci carrying antimicrobial mutations in *gyrA*, *parE* and *parC* genes from pre-existent resistant populations.

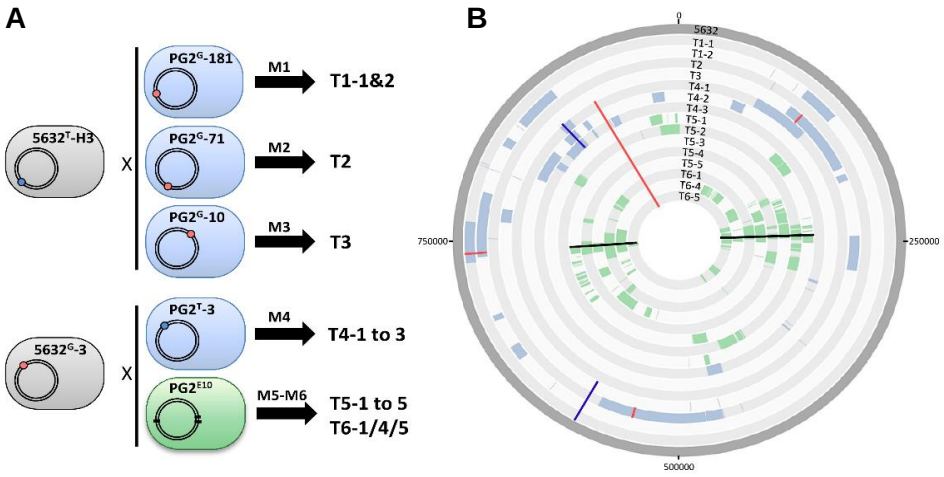

Figure 13. Mating experiments create a wide diversity of *M. agalactiae* transconjugants with mosaic genomes. (A) Mating experiments (M1 to M6) carried out to produce transconjugants. In mating experiments M5 and M6 the same parents were used. In the parental strains, 5632 and PG2, antibiotic markers providing resistance to tetracycline (blue dot) or gentamicin (red dot) had been previously introduced. These markers were in different loci depending on the mating. The parental PG2^{E10} (M5 and M6) had chromosomal mutations conferring resistance to enrofloxacin (fluoroquinolone). (B) Representation of the mosaic genome of the 15 transconjugants and the parental strain 5632. Genomic regions identical to 5632 are colored in grey and chromosomal fragments acquired from PG2 and PG2 ^{E10} in blue and green, respectively. In the genome of the transconjugants, the tetracycline and gentamycin resistance markers are indicated by blue and red lines, respectively, whereas the enrofloxacin resistance mutations are indicated by black lines. Adapted from Dordet-Frisoni et al., 2019.

Because ICEs encode the conjugative channel through which chromosomal fragments are transferred, MCT relies on the presence of at least one functional ICE in at least one partner, and the transfer occurs from ICE-negative to ICE-positive cells (Dordet-Frisoni et al., 2014, 2019; Faucher et al., 2019) (Figure 14).

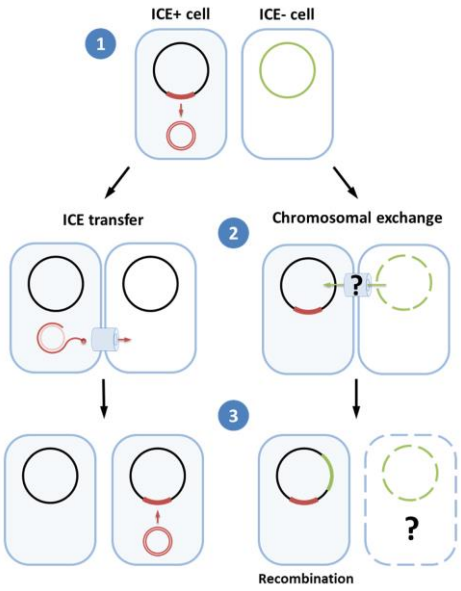

Figure 14. Conjugation in mycoplasmas: one ICE but two mechanisms. The ICE transfer occurs from ICE-positive to ICE-negative cells, whereas MCT occurs in the opposite direction (Citti et al., 2018).

Whether MCT occurs in the field, remains to be addressed. Concerning *M. bovis*, the widespread distribution of ICEs, together with their ability to induce conjugation, which

allows MCT, raises questions regarding the potential emergence of mosaic genomes in the field.

1.2.5. Genital transmission during artificial insemination

M. bovis has been isolated from bull commercial frozen semen (Amram et al., 2013). The pathogen can survive the *in vitro* fertilization process, infect embryos and reduce sperm penetration rates (Bielanski et al., 2000; Eaglesome & Garcia, 1990). Furthermore, insemination with *M. bovis* infected semen can cause chronic salpingitis, oophoritis and endometritis that result in infertility (Hirth et al., 1966).

Despite close contact with shedding carriers being the main source of *M. bovis* infection, the relative importance of semen used for artificial insemination could be increased in herds or areas in which external animals are not introduced. In Finland, *M. bovis* was introduced into two closed dairy herds through the use of contaminated semen during artificial insemination, leading to mastitis outbreaks in both farms (Haapala et al., 2018). In 2017, *M. bovis* was first detected in New Zealand, after samples collected from a dairy herd tested positive (Boyce et al., 2021). This country had not imported live cattle – the main source of cross-border infection – for about a decade. Although the origin of the outbreak was not definitively traced, imported frozen semen was pointed out as one of the potential sources (*Analysis of risk pathways for the introduction of Mycoplasma bovis into New Zealand*, 2017).

1.2.5.1. Antimicrobials in seminal doses

Several types of antimicrobials are added to seminal extenders before freezing to control bacterial contamination in semen. Gentamicin, lincomycin, penicillin, spectinomycin, streptomycin and tylosin are among the most common antimicrobials added to semen extenders, with the mixture containing gentamicin, lincomycin, tylosin and spectinomycin (GLTS) being widely used in bovine semen production (Bielanski, 2007; Vickram et al., 2017). Despite GLTS was shown to be effective to control mycoplasmas (Shin et al., 1988), some authors questioned its effectiveness to control *M. bovis* (Visser et al., 1999). In any case, the identification of semen used for artificial insemination as the source of *M. bovis*-mastitis outbreaks in Finland highlights (i) the need for re-evaluating antimicrobials added to semen or (ii) searching for alternative control measures.

1.2.5.2. *Lactobacillus* spp.

Lactobacillus is a genus of Gram-positive lactic acid bacteria (LAB) that habitually inhabit the vagina and the gastrointestinal and urinary tracts of humans and animals. *Lactobacillus* spp. are part of the vaginal saprophytic flora of cows, although at low

abundances (Swartz et al., 2014; Wang et al., 2013), and they have also been detected in bovine semen (González-Marín et al., 2011).

Lactobacillus spp.-based probiotics are commonly employed in human medicine to treat bacterial vaginosis, as they can compete for resources with pathogenic bacteria, stimulate the host immune system or produce organic acids that decrease pH (Charteris et al., 2001; Eschenbach et al., 1989; Mur et al., 2017). In periparturient cows, the intravaginal administration of LAB can reduce the incidence of purulent vaginal discharges and uterine infections, as well as accelerate uterine involution (Ametaj et al., 2014; Deng et al., 2014, 2015).

Several authors have studied the effect of medium acidification on mycoplasmas viability. Diluting goat semen contaminated with *M. mycoides* subsp. *capri* and *M. agalactiae* in an acidic semen extender (pH ≤ 6) had a detrimental effect on both mycoplasma species (Gómez-Martín et al., 2015). The acidification of contaminated milk to pH 4 for 1 hour was sufficient to kill *M. bovis* organisms (Parker et al., 2016). On the other side, an abnormally increased vaginal pH (>4.5) may enable the survival of *M. genitalium* in women with bacterial vaginosis (Huppert et al., 2013).

