MYCOPLASMA BOVIS MASTITIS: PREVALENCE, IDENTIFICATION OF VIRULENCE FACTORS AND HOST IMMUNE RESPONSE GENES

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ABSTRACT

Mycoplasma bovis is the most prevalent *Mycoplasma* species that causes mastitis in the United States (U.S.). *M. bovis* mastitis (MBM) is highly contagious and characterized by resistance to antimicrobial treatments. Reduction in milk yield and culling of infected cows are the major contributors to the substantial economic losses. Reports on MBM prevalence in Tennessee dairy farms are limited. To date, there is no effective commercial vaccine mainly due to lack of understanding about MBM pathogenesis and *M. bovis* virulence factors. The major objectives of this dissertation were to determine the prevalence of *M. bovis* in bulk tank milk in Tennessee dairy farms and identify *M. bovis* virulence factors and host immune response genes. Culture and PCR were used to determine the prevalence of *M. bovis* in bulk tank milk. Whole transcriptome analysis was performed following co-infection of *M. bovis* with bovine mammary epithelial cells and intramammary challenge of dairy cows by *M. bovis*. To investigate the virulence role of surface lipoproteins mnuA and 5'-nucleotidase, *M. bovis* mutants were developed and i*n vivo* intramammary cow and murine challenges were conducted. Prevalence of *M. bovis* in bulk tank milk in Tennessee dairy farms was found to be 5.3% and 76.7% using culture and PCR, respectively. Following co-infection with mammary epithelial cells, transposase activity was significantly upregulated in *M. bovis* whereas genes involved in protein translation were significantly downregulated. Genes associated with apoptosis pathways and pro-inflammatory cytokines were significantly upregulated in *M. bovis* infected bovine mammary epithelial cells whereas genes involved in cell cycle, ribosome biogenesis, and steroid biosynthesis were significantly down-regulated. Genes encoding pro-inflammatory pathways and formation of neutrophil extracellular traps were significantly upregulated in mammary glands of *M. bovis* challenged cows whereas steroid biosynthesis and metabolism was significantly down-regulated. The *5'-nucleotidase* gene was found to be a virulence factor of *M. bovis* which determines its fitness and survival. In conclusion, the high prevalence reported here is concerning and further studies involving repeated sampling from individual cows using combination of tests is highly recommended. The identified virulence factors can be used as an important intervention targets.

Keywords: *M. bovis*, mastitis, prevalence, whole genome sequencing, co-infection, intramammary challenge, transcriptome analysis, *5'-nucleotidase*

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INTRODUCTION

Disclosure

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Gelgie, A. E., Korsa, M. G., & Kerro Dego, O. (2022). Mycoplasma bovis Mastitis. *Current Research in Microbial Sciences, 3*, 100123.
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AG prepared the original draft. MGK and OKD reviewed and edited the manuscript.

Mollicutes are the smallest self-replicating microbes with unique features such as lack of cell-wall, small genome size (600 - 2,200 kb) , and low G+C content (24 - 33%) (Razin et al., 1998). They have diverse arrays of hosts including human, animals, plants, insects, reptiles, fish, and arthropods (Razin et al., 1998). The class *Mollicutes* have several genera, however the major ones include *Mycoplasma, Ureaplasma*, *Spiroplasma,* and *Acholeplasma* (Johansson & Pettersson, 2002). The genus *Mycoplasma* encompasses species that cause some of the most important animal diseases listed by the World Organization for Animal Health (WOAH) such as contagious bovine pleuropneumonia (CBPP), contagious caprine pleuropneumonia (CCPP), and avian mycoplasmosis (OIE, 2021). Furthermore, these diseases are designated as notifiable terrestrial diseases by the United States Department of Agriculture (USDA) (USDA-APHIS, 2024).

Mycoplasma bovis was originally detected from an outbreak of mastitis in the United States (U.S.) and has since been spreading to various countries including Israel, Spain, Australia, France, UK, Germany, Morocco, and other countries mainly through international animal trade (Hale et al., 1962; Nicholas et al., 2008). It is responsible for various types of illnesses in cattle involving multiple body organs (Caswell & Archambault, 2007; Guo et al., 2014; Haapala et al., 2018; Hananeh et al., 2018; Suwanruengsri et al., 2022). Additionally, *M. bovis* is the most prevalent *Mycoplasma* species that cause mastitis in the United States (U.S.) (USDA-APHIS, 2003), although it is generally believed the prevalence is underreported given the fastidious nature of the pathogen (Fox et al., 2005).

As *M. bovis* mastitis (MBM) continues to spread to previously *M. bovis*-free countries and become an emerging challenge (Jordan et al., 2021; Vähänikkilä et al., 2019), developing effective control measures needs more emphasis. Currently, there are no effective commercial vaccines and the control effort heavily relies on antimicrobial treatments. However, *M. bovis* is resistant to several classes of antimicrobials including β-lactams, rifampicin, polymixins and sulfonamides/trimethoprim due to lack of cell wall, mutation, and absence of the lipopolysaccharides, respectively (Gautier-Bouchardon, 2018). Therefore, only antimicrobial agents which target cell membrane, DNA, RNA, and protein synthesis such as macrolides,

tetracyclines, and fluoroquinolones are active against *Mycoplasma* species (Gautier-Bouchardon, 2018; McCormack, 1993).

Knowledge of the prevalence and molecular epidemiology of MBM plays a key role in determining disease magnitude and identifying circulating strains which ultimately contributes to disease prevention and control. In addition, understanding the virulence factors of *M. bovis* and elucidating host-pathogen interactions is critical in devising effective prevention and control measures. Once identified, virulence factors can be targeted to discover novel drugs and develop live-attenuated or protein-based subunit vaccines (Prysliak et al., 2018; Prysliak et al., 2013). Virulence factors are rarely expressed continuously and are only deployed under certain conditions such as during host interaction (Dorman, 2024). Therefore, systematic methods such as *in vitro* and *in vivo* infection models are crucial to generate real-time evidences of *M. bovis*-host interactions.

To that end, chapter 2 of this dissertation presents prevalence and molecular epidemiology of MBM in Tennessee dairy farms whereas chapter 3 and 4 deal with *in vitro* and *in vivo* infection of bovine mammary epithelial cells and dairy cows by *M. bovis* to identify candidate virulence factors and characterize host immune responses at the transcriptomic level.

References

Caswell, J. L., & Archambault, M. (2007). *Mycoplasma bovis* pneumonia in cattle. *Anim Health Res Rev*, *8*(2), 161-186. https://doi.org/10.1017/s1466252307001351

Dorman, C. J. (2024). Chapter 17 - Gene regulatory networks and hierarchies in bacterial pathogens. In Y.-W. Tang, M. Y. Hindiyeh, D. Liu, A. Sails, P. Spearman, & J.-R. Zhang (Eds.), *Molecular Medical Microbiology (Third Edition)* (pp. 337-355). Academic Press. https://doi.org/https://doi.org/10.1016/B978-0-12-818619-0.00108-8

Fox, L. K., Kirk, J. H., & Britten, A. (2005). Mycoplasma mastitis: a review of transmission and control. *J Vet Med B Infect Dis Vet Public Health*, *52*(4), 153-160. https://doi.org/10.1111/j.1439-0450.2005.00845.x

Gautier-Bouchardon, A. V. (2018). Antimicrobial Resistance in *Mycoplasma* spp. *Microbiol Spectr*, *6*(4). https://doi.org/10.1128/microbiolspec.ARBA-0030-2018

Guo, M., Wang, G., Lv, T., Song, X., Wang, T., Xie, G., Cao, Y., Zhang, N., & Cao, R. (2014). Endometrial inflammation and abnormal expression of extracellular matrix proteins induced by *Mycoplasma bovis* in dairy cows. *Theriogenology*, *81*(5), 669-674.

Haapala, V., Pohjanvirta, T., Vähänikkilä, N., Halkilahti, J., Simonen, H., Pelkonen, S., Soveri, T., Simojoki, H., & Autio, T. (2018). Semen as a source of *Mycoplasma bovis* mastitis in dairy herds. *Vet Microbiol*, *216*, 60-66. https://doi.org/10.1016/j.vetmic.2018.02.005

Hale, H. H., Helmboldt, C. F., Plastridge, W. N., & Stula, E. F. (1962). Bovine mastitis caused by a *Mycoplasma* species. *Cornell Vet*, *52*, 582-591. https://www.ncbi.nlm.nih.gov/pubmed/13952069

Hananeh, W. M., Momani, W. M. A., Ababneh, M. M., & Abutarbush, S. M. (2018). *Mycoplasma bovis* arthritis and pneumonia in calves in Jordan: An emerging disease. *Vet World*, *11*(12), 1663-1668. https://doi.org/10.14202/vetworld.2018.1663-1668

Johansson, K.-E., & Pettersson, B. (2002). Taxonomy of Mollicutes. In S. Razin & R. Herrmann (Eds.), *Molecular Biology and Pathogenicity of Mycoplasmas* (pp. 1-29). Springer US. https://doi.org/10.1007/0-306-47606-1_1

Jordan, A., Sadler, R. J., Sawford, K., van Andel, M., Ward, M., & Cowled, B. (2021). *Mycoplasma bovis* outbreak in New Zealand cattle: An assessment of transmission trends

using surveillance data. *Transbound Emerg Dis*, *68*(6), 3381-3395. https://doi.org/10.1111/tbed.13941

McCormack, W. M. (1993). Susceptibility of mycoplasmas to antimicrobial agents: clinical implications. *Clin Infect Dis*, *17 Suppl 1*, S200-201. https://doi.org/10.1093/clinids/17.supplement_1.s200

Nicholas, R., Ayling, R., & McAuliffe, L. (2008). *Mycoplasma diseases of ruminants*. CABI.

OIE. (2021). *Animal Diseases*. https://www.oie.int/en/what-we-do/animal-health-and-welfare/animal-diseases/

Prysliak, T., Maina, T., & Perez-Casal, J. (2018). Th-17 cell mediated immune responses to *Mycoplasma bovis* proteins formulated with Montanide ISA61 VG and curdlan are not sufficient for protection against an experimental challenge with *Mycoplasma bovis*. *Veterinary Immunology and Immunopathology*, *197*, 7-14. https://doi.org/https://doi.org/10.1016/j.vetimm.2018.01.004

Prysliak, T., van der Merwe, J., & Perez-Casal, J. (2013). Vaccination with recombinant *Mycoplasma bovis* GAPDH results in a strong humoral immune response but does not protect feedlot cattle from an experimental challenge with *M. bovis*. *Microb Pathog*, *55*, 1-8. https://doi.org/10.1016/j.micpath.2012.12.001

Razin, S., Yogev, D., & Naot, Y. (1998). Molecular biology and pathogenicity of mycoplasmas. *Microbiol Mol Biol Rev*, *62*(4), 1094-1156. https://doi.org/10.1128/MMBR.62.4.1094-1156.1998

Suwanruengsri, M., Uemura, R., Kanda, T., Fuke, N., Nueangphuet, P., Pornthummawat, A., Yasuda, M., Hirai, T., & Yamaguchi, R. (2022). Production of granulomas in *Mycoplasma bovis* infection associated with meningitis-meningoencephalitis, endocarditis, and pneumonia in cattle. *J Vet Diagn Invest*, *34*(1), 68-76. https://doi.org/10.1177/10406387211053254

Trachtenberg, S. (1998). Mollicutes-wall-less bacteria with internal cytoskeletons. *J Struct Biol*, *124*(2-3), 244-256. https://doi.org/10.1006/jsbi.1998.4063

USDA-APHIS. (2003). *Mycoplasma in Bulk Tank Milk on U.S. Dairies*. USA: Department of Agriculture

USDA-APHIS. (2024). National Animal Health Reporting System (NAHRS) Reportable Diseases, Infections, and Infestations List Retrieved Access Date from

https://www.aphis.usda.gov/aphis/ourfocus/animalhealth/monitoring-and-surveillance/nlrad/ct_national_list_reportable_animal_diseases

Vähänikkilä, N., Pohjanvirta, T., Haapala, V., Simojoki, H., Soveri, T., Browning, G. F., Pelkonen, S., Wawegama, N. K., & Autio, T. (2019). Characterisation of the course of *Mycoplasma bovis* infection in naturally infected dairy herds. *Vet Microbiol*, *231*, 107-115. https://doi.org/10.1016/j.vetmic.2019.03.007

CHAPTER 1: LITERATURE REVIEW

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Abstract

Mycoplasma bovis has recently been identified increasingly in dairy cows causing huge economic losses to the dairy industry. *M. bovis* is a causative agent for mastitis, pneumonia, endometritis, endocarditis, arthritis, otitis media, and many other clinical symptoms in cattle. However, some infected cows are asymptomatic or may not shed the pathogen for weeks to years. This characteristic of *M. bovis*, along with the lack of adequate testing and identification methods in many parts of the world until recently, has allowed the *M. bovis* to be largely undetected despite its increased prevalence in dairy farms. Due to growing levels of antibiotic resistance among wild-type *M. bovis* isolates and lack of cell walls in mycoplasmas that enable them to be intrinsically resistant to beta-lactam antibiotics that are widely used in dairy farms, there is no effective treatment for *M. bovis* mastitis. Similarly, there is no commercially available effective vaccine for *M. bovis* mastitis. The major constraint to developing effective intervention tools is limited knowledge of the virulence factors and mechanisms of the pathogenesis of *M. bovis* mastitis. There is a lack of quick and reliable diagnostic methods with high specificity and sensitivity for *M. bovis*. This review is a summary of the current state of knowledge of the virulence factors, pathogenesis, clinical manifestations, diagnosis, and control of *M. bovis* mastitis in dairy cows.

Keywords: *Mycoplasma bovis*, *M. bovis* mastitis, dairy cows, subclinical infection, virulence factors, pathogenesis

1.1. Introduction

Mycoplasma bovis, which was formerly known as *Mycoplasma agalactiae* subsp. *bovis* (Askaa & Ernø, 1976), is a causative agent of several diseases in cattle and other farmed ruminants including mastitis, pneumonia, endocarditis, arthritis, otitis, meningitis, and reproductive problems both in bulls and cows (Aebi et al., 2012; Foster et al., 2009; Hale et al., 1962; Kanda et al., 2019; Suwanruengsri et al., 2022). *M. bovis* mastitis is an emerging dairy cattle disease that poses a significant challenge globally due to its highly contagious nature and resistance to antibiotics (Nicholas, 2011). Despite the isolation of various *Mycoplasma* spp. from milk samples of cows with mastitis, *M. bovis* is the most common causative agent (Allan M. Britten, 2020; Gioia et al., 2021).

In the United States (U.S.) alone, economic losses due to *M. bovis* mastitis is estimated to be above $100 million annually (Nicholas & Ayling, 2003). This mainly results from increased somatic cell counts (SCC), decreased milk production, culling, and treatment costs. Since SCC is an indicator of milk quality and udder health, SCC beyond legal limits in U.S. results in regulatory measures such as suspension of operation permits (USDA-APHIS, 2019). Culling of infected cows has proven costly in U.S. with the rate reaching as high as 70% (Gonzalez et al., 1992). The prevalence of *M. bovis* mastitis is high in large dairy operations especially in the U.S. dairy industry where several large-scale dairy operations exist (Fernando Ulloa, 2021; USDA-APHIS, 2008). Currently adopted mastitis control programs which involve environmental sanitation, proper milking procedures and udder health (NMC, 2005) are partly ineffective due to transmission of *M. bovis* through respiratory route and semen or seminal fluid (Haapala et al., 2018; Pardon, 2020) as well as calf to cow or vice-versa (Timonen et al., 2020). Given the difficulty in diagnosis due to the fastidious growth nature and subclinical infection, with infected cows apparently appearing healthy, the disease can remain undetected for long time in dairy farms with significant economic losses (Nicholas et al., 2016).

Although *M. bovis* mastitis was first reported in the early 1960s (Hale et al., 1962) and has since been problematic, effective control tools such as vaccine or prophylactic therapy or treatment is

yet to be developed. This is largely attributed to the knowledge gap in the critically important virulence factors and pathogenesis mechanisms of *M. bovis* mastitis.

With increasing antibiotic resistance problem (Lysnyansky & Ayling, 2016), accurate diagnosis is crucial to avoid the use of broad-spectrum antimicrobial drugs and to conduct targeted treatment. Among the challenges in the control and prevention of *M. bovis* infections is developing effective and sustainable control tools and rapid and reliable pen-side diagnostic tool with high specificity and sensitivity that can be used at farm level (Calcutt et al., 2018). This review is a succinct summary of current state of knowledge in virulence factors, pathogenesis, clinical manifestations, diagnosis, and control measures of *M. bovis* mastitis in dairy cows.

1.2. Virulence factors

Adhesion and invasion

Mycoplasmas lack cell walls and have exposed membrane proteins. The exposed membrane proteins primarily interact with host surfaces and enable the bacteria to adhere to the host mucosal surfaces and are also necessary for the bacteria to acquire nutrients from their surroundings and evade their host's immune response (Razin et al., 1998).

Adhesion is an essential virulence attribute in mycoplasmas since adhesion mutants are avirulent (Krause et al., 1982). *M. bovis* utilizes a 48.8 kDa receptor TrmFO that binds to host fibronectin, an extracellular matrix glycoprotein (Guo et al., 2017). Other isolates also express key adhesins such as α-enolase, P27, vpmaX, and fructose-1,6-biophasphate aldolase (Huang et al., 2019; Shitamori et al., 2022). A cytoadhesive surface exposed protein in certain strains is expressed to surmount the highly tight epithelial junctions during infection in organs such as lung (Zhu et al., 2020). Attachment only, however, does not constitute internalization as some *M. bovis* cells were shown adhered onto and others internalized into calf turbinate cells in a single *in vitro* infection (Bürki et al., 2015; Rottem, 2003). Burki and co-authors shown that *M. bovis* enter host cells through non-classical endocytic pathway (Bürki et al., 2015) which involves invagination of plasma membrane to internalize pathogens (Cossart & Helenius, 2014). This pathway is used by host cells to uptake various fluids and solutes but is taken advantage of by *M. bovis.* Autophagy

is a highly conserved self-destructive process in eukaryotic cells aimed to remove faulty organelles, misfolded proteins, and pathogens (Glick et al., 2010). However, *M. bovis* prevents autophagy in bovine mammary epithelial cells to replicate in an intracellular environment while also avoiding host immune response and antimicrobial agents (Liu et al., 2021; Xu et al., 2022). *M. bovis* exerts this effect through blocking autophagic flux which involves recognition of intracellular *M. bovis* by receptors, delivery to enzyme-bound membranes and final transport to lysosomes, a degradation machinery (Liu et al., 2021).

Variable surface proteins (Vsps)

Among the characteristics that increases the virulence of *M. bovis* is the collection of immunodominant Vsps. These surface lipoproteins are highly variable in their size and coding sequences (Beier et al., 1998; Lysnyansky et al., 1996). Due to different surface lipoprotein variants potentially interacting with the host immune system at any given time, immune responses against these Vsps are not effective. Furthermore, the pathogenicity of *M. bovis* is significantly increased because these Vsps enable it to avoid detection and clearance by a host immune system (Buchenau et al., 2010).

Nucleases

Mycoplasmas do not have biosynthetic mechanisms for synthesizing nucleic acid precursors and depend on cellular nucleases to generate nucleotide precursors (Minion et al., 1993). Nucleases are components of the mycoplasmal membrane that hydrolytically cleave the phosphodiester backbone of DNA and they play important role in acquisition of the host nucleic acids. Various mycoplasma cellular nucleases have been characterized which are believed to be important for generating nucleotides and hence expected to contribute to virulence (Sharma et al., 2015). Endonucleases cleave the phosphodiester bond in the middle of chains within the polynucleotide whereas the exonucleases selectively cleave the polynucleotide chain either at the 5’ or 3’ ends (Mason & Cox, 2012).

Biofilm formation

It has been demonstrated that *M. bovis* produces biofilms; however, the level of adhesion and effectiveness of the biofilms vary between different strains depending on the surface lipoprotein (Vsp) expression. Biofilms of *M. bovis* increase heat resistance at 50°C and desiccation tolerance but are not any more resistant to antibiotics compared to planktonic cells (Laura McAuliffe et al., 2006).

Nucleomodulin secretion

Nucleomodulins are effector proteins secreted by bacteria that can interact with the host DNA and serve to regulate gene transcription to favor the pathogenesis of the bacteria (Hanford et al., 2021). The MbovP475 lipoprotein is secreted by *M. bovis* and binds the promoters of the cell cycle central regulatory genes, CRYAB and MCF2L2 genes and downregulates their expression in bovine macrophage cell line (Zhao et al., 2022) resulting in decreases in bovine macrophage cell line viability.

Metabolites

M. bovis synthesizes hydrogen peroxide (H_2O_2) which has the potential to react with iron and copper ions to produce cytotoxic hydroxyl radicals (Khan et al., 2005). This is possible through the NADH oxidase enzyme expressed by *M. bovis* which reduce oxygen to H_2O_2 on top of its adhesin role (Zhao et al., 2017). Reactive oxygen species produced by *M. bovis* can cause varying degree of damage in bovine mammary epithelial cells including apoptosis (Liu, Zhou, et al., 2020).

1.3. Pathogenesis of *M. bovis* mastitis and clinical symptoms

The most common clinical manifestations of *M. bovis* mastitis includes udder swelling and abnormal milk appearance ranging from watery and flaky milk to thick purulent inflammatory fluid (Bennett & Jasper, 1980). However, some cows may show no outward symptoms although having subclinical mastitis with or without shedding the bacterium (Biddle et al., 2003; M.

Hazelton et al., 2020; Punyapornwithaya et al., 2010; Vähänikkilä et al., 2019). *M. bovis* mastitis causes an increase in SCC. Among the initial reactions during intramammary challenge infection high SCC and acute phase proteins (Kauf et al., 2007). These authors reported increased production of serum amyloid A and LPS-binding protein in experimentally induced *M. bovis* intramammary infection. It is possible that *M. bovis* spreads from one site of infection to another via blood circulation. This was demonstrated by isolation of *M. bovis* from previously uninfected cows which were challenged experimentally by the intramammary route (Bennett & Jasper, 1980). Although shedding and re-infection is a possibility due to contagious nature of the organism, *M. bovis* from a milk of mastitic cows had identical pulsed field gel electrophoresis pattern with those isolated from other body parts such as eyes, nasal cavities and ears which is strong indication of internal dissemination (Biddle et al., 2005).

Transmission of *M. bovis* has been linked to colostrum, milk, semen, air-borne, and intrauterine routes (Gille et al., 2020; Haapala et al., 2018; Hermeyer et al., 2012; Kanci et al., 2017). Udder-to-udder is thought to be the primary route by which the infection is transmitted between cows (Gonzalez et al., 1992; González & Wilson, 2003). Although *M. bovis* intramammary infection is widespread in lactating dairy cows, it is also not uncommon in dry cows (Otter et al., 2015).

Several aspects of *M. bovis* mastitis differs from other major mastitis pathogens. Unlike coliform mastitis, which is environmental, *M. bovis* mastitis is contagious which means it can be transmitted from infected cows to healthy cows during milking time. The major contagious mastitis pathogens include *Staphylococcus aureus, Streptococcus agalactiae, and Mycoplasma* spp (USDA-APHIS, 2008). Compared to *Staphylococcus aureus* and *E. coli*, *M. bovis* weakly affects mRNA expression in bovine mammary epithelial cells (Gondaira et al., 2018). According to the USDA, *M. bovis* is more prevalent in large (500+) dairy operations compared to other contagious mastitis pathogens such as *Streptococcus agalactiae* (USDA-APHIS, 2008).

1.4. Host immune responses against *M. bovis* infection

Innate immunity

Epithelial cells are the major cell types in the bovine mammary gland and are the first line of defense. Upon first encounter, *M. bovis* adhere to and invade mammary gland epithelial cells (Josi et al., 2018) and bovine mammary epithelial cells respond by upregulated expression of proinflammatory cytokines such as interleukin (IL)-6 and IL-8 and TNF-α (Gondaira et al., 2018; Yang et al., 2022). Autophagy is one of the mechanism these cells employ to eliminate invading bacteria, however under-expression of autophagy related proteins has been demonstrated in *M. bovis* infected bovine mammary epithelial cells (Liu et al., 2021; Xu et al., 2022).

M. bovis mastitis is characterized by massive recruitment of neutrophils into the milk spaces of the mammary gland (Schneider et al., 2022). Similarly, *M. bovis* has been shown to stimulate production of neutrophils extracellular traps (NETs) and possess the membrane nuclease, MnuA which degrades the NETs through either exo- or endo-nuclease activity (Mitiku et al., 2018; Sharma et al., 2015). In addition, another membrane nuclease of *M. bovis (*MBOV_RS02825) has been demonstrated to degrade NETs and cause apoptosis in macrophages (Zhang et al., 2016). NETs are considered part of the innate immune response which have DNA as a major structural component to trap and stop pathogenic bacteria from spreading (Brinkmann et al., 2004). Degradation of the NETs by mycoplasmal nucleases have a bifold advantage including avoiding the entrapment and opsonophagocytic killing by neutrophils and scavenging the nucleotide precursors (Cacciotto & Alberti, 2022). Furthermore, *M. bovis* promote neutrophil apoptosis to ensure its persistence and systemic dissemination.

M. bovis has been observed to elicit proinflammatory cytokine and chemokine responses in infected hosts which may weaken the host and increase the pathogenicity of the bacteria (Valsala et al., 2017). Opsonization is necessary for phagocytosis of *M. bovis* by macrophages and neutrophils, but *M. bovis* can combat this through surface antigen variation, and biofilm formation (L. McAuliffe et al., 2006). Macrophages kill *M. bovis via* phagocytosis up on opsonization support from IgG1 and IgG2 (Howard & Taylor, 1983). In a study conducted to

determine *M. bovis*-bovine viral diarrhea virus (BVDV) synergism during infection, *M. bovis* induced apoptosis and cytotoxicity in bovine macrophages (Burgi et al., 2018). In contrast, another study reported significant reduction in apoptosis in macrophages induced by *M. bovis* possibly as a mechanism of survival (Maina et al., 2019).

M. bovis inhibits proliferation of peripheral blood mononuclear cells (PBMCs) proliferation to evade the immune system and cause chronic infections (Suleman et al., 2018). However, in contrary another study reported an increase in the expression of TNF-α, IL-12 and IFN-γ and increased proliferative responses in PBMCs stimulated with *M. bovis* (Gondaira et al., 2015). Following intramammary inoculation with *M. bovis*, milk from infected quarters exhibited increased SCCs, yet there was not a significant difference between levels of PBMCs or mononuclear cells in the stimulated and unstimulated mammary lymph nodes (Gondaira et al., 2020). In fact, there was a decrease in the mRNA levels of innate-immunity related genes from blood mononuclear cells (MNCs) following intramammary infection with *M. bovis*, such as complement factor D (CFD), ficolin 1 (FCN1), and tumor necrosis factor superfamily member 13 (TNFSF13) (Gondaira et al., 2020). This alteration of the host transcriptome likely contributes to the chronic nature of many *M. bovis* infections.

Adaptive immunity

M. bovis antigens activate host $CD4^+$, $CD8^+$, γδ T- cells, B lymphocytes, and leukocytosis (Dudek & Bednarek, 2017; Dudek et al., 2011). In addition, *M. bovis* also induces IgG1and IgG2 responses (Prysliak et al., 2017). IgG1, however, has a lower opsonin effect which does not activate a strong humoral immune response in the host and resulting in persistence of *M. bovis* infections for long periods of time (Askar et al., 2021). The effect of cytokines, like interferon gamma (IFN-γ), that encourage cell death but also cytokines that characterize the Th2 response and slow recovery of tissues likely contribute to the pathogenicity of *M. bovis* and make it more difficult for the host to recover from infections (Vanden Bush & Rosenbusch, 2003).

1.5. Diagnosis of *M. bovis*

Culture

Microbial culture has traditionally been used to definitively diagnose *M. bovis*. However, the longer time it takes to grow makes culture unfavorable to make a rapid diagnosis. Most common specimens to diagnose *M. bovis* includes milk, bronchioalveolar lavage, deep nasopharyngeal swabs, joint fluids, and semen. Mycoplasma plates are incubated at 37^{o}C in 5% CO_2 for 7-10 days and colonies are typically characterized by a 'fried egg appearance' when observed under light microscope (Parker et al., 2018). This is unfavorable where rapid diagnosis is needed to isolate affected animals and limit further dissemination of infections and commence appropriate antibiotic therapy (Fox, 2012). Due to the longer days required by *M. bovis*, sometimes the colonies are overgrown by other bacteria to the extent that they cover *M. bovis* colonies and makes it difficult to observe under microscope (Quinn et al., 2011). Not only they are overgrown on the plates, but they are also overgrown in the milk since mycoplasmas has limited capability to multiply in the milk (Parker et al., 2016).

Mycoplasmas have one of the smallest genome sizes (0.58-1.38 Mbp) which renders them needy of nutrients such as amino acids and fatty acids (Nicholas et al., 2008; Razin et al., 1998). The other challenge with *M. bovis* culture is its detection limit which is greater than or equal to 272 cfu/mL (Sachse et al., 2010) meaning any number of mycoplasma cells less than the detection limit could possibly be overlooked. Culture also fails to differentiate from other non-pathogenic mollicutes such as *Acholeplasma* which exhibit the same 'fried egg' appearance as *M. bovis* and can potentially contaminate *M. bovis* samples (M. S. Hazelton et al., 2020).

Bulk tank milk samples are used in several studies for estimating prevalence and other research purposes (Hurri et al., 2022; Justice-Allen et al., 2011; Liapi et al., 2021; McAloon et al., 2022). The major problem with bulk tank milk sampling is collecting a representative amount of sample out of 200 to 2,600 gallons of tanks which could massively dilute the amount of Mycoplasma cells. Usually, about 10 - 40 mL of milk sample is collected (Hurri et al., 2022; Liapi et al., 2021), centrifuged and only 100 - 200 µL are spread on the mycoplasma plates. Some

laboratories culture milk samples directly (Gonzalez et al., 1992), however it has been demonstrated that centrifugation and resuspension can potentially increase the rate of recovery (Punyapornwithaya et al., 2009). Occasionally, enrichment of milk samples in mycoplasma broth before culture is practiced (Boothby, Mueller, et al., 1986). Milk samples need to be processed immediately after collection to maximize the likelihood of positive diagnosis or need to be frozen although freezing has been shown to cause 1-2 $\log_{10}$ reduction (Biddle et al., 2004). In addition, multiple sampling is greatly advised due to intermittent shedding behavior of the pathogen in mastitis cases (Biddle et al., 2003; Dudek et al., 2013). However, there are also studies which were conducted based on one-time bulk tank milk sampling (Hurri et al., 2022; Liu, Xu, et al., 2020).

Polymerase chain reaction (PCR)

PCR is such a sensitive method in *M. bovis* diagnosis that it detects as low as 10 CFUs from broth cultures (Cremonesi et al., 2007; Hotzel et al., 1993). Compared to culture, enzyme-linked immunosorbent assay (ELISA), sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), and nucleic acid hybridization, PCR was found to be superior in terms of high specificity, sensitivity and rapidity (Sachse et al., 1993). However, expensive reagents and equipment are the major setbacks to apply in small-scale laboratories and farm environments. The application of PCR in *M. bovis* diagnosis brought about several advantages. It bypasses the tiresome culturing and samples can directly be screened for *M. bovis* DNA. In comparison to the culture method which only detects live organisms, PCR can detect the DNA from dead microorganisms which can have several implications. This is true, for instance, perhaps if milk samples are stored in a freezer for long time and *M. bovis* cells are no longer viable (Biddle et al., 2004). In some cases, pre-enrichment is recommended before performing PCR assays for as many as 4 days (Andrés-Lasheras et al., 2020). This might save time and resource in terms of avoiding culturing negative samples, however is not helpful when rapid diagnosis is needed.

UvrC gene has widely been used as a target in conventional and real-time PCR in *M. bovis* detection in bulk tank milk and lung samples (Arcangioli et al., 2011; Behera et al., 2018; Liapi et al., 2021). It is one of the house-keeping genes in *M. bovis* which encodes an enzyme that

mediates excision DNA repair system (Nicholas et al., 2008; Subramaniam et al., 1998). Furthermore, this gene has been recommended for use in routine laboratory diagnosis of *M. bovis* (Thomas et al., 2004). Realtime PCR has been shown to be more sensitive than conventional PCR and was able to amplify as low as 40 copies of the target gene (Behera et al., 2018). The CT values in real-time and quantitative PCR (qPCR) can be used as a predictive tool for *M. bovis* isolation (Andrés-Lasheras et al., 2020).

A multiplex PCR which separately detects different species such as *M. bovis*, *M. bovigenitalium*, and *M. californicum* in a same sample would greatly reduce efforts and time spent to perform different reactions (Parker, House, Hazelton, Bosward, & Sheehy, 2017). Furthermore, bacterial species such as *Mannheimia hemolytica* are usually isolated alongside *M. bovis* in respiratory diseases (Valeris-Chacin et al., 2022). Multiplex quantitative PCR has been proven to specifically detect and quantify these respiratory pathogens (Conrad et al., 2020; Pansri et al., 2020). Nested-PCR, where two sets of primers are employed, is preferred to increase sensitivity and specificity of mycoplasma detection (Baird et al., 1999; Justice-Allen et al., 2010). PCR has also been employed to identify antibiotic resistance genes in *M. bovis* (Amram et al., 2015; Lysnyansky et al., 2009).

Indirect and direct ELISA

Unlike culture and PCR, ELISA detects the anti-*M. bovis* antibodies in the host serum or milk from past or recent infections as a result of humoral immune responses. ELISA has widely been used on bulk tank milk samples to detect anti-*M. bovis* antibodies in the milk for diagnostic, prevalence, and retrospective studies (Hurri et al., 2022; McAloon et al., 2022; Parker, House, Hazelton, Bosward, Morton, et al., 2017; Petersen et al., 2018). A MilA ELISA with a specificity and sensitivity as high as 94.2% and 96.6%, respectively, has been used to estimate *M. bovis* mastitis prevalence from bulk tank milk samples (Salgadu et al., 2022). This ELISA, in which MilA membrane protein was used as a coating antigen, was first developed in Australia (Wawegama et al., 2014). Furthermore, the *milA* gene was expressed on the surface of a phage and was used as antigen in indirect ELISA which was reported to be inexpensive and convenient compared to the MilA peptide protein (Farzaneh et al., 2022). However, bulk tank milk is not a

fully representative sample for a herd since *M. bovis* can cause various disease manifestations in various age groups (Petersen et al., 2016). This necessitates considering blood samples in certain cases where the young stock is suspected to harbor the infection or they are being newly introduced to herd. ELISA might not necessarily indicate active *M. bovis* infection as positive ELISA results turns negative in PCR when both methods were used in same herds (Gogoi-Tiwari et al., 2022; Hurri et al., 2022). Thus, ELISA can be used as a biosecurity tool before introducing newcomers to the herd (Parker, House, Hazelton, Bosward, Morton, et al., 2017). ELISA has also been used as a surveillance tool to monitor and confirm eradication of *M. bovis* infections in some nations (Cowled et al., 2022). It also plays a role in testing immunogenicity of novel proteins in a study conducted to investigate *M. bovis* pathogenesis and its protective antigens (Chen et al., 2018).

The use of indirect ELISA to detect anti-*M. bovis* antibodies is challenged by cross-reactivity from other *Mycoplasma* spp. such as *M. agalactiae* (Sun et al., 2014). Indirect ELISA might not be as rapid as desired sometimes, since it takes 1-2 weeks for the animal to mount humoral immune response (seroconversion) and results could possibly turn negative in this time window (Parker et al., 2018), thus it needs to be used in conjunction with other tests. Once antibodies are produced by the host, however, ELSIA is not affected by the intermittent shedding behavior of *M. bovis* in the milk which indicates it is important to use culture, PCR and/or ELISA together, whenever possible, since they complement each other. Unlike indirect ELISA, the use of direct ELISA in *M. bovis* diagnosis is limited. There are not many direct ELISAs reported, however one study reported membrane protein P48 based monoclonal antibodies has been shown to be specifically detect *M. bovis* without cross-reactivity with related species such as *M. agalactiae* (Fu et al., 2014).

MALDI-TOF MS

Matrix-Assisted Laser Desorption Ionization – Time of Flight Mass Spectrometry (MALDI-TOF MS) has lately been adapted as a rapid tool to accurately diagnose microbes. The principle behind the method is formation of ions from intact bacterial cells using laser light ionization (Holland et al., 1996). Samples are obtained from colonies on agar plates, mixed with matrix

solution, and introduced to a mass spectrometer ion source. Results are analyzed against archived references or with colonies from known bacteria. Following a comparison with the 16S rDNA PCR as gold standard, MALDI-TOF has been in agreement for 97.8% species-level and 99.6% genus-level anaerobes and 95.3% species-level and 100% genus-level anaerobes which shows that it is a preferable method to diagnose bacteria of veterinary interest (Randall et al., 2015). The suitability of MALDI-TOF to distinguish between phylogenetically closest sub-species in human and ruminant mycoplasmas has been confirmed (Pereyre et al., 2013). Although some studies recommend MALDI-TOF as a promising test for routine diagnosis of *M. bovis,* the disadvantage lies in its dependence on enrichment which can take 2 to 4 days (Bokma et al., 2020; McDaniel & Derscheid, 2021). The starting material for MALDI-TOF could also be a secretome extraction or a protein from *M. bovis.* Zubair and co-workers used extracted secretome from *M. bovis* HB0801 strain to predict 8 proteins related to a virulence which signifies the importance of MALDI-TOF in identifying potential diagnostic and vaccine targets (Zubair et al., 2020).

Limited library of mycoplasmas in the MALDI-TOF databases has been reported as one of the challenges in using this method to detect *M. bovis* (McDaniel & Derscheid, 2021). Initial MALDI-TOF installation and instrumentation is also believed to be expensive which makes its application at large scale level difficult (Singhal et al., 2015). MALDI-TOF is commonly used more as a confirmatory technique rather than a routine diagnostic tool.

Loop-mediated isothermal amplification (LAMP)

The LAMP is a high sensitivity method which utilizes a set of 2 to 3 primers that can produce several copies (10^9) of target DNA in less than hour since it bypasses the denaturation step (Soroka et al., 2021). As the name indicates, LAMP amplification occurs within a constant temperature. Unlike the conventional PCR, LAMP does not need thermocyclers and can easily be done in heating block (Bai et al., 2011). Following amplification, results can be read in 2% agarose gel electrophoresis; using SYBR green I staining or based on turbidity of the reaction mixture (Bai et al., 2011; Higa et al., 2016). *UvrC*-based LAMP in *M. bovis* has been demonstrated to have 10-fold higher sensitivity compared to PCR with 100% and 74% sensitivity and specificity

respectively (Bai et al., 2011), however later on other researchers improved the specificity to 90.9% (Higa et al., 2016). Other *M. bovis* genes such as *oppD* (encodes oligopeptide permease D), *gltX* glutamate transfer RNA ligase), *gyrB* (gyrase B subunit) and 16s rRNA were also employed to show sensitive and specific detection of *M. bovis* using LAMP (Appelt et al., 2019; Ashraf et al., 2018).