Considering the above mentioned, the role of *Lactobacillus* spp. as competing agents and pH acidifiers may be of interest for the control of *M. bovis* transmission related to artificial insemination through two ways: (i) the treatment of seminal doses and (ii) the administration of *Lactobacillus* spp.-based probiotics in the genital tract of cows.

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2. Summary

2. Summary

This doctoral thesis is presented as a compendium of publications and applies for the mention of “International doctoral research component”. The work assesses the presence and circulation of *Mycoplasma bovis* in Spanish cattle herds, providing data about the epidemiological background, molecular typing and antimicrobial susceptibility of circulating isolates (2016-2019). Furthermore, the epidemiological role of *M. bovis* in the clinical respiratory disease of feedlot calves raised in this country is analyzed. On the other hand, this work demonstrates the occurrence of *in vivo* mycoplasma chromosomal transfer (MCT) between field strains of *M. bovis*. Finally, the work addresses the role of enrofloxacin, doxycycline and lactic acid bacteria (LAB) of the genus *Lactobacillus* in the control of *M. bovis* transmission linked to artificial insemination.

M. bovis is an important pathogen of cattle responsible for mastitis, pneumonia, arthritis, otitis media, keratoconjunctivitis and genital disorders. The agent is involved in the bovine respiratory disease complex (BRD), which especially affects calves raised in feedlots. In most countries, including Spain, there is no effective vaccine commercially available, and the control of *M. bovis* infections relies on good farming practices and antimicrobial treatments. However, many countries warn about the decreased antimicrobial susceptibility of circulating isolates and the identification of field isolates with mutations linked to resistance.

It is known, from previous studies, that *M. bovis* circulates in Spain, where the agent has been sporadically detected in nasal swabs and lung tissue of young calves with clinical respiratory disease. However, those studies do not provide complete, epidemiological background or molecular characteristics regarding the isolates. In this sense, understanding the distribution and features of circulating isolates is critical for establishing effective preventive and control measures. On the other hand, those studies neither address how important the role of circulating isolates is on the BRD.

The Spanish beef cattle industry usually imports a large number of animals from France, where two main subtypes (STs) of *M. bovis* currently circulate. Based on the sequence of a region of their *polC* gene, these are ST2 and ST3. These two STs differ in their ability to acquire fluoroquinolone resistance *in vitro*. While ST3 easily acquires mutations in the quinolone resistance-determining regions (QRDR) and becomes resistant, the genetic context of ST2 seems less prone to acquire this resistance. Field isolates of both STs were found to be resistant to the macrolides, tylosin and tilmicosin, and the tetracycline, oxytetracycline. The first multiresistant ST3 isolate reported in France was collected from

a calf born in Spain and raised in Southeast France. However, how widespread these two or other STs are in Spain and whether their antimicrobial susceptibility profiles are congruent with *polC* typing is unknown.

MCT is a novel mechanism of horizontal gene transfer (HGT) that has been recently documented *in vitro* using *M. agalactiae*, a small ruminant pathogen phylogenetically close to *M. bovis*. MCT involves the horizontal acquisition of small and large chromosomal fragments from any part of the donor genome, resulting in progenies composed of an infinite variety of mosaic genomes. For MCT to occur, at least one partner must carry a functional integrative conjugative element (ICE). Previous studies have reported a broad distribution of ICEs in *M. bovis* field isolates. However, whether MCT occurs in the field is unknown.

The introduction of asymptomatic carriers is thought to be the primary route of *M. bovis* entrance into a herd or area. In those that do not usually purchase external animals, the relative importance of diluted semen used for artificial insemination could increase. This may have recently occurred in Finland, where semen used for artificial insemination was pointed out as the source of *M. bovis* mastitis outbreaks in two closed dairy herds. Therefore, antimicrobials added to semen extenders should be re-evaluated or measures alternative to antimicrobials should be tested. In this sense, the role of *Lactobacillus* spp. as competing agents and pH acidifiers may be of interest for the control of *M. bovis* transmission related to artificial insemination through two ways: (i) the treatment of seminal doses and (ii) the administration of *Lactobacillus* spp.-based probiotics in the genital tract of cows.

All in all motivated the objectives of the present doctoral thesis, which are presented hereafter.

2.1. Objectives

The **overall goal** of this thesis is to contribute to a better understanding of *Mycoplasma bovis* infection and the biology of the pathogen, as well as to the establishment of new control measures. The **specific objectives** are:

FIRST To assess the presence and circulation of *M. bovis* in Spanish cattle herds using a large collection of samples collected from beef and dairy cattle and from different sources. This objective will be examined in **Study 1**.

SECOND To investigate the circulation of different subtypes by single-locus sequencing of the *polC* gene, studying differences in antimicrobial susceptibility between subtypes and the presence of genetic mutations conferring resistance. This objective will be examined in **Study 1**.

THIRD To address the role of *M. bovis* in clinical respiratory disease unresponsive to antimicrobials in feedlot calves in Spain through bacteriology, histopathology and immunohistochemistry, and to determine the minimum inhibitory concentration values of the isolates recovered against the specific set of antimicrobials used for the therapy *in vivo*. This objective will be examined in **Study 2**.

FOURTH To investigate whether mycoplasma chromosomal transfer occurs in the field, analyzing the occurrence of mosaic genomes among *M. bovis* isolates by whole-genome sequencing. This objective will be examined in **Study 3**.

FIFTH To assess the viability of *M. bovis* in diluted semen after the addition of an antimicrobial (enrofloxacin or doxycycline), or a *Lactobacillus* spp.- based probiotic at different concentrations under *in vitro* conditions. This objective will be examined in **Study 4**.

SIXTH To assess the viability of *M. bovis* in cervical mucus after the addition of a *Lactobacillus* spp.-based probiotic at different concentrations under *in vitro* conditions. This objective will be examined in **Study 5**.

2.2. Study 1. *Mycoplasma bovis* in Spanish cattle herds: two groups of multiresistant isolates predominate, with one remaining susceptible to fluoroquinolones

M. bovis is an important bovine pathogen causing pneumonia, mastitis and arthritis, and is responsible for major economic losses worldwide. In the absence of an efficient vaccine, control of *M. bovis* infections mainly relies on antimicrobial treatments, but resistance is reported in an increasing number of countries. To address the situation in Spain, *M. bovis* was searched in 436 samples collected from beef and dairy cattle (2016-2019) and 28% were positive. Single-locus typing using *polC* sequences further revealed that two subtypes, ST2 and ST3, circulate in Spain both in beef and dairy cattle, regardless of the regions or the clinical signs. Monitoring of ST2 and ST3 isolates minimum inhibitory concentration (MIC) to a panel of antimicrobials revealed one major difference when using fluoroquinolones: ST2 is more susceptible than ST3. Genome analyses further revealed mutations in the quinolone resistance-determining regions (QRDR) of the *gyrA* and *parC* genes, only in ST3 isolates. This situation shows the capacity of ST3 to accumulate mutations in QRDR and might reflect the selective pressure imposed by the extensive use of these antimicrobials. MIC values and detection of mutations by sequence analysis also showed that most Spanish isolates are resistant to macrolides, lincosamides, and tetracyclines. Valnemulin was the only one effective, at least *in vitro*, against both STs.