1.6. Control and prevention measures

Globally, DNA amplification techniques used for detection and identification of bacteria have only become widely globally accessible within the last 30 years, making it difficult to trace the exact time and route by which *M. bovis* first spread around the world (Hotzel et al., 1993). The first definitive identification of *M. bovis* infection was in 1961 in the United States (Hale et al., 1962). From there, the pathogen was thought to have been spread to other countries through movement of cattle and cattle products (Nicholas, 2011).

The strategy towards the control and prevention of *M. bovis* mastitis, or *M. bovis* infection in general, depends on the country. New Zealand, which is the latest country to report *M. bovis* mastitis in 2017, prefers a nationwide complete eradication program (Cowled et al., 2022). However, other endemic countries endeavor to contain infections at the farm level through culling or isolating infected animals (Haapala et al., 2021). Finland pursues a voluntary control program involving farmers since *M. bovis* is regarded as one of less serious diseases (Autio et al., 2021).

Currently, the best-known method for controlling *M. bovis* is mere prevention of exposure to the pathogen and other infected cows. Screening of original herd before purchasing new cows is worthwhile as well as quarantine of new cows which adds extra layer of security. In addition, isolation and culling of infected cows are necessary measures to effectively control the disease and minimize outbreaks (Zhao et al., 2022). However, advances in rapid and accessible tests for detecting *M. bovis* on dairy farms are necessary to control and prevent outbreaks from occurring more effectively.

Use of antimicrobials

There are currently no known effective treatments for *M. bovis* available for use. One of the main reasons for this is the growing incidence of antibiotic resistant bacterial strains. Drugs such as tiamulin, enrofloxacin, danofloxacin, and florfenicol has been reported to have low minimum inhibitory concentration against *M. bovis* (Lysnyansky & Ayling, 2016). However, in the last 2 decades, *M. bovis* have shown less susceptibility to antimicrobial agents like fluoroquinolones (Klein et al., 2019). Furthermore, lack of cell wall makes the organism resistant to commonly used antibiotics such as penicillin and cephalosporins. Macrolides such as tylosin and tilmicosin which were traditionally used to treat mycoplasma infections has gradually become less effective (Lerner et al., 2014). Recent trends indicate that antimicrobial resistance against other common antibiotics, such as tetracyclines, has been increasing as broadly reviewed elsewhere (Lysnyansky & Ayling, 2016). Some natural compounds have been shown to be promising and may further be developed to produce effective therapeutic options (M. K. Soehnlen et al., 2011).

Vaccines

Many potential vaccine candidates have been developed but are not available for widespread commercial use. For example, autogenous vaccines have been developed (Dudek et al., 2016); however, such vaccines are useful only for a single farm, limiting their potential for large-scale use. Other vaccines showed efficacy in studies but failed to elicit any protective effects in field trials as they failed to reduce the incidence of *M. bovis* cases (Boothby, Jasper, et al., 1986; Marty K. Soehnlen et al., 2011). There are currently no commercially available effective vaccines prevent the incidence of *M. bovis* infection. Given the fact that *M. bovis* causes pneumonia in the feedlot cattle (Arcangioli et al., 2008), considerable number of vaccine works has been done using feedlot cattle as a model. Prysliak and co-authors developed a sub-unit vaccine using the highly conserved glyceraldehyde-3-phosphate dehydrogenase (GAPDH) protein; however, subsequent controlled experimental efficacy evaluation showed that it did not confer protection against experimental challenge (Prysliak et al., 2013). Similarly, intranasal inoculation of total protein extract and membrane fractions from *M. bovis* triggered strong humoral immune responses but failed to protect against experimental challenge infection

(Mulongo et al., 2013). Another challenge in vaccine development against *M. bovis* mastitis is strain variations of *M. bovis* (Aebi et al., 2012). *M. bovis* also expresses antigenically variable surface proteins (Li et al., 2011; Lysnyansky et al., 1996) and thus necessitates developing vaccines from highly conserved immunogenic proteins. Despite several trials in the past, conserved immunogenic molecule which can elicit protective immune responses against *M. bovis* mastitis are yet to be identified.

1.7. Perspectives and future directions

In summary, effective control tools such as vaccine or prophylactic or therapeutic drugs against *M. bovis* mastitis are not available. Currently adopted mastitis control programs which involve environmental sanitation, proper milking procedures, and udder health are partly ineffective due to transmission of *M. bovis* through the respiratory route, semen or seminal fluid as well as calf to cow or *vice-versa*.

Rapid diagnosis is extremely important for the identification and culling of infected animals before infection spreads through the herd. This is particularly true in farm settings where purchased animals need to be screened or where segregation and culling of infected animals is much needed. Therefore, rapid and accurate pen-side diagnostic tests or combination of tests are needed. Given the difficulty in diagnosis due to the fastidious growth nature and subclinical infection, the disease can remain undetected for a long time in dairy farms with significant economic losses. Accurate diagnosis is also important to conduct targeted treatment which reduces the emergence of antibiotic resistance. Whenever microbial culture is used for diagnosis, suspected animals awaiting results should be segregated from other herd members. Immediate culturing of milk samples is highly encouraged to increase the likelihood of detection since refrigeration and freezing lowers the survival of Mycoplasma cells (Boonyayatra et al., 2010). Cows with mastitis are usually asymptomatic and sometimes *M. bovis* is detected from healthy animals (Penterman et al., 2022). This is also best exemplified by sudden occurrence of infection signs such as lameness and mastitis in yet closed dairy herds (Wilson et al., 2007). Therefore, multiple sampling and regular screening of existing herd members is extremely important depending on how often new heifers are purchased. To overcome the problem of intermittent

shedding of the *Mycoplasma* in the milk, indirect ELISA could be of great help since it depends on the antibodies rather than the detection of the antigen itself. MALDI-TOF is an advanced, rapid, and accurate method to detect *M. bovis* from various clinical samples. Its expensiveness and requiring sophisticated instrumentation and expertise renders it difficult to recommend for large-scale use. Finally, uniform recommendation should be put forward regarding which tests can be bundled together and yield complete diagnosis of *M. bovis* for different clinical samples collected from the animal body organs affected.

One of the major reasons for the failure to develop an effective control tool is limited knowledge of the virulence factors of *M. bovis* and the pathogenesis of *M. bovis* mastitis in dairy cows. Therefore, developing effective and sustainable control tools such as vaccines or prophylactic or therapeutic drugs, or any other innovative intervention tool using advanced molecular biology and cellular and molecular immunology approaches is required.

The combined economic and welfare impact of *M. bovis* infections prompted extensive search for effective and sustainable control tools such as vaccines, prophylactic and therapeutic solutions (Calcutt et al., 2018) while also antibiotic resistance is increasing (Lysnyansky & Ayling, 2016). Vaccine attempts has been futile due to mainly the knowledge gap in the pathogenesis and virulence mechanism of *M. bovis.* Novel vaccine or antimicrobial drugs will be out of reach if conserved immunogenic antigens or therapeutic agents are not discovered.

Hygienic husbandry practices during milking, feeding, and overall rearing is of paramount significance.

References

Aebi, M., Bodmer, M., Frey, J., & Pilo, P. (2012). Herd-specific strains of *Mycoplasma bovis* in outbreaks of mycoplasmal mastitis and pneumonia. *Vet Microbiol*, *157*(3-4), 363-368. https://doi.org/10.1016/j.vetmic.2012.01.006

Allan M. Britten, E. D. T., and Justine E. Britten. (2020). Why Species Matter? Dramatic Revelations in Mycoplasma Mastitis Management with Molecular Diagnostics. NMC Annual Meeting Proceedings,

Amram, E., Mikula, I., Schnee, C., Ayling, R. D., Nicholas, R. A., Rosales, R. S., Harrus, S., & Lysnyansky, I. (2015). 16S rRNA gene mutations associated with decreased susceptibility to tetracycline in *Mycoplasma bovis*. *Antimicrob Agents Chemother*, *59*(2), 796-802. https://doi.org/10.1128/aac.03876-14

Andrés-Lasheras, S., Zaheer, R., Ha, R., Lee, C., Jelinski, M., & McAllister, T. A. (2020). A direct qPCR screening approach to improve the efficiency of *Mycoplasma bovis* isolation in the frame of a broad surveillance study. *Journal of Microbiological Methods*, *169*, 105805. https://doi.org/https://doi.org/10.1016/j.mimet.2019.105805

Appelt, S., Aly, S. S., Tonooka, K., Glenn, K., Xue, Z., Lehenbauer, T. W., & Marco, M. L. (2019). Development and comparison of loop-mediated isothermal amplification and quantitative polymerase chain reaction assays for the detection of *Mycoplasma bovis* in milk. *J Dairy Sci*, *102*(3), 1985-1996. https://doi.org/10.3168/jds.2018-15306

Arcangioli, M. A., Chazel, M., Sellal, E., Botrel, M. A., Bezille, P., Poumarat, F., Calavas, D., & Le Grand, D. (2011). Prevalence of *Mycoplasma bovis* udder infection in dairy cattle: preliminary field investigation in southeast France. *N Z Vet J*, *59*(2), 75-78. https://doi.org/10.1080/00480169.2011.552856

Arcangioli, M. A., Duet, A., Meyer, G., Dernburg, A., Bézille, P., Poumarat, F., & Le Grand, D. (2008). The role of *Mycoplasma bovis* in bovine respiratory disease outbreaks in veal calf feedlots. *Vet J*, *177*(1), 89-93. https://doi.org/10.1016/j.tvjl.2007.03.008

Ashraf, A., Imran, M., Yaqub, T., Tayyab, M., Shehzad, W., Mingala, C. N., & Chang, Y. F. (2018). Development and validation of a loop-mediated isothermal amplification assay for the detection of *Mycoplasma bovis* in mastitic milk. *Folia Microbiol (Praha)*, *63*(3), 373-380. https://doi.org/10.1007/s12223-017-0576-x

Askaa, G., & Ernø, H. (1976). Note: Elevation of *Mycoplasma agalactiae* subsp. *bovis* to Species Rank: Mycoplasma bovis (Hale et al.) comb. nov. *International Journal of Systematic and Evolutionary Microbiology*, *26*(3), 323-325. https://doi.org/https://doi.org/10.1099/00207713-26-3-323

Askar, H., Chen, S., Hao, H., Yan, X., Ma, L., Liu, Y., & Chu, Y. (2021). Immune Evasion of *Mycoplasma bovis*. *Pathogens*, *10*(3), 297. https://doi.org/10.3390/pathogens10030297

Autio, T., Tuunainen, E., Nauholz, H., Pirkkalainen, H., London, L., & Pelkonen, S. (2021). Overview of Control Programs for Cattle Diseases in Finland. *Front Vet Sci*, *8*, 688936. https://doi.org/10.3389/fvets.2021.688936

Bai, Z., Shi, L., Hu, C., Chen, X., Qi, J., Ba, X., Peng, Q., Chen, Y., Chen, H., & Guo, A. (2011). Development of a loop-mediated isothermal amplification assay for sensitive and rapid detection of *Mycoplasma bovis*. *African Journal of Biotechnology*, *10*(57), 12333-12338.

Baird, S., Carman, J., & Dinsmore, R. (1999). Detection and identification of Mycoplasma from bovine mastitis infections using a nested polymerase chain reaction. *J Vet Diagn Invest*, *11*, 432-435. https://doi.org/10.1177/104063879901100507

Behera, S., Rana, R., Gupta, P. K., Kumar, D., Sonal, Rekha, V., Arun, T. R., & Jena, D. (2018). Development of real-time PCR assay for the detection of *Mycoplasma bovis*. *Trop Anim Health Prod*, *50*(4), 875-882. https://doi.org/10.1007/s11250-018-1510-1

Beier, T., Hotzel, H., Lysnyansky, I., Grajetzki, C., Heller, M., Rabeling, B., Yogev, D., & Sachse, K. (1998). Intraspecies polymorphism of *vsp* genes and expression profiles of variable surface protein antigens (Vsps) in field isolates of *Mycoplasma bovis*. *Vet Microbiol*, *63*(2-4), 189-203. https://doi.org/10.1016/s0378-1135(98)00238-7

Bennett, R. H., & Jasper, D. E. (1980). Bovine mycoplasmal mastitis from intramammary inoculations of small numbers of *Mycoplasma bovis*: local and systemic antibody response. *Am J Vet Res*, *41*(6), 889-892.

Biddle, M. K., Fox, L. K., Evans, M. A., & Gay, C. C. (2005). Pulsed-field gel electrophoresis patterns of Mycoplasma isolates from various body sites in dairy cattle with Mycoplasma mastitis. *J Am Vet Med Assoc*, *227*(3), 455-459. https://doi.org/10.2460/javma.2005.227.455

Biddle, M. K., Fox, L. K., & Hancock, D. D. (2003). Patterns of mycoplasma shedding in the milk of dairy cows with intramammary mycoplasma infection. *J Am Vet Med Assoc*, *223*(8), 1163-1166. https://doi.org/10.2460/javma.2003.223.1163

Biddle, M. K., Fox, L. K., Hancock, D. D., Gaskins, C. T., & Evans, M. A. (2004). Effects of storage time and thawing methods on the recovery of *Mycoplasma* species in milk samples from cows with intramammary infections. *J Dairy Sci*, *87*(4), 933-936. https://doi.org/10.3168/jds.S0022-0302(04)73237-3

Bokma, J., Van Driessche, L., Deprez, P., Haesebrouck, F., Vahl, M., Weesendorp, E., Deurenberg, R. H., Pardon, B., & Boyen, F. (2020). Rapid Identification of *Mycoplasma bovis* Strains from Bovine Bronchoalveolar Lavage Fluid with Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry after Enrichment Procedure. *J Clin Microbiol*, *58*(6). https://doi.org/10.1128/jcm.00004-20

Boonyayatra, S., Fox, L. K., Besser, T. E., Sawant, A., & Gay, J. M. (2010). Effects of storage methods on the recovery of *Mycoplasma* species from milk samples. *Vet Microbiol*, *144*(1-2), 210-213. https://doi.org/10.1016/j.vetmic.2009.12.014

Boothby, J. T., Jasper, D. E., & Thomas, C. B. (1986). Experimental intramammary inoculation with *Mycoplasma bovis* in vaccinated and unvaccinated cows: effect on milk production and milk quality. *Can J Vet Res*, *50*(2), 200-204. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1255190/pdf/cjvetres00062-0058.pdf

Boothby, J. T., Mueller, R., Jasper, D. E., & Thomas, C. B. (1986). Detecting *Mycoplasma bovis* in milk by enzyme-linked immunosorbent assay, using monoclonal antibodies. *Am J Vet Res*, *47*(5), 1082-1084.

Brinkmann, V., Reichard, U., Goosmann, C., Fauler, B., Uhlemann, Y., Weiss, D. S., Weinrauch, Y., & Zychlinsky, A. (2004). Neutrophil extracellular traps kill bacteria. *Science*, *303*(5663), 1532-1535. https://doi.org/10.1126/science.1092385

Buchenau, I., Poumarat, F., Le Grand, D., Linkner, H., Rosengarten, R., & Hewicker-Trautwein, M. (2010). Expression of *Mycoplasma bovis* variable surface membrane proteins in the respiratory tract of calves after experimental infection with a clonal variant of *Mycoplasma bovis* type strain PG45. *Res Vet Sci*, *89*(2), 223-229. https://doi.org/10.1016/j.rvsc.2010.03.014

Burgi, N., Josi, C., Burki, S., Schweizer, M., & Pilo, P. (2018). *Mycoplasma bovis* co-infection with bovine viral diarrhea virus in bovine macrophages. *Vet Res*, *49*(1), 2. https://doi.org/10.1186/s13567-017-0499-1

Bürki, S., Gaschen, V., Stoffel, M. H., Stojiljkovic, A., Frey, J., Kuehni-Boghenbor, K., & Pilo, P. (2015). Invasion and persistence of *Mycoplasma bovis* in embryonic calf turbinate cells. *Vet Res*, *46*(1), 53. https://doi.org/10.1186/s13567-015-0194-z

Cacciotto, C., & Alberti, A. (2022). Eating the Enemy: *Mycoplasma* Strategies to Evade Neutrophil Extracellular Traps (NETs) Promoting Bacterial Nucleotides Uptake and Inflammatory Damage. *Int J Mol Sci*, *23*(23). https://doi.org/10.3390/ijms232315030

Calcutt, M. J., Lysnyansky, I., Sachse, K., Fox, L. K., Nicholas, R. A. J., & Ayling, R. D. (2018). Gap analysis of *Mycoplasma bovis* disease, diagnosis and control: An aid to identify future development requirements. *Transbound Emerg Dis*, *65 Suppl 1*, 91-109. https://doi.org/10.1111/tbed.12860

Chen, X., Huang, J., Zhu, H., Guo, Y., Khan, F. A., Menghwar, H., Zhao, G., & Guo, A. (2018). P27 (MBOV_RS03440) is a novel fibronectin binding adhesin of *Mycoplasma bovis*. *Int J Med Microbiol*, *308*(7), 848-857. https://doi.org/10.1016/j.ijmm.2018.07.006

Conrad, C. C., Daher, R. K., Stanford, K., Amoako, K. K., Boissinot, M., Bergeron, M. G., Alexander, T., Cook, S., Ralston, B., Zaheer, R., Niu, Y. D., & McAllister, T. (2020). A Sensitive and Accurate Recombinase Polymerase Amplification Assay for Detection of the Primary Bacterial Pathogens Causing Bovine Respiratory Disease. *Front Vet Sci*, *7*, 208. https://doi.org/10.3389/fvets.2020.00208

Cossart, P., & Helenius, A. (2014). Endocytosis of viruses and bacteria. *Cold Spring Harb Perspect Biol*, *6*(8). https://doi.org/10.1101/cshperspect.a016972

Cowled, B. D., Sergeant, E. S. G., Leslie, E. E. C., Crosbie, A., Burroughs, A., Kingston, O., Neill, M., Sawford, K., & van Andel, M. (2022). Use of scenario tree modelling to plan freedom from infection surveillance: *Mycoplasma bovis* in New Zealand. *Prev Vet Med*, *198*, 105523. https://doi.org/10.1016/j.prevetmed.2021.105523

Cremonesi, P., Vimercati, C., Pisoni, G., Perez, G., Ribera, A. M., Castiglioni, B., Luzzana, M., Ruffo, G., & Moroni, P. (2007). Development of DNA extraction and PCR amplification protocols for detection of *Mycoplasma bovis* directly from milk samples. *Vet Res Commun*, *31 Suppl 1*, 225-227. https://doi.org/10.1007/s11259-007-0011-x

Dudek, K., & Bednarek, D. (2017). T- and B-cell Response Analysis Following Calf Immunisation with Experimental *Mycoplasma Bovis* Vaccine Containing Saponin and Lysozyme Dimer. *J Vet Res*, *61*(4), 433-437. https://doi.org/10.1515/jvetres-2017-0060

Dudek, K., Bednarek, D., Ayling, R. D., Kycko, A., Szacawa, E., & Karpinska, T. A. (2016). An experimental vaccine composed of two adjuvants gives protection against *Mycoplasma bovis* in calves. *Vaccine*, *34*(27), 3051-3058. https://doi.org/10.1016/j.vaccine.2016.04.087

Dudek, K., Bednarek, D., Ayling, R. D., & Szacawa, E. (2013). Immunomodulatory effect of *Mycoplasma bovis* in experimentally infected calves. *Bull Vet Inst Pulawy*, *57*(4), 499-506.

Dudek, K., Bednarek, D., & Szacawa, E. (2011). Evaluation of immune response in seropositive cattle for *Mycoplasma bovis*. *Bull Vet Inst Pulawy*, *55*, 631-634.

Farzaneh, M., Derakhshandeh, A., Al-Farha, A. A. A., Petrovski, K., & Hemmatzadeh, F. (2022). A novel phage-displayed MilA ELISA for detection of antibodies against *M. bovis* in bovine milk. *J Appl Microbiol*, *133*(3), 1496-1505. https://doi.org/10.1111/jam.15655

Fernando Ulloa, D. B., Ronald Jara, Eduardo García, Juan Kruze, and Armin Mella. (2021). Case Report: *Mycoplasma* Mastitis Outbreaks in Two Chilean Dairy Herds. NMC Annual Meeting Proceedings,

Foster, A. P., Naylor, R. D., Howie, N. M., Nicholas, R. A., & Ayling, R. D. (2009). *Mycoplasma bovis* and otitis in dairy calves in the United Kingdom. *Vet J*, *179*(3), 455-457. https://doi.org/10.1016/j.tvjl.2007.10.020

Fox, L. K. (2012). Mycoplasma mastitis: causes, transmission, and control. *Vet Clin North Am Food Anim Pract*, *28*(2), 225-237. https://doi.org/10.1016/j.cvfa.2012.03.007

Fu, P., Sun, Z., Zhang, Y., Yu, Z., Zhang, H., Su, D., Jiang, F., & Wu, W. (2014). Development of a direct competitive ELISA for the detection of *Mycoplasma bovis* infection based on a monoclonal antibody of P48 protein. *BMC Vet Res*, *10*, 42. https://doi.org/10.1186/1746-6148-10-42

Gille, L., Evrard, J., Callens, J., Supré, K., Grégoire, F., Boyen, F., Haesebrouck, F., Deprez, P., & Pardon, B. (2020). The presence of *Mycoplasma bovis* in colostrum. *Vet Res*, *51*(1), 54. https://doi.org/10.1186/s13567-020-00778-w

Gioia, G., Addis, M. F., Santisteban, C., Gross, B., Nydam, D. V., Sipka, A. S., Virkler, P. D., Watters, R. D., Wieland, M., Zurakowski, M. J., & Moroni, P. (2021). *Mycoplasma* species isolated from bovine milk collected from US dairy herds between 2016 and 2019. *J Dairy Sci*, *104*(4), 4813-4821. https://doi.org/10.3168/jds.2020-19171

Glick, D., Barth, S., & Macleod, K. F. (2010). Autophagy: cellular and molecular mechanisms. *J Pathol*, *221*(1), 3-12. https://doi.org/10.1002/path.2697

Gogoi-Tiwari, J., Tiwari, H. K., Wawegama, N. K., Premachandra, C., Robertson, I. D., Fisher, A. D., Waichigio, F. K., Irons, P., & Aleri, J. W. (2022). Prevalence of *Mycoplasma bovis* Infection in Calves and Dairy Cows in Western Australia. *Vet Sci*, *9*(7). https://doi.org/10.3390/vetsci9070351

Gondaira, S., Higuchi, H., Iwano, H., Nakajima, K., Kawai, K., Hashiguchi, S., Konnai, S., & Nagahata, H. (2015). Cytokine mRNA profiling and the proliferative response of bovine peripheral blood mononuclear cells to *Mycoplasma bovis*. *Vet Immunol Immunopathol*, *165*(1-2), 45-53. https://doi.org/10.1016/j.vetimm.2015.03.002

Gondaira, S., Higuchi, H., Iwano, H., Nishi, K., Nebu, T., Nakajima, K., & Nagahata, H. (2018). Innate immune response of bovine mammary epithelial cells to *Mycoplasma bovis*. *J Vet Sci*, *19*(1), 79-87. https://doi.org/10.4142/jvs.2018.19.1.79

Gondaira, S., Nishi, K., Tanaka, T., Yamamoto, T., Nebu, T., Watanabe, R., Konnai, S., Hayashi, T., Kiku, Y., Okamoto, M., Matsuda, K., Koiwa, M., Iwano, H., Nagahata, H., & Higuchi, H. (2020). Immunosuppression in Cows following Intramammary Infusion of *Mycoplasma bovis*. *Infect Immun*, *88*(3). https://doi.org/10.1128/iai.00521-19

Gonzalez, R. N., Sears, P. M., Merrill, R. A., & Hayes, G. L. (1992). Mastitis due to Mycoplasma in the state of New York during the period 1972-1990. *Cornell Vet*, *82*(1), 29-40.

González, R. N., & Wilson, D. J. (2003). Mycoplasmal mastitis in dairy herds. *Vet Clin North Am Food Anim Pract*, *19*(1), 199-221. https://doi.org/10.1016/s0749-0720(02)00076-2

Guo, Y., Zhu, H., Wang, J., Huang, J., Khan, F. A., Zhang, J., Guo, A., & Chen, X. (2017). TrmFO, a Fibronectin-Binding Adhesin of *Mycoplasma bovis*. *International Journal of Molecular Sciences*, *18*(8), 1732. https://www.mdpi.com/1422-0067/18/8/1732

Haapala, V., Pohjanvirta, T., Vähänikkilä, N., Halkilahti, J., Simonen, H., Pelkonen, S., Soveri, T., Simojoki, H., & Autio, T. (2018). Semen as a source of *Mycoplasma bovis* mastitis in dairy herds. *Vet Microbiol*, *216*, 60-66. https://doi.org/10.1016/j.vetmic.2018.02.005

Haapala, V., Vähänikkilä, N., Kulkas, L., Tuunainen, E., Pohjanvirta, T., Autio, T., Pelkonen, S., Soveri, T., & Simojoki, H. (2021). *Mycoplasma bovis* infection in dairy herds-Risk factors and effect of control measures. *J Dairy Sci*, *104*(2), 2254-2265. https://doi.org/10.3168/jds.2020-18814

Hale, H. H., Helmboldt, C. F., Plastridge, W. N., & Stula, E. F. (1962). Bovine mastitis caused by a *Mycoplasma* species. *Cornell Vet*, *52*, 582-591. https://www.ncbi.nlm.nih.gov/pubmed/13952069

Hanford, H. E., Von Dwingelo, J., & Abu Kwaik, Y. (2021). Bacterial nucleomodulins: A coevolutionary adaptation to the eukaryotic command center. *PLoS pathogens*, *17*(1), e1009184. https://doi.org/10.1371/journal.ppat.1009184

Hazelton, M., Morton, J., Parker, A., Sheehy, P., Bosward, K., Malmo, J., & House, J. (2020). Whole dairy herd sampling to detect subclinical intramammary *Mycoplasma bovis* infection after clinical mastitis outbreaks. *Veterinary Microbiology*, *244*, 108662.

Hazelton, M. S., Morton, J. M., Parker, A. M., Bosward, K. L., Sheehy, P. A., Dwyer, C. J., Niven, P. G., & House, J. K. (2020). *Mycoplasma bovis* and other Mollicutes in replacement dairy heifers from Mycoplasma bovis-infected and uninfected herds: A 2-year longitudinal study. *Journal of Dairy Science*, *103*(12), 11844-11856. https://doi.org/https://doi.org/10.3168/jds.2020-18921

Hermeyer, K., Peters, M., Brügmann, M., Jacobsen, B., & Hewicker-Trautwein, M. (2012). Demonstration of *Mycoplasma bovis* by immunohistochemistry and in situ hybridization in an aborted bovine fetus and neonatal calf. *J Vet Diagn Invest*, *24*(2), 364-369. https://doi.org/10.1177/1040638711435145

Higa, Y., Uemura, R., Yamazaki, W., Goto, S., Goto, Y., & Sueyoshi, M. (2016). An improved loop-mediated isothermal amplification assay for the detection of *Mycoplasma bovis*. *J Vet Med Sci*, *78*(8), 1343-1346. https://doi.org/10.1292/jvms.15-0459

Holland, R. D., Wilkes, J. G., Rafii, F., Sutherland, J. B., Persons, C. C., Voorhees, K. J., & Lay, J. O., Jr. (1996). Rapid identification of intact whole bacteria based on spectral patterns using matrix-assisted laser desorption/ionization with time-of-flight mass spectrometry.

Rapid Commun Mass Spectrom, *10*(10), 1227-1232. https://doi.org/10.1002/(sici)1097-0231(19960731)10:10<1227::Aid-rcm659>3.0.Co;2-6

Hotzel, H., Demuth, B., Sachse, K., Pflitsch, A., & Pfützner, H. (1993). Detection of *Mycoplasma bovis* using *in vitro* deoxyribonucleic acid amplification. *Rev Sci Tech*, *12*(2), 581-591. https://doi.org/10.20506/rst.12.2.700

Howard, C. J., & Taylor, G. (1983). Interaction of mycoplasmas and phagocytes. *Yale J Biol Med*, *56*(5-6), 643-648.

Huang, J., Zhu, H., Wang, J., Guo, Y., Zhi, Y., Wei, H., Li, H., Guo, A., Liu, D., & Chen, X. (2019). Fructose-1,6-bisphosphate aldolase is involved in *Mycoplasma bovis* colonization as a fibronectin-binding adhesin. *Research in Veterinary Science*, *124*, 70-78. https://doi.org/https://doi.org/10.1016/j.rvsc.2019.02.010

Hurri, E., Ohlson, A., Lundberg, Å., Aspán, A., Pedersen, K., & Tråvén, M. (2022). Herd-level prevalence of *Mycoplasma bovis* in Swedish dairy herds determined by antibody ELISA and PCR on bulk tank milk and herd characteristics associated with seropositivity. *J Dairy Sci*, *105*(9), 7764-7772. https://doi.org/10.3168/jds.2021-21390

Josi, C., Bürki, S., Stojiljkovic, A., Wellnitz, O., Stoffel, M. H., & Pilo, P. (2018). Bovine Epithelial *in vitro* Infection Models for *Mycoplasma bovis*. *Front Cell Infect Microbiol*, *8*, 329. https://doi.org/10.3389/fcimb.2018.00329

Justice-Allen, A., Trujillo, J., Corbett, R., Harding, R., Goodell, G., & Wilson, D. (2010). Survival and replication of *Mycoplasma* species in recycled bedding sand and association with mastitis on dairy farms in Utah. *J Dairy Sci*, *93*(1), 192-202. https://doi.org/10.3168/jds.2009-2474

Justice-Allen, A., Trujillo, J., Goodell, G., & Wilson, D. (2011). Detection of multiple *Mycoplasma* species in bulk tank milk samples using real-time PCR and conventional culture and comparison of test sensitivities. *J Dairy Sci*, *94*(7), 3411-3419. https://doi.org/10.3168/jds.2010-3940

Kanci, A., Wawegama, N. K., Marenda, M. S., Mansell, P. D., Browning, G. F., & Markham, P. F. (2017). Reproduction of respiratory mycoplasmosis in calves by exposure to an aerosolised culture of *Mycoplasma bovis*. *Vet Microbiol*, *210*, 167-173. https://doi.org/10.1016/j.vetmic.2017.09.013

Kanda, T., Tanaka, S., Suwanruengsri, M., Sukmawinata, E., Uemura, R., Yamaguchi, R., & Sueyoshi, M. (2019). Bovine Endocarditis Associated with *Mycoplasma bovis*. *J Comp Pathol*, *171*, 53-58. https://doi.org/10.1016/j.jcpa.2019.07.003

Kauf, A. C., Rosenbusch, R. F., Paape, M. J., & Bannerman, D. D. (2007). Innate immune response to intramammary *Mycoplasma bovis* infection. *J Dairy Sci*, *90*(7), 3336-3348. https://doi.org/10.3168/jds.2007-0058

Khan, L. A., Miles, R. J., & Nicholas, R. A. (2005). Hydrogen peroxide production by *Mycoplasma bovis* and *Mycoplasma agalactiae* and effect of *in vitro* passage on a *Mycoplasma bovis* strain producing high levels of H2O2. *Vet Res Commun*, *29*(3), 181-188. https://doi.org/10.1023/b:verc.0000047506.04096.06

Klein, U., de Jong, A., Youala, M., El Garch, F., Stevenin, C., Moyaert, H., Rose, M., Catania, S., Gyuranecz, M., Pridmore, A., & Ayling, R. D. (2019). New antimicrobial susceptibility data from monitoring of *Mycoplasma bovis* isolated in Europe. *Vet Microbiol*, *238*, 108432. https://doi.org/10.1016/j.vetmic.2019.108432

Krause, D. C., Leith, D. K., Wilson, R. M., & Baseman, J. B. (1982). Identification of *Mycoplasma pneumoniae* proteins associated with hemadsorption and virulence. *Infect Immun*, *35*(3), 809-817. https://doi.org/10.1128/iai.35.3.809-817.1982

Lerner, U., Amram, E., Ayling, R. D., Mikula, I., Gerchman, I., Harrus, S., Teff, D., Yogev, D., & Lysnyansky, I. (2014). Acquired resistance to the 16-membered macrolides tylosin and tilmicosin by *Mycoplasma bovis*. *Vet Microbiol*, *168*(2-4), 365-371. https://doi.org/10.1016/j.vetmic.2013.11.033

Li, Y., Zheng, H., Liu, Y., Jiang, Y., Xin, J., Chen, W., & Song, Z. (2011). The complete genome sequence of *Mycoplasma bovis* strain Hubei-1. *PLoS One*, *6*(6), e20999. https://doi.org/10.1371/journal.pone.0020999

Liapi, M., Botsaris, G., Arsenoglou, C., Markantonis, N., Michael, C., Antoniou, A., & Pipis, C. (2021). Rapid Detection of *Mycoplasma bovis*, *Staphylococcus aureus* and *Streptococcus agalactiae* in Cattle Bulk Tank Milk in Cyprus and Relations with Somatic Cell Counts. *Pathogens*, *10*(7). https://doi.org/10.3390/pathogens10070841

Liu, Y., Deng, Z., Xu, S., Liu, G., Lin, Y., Khan, S., Gao, J., Qu, W., Kastelic, J. P., & Han, B. (2021). *Mycoplasma bovis* subverts autophagy to promote intracellular replication in

bovine mammary epithelial cells cultured *in vitro*. *Vet Res*, *52*(1), 130. https://doi.org/10.1186/s13567-021-01002-z

Liu, Y., Xu, S., Li, M., Zhou, M., Huo, W., Gao, J., Liu, G., Kastelic, J. P., & Han, B. (2020). Molecular characteristics and antibiotic susceptibility profiles of *Mycoplasma bovis* associated with mastitis on dairy farms in China. *Prev Vet Med*, *182*, 105106. https://doi.org/10.1016/j.prevetmed.2020.105106

Liu, Y., Zhou, M., Xu, S., Khan, M. A., Shi, Y., Qu, W., Gao, J., Liu, G., Kastelic, J. P., & Han, B. (2020). *Mycoplasma bovis*-generated reactive oxygen species and induced apoptosis in bovine mammary epithelial cell cultures. *J Dairy Sci*, *103*(11), 10429-10445. https://doi.org/10.3168/jds.2020-18599

Lysnyansky, I., & Ayling, R. D. (2016). *Mycoplasma bovis*: Mechanisms of Resistance and Trends in Antimicrobial Susceptibility. *Front Microbiol*, *7*, 595. https://doi.org/10.3389/fmicb.2016.00595

Lysnyansky, I., Mikula, I., Gerchman, I., & Levisohn, S. (2009). Rapid detection of a point mutation in the parC gene associated with decreased susceptibility to fluoroquinolones in Mycoplasma bovis. *Antimicrob Agents Chemother*, *53*(11), 4911-4914. https://doi.org/10.1128/aac.00703-09

Lysnyansky, I., Rosengarten, R., & Yogev, D. (1996). Phenotypic switching of variable surface lipoproteins in *Mycoplasma bovis* involves high-frequency chromosomal rearrangements. *J Bacteriol*, *178*(18), 5395-5401. https://doi.org/10.1128/jb.178.18.5395-5401.1996

Maina, T., Prysliak, T., & Perez-Casal, J. (2019). *Mycoplasma bovis* delay in apoptosis of macrophages is accompanied by increased expression of anti-apoptotic genes, reduced cytochrome C translocation and inhibition of DNA fragmentation. *Vet Immunol Immunopathol*, *208*, 16-24. https://doi.org/10.1016/j.vetimm.2018.12.004

Mason, P. A., & Cox, L. S. (2012). The role of DNA exonucleases in protecting genome stability and their impact on ageing. *Age (Dordr)*, *34*(6), 1317-1340. https://doi.org/10.1007/s11357-011-9306-5

McAloon, C. I., McAloon, C. G., Tratalos, J., O'Grady, L., McGrath, G., Guelbenzu, M., Graham, D. A., O'Keeffe, K., Barrett, D. J., & More, S. J. (2022). Seroprevalence of *Mycoplasma bovis* in bulk milk samples in Irish dairy herds and risk factors associated

with herd seropositive status. *Journal of Dairy Science*, *105*(6), 5410-5419. https://doi.org/10.3168/jds.2021-21334

McAuliffe, L., Ellis, R. J., Miles, K., Ayling, R. D., & Nicholas, R. A. J. (2006). Biofilm formation by mycoplasma species and its role in environmental persistence and survival. *Microbiology*, *152*(4), 913-922. https://doi.org/10.1099/mic.0.28604-0

McAuliffe, L., Ellis, R. J., Miles, K., Ayling, R. D., & Nicholas, R. A. J. (2006). Biofilm formation by mycoplasma species and its role in environmental persistence and survival. *Microbiology (Reading)*, *152*(Pt 4), 913-922. https://doi.org/10.1099/mic.0.28604-0

McDaniel, A. J., & Derscheid, R. J. (2021). MALDI-TOF mass spectrometry and high-resolution melting PCR for the identification of *Mycoplasma bovis* isolates. *BMC Vet Res*, *17*(1), 170. https://doi.org/10.1186/s12917-021-02870-5

Minion, F. C., Jarvill-Taylor, K. J., Billings, D. E., & Tigges, E. (1993). Membrane-associated nuclease activities in mycoplasmas. *J Bacteriol*, *175*(24), 7842-7847. https://doi.org/10.1128/jb.175.24.7842-7847.1993

Mitiku, F., Hartley, C. A., Sansom, F. M., Coombe, J. E., Mansell, P. D., Beggs, D. S., & Browning, G. F. (2018). The major membrane nuclease MnuA degrades neutrophil extracellular traps induced by *Mycoplasma bovis*. *Vet Microbiol*, *218*, 13-19. https://doi.org/10.1016/j.vetmic.2018.03.002

Mulongo, M., Prysliak, T., & Perez-Casal, J. (2013). Vaccination of feedlot cattle with extracts and membrane fractions from two *Mycoplasma bovis* isolates results in strong humoral immune responses but does not protect against an experimental challenge. *Vaccine*, *31*(10), 1406-1412. https://doi.org/10.1016/j.vaccine.2012.12.055

Nicholas, R., Ayling, R., & McAuliffe, L. (2008). *Mycoplasma diseases of ruminants*. CABI.

Nicholas, R. A. (2011). Bovine mycoplasmosis: silent and deadly. *Vet Rec*, *168*(17), 459-462. https://doi.org/10.1136/vr.d2468

Nicholas, R. A., & Ayling, R. D. (2003). *Mycoplasma bovis*: disease, diagnosis, and control. *Res Vet Sci*, *74*(2), 105-112. https://doi.org/10.1016/s0034-5288(02)00155-8

Nicholas, R. A., Fox, L. K., & Lysnyansky, I. (2016). Mycoplasma mastitis in cattle: To cull or not to cull. *Vet J*, *216*, 142-147. https://doi.org/10.1016/j.tvjl.2016.08.001

NMC. (2005). *The cost of mastitis: Dairy insight research 2005/2006: Final report.* (National Mastitis Advisory Committee, Issue.

Otter, A., Wright, T., Leonard, D., Richardson, M., & Ayling, R. (2015). *Mycoplasma bovis* mastitis in dry dairy cows. *Vet Rec*, *177*(23), 601-602. https://doi.org/10.1136/vr.h6663

Pansri, P., Katholm, J., Krogh, K. M., Aagaard, A. K., Schmidt, L. M. B., Kudirkiene, E., Larsen, L. E., & Olsen, J. E. (2020). Evaluation of novel multiplex qPCR assays for diagnosis of pathogens associated with the bovine respiratory disease complex. *Vet J*, *256*, 105425. https://doi.org/10.1016/j.tvjl.2020.105425

Pardon, B. (2020). Mastitis Pathogens Revisited: *Mycoplasma* spp. NMC Annual Meeting Proceedings (2020),

Parker, A. M., House, J. K., Hazelton, M. S., Bosward, K. L., Mohler, V. L., Maunsell, F. P., & Sheehy, P. A. (2016). Milk acidification to control the growth of *Mycoplasma bovis* and Salmonella Dublin in contaminated milk. *J Dairy Sci*, *99*(12), 9875-9884. https://doi.org/10.3168/jds.2016-11537

Parker, A. M., House, J. K., Hazelton, M. S., Bosward, K. L., Morton, J. M., & Sheehy, P. A. (2017). Bulk tank milk antibody ELISA as a biosecurity tool for detecting dairy herds with past exposure to *Mycoplasma bovis*. *J Dairy Sci*, *100*(10), 8296-8309. https://doi.org/10.3168/jds.2016-12468

Parker, A. M., House, J. K., Hazelton, M. S., Bosward, K. L., & Sheehy, P. A. (2017). Comparison of culture and a multiplex probe PCR for identifying *Mycoplasma* species in bovine milk, semen and swab samples. *PLoS One*, *12*(3), e0173422. https://doi.org/10.1371/journal.pone.0173422

Parker, A. M., Sheehy, P. A., Hazelton, M. S., Bosward, K. L., & House, J. K. (2018). A review of mycoplasma diagnostics in cattle. *J Vet Intern Med*, *32*(3), 1241-1252. https://doi.org/10.1111/jvim.15135

Penterman, P. M., Holzhauer, M., van Engelen, E., Smits, D., & Velthuis, A. G. J. (2022). Dynamics of *Mycoplasma bovis* in Dutch dairy herds during acute clinical outbreaks. *Vet J*, *283-284*, 105841. https://doi.org/10.1016/j.tvjl.2022.105841

Pereyre, S., Tardy, F., Renaudin, H., Cauvin, E., Del Pra Netto Machado, L., Tricot, A., Benoit, F., Treilles, M., & Bebear, C. (2013). Identification and subtyping of clinically relevant human and ruminant mycoplasmas by use of matrix-assisted laser desorption ionization-time of flight mass spectrometry. *J Clin Microbiol*, *51*(10), 3314-3323. https://doi.org/10.1128/JCM.01573-13

Petersen, M. B., Krogh, K., & Nielsen, L. R. (2016). Factors associated with variation in bulk tank milk *Mycoplasma bovis* antibody-ELISA results in dairy herds. *J Dairy Sci*, *99*(5), 3815-3823. https://doi.org/10.3168/jds.2015-10056

Petersen, M. B., Wawegama, N. K., Denwood, M., Markham, P. F., Browning, G. F., & Nielsen, L. R. (2018). *Mycoplasma bovis* antibody dynamics in naturally exposed dairy calves according to two diagnostic tests. *BMC Vet Res*, *14*(1), 258. https://doi.org/10.1186/s12917-018-1574-1

Prysliak, T., Maina, T., Yu, L., Suleman, M., Jimbo, S., & Perez-Casal, J. (2017). Induction of a balanced IgG1/IgG2 immune response to an experimental challenge with *Mycoplasma bovis* antigens following a vaccine composed of Emulsigen™, IDR peptide1002, and poly I:C. *Vaccine*, *35*(48 Pt B), 6604-6610. https://doi.org/10.1016/j.vaccine.2017.10.037

Prysliak, T., van der Merwe, J., & Perez-Casal, J. (2013). Vaccination with recombinant *Mycoplasma bovis* GAPDH results in a strong humoral immune response but does not protect feedlot cattle from an experimental challenge with *M. bovis*. *Microb Pathog*, *55*, 1-8. https://doi.org/10.1016/j.micpath.2012.12.001

Punyapornwithaya, V., Fox, L. K., Gay, G. M., Hancock, D. D., & Alldredge, J. R. (2009). Short communication: The effect of centrifugation and resuspension on the recovery of *Mycoplasma* species from milk. *J Dairy Sci*, *92*(9), 4444-4447. https://doi.org/10.3168/jds.2009-2182

Punyapornwithaya, V., Fox, L. K., Hancock, D. D., Gay, J. M., & Alldredge, J. R. (2010). Association between an outbreak strain causing *Mycoplasma bovis* mastitis and its asymptomatic carriage in the herd: a case study from Idaho, USA. *Prev Vet Med*, *93*(1), 66-70. https://doi.org/10.1016/j.prevetmed.2009.08.008

Quinn, P. J., Markey, B. K., Leonard, F. C., Hartigan, P., Fanning, S., & Fitzpatrick, E. (2011). *Veterinary microbiology and microbial disease*. John Wiley & Sons.

Randall, L. P., Lemma, F., Koylass, M., Rogers, J., Ayling, R. D., Worth, D., Klita, M., Steventon, A., Line, K., Wragg, P., Muchowski, J., Kostrzewa, M., & Whatmore, A. M. (2015). Evaluation of MALDI-ToF as a method for the identification of bacteria in the veterinary diagnostic laboratory. *Research in Veterinary Science*, *101*, 42-49. https://doi.org/https://doi.org/10.1016/j.rvsc.2015.05.018

Razin, S., Yogev, D., & Naot, Y. (1998). Molecular biology and pathogenicity of mycoplasmas. *Microbiol Mol Biol Rev*, *62*(4), 1094-1156. https://doi.org/10.1128/MMBR.62.4.1094-1156.1998

Rottem, S. (2003). Interaction of mycoplasmas with host cells. *Physiol Rev*, *83*(2), 417-432. https://doi.org/10.1152/physrev.00030.2002

Sachse, K., Pfützner, H., Hotzel, H., Demuth, B., Heller, M., & Berthold, E. (1993). Comparison of various diagnostic methods for the detection of *Mycoplasma bovis*. *Rev Sci Tech*, *12*(2), 571-580. https://doi.org/10.20506/rst.12.2.701

Sachse, K., Salam, H. S., Diller, R., Schubert, E., Hoffmann, B., & Hotzel, H. (2010). Use of a novel real-time PCR technique to monitor and quantitate *Mycoplasma bovis* infection in cattle herds with mastitis and respiratory disease. *Vet J*, *186*(3), 299-303. https://doi.org/10.1016/j.tvjl.2009.10.008

Salgadu, A., Firestone, S. M., Watt, A., Thilakarathne, D. S., Condello, A. K., Siu, D., Masukagami, Y., Tivendale, K. A., Stevenson, M. A., Mansell, P. D., Browning, G. F., & Wawegama, N. K. (2022). Evaluation of the MilA ELISA for the diagnosis of herd infection with *Mycoplasma bovis* using bulk tank milk and estimation of the prevalence of *M. bovis* in Australia. *Vet Microbiol*, *270*, 109454. https://doi.org/10.1016/j.vetmic.2022.109454

Schneider, P., Brill, R., Schouten, I., Nissim-Eliraz, E., Lysnyansky, I., & Shpigel, N. Y. (2022). Lipoproteins Are Potent Activators of Nuclear Factor Kappa B in Mammary Epithelial Cells and Virulence Factors in *Mycoplasma bovis* Mastitis. *Microorganisms*, *10*(11). https://doi.org/10.3390/microorganisms10112209

Sharma, S., Tivendale, K. A., Markham, P. F., & Browning, G. F. (2015). Disruption of the membrane nuclease gene (MBOVPG45_0215) of *Mycoplasma bovis* greatly reduces cellular nuclease activity. *J Bacteriol*, *197*(9), 1549-1558. https://doi.org/10.1128/jb.00034-15

Shitamori, F., Uemura, R., Kanda, T., & Sueyoshi, M. (2022). The presence of adhesion factors NOX, α-enolase, TrmFO, P27, and VpmaX in *Mycoplasma bovis* wild isolates in Japan. *Open Vet J*, *12*(6), 782-786. https://doi.org/10.5455/OVJ.2022.v12.i6.1

Singhal, N., Kumar, M., Kanaujia, P. K., & Virdi, J. S. (2015). MALDI-TOF mass spectrometry: an emerging technology for microbial identification and diagnosis [Review]. *Frontiers in Microbiology*, *6*. https://doi.org/10.3389/fmicb.2015.00791

Soehnlen, M. K., Aydin, A., Lengerich, E. J., Houser, B. A., Fenton, G. D., Lysczek, H. R., Burns, C. M., Byler, L. I., Hattel, A. L., Wolfgang, D. R., & Jayarao, B. M. (2011). Blinded, controlled field trial of two commercially available *Mycoplasma bovis* bacterin vaccines in veal calves. *Vaccine*, *29*(33), 5347-5354. https://doi.org/https://doi.org/10.1016/j.vaccine.2011.05.092

Soehnlen, M. K., Tran, M. A., Lysczek, H. R., Wolfgang, D. R., & Jayarao, B. M. (2011). Identification of novel small molecule antimicrobials targeting *Mycoplasma bovis*. *J Antimicrob Chemother*, *66*(3), 574-577. https://doi.org/10.1093/jac/dkq503

Soroka, M., Wasowicz, B., & Rymaszewska, A. (2021). Loop-Mediated Isothermal Amplification (LAMP): The Better Sibling of PCR? *Cells*, *10*(8). https://doi.org/10.3390/cells10081931

Subramaniam, S., Bergonier, D., Poumarat, F., Capaul, S., Schlatter, Y., Nicolet, J., & Frey, J. (1998). Species identification of Mycoplasma bovis and *Mycoplasma agalactiae* based on the uvrC genes by PCR. *Mol Cell Probes*, *12*(3), 161-169. https://doi.org/10.1006/mcpr.1998.0160

Suleman, M., Cyprian, F. S., Jimbo, S., Maina, T., Prysliak, T., Windeyer, C., & Perez-Casal, J. (2018). *Mycoplasma bovis*-Induced Inhibition of Bovine Peripheral Blood Mononuclear Cell Proliferation Is Ameliorated after Blocking the Immune-Inhibitory Programmed Death 1 Receptor. *Infect Immun*, *86*(3). https://doi.org/10.1128/iai.00921-17

Sun, Z., Fu, P., Wei, K., Zhang, H., Zhang, Y., Xu, J., Jiang, F., Liu, X., Xu, W., & Wu, W. (2014). Identification of novel immunogenic proteins from *Mycoplasma bovis* and establishment of an indirect ELISA based on recombinant E1 beta subunit of the pyruvate dehydrogenase complex. *PLoS One*, *9*(2), e88328. https://doi.org/10.1371/journal.pone.0088328

Suwanruengsri, M., Uemura, R., Kanda, T., Fuke, N., Nueangphuet, P., Pornthummawat, A., Yasuda, M., Hirai, T., & Yamaguchi, R. (2022). Production of granulomas in Mycoplasma bovis infection associated with meningitis-meningoencephalitis,

endocarditis, and pneumonia in cattle. *J Vet Diagn Invest*, *34*(1), 68-76. https://doi.org/10.1177/10406387211053254

Thomas, A., Dizier, I., Linden, A., Mainil, J., Frey, J., & Vilei, E. M. (2004). Conservation of the uvrC gene sequence in *Mycoplasma bovis* and its use in routine PCR diagnosis. *Vet J*, *168*(1), 100-102. https://doi.org/10.1016/j.tvjl.2003.10.006

Timonen, A. A. E., Autio, T., Pohjanvirta, T., Häkkinen, L., Katholm, J., Petersen, A., Mõtus, K., & Kalmus, P. (2020). Dynamics of the within-herd prevalence of *Mycoplasma bovis* intramammary infection in endemically infected dairy herds. *Vet Microbiol*, *242*, 108608. https://doi.org/10.1016/j.vetmic.2020.108608

USDA-APHIS. (2008). *Prevalence of Contagious Mastitis Pathogens on U.S. Dairy Operations*. USA: Department of Agriculture

USDA-APHIS. (2019). *Determining U.S. Milk Quality Using Bulk Tank Somatic Cell Counts, 2018*.

Vähänikkilä, N., Pohjanvirta, T., Haapala, V., Simojoki, H., Soveri, T., Browning, G. F., Pelkonen, S., Wawegama, N. K., & Autio, T. (2019). Characterisation of the course of *Mycoplasma bovis* infection in naturally infected dairy herds. *Vet Microbiol*, *231*, 107-115. https://doi.org/10.1016/j.vetmic.2019.03.007

Valeris-Chacin, R., Powledge, S., McAtee, T., Morley, P. S., & Richeson, J. (2022). *Mycoplasma bovis* is associated with *Mannheimia haemolytica* during acute bovine respiratory disease in feedlot cattle. *Front Microbiol*, *13*, 946792. https://doi.org/10.3389/fmicb.2022.946792

Valsala, R., Rana, R., Remesh, A. T., Thankappan, S., & Behera, S. (2017). Effect of *Mycoplasma bovis* on production of pro-inflammatory cytokines by peripheral blood mononuclear cells. *Adv. Anim. Vet. Sci*, *5*(10), 400-404.

Vanden Bush, T. J., & Rosenbusch, R. F. (2003). Characterization of the immune response to *Mycoplasma bovis* lung infection. *Vet Immunol Immunopathol*, *94*(1-2), 23-33. https://doi.org/10.1016/s0165-2427(03)00056-4

Wawegama, N. K., Browning, G. F., Kanci, A., Marenda, M. S., & Markham, P. F. (2014). Development of a recombinant protein-based enzyme-linked immunosorbent assay for diagnosis of *Mycoplasma bovis* infection in cattle. *Clin Vaccine Immunol*, *21*(2), 196-202. https://doi.org/10.1128/cvi.00670-13

Wilson, D. J., Skirpstunas, R. T., Trujillo, J. D., Cavender, K. B., Bagley, C. V., & Harding, R. L. (2007). Unusual history and initial clinical signs of *Mycoplasma bovis* mastitis and arthritis in first-lactation cows in a closed commercial dairy herd. *J Am Vet Med Assoc, 230*(10), 1519-1523. https://doi.org/10.2460/javma.230.10.1519

Xu, M., Liu, Y., Mayinuer, T., Lin, Y., Wang, Y., Gao, J., Wang, D., Kastelic, J. P., & Han, B. (2022). *Mycoplasma bovis* inhibits autophagy in bovine mammary epithelial cells via a PTEN/PI3K-Akt-mTOR-dependent pathway. *Front Microbiol, 13*, 935547. https://doi.org/10.3389/fmicb.2022.935547

Yang, J., Liu, Y., Lin, C., Yan, R., Li, Z., Chen, Q., Zhang, H., Xu, H., Chen, X., Chen, Y., Guo, A., & Hu, C. (2022). Regularity of Toll-Like Receptors in Bovine Mammary Epithelial Cells Induced by *Mycoplasma bovis*. *Front Vet Sci*, *9*, 846700. https://doi.org/10.3389/fvets.2022.846700

Zhang, H., Zhao, G., Guo, Y., Menghwar, H., Chen, Y., Chen, H., & Guo, A. (2016). *Mycoplasma bovis* MBOV_RS02825 Encodes a Secretory Nuclease Associated with Cytotoxicity. *Int J Mol Sci, 17*(5). https://doi.org/10.3390/ijms17050628

Zhao, G., Lu, D., Wang, S., Zhang, H., Zhu, X., Hao, Z., Dawood, A., Chen, Y., Schieck, E., Hu, C., Chen, X., Yang, L., & Guo, A. (2022). Novel mycoplasma nucleomodulin MbovP475 decreased cell viability by regulating expression of CRYAB and MCF2L2. *Virulence, 13*(1), 1590-1613. https://doi.org/10.1080/21505594.2022.2117762

Zhao, G., Zhang, H., Chen, X., Zhu, X., Guo, Y., He, C., Anwar Khan, F., Chen, Y., Hu, C., Chen, H., & Guo, A. (2017). *Mycoplasma bovis* NADH oxidase functions as both a NADH oxidizing and O(2) reducing enzyme and an adhesin. *Sci Rep, 7*(1), 44. https://doi.org/10.1038/s41598-017-00121-y

Zhu, X., Dong, Y., Baranowski, E., Li, X., Zhao, G., Hao, Z., Zhang, H., Chen, Y., Hu, C., Chen, H., Citti, C., & Guo, A. (2020). Mbov_0503 Encodes a Novel Cytoadhesin that Facilitates *Mycoplasma bovis* Interaction with Tight Junctions. *Microorganisms, 8*(2). https://doi.org/10.3390/microorganisms8020164

Zubair, M., Muhamed, S. A., Khan, F. A., Zhao, G., Menghwar, H., Faisal, M., Zhang, H., Zhu, X., Rasheed, M. A., Chen, Y., Marawan, M. A., Chen, H., & Guo, A. (2020). Identification of 60 secreted proteins for *Mycoplasma bovis* with secretome assay. *Microb Pathog*, *143*, 104135. https://doi.org/10.1016/j.micpath.2020.104135

CHAPTER 2: PREVALENCE AND WHOLE GENOME SEQUENCE ANALYSIS OF *MYCOPLASMA BOVIS* FROM BULK TANK MILK OF DAIRY FARMS IN TENNESSEE, USA

Disclosure

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Authors' contribution

AG performed sample collection and lab work. BDG performed sample collection. *Mycoplasma bovis* isolate from the State of Virginia was kindly provided by DC. TF performed whole genome sequence analysis and drafted associated methods. JB provided fund for whole genome sequencing. OKD initiated and supervised the study. AG prepared the original draft manuscript and all the authors revised, read, and approved the final manuscript.

Abstract

Mycoplasma bovis mastitis is an important disease of dairy cows that causes huge economic losses to dairy farmers due to milk production loss and culling of infected cows. *Mycoplasma bovis* mastitis is responsible for an annual economic loss of more than $100 million in the United States (U.S.). There are no effective control measures and antimicrobial drugs are less effective due to increased resistance among field isolates and intrinsic resistance of *Mycoplasma* species to commonly used beta-lactam antibiotics in dairy farms. Despite the significant effects of *M. bovis* mastitis on dairy farms' productivity, its prevalence in different States in the U.S. including Tennessee is not well known. Similarly, recent studies showed a high prevalence of bovine hemotropic mycoplasmas such as *Mycoplasma wenyonii* and *Candidatus Mycoplasma haemobos* in dairy cattle in the U.S. The prevalence of these hemoplasmas in the bulk tank milk remains unknown.

The objectives of this study were to determine the herd-level prevalence of *M. bovis* intramammary infection (IMI) in dairy farms in Tennessee and determine the genetic diversity and virulence factors of the isolates. Overall, 75 bulk tank milk (BTM) samples were collected from 59 dairy farms located in the Eastern, Middle, and Western regions at different times from 2022 to 2023. The BTM samples were tested for the presence of *M. bovis* by bacterial culture and PCR. Out of 59 farms, 56 are in Tennessee and the remaining 3 farms are in the neighboring states, Georgia (n=2) and Alabama (n=1). From the 56 farms, 3 (5.3%) were positive for *M. bovis* by bacterial culture and 43 (76.7%) were positive by PCR. We conducted whole genome sequencing of 5 *M. bovis* isolates (3 field isolates from Tennessee, 1 from Viriginia and 1 from American Type Culture Collection (ATCC 25523). Pangenome analysis showed clustering of current isolates with isolates from U.S., Israel, and Europe as well as clustering with isolates which cause mastitis rather than strains isolated from cases of other diseases caused by *M. bovis*. From 75 BTM samples tested by qPCR, 42 (56%) and 51 (68%) were positive for *M. wenyonii* and *C. M. haemobos*, respectively.

To our knowledge, this is the first study which fully focused on prevalence of *M. bovis* prevalence in BTM in Tennessee and is the first report of *M. bovis* detection in bulk tank milk in East Tennessee. Future detailed studies involving repeated sampling of individual quarters of

each cow from all culture and PCR-positive farms and further screening of negative farms with a combination of different tests including PCR, culture and enzyme-linked immunosorbent assay (ELISA) is highly recommended to avoid missing positive farms. The widespread presence of *M. bovis* in BTM, detected by PCR, is concerning suggesting the potential extensive spread of the disease. Urgent action is needed to implement enhanced testing of individual cows on farms using repeated sampling and parallel testing with different methods.

Keywords: Bulk tank milk, Hemoplasmas, *Mycoplasma bovis*, mastitis, prevalence and whole genome sequence analysis

2.1. Introduction

Mycoplasma bovis mastitis (MBM) is one of major dairy health problems in North America, Canada, Europe, Australia and recently in New Zealand (Dudek et al., 2020; Jordan et al., 2021; Maunsell et al., 2011; Tardy et al., 2020). In the United States (U.S.), economic losses due to *M. bovis* mastitis are estimated to be more than $100 million annually (Nicholas & Ayling, 2003). Given the limited availability of information on the prevalence of *M. bovis* intramammary infection (IMI) in the U.S., this figure likely underestimates the current realistic losses caused by the disease. The losses are mainly attributed to the reduction in milk yield, veterinary care and more importantly culling of infected cows which can reach as high as 70% of the affected herd (Gonzalez et al., 1992). However, culling does not guarantee early clearance of *M. bovis* from the herd which exacerbate financial losses (Punyapornwithaya et al., 2012).

In the U.S., more than 75% of MBM cases are associated with *M. bovis* although several *Mycoplasma* species (*M. californicum, M. alkalescens, M. canadense, and M. bovigenitalium*) have also been isolated from milk (Gioia et al., 2021; USDA-APHIS, 2003). *M. bovis* causes highly contagious mastitis characterized by involvement of multiple quarters (Bennett & Jasper, 1980), high milk production loss (Al-Farha et al., 2017; Timonen et al., 2017), and more importantly antimicrobial drug resistance (Lysnyansky & Ayling, 2016). Infected quarters show swelling, abnormal milk secretion, and an increase in somatic cell count (SCC) (Fox et al., 2003; Gondaira et al., 2020; Kauf et al., 2007). Infected cows usually remain asymptomatic which allows silent spread of the infection and late antimicrobial treatment is almost always futile. Although hygienic practices during milking are highly recommended, infection sometimes occurs regardless of hygienic status due to non-intramammary transmissions. Colonization of other body sites such as mucosal surfaces of eyes, nasal cavities, ears, and vestibules can occur from mastitis (Punyapornwithaya et al., 2010). Other transmission routes include milk consumption (Aebi et al., 2012; Walz et al., 1997), nose-to-nose contact (Nicholas et al., 2016) and artificial insemination (Haapala et al., 2018).

Hemoplasmas are unculturable mycoplasmas which have tropism for mammalian red blood cells (Messick, 2004). *Mycoplasma wenyonii* and *Candidatus Mycoplasma haemobos* are the major

hemoplasmas which cause morbidity in cattle worldwide, although they are also detected in apparently healthy animals (de Souza Ferreira et al., 2024; Murugesan et al., 2023). The main clinical signs in dairy cattle include severe hemolytic anemia (Genova et al., 2011), reduction in milk yield (Tagawa et al., 2013), and swelling of mammary gland and hind limbs (Smith et al., 1990). To our knowledge, there is no report on the prevalence of these mycoplasmas in bulk tank milk in Tennessee dairy farms.

One of the major constraints to developing effective control measures against *M. bovis* mastitis is its virulence factors and pathogenic mechanisms are poorly understood. Evaluation of its genome for virulence factors and antimicrobial resistance genes is important to determine virulence factors responsible for *M. bovis* IMI. Several techniques have been employed for genetic characterization of *M. bovis.* Traditionally, sequencing of highly conserved genes such as 16S rRNA is used to differentiate *M. bovis* from other mycoplasmas (Mattsson et al., 1994). Labor-intensive methods such as pulsed-field gel electrophoresis have been used to study relatedness among *Mycoplasma* strains (Biddle et al., 2005). Conventional multi-locus sequence typing, which typically utilizes seven housekeeping genes (Register et al., 2015) has limited discriminatory capability since the variation outside the housekeeping genes is overlooked. Whole genome sequencing, however, offers high resolution and large amounts of data in a short time. Furthermore, it utilizes core genomes (larger number of genes/loci) and hence enables robust epidemiological surveillance and comparative genome analysis to determine differences and similarities among different field isolates of *M. bovis* (Kumar et al., 2020; Menghwar et al., 2022; Yair et al., 2020).

To date, there is no specific report regarding the prevalence of *M. bovis* IMI in the State of Tennessee, except the USDA report from the Southeast part of the U.S. including Tennessee that reported a prevalence of 6.6% in 2003 (USDA-APHIS, 2003). The current study was aimed to determine the prevalence of *M. bovis* IMI in Tennessee dairy farms and genetic diversity, virulence factors and antimicrobial resistance genes of the field isolates.

2.2. Materials and methods

Study population

According to the Tennessee Department of Agriculture, there were 140 dairy farms in the State of Tennessee until August 2022 (Fig. A2.1; Table A2.1). Out of the total of 95 counties in the State of Tennessee, 47 counties have at least one dairy farm. Out of the 47 counties, 22, 19, and 6 are in the East, Middle, and West regions of Tennessee, respectively (Fig. A2.1). The sample size was calculated using WIN EPISCOPE (WinEpi ©2006 Working in Epidemiology) (Passchyn et al., 2012; Thrusfield et al., 2001) considering 95% confidence level, 6.6% expected prevalence (USDA-APHIS, 2003) and 5% accepted error which resulted in an adjusted sample size of 57. In total, 75 bulk tank milk (BTM) samples were collected from 59 dairy farms between 2022 and 2023 (Fig. A2.2; Table A2.2). Fifty-six dairy farms are in Tennessee and the remaining 3 farms are in the neighboring states of Georgia (n=2) and Alabama (n=1). However, herd-level prevalence was calculated based on samples from Tennessee only. The initial plan was to collect samples from all the 47 counties but that was practically impossible due to some producers declining to participate in the study. Dairy producers were requested for consent to participate in the study either through phone calls, email, or dairy extension agents.

Isolation and identification of *M. bovis* from milk samples

A 50 mL of BTM was collected into sterile 50 mL Falcon tubes (Thermo Fischer Scientific, Waltham, MA) and transported on ice to a lab in the Animal Science Department, University of Tennessee Institute of Agriculture (UTIA). Samples were centrifuged at 5,000 g for 30 min (Punyapornwithaya et al., 2009) using Beckman high speed centrifuge (Beckman Instruments Inc., Palo Alto, CA). The pellet was resuspended in a sterile 1 mL solution of 1× phosphate-buffered saline (PBS). About 200 µL was inoculated into a 5 mL Friis broth (Kauf et al., 2007; Knudtson et al., 1986) and incubated at 37ºC, 5% CO_2 :95% air incubator for 72 h for enrichment. From the enriched broth culture, 100 µL was plated onto a mycoplasma agar (Hardy Diagnostics, Santa Maria, CA) and incubated at 37ºC in the 5% CO_2:95% air incubator. Agar plates were monitored every 3 days for 12 d for mycoplasma growth. *M. bovis* colonies were

further confirmed using matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) (Bruker Daltonics, Billerica, MA) at the UT College of Veterinary Medicine, Diagnostic Bacteriology and Mycology Lab. MALDI-TOF MS confirmed isolates were inoculated into Friis broth and grown at 37^{o}C in the 5% CO_2:95% air incubator for 5 d. About 1 mL of the culture suspension was mixed with an equal volume of sterile 80% glycerol and stocked at -80^{o}C until further use. One *M. bovis* isolate from Consolidated Lab Services, Knoxville, which was originally isolated from a dairy farm in Virginia, USA, and an ATCC 25523 strain *M. bovis* PG45 were also included in this study.

DNA extraction, PCR and qPCR

Mycoplasma bovis DNA was extracted from a culture grown in the Friis broth using boiling method as described elsewhere with minor modifications (Abd El Tawab et al., 2019). Briefly, 1 mL of 72 h grown culture was centrifuged at 15,000 g for 10 min (Brinkmann Instruments Inc. Westbury, NY). The pellet was washed once in a 1× Tris-Borate EDTA (TBE) buffer at 10,300 g and resuspended in a 100 µL of 1× TBE. The mixture was boiled in a heating block at 100^{o}C for 20 min and cooled at -20^{o}C for 10 min. The mixture was centrifuged at 14,800 g for 10 min and the supernatant was collected and stored at -20^{o}C.

For PCR, the forward (5’ GAA TTT ACG CAA GAG AAT GCT TCA 3’) and reverse (5’ GCA ATG CCT CTT TAT TTG TTT TAC A 3’) primer pair were designed using Clone Manager 9 Professional Edition, (Sci Ed Software LLC, Westminster, Co) to target 125 bp of *uvrC* gene (Acc. No. AF003959.1). PCR was performed in 20 µL total volume reaction containing 10 µL of the 2× master mix (Thermo Fisher Scientific)), 1 µL of 10 µM of each F and R primer (Integrated DNA Technologies, Morrisville, NC). The PCR fragments were analyzed by electrophoresis in 1.3% agarose gel using TBE buffer. PCR products from weak and strong bands were further purified using QIAquick PCR purification kit (Qiagen, Germantown, MD) and sequenced at Eurofins (Eurofins Genomics LLC, Louisville, KY). The DNA sequence was used to perform BLAST search in the NCBI nucleotide database using Geneious software (Geneious Prime® 2024.0.2, Boston, MA) (Fig. 2.1).

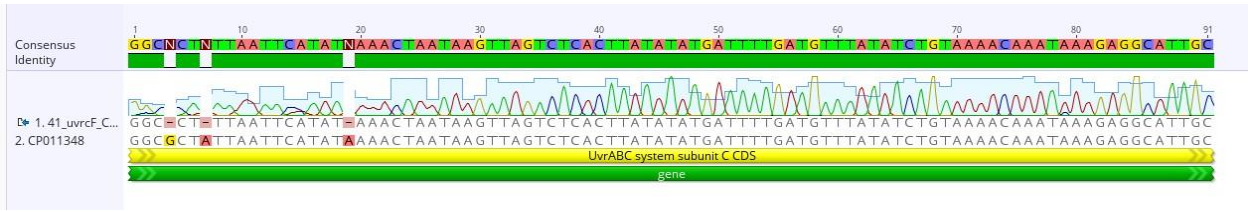

Figure 2.1. Basic Local Alignment Search Tool (BLAST) analysis showed amplification of *uvrC* gene from one of *M. bovis* isolates. (MB41_uvrCF_C: Sanger sequence read; CP011348: GenBank Accession number for *M. bovis*)

Real-time PCR was performed in 20 µL total volume reaction containing 10 µL PowerTrack™ SYBR Green master mix and 0.5 µL sample buffer (Thermo Fischer Scientific), and 1 µL of 10 µM of each primer. For screening of hemotropic mycoplasmas previously used primer pairs (*M. wenyonii*: F 5′-GAAAGTCTGATGGAGCAATA-3′ and R 5'-CCTTTACGCCCAATAAATC-3′ ;*C. M. Haemobos*: F 5′ - ACGAAAGTCTGATGGAGCAATAC-3′ and R 5′-ACGCCCAATAAATCCGRATAATG-3′) were used (de Souza Ferreira et al., 2024).

The primers targeted to amplify certain region of the 16S rRNA gene of their respective organisms and *M. bovis* PG45 genomic DNA was used as control to determine if these primer pairs bind to *M. bovis.* Since hemotropic mycoplasmas are unculturable, we performed *in silico* PCR on *M. wenyonii* (Acc. No. NC_018149) and *C.M. haemobos* (Acc. No. NZ_LWUJ01000000) genomes using uvrC Fand R primer pair (Geneious Prime® 2024.0.2).

We only tested the presence of these Hemoplasmas in bulk tank milk to determine if their presence interfere with the PCR test for *M. bovis*. We did not test their presence in the blood samples from individual dairy cows from these dairy farms since this was out of the scope of our study.

Whole genome sequencing

M. bovis genomic DNA was extracted using MagAttract® HMW DNA kit (Qiagen, Germantown, MD) following manufacturer's instructions with minor modifications. Briefly, 2 mL of *Mycoplasma bovis* culture suspension at about 1.5×10^8 was harvested by centrifugation at 5000 g for 15 min. The pellet was resuspended in buffer ATL with Proteinase K and incubated on shaker incubator (500 RPM) at 56^{o}C for 45 min. Subsequently, 4 µL of RNase, 15 µL MagAttract Suspension G and 280 µL of buffer MB were added and incubated at room temperature for 10 min while shaking. The magnetic beads and 700 µL Buffer MW1 were added to the mixture to separate DNA followed by shaking incubation at room temperature for 5 min. About 700 µL of Buffer PE was added and the mixture was incubated for 5 min while shaking. The tubes were placed on a magnetic base and the magnetic particles were rinsed twice with 700 µL of distilled water. High molecular weight DNA was eluted using 100 µL of buffer AE. The

DNA was further purified using DNA Clean and Concentrator™-5 kit (Zymo Research, Irvine, CA). The quality of the DNA was assessed using Nanodrop™ One Spectrophotometer (Thermo Fisher Scientific) and TapeStation (4200 Tapestation System, Santa Clara, CA). The average 260/230 and 260/280 ratios and DNA integrity number (DIN) of sequenced DNA samples were 2, 1.8 and 9 respectively.

DNA-Seq library was prepared using the automated MagicPrep™ NGS system (TECAN, Redwood City, CA) according to the manufacturer's instructions. Briefly, 200 ng of total DNA was prepared in 40 µL nuclease-free water and mixed thoroughly with 70 µL homogenized magnetic beads. The system requires 15 min of hands-on tasks including sample preparation, loading and retrieval whereas the rest of the workflow such as DNA fragmentation, adaptor ligation, and amplification were fully automated. The final library pool of 861 bp size, 25 ng/µL concentration, and 67 nmol/L molarity was prepared from the 5 samples and submitted to University of Tennessee Genomics Core for sequencing using MiSeq platform.

Genome assembly

Raw reads were trimmed and quality filtered using fastp (v0.23.4) as described elsewhere (Chen, 2023; Chen et al., 2018). Raw and trimmed reads quality was assessed with FastQC (Andrews, 2010). *De novo* assemblies were produced from trimmed reads by SPAdes (v3.15.5) with the '—isolate' argument set (Prjibelski et al., 2020). SPAdes determined optimal k-mer length as 127 for all assemblies. Quality control (QC) for assemblies was performed with QUAST (v5.2.0) for all contigs and scaffolds using *M. bovis* PG45 reference genome (GCF_000183385.1) and annotations (Gurevich et al., 2013). MultiQC was used to summarize QC reports (Ewels et al., 2016).

Pangenome construction and analysis

PPanGGOLiN (v2.0.3) was used for the construction and analysis of *M. bovis* pangenome (Gautreau et al., 2020). The pangenome was constructed from 221 *M. bovis* genome sequences in fasta format: the 5 scaffold-level genomes assembled from *M. bovis* isolates in this study (filtered to remove scaffolds with k-mer coverage less than 100), 8 *M. bovis* assemblies available

through NCBI RefSeq (PG45, GCF_000183385.1; Hubei-1, GCF_000219375.1; HB0801, GCF_000270525.1; CQ-W70, GCF_000696015.1; NM_2012, GCF_001043135.1; 08M, GCF_002009275.1; Ningxia-1, GCF_002749575.1; JF4278, GCF_900088685.1), a panel of 175 *M. bovis* isolates assemblies (PRJNA564939) (Yair et al., 2020), a panel of 32 *M. bovis* isolate assemblies (PRJNA954308) (Triebel et al., 2023), and *M. agalactiae* (PG2) assembly as an outgroup available through NCBI RefSeq (PG2, GCF_000063605.1). The complete workflow is available through the 'ppanggolin all' command with arguments '--translation_table 4 --kingdom bacteria'.

The selection of gene sets of interest based on presence/absence of gene families in specific genomes was performed by a custom R script on the gene presence/absence table produced by PPanGGOLiN. All pangenome protein family sequences were used for functional annotation. These sequences were written from the pangenome using the 'ppanggolin fasta --prot_families all' command. Functional annotation of these protein family sequences was performed using InterProScan (v5.66-98.0) (Blum et al., 2020). All member databases were used, and GO terms were annotated with the '–goterms' argument.

Phylogenetic tree construction

Multiple sequence alignment for the pangenome was used as an input for phylogenetic tree re-construction. This was performed using the 'ppanggolin msa' command with argument '--translation_table 4', which writes the multiple sequence alignment for each strict core gene family (genes present in every genome represented in the pangenome) with duplicate genes removed. Phylogenetic tree reconstruction was performed with IQ-TREE 2 (v2.2.6) (Minh et al., 2020). Model selection was performed using ModelFinder within IQ-TREE (Kalyaanamoorthy et al., 2017) which selected JTT+F+I+R3 as the best-fit model according to BIC. The phylogenetic tree was rooted on the outgroup strain *M. agalactiae* PG2 and visualized using the EMBL Interactive Tree of Life (v6.9) (Letunic & Bork, 2021).

MLST analysis

MLST analysis was performed using PubMLST (https://pubmlst.org/) with the DNA sequences assembled draft genome in FASTA format. Seven loci/genes curated by PubMLST for *M. bovis* typing including *dnaA, gltX, gpsA, gyrB, pta2, tdk,* and *tkt* were used to compare the isolates from this study.

Virulence genes, antimicrobial resistance genes and insertion sequences (ISs)

To identify virulence factors, the DNA sequences of draft genomes assembled in this study were used as a query in virulence factors database (VFDB) (Liu et al., 2022). Similarly, the genomes were used as a query in the comprehensive antibiotic resistance database (CARD) (Alcock et al., 2023) and ISFinder (Siguier et al., 2006) to identify antimicrobial resistance genes and insertion sequences. The ISFinder blastn programme was set at a cutoff E-value of 0.05.

2.3. Results

Culture, PCR, and qPCR

Three out of 56 (5.3%) dairy farms in Tennessee were culture-positive for *M. bovis*. The colonies were recognized by typical fried egg appearance under EVOS M7000 imaging system (Life Technologies Corporation, Bothell, WA) captured at UTIA OMICS Hub Lab in the Plan Biotech Building, Knoxville, TN (Fig. 2.2). Remarkably, 43 of the 56 (76.7%) farms were PCR-positive for *M. bovis.* Out of the 75 BTM samples, 60 (80%) were PCR-positive for *M. bovis* (Fig. 2.3). Based on fried egg colony appearance, 13 samples were tested by MALDI-TOF MS and 3 isolates were identified as *M. bovis* with a high confidence identification score value of 2.17. Other organisms identified by the MALDI-TOF MS include *Enterococcus faecalis* (n=5), *Acholeplasma laidlawii* (n=1), *Pseudomonas patida* (n=1), *Cyberlindnera fabianii* (n=1), *Orchrobactrum* spp. (n=1), and no organism (n=1). The BLAST analysis of the sequenced PCR products showed binding only to *M. bovis* genomes with high (98-100%) percent identity.

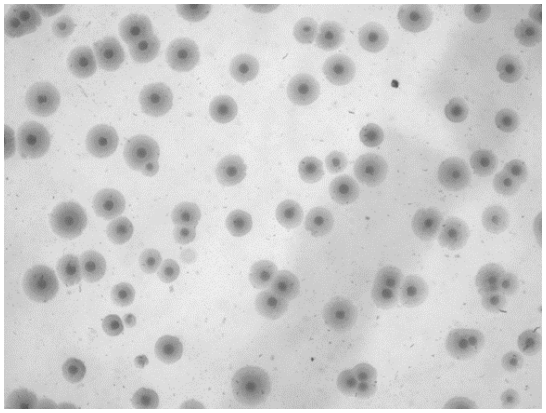

Figure 2.2. Typical 'fried egg appearance' of *M. bovis* isolated in this study (ID No. MB40). (Photo captured by EVOSM7000 Microscope, Invitrogen, Thermo Fisher Scientific).