2.3. Study 2. Importance and antimicrobial resistance of *Mycoplasma bovis* in clinical respiratory disease in feedlot calves

Bovine respiratory disease complex (BRD) is a leading cause of economic losses in the global beef cattle industry and especially affects feedlot calves. The involvement of *M. bovis* in BRD can lead to chronic pneumonia poorly responsive to antimicrobial treatment. Caseonecrotic bronchopneumonia is a pulmonary lesion typically associated with *M. bovis*. In Spain, *M. bovis* is widely distributed in the feedlots and circulating isolates are resistant to most antimicrobials *in vitro*. However, the role of this species in clinical respiratory disease of feedlot calves remains unknown. Furthermore, available data are relative to a fixed panel of antimicrobials commonly used to treat BRD, but not to the specific set of antimicrobials that have been used for treating each animal. This study examined 23 feedlot calves raised in southeast Spain (2016-2019) with clinical signs of respiratory disease unresponsive to treatment. The presence of *M. bovis* was investigated through bacteriology (culture and subsequent PCR), histopathology and immunohistochemistry. The pathogen was found in 86.9% (20/23) of the calves, mainly in the lungs (78.26%; 18/23). Immunohistochemistry revealed *M. bovis* antigens in 73.9% (17/23) of the calves, in which caseonecrotic bronchopneumonia was the most frequent lesion (16/17). Minimum inhibitory concentration assays confirmed the resistance of a selection of 12 isolates to most of the antimicrobials specifically used for treating the animals *in vivo*. These results stress the importance of *M. bovis* in the BRD affecting feedlot calves in Spain.

2.4. Study 3. Genome mosaicism in field strains of *Mycoplasma bovis* as footprints of in-host horizontal chromosomal transfer

Horizontal gene transfer (HGT) was long thought to be marginal in *Mollicutes*, but the capacity of some of these wall-less bacteria to exchange large chromosomal regions has been recently documented. Mycoplasma chromosomal transfer (MCT) is an unconventional mechanism of HGT that relies on the presence of a functional integrative conjugative element (ICE) in at least one partner and involves the horizontal acquisition of small and large chromosomal fragments from any part of the donor genome, which results in progenies composed of an infinitive variety of mosaic genomes. By combining phylogenetic tree reconstructions and detailed comparative genome analyses of 36 *M. bovis* isolates collected in Spain (2016-2018) the mosaic nature of 16 field isolates was confirmed and chromosomal transfers exchanged between their hypothetical ancestors were mapped. This study provides evidence that MCT can take place in the field, most likely during co-infections by multiple strains. Because mobile genetic elements (MGEs) are classical contributors of genome plasticity, the presence of phages, insertion sequences (ISs) and ICEs was also investigated. Data revealed that these elements are widespread within the *M. bovis* species and evidenced classical horizontal transfer of phages and ICEs in addition to MCT. These events contribute to wide-genome diversity and reorganization within this species and may have a tremendous impact on diagnosis and disease control.

2.5. Study 4. The addition of *Lactobacillus* spp., enrofloxacin or doxycycline negatively affects the viability of *Mycoplasma bovis* in diluted bovine semen

Different transmission routes of *M. bovis* have been described, including those related to reproduction. The presence *M. bovis* in semen has led to its appearance in infection-free areas through artificial insemination. Semen was recently reported to be the initial source of two *M. bovis* mastitis outbreaks in two closed dairy herds in Finland. This questions the effectiveness of the antimicrobials currently used in semen extenders to control the pathogen in contaminated semen. They should be re-evaluated, or alternative measures to antimicrobials should be tested to obtain *M. bovis*-free semen. This *in vitro* study aimed to assess different strategies to reduce the risk of transmission of *M. bovis* through artificial insemination technologies. The viability of *M. bovis* (PG45, NCTC 10131) in bull semen diluted in a Tris-citrate-fructose solution was tested, after the addition of enrofloxacin, doxycycline or a *Lactobacillus* spp.-based probiotic. The data show the susceptibility of the pathogen to the addition of 0.125 µg/mL of enrofloxacin or 0.0625 µg/mL of doxycycline and to the addition of the probiotic at a concentration of 3.24×10^6 colony-forming units (CFU)/mL or 3.24×10^8 CFU/mL in diluted semen. The Tris-citrate-fructose medium negatively affected the viability of *M. bovis*, although this effect was lower than that observed after the addition of the probiotic and antimicrobials ($p < 0.05$). Our results may support new strategies for reducing the risk of *M. bovis* transmission through artificial insemination.

2.6. Study 5. The addition of *Lactobacillus* spp. negatively affects *Mycoplasma bovis* viability in bovine cervical mucus

M. bovis can colonize the female reproductive tract after artificial insemination with contaminated semen. *Lactobacillus* spp.-based probiotics have been used for vaginal dysbiosis treatment in women and cows although their role in controlling cervico-vaginal infections due to *M. bovis* is unknown. The objective of this study was to assess the viability of *M. bovis* (PG45, NCTC 10131) in experimentally contaminated cervical mucus after the addition of *Lactobacillus* spp. at different concentrations as competing agents and pH acidifiers. The addition of probiotic at a concentration higher than 10^8 colony-forming units (CFU)/mL had a detrimental effect ($p < 0.05$) on mycoplasma viability in cervical mucus. This coincided with a significant lactic acid bacteria (LAB) growth and an important decrease in pH from 8.4 to 5.6 ($p < 0.05$). However, after the addition of less concentrated probiotic, *M. bovis* survival was not affected and there was no significant LAB growth despite the drop in pH from 8.4 to 6.73 ($p < 0.05$). The addition of concentrations higher than 10^8 CFU/mL of *Lactobacillus* spp. negatively affects *M. bovis* viability in bovine cervical mucus under *in vitro* conditions. Although the effect observed on the pathogen viability seems to be related to the pH decrease after LAB proliferation in cervical mucus, further studies are necessary to elucidate if other factors are implicated. Nevertheless, the administration of *Lactobacillus* spp.-based probiotics might be used in the future to control *M. bovis* proliferation in the cervico-vaginal tract of cows.

2.7. Conclusions

FIRST *M. bovis* is widely extended in Spanish beef cattle herds and it is also implicated in mastitis cases affecting dairy herds.

SECOND Spanish *M. bovis* isolates analyzed are divided into two groups, ST2 and ST3, both being resistant to macrolides, lincosamides and tetracyclines. Most ST3 isolates are resistant to fluoroquinolones, a situation that illustrates the remarkable capacity of ST3 to accumulate mutations in the quinolone resistance-determining regions and the selective pressure imposed by the indiscriminate use of these antimicrobials. Valnemulin has been shown to be effective against both subtypes *in vitro*.

THIRD *M. bovis* plays a significant role in cases of clinical respiratory disease affecting feedlot calves in Spain. Caseonecrotic bronchopneumonia is the morphological pattern most frequently observed in animals infected with *M. bovis*, and patterns indicative of other bacteria species and viruses can be concurrently detected.

FOURTH *M. bovis* isolates recovered from animals with clinical respiratory disease are resistant *in vitro* to most of the antimicrobials specifically used for therapy *in vivo*.

FIFTH The co-existence of *M. bovis* lineages at the herd level and even at the animal level enables events of horizontal gene transfer like mycoplasma chromosomal transfer. These events contribute to wide-genome diversity and may have a negative impact on diagnosis and disease control.

SIXTH Under the conditions analyzed, the addition of *Lactobacillus* spp., enrofloxacin or doxycycline negatively affects *M. bovis* viability in diluted semen, which could be useful to treat seminal doses and reduce the risk of transmission of *M. bovis* during artificial insemination. Dilution with the semen extender, Tris-citrate-fructose also negatively affects the viability of the agent, although to a lesser extent than the addition of the probiotic and antimicrobials.

SEVENTH Under the conditions analyzed, the addition of *Lactobacillus* spp. negatively affects *M. bovis* viability in cervical mucus, which could be useful to control *M. bovis* proliferation in the cervico-vaginal tract of cows.

3. Resumen

3. Resumen

Esta tesis doctoral se presenta como un compendio de publicaciones y opta a la mención de “Doctorado Internacional”. El trabajo presentado evalúa la presencia y circulación de *Mycoplasma bovis* en rebaños bovinos españoles, aportando datos sobre antecedentes epidemiológicos, tipificación molecular y susceptibilidad antimicrobiana de los aislamientos circulantes (2016-2019). Además, analiza el papel epidemiológico de *M. bovis* en la enfermedad respiratoria clínica de terneros de cebo criados en España. Por otro lado, este trabajo demuestra fenómenos de transferencia cromosómica *in vivo* entre cepas de campo de *M. bovis.* Finalmente, el trabajo aborda el papel del enrofloxacino, la doxiciclina y bacterias ácido lácticas (BAL) del género *Lactobacillus* en el control de la transmisión de *M. bovis* ligada a la inseminación artificial.

M. bovis es un importante patógeno del ganado bovino que causa mastitis, neumonía, artritis, otitis media, queratoconjuntivitis y alteraciones genitales. El agente participa en el complejo respiratorio bovino (CRB), que afecta especialmente a los terneros criados en cebaderos. En la mayoría de los países, incluyendo España, no existen vacunas efectivas disponibles en el mercado, por lo que el control de la infección se basa en buenas prácticas de manejo y tratamiento con antimicrobianos. Sin embargo, muchos países alertan sobre la disminución de la susceptibilidad antimicrobiana de los aislamientos circulantes y la identificación de aislamientos de campo con mutaciones ligadas a resistencia.

Se sabe, por estudios previos, que *M. bovis* circula en España, donde el agente ha sido esporádicamente detectado en hisopos nasales y tejido pulmonar de animales jóvenes con signos clínicos de enfermedad respiratoria. Sin embargo, esos trabajos no aportan información completa sobre los antecedentes epidemiológicos o las características moleculares de los aislamientos. En este sentido, conocer la distribución y características de los aislamientos circulantes es importante de cara a establecer medidas efectivas de prevención y control de la infección. Por otro lado, estos estudios tampoco abordan la importancia del papel de los aislamientos circulantes en el CRB.

La industria ganadera española de bovino de carne importa un gran número de animales de Francia, donde actualmente circulan, principalmente, dos subtipos (STs) de aislamientos de *M. bovis.* En base a la secuencia de una región de su gen *polC*, éstos son el ST2 y el ST3. Estos dos STs difieren en su capacidad de adquirir resistencia a las fluoroquinolonas *in vitro.* Mientras que el ST3 adquiere mutaciones fácilmente en las regiones determinantes de resistencia a quinolonas (QRDR), volviéndose resistente, el

contexto genético del ST2 parece menos propenso a la adquisición de esta resistencia. Por otro lado, se ha observado que aislamientos de campo de ambos STs son resistentes a los macrólidos, tilosina y tilmicosina, y a la tetraciclina, oxitetraciclina. El primer aislamiento multirresistente del ST3 notificado en Francia fue obtenido de un ternero nacido en España y criado en el sudeste de Francia. Sin embargo, se desconoce si estos u otros STs se encuentran en España y si su perfil de susceptibilidad antimicrobiana coincide con su tipaje en base al gen *polC*.

La transferencia cromosómica de los micoplasmas (MCT) es un mecanismo de transferencia genética horizontal (HGT) que ha sido recientemente documentado *in vitro* en *M. agalactiae*, un patógeno de pequeños rumiantes filogenéticamente cercano a *M. bovis.* La MCT consiste en la adquisición horizontal de pequeños y grandes fragmentos cromosómicos de cualquier parte del genoma donante, dando lugar a progenies compuestas de una variedad infinita de genomas mosaico. Para que la MCT pueda ocurrir, al menos uno de los parentales debe portar en su genoma un elemento integrativo conjugativo (ICE) funcional. Estudios previos han documentado una amplia distribución de ICEs en aislamientos de campo de *M. bovis.* Sin embargo, se desconoce si la MCT puede producirse en el campo.

Se cree que la introducción de portadores asintomáticos es la ruta principal de entrada de *M. bovis* en una explotación o región. En aquellas que no suelen comprar animales externos, la importancia relativa del semen diluido empleado para la inseminación artificial podría incrementarse. Ello podría haber sucedido recientemente en Finlandia, donde el semen empleado para la inseminación artificial fue señalado como la fuente de brotes de mastitis por *M. bovis* en dos granjas lecheras. Por tanto, los antimicrobianos añadidos a los diluyentes espermáticos deberían ser revaluados, o medidas alternativas a los antimicrobianos deberían ser contempladas. En este sentido, el papel de *Lactobacillus* spp. como agentes competidores y acidificantes del pH podría ser de interés en el control de la transmisión de *M. bovis* ligada a la inseminación artificial a través de dos vías: (i) el tratamiento de las dosis seminales y (ii) la administración de probióticos a base de *Lactobacillus* spp. en el tracto genital de las vacas.

Todo ello motivó los objetivos de la presente tesis doctoral, que se presentan a continuación.

3.1. Objetivos

El **objetivo general** de esta tesis es contribuir a una mejor comprensión de la infección por *Mycoplasma bovis* y de la biología del patógeno, así como al establecimiento de nuevas medidas de control. Los **objetivos específicos** son:

PRIMERO Evaluar la presencia y circulación de *M. bovis* en rebaños bovinos españoles, empleando una gran colección de muestras obtenidas de ganado bovino de carne y de leche, y de distintas fuentes. Este objetivo será abordado en el **Estudio 1**.

SEGUNDO Investigar la circulación de diferentes subtipos mediante secuenciación unilocus del gen *polC*, estudiando diferencias de susceptibilidad antimicrobiana entre los subtipos y la presencia de mutaciones genéticas que confieren resistencia. Este objetivo será abordado en el **Estudio 1**.

TERCERO Evaluar el papel de *M. bovis* en la enfermedad respiratoria clínica sin respuesta al tratamiento antimicrobiano en terneros de cebo en España, mediante bacteriología, histopatología e inmunohistoquímica, y determinar los valores de concentración mínima inhibitoria de los aislamientos obtenidos al set de antimicrobianos empleados para el tratamiento *in vivo*. Este objetivo será abordado en el **Estudio 2**.

CUARTO Investigar si la transferencia cromosómica de los micoplasmas puede ocurrir en el campo, analizando la presencia de genomas en mosaico en aislamientos de *M. bovis* mediante secuenciación del genoma completo. Este objetivo será abordado en el **Estudio 3**.

QUINTO Evaluar la viabilidad de *M. bovis* en semen diluido tras la adición de un antimicrobiano (enrofloxacino o doxiciclina), o un probiótico a base de *Lactobacillus* spp. a diferentes concentraciones en condiciones *in vitro.* Este objetivo será abordado en el **Estudio 4**.

SEXTO Evaluar la viabilidad de *M. bovis* en moco cervical tras la adición de un probiótico a base de *Lactobacillus* spp. a diferentes concentraciones en condiciones *in vitro.* Este objetivo será abordado en el **Estudio 5**.

3.2. Artículo 1. *Mycoplasma bovis* en rebaños bovinos españoles: dos grupos de aislamientos multirresistentes predominan, permaneciendo uno de ellos susceptible a fluoroquinolonas

M. bovis es un importante patógeno bovino que causa neumonía, mastitis y artritis, y es responsable de grandes pérdidas económicas a nivel mundial. Dada la ausencia de vacunas eficaces disponibles, el control de las infecciones de *M. bovis* depende de los tratamientos antimicrobianos, aunque un número elevado de países ha comunicado resistencia a estos tratamientos. Para evaluar la situación en España, se buscó *M. bovis* en 436 muestras procedentes de ganado bovino de carne y de leche (2016–2019) y 28% fueron positivas. Además, la tipificación unilocus utilizando secuencias de *polC* reveló que en España circulan dos subtipos, ST2 y ST3, tanto en bovino de carne como de leche, e independientemente de las regiones o los signos clínicos. La monitorización de la concentración mínima inhibitoria (CMI) de los aislamientos del ST2 y del ST3 reveló una importante diferencia con respecto al uso de las fluoroquinolonas: el ST2 es más susceptible que el ST3. El análisis de los genomas identificó mutaciones en las regiones determinantes de resistencia a quinolonas (QRDR) de los genes *gyrA* y *parC*, sólo en los aislamientos del ST3. Esta situación refleja la capacidad del ST3 para acumular mutaciones en las regiones QRDR y podría indicar la presión selectiva impuesta por el uso masivo de estos antimicrobianos. Además, los valores de CMI y la detección de mutaciones mediante el análisis de la secuencia genética mostró que la mayoría de los aislamientos españoles son resistentes a los macrólidos, lincosamidas y tetraciclinas. La valnemulina fue el único antimicrobiano eficaz, al menos *in vitro*, frente a ambos STs.