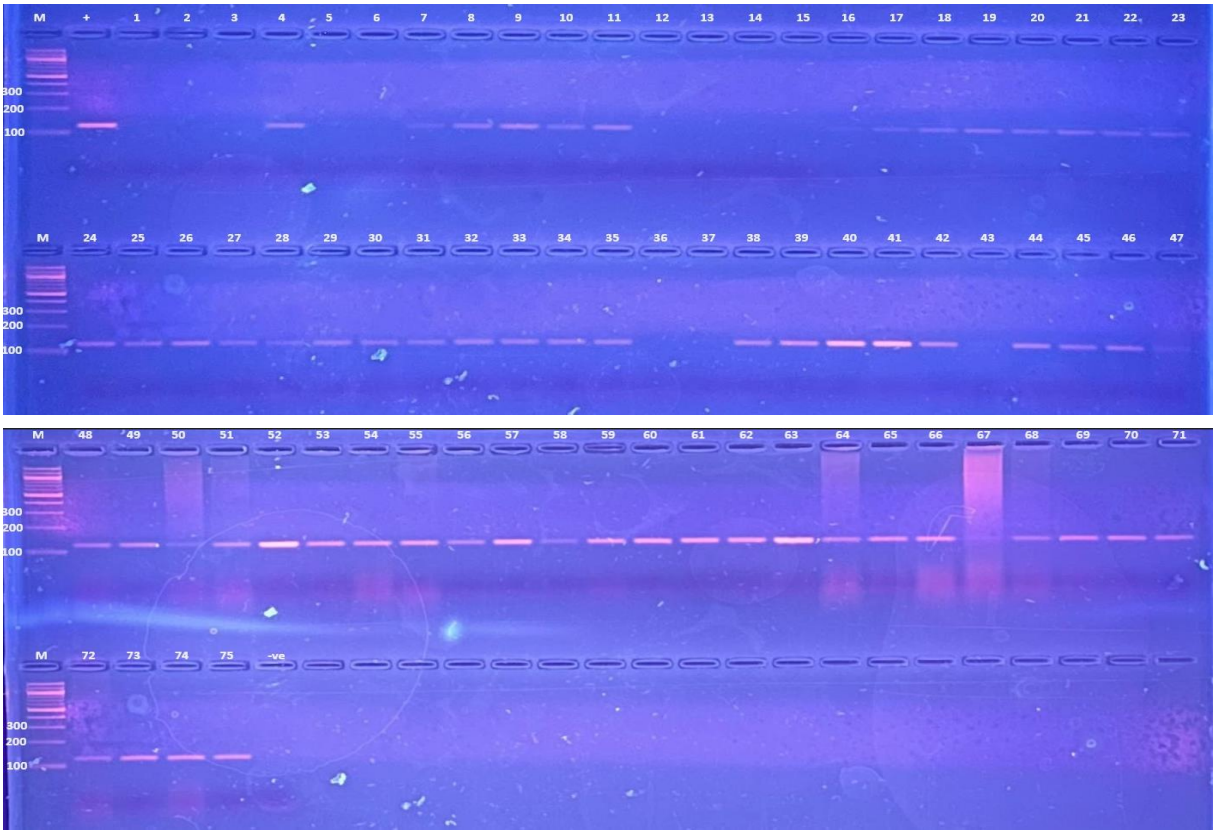

Figure 2.3. PCR amplification of *uvrC* gene (125 bp product) from genomic DNAs of 75 BTM samples. (M: marker, 100 bp DNA ladder visualized by Ethidium Bromide; +: positive control, *M. bovis* PG45; -ve: negative control; 40 = MB40; 41 = MB41; 63 = MB63).

Of the 75 milk samples tested by qPCR, 42 (56%) and 51 (68%) were positive for *M. wenyonii* and *C. M. haemobos*, respectively. *M. wenyonii* and *C. M. haemobos* were co-detected in 40 (53%) samples. *M. bovis*, *M. wenyonii* and *C. M. haemobos* were co-detected in 34 (46.5%) samples whereas *M. bovis* and *C. M. haemobos* were co-detected in 42 (56%) samples. Interestingly, the *C. M. haemobos* primer amplified a region of *M. bovis* PG45 16S rRNA gene but the *M. wenyonii* primer does not amplify *M. bovis*. In addition, pairwise alignment between the 16S rRNA genes of *M. bovis* PG45 and *C. M. haemobos* showed 70% sequence identity.

We found that uvrC F & R primers only bind 118, 000 and 311, 000 bases apart from each other to *M. wenyonii* and *C. M. haemobos*, respectively, to certain hypothetical proteins with only up to 75% identity which shows that it is a random binding.

Genome assembly

MiSeq sequencing produced an average of 6,712,946 reads from 5 samples with an average GC content of 29%. The average genome size from the 5 assemblies was 0.9 Mbp. The average number of contigs identified from the assemblies in this study was 370 whereas the average number of protein-coding genes identified in the assemblies in this study was 773, which is consistent with the overall average number of genes (791) identified from all the isolates (n=221) used in the analysis.

Pangenome and MLST analysis

We constructed a phylogenetic tree based on core gene sets to compare genetic relatedness among the isolates from this study and their relationship to other *M. bovis* isolates from the global perspective. Three of our isolates (MB40, MB41, and MB63) clustered with isolates that cause mastitis in various countries including the U.S., Israel, Lithuania, and Germany (Fig. 2.4, Clade 4). One of our isolates (MBDFA) also clustered with strains that cause mastitis in the U.S. in a different clade (Fig. 2.4, Clade 12). Furthermore, MB40, MB41, and MB63 clustered with some of the oldest isolates from the U.S. including *M. bovis* PG45 and KS-1 and KS-11 from Israel whereas MBDFA clustered with isolates reported in 2015 or later. In MLST analysis,

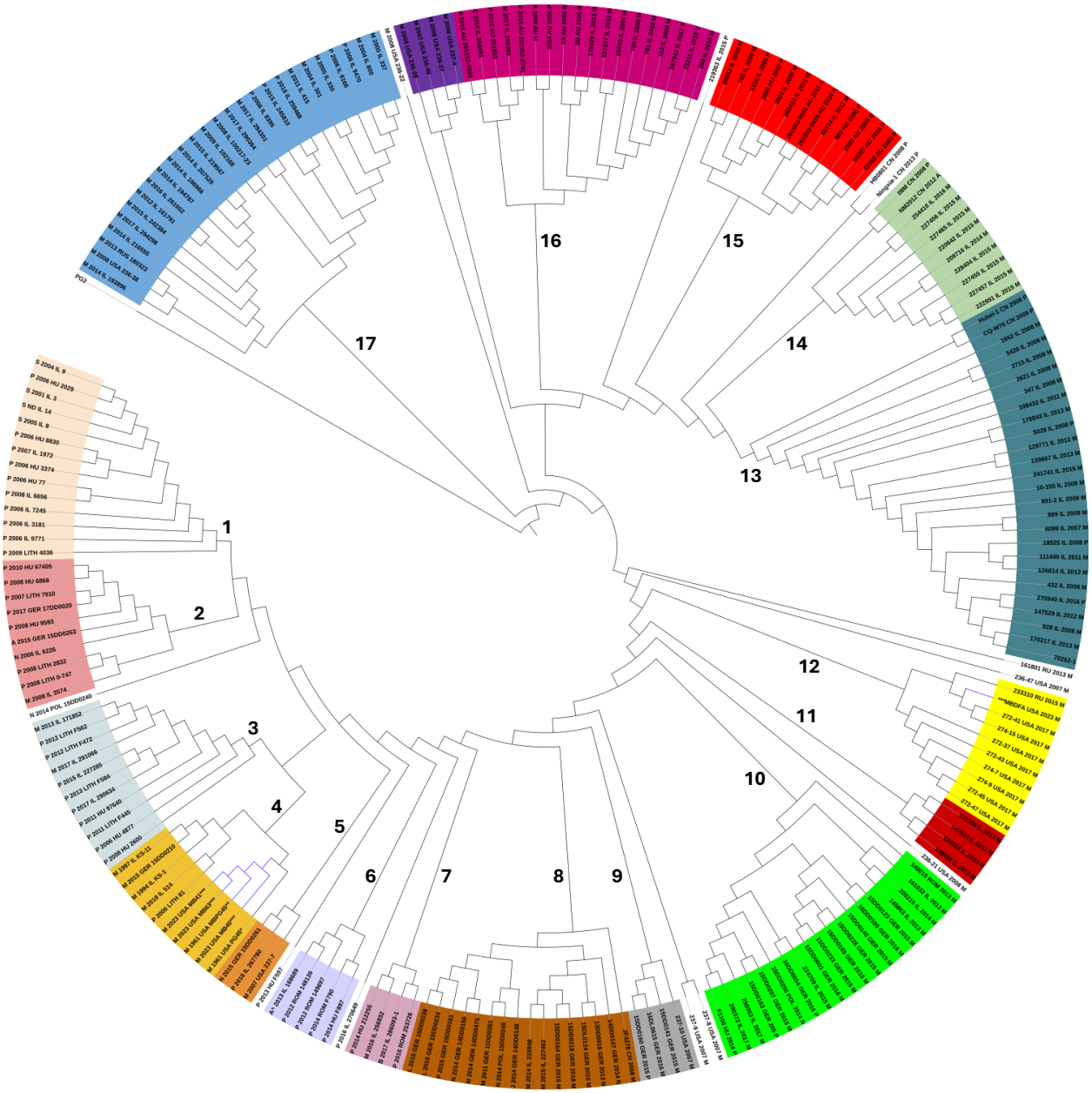

Figure 2.4. Phylogeny of 221 *M. bovis* isolates developed using PPanGGOLiN. The isolates from this study belongs to clade 4 and 12. The branch labels include isolate ID, country, year of isolation and disease. (M: Mastitis; P: Pneumonia; B: Bovine respiratory disease; C: Conjunctivitis; A: Arthritis; A^: Abortion; V: Vulvitis, N: Nasal swab; J: Joint; L: Lung; S: Semen; USA: United States of America; RU: Russia; IL: Israel; AU: Australia; HU: Hungary; CN: China; GER: Germany; LITH: Lithuania; ROM: Romania; POL: Poland; CH: Switzerland; PG2: *Mycoplasma agalactiae*, outgroup; ND: No Data; ***This study; **Positive control; * Reference strain).

MB40, MB41, and MB63 were grouped under sequence type (ST)-12 whereas MBDFA was grouped under ST-45.

Virulence, antimicrobial resistance genes and insertion sequences

Virulence associated multifunctional proteins encoding genes such as elongation factor Tu (EF-Tu), alpha-ketoacid dehydrogenase subunit beta (PdhB), BMP family ABC transporter substrate-binding protein (P48), variable surface lipoproteins (Vpma, P40), Glyceraldehyde 3-phosphate dehydrogenase (GapA/GAPDH), peptide ABC transporter ATP-binding protein (OppF), endonuclease/exonuclease phosphatase family protein (Nuc), which are involved in adherence, invasion, immune modulation, and nuclease activity were identified in all 4 isolates and the *M. bovis* strain PG45 (ATCC 25523). The highest similarity (83 - 96%) and best alignment score (≥200) were observed in EF-Tu, PhdB, and P48 in all isolates. Resistance gene families including EF-Tu, rpsE, gyrB, and gyrA which encode for resistance against elfamaycin, spectinomycin, and fluoroquinolone were identified with up to 71% identity with *M. agalactiae* (PG2) in all 4 isolates and the PG45 ATCC 25523 strain. Antibiotic target modification was the predicted resistance mechanism in all resistance genes identified in this study.

All IS types ISMbov1, 2, 3, 4, 5, 6, 8 and 9, except ISMbov 7, were identified in all isolates (S2). Other IS types from other *Mycoplasma* species such as ISMag1 and ISMmy1 were also identified with a high percent identity.

2.4. Discussion

To our knowledge, this is the first report of *M. bovis* detection in bulk tank milk samples specifically focusing on dairy farms in Tennessee. Current report on the prevalence of *M. bovis* mastitis are limited in the U.S. at large and Tennessee in particular. In 2002, Animal and Plant Health Inspection Services (APHIS) of USDA conducted BTM screening for *Mycoplasma* spp. in selected states from four regions of the U.S. (USDA-APHIS, 2003). In this study, a prevalence of 5.3% was observed using culture. Closely similar to our finding, a prevalence of 6.6% was reported in the Southeast states including Tennessee, Florida, Kentucky, and Virginia using standard culture method by USDA APHIS (USDA-APHIS, 2003). Likewise, another study

which involved 17 states, 1.8%, 4.2% and 14.4% of *Mycoplasma* spp. associated mastitis prevalence was reported in small, medium, and large dairy operations, respectively, using culture (USDA-APHIS, 2008).

In general, low prevalence of *M. bovis* mastitis are disputable due to several reasons. Chronically infected and subclinical cows shed *Mycoplasma* intermittently increasing the possibility of false negative cows if screening was performed only once using culture (Biddle et al., 2003; Parker et al., 2018). In addition, *Mycoplasmas* are the smallest self-replicating (580 – 1,350 Kb) organisms lacking essential synthetic pathways for growth (Razin et al., 1998). This necessitates a special media for growth coupled with long incubation time (7-10 days) (Parker et al., 2018). This time can possibly be extended if pre-enrichment is needed (Thurmond et al., 1989). Therefore, *M. bovis* is commonly not part of regular mastitis testing scheme conducted in dairy operations. Another challenge with *Mycoplasma* culture from milk is the detection limit. Unlike the molecular techniques which only need a trace of *Mycoplasma* DNA, culture requires at least 272 CFU/mL for successful isolation (Sachse et al., 2010). This possibly explains the big difference in prevalence between culture and PCR screening observed in this study. Many studies reported the use of BTM samples to estimate prevalence (Hurri et al., 2022; Justice-Allen et al., 2011; Liapi et al., 2021; McAloon et al., 2022). Positive BTM to *M. bovis* indicates that there is at least one cow in the herd which is shedding the bacterium. However, obtaining a positive result in small sample volume from the large volume milk tank is possibly another limitation.

Two of the *M. bovis* culture positive farms were designated as large dairy operations since they had 500+ heads of dairy cows. It has been reported that *M. bovis* IMI is common in large dairy operations and in herds shipping more milk frequently (Fox et al., 2003; USDA-APHIS, 2003, 2008). The producer of farm B complained about prevalent pneumonia cases among the calves and pink eye cases in the herd. This is consistent with other studies which reported *M. bovis* association with keratoconjunctivitis (Jack et al., 1977; Kirby & Nicholas, 1996; Zheng et al., 2019), and mastitis and pneumonia outbreaks (Aebi et al., 2012).

M. bovis isolation and growth needs meticulous optimization. The centrifugation and resuspension of 50 mL BTM samples into 400 µL PBS increases the likelihood of *M. bovis*

isolation (Punyapornwithaya et al., 2009). It is common that mycoplasmas are overgrown by other bacteria if the agar plates are not used within 2-3 weeks after preparation due to loss of the antibiotic effect. *M. bovis* colony detection on agar plates should be followed by MALDI-TOF MS confirmation since the L-forms of other bacteria usually exhibit the fried-egg appearance. This is also true for other mollicutes such as *Acholeplasma laidlawii*, as one of our BTM sample was MALDI-TOF-positive to *Acholeplasma laidlawii*. Immediate inoculation of milk samples is highly recommended since long refrigeration and thawing reduces recoverability of mycoplasmas which might be a case with culture-negative 38 BTM samples in this study (Biddle et al., 2004).

The high prevalence of hemotropic mycoplasmas in dairy cattle due to *M. wenyonii* and *C. M. haemobos* have been reported in several countries as reviewed by De Souza and Ruegg (De Souza & Ruegg, 2023). Schambow et al. reported 100% prevalence of *M. wenyonii* and *C. M. haemobos* in a dairy herd in Wisconsin and Michigan, USA (Schambow et al., 2021) which is higher than the prevalence reported in this study. This is possibly due to the sample differences which was blood in Schambow et. al. study and these hemoplasmas have tropism towards blood cells. In this study, we showed that *M. wenyonii* and *C. M. haemobos* might shed in the milk as well as milk samples can alternatively be used to detect these hemoplasmas.

The pangenome analysis showed geographical clustering of isolates from this study with isolates from USA, Israel, and Europe in agreement with previous reports. This might be attributed to transmission of the same strain through international cattle trade between these countries or transmission through other routes among these countries (Triebel et al., 2023; Yair et al., 2020). Furthermore, isolates from this study clustered with isolates from cases of mastitis or isolates from milk samples. Based on the genome sequencing and pangenome and MLST analyses and identification of virulence genes, antimicrobial resistance genes, and insertion sequences (IS), isolates from this study tend to have high genomic relatedness.

Several virulence and antibiotic resistance genes were identified by VFDB and CARD blast search. In the complement activation system, the host immune system utilizes plasma glycoprotein factor H to interact with receptors on host cells and inhibit complement attack on

host tissue (Weiler et al., 1976). In an attempt to evade the complement system, microbes simulate the host receptors by expressing surface proteins. Based on VFDB analysis in this study, we found that, virulence associated multifunctional proteins encoding genes such as elongation factor Tu (EF-Tu) (Harvey et al., 2019; Jiang et al., 2016; Yu et al., 2018; Yanfei Yu et al., 2020) and alpha-ketoacid dehydrogenase subunit beta (PdhB) (Byron & Lindsay, 2017; Hegde et al., 2015; Paredes Felipe, 2022) were identified in all isolates. In agreement with our findings, Yu et al. reported *M. hyopneumoniae* expresses elongation factor Tu (EF-Tu) to interact with the factor H and further recounted that EF-Tu is also expressed by *M. bovis* (Y. Yu et al., 2020). This is possibly a contributing factor why *M. bovis* is resistant to the alternative complement pathway (Howard, 1980). Consistent to our finding, it was shown that PdhB of *M. bovis* is highly antigenic (Sun et al., 2014). Similar to EF-Tu, PhdB interacts with factor H which possibly plays role in the complement system evasion (Y. Yu et al., 2020). In *M. gallisepticum,* PdhB is a membrane lipoprotein which binds to the plasminogen of the host cells to facilitate adherence (Akbarzadeh-Niaki et al., 2020; Qi et al., 2018). The BMP family ABC transporter substrate-binding protein (P48) (Lee, 2009; Lysnyansky et al., 2008) was present in all isolates. In agreement with our finding, P48 which is a membrane-bound protein, was reported to be a virulence factor and induced apoptosis in embryonic bovine lung cells (Wu et al., 2021). Variable surface lipoproteins (Vpma, P40) were present in all isolates and are well established virulence factors in *M. bovis* (Chopra-Dewasthaly et al., 2017; Davis, 2003; Hegde et al., 2018; Jingyun Wang et al., 2023). The variable surface proteins (Vsp) of publicly available *M. bovis* PG45 reference strain and isolates from current study had 65% and 55% sequence identity to Vpma of *M. agalactiae,* respectively. *M. agalactiae* Vpma plays key role in fitness and survival in the host and likewise the Vsp in *M. bovis* is responsible for chronic and persistent respiratory infections possibly because of difficulty of clearance by host immune response due to frequent variation or change in epitope (Buchenau et al., 2010; Lysnyansky et al., 1996).

The GapA/GAPDH was one of multifunctional protein (Khan et al., 2016; Prysliak et al., 2013; Rasheed et al., 2017; Xu et al., 2022; Zhao et al., 2017) present in all isolates in this study. Similarly, GAPDH plays virulence role in other mycoplasmas and bacterial species such as *Mycoplasma pneumoniae, Streptococcus pyogenes*, and *Streptococcus agalactiae* (Jin et al., 2011; Madureira et al., 2007; J. Wang et al., 2023). *M. bovis* GAPDH protein has been shown to

induce robust humoral response, however failed to provide protection following experimental challenge (Perez-Casal & Prysliak, 2007; Prysliak et al., 2013). Peptide ABC transporter ATP-binding protein (OppF) (Zhao et al., 2021) was present in all isolates. Similarly, oppF contributes to the pathogenicity of *Mycoplasma synoviae* along with other proteins (Klose et al., 2022).

Endonuclease/exonuclease phosphatase family protein (Nuc), which are involved in adherence, invasion, immune modulation, and nuclease activity were identified in all 4 isolates and the *M. bovis* strain PG45 (ATCC 25523) (Liao et al., 2022; Masukagami et al., 2017; Mitiku et al., 2018; Sharma et al., 2015). The endonuclease/exonuclease/phosphatase family protein (Nuc) had 63% sequence identity to 5'-nucleotidase in this study. Recently, through a combination of genomic and metabolomic analyses, Masukagami et al. identified and annotated a putative 5'-nucleotidase (MBOVPG45_0690) in the genome of *M. bovis* (Masukagami et al., 2017). In our recent work, we found that 5'-nucleotidase play a key role in *M. bovis* PG45 survival and fitness in mice and dairy cows *M. bovis* mastitis models (Unpublished).

The EF-Tu gene encodes a multifunctional protein that involve in translation associated with virulence and resistance. Antimicrobial drugs such as elfamycin, enacyloxin, and kirromycin prevent bacterial protein synthesis by binding to EF-Tu blocking its protein translational activity. The presence of this protein, if in fact responsible for phenotypic resistance in *Mycoplasma* species is interesting. However, the EF-Tu associated predicted resistance requires further in vitro validation of phenotypic resistance.

Insertion sequences are transposable elements which can induce genetic rearrangements, deletions or duplications and play a role in genome plasticity and adaptation (Touchon & Rocha, 2007). ISMbov1, 2, 3, 4, 5, 6, 8 and 9 have previously been identified in *M. bovis* field isolates (Li et al., 2011; Qi et al., 2012). Lysnyansky et al. identified ISMbov7 in *M. bovis* (Lysnyansky et al., 2009), however ISMbov7 was not identified in any of our isolates. Furthermore, the sequence of the ISMbov7 is not available in ISFinder until this dissertation was compiled. ISMag1 of *M. agalactiae* and ISMmy1 of *M. mycoides subsp. mycoides SC* which are closely related to ISMbov1, 2 and 3 (Thomas et al., 2005) were identified with high sequence identity (85-100%) as expected. In our recent work, we found upregulation of ISs in *M. bovis* following

in vitro and in vivo infections which possibly indicates role of these mobile genetic elements in modifying genomes of *M. bovis* (Unpublished).

In conclusion, *M. bovis* IMI is prevalent in Tennessee dairy farms. The isolates which were identified from bulk tank milk in Tennessee dairy farms clustered with isolates from U.S., Israel and Europe that are isolated from mastitis cases. The high *M. bovis* prevalence in BTM by PCR need further detailed study to determine the true status of *M. bovis* IMI. We highly recommend further studies involving individual quarter milk sample testing using a combination of tests including qPCR, culture and ELISA with further confirmation of isolates with MALDI-TOF. Hygienic practice during milking must be strictly followed to limit contagious transmission by contaminated milking machine and milkers' hands during milking. If possible, maintaining closed herd and raising dairy heifers on-farm is recommended. Producers will benefit from milk culture before purchasing dairy cows and immediate isolation of suspected cows from the herd. According to our pangenome and MLST analyses, there are no significant difference among field isolates of *M. bovis* from bulk tank milk samples in Tennessee. Unlike MBDFA, MB40, MB41, and MB63 are genetically closely related. The virulence factors identified in this study can be targeted to develop vaccine-based intervention measures or immunotherapy.

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References

Abd El Tawab, A. A., El-hofy, F. I., Hassan, N. I., & El-khayat, M. E. (2019). Prevalence of *Mycoplasma bovis* in bovine clinical mastitis milk in Egypt. *Benha Veterinary Medical Journal*, *36*(2), 57-65.

Aebi, M., Bodmer, M., Frey, J., & Pilo, P. (2012). Herd-specific strains of *Mycoplasma bovis* in outbreaks of mycoplasmal mastitis and pneumonia. *Vet Microbiol*, *157*(3-4), 363-368. https://doi.org/10.1016/j.vetmic.2012.01.006

Akbarzadeh-Niaki, M., Derakhshandeh, A., Kazemipour, N., Eraghi, V., & Hemmatzadeh, F. (2020). A novel chimeric recombinant protein PDHB-P80 of *Mycoplasma agalactiae* as a potential diagnostic tool. *Mol Biol Res Commun*, *9*(3), 123-128. https://doi.org/10.22099/mbrc.2020.37684.1513

Al-Farha, A. A., Hemmatzadeh, F., Khazandi, M., Hoare, A., & Petrovski, K. (2017). Evaluation of effects of Mycoplasma mastitis on milk composition in dairy cattle from South Australia. *BMC Vet Res*, *13*(1), 351. https://doi.org/10.1186/s12917-017-1274-2

Alcock, B. P., Huynh, W., Chalil, R., Smith, K. W., Raphenya, A. R., Wlodarski, M. A., Edalatmand, A., Petkau, A., Syed, S. A., Tsang, K. K., Baker, S. J. C., Dave, M., McCarthy, M. C., Mukiri, K. M., Nasir, J. A., Golbon, B., Imtiaz, H., Jiang, X., Kaur, K., McArthur, A. G. (2023). CARD 2023: expanded curation, support for machine learning, and resistome prediction at the Comprehensive Antibiotic Resistance Database. *Nucleic Acids Res*, *51*(D1), D690-d699. https://doi.org/10.1093/nar/gkac920

Andrews, S. (2010). FastQC: a quality control tool for high throughput sequence data. In: Babraham Bioinformatics, Babraham Institute, Cambridge, United Kingdom.

Bennett, R. H., & Jasper, D. E. (1980). Bovine mycoplasmal mastitis from intramammary inoculations of small numbers of *Mycoplasma bovis*: local and systemic antibody response. *Am J Vet Res*, *41*(6), 889-892.

Biddle, M. K., Fox, L. K., Evans, M. A., & Gay, C. C. (2005). Pulsed-field gel electrophoresis patterns of Mycoplasma isolates from various body sites in dairy cattle with Mycoplasma mastitis. *J Am Vet Med Assoc*, *227*(3), 455-459. https://doi.org/10.2460/javma.2005.227.455

Biddle, M. K., Fox, L. K., & Hancock, D. D. (2003). Patterns of mycoplasma shedding in the milk of dairy cows with intramammary mycoplasma infection. *J Am Vet Med Assoc*, *223*(8), 1163-1166. https://doi.org/10.2460/javma.2003.223.1163

Biddle, M. K., Fox, L. K., Hancock, D. D., Gaskins, C. T., & Evans, M. A. (2004). Effects of storage time and thawing methods on the recovery of *Mycoplasma* species in milk samples from cows with intramammary infections. *J Dairy Sci*, *87*(4), 933-936. https://doi.org/10.3168/jds.S0022-0302(04)73237-3

Blum, M., Chang, H.-Y., Chuguransky, S., Grego, T., Kandasaamy, S., Mitchell, A., Nuka, G., Paysan-Lafosse, T., Qureshi, M., Raj, S., Richardson, L., Salazar, G. A., Williams, L., Bork, P., Bridge, A., Gough, J., Haft, D. H., Letunic, I., Marchler-Bauer, A., Finn, R. D. (2020). The InterPro protein families and domains database: 20 years on. *Nucleic Acids Research*, *49*(D1), D344-D354. https://doi.org/10.1093/nar/gkaa977

Buchenau, I., Poumarat, F., Le Grand, D., Linkner, H., Rosengarten, R., & Hewicker-Trautwein, M. (2010). Expression of *Mycoplasma bovis* variable surface membrane proteins in the respiratory tract of calves after experimental infection with a clonal variant of *Mycoplasma bovis* type strain PG45. *Res Vet Sci*, *89*(2), 223-229. https://doi.org/10.1016/j.rvsc.2010.03.014

Byron, O., & Lindsay, J. G. (2017). The Pyruvate Dehydrogenase Complex and Related Assemblies in Health and Disease. In J. R. Harris & J. Marles-Wright (Eds.), *Macromolecular Protein Complexes: Structure and Function* (pp. 523-550). Springer International Publishing. https://doi.org/10.1007/978-3-319-46503-6_19

Chen, S. (2023). Ultrafast one-pass FASTQ data preprocessing, quality control, and deduplication using fastp. *iMeta*, *2*(2), e107. https://doi.org/https://doi.org/10.1002/imt2.107

Chen, S., Zhou, Y., Chen, Y., & Gu, J. (2018). fastp: an ultra-fast all-in-one FASTQ preprocessor. *Bioinformatics*, *34*(17), i884-i890. https://doi.org/10.1093/bioinformatics/bty560

Chopra-Dewasthaly, R., Spergser, J., Zimmermann, M., Citti, C., Jechlinger, W., & Rosengarten, R. (2017). Vpma phase variation is important for survival and persistence of *Mycoplasma agalactiae* in the immunocompetent host. *PLoS pathogens*, *13*(9), e1006656. https://doi.org/10.1371/journal.ppat.1006656

Davis, K. L. (2003). *Mycoplasma fermentans MALP -404: A new paradigm for surface variation of mycoplasmas* (Publication Number 3091915) Ph.D., University of Missouri - Columbia. ProQuest Dissertations & Theses Global. United States -- Missouri. https://utk.idm.oclc.org/login?url=https://www.proquest.com/dissertations-theses/i-mycoplasma-fermentans-malp-404-new-paradigm/docview/305308879/se-2?accountid=14766

De Souza Ferreira, L., Bolin, S., Abuelo, A., Norby, B., & Ruegg, P. L. (2024). Apparent prevalence of transplacental transmission of hemotropic mycoplasmas in Holstein dairy calves. *JDS Communications*. https://doi.org/https://doi.org/10.3168/jdsc.2023-0518

De Souza, F. L., & Ruegg, P. L. (2023). Graduate Student Literature Review: Hemotropic mycoplasmas in cattle. *J Dairy Sci*. https://doi.org/10.3168/jds.2023-24120

Dudek, K., Nicholas, R. A. J., Szacawa, E., & Bednarek, D. (2020). *Mycoplasma bovis* Infections-Occurrence, Diagnosis and Control. *Pathogens, 9*(8). https://doi.org/10.3390/pathogens9080640

Ewels, P., Magnusson, M., Lundin, S., & Käller, M. (2016). MultiQC: summarize analysis results for multiple tools and samples in a single report. *Bioinformatics*, *32*(19), 3047-3048. https://doi.org/10.1093/bioinformatics/btw354

Fox, L. K., Hancock, D. D., Mickelson, A., & Britten, A. (2003). Bulk tank milk analysis: factors associated with appearance of *Mycoplasma* sp. in milk. *J Vet Med B Infect Dis Vet Public Health*, *50*(5), 235-240. https://doi.org/10.1046/j.1439-0450.2003.00668.x

Gautreau, G., Bazin, A., Gachet, M., Planel, R., Burlot, L., Dubois, M., Perrin, A., Médigue, C., Calteau, A., Cruveiller, S., Matias, C., Ambroise, C., Rocha, E. P. C., & Vallenet, D. (2020). PPanGGOLiN: Depicting microbial diversity via a partitioned pangenome graph. *PLoS Comput Biol*, *16*(3), e1007732. https://doi.org/10.1371/journal.pcbi.1007732

Genova, S. G., Streeter, R. N., Velguth, K. E., Snider, T. A., Kocan, K. M., & Simpson, K. M. (2011). Severe anemia associated with *Mycoplasma wenyonii* infection in a mature cow. *Can Vet J*, *52*(9), 1018-1021.

Gioia, G., Addis, M. F., Santisteban, C., Gross, B., Nydam, D. V., Sipka, A. S., Virkler, P. D., Watters, R. D., Wieland, M., Zurakowski, M. J., & Moroni, P. (2021). *Mycoplasma* species isolated from bovine milk collected from US dairy herds between 2016 and 2019. *J Dairy Sci*, *104*(4), 4813-4821. https://doi.org/10.3168/jds.2020-19171

Gondaira, S., Nishi, K., Tanaka, T., Yamamoto, T., Nebu, T., Watanabe, R., Konnai, S., Hayashi, T., Kiku, Y., Okamoto, M., Matsuda, K., Koiwa, M., Iwano, H., Nagahata, H., & Higuchi, H. (2020). Immunosuppression in Cows following Intramammary Infusion of *Mycoplasma bovis*. *Infect Immun*, *88*(3). https://doi.org/10.1128/iai.00521-19

Gonzalez, R. N., Sears, P. M., Merrill, R. A., & Hayes, G. L. (1992). Mastitis due to Mycoplasma in the state of New York during the period 1972-1990. *Cornell Vet*, *82*(1), 29-40.

Gurevich, A., Saveliev, V., Vyahhi, N., & Tesler, G. (2013). QUAST: quality assessment tool for genome assemblies. *Bioinformatics*, *29*(8), 1072-1075. https://doi.org/10.1093/bioinformatics/btt086

Haapala, V., Pohjanvirta, T., Vähänikkilä, N., Halkilahti, J., Simonen, H., Pelkonen, S., Soveri, T., Simojoki, H., & Autio, T. (2018). Semen as a source of *Mycoplasma bovis* mastitis in dairy herds. *Vet Microbiol*, *216*, 60-66. https://doi.org/10.1016/j.vetmic.2018.02.005

Harvey, K. L., Jarocki, V. M., Charles, I. G., & Djordjevic, S. P. (2019). The Diverse Functional Roles of Elongation Factor Tu (EF-Tu) in Microbial Pathogenesis [Review]. *Frontiers in Microbiology*, *10*. https://doi.org/10.3389/fmicb.2019.02351

Hegde, S., Rosengarten, R., & Chopra-Dewasthaly, R. (2015). Disruption of the pdhB Pyruvate Dehyrogenase Gene Affects Colony Morphology, In Vitro Growth and Cell Invasiveness of *Mycoplasma agalactiae*. *PLoS ONE*, *10*(3). https://doi.org/doi:10.1371/journal.pone.0119706 PMID: 25799063

Hegde, S., Zimmermann, M., Rosengarten, R., & Chopra-Dewasthaly, R. (2018). Novel role of Vpmas as major adhesins of *Mycoplasma agalactiae* mediating differential cell adhesion and invasion of Vpma expression variants. *Int J Med Microbiol*, *308*(2), 263-270. https://doi.org/10.1016/j.ijmm.2017.11.010

Howard, C. J. (1980). Variation in the susceptibility of bovine mycoplasmas to killing by the alternative complement pathway in bovine serum. *Immunology*, *41*(3), 561-568.

Hurri, E., Ohlson, A., Lundberg, Å., Aspán, A., Pedersen, K., & Tråvén, M. (2022). Herd-level prevalence of *Mycoplasma bovis* in Swedish dairy herds determined by antibody ELISA and PCR on bulk tank milk and herd characteristics associated with seropositivity. *J Dairy Sci*, *105*(9), 7764-7772. https://doi.org/10.3168/jds.2021-21390

Jack, E., Moring, J., & Boughton, E. (1977). Isolation of *Mycoplasma bovis* from an outbreak of infectious bovine kerato conjunctivitis. *The Veterinary record*, *101*(14), 287.

Jiang, F., He, J., Navarro-Alvarez, N., Xu, J., Li, X., Li, P., & Wu, W. (2016). Elongation Factor Tu and Heat Shock Protein 70 Are Membrane-Associated Proteins from *Mycoplasma ovipneumoniae* Capable of Inducing Strong Immune Response in Mice. *PLoS ONE*, *11*(8), e0161170. https://doi.org/10.1371/journal.pone.0161170

Jin, H., Agarwal, S., Agarwal, S., & Pancholi, V. (2011). Surface export of GAPDH/SDH, a glycolytic enzyme, is essential for *Streptococcus pyogenes* virulence. *mBio*, *2*(3), e00068-00011. https://doi.org/10.1128/mBio.00068-11

Jordan, A., Sadler, R. J., Sawford, K., van Andel, M., Ward, M., & Cowled, B. (2021). *Mycoplasma bovis* outbreak in New Zealand cattle: An assessment of transmission trends using surveillance data. *Transbound Emerg Dis*, *68*(6), 3381-3395. https://doi.org/10.1111/tbed.13941

Justice-Allen, A., Trujillo, J., Goodell, G., & Wilson, D. (2011). Detection of multiple *Mycoplasma* species in bulk tank milk samples using real-time PCR and conventional culture and comparison of test sensitivities. *J Dairy Sci*, *94*(7), 3411-3419. https://doi.org/10.3168/jds.2010-3940

Kalyaanamoorthy, S., Minh, B. Q., Wong, T. K. F., von Haeseler, A., & Jermiin, L. S. (2017). ModelFinder: fast model selection for accurate phylogenetic estimates. *Nature Methods*, *14*(6), 587-589. https://doi.org/10.1038/nmeth.4285

Kauf, A. C., Rosenbusch, R. F., Paape, M. J., & Bannerman, D. D. (2007). Innate immune response to intramammary *Mycoplasma bovis* infection. *J Dairy Sci*, *90*(7), 3336-3348. https://doi.org/10.3168/jds.2007-0058

Khan, F. A., Faisal, M., Chao, J., Liu, K., Chen, X., Zhao, G., Menghwar, H., Zhang, H., Zhu, X., Rasheed, M. A., He, C., Hu, C., Chen, Y., Baranowski, E., Chen, H., & Guo, A. (2016). Immunoproteomic identification of MbovP579, a promising diagnostic biomarker for serological detection of *Mycoplasma bovi* infection. *Oncotarget*, *7*(26), 39376-39395. https://doi.org/10.18632/oncotarget.9799

Kirby, F., & Nicholas, R. (1996). Isolation of *Mycoplasma bovis* from bullocks' eyes. *The Veterinary record*, *138*(22), 552-552.

Klose, S. M., Omotainse, O. S., Zare, S., Vaz, P. K., Armat, P., Shil, P., Wawegama, N., Kanci Condello, A., O'Rourke, D., Disint, J. F., Andrews, D. M., Underwood, G. J., Morrow, C. J., Marenda, M. S., & Noormohammadi, A. H. (2022). Virulence factors of *Mycoplasma synoviae*: Three genes influencing colonization, immunogenicity, and transmissibility. *Front Microbiol, 13*, 1042212. https://doi.org/10.3389/fmicb.2022.1042212

Knudtson, W., Reed, D., & Daniels, G. (1986). Identification of Mycoplasmatales in pneumonic calf lungs. *Veterinary Microbiology, 11*(1-2), 79-91.

Kumar, R., Register, K., Christopher-Hennings, J., Moroni, P., Gioia, G., Garcia-Fernandez, N., Nelson, J., Jelinski, M. D., Lysnyansky, I., Bayles, D., Alt, D., & Scaria, J. (2020). Population Genomic Analysis of *Mycoplasma bovis* Elucidates Geographical Variations and Genes associated with Host-Types. *Microorganisms, 8*(10), 1561. https://www.mdpi.com/2076-2607/8/10/1561

Lee, N. (2009). Characterization of an ATP-binding cassette (ABC) transport system involved in nucleoside uptake in *Mycoplasma bovis* strain M23, and discovery of its pathogenicity genes. Iowa State University.