3.3. Artículo 2. Importancia y resistencia antimicrobiana de *Mycoplasma bovis* en la enfermedad respiratoria clínica en terneros de cebo

El complejo respiratorio bovino (CRB) es una de las principales causas de pérdidas económicas en la industria global de bovino de carne y afecta especialmente a los terneros de cebo. La implicación de *M. bovis* en el CRB puede conducir a una neumonía crónica que no responde al tratamiento antimicrobiano. La bronconeumonía caseonecrótica es una lesión pulmonar típicamente asociada con *M. bovis*. Este patógeno está ampliamente distribuido en España y los aislamientos circulantes son resistentes a la mayoría de antimicrobianos *in vitro*. No obstante, se desconoce el papel de esta especie en la enfermedad respiratoria clínica de los terneros de cebo. Además, los datos disponibles son relativos a un panel fijo de antimicrobianos utilizados comúnmente para el tratamiento del CRB, pero no a los que han sido específicamente utilizados para el tratamiento de cada animal. Este estudio examinó 23 terneros de cebo criados en el sudeste de España (2016-2019) con signos clínicos de enfermedad respiratoria sin respuesta al tratamiento. Se investigó la presencia de *M. bovis* mediante bacteriología (cultivo y subsecuente PCR), histopatología e inmunohistoquímica. El patógeno fue detectado en 86.9% (20/23) de los terneros, principalmente en los pulmones (78.26%; 18/23). La inmunohistoquímica reveló antígenos de *M. bovis* en 73.9% (17/23) de los terneros y, en éstos, la bronconeumonía caseonecrótica fue la lesión más frecuente (16/17). Los ensayos de concentración mínima inhibitoria confirmaron la resistencia de una selección de 12 aislamientos a la mayoría de antimicrobianos específicamente utilizados para el tratamiento de los animales *in vivo*. Estos resultados destacan la importancia de *M. bovis* en el CRB que afecta a los terneros de cebo en España.

3.4. Artículo 3. Mosaicismo del genoma en cepas de campo de *Mycoplasma bovis* como huella de transferencia cromosómica horizontal en el hospedador

El fenómeno de transferencia genética horizontal (HGT) fue, durante mucho tiempo, considerado marginal en *Mollicutes*. Recientemente, se ha documentado la capacidad de algunas de estas bacterias carentes de pared celular para intercambiar grandes regiones cromosómicas. La transferencia cromosómica de los micoplasmas (MCT) es un mecanismo poco convencional de HGT que depende de la presencia de un elemento integrativo conjugativo (ICE) funcional en, al menos, un parental e implica la adquisición horizontal de fragmentos cromosómicos pequeños y grandes, dando lugar a progenies compuestas de una variedad infinita de genomas en mosaico. Combinando reconstrucciones de árboles filogenéticos y detallados análisis genómicos comparativos de 36 aislamientos de *M. bovis* obtenidos en España (2016-2018) se confirmó la naturaleza en mosaico de 16 aislamientos de campo y se mapearon las transferencias cromosómicas intercambiadas entre sus ancestros hipotéticos. Este estudio proporciona evidencias de que la MCT puede ocurrir en el campo, probablemente durante co-infecciones con múltiples cepas. Además, se investigó la presencia de elementos genéticos móviles (EGM) como fagos, secuencias de inserción (ISs) y ICEs, ya que son clásicos contribuyentes a la plasticidad del genoma. Los datos revelaron que estos elementos están ampliamente distribuidos en la especie *M. bovis* y evidenciaron la clásica transferencia horizontal de fagos y ICEs además de MCT. Estos eventos contribuyen a la amplia diversidad y reorganización genómica en esta especie y podrían tener un tremendo impacto en el diagnóstico y control de la enfermedad.

3.5. Artículo 4. La adición de *Lactobacillus* spp., enrofloxacino o doxiciclina afecta negativamente a la viabilidad de *Mycoplasma bovis* en semen bovino diluido

Se han descrito distintas vías de transmisión de *M. bovis*, incluyendo aquellas relacionadas con la reproducción. La presencia de *M. bovis* en el semen ha propiciado su aparición en zonas libres de infección a través de la inseminación artificial. Recientemente, se informó de que el semen fue la fuente inicial de dos brotes de mastitis por *M. bovis* en dos explotaciones de vacuno de leche cerradas en Finlandia. Ello cuestiona la efectividad de los antimicrobianos añadidos actualmente a los diluyentes seminales para controlar el patógeno en semen contaminado. Para obtener semen libre de *M. bovis,* los antimicrobianos utilizados deberían ser revaluados, o se deberían estudiar medidas alternativas a éstos. Este estudio *in vitro* evalúa diferentes estrategias para reducir el riesgo de transmisión de *M. bovis* a través de las tecnologías de la inseminación artificial. Se evaluó la viabilidad de *M. bovis* (PG45, NCTC 10131) en semen de toro diluido en una solución de Tris-citrato-fructosa tras la adición de enrofloxacino, doxiciclina o un probiótico a base de *Lactobacillus* spp. Los datos revelaron la susceptibilidad del patógeno a la adición de 0.125 µg/mL de enrofloxacino o 0.0625 µg/mL de doxiciclina y a la adición del probiótico a una concentración de 3.24×10^6 unidades formadoras de colonias (UFC)/mL o 3.24×10^8 UFC/mL en semen diluido. El medio Tris-citrato-fructosa afectó negativamente a la viabilidad de *M. bovis*, aunque este efecto fue menor que el observado tras la adición del probiótico y antimicrobianos ($p < 0.05$). Estos resultados podrían respaldar el uso de nuevas estrategias para controlar el riesgo de transmisión de *M. bovis* a través de la inseminación artificial.

3.6. Artículo 5. La adición de *Lactobacillus* spp. afecta negativamente a la viabilidad de *Mycoplasma bovis* en moco cervical bovino

M. bovis puede colonizar el tracto reproductivo femenino tras la inseminación artificial con semen contaminado. Se han utilizado probióticos a base de *Lactobacillus* spp. para el tratamiento de la disbiosis vaginal en mujeres y vacas, aunque se desconoce su rol en el control de las infecciones cérvico-vaginales debidas a *M. bovis.* El objetivo de este estudio fue evaluar la viabilidad de *M. bovis* (PG45, NCTC 10131) en moco cervical contaminado experimentalmente tras la adición de *Lactobacillus* spp. a diferentes concentraciones como agentes competidores y acidificantes del pH. La adición del probiótico a una concentración superior a 10^8 unidades formadoras de colonias (UFC)/mL tuvo un efecto perjudicial ($p < 0.05$) sobre la viabilidad del micoplasma en el moco cervical. Esto coincidió con un crecimiento significativo de bacterias ácido lácticas (BAL) y un importante descenso del pH desde 8.4 hasta 5.6 ($p < 0.05$). Sin embargo, tras la adición de probiótico menos concentrado, la viabilidad de *M. bovis* no se vio afectada y no hubo crecimiento significativo de BAL a pesar de la bajada de pH desde 8.4 hasta 6.73 ($p < 0.05$). En conclusión, la adición de concentraciones de *Lactobacillus* spp. superiores a 10^8 UFC/mL afecta negativamente a la viabilidad de *M. bovis* en moco cervical bovino bajo condiciones *in vitro*. Aunque el efecto observado sobre la viabilidad del patógeno parece estar relacionado con el descenso de pH tras la proliferación de BAL en el moco cervical, son necesarios estudios adicionales para elucidar si existen otros factores implicados. No obstante, la administración de probióticos a base de *Lactobacillus* spp. podría ser utilizada en el futuro para controlar la proliferación de *M. bovis* en tracto cérvico-vaginal de las vacas.

3.7. Conclusiones

PRIMERA *M. bovis* se encuentra ampliamente distribuido en los rebaños españoles de bovino de carne y está implicado en casos de mastitis en los rebaños de leche.

SEGUNDA Los aislamientos españoles de *M. bovis* se dividen en dos grupos, el ST2 y el ST3, siendo ambos resistentes a los macrólidos, lincosamidas y tetraciclinas. La mayoría de los aislamientos del ST3 son resistentes a las fluoroquinolonas, situación que ilustra la notable capacidad del ST3 de acumular mutaciones en las regiones determinantes de resistencia a quinolonas y la presión selectiva impuesta por el uso indiscriminado de estos antimicrobianos. La valnemulina ha mostrado ser eficaz frente a ambos subtipos *in vitro.*

TERCERA *M. bovis* juega un papel importante en casos clínicos de enfermedad respiratoria en terneros de cebo en España. La bronconeumonía caseonecrótica es el patrón morfológico más frecuentemente observado en los animales infectados con *M. bovis* y los patrones indicativos de otras especies bacterianas y virus pueden ser detectados simultáneamente.

CUARTA Los aislamientos de *M. bovis* recuperados de animales con enfermedad respiratoria clínica son resistentes *in vitro* a la mayoría de los antimicrobianos empleados para el tratamiento *in vivo.*

QUINTA La coexistencia de linajes de *M. bovis* a nivel de rebaño e incluso a nivel de animal permite eventos de transferencia genética horizontal como la transferencia cromosómica de los micoplasmas. Estos eventos contribuyen a la amplia diversidad del genoma y podrían tener un impacto negativo en el diagnóstico y control de la enfermedad.

SEXTA En las condiciones analizadas, la adición de *Lactobacillus* spp., enrofloxacino o doxiciclina afecta negativamente a la viabilidad de *M. bovis* en semen diluido, lo que podría ser de utilidad para el tratamiento de las dosis seminales y reducir el riesgo de transmisión de *M. bovis* durante la inseminación artificial. La dilución con el diluyente seminal Tris-citrato-fructosa también afecta negativamente a la viabilidad del agente, aunque en menor medida que la adición del probiótico y antimicrobianos.

SÉPTIMA En las condiciones analizadas, la adición de *Lactobacillus* spp. afecta negativamente a la viabilidad de *M. bovis* en moco cervical, lo que podría ser de utilidad para controlar la proliferación de *M. bovis* el tracto cérvico-vaginal de las vacas.

4. Published articles

4. Published articles

4.1. Article 1. *Mycoplasma bovis* in Spanish cattle herds: two groups of multiresistant isolates predominate, with one remaining susceptible to fluoroquinolones

- **Journal:** Pathogens
- **Abstract:** *Mycoplasma bovis* is an important bovine pathogen causing pneumonia, mastitis, and arthritis and is responsible for major economic losses worldwide. In the absence of an efficient vaccine, control of *M. bovis* infections mainly relies on antimicrobial treatments, but resistance is reported in an increasing number of countries. To address the situation in Spain, *M. bovis* was searched in 436 samples collected from beef and dairy cattle (2016–2019) and 28% were positive. Single-locus typing using *polC* sequences further revealed that two subtypes ST2 and ST3, circulate in Spain both in beef and dairy cattle, regardless of the regions or the clinical signs. Monitoring of ST2 and ST3 isolates minimum inhibitory concentration (MIC) to a panel of antimicrobials revealed one major difference when using fluoroquinolones (FQL): ST2 is more susceptible than ST3. Accordingly, whole-genome sequencing (WGS) further identified mutations in the *gyrA* and *parC* regions, encoding quinolone resistance-determining regions (QRDR) only in ST3 isolates. This situation shows the capacity of ST3 to accumulate mutations in QRDR and might reflect the selective pressure imposed by the extensive use of these antimicrobials. MIC values and detection of mutations by WGS also showed that most Spanish isolates are resistant to macrolides, lincosamides, and tetracyclines. Valnemulin was the only one effective, at least in vitro, against both STs.
- **URL:** https://doi.org/10.3390/pathogens9070545

4.2. Article 2. Importance and antimicrobial resistance of *Mycoplasma bovis* in clinical respiratory disease in feedlot calves

- **Journal:** Animals
- **Abstract:** Bovine respiratory disease (BRD) is an important viral and/or bacterial disease that mainly affects feedlot calves. The involvement of *Mycoplasma bovis* in BRD can lead to chronic pneumonia poorly responsive to antimicrobial treatment. Caseonecrotic bronchopneumonia is a pulmonary lesion typically associated with *M. bovis*. In Spain, *M. bovis* is widely distributed in the feedlots and circulating isolates are resistant to most antimicrobials in vitro. However, the role of this species in clinical respiratory disease of feedlot calves remains unknown. Furthermore, available data are relative to a fixed panel of antimicrobials commonly used to treat BRD, but not to the specific set of antimicrobials that have been used for treating each animal. This study examined 23 feedlot calves raised in southeast Spain (2016–2019) with clinical signs of respiratory disease unresponsive to treatment. The presence of *M. bovis* was investigated through bacteriology (culture and subsequent PCR), histopathology and immunohistochemistry. The pathogen was found in 86.9% (20/23) of the calves, mainly in the lungs (78.26%; 18/23). Immunohistochemistry revealed *M. bovis* antigens in 73.9% (17/23) of the calves in which caseonecrotic bronchopneumonia was the most frequent lesion (16/17). Minimum inhibitory concentration assays confirmed the resistance of a selection of 12 isolates to most of the antimicrobials specifically used for treating the animals in vivo. These results stress the importance of *M. bovis* in the BRD affecting feedlot calves in Spain.
- **URL:** https://doi.org/10.3390/ani11051470

4.3. Article 3. Genome mosaicism in field strains of *Mycoplasma bovis* as footprints of in-host horizontal chromosomal transfer

- **Journal:** Applied and Environmental Microbiology
- **Abstract:** Horizontal gene transfer was long thought to be marginal in *Mollicutes*, but the capacity of some of these wall-less bacteria to exchange large chromosomal regions has been recently documented. Mycoplasma chromosomal transfer (MCT) is an unconventional mechanism that relies on the presence of a functional integrative conjugative element (ICE) in at least one partner and involves the horizontal acquisition of small and large chromosomal fragments from any part of the donor genome, which results in progenies composed of an infinite variety of mosaic genomes. The present study focuses on *Mycoplasma bovis*, an important pathogen of cattle responsible for major economic losses worldwide. By combining phylogenetic tree reconstructions and detailed comparative genome analyses of 36 isolates collected in Spain (2016 to 2018), we confirmed the mosaic nature of 16 field isolates and mapped chromosomal transfers exchanged between their hypothetical ancestors. This study provides evidence that MCT can take place in the field, most likely during coinfections by multiple strains. Because mobile genetic elements (MGEs) are classical contributors of genome plasticity, the presence of phages, insertion sequences (ISs), and ICEs was also investigated. Data revealed that these elements are widespread within the *M. bovis* species and evidenced classical horizontal transfer of phages and ICEs in addition to MCT. These events contribute to wide-genome diversity and reorganization within this species and may have a tremendous impact on diagnostic and disease control.
- **URL:** https://doi.org/10.1128/AEM.01661-21

4.4. Article 4. The addition of *Lactobacillus* spp., enrofloxacin or doxycycline negatively affects the viability of *Mycoplasma bovis* in diluted bovine semen