Letunic, I., & Bork, P. (2021). Interactive Tree Of Life (iTOL) v5: an online tool for phylogenetic tree display and annotation. *Nucleic Acids Research, 49*(W1), W293-W296. https://doi.org/10.1093/nar/gkab301

Li, Y., Zheng, H., Liu, Y., Jiang, Y., Xin, J., Chen, W., & Song, Z. (2011). The complete genome sequence of *Mycoplasma bovis* strain Hubei-1. *PLoS One, 6*(6), e20999. https://doi.org/10.1371/journal.pone.0020999

Liao, C., Mao, F., Qian, M., & Wang, X. (2022). Pathogen-Derived Nucleases: An Effective Weapon for Escaping Extracellular Traps. *Frontiers in Immunology, 13*. https://doi.org/10.3389/fimmu.2022.899890

Liapi, M., Botsaris, G., Arsenoglou, C., Markantonis, N., Michael, C., Antoniou, A., & Pipis, C. (2021). Rapid Detection of *Mycoplasma bovis*, *Staphylococcus aureus* and *Streptococcus agalactiae* in Cattle Bulk Tank Milk in Cyprus and Relations with Somatic Cell Counts. *Pathogens, 10*(7). https://doi.org/10.3390/pathogens10070841

Liu, B., Zheng, D., Zhou, S., Chen, L., & Yang, J. (2022). VFDB 2022: a general classification scheme for bacterial virulence factors. *Nucleic Acids Res, 50*(D1), D912-d917. https://doi.org/10.1093/nar/gkab1107

Lysnyansky, I., & Ayling, R. D. (2016). *Mycoplasma bovis*: Mechanisms of Resistance and Trends in Antimicrobial Susceptibility. *Front Microbiol*, *7*, 595. https://doi.org/10.3389/fmicb.2016.00595

Lysnyansky, I., Calcutt, M. J., Ben-Barak, I., Ron, Y., Levisohn, S., Methé, B. A., & Yogev, D. (2009). Molecular characterization of newly identified IS3, IS4 and IS30 insertion sequence-like elements in *Mycoplasma bovis* and their possible roles in genome plasticity. *FEMS Microbiol Lett*, *294*(2), 172-182. https://doi.org/10.1111/j.1574-6968.2009.01562.x

Lysnyansky, I., Rosengarten, R., & Yogev, D. (1996). Phenotypic switching of variable surface lipoproteins in *Mycoplasma bovis* involves high-frequency chromosomal rearrangements. *J Bacteriol*, *178*(18), 5395-5401. https://doi.org/10.1128/jb.178.18.5395-5401.1996

Lysnyansky, I., Yogev, D., & Levisohn, S. (2008). Molecular characterization of the *Mycoplasma bovis* p68 gene, encoding a basic membrane protein with homology to P48 of *Mycoplasma agalactiae*. *FEMS microbiology letters*, *279*(2), 234-242. https://doi.org/10.1111/j.1574-6968.2007.01040.x

Madureira, P., Baptista, M., Vieira, M., Magalhães, V., Camelo, A., Oliveira, L., Ribeiro, A., Tavares, D., Trieu-Cuot, P., Vilanova, M., & Ferreira, P. (2007). *Streptococcus agalactiae* GAPDH is a virulence-associated immunomodulatory protein. *J Immunol*, *178*(3), 1379-1387. https://doi.org/10.4049/jimmunol.178.3.1379

Masukagami, Y., De Souza, D. P., Dayalan, S., Bowen, C., O'Callaghan, S., Kouremenos, K., Nijagal, B., Tull, D., Tivendale, K. A., Markham, P. F., McConville, M. J., Browning, G. F., & Sansom, F. M. (2017). Comparative Metabolomics of *Mycoplasma bovis* and *Mycoplasma gallisepticum* Reveals Fundamental Differences in Active Metabolic Pathways and Suggests Novel Gene Annotations. *mSystems*, *2*(5). https://doi.org/10.1128/mSystems.00055-17

Mattsson, J. G., Guss, B., & Johansson, K. E. (1994). The phylogeny of *Mycoplasma bovis* as determined by sequence analysis of the 16S rRNA gene. *FEMS Microbiol Lett*, *115*(2-3), 325-328. https://doi.org/10.1111/j.1574-6968.1994.tb06658.x

Maunsell, F. P., Woolums, A. R., Francoz, D., Rosenbusch, R. F., Step, D. L., Wilson, D. J., & Janzen, E. D. (2011). *Mycoplasma bovis* infections in cattle. *J Vet Intern Med*, *25*(4), 772-783. https://doi.org/10.1111/j.1939-1676.2011.0750.x

McAloon, C. I., McAloon, C. G., Tratalos, J., O'Grady, L., McGrath, G., Guelbenzu, M., Graham, D. A., O'Keeffe, K., Barrett, D. J., & More, S. J. (2022). Seroprevalence of *Mycoplasma bovis* in bulk milk samples in Irish dairy herds and risk factors associated with herd seropositive status. *Journal of Dairy Science*, *105*(6), 5410-5419. https://doi.org/10.3168/jds.2021-21334

Menghwar, H., Guo, A., Chen, Y., Lysnyansky, I., Parker, A. M., Prysliak, T., & Perez-Casal, J. (2022). A core genome multilocus sequence typing (cgMLST) analysis of *Mycoplasma bovis* isolates. *Vet Microbiol*, *273*, 109532. https://doi.org/10.1016/j.vetmic.2022.109532

Messick, J. B. (2004). Hemotrophic mycoplasmas (hemoplasmas): a review and new insights into pathogenic potential. *Vet Clin Pathol*, *33*(1), 2-13. https://doi.org/10.1111/j.1939-165x.2004.tb00342.x

Minh, B. Q., Schmidt, H. A., Chernomor, O., Schrempf, D., Woodhams, M. D., von Haeseler, A., & Lanfear, R. (2020). IQ-TREE 2: New Models and Efficient Methods for Phylogenetic Inference in the Genomic Era. *Molecular Biology and Evolution*, *37*(5), 1530-1534. https://doi.org/10.1093/molbev/msaa015

Mitiku, F., Hartley, C. A., Sansom, F. M., Coombe, J. E., Mansell, P. D., Beggs, D. S., & Browning, G. F. (2018). The major membrane nuclease MnuA degrades neutrophil extracellular traps induced by *Mycoplasma bovis*. *Vet Microbiol*, *218*, 13-19. https://doi.org/10.1016/j.vetmic.2018.03.002

Murugesan, A. C., Kumaragurubaran, K., Gunasekaran, K., Murugasamy, S. A., Arunachalam, S., Annamalai, R., Ragothaman, V., & Ramaswamy, S. (2023). Molecular Detection of Hemoplasma in animals in Tamil Nadu, India and Hemoplasma genome analysis. *Vet Res Commun*. https://doi.org/10.1007/s11259-023-10263-2

Nicholas, R. A., & Ayling, R. D. (2003). *Mycoplasma bovis*: disease, diagnosis, and control. *Res Vet Sci*, *74*(2), 105-112. https://doi.org/10.1016/s0034-5288(02)00155-8

Nicholas, R. A., Fox, L. K., & Lysnyansky, I. (2016). Mycoplasma mastitis in cattle: To cull or not to cull. *Vet J*, *216*, 142-147. https://doi.org/10.1016/j.tvjl.2016.08.001

Paredes Felipe, N. (2022). Determinació de les proteïnes multifuncionals de Mycoplasma genitalium.

Parker, A. M., Sheehy, P. A., Hazelton, M. S., Bosward, K. L., & House, J. K. (2018). A review of mycoplasma diagnostics in cattle. *J Vet Intern Med*, *32*(3), 1241-1252. https://doi.org/10.1111/jvim.15135

Passchyn, P., Piepers, S., De Meulemeester, L., Boyen, F., Haesebrouck, F., & De Vliegher, S. (2012). Between-herd prevalence of *Mycoplasma bovis* in bulk milk in Flanders, Belgium. *Res Vet Sci*, *92*(2), 219-220. https://doi.org/10.1016/j.rvsc.2011.03.016

Perez-Casal, J., & Prysliak, T. (2007). Detection of antibodies against the *Mycoplasma bovis* glyceraldehyde-3-phosphate dehydrogenase protein in beef cattle. *Microb Pathog*, *43*(5-6), 189-197. https://doi.org/10.1016/j.micpath.2007.05.009

Prjibelski, A., Antipov, D., Meleshko, D., Lapidus, A., & Korobeynikov, A. (2020). Using SPAdes De Novo Assembler. *Current Protocols in Bioinformatics*, *70*(1), e102. https://doi.org/https://doi.org/10.1002/cpbi.102

Prysliak, T., van der Merwe, J., & Perez-Casal, J. (2013). Vaccination with recombinant *Mycoplasma bovis* GAPDH results in a strong humoral immune response but does not protect feedlot cattle from an experimental challenge with *M. bovis*. *Microb Pathog*, *55*, 1-8. https://doi.org/10.1016/j.micpath.2012.12.001

Punyapornwithaya, V., Fox, L. K., Gay, G. M., Hancock, D. D., & Alldredge, J. R. (2009). Short communication: The effect of centrifugation and resuspension on the recovery of *Mycoplasma* species from milk. *J Dairy Sci*, *92*(9), 4444-4447. https://doi.org/10.3168/jds.2009-2182

Punyapornwithaya, V., Fox, L. K., Hancock, D. D., Gay, J. M., & Alldredge, J. R. (2010). Association between an outbreak strain causing *Mycoplasma bovis* mastitis and its asymptomatic carriage in the herd: a case study from Idaho, USA. *Prev Vet Med*, *93*(1), 66-70. https://doi.org/10.1016/j.prevetmed.2009.08.008

Punyapornwithaya, V., Fox, L. K., Hancock, D. D., Gay, J. M., & Alldredge, J. R. (2012). Time to clearance of mycoplasma mastitis: the effect of management factors including milking time hygiene and preferential culling. *Can Vet J*, *53*(10), 1119-1122. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3447320/pdf/cvj_10_1119.pdf

Qi, J., Guo, A., Cui, P., Chen, Y., Mustafa, R., Ba, X., Hu, C., Bai, Z., Chen, X., Shi, L., & Chen, H. (2012). Comparative geno-plasticity analysis of *Mycoplasma bovis* HB0801 (Chinese isolate). *PLoS One*, *7*(5), e38239. https://doi.org/10.1371/journal.pone.0038239

Qi, J., Zhang, F., Wang, Y., Liu, T., Tan, L., Wang, S., Tian, M., Li, T., Wang, X., Ding, C., & Yu, S. (2018). Characterization of *Mycoplasma gallisepticum* pyruvate dehydrogenase alpha and beta subunits and their roles in cytoadherence. *PLoS One, 13*(12), e0208745. https://doi.org/10.1371/journal.pone.0208745

Rasheed, M. A., Qi, J., Zhu, X., Chenfei, H., Menghwar, H., Khan, F. A., Zhao, G., Zubair, M., Hu, C., Chen, Y., Chen, H., & Guo, A. (2017). Comparative Genomics of *Mycoplasma bovis* Strains Reveals That Decreased Virulence with Increasing Passages Might Correlate with Potential Virulence-Related Factors. *Frontiers in Cellular and Infection Microbiology*, *7*. https://doi.org/10.3389/fcimb.2017.00177

Razin, S., Yogev, D., & Naot, Y. (1998). Molecular biology and pathogenicity of Mycoplasmas. *Microbiol Mol Biol Rev, 62*(4), 1094-1156. https://doi.org/10.1128/MMBR.62.4.1094-1156.1998

Register, K. B., Thole, L., Rosenbush, R. F., & Minion, F. C. (2015). Multilocus sequence typing of *Mycoplasma bovis* reveals host-specific genotypes in cattle versus bison. *Vet Microbiol*, *175*(1), 92-98. https://doi.org/10.1016/j.vetmic.2014.11.002

Sachse, K., Salam, H. S., Diller, R., Schubert, E., Hoffmann, B., & Hotzel, H. (2010). Use of a novel real-time PCR technique to monitor and quantitate *Mycoplasma bovis* infection in cattle herds with mastitis and respiratory disease. *Vet J, 186*(3), 299-303. https://doi.org/10.1016/j.tvjl.2009.10.008

Schambow, R. A., Poulsen, K., Bolin, S., Krahn, D., Norby, B., Sockett, D., & Ruegg, P. L. (2021). Apparent prevalence of *Mycoplasma wenyonii*, *Candidatus Mycoplasma haemobos*, and bovine leukemia virus in Wisconsin and Michigan dairy cattle herds. *JDS Commun*, *2*(2), 61-66. https://doi.org/10.3168/jdsc.2020-0033

Sharma, S., Tivendale, K. A., Markham, P. F., & Browning, G. F. (2015). Disruption of the Membrane Nuclease Gene (MBOVPG45_0215) of *Mycoplasma bovis* Greatly Reduces Cellular Nuclease Activity. *Journal of bacteriology*, *197*(9), 1549-1558. https://doi.org/10.1128/jb.00034-15

Siguier, P., Perochon, J., Lestrade, L., Mahillon, J., & Chandler, M. (2006). ISfinder: the reference centre for bacterial insertion sequences. *Nucleic Acids Res*, *34*(Database issue), D32-36. https://doi.org/10.1093/nar/gkj014

Smith, J. A., Thrall, M. A., Smith, J. L., Salman, M. D., Ching, S. V., & Collins, J. K. (1990). *Eperythrozoon wenyonii* infection in dairy cattle. *Journal of the American Veterinary Medical Association*, *196*(8), 1244-1250. https://doi.org/10.2460/javma.1990.196.08.1244

Sun, Z., Fu, P., Wei, K., Zhang, H., Zhang, Y., Xu, J., Jiang, F., Liu, X., Xu, W., & Wu, W. (2014). Identification of novel immunogenic proteins from *Mycoplasma bovis* and establishment of an indirect ELISA based on recombinant E1 beta subunit of the pyruvate dehydrogenase complex. *PLoS One*, *9*(2), e88328. https://doi.org/10.1371/journal.pone.0088328

Tagawa, M., Yamakawa, K., Aoki, T., Matsumoto, K., Ishii, M., & Inokuma, H. (2013). Effect of chronic hemoplasma infection on cattle productivity. *J Vet Med Sci*, *75*(10), 1271-1275. https://doi.org/10.1292/jvms.13-0119

Tardy, F., Aspan, A., Autio, T., Ridley, A., Tricot, A., Colin, A., Pohjanvirta, T., Smid, B., Harders, F., Lindegaard, M., Tølbøll Lauritsen, K., Lyhs, U., Wisselink, H. J., & Strube, M. L. (2020). *Mycoplasma bovis* in Nordic European Countries: Emergence and Dominance of a New Clone. *Pathogens*, *9*(11). https://doi.org/10.3390/pathogens9110875

Thomas, A., Linden, A., Mainil, J., Bischof, D. F., Frey, J., & Vilei, E. M. (2005). *Mycoplasma bovis* shares insertion sequences with *Mycoplasma agalactiae* and *Mycoplasma mycoides* subsp. *mycoides* SC: Evolutionary and developmental aspects. *FEMS Microbiol Lett*, *245*(2), 249-255. https://doi.org/10.1016/j.femsle.2005.03.013

Thrusfield, M., Ortega, C., de Blas, I., Noordhuizen, J. P., & Frankena, K. (2001). WIN EPISCOPE 2.0: improved epidemiological software for veterinary medicine. *Vet Rec*, *148*(18), 567-572. https://doi.org/10.1136/vr.148.18.567

Thurmond, M. C., Tyler, J. W., Luiz, D. M., Holmberg, C. A., & Picanso, J. P. (1989). The effect of pre-enrichment on recovery of *Streptococcus agalactiae*, *Staphylococcus aureus* and mycoplasma from bovine milk. *Epidemiol Infect*, *103*(3), 465-474. https://doi.org/10.1017/s0950268800030879

Timonen, A. A. E., Katholm, J., Petersen, A., Mõtus, K., & Kalmus, P. (2017). Within-herd prevalence of intramammary infection caused by *Mycoplasma bovis* and associations

between cow udder health, milk yield, and composition. *J Dairy Sci*, *100*(8), 6554-6561. https://doi.org/10.3168/jds.2016-12267

Touchon, M., & Rocha, E. P. (2007). Causes of insertion sequences abundance in prokaryotic genomes. *Mol Biol Evol*, *24*(4), 969-981. https://doi.org/10.1093/molbev/msm014

Triebel, S., Sachse, K., Weber, M., Heller, M., Diezel, C., Hölzer, M., Schnee, C., & Marz, M. (2023). De novo genome assembly resolving repetitive structures enables genomic analysis of 35 European *Mycoplasmopsis bovis* strains. *BMC Genomics*, *24*(1), 548. https://doi.org/10.1186/s12864-023-09618-5

USDA-APHIS. (2003). *Mycoplasma in Bulk Tank Milk on U.S. Dairies*. USA: Department of Agriculture

USDA-APHIS. (2008). *Prevalence of Contagious Mastitis Pathogens on U.S. Dairy Operations*. USA: Department of Agriculture

Walz, P. H., Mullaney, T. P., Render, J. A., Walker, R. D., Mosser, T., & Baker, J. C. (1997). Otitis media in preweaned Holstein dairy calves in Michigan due to *Mycoplasma bovis*. *J Vet Diagn Invest*, *9*(3), 250-254. https://doi.org/10.1177/104063879700900305

Wang, J., Li, S., Chen, J., Gan, L., Wang, J., Xiong, Q., Feng, Z., Li, Q., Deng, Z., Yuan, X., & Yu, Y. (2023). Hijacking of Host Plasminogen by *Mesomycoplasma* (*Mycoplasma*) *hyopneumoniae* via GAPDH: an Important Virulence Mechanism To Promote Adhesion and Extracellular Matrix Degradation. *Microbiol Spectr*, *11*(3), e0021823. https://doi.org/10.1128/spectrum.00218-23

Wang, J., Liang, K., Chen, L., Su, X., Liao, D., Yu, J., & He, J. (2023). Unveiling the stealthy tactics: mycoplasma's immune evasion strategies. *Frontiers in Cellular and Infection Microbiology*, *13*. https://doi.org/10.3389/fcimb.2023.1247182

Weiler, J. M., Daha, M. R., Austen, K. F., & Fearon, D. T. (1976). Control of the amplification convertase of complement by the plasma protein beta1H. *Proc Natl Acad Sci U S A*, *73*(9), 3268-3272. https://doi.org/10.1073/pnas.73.9.3268

Wu, X., Zhang, S., Long, C., An, Z., Xing, X., Wen, F., & Bao, S. (2021). *Mycoplasmas bovis* P48 induces apoptosis in EBL cells via an endoplasmic reticulum stress-dependent signaling pathway. *Vet Microbiol*, *255*, 109013. https://doi.org/10.1016/j.vetmic.2021.109013

Xu, Q., Pan, Q., Wu, Q., & Xin, J. (2022). *Mycoplasma Bovis* adhesins and their target proteins. *Frontiers in Immunology*, *13*. https://doi.org/10.3389/fimmu.2022.1016641

Yair, Y., Borovok, I., Mikula, I., Falk, R., Fox, L. K., Gophna, U., & Lysnyansky, I. (2020). Genomics-based epidemiology of bovine *Mycoplasma bovis* strains in Israel. *BMC Genomics*, *21*(1), 70. https://doi.org/10.1186/s12864-020-6460-0

Yu, Y., Wang, H., Wang, J., Xiong, Q., Feng, Z., Wu, M., Liu, B., Xin, J., Liu, M., & Shao, G. (2018). Elongation factor thermo unstable (EF-Tu) moonlights as an adhesin on the surface of *Mycoplasma hyopneumoniae* by binding to fibronectin. *Frontiers in microbiology,*, *9*, 974.

Yu, Y., Wang, J., Han, R., Wang, L., Zhang, L., Zhang, A. Y., Xin, J., Li, S., Zeng, Y., Shao, G., Feng, Z., & Xiong, Q. (2020). *Mycoplasma hyopneumoniae* evades complement activation by binding to factor H via elongation factor thermo unstable (EF-Tu). *Virulence*, *11*(1), 1059-1074. https://doi.org/10.1080/21505594.2020.1806664

Yu, Y., Wang, J., Han, R., Wang, L., Zhang, L., Zhang, A. Y., Xin, J., Li, S., Zeng, Y., Shao, G., Feng, Z., & Xiong, Q. (2020). *Mycoplasma hyopneumoniae* evades complement activation by binding to factor H via elongation factor thermo unstable (EF-Tu). *Virulence*, *11*(1), 1059-1074. https://doi.org/10.1080/21505594.2020.1806664

Zhao, G., Zhu, X., Zhang, H., Chen, Y., Schieck, E., Hu, C., Chen, H., & Guo, A. (2021). Novel Secreted Protein of *Mycoplasma bovis* MbovP280 Induces Macrophage Apoptosis Through CRYAB. *Frontiers in Immunology*, *12*. https://doi.org/10.3389/fimmu.2021.619362

Zhao, S., Gao, Y., Xia, X., Che, Y., Wang, Y., Liu, H., Sun, Y., Ren, W., Han, W., & Yang, J. (2017). TGF-β1 promotes *Staphylococcus aureus* adhesion to and invasion into bovine mammary fibroblasts via the ERK pathway. *Microbial pathogenesis*, *106*, 25-29.

Zheng, W., Porter, E., Noll, L., Stoy, C., Lu, N., Wang, Y., Liu, X., Purvis, T., Peddireddi, L., Lubbers, B., Hanzlicek, G., Henningson, J., Liu, Z., & Bai, J. (2019). A multiplex real-time PCR assay for the detection and differentiation of five bovine pinkeye pathogens. *J Microbiol Methods*, *160*, 87-92. https://doi.org/10.1016/j.mimet.2019.03.024

Appendices

Figure A2.1. Spatial distribution of dairy farms in Tennessee.

Source: Adopted from Tennessee Department of Agriculture (TNDA) data

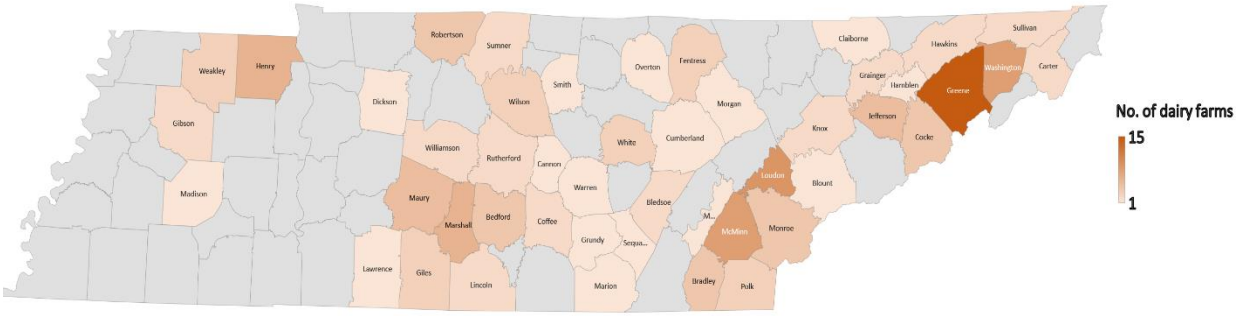

Figure A2.2. Spatial distribution of sampled counties. NB: The farm in Hardin County possibly started operation after TNDA generated the data.

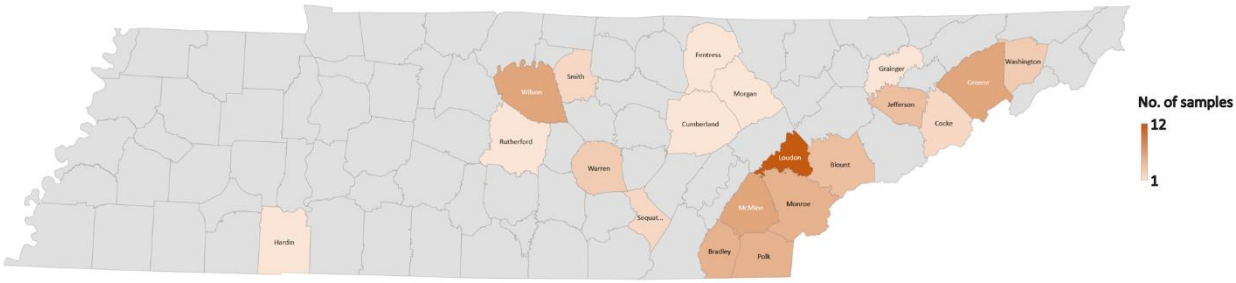

Table A2.1. Distribution of dairy farms in various counties in the State of Tennessee (Source: Tennessee Department of Agriculture)

County	Region	No. of dairy farms (%)
Greene	East	15 (11)
Loudon	East	9 (6.5)
McMinn	East	8 (6)
Washington	East	8 (6)
Henry	West	6 (4)
Marshall	Central	6 (4)
Maury	Central	5 (3.5)
Jefferson	East	5 (3.5)
Robertson	Central	4 (3)
Monroe	East	4 (3)
Cocke	East	4 (3)
Bradley	East	4 (3)
Bedford	Central	4 (3)
Giles	Central	3 (2)
Fentress	East	3 (2)
Weakley	West	3 (2)
Polk	East	3 (2)
White	Central	3 (2)
Wilson	Central	3 (2)
Grainger	East	2 (1.5)
Sullivan	East	2 (1.5)
Hawkins	East	2 (1.5)
Gibson	West	2 (1.5)
Sumner	Central	2 (1.5)
Williamson	Central	2 (1.5)
Coffee	Central	2 (1.5)
Bledsoe	East	2 (1.5)
Carter	East	2 (1.5)
Rutherford	Central	2 (1.5)
Knox	East	2 (1.5)
Lincoln	Central	2 (1.5)
Meigs	East	1 (0.7)
Marion	Central	1 (0.7)
Madison	West	1 (0.7)
Morgan	East	1 (0.7)
Claiborne	East	1 (0.7)
Overton	Central	1 (0.7)
Grundy	Central	1 (0.7)
Cumberland	East	1 (0.7)
Warren	Central	1 (0.7)
Lawrence	West	1 (0.7)
Hamblen	East	1 (0.7)
Dickson	West	1 (0.7)
Cannon	Central	1 (0.7)
Sequatchie	Central	1 (0.7)
Blount	East	1 (0.7)
Smith	Central	1 (0.7)
Grand Total		**140**

Table A2.2. Number of bulk tank milk samples collected from various counties in the State of Tennessee and their respective regions.

County	**Region**	**No. of samples (%)**
Loudon	East	12 (17)
McMinn	East	6 (8.5)
Greene	East	6 (8.5)
Wilson	Central	6 (8.5)
Polk	East	5 (7)
Monroe	East	5 (7)
Bradley	East	5 (7)
Jefferson	East	4 (5.5)
Blount	East	4 (5.5)
Washington	East	3 (4)
Warren	Central	3 (4)
Smith	Central	2 (2.8)
Sequatchie	Central	2 (2.8)
Cocke	East	2 (2.8)
Fentress	East	1 (1.4)
Morgan	East	1 (1.4)
Grainger	East	1 (1.4)
Cumberland	East	1 (1.4)
Rutherford	Central	1 (1.4)
Hardin	West	1 (1.4)
Grand Total		**71**

CHAPTER 3: WHOLE TRANSCRIPTOME ANALYSIS OF *MYCOPLASMA BOVIS*-HOST INTERACTIONS UNDER *IN VITRO* AND *IN VIVO* CONDITIONS

Disclosure

This chapter has been prepared for submission to the Journal of Veterinary Microbiology.

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Authors' contribution

AG performed laboratory tests and intramammary challenge and prepared the original draft manuscript. BDG performed intramammary challenge. TF performed bioinformatic analysis. JB provided the sequencing fund. JB and TS performed library preparation and RNA quantitation. OKD involved in the intramammary challenge and overall supervision of the study. All the authors read and revised the manuscript.

Abstract

Mycoplasma bovis mastitis is becoming increasingly problematic for dairy cattle farming in different parts of the world. *M. bovis* is inherently resistant to several antimicrobial classes and there is no effective vaccine. The major constraints to developing effective control tools are limited knowledge of *M. bovis* virulence factors and the underlying pathogenic mechanisms. The objective of this study was to determine virulence-associated genes of *M. bovis* and host immune response genes expressed during the early stages of host-pathogen interactions. In this study, we conducted *in vitro* infection of mammary epithelial cell (MAC-T) lines and *in vivo* intramammary infection of dairy cows with *M. bovis* strain PG45 and evaluated whole transcriptome differential gene expression and pathway enrichment analysis. We found that a total of 614 (304 up-regulated, 310 down-regulated) and 7161 (3935 up-regulated, 3226 down-regulated) genes of *M. bovis* and bovine host responses were differentially expressed, respectively. Of the up-regulated genes of *M. bovis*, insertion sequence (IS) genes that are involved in transposase activity such as ISMbov1, ISMbov2, ISMbov3, and ISMbov9 were significantly up-regulated whereas protein translation-associated genes were significantly down-regulated. In MAC-T cells, genes involved in apoptosis pathways and proinflammatory cytokines were significantly up-regulated whereas genes involved in cell cycle, ribosome biogenesis, and steroid biosynthesis were significantly down-regulated. Genes encoding formation of neutrophil extracellular traps and proinflammatory cytokines, were significantly up-regulated in the mammary gland of *M. bovis* challenged cows whereas genes involved in steroid biosynthesis and metabolism were significantly down-regulated. In conclusion, there were increased expressions of IS elements which are involved in transposase activity during the early stages of *M. bovis* infection. This observation warrants further detailed investigation to determine important specific potential target involved in the transposition for intervention. Apoptosis might be in favor of the progression of *M. bovis* infection, but intervention that reduces apoptosis and enhances quick and effective polymorphonuclear neutrophilic recruitment with increased extracellular trap activity may improve *M. bovis* clearance. Our findings are key to determining important targets for immunity-based intervention. To our knowledge, this is the first whole transcriptome analysis in both *M. bovis* and the host under the same *in vitro* and *in vivo* conditions. Therefore, we recommend additional metatranscriptomic, metaproteomic,

metabolomic and metagenomic evaluation of *M. bovis* and other potentially non-culturable mycoplasmas in the mammary glands during the pathogenesis of *M. bovis mastitis* in the bovine host to understand a complete picture of these interactions in maintaining healthy homeostatic state or mastitis.

Keywords: *M. bovis* mastitis, MAC-T cells, dual RNA-Seq, virulence genes, and host responses

3.1. Introduction

Mycoplasma bovis is a mollicute with greatly reduced genome size (~1 Mbp) and limited metabolic capabilities (Razin et al., 1998). However, it successfully causes bovine diseases with global occurrence and economic significance such as mastitis and pneumonia (Dudek et al., 2020). Epithelial cells in the mammary glands and lungs are common predilection sites for *M. bovis* (Gondaira et al., 2018; Liu et al., 2020; Thomas et al., 2003). On top of milk synthesis and secretion, mammary epithelial cells play a major role in the host immune responses against various invading microbes (Schukken et al., 2011).

Traditionally, transcriptomic analysis dealt either with the pathogen or the host. Dual RNA-sequencing, however, is the best approach to gauge host and pathogen genome-wide transcriptomic changes during the infection process (Westermann et al., 2012). By capturing both prokaryotic and eukaryotic expression profiles, dual RNA-seq helps to understand the complex host-pathogen interactions. However, separation of bacterial and host total RNA from the mixed eukaryotic and prokaryotic cell populations can be challenging. Usually, bacterial RNA makes up only <1% of the total RNA following co-culture with host cells and 98% of the total RNA represents eukaryotic ribosomal RNA (rRNA) which necessitates rRNA depletion (Marsh et al., 2017). The imbalance in RNA proportion can partly be resolved by increasing the multiplicity of infection (MOI); however, it requires meticulous prior optimization or an established protocol from previous studies since MOI needs to represent the natural infection dynamics (Humphrys et al., 2013). *M. bovis* lacks cell wall and conventional cell lysis protocols designed for other bacteria can impact RNA quality. In addition, the co-culture medium should simultaneously support the growth of *M. bovis* and host cells when conducting *in vitro* studies. Therefore, careful selection of appropriate methodology is a key to success.

The pathogenicity of *M. bovis* to bovine mammary epithelial cells has been established (Josi et al., 2018; Liu et al., 2020). However, the underlying virulence mechanisms and cascades of gene expression occurring in epithelial cells during the early stages of the *M. bovis*-mammary epithelial cell co-culture or infection are not well defined. Therefore, this study was aimed to determine important virulence-associated genes of *M. bovis* and host immune response genes expressed during the early stages of host-pathogen interactions from the same biological sample.

3.2. Materials and methods

Bacterial strain

M. bovis strain PG45 (ATCC 25523) (250 µL of 10^8 CFU/mL) was inoculated into 5 mL of Friis broth (Kauf et al., 2007b; Knudtson et al., 1986b) and incubated at 37ºC in 5% CO_2:95% air incubator for 24 h. This growth condition was used throughout the study unless otherwise stated. The bacterial suspension was aliquoted into sterile 1 mL cryovials and stored at -80ºC until further use. Viable *Mycoplasma* counts in CFU/mL were determined using 10-fold serial dilutions in sterile 1× phosphate-buffered saline (PBS; pH 7.4). Each dilution was plated on agar in triplicates and the agar plates were incubated for 4 - 7 d (Rodwell and Whitcomb, 1983).

Bovine mammary epithelial cell (MAC-T) cells

Bovine mammary epithelial cell lines (Huynh et al., 1991) were seeded in 75 cm^2 tissue culture flask (Fisher Scientific, Pittsburgh, PA) at a concentration of 2.0×10^6 cells/mL in 37ºC, 5% CO_2:95% air incubator for 24 h. Dulbecco's Modified Eagle Medium (DMEM) (Lonza, Walkersville, MD), supplemented with 10% heat-inactivated fetal bovine serum (Gibco, Thermo Fischer Scientific, Waltham, MA), 5 µg/mL insulin (Sigma Aldrich, St. Louis, MO), 1 µg/mL hydrocortisone (Sigma-Aldrich), 25 mM HEPES (Cytiva, Hyclone Laboratories, South Logan, UT), 0.002 g/mL sodium bicarbonate (Thermo Fischer Scientific), and 40 mM L-glutamine (Corning, MediaTech Inc, Manassas, VA). After growth reached 85-95% confluency, cells were dissociated using 0.25% Trypsin EDTA (Thermo Fisher Scientific) and collected by centrifugation at 1600 g for 10 min and counted using hemacytometer (Hausser Scientific, Horsham, PA). Cell viability was assessed by adding equal volume of Trypan Blue staining (Avantor, Suwanee, GA).

MAC-T cell infection

For infection, 400 µL of *M. bovis* PG45 (1×10^8 CFU/mL) stock was inoculated into 40 mL Friis broth (Kauf et al., 2007a; Knudtson et al., 1986a) until the exponential phase was reached within approximately 24 h. Mycoplasma cells were harvested by centrifugation at 5000 g for 30 min

(Liu et al., 2020) and the pellet was resuspended in 15 mL Friis broth (Josi et al., 2018). MAC-T cells were sub-seeded 24 h prior to the infection in 75 cm^2 flask in duplicate at a concentration of 2.0×10^7 cells/mL (Liu et al., 2020). Once MAC-T cells growth reached 85 - 95% confluency, cells were infected at a MOI of 150 (150 *M.* bovis : 1 cell) for 24 h (Zbinden et al., 2015). *M. bovis* PG45 and MAC-T cells were grown independently in duplicate in a similar manner in Friis broth and DMEM medium, respectively and used as controls.

In vivo cow intramammary challenge

Study animals

Holstein dairy cows from East Tennessee Ag-Research and Education Center-Little River Animal and Environmental Unit (ETREC-LRAEU) dairy farm were screened based on intramammary infection (IMI) by major mastitis pathogens and somatic cell count (SCC). A total of 3 healthy cows free of mastitis with SCC of ≤ 200, 000 cells/mL milk and negative individual quarter milk culture for *M. bovis* and other major mastitis pathogens (*Staphylococcus aureus*, *Streptococcus uberis*, *Streptococcus agalactiae*, *Streptococcus dysgalactiae* and *Escherichia coli* and *Klebsiella* spp.) were enrolled in this study.

Intramammary challenge

Dairy cows were challenged as previously described with minor modifications (Gondaira et al., 2020; Kauf et al., 2007a). Briefly, 400 µL (1×10^8 CFU/mL) of stock suspension was inoculated into 40 mL Friis broth and incubated for 24 h until the exponential phase was reached. The growing culture was centrifuged at 5000 g for 30 min at 4°C. The bacterial pellet was washed in sterile 1× PBS (pH 7.4) twice and the final pellet was resuspended in 40 mL sterile endotoxin free 1× PBS. The suspension was divided into aliquots of 5 mL containing 1×10^6 CFU and two contralateral quarters (right-front and left-rear) of 2 cows were infused. One cow received intramammary infusion of 5 mL sterile endotoxin free 1× PBS (pH 7.4) into similar quarters and used as a control. Cows were monitored for the onset of mastitis for 7 days post challenge. Following euthanasia, mammary tissue samples were collected in 10 mL RNALater (Thermo

Fisher Scientific) from the secretory region of the right-front (RF) and Left rear (LR) quarters infused with *M. bovis* from challenged cows. For the control cow, mammary tissue samples were collected from the right-front (RF) and left- rear (LR) PBS challenged quarters and all samples were stored at -80^{o}C until further use.

Total RNA extraction and library preparation

***In vitro* study**

Total RNA was extracted using RNeasy Mini Kit (Qiagen GmbH, Germantown, MD) according to the manufacturer's instructions with few modifications. MAC-T cells were detached from the flask using 0.25% Trypsin EDTA (Thermo Fisher Scientific). About 600 µL of cell suspension (1×10^7 cells) from *M. bovis* infected MAC-T cells, uninfected control, and *M. bovis* grown alone in tissue culture flask without MAC-T cells were pelleted and mixed with 600 µL (1:1) of Buffer RLT thoroughly by vertexing. An equal volume of 70% ethanol was added and the solution was transferred to spin column and centrifuged at 15000 g for 20 s at room temperature. DNase I (250 U, 5 µL) (Zymo Research, Irvine, CA) was used for on-column digestion and RNA was eluted with 50 µL RNase-free water.

***In vivo* study**

For mammary tissues, 25 milligram (mg) of frozen tissues were homogenized with 600 µL of Buffer RLT using tissue homogenizer (Qiagen) and the same procedures was followed as described above under in vitro study. The extracted total RNA was purified using RNA Clean and Concentrator Kit (Zymo Research). RNA-seq libraries were prepared using Zymo-Seq RiboFree Total RNA Library Kit (Zymo Research) following the manufacturer's procedure. The quantity and quality of total RNA was evaluated using Nanodrop (Thermo Fisher Scientific) and 4200 Tapestation System (Agilent Tech, Santa Clara, CA), respectively. A total of 10 samples (Fig. 3.1), 2 from each condition, were sequenced at the Genomics Core of the University of Tennessee, Knoxville (https://research.utk.edu/iamm/about/core-facilities/genomics-core/).