- **Journal:** Animals
- **Abstract:** *Mycoplasma bovis* is an important etiologic agent of bovine mycoplasmosis in cattle. Different transmission routes have been described, including those related to reproduction. The presence of mycoplasma in semen has led to its appearance in infection-free areas through artificial insemination (AI). Semen was recently reported to be the initial source of two *M. bovis* mastitis outbreaks in two closed dairy herds in Finland. This questions the effectiveness of the antimicrobials currently used in semen extenders to control the pathogens in contaminated semen. They should be re-evaluated, or alternative measures to antimicrobials should be tested to obtain *M. bovis*-free semen. This in vitro study aimed to assess different strategies to reduce the risk of transmission of *M. bovis* through AI technologies. The viability of *M. bovis* (PG45, NCTC 10131) in bull semen diluted (DS) in a Tris-citrate-fructose solution was tested, after the addition of enrofloxacin, doxycycline or a *Lactobacillus* spp.-based probiotic. The data show the susceptibility of the pathogen to the addition of 0.125 µg/mL of enrofloxacin or 0.0625 µg/mL of doxycycline and to the addition of the probiotic at a concentration of 3.24×10^6 colony forming units (CFU)/mL or 3.24×10^8 CFU/mL in DS. The Tris-citrate-fructose medium negatively affected the viability of *M. bovis*, although this effect was lower than that observed after the addition of the probiotic and antimicrobials ($p < 0.05$). Our results may support new strategies for reducing the risk of *M. bovis* transmission through AI.
- **URL:** https://doi.org/10.3390/ani10050837

4.5. Article 5. The addition of *Lactobacillus* spp. negatively affects *Mycoplasma bovis* viability in bovine cervical mucus

- **Journal:** BMC Veterinary Research
- **Abstract: Background** *Mycoplasma bovis* is an important pathogen for the cattle industry worldwide causing significant economic losses. Several transmission routes, including those related to reproduction, have been described. Indeed, the pathogen can colonize the female reproductive tract after artificial insemination (AI) with contaminated semen. *Lactobacillus* spp.-based probiotics have been used for vaginal dysbiosis treatment in women and cows although their role in controlling cervico-vaginal infections due to *M. bovis* is unknown. The objective of the present work is to assess the viability of *M. bovis* (PG45, NCTC 10131) in experimentally contaminated cervical mucus after the addition of *Lactobacillus* spp. at different concentrations as a competing agent and pH acidifier.

  Results The addition of probiotic at a concentration higher than 10^8 colony forming units (CFU/mL had a detrimental effect ($P < 0.05$) on mycoplasma viability in cervical mucus. This coincided with a significant LAB growth and an important decrease in pH from 8.4 to 5.6 ($P < 0.05$). However, after the addition of less concentrated probiotic, *M. bovis* survival was not affected and there was no significant LAB growth despite the drop of pH from 8.4 to 6.73 ($P < 0.05$).

  Conclusion The addition of concentrations higher than 10^8 CFU/mL of *Lactobacillus* spp. negatively affects *M. bovis* viability in bovine cervical mucus under in vitro conditions. Although the effect observed on the pathogen viability seems to be related to the pH decrease after LAB proliferation in cervical mucus, further studies are necessary to elucidate if other factors are implicated. Nevertheless, the administration of *Lactobacillus* spp.-based probiotics might be used in the future to control *M. bovis* proliferation in the cervico-vaginal tract of cows.
- **URL:** https://doi.org/10.1186/s12917-020-02454-9

4.6. Contributions of the PhD student to the articles

Ana García-Galán Pérez, PhD student of the Ruminant Health Research Group of the Department of Animal Health of the Faculty of Veterinary Sciences of the University of Murcia, states that her work in the following scientific articles has been:

García-Galán, A., Nouvel, L.-X., Baranowski, E., Gómez-Martín, Á., Sánchez, A., Citti, C., & de la Fe, C. (2020). *Mycoplasma bovis* in Spanish cattle herds: Two groups of multiresistant isolates predominate, with one remaining susceptible to fluoroquinolones. *Pathogens*, *9*(7), 545. https://doi.org/10.3390/pathogens9070545

- Sample reception and processing, laboratory and bioinformatic analyses execution, data analysis and writing of the article.

García-Galán, A., Seva, J., Gómez-Martín, Á., Ortega, J., Rodríguez, F., García-Muñoz, Á., & De la Fe, C. (2021). Importance and antimicrobial resistance of *Mycoplasma bovis* in clinical respiratory disease in feedlot calves. *Animals*, *11*(5), 1470. https://doi.org/10.3390/ani11051470

- Culture reception and processing, laboratory analyses execution, data analysis and writing of the article.

García-Galán, A., Baranowski, E., Hygonenq, M.-C., Walch, M., Croville, G., Citti, C., De la Fe, C., & Nouvel, L.-X. (2022). Genome mosaicism in field strains of *Mycoplasma bovis* as footprints of in-host horizontal chromosomal transfer. *Applied and Environmental Microbiology*, *88*(1), e0166121. https://doi.org/10.1128/AEM.01661-21

- Participation in sample preparation for sequencing, bioinformatic analyses execution, data analysis and writing of the article.

García-Galán, A., Gómez-Martín, Á., Bataller, E., Gomis, J., Sánchez, A., Gadea, J., Vieira, L. A., García-Roselló, E., & De la Fe, C. (2020). The addition of *Lactobacillus* spp., enrofloxacin or doxycycline negatively affects the viability of *Mycoplasma bovis* in diluted bovine semen. *Animals*, *10*(5), 837. https://doi.org/10.3390/ani10050837

- Sample reception and processing, laboratory analyses execution, data analysis and writing of the article.

García-Galán, A., De la Fe, C., Gomis, J., Bataller, E., Sánchez, A., Quereda, J. J., García-Roselló, E., & Gómez-Martín, A. (2020). The addition of *Lactobacillus* spp. negatively affects *Mycoplasma bovis* viability in bovine cervical mucus. *BMC Veterinary Research*, *16*(1), 251. https://doi.org/10.1186/s12917-020-02454-9

- Sample reception and processing, laboratory analyses execution, data analysis and writing of the article.

5. Appendices

5. Appendices

5.1. Supporting information: article 1

The supplementary material corresponding to article 1 can be found online at: http://www.mdpi.com/2076-0817/9/7/545/s1.

Due to its length, Table S1 was prepared in an excel file and is not attached to the present manuscript. It can be accessed through the link.

5.2. Supporting information: article 3

The supplementary material corresponding to article 3 can be found online through the article DOI https://doi.org/10.1128/AEM.01661-21

Due to their length, Data Set S1 and S2 were prepared in an excel file and are not attached to the present manuscript. They can be accessed through the link.

5.3. Research stays abroad

1. Stay 1.
    - Center: Unité mixte de recherche 1225 de l´Institut National de Recherche pour l´Alimentation et l´Environment (INRAE) and École Nationale Vétérinaire de Toulouse (ENVT).
    - Dates: 01/10/2019 – 27/07/2020 (10 months).
    - Research supervisor: Laurent Xavier Nouvel.
    - Activities related to the doctoral thesis:
        I. First, the PhD student learned how to use bioinformatics tools for whole-genome sequence (WGS) analysis, including Galaxy Platform, Integrative Genome Viewer (IGV 2.7.0), Artemis (16.0.0), Artemis Comparison Tool (ACT 13.0.0), BLAST and MEGA X.
        II. Then, 36 *M. bovis* isolates were selected and mutations associated with antimicrobial resistance were studied.
        III. The data obtained in (II) completed the work previously carried out by the PhD student and allowed her to write and publish a scientific article during the period of stay (Article 1).
        IV. The molecular typing of the strains was carried out based on two multilocus sequence typing systems (MLST) and based on the WGS-single nucleotide polymorphisms (SNPs) analysis. Phylogenetic trees obtained orientated the research towards the demonstration of chromosomal exchanges between *M. bovis* strains *in vivo* (Article 3).

5.4. Communications to congresses derived from this doctoral thesis

1. Genome mosaicism in *Mycoplasma bovis* isolates: a footprint of mycoplasma chromosomal transfer in the field.
    - Authors: Ana García-Galán, Eric Baranowski, Marie-Claude Hygonenq, Mathilda Walch, Guillaume Croville, Christine Citti, Christian De la Fe and Laurent-Xavier Nouvel.
    - Congress: XXIII Biennial Congress of the International Organization for Mycoplasmology.
    - Place: On a virtual online platform.
    - Date: November 1st – 4th, 2021.
    - Type of communication: Poster.

2. *Mycoplasma bovirhinis* está presente en el tracto respiratorio de terneros españoles al inicio del cebo.
    - Authors: Ana García-Galán, Ángel Gómez-Martín, Ángel García, Juan Tatay-Dualde, Miranda Prats-van der Ham, Juan Álcazar, Antonio Sánchez, Antonio Contreras, Alberto Mas and Christian De la Fe.
    - Congress: XXIV Congreso Internacional ANEMBE de Medicina Bovina.
    - Place: Seville, Spain.
    - Date: May 22nd – 24th, 2019.
    - Type of communication: Poster.

3. *Mycoplasma bovis* y mastitis: susceptibilidad antimicrobiana frente a fluoroquinolonas.
    - Authors: Ana García-Galán, Ángel Gómez-Martín, Ángel García, Juan Tatay-Dualde, Miranda Prats-van der Ham, Juan Álcazar, Mercè Lázaro, Juan Carlos Corrales, Antonio Sánchez, Antonio Contreras and Christian De la Fe.
    - Congress: XXIV Congreso Internacional ANEMBE de Medicina Bovina.
    - Place: Seville, Spain.
    - Date: May 22nd – 24th, 2019.
    - Type of communication: Poster.

4. ¿Son eficaces los macrólidos en la lucha frente a *Mycoplasma bovis*?
    - Authors: Ana García-Galán, Ángel Gómez-Martín, Ángel García, Juan Tatay-Dualde, Miranda Prats-van der Ham, Juan Álcazar, Juan Carlos Corrales, Antonio Sánchez, Antonio Contreras and Christian De la Fe.
    - Congress: XXIV Congreso Internacional ANEMBE de Medicina Bovina.
    - Place: Seville, Spain.
    - Date: May 22nd – 24th, 2019.
    - Type of communication: Poster.

5. Susceptibilidad antibiótica de aislamientos de *Mycoplasma bovis* procedentes de cebaderos españoles.
    - Authors: Ana García-Galán, Ángel Gómez-Martín, Ángel García, Juan Tatay-Dualde, Miranda Prats-van der Ham, Juan Álcazar and Christian De la Fe.
    - Congress: XXIII Congreso Internacional ANEMBE de Medicina Bovina.
    - Place: Vigo, Spain.
    - Date: June 6th – 8th, 2018.
    - Type of communication: Oral.

6. Presencia de *Mycoplasma bovis* en bovino de cebo y lechero en explotaciones españolas.
    - Authors: Ana García-Galán, Ángel Gómez-Martín, Ángel García, Juan Tatay-Dualde, Miranda Prats-van der Ham, Juan Álcazar, Alberto Mas and Christian De la Fe.
    - Congress: XXIII Congreso Internacional ANEMBE de Medicina Bovina.
    - Place: Vigo, Spain.
    - Date: June 6th – 8th, 2018.
    - Type of communication: Poster.

7. Presencia de *Mycoplasma bovis* en terneros cebados sin respuesta al tratamiento antibiótico.
    - Authors: Ana García-Galán, Joaquín Ortega, Jorge Rosell, Laura Sylvie Danielle Vautier, Jaume Alomar, Juan José Quereda, Juan Tatay-Dualde, Miranda Prats-van der Ham, Christian De la Fe and Ángel Gómez-Martín.
    - Congress: XXIII Congreso Internacional ANEMBE de Medicina Bovina.
    - Place: Vigo, Spain.
    - Date: June 6th – 8th, 2018.
    - Type of communication: Poster.

8. Vigilancia de *Mycoplasma bovis* en un lote de terneros recién llegado a un cebadero del sureste español.
    - Authors: Ana García-Galán, Miranda Prats-van der Ham, Juan Tatay-Dualde, Antonio Contreras, Antonio Sánchez, Juan Carlos Corrales, Ángel Gómez-Martín, Alberto Mas and Christian De la Fe.
    - Congress: IV Jornadas Doctorales de la Universidad de Murcia.
    - Place: Murcia, Spain.
    - Date: May 29th – 31st, 2018.
    - Type of communication: Poster.

5.5. Publications in national journals

1. Dos subtipos de *Mycoplasma bovis* circulan de forma endémica en España.
    - Authors: Ana García-Galán, Laurent-Xavier Nouvel, Eric Baranowski, Ángel Gómez-Martín, Antonio Sánchez, Juan Alcázar, Edgar García-Romero, Ángel García-Muñoz, Xóchitl Hernández, Juan Carlos Corrales, Antonio Contreras, Christine Citti and Christian De la Fe.
    - Journal: rumiNews.
    - Date of publication: March 2021.
    - Pages: 6 – 14.

2. Las mastitis por *Mycoplasma bovis* en el ganado vacuno.
    - Authors: Ana García-Galán, Ángel Gómez-Martín, Xavier Nouvel, Antonio Sánchez, Ángel García-Muñoz, Juan Carlos Corrales, Eric Baranowski, Antonio Contreras, Edgar García and Christian De la Fe.
    - Journal: Ganadería.
    - Date of publication: March – April 2020.
    - Pages: 28 – 32.

3. La infección por *Mycoplasma bovis* en terneros de cebo.
    - Authors: Ana García-Galán, Ángel Gómez-Martín, Ángel García, Miranda Prats-van der Ham, Juan Tatay-Dualde, Juan Carlos Corrales, Antonio Sánchez, Antonio Contreras and Christian De la Fe.
    - Journal: Vacuno de élite.
    - Date of publication: Spring 2018.
    - Pages: 16 – 18.

6. Abbreviations

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ABC: avidin biotinylated enzyme complex

AI: artificial insemination

ACT: Artemis Comparison Tool

ANI: average nucleotide identity

BALT: bronchus-associated lymphoid tissue

BCOV: bovine coronavirus

BHV-1: bovine herpesvirus type 1

BRD / CRB: bovine respiratory disease complex / complejo respiratorio bovino

BRSV: bovine respiratory syncytial virus

BTM: bulk tank milk

BVDV: bovine viral diarrhea virus

CDS: coding DNA sequence

CFU/ UFC: colony-forming units / unidades formadoras de colonias

DS: diluted semen

ELISA: enzyme-linked immunoabsorbent assay

ENA: European Nucleotide Archive database

FQN: fluoroquinolones

GLTS: gentamycin, lincomycin, tylosin, spectinomycin

H-E: hematoxylin-eosin

HGT: horizontal gene transfer / transferencia genética horizontal

HIHS: heat-inactivated horse serum

ICE: integrative conjugative element / elemento integrativo conjugativo

IHC: immunohistochemistry

IGV: Integrative Genomics Viewer

IS: insertion sequence / secuencia de inserción

LAB / BAL: lactic acid bacteria / bacterias ácido lácticas

L1: *Lactobacillus* spp. at a concentration of 3.24 x 10^6 CFU/mL

L2: *Lactobacillus* spp. at a concentration of 3.24 x 10^8 CFU/mL

MCT: mycoplasma chromosomal transfer / transferencia cromosómica de los micoplasmas

MGE / EGM: mobile genetic element / elemento genético móvil

MIC / CMI: minimum inhibitory concentration / concentración minima inhibitoria

MLST: multilocus sequence typing

MST: minimum spanning tree

PCR: polymerase chain reaction

PI-3: parainfluenza-3 virus

QRDR: quinolone resistance-determining regions / regiones determinantes de resistencia a quinolonas

SAS: Statistical Analysis System

SNP: single nucleotide polymorphism

ST: subtype / subtipo

vICE: vestige of ICE

Vsp: variable surface protein

WGS: whole-genome sequence/sequencing

7. Acknowledgments

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