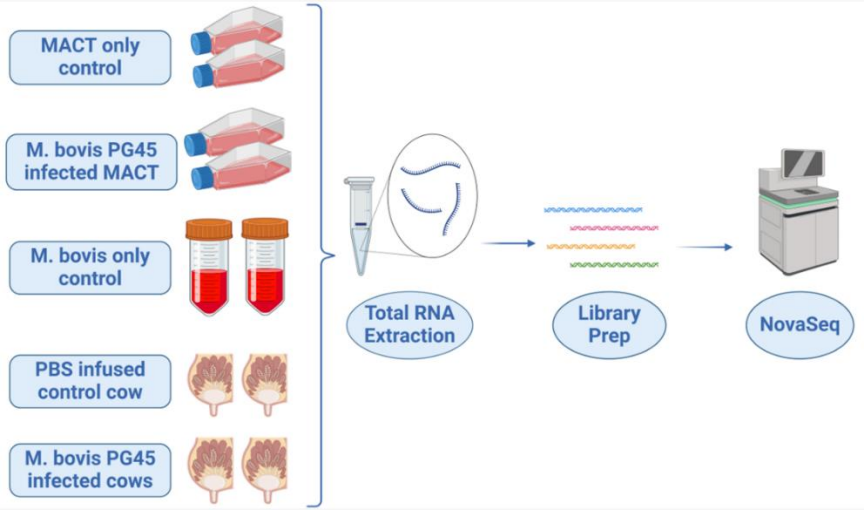

Figure 3.1. Experimental design and treatment conditions.

RNA-Seq data analysis

Raw reads were trimmed and quality filtered using fastp (v0.23.4) as described elsewhere (Chen, 2023; Chen et al., 2018). For read mapping, a custom reference genome was developed by appending *M. bovis* PG45 (Accession No. NC_014760) reference genome to *Bos taurus* (ARS-UCD2.0 ; RefSeq accession GCF_002263795.3). Spliced Transcripts Alignment to a Reference (STAR) (Dobin et al., 2013) and BWA-MEM2 (Vasimuddin et al., 2019) were used to map reads to the combined *Bos taurus* and *M. bovis*. Reads were counted within genes for the separate *Bos taurus* and *M. bovis* annotations, respectively, using featureCounts (Liao et al., 2014). Differentially expressed genes (DEGs) were called using DESEq2 with log fold changes shrunken by apeglm method (Love et al., 2014; Zhu et al., 2018). An alpha level of 0.001was used for DESeq2 independent filtering and as the false discovery rate (FDR)-adjusted p-value cutoff for calling DEGs. Functional overrepresentation analysis of up-regulated and down-regulated genes were identified using Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) with the clusterProfiler R package (Kanehisa et al., 2017; Wu et al., 2021). Pathways with and FDR adjusted *P* value (q value) of < 0.05 were considered significantly enriched.

3.3. Results

A total of 223,017,748 reads were obtained from the sequencing (Table A3.1). We found that 11% and 89% of the reads from *M. bovis*-MAC-T co-infection samples mapped to *M. bovis* and *Bos taurus* reference genomes, respectively (Table A3.1). The reads from control groups that only consisted of *M. bovis*, MAC-T and PBS challenged cow mapped ~95 - 100% to their respective reference genomes. Counts by biotypes shows effective removal of ribosomal RNA and sufficient percentage of protein coding genes for analysis (Fig. 3.2). The Euclidean distance between the biological replicates showed a high degree of similarity (Fig. 3.3). Sample to sample spearman correlation of RNA-seq libraries was confirmed (Fig. A3.1).

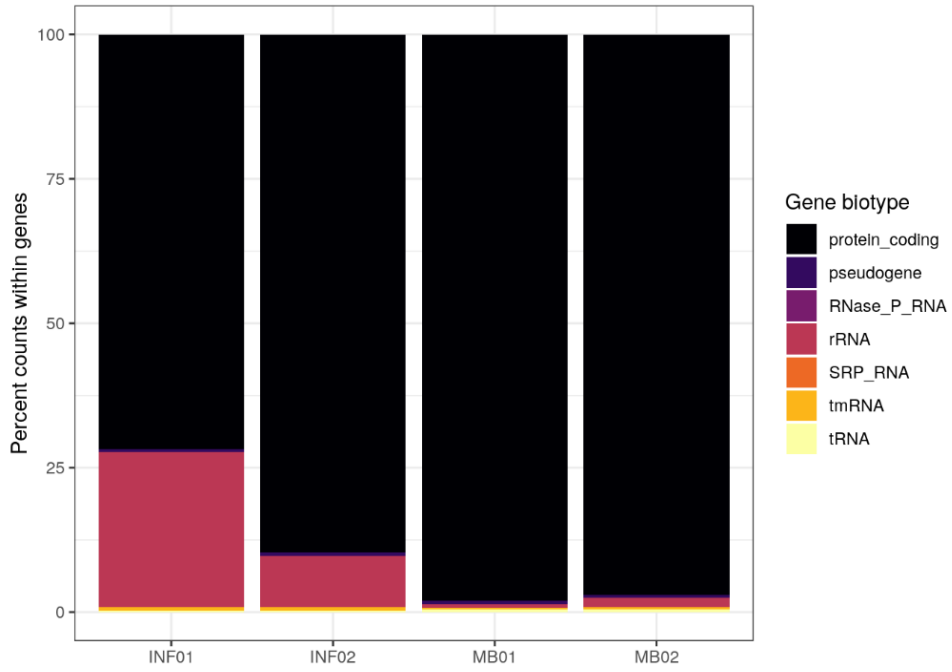

Figure 3.2. Breakdown of reads by gene biotypes (INF01 and INF02: co-infection samples; MB01 and MB02: *M. bovis* only control).

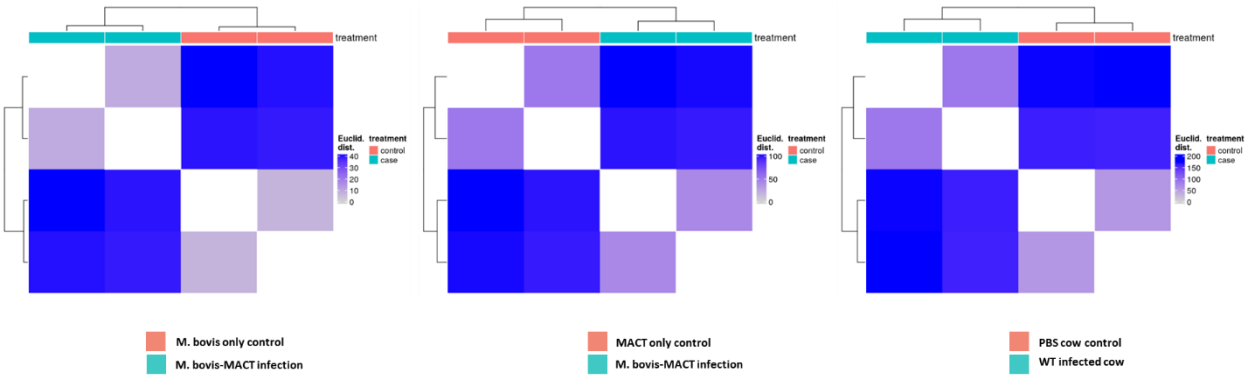

Figure 3.3. Sample-sample Euclidean distances between the biological replicates.

Differentially expressed genes (DEGs)

We analyzed the DEG profiles of *M. bovis*-MAC-T co-infection samples in comparison to the *M. bovis* and MAC-T only controls (Fig. 3.4). Similarly, DEGs were also identified by comparing expression of host response genes from *M. bovis* infected cows against uninfected control cow. A total of 614 and 7161 genes of *M. bovis* and bovine host responses were differentially expressed, respectively. From the differentially expressed *M. bovis* genes, 304 genes were up-regulated whereas 310 genes were down-regulated. Out of the differentially expressed bovine host response genes, 3935 genes were up-regulated whereas 3226 genes were down-regulated (Fig. 3.5A-C).

KEGG and GO enrichment analysis of DEGs

M. bovis

Comparing expression of *M. bovis* genes during *M. bovis*-MAC-T cells co-infection to *M. bovis* control, enrichment analysis identified a total of 31 genes associated with transposase activity that were significantly up-regulated in *M. bovis* (Fig. 3.6A). Insertion sequences IS*Mbov1* (IS30), IS*Mbov2* (IS1634), IS*Mbov3* (IS1634), and IS*Mbov9* (IS1634) were significantly up-regulated. On the other hand, pathways associated with ribosomal translation were significantly down-regulated (Fig. 3.6B).

MAC-T cells

KEGG enrichment analysis identified significantly up-regulated pathways including mitogen-activated protein kinase (MAPK) signaling pathway (Fig. 3.6C). In addition, genes encoding hypoxia-inducible factor 1 (HIF-1) signaling pathway were significantly up-regulated. Furthermore, several other pathways such as interleukin 17 (IL-17), IL-1, and IL-6 signaling, Forkhead box O (FoxO) signaling, autophagy, and chemokine signaling pathways were significantly upregulated. On the other hand, pathways such as cell cycle, ribosome biogenesis, and antigen processing and presenting pathways were significantly downregulated (Fig. 3.6D).

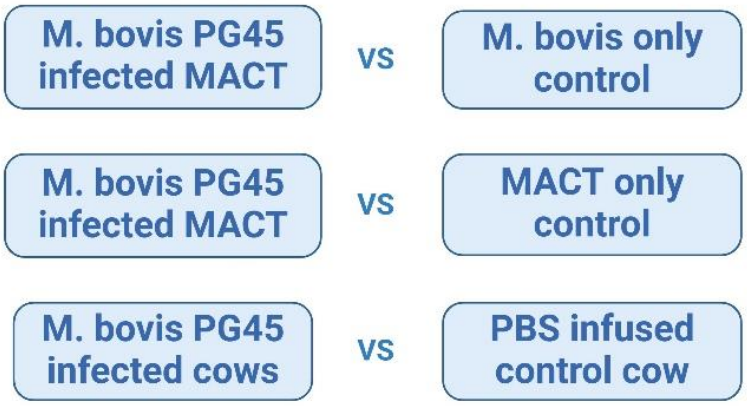

Figure 3.4. Comparison scheme of samples to identify differentially expressed genes.

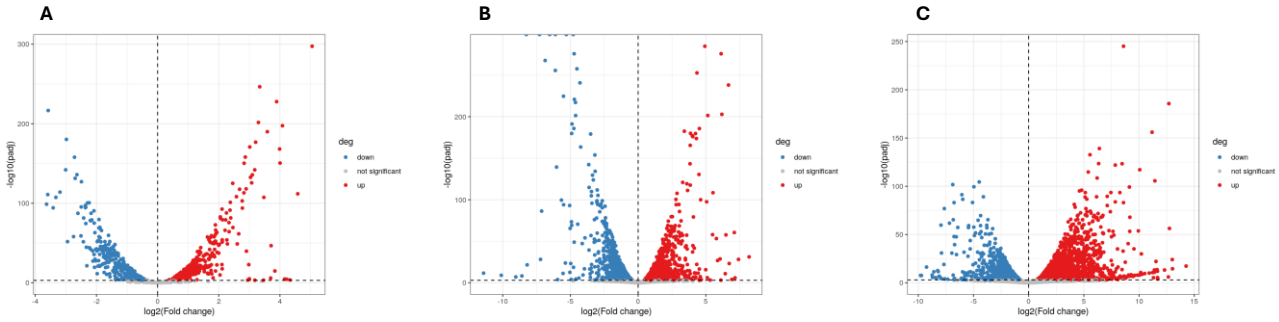

Figure 3.5. Volcano plots of differentially expressed genes in M. bovis (A), MAC-T cells (B), and mammary tissues (C).

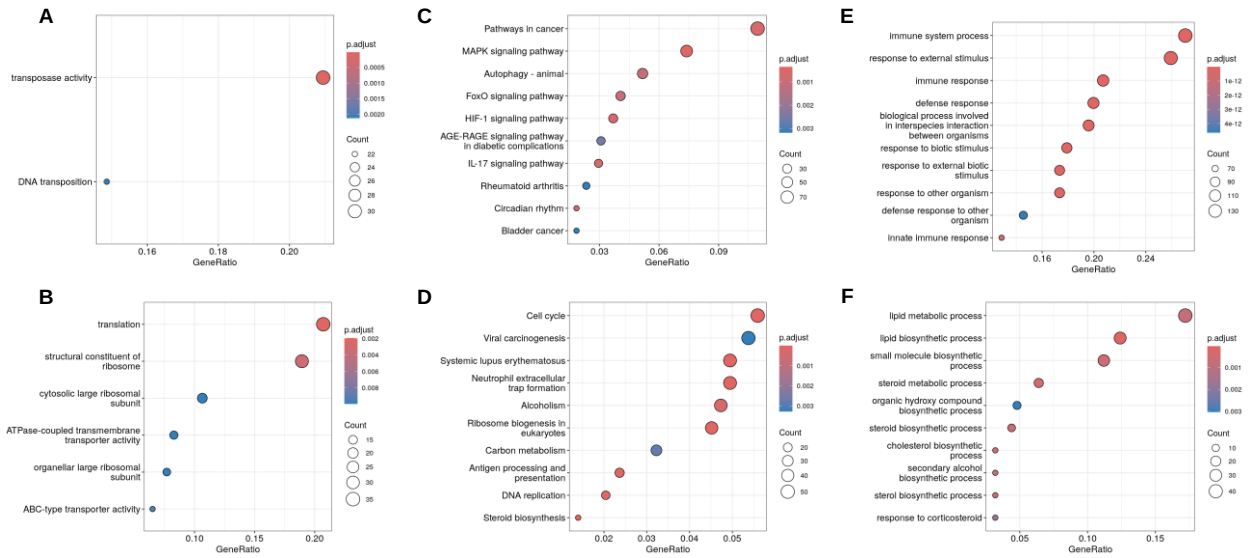

Figure 3.6. GO/KEGG enrichments of top 10 upregulated (A, C & E) and downregulated (B, D, &F) pathways in *M. bovis* (A & B), MAC-T cells (C & D) and mammary gland tissues (E & F). The size of the dots indicates the gene count and the color of the dots corresponds the adjusted p value (p.adjust).

Mammary gland tissues

GO enrichment analysis showed immune response dominated significantly up-regulated pathways in infected mammary gland tissues (Fig. 3.6E). These include defense response to other organisms, response to stress, innate immune response, adaptive immune response, leukocyte activation, and cytokine production, among many. In particular, genes such as interferon alpha receptor 1 (IFNAR1), tumor necrosis factor (TNF), IL-1β, IL-6, IL-10, IL-12, IL-18, IL-34, toll-like receptor 2 (TLR-2), TLR-4, TLR-10, and hypoxia inducible factor 1 (HIF-1) were significantly up-regulated. Lipid and steroid metabolic pathways were significantly down-regulated (Fig. 3.6F).

3.4. Discussion

Several *in vitro* studies involving *M. bovis* and epithelial cells co-infection have been conducted, however, most of these studies mainly focused on responses of the epithelial cells against *M. bovis* infection (Gondaira et al., 2018; Josi et al., 2018; Liu et al., 2021; Liu et al., 2020; Zbinden et al., 2015). To our knowledge, this is the first comprehensive (bacteria and host) RNA-seq analysis in both *M. bovis* and host/host cells under the same *in vitro* and *in vivo* conditions.

Up-regulation of transposase activity in *M. bovis* co-cultured with MAC-T cells

Insertion sequences are transposable elements that can relocate within or among genomes in such a way that causes deletions, duplications, rearrangements, and genetic information transfer coding fitness, virulence, and other adaptive traits (Touchon and Rocha, 2007). Various families of ISs have been reported in several *Mycoplasma* species (Bhugra and Dybvig, 1993; Calcutt et al., 1999; Frey et al., 1995; Pilo et al., 2003). Consistent with our finding, IS*Mbov1*, IS*Mbov2*, and IS*Mobv3* have previously been reported in clinical *M. bovis* field isolates in high number of copies with varying size and genome proportion (5-6%) (Ambroset et al., 2022; Li et al., 2011; Miles et al., 2005; Thomas et al., 2005). Similarly, closely related ISs have been found in *Mycoplasma* agalactiae (IS*Mag1*) and *Mycoplasma mycoides* subsp. *mycoides* small colony (ISMmy1) and are believed to be horizontally transmitted between *M. bovis* and these *Mycoplasma* species (Pilo et al., 2003; Westberg et al., 2002).

Although there are multiple reports of various isoforms of ISs in *Mycoplasma* species, there is limited information on their biological functions (Bischof et al., 2006; Thomas et al., 2005; Vilei et al., 1999). Spontaneous high-frequency rearrangement in variable surface proteins (Vsp) locus of *M. bovis* which is associated with phenotypic switching of these proteins might be due to the transposase activity of ISs (Lysnyansky et al., 1996; Lysnyansky et al., 1999). In addition, IS*Mbov1* has been found adjacent to the Vsp gene in the genome of clinical *M. bovis* isolates (Li et al., 2011). IS3 and IS30 families are associated with antimicrobial resistance genes in various bacterial genera (Razavi et al., 2020). Fluoroquinolone resistant *M. bovis* strains with mutations in *parC*, quinolone resistance-determining gene, have been identified harboring different IS (IS*Mbov3* and *4*) patterns (Lysnyansky et al., 2009). The IS1634 has been associated with macrolide resistance gene in *Trueperella pyogenes* and is speculated to play role in horizontal transfer of resistance genes (Dong et al., 2020) and IS3 is identified in adhesion-related genes in multi-drug resistant *Escherichia coli* (Mbanga et al., 2021).

Down-regulation of ribosomal translation in *M. bovis* co-cultured with MAC-T cells

Our findings indicated that the overall ribosomal translation processes in *M. bovis* were down-regulated. It has been shown that translation-related genes are down-regulated in *M. bovis* under stressful conditions such as in *in vitro* interaction with antimicrobials (Ranjitkar et al., 2022). Similarly, *Mycoplasma genitalium* have shown downregulation of its protein translation processes upon exposure to stressful conditions in the form of osmotic shock (Zhang and Baseman, 2011). This is true in other bacteria also, where microbes reduce energy-demanding processes such as protein synthesis and transport possibly to shunt resources towards virulence (Njenga et al., 2023; Starosta et al., 2014). Based on our findings, it is possible that *M. bovis* is facing certain stressful conditions whether it is the cellular response and/or as a result of nutrient depletion in the medium.

Up-regulation of apoptosis pathways in *M. bovis* infected MAC-T cells

The KEGG enrichment of MAC-T cell samples showed up-regulation of pathways mainly associated with apoptosis. This is also supported by the GO enrichment result in this study,

where several genes associated with apoptosis/programmed cell death biological processes were significantly up-regulated. *M. bovis* induces apoptosis in several cell types including epithelial cells, macrophages, lymphocytes, neutrophils, and embryonic bovine lung cells (Liu et al., 2020; S. Jimbo et al., 2017; Vanden Bush and Rosenbusch, 2002; Wu et al., 2021; Zhao et al., 2021). It has been shown that apoptosis can be induced in bovine mammary epithelial cells via the MAPK pathway (An et al., 2023). The up-regulation of the MAPK signaling pathway has also been reported in bovine macrophages following infection by *M. bovis* (Zhang et al., 2022b), and by *M. genitalium* in human monocytic cell line (You et al., 2008). *M. bovis* mastitis results in reduced milk yield (Torres et al., 2022) and while apoptosis may be one cause, further detailed study is required if apoptosis is one of many mechanisms for cell death or the only mechanism during *M. bovis* mastitis or a widespread event in the epithelial cells that resulted in cell death and loss of milk production.

Bovine mammary epithelial cells have a key involvement in the innate immune response (Gondaira et al., 2021a), in addition to their secretory role. This involves production of proinflammatory cytokines such as IL-1β, IL-6, and tumor necrosis factor-alpha (TNF-α) mediated by various pathways including mitogen-activated protein kinases (MAPKs) (Jiang et al., 2017). Similarly, up-regulation of IL-1 and IL-6 was observed in MAC-T cells in this study.

Autophagy, which is a degradation mechanism in eukaryotes to eliminate pathogens was significantly up-regulated in MAC-T cells in this study (Glick et al., 2010). In agreement with our findings, Liu et al. reported the induction of autophagy by *M. bovis* in bovine mammary epithelial cells (Liu et al., 2021). However, *M. bovis* and other *Mycoplasma* species have evolved to interfere with autophagy to enhance their intracellular replication (Hu et al., 2014; Liu et al., 2021; Luo et al., 2020; Shimizu et al., 2014; Xu et al., 2022) which might be responsible for persistent IMI of *M. bovis* in dairy cows.

The forkhead box O (FoxO) signaling pathway was significantly enriched in infected MAC-T cells compared to the non-infected controls. FoxO signaling pathway mediates several cellular biological processes in mammals such as apoptosis, oxidative stress resistance, and immune responses (Eijkelenboom and Burgering, 2013). In agreement with our finding, Zhang et al.

reported that *M. bovis* induces FoxO pathway up-regulation in bovine macrophages (Zhang et al., 2022b). On the other hand, hydrogen peroxide (H_2O_2) synthesis is one of the virulence factors in *M. bovis* which can cause oxidative stress and apoptosis in bovine mammary epithelial cells and also responsible for caseo-necrotic lung lesions in calves (Khan et al., 2005; Liu et al., 2020; Schott et al., 2014). Furthermore, reactive oxygen species (ROS) such as H_2O_2 cause the accumulation of hypoxia-inducible factor-1 (HIF-1) (Movafagh et al., 2015) which was significantly up-regulated in our study. HIF-1 is a major pathway involved in hypoxic conditions of cells, however it can be initiated by several conditions independent of hypoxia (Movafagh et al., 2015).

Interleukin-17 (IL-17) is a cytokine produced by Th17 lymphocytes which are specialized in the recruitment of neutrophils (Rainard et al., 2013; Riollet et al., 2006). It has been shown that *M. bovis* stimulated increased expression of IL-17 in macrophages, peripheral mononuclear and bovine lung epithelial cells (Gondaira et al., 2021b; Zhang et al., 2022a; Zhang et al., 2022b). This agrees with our findings as the role of IL-17 in neutrophil recruitment in animal and human *Mycoplasma* infections has been established (Bai et al., 2021; Grossman et al., 2021; Kurata et al., 2014). There are innate and adaptive cell mediated type 3 effector immunity that is recently shown to have effector activity in epithelial and mucosal surfaces (Annunziato et al., 2015; Eberl et al., 2015). Type 3 immunity is characterized by recruitment of neutrophils, production of antimicrobial defenses by epithelial cells, involvement of type 3 innate lymphoid cells (ILC3s), expression of cytokines (IL-17A, IL-17F, IL-22) and transcription factors (Annunziato et al., 2015; Gaffen, 2008). Further investigation is required to determine if increased expression of IL-17 is associated with type 3 immunity in the mammary glands.

Up-regulation of immune response pathways in *M. bovis* infected mammary glands

The up-regulation of immune response related genes in udder tissue shown by GO enrichment is a strong indicator of *M. bovis* replication and an encounter with the immune system. However, the increased multiplication of *M. bovis* in the udder tissue in the presence of increased expression of proinflammatory cytokines, which are expected to increase recruitment of immune cells to udder tissue that clear infection, need further detailed investigation. In agreement with

our in vivo finding, it has been shown that *M. bovis* elicits significant up-regulation of pro-inflammatory cytokines including IL-1β, IL-6, and TNF in bovine mammary epithelial cells (Gondaira et al., 2018; Rodríguez et al., 2015; Yang et al., 2022; Zbinden et al., 2015). Interestingly, IL-1 and IL-6 were also up-regulated in MAC-T cells in this study. Up-regulation of IL-6 due to *M. bovis* stimulation of peripheral blood mononuclear cells has also been reported (Valsala et al., 2017). In agreement with our findings, upregulation of IL-10 was reported in *M. bovis* IMI that lasted for 9 days in cows and calves naturally infected by *M. bovis* (Kauf et al., 2007a; Rodríguez et al., 2015). Expression of immunosuppressive cytokines such as IL-10, TLR-10 and IFNAR1 along with the pro-inflammatory cytokines might be a time-dependent phenomenon since mammary tissue samples in this study were collected on the 7^{th} day of the challenge. Based on this finding it seems that the initial expression of proinflammatory cytokine is followed by expression of immunosuppressive cytokines and survival of *M. bovis* rather than recruitment of phagocytic cells and clearance of *M. bovis* IMI. Furthermore, co-upregulation of pro- and anti-inflammatory cytokines such as IL-6 and IL-10 have been reported in another mastitis pathogen *Streptococcus uberis* in *in vivo* intramammary challenge (Moyes et al., 2009). *M. bovis* induced up-regulation of TLR-2 in bovine mammary epithelial cells have been confirmed (Yang et al., 2022; Zbinden et al., 2015). Furthermore, TLR-2 and TLR-6 play an important role in recognizing proinflammatory lipopeptide in *Mycoplasma fermentans* (Omueti et al., 2005). Interestingly, HIF-1 was one of the significantly up-regulated pathways in both MAC-T cells and mammary tissues in this study.

Among the significantly up-regulated pathways in KEGG enrichment was the neutrophil extracellular trap (NET) formation. Reports indicate that *M. bovis* induces NET formation in (Mitiku et al., 2018). Although not confirmed, this is likely through the reactive oxygen species produced by *M. bovis* as demonstrated in human neutrophils (Keshari et al., 2013). During *M. bovis* mastitis, neutrophils are the major phagocytic cells recruited as a first line of defense (Gondaira et al., 2020; Kauf et al., 2007a) and it is possible that this is why up-regulated NET formation was observed in this study. As the name indicates, NETs are mainly produced by neutrophils and to some extent by eosinophils, basophils, monocytes, and macrophages (Vorobjeva and Chernyak, 2020), however further investigation is required to determine why NET formation is not followed by clearance of *M. bovis* IMI.

Down-regulation of lipid and steroid biosynthesis in *M. bovis* infected MAC-T cells and mammary glands

Lipid/steroid biosynthesis and metabolism were significantly downregulated in both MAC-T cells and in mammary tissues. This might be associated with significant decline in milk yield, fat percentage and total milk solids (Al-Farha et al., 2017; Timonen et al., 2017) which is clinically manifested as watery or flaky milk in *M. bovis* mastitis in dairy cows (Bennett and Jasper, 1980). In a similar manner, down-regulation of lipid biosynthesis enzymes involved in milk production have been shown in primary bovine mammary epithelial cells infected by *E. coli* and dairy cows challenged by *S. uberis* (Moyes et al., 2009; Younis et al., 2016).

In conclusion, the significant up-regulation of insertion sequence elements in *M. bovis* among other genes warrants further detailed investigation of transposase activity associated genes involved in the *M. bovis* mastitis to determine potential targets for intervention. Our wholistic approach of evaluating the expression of both *M. bovis* and bovine host response genes during early stages of *M. bovis*-host/cell interactions is key to determine important targets for immunity-based intervention or to determine intramammary microbiome-based homeostasis. We recommend additional metatranscriptomic, metaproteomic, metabolomic and metagenomic evaluation of both intramammary microbiota (*M. bovis* and other culturable and non-culturable microbes) and bovine host to get a complete picture of these interactions.

Ethical consideration

The IACUC approval for intramammary challenge was obtained from the University of Tennessee Knoxville (Registration # 2870-0322).

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References

Al-Farha, A. A., Hemmatzadeh, F., Khazandi, M., Hoare, A., & Petrovski, K. (2017). Evaluation of effects of Mycoplasma mastitis on milk composition in dairy cattle from South Australia. *BMC Vet Res*, *13*(1), 351. https://doi.org/10.1186/s12917-017-1274-2

Ambroset, C., Peticca, A., Tricot, A., & Tardy, F. (2022). Genomic features of *Mycoplasma bovis* subtypes currently circulating in France. *BMC Genomics*, *23*(1), 603. https://doi.org/10.1186/s12864-022-08818-9

An, G., Park, J., Lim, W., & Song, G. (2023). Folpet promotes apoptosis of bovine mammary epithelial cells via disruption of redox homeostasis and activation of MAPK cascades. *Food Chem Toxicol*, *175*, 113709. https://doi.org/10.1016/j.fct.2023.113709

Annunziato, F., Romagnani, C., & Romagnani, S. (2015). The 3 major types of innate and adaptive cell-mediated effector immunity. *Journal of Allergy and Clinical Immunology*, *135*(3), 626-635. https://doi.org/https://doi.org/10.1016/j.jaci.2014.11.001

Bai, S., Wang, W., Ye, L., Fang, L., Dong, T., Zhang, R., Wang, X., Gao, H., Shen, B., & Ding, S. (2021). IL-17 stimulates neutrophils to release S100A8/A9 to promote lung epithelial cell apoptosis in *Mycoplasma pneumoniae*-induced pneumonia in children. *Biomed Pharmacother*, *143*, 112184. https://doi.org/10.1016/j.biopha.2021.112184

Bennett, R. H., & Jasper, D. E. (1980). Bovine mycoplasmal mastitis from intramammary inoculations of small numbers of *Mycoplasma bovis*: local and systemic antibody response. *Am J Vet Res*, *41*(6), 889-892.

Bhugra, B., & Dybvig, K. (1993). Identification and characterization of IS1138, a transposable element from *Mycoplasma pulmonis* that belongs to the IS3 family. *Mol Microbiol*, *7*(4), 577-584. https://doi.org/10.1111/j.1365-2958.1993.tb01148.x

Bischof, D. F., Vilei, E. M., & Frey, J. (2006). Genomic differences between type strain PG1 and field strains of *Mycoplasma mycoides* subsp. *mycoides* small-colony type. *Genomics*, *88*(5), 633-641. https://doi.org/10.1016/j.ygeno.2006.06.018

Calcutt, M. J., Lavrrar, J. L., & Wise, K. S. (1999). IS1630 of *Mycoplasma fermentans*, a novel IS30-type insertion element that targets and duplicates inverted repeats of variable length and sequence during insertion. *J Bacteriol*, *181*(24), 7597-7607. https://doi.org/10.1128/jb.181.24.7597-7607.1999

Chen, S. (2023). Ultrafast one-pass FASTQ data preprocessing, quality control, and deduplication using fastp. *iMeta*, *2*(2), e107. https://doi.org/https://doi.org/10.1002/imt2.107

Chen, S., Zhou, Y., Chen, Y., & Gu, J. (2018). fastp: an ultra-fast all-in-one FASTQ preprocessor. *Bioinformatics*, *34*(17), i884-i890. https://doi.org/10.1093/bioinformatics/bty560

Dobin, A., Davis, C. A., Schlesinger, F., Drenkow, J., Zaleski, C., Jha, S., Batut, P., Chaisson, M., & Gingeras, T. R. (2013). STAR: ultrafast universal RNA-seq aligner. *Bioinformatics*, *29*(1), 15-21. https://doi.org/10.1093/bioinformatics/bts635

Dong, W. L., Xu, Q. J., Atiah, L. A., Odah, K. A., Gao, Y. H., Kong, L. C., & Ma, H. X. (2020). Genomic island type IV secretion system and transposons in genomic islands involved in antimicrobial resistance in *Trueperella pyogenes*. *Vet Microbiol*, *242*, 108602. https://doi.org/10.1016/j.vetmic.2020.108602

Dudek, K., Nicholas, R. A. J., Szacawa, E., & Bednarek, D. (2020). *Mycoplasma bovis* Infections-Occurrence, Diagnosis and Control. *Pathogens*, *9*(8). https://doi.org/10.3390/pathogens9080640

Eberl, G., Colonna, M., Di Santo, J. P., & McKenzie, A. N. J. (2015). Innate lymphoid cells: A new paradigm in immunology. *Science*, *348*(6237), aaa6566. https://doi.org/doi:10.1126/science.aaa6566

Eijkelenboom, A., & Burgering, B. M. T. (2013). FOXOs: signalling integrators for homeostasis maintenance. *Nature Reviews Molecular Cell Biology*, *14*(2), 83-97. https://doi.org/10.1038/nrm3507

Ewels, P., Magnusson, M., Lundin, S., & Käller, M. (2016). MultiQC: summarize analysis results for multiple tools and samples in a single report. *Bioinformatics*, *32*(19), 3047-3048. https://doi.org/10.1093/bioinformatics/btw354

Frey, J., Cheng, X., Kuhnert, P., & Nicolet, J. (1995). Identification and characterization of IS1296 in *Mycoplasma mycoides* subsp. *mycoides* SC and presence in related mycoplasmas. *Gene*, *160*(1), 95-100. https://doi.org/10.1016/0378-1119(95)00195-c

Gaffen, S. L. (2008). An overview of IL-17 function and signaling. *Cytokine*, *43*(3), 402-407. https://doi.org/10.1016/j.cyto.2008.07.017

Glick, D., Barth, S., & Macleod, K. F. (2010). Autophagy: cellular and molecular mechanisms. *J Pathol*, *221*(1), 3-12. https://doi.org/10.1002/path.2697

Gondaira, S., Higuchi, H., Iwano, H., Nishi, K., Nebu, T., Nakajima, K., & Nagahata, H. (2018). Innate immune response of bovine mammary epithelial cells to *Mycoplasma bovis*. *J Vet Sci*, *19*(1), 79-87. https://doi.org/10.4142/jvs.2018.19.1.79

Gondaira, S., Nishi, K., Fujiki, J., Iwano, H., Watanabe, R., Eguchi, A., Hirano, Y., Higuchi, H., & Nagahata, H. (2021). Innate immune response in bovine neutrophils stimulated with *Mycoplasma bovis*. *Vet Res*, *52*(1), 58. https://doi.org/10.1186/s13567-021-00920-2

Gondaira, S., Nishi, K., Iwano, H., Fujiki, J., Watanabe, R., Eguchi, A., Hirano, Y., Higuchi, H., & Nagahata, H. (2021). Transcriptome analysis of *Mycoplasma bovis* stimulated bovine peripheral blood mononuclear cells. *Veterinary Immunology and Immunopathology*, *232*, 110166. https://doi.org/https://doi.org/10.1016/j.vetimm.2020.110166

Gondaira, S., Nishi, K., Tanaka, T., Yamamoto, T., Nebu, T., Watanabe, R., Konnai, S., Hayashi, T., Kiku, Y., Okamoto, M., Matsuda, K., Koiwa, M., Iwano, H., Nagahata, H., & Higuchi, H. (2020). Immunosuppression in Cows following Intramammary Infusion of *Mycoplasma bovis*. *Infect Immun*, *88*(3). https://doi.org/10.1128/iai.00521-19

Grossman, P. C., Schneider, D. A., Herndon, D. R., Knowles, D. P., & Highland, M. A. (2021). Differential pulmonary immunopathology of domestic sheep (Ovis aries) and bighorn sheep (Ovis canadensis) with *Mycoplasma ovipneumoniae* infection: A retrospective study. *Comp Immunol Microbiol Infect Dis*, *76*, 101641. https://doi.org/10.1016/j.cimid.2021.101641

Hu, X., Yu, J., Zhou, X., Li, Z., Xia, Y., Luo, Z., & Wu, Y. (2014). Synergism between upregulation of Rab7 and inhibition of autophagic degradation caused by mycoplasma facilitates intracellular mycoplasma infection. *Mol Med Rep*, *9*(3), 793-800. https://doi.org/10.3892/mmr.2014.1907

Humphrys, M. S., Creasy, T., Sun, Y., Shetty, A. C., Chibucos, M. C., Drabek, E. F., Fraser, C. M., Farooq, U., Sengamalay, N., Ott, S., Shou, H., Bavoil, P. M., Mahurkar, A., & Myers, G. S. (2013). Simultaneous transcriptional profiling of bacteria and their host cells. *PLoS One*, *8*(12), e80597. https://doi.org/10.1371/journal.pone.0080597

Huynh, H. T., Robitaille, G., & Turner, J. D. (1991). Establishment of bovine mammary epithelial cells (MAC-T): an in vitro model for bovine lactation. *Exp Cell Res*, *197*(2), 191-199.

Jiang, K.-f., Zhao, G., Deng, G.-z., Wu, H.-c., Yin, N.-n., Chen, X.-y., Qiu, C.-w., & Peng, X.-l. (2017). Polydatin ameliorates *Staphylococcus aureus*-induced mastitis in mice via inhibiting TLR2-mediated activation of the p38 MAPK/NF-κB pathway. *Acta Pharmacologica Sinica*, *38*(2), 211-222. https://doi.org/10.1038/aps.2016.123

Josi, C., Bürki, S., Stojiljkovic, A., Wellnitz, O., Stoffel, M. H., & Pilo, P. (2018). Bovine Epithelial in vitro Infection Models for *Mycoplasma bovis*. *Front Cell Infect Microbiol*, *8*, 329. https://doi.org/10.3389/fcimb.2018.00329

Kanehisa, M., Furumichi, M., Tanabe, M., Sato, Y., & Morishima, K. (2017). KEGG: new perspectives on genomes, pathways, diseases and drugs. *Nucleic Acids Res*, *45*(D1), D353-d361. https://doi.org/10.1093/nar/gkw1092

Kauf, A. C., Rosenbusch, R. F., Paape, M. J., & Bannerman, D. D. (2007). Innate immune response to intramammary *Mycoplasma bovis* infection. *J Dairy Sci*, *90*(7), 3336-3348. https://doi.org/10.3168/jds.2007-0058

Kauf, A. C. W., Rosenbusch, R. F., Paape, M. J., & Bannerman, D. D. (2007). Innate immune response to intramammary *Mycoplasma bovis* infection. *J. Dairy Sci.*, *90*(7), 3336-3348. http://jds.fass.org/cgi/content/abstract/90/7/3336

Keshari, R. S., Verma, A., Barthwal, M. K., & Dikshit, M. (2013). Reactive oxygen species-induced activation of ERK and p38 MAPK mediates PMA-induced NETs release from human neutrophils. *J Cell Biochem*, *114*(3), 532-540. https://doi.org/10.1002/jcb.24391

Khan, L. A., Miles, R. J., & Nicholas, R. A. (2005). Hydrogen peroxide production by *Mycoplasma bovis* and *Mycoplasma agalactiae* and effect of in vitro passage on a *Mycoplasma bovis* strain producing high levels of H2O2. *Vet Res Commun*, *29*(3), 181-188. https://doi.org/10.1023/b:verc.0000047506.04096.06

Knudtson, W., Reed, D., & Daniels, G. (1986). Identification of Mycoplasmatales in pneumonic calf lungs. *Veterinary Microbiology*, *11*(1-2), 79-91.

Knudtson, W. U., Reed, D. E., & Daniels, G. (1986). Identification of Mycoplasmatales in pneumonic calf lungs. *Vet Microbiol*, *11*(1-2), 79-91.

Kurata, S., Osaki, T., Yonezawa, H., Arae, K., Taguchi, H., & Kamiya, S. (2014). Role of IL-17A and IL-10 in the antigen induced inflammation model by *Mycoplasma pneumoniae*. *BMC Microbiol*, *14*, 156. https://doi.org/10.1186/1471-2180-14-156

Li, Y., Zheng, H., Liu, Y., Jiang, Y., Xin, J., Chen, W., & Song, Z. (2011). The complete genome sequence of *Mycoplasma bovis* strain Hubei-1. *PLoS One*, *6*(6), e20999. https://doi.org/10.1371/journal.pone.0020999

Liao, Y., Smyth, G. K., & Shi, W. (2014). featureCounts: an efficient general purpose program for assigning sequence reads to genomic features. *Bioinformatics*, *30*(7), 923-930. https://doi.org/10.1093/bioinformatics/btt656

Liu, Y., Deng, Z., Xu, S., Liu, G., Lin, Y., Khan, S., Gao, J., Qu, W., Kastelic, J. P., & Han, B. (2021). *Mycoplasma bovis* subverts autophagy to promote intracellular replication in bovine mammary epithelial cells cultured in vitro. *Vet Res*, *52*(1), 130. https://doi.org/10.1186/s13567-021-01002-z

Liu, Y., Zhou, M., Xu, S., Khan, M. A., Shi, Y., Qu, W., Gao, J., Liu, G., Kastelic, J. P., & Han, B. (2020). *Mycoplasma bovis*-generated reactive oxygen species and induced apoptosis in bovine mammary epithelial cell cultures. *J Dairy Sci*, *103*(11), 10429-10445. https://doi.org/10.3168/jds.2020-18599

Love, M. I., Huber, W., & Anders, S. (2014). Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol*, *15*(12), 550. https://doi.org/10.1186/s13059-014-0550-8

Luo, H., Wu, X., Xu, Z., Hao, X., Wang, Y., & Li, M. (2020). NOD2/c-Jun NH(2)-Terminal Kinase Triggers *Mycoplasma ovipneumoniae*-Induced Macrophage Autophagy. *J Bacteriol*, *202*(20). https://doi.org/10.1128/jb.00689-19

Lysnyansky, I., Mikula, I., Gerchman, I., & Levisohn, S. (2009). Rapid detection of a point mutation in the parC gene associated with decreased susceptibility to fluoroquinolones in *Mycoplasma bovis*. *Antimicrob Agents Chemother*, *53*(11), 4911-4914. https://doi.org/10.1128/aac.00703-09

Lysnyansky, I., Rosengarten, R., & Yogev, D. (1996). Phenotypic switching of variable surface lipoproteins in *Mycoplasma bovis* involves high-frequency chromosomal rearrangements. *J Bacteriol*, *178*(18), 5395-5401. https://doi.org/10.1128/jb.178.18.5395-5401.1996

Lysnyansky, I., Sachse, K., Rosenbusch, R., Levisohn, S., & Yogev, D. (1999). The vsp Locus of *Mycoplasma bovis*: Gene Organization and Structural Features. *Journal of Bacteriology*, *181*(18), 5734-5741. https://doi.org/doi:10.1128/jb.181.18.5734-5741.1999

Marsh, J. W., Humphrys, M. S., & Myers, G. S. A. (2017). A Laboratory Methodology for Dual RNA-Sequencing of Bacteria and their Host Cells In Vitro. *Front Microbiol*, *8*, 1830. https://doi.org/10.3389/fmicb.2017.01830

Mbanga, J., Amoako, D. G., Abia, A. L. K., Allam, M., Ismail, A., & Essack, S. Y. (2021). Genomic Insights of Multidrug-Resistant *Escherichia coli* From Wastewater Sources and Their Association With Clinical Pathogens in South Africa. *Front Vet Sci*, *8*, 636715. https://doi.org/10.3389/fvets.2021.636715

Miles, K., McAuliffe, L., Persson, A., Ayling, R. D., & Nicholas, R. A. (2005). Insertion sequence profiling of UK *Mycoplasma bovis* field isolates. *Vet Microbiol*, *107*(3-4), 301-306. https://doi.org/10.1016/j.vetmic.2005.02.003

Mitiku, F., Hartley, C. A., Sansom, F. M., Coombe, J. E., Mansell, P. D., Beggs, D. S., & Browning, G. F. (2018). The major membrane nuclease MnuA degrades neutrophil extracellular traps induced by *Mycoplasma bovis*. *Vet Microbiol*, *218*, 13-19. https://doi.org/10.1016/j.vetmic.2018.03.002

Movafagh, S., Crook, S., & Vo, K. (2015). Regulation of hypoxia-inducible factor-1a by reactive oxygen species: new developments in an old debate. *J Cell Biochem*, *116*(5), 696-703. https://doi.org/10.1002/jcb.25074

Moyes, K. M., Drackley, J. K., Morin, D. E., Bionaz, M., Rodriguez-Zas, S. L., Everts, R. E., Lewin, H. A., & Loor, J. J. (2009). Gene network and pathway analysis of bovine mammary tissue challenged with *Streptococcus uberis* reveals induction of cell proliferation and inhibition of PPARgamma signaling as potential mechanism for the negative relationships between immune response and lipid metabolism. *BMC Genomics*, *10*, 542. https://doi.org/10.1186/1471-2164-10-542

Njenga, R., Boele, J., Öztürk, Y., & Koch, H. G. (2023). Coping with stress: How bacteria fine-tune protein synthesis and protein transport. *J Biol Chem*, *299*(9), 105163. https://doi.org/10.1016/j.jbc.2023.105163

Omueti, K. O., Beyer, J. M., Johnson, C. M., Lyle, E. A., & Tapping, R. I. (2005). Domain Exchange between Human Toll-like Receptors 1 and 6 Reveals a Region Required for

Lipopeptide Discrimination*. *Journal of Biological Chemistry*, *280*(44), 36616-36625. https://doi.org/https://doi.org/10.1074/jbc.M504320200

Pilo, P., Fleury, B., Marenda, M., Frey, J., & Vilei, E. M. (2003). Prevalence and distribution of the insertion element ISMag1 in *Mycoplasma agalactiae*. *Vet Microbiol*, *92*(1-2), 37-48. https://doi.org/10.1016/s0378-1135(02)00311-5

Rainard, P., Cunha, P., Bougarn, S., Fromageau, A., Rossignol, C., Gilbert, F. B., & Berthon, P. (2013). T helper 17-associated cytokines are produced during antigen-specific inflammation in the mammary gland. *PLoS One*, *8*(5), e63471. https://doi.org/10.1371/journal.pone.0063471

Ranjitkar, S., Duan, J. E., Srirattana, K., Alqahtani, F., Tulman, E. R., Mandoiu, I., Venkitanarayanan, K., & Tian, X. (2022). Transcriptomic Responses of *Mycoplasma bovis* Upon Treatments of trans-Cinnamaldehyde, Carvacrol, and Eugenol. *Front Microbiol*, *13*, 888433. https://doi.org/10.3389/fmicb.2022.888433

Razavi, M., Kristiansson, E., Flach, C.-F., & Larsson, D. G. J. (2020). The Association between Insertion Sequences and Antibiotic Resistance Genes. *mSphere*, *5*(5), 10.1128/msphere.00418-00420. https://doi.org/doi:10.1128/msphere.00418-20

Razin, S., Yogev, D., & Naot, Y. (1998). Molecular biology and pathogenicity of mycoplasmas. *Microbiol Mol Biol Rev*, *62*(4), 1094-1156. https://doi.org/10.1128/MMBR.62.4.1094-1156.1998

Riollet, C., Mutuel, D., Duonor-Cérutti, M., & Rainard, P. (2006). Determination and characterization of bovine interleukin-17 cDNA. *J Interferon Cytokine Res*, *26*(3), 141-149. https://doi.org/10.1089/jir.2006.26.141

Rodríguez, F., González, J. F., Arbelo, M., Zucca, D., & Fernández, A. (2015). Cytokine expression in lungs of calves spontaneously infected with *Mycoplasma bovis*. *Vet Res Commun*, *39*(1), 69-72. https://doi.org/10.1007/s11259-014-9620-3

Rodwell, A., & Whitcomb, R. (1983). Methods for direct and indirect measurement of mycoplasma growth. *Methods in mycoplasmology*, *1*, 185-196.

S. Jimbo, Suleman, M., Maina, T., Prysliak, T., Mulongo, M., & Perez-Casal, J. (2017). Effect of *Mycoplasma bovis* on bovine neutrophils. *Vet Immunol Immunopathol*, *188*, 27-33. https://doi.org/10.1016/j.vetimm.2017.04.011

Schott, C., Cai, H., Parker, L., Bateman, K. G., & Caswell, J. L. (2014). Hydrogen peroxide production and free radical-mediated cell stress in *Mycoplasma bovis* pneumonia. *J Comp Pathol*, *150*(2-3), 127-137. https://doi.org/10.1016/j.jcpa.2013.07.008

Schukken, Y. H., Günther, J., Fitzpatrick, J., Fontaine, M. C., Goetze, L., Holst, O., Leigh, J., Petzl, W., Schuberth, H. J., Sipka, A., Smith, D. G., Quesnell, R., Watts, J., Yancey, R., Zerbe, H., Gurjar, A., Zadoks, R. N., & Seyfert, H. M. (2011). Host-response patterns of intramammary infections in dairy cows. *Vet Immunol Immunopathol*, *144*(3-4), 270-289. https://doi.org/10.1016/j.vetimm.2011.08.022

Shimizu, T., Kimura, Y., Kida, Y., Kuwano, K., Tachibana, M., Hashino, M., & Watarai, M. (2014). Cytadherence of *Mycoplasma pneumoniae* induces inflammatory responses through autophagy and toll-like receptor 4. *Infect Immun*, *82*(7), 3076-3086. https://doi.org/10.1128/iai.01961-14

Starosta, A. L., Lassak, J., Jung, K., & Wilson, D. N. (2014). The bacterial translation stress response. *FEMS Microbiology Reviews*, *38*(6), 1172-1201. https://doi.org/10.1111/1574-6976.12083

Thomas, A., Linden, A., Mainil, J., Bischof, D. F., Frey, J., & Vilei, E. M. (2005). *Mycoplasma bovis* shares insertion sequences with *Mycoplasma agalactiae* and *Mycoplasma mycoides* subsp. *mycoides* SC: Evolutionary and developmental aspects. *FEMS Microbiol Lett*, *245*(2), 249-255. https://doi.org/10.1016/j.femsle.2005.03.013

Thomas, A., Sachse, K., Farnir, F., Dizier, I., Mainil, J., & Linden, A. (2003). Adherence of *Mycoplasma bovis* to bovine bronchial epithelial cells. *Microb Pathog*, *34*(3), 141-148. https://doi.org/10.1016/s0882-4010(03)00003-2

Timonen, A. A. E., Katholm, J., Petersen, A., Mõtus, K., & Kalmus, P. (2017). Within-herd prevalence of intramammary infection caused by *Mycoplasma bovis* and associations between cow udder health, milk yield, and composition. *J Dairy Sci*, *100*(8), 6554-6561. https://doi.org/10.3168/jds.2016-12267

Torres, F. D., Madureira, K. M., Nascimento da Silva, K., Azevedo, C., de Moura, T. M., Skorei, M. R., & Gomes, V. (2022). Influence of *Mycoplasma bovis* infection on milk production and quality of Holstein dairy cows. *J Dairy Res*, *89*(4), 416-418. https://doi.org/10.1017/s0022029922000814

Touchon, M., & Rocha, E. P. (2007). Causes of insertion sequences abundance in prokaryotic genomes. *Mol Biol Evol*, *24*(4), 969-981. https://doi.org/10.1093/molbev/msm014

Valsala, R., Rana, R., Remesh, A. T., Thankappan, S., & Behera, S. (2017). Effect of *Mycoplasma bovis* on production of pro-inflammatory cytokines by peripheral blood mononuclear cells. *Adv. Anim. Vet. Sci*, *5*(10), 400-404.

Vanden Bush, T. J., & Rosenbusch, R. F. (2002). *Mycoplasma bovis* induces apoptosis of bovine lymphocytes. *FEMS Immunol Med Microbiol*, *32*(2), 97-103. https://doi.org/10.1111/j.1574-695X.2002.tb00540.x

Vasimuddin, M., Misra, S., Li, H., & Aluru, S. (2019). Efficient architecture-aware acceleration of BWA-MEM for multicore systems. 2019 IEEE international parallel and distributed processing symposium (IPDPS),

Vilei, E. M., Nicolet, J., & Frey, J. (1999). IS1634, a novel insertion element creating long, variable-length direct repeats which is specific for *Mycoplasma mycoides* subsp. *mycoides* small-colony type. *J Bacteriol*, *181*(4), 1319-1323. https://doi.org/10.1128/jb.181.4.1319-1323.1999

Vorobjeva, N. V., & Chernyak, B. V. (2020). NETosis: Molecular Mechanisms, Role in Physiology and Pathology. *Biochemistry (Mosc)*, *85*(10), 1178-1190. https://doi.org/10.1134/s0006297920100065

Westberg, J., Persson, A., Pettersson, B., Uhlén, M., & Johansson, K. E. (2002). ISMmy1, a novel insertion sequence of *Mycoplasma mycoides* subsp. *mycoides* small colony type. *FEMS Microbiol Lett*, *208*(2), 207-213. https://doi.org/10.1111/j.1574-6968.2002.tb11083.x

Westermann, A. J., Gorski, S. A., & Vogel, J. (2012). Dual RNA-seq of pathogen and host. *Nature Reviews Microbiology*, *10*(9), 618-630. https://doi.org/10.1038/nrmicro2852

Wu, X., Zhang, S., Long, C., An, Z., Xing, X., Wen, F., & Bao, S. (2021). *Mycoplasmas bovis* P48 induces apoptosis in EBL cells via an endoplasmic reticulum stress-dependent signaling pathway. *Vet Microbiol*, *255*, 109013. https://doi.org/10.1016/j.vetmic.2021.109013

Xu, M., Liu, Y., Mayinuer, T., Lin, Y., Wang, Y., Gao, J., Wang, D., Kastelic, J. P., & Han, B. (2022). *Mycoplasma bovis* inhibits autophagy in bovine mammary epithelial cells via a

PTEN/PI3K-Akt-mTOR-dependent pathway. *Front Microbiol*, *13*, 935547. https://doi.org/10.3389/fmicb.2022.935547

Yang, J., Liu, Y., Lin, C., Yan, R., Li, Z., Chen, Q., Zhang, H., Xu, H., Chen, X., Chen, Y., Guo, A., & Hu, C. (2022). Regularity of Toll-Like Receptors in Bovine Mammary Epithelial Cells Induced by *Mycoplasma bovis*. *Front Vet Sci*, *9*, 846700. https://doi.org/10.3389/fvets.2022.846700

You, X., Wu, Y., Zeng, Y., Deng, Z., Qiu, H., & Yu, M. (2008). *Mycoplasma genitalium*-derived lipid-associated membrane proteins induce activation of MAPKs, NF-kappaB and AP-1 in THP-1 cells. *FEMS Immunol Med Microbiol*, *52*(2), 228-236. https://doi.org/10.1111/j.1574-695X.2007.00366.x

Younis, S., Javed, Q., & Blumenberg, M. (2016). Meta-Analysis of Transcriptional Responses to Mastitis-Causing *Escherichia coli*. *PLoS One*, *11*(3), e0148562. https://doi.org/10.1371/journal.pone.0148562

Zbinden, C., Pilo, P., Frey, J., Bruckmaier, R. M., & Wellnitz, O. (2015). The immune response of bovine mammary epithelial cells to live or heat-inactivated *Mycoplasma bovis*. *Vet Microbiol*, *179*(3-4), 336-340. https://doi.org/10.1016/j.vetmic.2015.07.007

Zhang, H., Lu, D., Zhang, Y., Zhao, G., Raheem, A., Chen, Y., Chen, X., Hu, C., Chen, H., Yang, L., & Guo, A. (2022). Annexin A2 regulates *Mycoplasma bovis* adhesion and invasion to embryo bovine lung cells affecting molecular expression essential to inflammatory response. *Front Immunol*, *13*, 974006. https://doi.org/10.3389/fimmu.2022.974006

Zhang, H., Lu, S., Chao, J., Lu, D., Zhao, G., Chen, Y., Chen, H., Faisal, M., Yang, L., Hu, C., & Guo, A. (2022). The attenuated *Mycoplasma bovis* strain promotes apoptosis of bovine macrophages by upregulation of CHOP expression. *Front Microbiol*, *13*, 925209. https://doi.org/10.3389/fmicb.2022.925209

Zhang, W., & Baseman, J. B. (2011). Transcriptional response of *Mycoplasma genitalium* to osmotic stress. *Microbiology (Reading)*, *157*(Pt 2), 548-556. https://doi.org/10.1099/mic.0.043984-0

Zhao, G., Zhu, X., Zhang, H., Chen, Y., Schieck, E., Hu, C., Chen, H., & Guo, A. (2021). Novel Secreted Protein of *Mycoplasma bovis* MbovP280 Induces Macrophage Apoptosis

Through CRYAB. *Front Immunol*, *12*, 619362. https://doi.org/10.3389/fimmu.2021.619362

Zhu, A., Ibrahim, J. G., & Love, M. I. (2018). Heavy-tailed prior distributions for sequence count data: removing the noise and preserving large differences. *Bioinformatics*, *35*(12), 2084-2092. https://doi.org/10.1093/bioinformatics/bty895

Appendices

Table A3.1 Total number of reads and the percentage mapped to *M. bovis* and *Bos taurus* reference genomes. (INF: co-infection; MACT: MAC-T cells only control; MB: *M. bovis* only control; PBS: PBS infused cow samples; WT: *M. bovis* challenged cows samples; *very small percentage)

Sample name	Total no. of reads	Reads mapped to combined genome	Reads mapped to *M. bovis* reference genome (%)	Reads mapped to *Bos taurus* reference genome
INF01	230511679	230511372	24917942 (10.8)	205593430
INF02	209960724	209960476	23651926 (11.2)	186308550
MACT01	154729573	154729404	10876 (0.0070)	154718528
MACT02	213353567	213353292	836 (0.00039)	213352456
MB01	212838922	212838828	212500290 (99.8)	338538
MB02	190107930	190107846	189628322 (99.7)	479524
PBS01	256434974	256434748	114 (VS*)	256434634
PBS02	254771918	254771722	22 (VS)	254771700
WT01	259448165	259447948	661844 (0.25)	258786104
WT02	248020030	248019792	639384 (0.25)	247380408

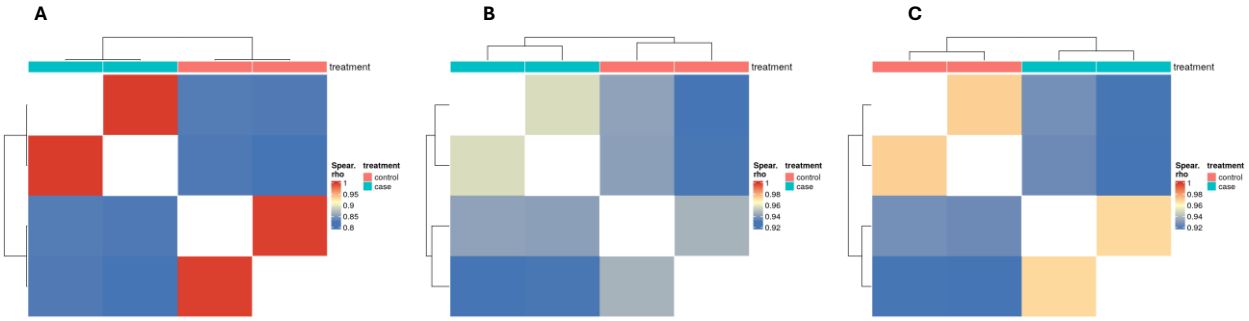

Figure A3.1. Spearman correlation between experimental and control RNA-Seq libraries of *M. bovis* (A), MAC-T cells (B) and mammary tissue (C) groups.

CHAPTER 4: *MYCOPLASMA BOVIS* 5'-NUCLEOTIDASE IS A VIRULENCE FACTOR CONFERRING MAMMARY FITNESS IN BOVINE MASTITIS

Disclosure

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Authors' contribution

The conception and design of the study were collaboratively contributed by OKD, RAA, NS, and IL. Tn-transformation of *M. bovis* was carried out by EDF and CC. PS undertook the *in vitro* characterization of the mutants and conducted the murine mastitis challenge. Immunostaining was performed by YA. The intramammary cow challenge and related experiments were carried out by AG, BEG, RAA, and OKD. GEA performed the statistical analysis of intramammary cow challenge data. IL took the lead in drafting the initial manuscript. All authors actively contributed critical feedback, shaping the research, analysis, and manuscript. They thoroughly read and approved the final manuscript.

Abstract

Nucleases and 5' nucleotidase (5'-NT) play essential roles in cell biology and are often associated with bacterial virulence. In *Mycoplasma* spp., which have limited metabolic capacities and rely on nutrient availability, these enzymes are of significant importance for nucleotide salvage. This study explores the potential role of 2 membrane-associated lipoproteins, the major nuclease MnuA and 5'-NT, in *Mycoplasma bovis* mastitis. Mutants deficient in MnuA (Δ0215) and 5'-NT (Δ0690) were identified through genome-wide transposon mutagenesis of *M. bovis* PG45 type strain and their fitness and virulence were assessed both *in vitro*, in axenic medium, and *in vivo*, using murine and cow mastitis models. The Δ0215 mutant demonstrated reduced nuclease activity, while Δ0690 exhibited slow log-phase growth and impaired hydrolase activity towards ATP and 5' / 3' AMPs. In comparison to the parent strain, the Δ0690 mutant displayed markedly reduced fitness, as evidenced by a significant decrease or even absence in post-challenge mycoplasma counts in murine and cow mammary tissues, respectively. Moreover, the Δ0690 mutant failed to induce mastitis in both experimental models. Conversely, the Δ0215 mutant induced inflammation in murine mammary glands, characterized by neutrophil infiltration and increased expression of major inflammatory genes. In cows, the Δ0215 was able to cause an increase in somatic cell counts in a manner comparable to the wild type, recruit neutrophils, and induce mastitis. Collectively, these findings provide complementary insights, revealing that disruption of 5'-NT significantly attenuated *M. bovis* pathogenicity, whereas a MnuA-deficient mutant retained the ability to cause mastitis.

Keywords: *M. bovis*, nuclease, 5'-nucleotidase, fitness, virulence factors, mastitis

4.1. Introduction

Mycoplasma bovis belongs to the class *Mollicutes*, a fastidious group of bacteria that is characterized by the lack of a cell wall, a reduced genome, limited metabolic capabilities and numerous nutritional requirements (Razin et al., 1998). Despite these limitations, *M. bovis* is a successful pathogen causing a variety of diseases in bovines resulting in significant economic losses (Calcutt et al., 2018). For successful survival and continuous multiplication, mycoplasma must compete with its host for nutrition; hence nutritional resources become a focal point.

Nucleotides, encompassing purines and pyrimidines along with their associated metabolic products, hold indispensable importance for all living organisms. They play crucial roles in vital cellular functions, including nucleic acid synthesis, DNA replication, RNA transcription, DNA repair, energy storage and metabolism, and cell signaling (Hengge et al., 2016; Leiva et al., 2023; Thomas et al., 1970). Additionally, nucleotides serve as constituents of essential coenzymes and assume key roles as activated intermediates in lipid and carbohydrate synthesis (Mikkola, 2020). Due to their critical functions, nucleotides and their metabolites affect bacterial pathogenesis. Indeed, various genes involved in nucleotide biosynthesis are essential for bacterial growth, survival within the host, especially during intracellular replication, and for virulence (reviewed in (Goncheva et al., 2022)). Furthermore, disruptions in nucleotide biosynthesis may induce antibiotic persistence, thereby influencing antibiotic efficacy and contributing to antibiotic treatment failure (Li et al., 2018; Yang et al., 2017).

In most bacteria, nucleotides are synthesized through both *de novo* and salvage pathways. However, the majority of mycoplasmas lack the biosynthetic machinery for *de novo* synthesis (Bizarro & Schuck, 2007; Pollack et al., 1997) and instead rely on pre-formed nucleobases or nucleosides, sourced either within the cell or transported from the external environment, to construct nucleotides. Nucleic acids serve as the primary source for the uptake of nucleotide precursors (Samant et al., 2008), and extracellular DNA (e-DNA) has been identified as a limiting nutrient for the proliferation of *M. bovis* in eukaryotic cells. Supplementation with e-DNA enhances bacterial growth, leading to a cytopathic effect due to H_2O_2 production (Zhu et al., 2019). The ability to non-specifically degrade and process nucleic acids is evidently

advantageous for bacteria, and many *Mycoplasma* species express external membrane-associated nucleases (Minion et al., 1993). A recent study demonstrated that inactivation of *M. bovis* major membrane-associated nuclease MnuA (MBOVPG45_0215) abolished most of the nuclease activity in this pathogen (Sharma et al., 2015). Moreover, it was suggested that MnuA degrades the DNA component of neutrophil extracellular traps (NETs); NETs were observed *in vitro* in bovine neutrophils exposed to the *mnu*A mutant but not in neutrophils exposed to *M. bovis* wild type (WT) or the *mnuA*-complemented strain (Mitiku et al., 2018). It is likely that the breakdown of structural DNA in NETs allows *M. bovis* to evade the host immune response and provides nutrients crucial for bacterial survival and pathogenesis. The degradation of NETs, as well as biofilm DNA backbone, by extracellular nucleases is a known bacterial strategy for survival fitness and virulence, documented in *Streptococcus pyogenes* (Buchanan et al., 2006), *Streptococcus pneumoniae* (Beiter et al., 2006), *Vibrio cholerae* (Pressler et al., 2019), *Staphylococcus aureus* (Kiedrowski et al., 2011; Thammavongsa et al., 2013), *Serratia marcescens* (Eaves & Jeffries, 1963) and several mycoplasmas (Cacciotto & Alberti, 2022; Cacciotto et al., 2019; Li et al., 2019; Yamamoto et al., 2017).

Another enzyme involved in nucleotide salvage is the 5'-nucleotidase (5'-NT), which catalyzes the hydrolysis of ribonucleoside and deoxyribonucleoside phosphates into nucleosides and orthophosphate. 5'-NTs are diverse and multifunctional enzymes widely distributed among mammals, plants, fungi, and bacteria (Zimmermann, 1992). 5'-NTs are classified based on cellular localization, hydrolysis mechanism, and nucleobase specificity (Zakataeva, 2021). Surface-located 5'-NTs, also known as ecto-5′-nucleotidases, have emerged as central regulators of extracellular balance between proinflammatory e-ATP and anti-inflammatory adenosine (Ado) molecules, both are known as important controllers of the immune cell functions acting via purinergic receptors present on various host immune cells (Cekic & Linden, 2016; Idzko et al., 2014). Several extracellular nucleotidases have been identified in Gram-positive and Gram-negative bacteria, serving as important virulence factors that facilitate the pathogen's evasion of the host immune defense (Soh et al., 2020; Zakataeva, 2021). In some pathogens, a synergy between 5'-NTs and secreted nucleases has been reported (Chang et al., 2011; Thammavongsa et al., 2013; Zheng et al., 2015). Recently, through a combination of genomic and metabolomic

analyses, Masukagami et al. identified and annotated a putative 5'-NT (MBOVPG45_0690) in the genome of *M. bovis* (Masukagami et al., 2017).

M. bovis is an important pathogen that causes pneumonia, mastitis, arthritis, otitis media and other diseases in bovines. However, virulence factors underlying its pathogenicity remain poorly understood. Considering the important roles of exonucleases and 5'-NT play in fundamental cellular processes and their association with bacterial virulence, this study aimed to investigate the potential roles of MnuA and 5'-NT in the pathogenesis of *M. bovis* mastitis. Using genome-wide transposon (Tn) mutagenesis, we identified and characterized mutants in MBOVPG45_0215 and MBOVPG45_0690. These mutant clones were further validated under *in vitro* condition, in axenic medium, and in *in vivo*, using mice and dairy cows *M. bovis* mastitis models.

4.2. Materials and methods

Bacterial strains and growth conditions

The *M. bovis* PG45 type strain (ATCC 25523 / NCTC 10131 (Hale et al., 1962) as well as two local *M. bovis* field isolates 514 and 4877, isolated from the milk of a dairy cow with mastitis and the lungs of a calf with pneumonia, respectively (Yair et al., 2020) were used in this study. *M. bovis* was grown at 37 °C in Friis (FF) modified broth medium (Friis, 1975) with 0.5% (w/v) sodium pyruvate and 0.005% (w/v) phenol red (Thermo Fisher Scientific, Waltham, MA, USA). Gentamicin (100 μg/mL; Sigma-Aldrich) was added to the medium for the culture of *M. bovis* mutants created by Tn mutagenesis and puromycin (5 μg/mL; Sigma-Aldrich, Rehovot, Israel) was added for the propagation of the constructs used for complementation study. Stock cultures were grown on FF-modified agar to the titers of 10^8-10^9 colony forming units (CFU)/mL, aliquoted and stocked at -80 °C. The CFU/mL was determined by 10-fold serial dilutions in FF broth and the dilution was plated on FF agar (Rodwell & Whitcomb, 1983). The agar plates were incubated at 37 °C, 5% CO2 and 95% air for 4-7 days.

Construction of a random, genome-wide mutant collection

Transposon mutagenesis was carried out as previously described (Zhu et al., 2020) using *M. bovis* PG45 type strain and a modified version of transposon Tn*4001* (mTn) inserted into the plasmid pMT85 (Chopra-Dewasthaly et al., 2005; Dordet Frisoni et al., 2013; Zimmerman & Herrmann, 2005). Individual colonies of *M. bovis* PG45-mTn mutants were collected from several transformants, grown in 1 mL of selective FF medium supplemented with gentamicin and stored at −80 °C in 96 well-plate format. To identify the specific gene knockouts (Δ0215 and Δ0690), the pools of DNA related to each 96 well plate was subjected to PCR screening using gene-specific as well as pMT85-specific primers either 195R or 3192F, located close to the 5′ and 3′ mTn-inverted repeat sequences, respectively (Aboklaish et al., 2014). In some cases, for example, to identify Δ0215 mutant, primer complementary to the upstream-located MBOVPG45_0216 gene was used. Oligonucleotide synthesis was carried out at Sigma-Aldrich (Rehovot, Israel).

PCR assays were conducted in 25 µL volumes containing 100 ng of template DNA, 0.25 µL of Phire Hot Start II DNA polymerase (Thermo Fisher Scientific) in 1× buffer supplied by the manufacturer, 0.4 µM each primer and 0.2 µM of dNTP mix. PCR amplifications were performed in a C1000 series thermocycler (Bio-Rad, Hercules, CA, USA). The final PCR amplicons were purified using MEGAquick-spin^{TM} plus total fragment PCR Purification Kit (iNtRON, Biotechnology, Gyeonggi, South Korea) and submitted to Sanger sequencing (Hylabs Ltd, Rehovot, Israel). The location of each transposon was identified using the resulting DNA sequence (Wise et al., 2011).

Illumina whole-genome sequencing and bioinformatic analyses

To confirm a single integration site of mTn insertion, whole genome sequences (WGS) of the *M. bovis* PG45 parental strain as well as its isogenic Δ0215 and Δ0690 mutants were performed using Illumina HiSeq (Genotypic Technology Pvt. Ltd., Bangalore, India). The bioinformatic analysis was done using bioinformatic unit services at the Agricultural Research Organization (Volcani Center, Israel) using dedicated pipelines. Briefly, the quality of Illumina raw reads was

checked using FastQC software (Babraham Bioinformatics, Cambridge, UK) and low-quality reads were removed. Quality-tested and filtered reads were mapped to the reference *M. bovis* PG45 genome (Wise et al., 2011). Transposon insertion sites were mapped onto the chromosome of *M. bovis* PG45 genome using BLAST alignment search tool for nucleotides (https://blast.ncbi.nlm.nih.gov/Blast.cgi), Geneious software version R9 (https://www.geneious.com/academic/) and DNASTAR software, version 5.06/5.51, 2003 (Lasergene Inc., Madison, WI, USA). The different functional domains were identified using SwissProt database (https://www.uniprot.org/).

Complementation assay

Plasmid pOH/P was used as a vector for complementation studies as previously described (Baranowski et al., 2010) with some modifications. Briefly, for complementation of the Δ0215 and Δ0690 mutants, MBOVPG45_0215 and MBOVPG45_0690 coding and noncoding regions (NCRs) positions 248826-250217 and 791010-793246 in *M. bovis* PG45 genome (NC_014760), respectively, were cloned into *Not*I restriction site of pOH/P plasmid. The resulting plasmids were transformed into *E. coli* strain DH10β (Thermo Fisher Scientific). Selection of the pOH/P_p0215 and pOH/P_p0690 recombinant plasmids was done on LB plates supplemented with puromycin (5 μg/mL; Sigma-Aldrich). The pOH_pur_F1, pOH_T7_R1 or internal primers were used to amplify and to sequence the inserted fragments (Hylabs).

M. bovis Δ0215 and Δ0690 mutants were transformed with recombinant pOH/P_p0215 and pOH/P_p0690 plasmids, respectively to generate mutation-complemented strains Δ0215::pOH/P_p0215 and Δ0690::pOH/P_p0690 as previously described (Baranowski et al., 2010). A single colony was selected by growing on modified FF agar containing 5 μg/mL puromycin (Sigma-Aldrich) and verified by PCR.

Growth rate assessment of *M. bovis* PG45 and its mutants

Growth rates of the Δ0215 and Δ0690 mutants as well complemented mutant Δ0690::pOH/P_p0690 were compared to the growth rate of WT *M. bovis* PG45 type strain. Briefly, bacterial starters (10^5 CFU/mL) were grown in modified FF broth medium supplemented

with the appropriate antibiotics at 37 °C. Every 24 h, 10 µL of each culture were ten-fold diluted in FF broth, plated on FF agar and incubated at 37 °C with 5% CO2/95%. The number of colonies was counted to determine the concentrations at each time point. Three independent growth trials were performed and cultures were plated in duplicates.

Preparation of *M. bovis* lipoprotein fraction by Triton X-114 phase partitioning

The lipoprotein fraction of *M. bovis* was obtained by the TX-114 fractionation method as previously described (Kornspan et al., 2010). Briefly, mycoplasma cells were pelleted from 1 L of a mid-log-phase broth culture by centrifugation at 8,000 ×*g* for 30 min at 4 ºC, washed three times with Tris-buffered saline (TBS; Sigma-Aldrich) and resuspended in 1 mL TBS containing 1% Triton X-114 and x1 Complete Mini protease inhibitor (both from Sigma-Aldrich) at 4 ºC for 1 h with moderate shaking. Following centrifugation at 5000 ×*g* for 30 min at 4 ºC, the supernatant with soluble proteins was fractionated in three phases with incubation at 37 ºC for 5 min for micelle formation. Phase separation was performed using centrifugation at room temperature for 5 min at 12,000 ×*g*. The upper aqueous phase and a lower detergent phase containing Triton X-114 and lipoproteins. The concentration of the lipoproteins was determined by Pierce BCA Protein Assay Kit (Thermo Fisher Scientific). The lipoproteins were kept at −20 ºC until used.

Measurement of *M. bovis* nuclease activity

Nuclease activity was measured using the Quant-It PicoGreen dsDNA assay kit (Thermo Fisher Scientific) according to the manufacturer's instructions. In brief, the total amount of lipoproteins in each well was normalized to 32 µg while the total amount of DNA in each well was normalized to 2 µg/mL by combining lysate's DNA with λDNA (Sigma-Aldrich) in a total volume of 100 µL in a 96-well black polystyrene plate (Greiner Bio-One, Kremsmünster, Österreich). As a positive control, 1 µg/mL DNase I (Sigma-Aldrich) was added to 2 µg/mL of λDNA. PicoGreen x200 (Sigma-Aldrich, Rehovot, Israel) was diluted in a 1:200 ratio in DNase buffer, and 100 µL were added to each well. For negative control, 2 µg/mL λDNA without mycoplasmal lipoproteins or DNase I was used. The fluorescence signal was measured for 5 h at

37 °C using the SpectraMax i3 multiple detection microplate reader with Ex485nm/Em530nm filter (Molecular Devices, San Jose, California, USA). Results were repeated by three independent experiments, with duplicates per lysate in each experiment.

Measurement of *M. bovis* 5'-NT hydrolyzing activity

Mycoplasma cells, harvested at stationary phase, were washed twice with nucleosidase buffer (50 mM Tris-HCl and 5 mM $MgCl_2$; pH 7.4) and centrifuged at 4000 ×*g* for 10 min at 4 °C. Pellets were resuspended in 1 mL of nucleosidase buffer containing either 1 mM of 5'AMP or ATP or 0.5 mM of 3'AMP (Sigma-Aldrich) to final concentration of 10^9 CFUs/mL of bacterial cells. Reaction samples were incubated at 37 °C for 30 min with shaking at 60 rpm. Bacteria were incubated in nucleosidase buffer without nucleotides as control and the reaction mixture was incubated without bacteria. The reactions were stopped by adding EDTA to a final concentration of 50 mM. The reaction samples were centrifuged at 10,000 ×*g* for 5 minutes at 4 °C and the supernatants were taken and diluted 4-fold with molecular biology grade water (Sartorius, Kibbutz Beit Haemek, Israel). The inorganic phosphate (Pi) was quantified using a malachite green phosphate colorimetric assay kit (Sigma-Aldrich) according to the manufacturer's instruction. In brief, 20 µL of working reagent was mixed with 80 µL of the diluted supernatant was transferred into a 96-well plate (Corning, Corning, NY, USA) and incubated at room temperature for 30 min until color development. The release of Pi was measured at A620 nm and its concentration was calculated against a standard Pi curve.

Murine mastitis model system

Mice challenge of mice was performed as previously described (Schneider et al., 2022; Schneider et al., 2023). Briefly, 12 – 14 week-old BALB/c female mice were used in this study (Envigo, Jerusalem, Israel). IMM challenge with ≈10^9 CFUs /50 µL of *M. bovis* PG45 and its isogenic Δ0215 and Δ0690 mutants was performed 8 days after parturition. IMM infusion was delivered through the teat canal in L4 and R4 abdominal mammary glands. Three mice (six glands) were used in each experiment. Mice were sacrificed 48 h after the challenge, and mammary tissues were collected for histology, bacterial count and RNA extraction. Glands harvested from normal

unchallenged mice were used for control. Mice were maintained under specific pathogen-free (SPF) conditions.

Bacterial counts and histological analysis

Udder tissues were collected for histopatholgy, bacterial count and RNA extraction as previously described (Salamon et al., 2020). Immediately following the harvest, tissue samples were weighed and homogenized in ice-cold PBS. The homogenized samples were plated on FF-modified agar plates with or without gentamicin. After 5 days of incubation at 37 °C grown bacterial colonies were counted as CFU/g of tissue. For histopathological evaluation, samples were fixed in 4% paraformaldehyde (PFA) (Santa Cruz Biotechnology, Inc. Dallas, Texas, USA) and embedded in formalin-fixed paraffin-embedded (FFPE) blocks. Tissue sections were cut at 5 μm thickness and stained with hematoxylin and eosin (H&E) following the standard procedures. For immunohistochemistry (IHC), sections were deparaffinized and hydrated from 100% ethanol to Double Distilled Water (DDW), followed by antigen retrieval, blocking, and immunostaining with anti Ly6g (Lymphocyte antigen 6 complex locus G6D; a marker for monocytes, granulocytes, and neutrophils) antibody. DAB (3,3'-diaminobenzidine) horseradish peroxidase (HRP) substrate (Vector Laboratories, ImmPACT DAB, SK-4105, Burlingame, CA, USA) was used to visualize antibody binding and counterstaining with haematoxylin. After slides were dehydrated and mounted (HistoLab, PERTEX, #00801, Askim, Sweden), 3D HISTECH Pannoramic-250 microscope slide-scanner (3D HISTECH, Budapest, Hungary) was used to capture the image. Case Viewer software was used to take the snapshots (3D HISTECH, Budapest, Hungary).

Relative quantitative real-time PCR

From the harvested lymph node draining L4 or R4 mammary glands of unchallenged and challenged mice RNA was extracted for qPCR (Schneider et al., 2022). Briefly, total RNA was purified from tissue samples using GeneElute Kit (Sigma-Aldrich) and DNase I digestion was performed on-column (Sigma-Aldrich). QScript cDNA Synthesis Kit was used for reverse transcription PCR (Quanta BioSciences, Gaithersburg, MD, USA). The StepOne Plus PCR

instrument was used for PCR (Applied Biosystems, Thermo Fisher Scientific) using the SYBR™ Green PCR Master Mix (Applied Biosystems, Thermo Fisher Scientific). The reactions were performed in triplicates and ΔΔCT method was used to quantify gene expression levels with normalization against prothymosin alpha encoding gene (*ptma*) (Livak & Schmittgen, 2001).

Intramammary challenge of dairy cows

For enrollment into the study, cows from East Tennessee AgResearch and Education Center-Little River Animal and Environmental Unit (ETREC-LRAEU) dairy herd were screened for mastitis by milk somatic cell count (SCC) and bacteriological culture weekly at 28, 21, 14 and 7 days before challenge. Twelve lactating Holstein dairy cows in their 1^{st} to 4^{th} lactations and at 25 – 301 days in milk that fulfilled enrolment criteria (SCC of ≤ 200,000 cells/mL of milk and negative for major bacterial mastitis pathogens and coliform bacteria) were divided into 4 groups of 3 cows each. Each group was transferred from the ETREC-LRAEU to the Johnson Animal Research and Teaching Unit (JARTU) at a time and given one day acclimatization before the challenge. Each animal was confined in its own pen covering 15 m^2 area with separate feed trough and automatic freshwater bowl in the same hall with no contact with each other. Feed and water were given ad libitum following the standard protocol of ETREC-LRAEU dairy farm. Each group was monitored for 7 days post-challenge and euthanized on the 7^{th} day after clinical evaluation and sample collection. There was a one-week interval between completion of the challenge infection of one group and the beginning of the next group for proper cleaning and disinfection of the pens to avoid cross contamination among the cows and the groups.

Challenge dose preparation and challenge infection

The challenge dose preparation was performed as described previously (Gondaira et al., 2020; Kauf et al., 2007) with modifications. Briefly, 250 µL of 10^8 CFU/mL of the wild-type *M. bovis* strain PG45 and its isogenic mutants (Δ0215 and Δ0690) were added to 5 mL of FF broth (Kauf et al., 2007; Knudtson et al., 1986) and incubated at 37 ^oC for 24 h. The bacterial suspension was aliquoted into sterile 1 mL cryovials and stored in -80 ^oC freezer. The titers of the cultures (10^8

CFU/mL) were determined as described above. The viability of the stock cultures at -80 ^{o}C was established ahead of each challenge experiment.

Before actual challenge date, 400 µL (10^8 CFU/mL) stock culture either of wild-type *M. bovis* strain PG45 or the mutants, was inoculated into 40 mL FF broth and grown at 37 ^{o}C until mid-log phase. The growing bacterial culture was centrifuged at 17000 g for 40 min at 4 ^{o}C. The bacterial pellet was resuspended in 40 mL 1X PBS (pH 7.4) and centrifuged at 17000 g for 30 minutes; this step was repeated twice. The final pellet was resuspended in 40 mL sterile endotoxin-free 1X PBS. A 1 mL of *M. bovis* suspension at 10^8 CFU/mL was transferred to 40 mL of sterile endotoxin free PBS. Cows received 1 X 10^6 CFU/mL of WT, Δ0215, or Δ0690 in 5 mL of PBS into two contralateral quarters of each cow (the right front and left rear quarters). Cows in the control group were injected with 5 mL of 1X PBS (pH 7.4) in a similar manner. To determine the challenge dose, immediately after infusion the remaining bacterial suspension was plated and CFU was determined by viable colony counts as described above.

Post-challenge health monitoring

Following the challenge, cows were monitored for 7 days for the development of mastitis or any other health problems. Cows were milked twice a day at 5 AM and 5 PM using Melasty portable milking machine (Melasty, Bursa, Turkey). Clinical mastitis was detected by abnormal changes in the milk and mammary glands tissue were recorded following previously described scoring system (Merrill et al., 2019). In addition, individual quarter milk samples were tested by California Mastitis Test (CMT) (ImmuCell Corporation, Portland, Maine, USA) and SCC.

Milk samples collection and processing

Before sample collection, teats were pre-dipped in antiseptic solution (OPI blue, DeLaval, Inc., Kansas, MO) and wiped with paper towels to remove bacteria on teat skin and teat opening. Individual udder milk samples (3 mL) used for bacteriological analysis were aseptically collected into sterile 15 mL falcon centrifuge tubes (Thermo Fisher Scientific), transported to the lab on ice and cultured immediately upon arrival in about 1-2 h of collection; the remaining samples were stored at -20 °C until culture results were obtained.

Individual udder milk samples were collected daily for SCC into 50 mL Capitol Vials (Thermo Fisher Scientific) tubes containing potassium dichromate pills as preservative (Capitol Vial™, Auburn, AL, USA). The SCC was measured at the Center for dairy Advancement and sustainability Precision Dairy Quality Laboratory, Knoxville, TN, USA using the Foss Foss Instruments (The Foss Instruments Inc., Eden Prairie, MN, USA). All udder quarters were dipped in an antiseptic solution (Bovadine: 1% iodine solution, WESTAGRO, Kansas, MO, USA) post-milking daily to prevent the spread of mycoplasma or entry of other bacterial mastitis pathogens during milking.

Milk bacteriological culture for isolation and identification of *M. bovis*

Bacteriological culture and analysis was conducted as described by National Mastitis Council (NMC) (Adkins, et al. 2017) with slight modification. Briefly, 100 µL of milk sample was spread onto individual FF agar plates and tryptic soy agar plates (TSA) with 5% sheep blood (blood agar) (BD Difco™, Sparks, MD, USA) and incubated at 37 °C, 5% CO_2:95% incubator for 7 days for FF and for 24 to 48 h for TSA plates. Representative mycoplasma colonies were tested by matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF) for confirmation. The TSA plates were examined for colony growth visually and characteristics of each colony such as size, morphology, hemolysis, and pigment production. Colonies were differentiated to genus/species level using biochemical tests.

Euthanasia procedure

On the seventh day after the challenge, cows were transported to a necropsy facility of Anatomic Pathology Laboratory, Department of Biomedical and Diagnostic Sciences, College of Veterinary Medicine, the University of Tennessee. Cows were sedated with intramuscular injection of xylazine at 0.73 mg/kg of body weight (100 mg/mL conc.) and euthanized with pentobarbital at 43 mg/kg of body weight (Euthasol 390 mg/mL conc.; Virbac AH, Inc., Fort Worth, TX, USA).

Necropsy and sample collection

Samples of secretory region of mammary gland tissue were collected in 10% neutral buffered formalin (NBF) (Thermo Fischer Scientific) and submitted to the diagnostic laboratory services of the College of Veterinary Medicine, the University of Tennessee for H&E staining using their standard procedure (https://vetmed.tennessee.edu/vmc/dls/dls-forms-faqs-resources/). In addition, swabs of other organs that are known to be the predilection sites for *M. bovis* infection were collected for bacterial isolation. Briefly, swab samples of both hock and knee (carpal) joint fluid, bronchioles and lower respiratory tract regions of both lungs, uterus and middle ear were collected and transferred into sterile 15 mL Falcon tubes containing 5 mL FF broth and transported on ice to the laboratory. Each swab sample was rolled onto one FF agar plate and incubated at 37 ^{o}C in 5% CO_2:95% for 7 days. Representative colonies were tested by MALDI-TOF to confirm *M. bovis*.

Statistical analysis

Statistical differences between *M. bovis* PG45, the Δ0215 and Δ0690 mutants and complemented strains in growth rate, nuclease and hydrolytic activities were determined using unpaired t-test, while mean bacterial loads and genes' relative expression (murine mastitis model) were calculated using non-parametric Mann–Whitney two-independent-samples test by comparing experimental groups. The values were tested by a square root transformation and failed to follow a normal distribution; thus, non-parametric statistics were used instead. Statistical analyses were performed in GraphPad Prism 9.1.2 (GraphPad Software, Inc.), and a P value of <0.05 was considered significant.

Experimental intramammary challenge infection in dairy cows

Comparing challenge groups against the control group

For the analysis of quantitative outcomes (SCC, bacterial count), mixed effects linear regression with maximum likelihood estimation method was first fitted by including cow ID as a random effect. The mixed effects model was then compared against fixed effects linear regression by

likelihood ratio test (LRT); when LRT was not significant, the fixed effects linear regression model was used. In all models, a full factorial design was used that evaluated the interaction between treatment and day, and their main effects. Starting with the full model, the significance of each term was assessed with the LRT by sequentially removing one term at a time. Inferences and graphs are based on the final model. The PBS group, the non-challenged quarters and the baseline (Ch+1) measurements served as references in all the analyses. Quarter SCC and *M. bovis* count data were $\log_{10}$ transformed and analyzed with mixed effects linear regression by including the random effect of animals and the fixed effects of treatment, day, and quarter challenge status (yes/no) and all interaction terms as full factorial analysis. Since the three-way interaction was significant for both outcomes (SCC and CFU/mL), contrasts of marginal means between two dependent variables were obtained at the levels of the third variable. Contrasts were adjusted for multiple comparisons by Sidak method. Individual quarter milk CMT scores of abnormal changes in individual quarter gland tissue, scores of abnormal changes in individual quarter milk, and mastitis were compared among the treatment groups and quarter challenge status by Fisher's exact test.

Comparison between mutants and the wild-type

The $\log_{10}$ SCC and $\log_{10}$ CFU were also compared in a similar manner except that the effects were also adjusted for challenged and unchallenged quarters. To compare udder quarters' CMT scores, individual quarter tissue abnormality scores and individual quarter milk abnormality scores, the data were binary transformed by re-categorizing scores > 0 into one category. Binary transformed data were then compared between the WT and the mutant strains either by logistic regression modelling or Fisher's exact test, as appropriate. Similarly, quarter mastitis status was binary transformed by recategorizing the Subclinical mastitis (SCM) and Clinical mastitis (CM) cases together and analyzed with logistic regression modelling. Dairy cow challenge experiment data were analyzed using STATA 18 (StataCorp LLC, College Station, TX, USA).

4.3. Results

Identification of *M. bovis* PG45 mutants deficient in the major membrane nuclease MnuA and the 5'-NT

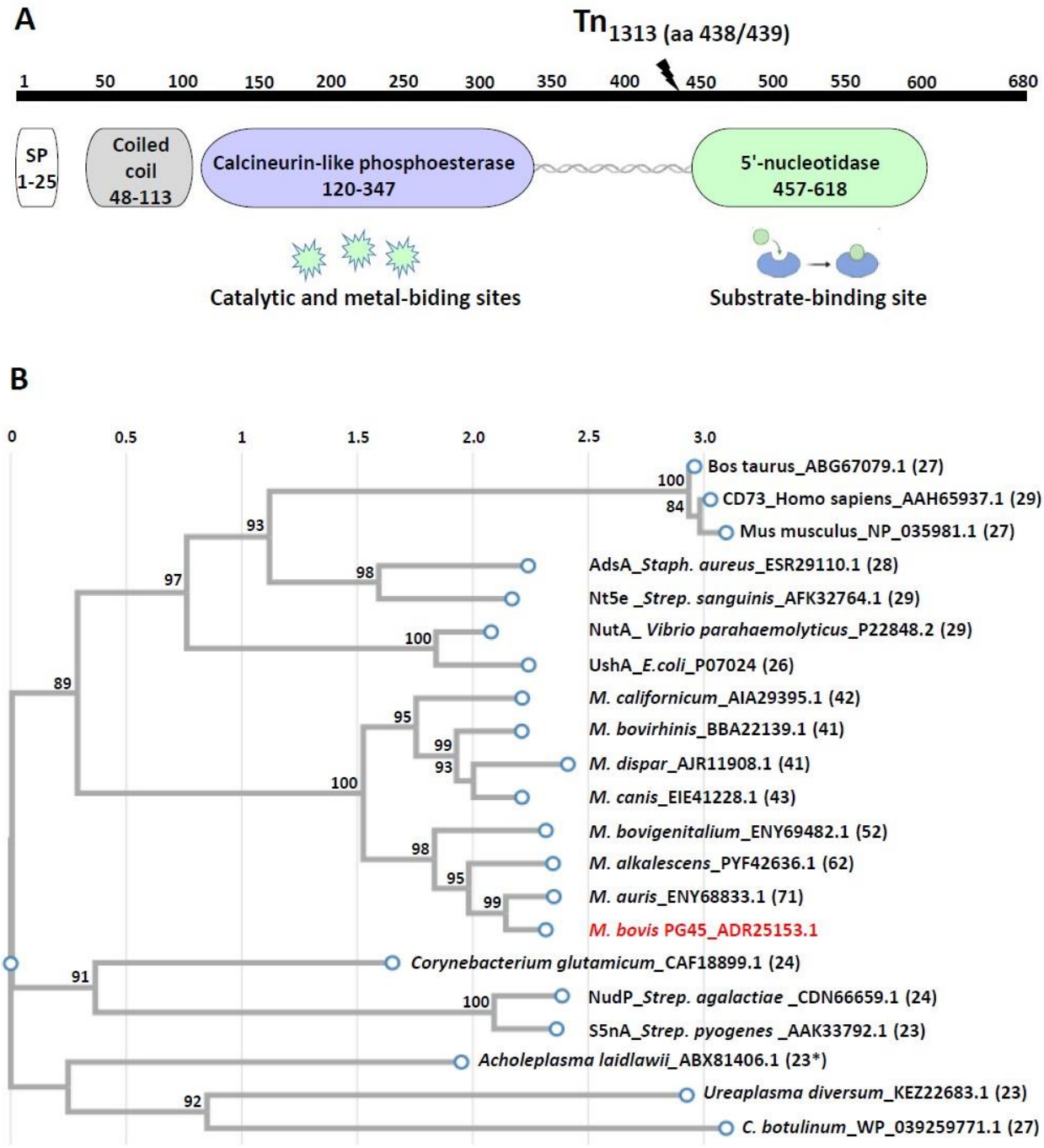
A library of 1700 individual mutants of the *M. bovis* PG45 was screened using MBOVPG45_0215 (*mnuA*) or MBOVPG45_0690 (5'-NT) gene-specific primers, coupled with mTn-specific primers, as detailed in the Materials and Methods section. The Δ0215 mutant, upon identification, exhibited a single mTn insertion in the *mnuA* gene, precisely 295 bp from the predicted start codon and the Δ0690 mutant displayed an insertion located 1313 bp into the 5'-NT gene. The phenotypic features of these mutants along with their complemented strains are described below, both *in vitro* and *in vivo*.

Domain structure and conserved amino acid residues in 5'-NT of *M. bovis*

While the detailed characterization of MnuA has been previously documented (Sharma et al., 2015), limited information exists about 5'-NT. MBOVPG45_0690 encodes a putative surface-exposed lipoprotein spanning 680 amino acids (aa) (Fig. 4.1A). The protein has an N-terminal signal peptide (SP; 1-25 aa) and is initially synthesized as a precursor, undergoing subsequent processing into a mature lipoprotein. The estimated molecular mass of the predicted mature protein is 73.3 kDa, with an isoelectric constant (pI) of 8.13.

M. bovis 5'-NT is a homolog of *Escherichia coli*'s UshA protein exhibiting a shared 26% identity. It belongs to the cluster of orthologous groups (COG) coding for 2',3'-cyclic-nucleotide 2'-phosphodiesterase/5'- or 3'-nucleotidase, 5'-nucleotidase (COG0737). This protein is associated with various superfamilies, such as UshA (cl34025), metallophosphatase (cl13995), and bifunctional UDP-sugar hydrolase/5'-nucleotidase periplasmic precursor (cl35858). According to the SwissProt database (https://www.uniprot.org/), *M. bovis* 5'-NT displays a coiled-coil region (48-113 aa) and two domains – an N-terminal calcineurin-like phosphoesterase domain, also known as the metallophos domain (120-347 aa; Pfam ID PF00149) and a 5'-nucleotidase, C-terminal domain (457-618 aa; 5_nucleotid_C, _PF02872) (Fig. 4.1A). Similar to UshA, the putative catalytic and metal-binding sites are located in the N-terminal domain of

Figure 4.1. Schematic presentation of *M. bovis* PG45 5'-NT and phylogenetic tree of its homologs. (A) Schematic diagram of the surface-exposed *M. bovis* 5'-NT lipoprotein is depicted based on prediction from UniProtKB software at Swiss-Prot (https://www.uniprot.org/). The diagram highlights key features, including the coiled coil (gray) sequence, calcineurin-like phosphoesterase (Metallophos, PF00149; violet), and 5'-nucleotidase (5_nucleotide_C, PF02872; green) domains. The grey helix signifies the region linking the calcineurin-like phosphoesterase and 5'-nucleotidase domains of *M. bovis* 5'-NT. The position of mTn insertion in the 15F3 mutant is shown by a black vertical arrow. SP, signal peptide sequence. The numbers on top indicate amino acid position. (B) Phylogenetic tree construction of 5'-NTs. The rooted phylogenetic tree of the *M. bovis* PG45 5′-NT was generated through multiple sequence alignment and phylogenetic reconstructions. ClustalW and the 'build' function of ETE3 3.1.2 by Huerta-Cepas (Huerta-Cepas et al., 2016) were employed for these analyses, as implemented on GenomeNet (https://www.genome.jp/tools/ete/). The tree construction utilized fast tree with slow NNI and MLACC=3 (Price et al., 2010). Bootstrap values are indicated at branching points, providing confidence in the tree topology. Accession numbers follow the species' names, and the percent identity with the 5'-NT of *M. bovis* PG45 is presented in parentheses. * - percent identity with the 5'-NT of *A. laidlawii* (total 574 aa) was calculated over a 243-aa alignment only. Bovine mycoplasma species without identified 5'-NT available in genomes databases include *M. alvi, M. tauri, M. testudinis, M. canadense, M. bovoculi, M. arginine, M. leachii, M. mycoides* subsp. *mycoides* SC and *M. wenyonii*.


A
Tn1313 (aa 438/439)
1
50
100
150
200
250
300
350
400
450
500
550
600
680
SP
1-25
Coiled
coil
48-113
Calcineurin-like phosphoesterase
120-347
5'-nucleotidase
457-618
Catalytic and metal-biding sites
Substrate-binding site
B
0
0.5
1
1.5
2.0
2.5
3.0
Bos taurus_ABG67079.1 (27)
CD73_Homo sapiens_AAH65937.1 (29)
Mus musculus_NP_035981.1 (27)
AdsA_Staph. aureus_ESR29110.1 (28)
Nt5e _Strep. sanguinis_AFK32764.1 (29)
NutA_ Vibrio parahaemolyticus_P22848.2 (29)
UshA_E.coli_P07024 (26)
M. californicum_AIA29395.1 (42)
M. bovirhinis_BBA22139.1 (41)
M. dispar_AJR11908.1 (41)
M. canis_EIE41228.1 (43)
M. bovigenitalium_ENY69482.1 (52)
M. alkalescens_PYF42636.1 (62)
M. auris_ENY68833.1 (71)
M. bovis PG45_ADR25153.1
Corynebacterium glutamicum_CAF18899.1 (24)
NudP_Strep. agalactiae _CDN66659.1 (24)
S5nA_Strep. pyogenes _AAK33792.1 (23)
Acholeplasma laidlawii_ABX81406.1 (23*)
Ureaplasma diversum_KEZ22683.1 (23)
C. botulinum_WP_039259771.1 (27)
100
84
93
98
97
100
89
95
99
93
100
98
95
99
91
100
92

M. bovis 5'-NT, while the substrate-binding pocket is present in the C-terminal domain (Fig. 4.1A). The N- and C-terminal domains of *M. bovis* 5'-NT are linked by a 109 aa (348-456 aa), in contrast to the 19 aa in UshA of *E. coli* (Fig. 4.1A).

Within the group of bovine-related *Mycoplasma* spp., homologs to the *M. bovis* 5'-NT were primarily identified in species related to the same phylogenetic group, namely the Hominis group. The closest bovine-related *Mycoplasma* spp., *M. auris* and *M. alkalescens*, exhibited 71% and 62% identity, respectively to the *M. bovis* PG45 5'-NT. Additionally, limited but noteworthy amino acid identity was observed between *M. bovis* 5'-NT and nucleotidases present in the genomes of other pathogenic bacteria, as well as mammals (Fig. 4.1B).

Identification of *M. bovis* field isolates with truncated MnuA protein

To identify *M. bovis* strains harboring naturally truncated MnuA or 5'-NT coding genes, we performed a BLAST search analysis using the MBOVPG45_0215 and MBOVPG45_0690 genes as query sequences. Although no field strains of *M. bovis* with a truncated 5'-NT were identified, several had alterations in the *mnuA* encoding region, causing a shift in the translational reading frame of MnuA. Specifically, two strains, 4877 (WFEJ01000016.1) and 514 (WFAW01000057.1) were identified in the cohort of Israeli isolates (Yair et al., 2020) and their *mnuA*-encoding regions were confirmed through PCR and Sanger sequencing. The *M. bovis* 4877 and 514 strains exhibited one (A) or four (AAAG)-base deletions within the $_{164}$AAAAAAAAGAA$_{174}$ region of the MBOVPG45_0215, respectively resulting in truncated proteins. Furthermore, *M. bovis* 17DD0020 (NZ_JASFAP010000001.1, isolated in Germany (Triebel et al., 2023) with a *mnuA* sequence identical to that of *M. bovis* 4877, and *M. bovis* F9160 (CP092777.1, isolated in France (Ambroset et al., 2022) containing two-bases (AG) deletion within the same region of the *mnuA* gene, leading to the truncation of MnuA were also identified. Additionally, several *M. bovis* strains, isolated in Belgium (Bokma et al., 2020), contained single or multiple changes in other regions of the *mnuA* gene, resulting in the production of abortive MnuA were identified.

In vitro validation and characterization of *M. bovis* mutants Δ0690 and Δ0215

The Δ0690 mutant displayed a reduced growth rate and smaller colony size compared to the wild type

To assess the impact of *mnu*A and MBOVPG45_0690 disruption on the growth of mutants in axenic (modified FF) medium, we compared their growth rates and colony sizes with those of the *M. bovis* PG45 wild-type. The growth rate of the Δ0690 mutant was significantly slower during the logarithmic phase, while no differences were observed between the Δ0215 mutant and *M. bovis* PG45 (Fig. 4.2A and Fig. 4.2B). Additionally, the Δ0690 mutant displayed a smaller colony size on agar compared to the WT and Δ0215 (Fig. 42C). The growth rate as well as colony size were fully restored upon complementation of the Δ0690 mutant using a pOH/P plasmid containing an intact MBOVPG45_0690 gene as well as its upstream and downstream noncoding regions (NCRs; Δ0690::pOH/P_p0690). This restoration indicates that these phenotypes are directly linked to the MBOVPG45_0690 gene (Fig. 4.2).

The Δ0690 mutant exhibited significant impairment in the hydrolysis of ATP, 5'AMP, and 3'AMP

To assess whether the disruption of MBOVPG45_0690 results in a loss of hydrolytic activity by 5'-NT, bacterial cells of *M. bovis* WT and the Δ0690 mutant were incubated with ATP, 5'AMP, or 3'AMP, and the release of inorganic phosphate (Pi) was measured using the malachite green colorimetric assay, as described in Materials and Methods. Compared to the WT, the total release of Pi was significantly lower for all substrates, particularly for 5'/3'AMP, upon incubation with the Δ0690 mutant (Fig. 4.3A). Complementation of the Δ0690 mutant restored the hydrolytic activity of *M. bovis* against ATP, 5'AMP, and 3'AMP substrates (Fig. 4.3A).

The Δ0215 mutant exhibited a loss of nuclease activity

Using real-time picogreen DNase assay, we tested the nuclease activity of Triton X-114 partitioned hydrophobic protein fractions of *M. bovis* PG45 and its two mutants in the presence of double-

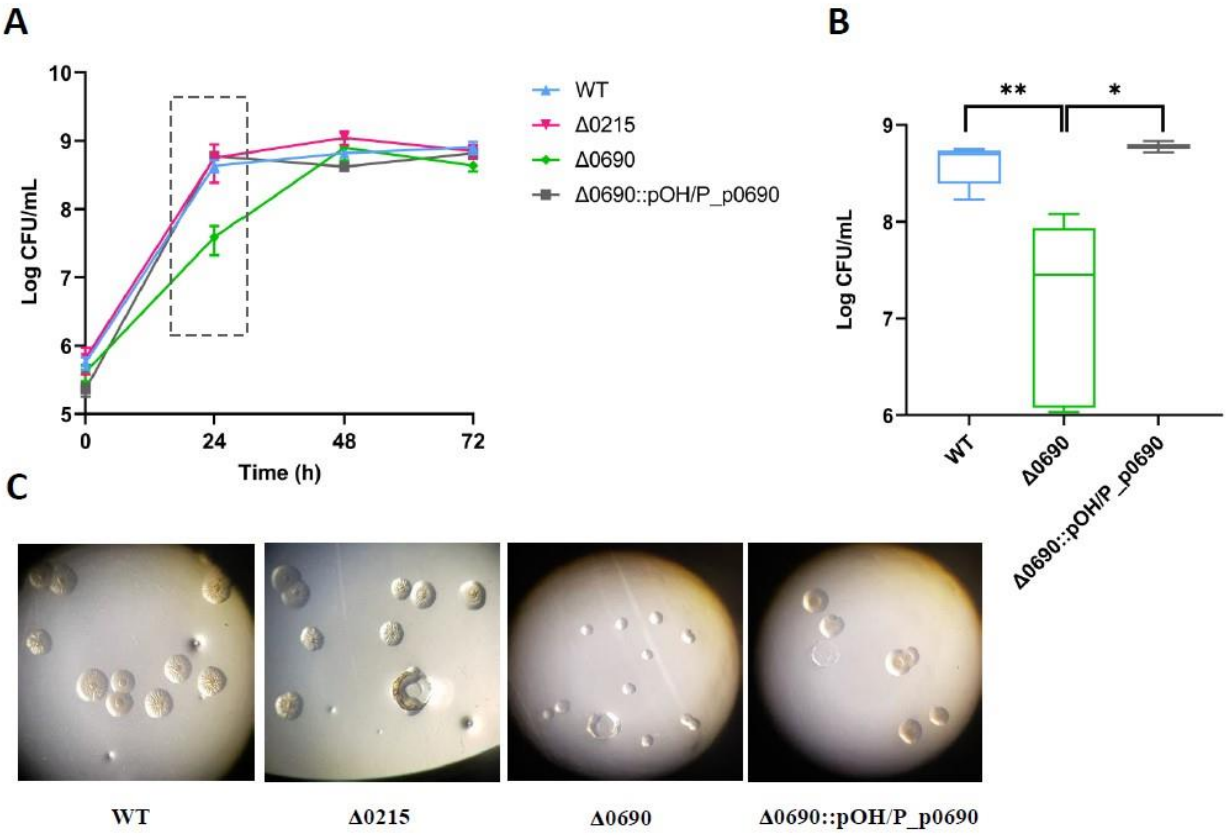


Figure 4.2. Impact of 5'-NT disruption on *M. bovis* growth phenotype in axenic conditions. (A) Growth curve analysis of *M. bovis* PG45 WT, mutants Δ0215 and Δ0690 and complemented strain Δ0690::pOH/P_p0690 under cultivation in modified FF medium. Mycoplasma titers were determined every 24 hours over a total incubation period of 72 hours. The data are presented as the means of three independent assays, with standard deviations indicated by error bars. The hatched rectangle shows a delay in the growth rate of the Δ0690 mutant observed during first 24 hours. (B) Statistical significance between the growth rates of the WT, the Δ0690 and its complementation at 24-hour time point was assessed using an unpaired t-test. P values are indicated by asterisks (*P<0.05; **P<0.001). (C) Micrographs of *M. bovis* PG45 (WT), Δ0215 and Δ0690 mutants, and the complemented Δ0690::pOH/P_p0690 colonies, grown on modified FF agar for 6 days. The images were captured under a light microscope using the same settings and magnification (x 2.5).

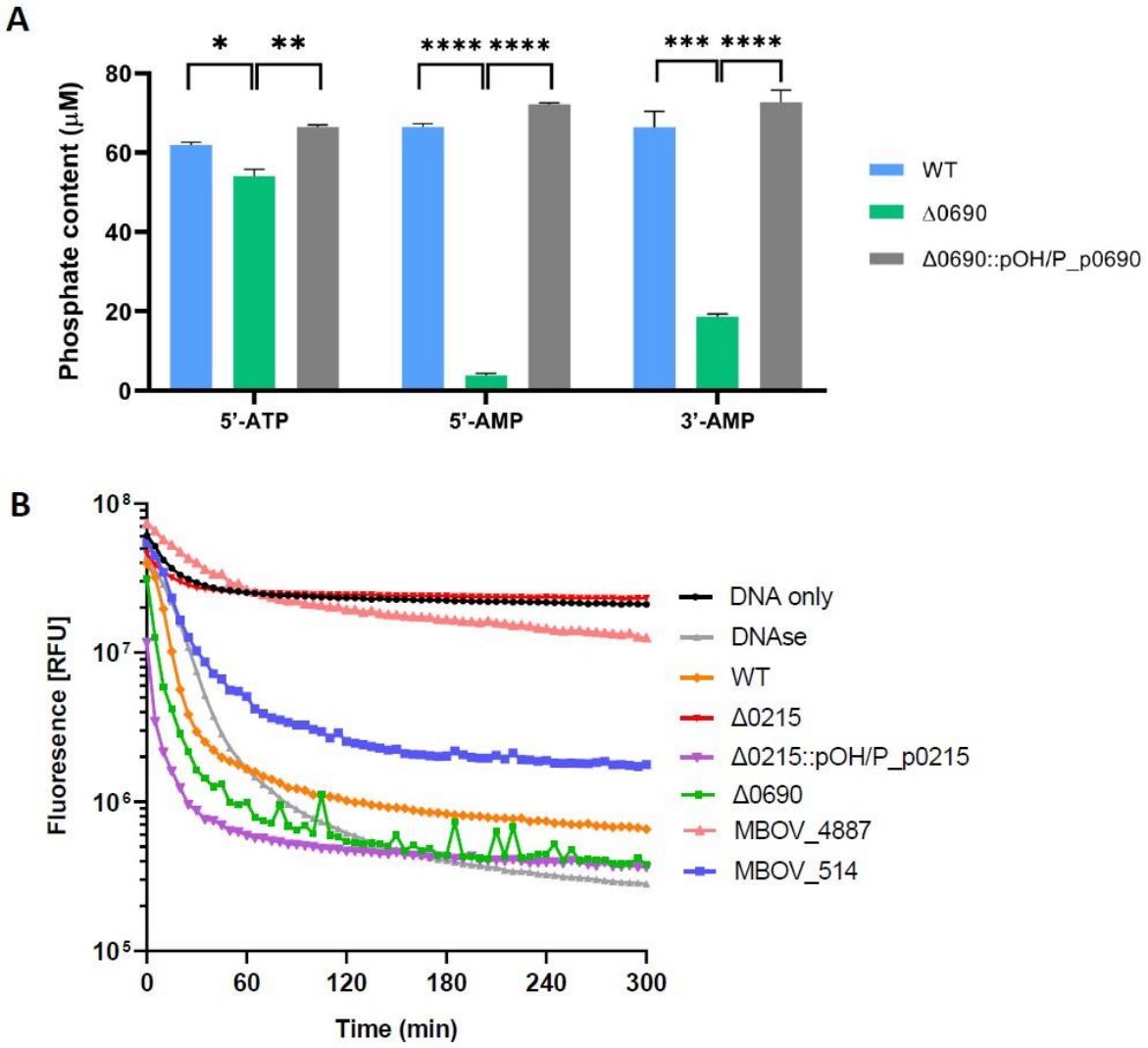


Figure 4.3. *In vitro* characterization of the Δ0215 and Δ0690 mutants. (A) The hydrolytic activity of the Δ0690 mutant towards ATP, 5' AMP and 3' AMP was evaluated by assessing phosphate content (inorganic phosphate release) after incubation of mycoplasma cultures with the indicated substrates for 30 min at 37°C. The release of inorganic phosphate was measured using a malachite green phosphate colorimetric assay kit. Data represent results from 3 independent experiments conducted in triplicates, presented as the mean + SEM. Statistical significance was determined by an unpaired t-test. *P<0.05 **P<0.01 ****P<0.0001. (B) Nuclease activity of *M. bovis* Triton X-114-fractionated hydrophobic protein fractions measured by Real-time PicoGreen DNase assay. The assay conditions are described in Materials and Methods. The fluorescence signal expresses the amount of the dsDNA measured over 5 hours at 37 °C using the SpectraMax i3 multiple detection microplate reader. Data represent the mean + SEM.

stranded (ds) phage λ-DNA. The results revealed that both the WT and the Δ0690 mutant efficiently degraded 500 ng of dsDNA as evidenced by a decrease in picogreen fluorescence. In contrast, the Δ0215 mutant failed to digest dsDNA (Fig. 4.3B). The nuclease activity of the Δ0215 mutant was restored upon complementation using a pOH/P plasmid containing an intact *mnuA* gene and its NCRs (Δ0215::pOH/P_p0215; Fig. 4.3B). The nuclease activity of the *M. bovis* 4877 and 514 field isolates, which contained truncated MnuA protein, was also compared with that of *M. bovis* PG45 WT and the Δ0215 mutant. While the *M. bovis* 514 strain demonstrated reduced nuclease activity, the *M. bovis* 4877 strain exhibited a loss of nuclease activity, similar to that of the Δ0215 mutant (Fig. 4.3B).

In vivo validation and characterization of *M. bovis* mutants Δ0690 and Δ0215 using murine and bovine mastitis models

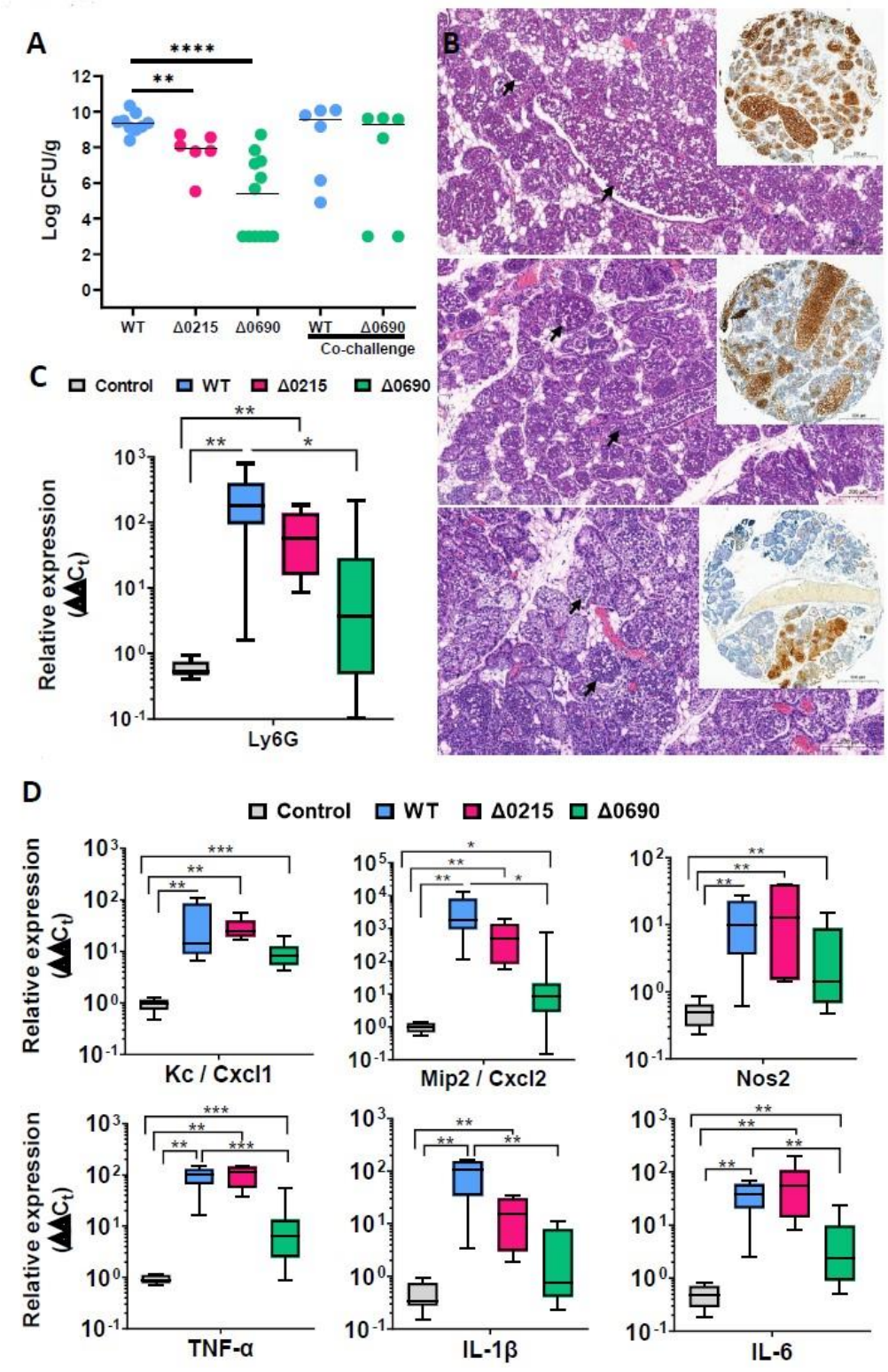
Inactivation of MnuA and 5'-NT led to reduced fitness of *M. bovis* PG45 in murine mammary glands

The loss of nuclease activity in the Δ0215 mutant and the inability to hydrolyze nucleosides in the Δ0690 mutant may be associated with a loss of mammary virulence and attenuation of *M. bovis* PG45. To investigate this hypothesis, we utilized the recently established mycoplasma murine mastitis model in lactating mice (Schneider et al., 2022; Schneider et al., 2023) and compared the disease induced by *M. bovis* PG45 WT and the Δ0215 or Δ0690 mutants (Fig. 4.4).

The results demonstrated that the inactivation of *mnu*A and MBOVPG45_0690 significantly reduced the colonization of *M. bovis* in the mammary gland, as evidenced by a significant reduction in bacterial counts for the Δ0215 and especially the Δ0690 mutants compare to the WT (Fig. 4.4A). Mammary gland colonization of the Δ0690 mutant was rescued following co-challenge with *M. bovis* PG45 WT (Fig. 4.4A).

Murine infected glands that developed mastitis were characterized by perfuse neutrophil recruitment into milk spaces, which was also quantified using immunofluorescence staining and relative qPCR of the lymphocyte antigen 6 complex, locus G gene (LY6G; Fig. 4.4B and Fig. 4.4D). Notably, while no significant differences in the expression of inflammatory marker genes

Figure 4.4. Inactivation of MnuA and 5'-NT reduced bacterial fitness and virulence of *M. bovis* PG45 in mammary glands. Mammary virulence of *M. bovis* PG45 and attenuation of the Δ0215 and Δ0690 mutants were demonstrated in lactating BALB/c mice following intramammary challenge of L4 and R4 glands with approximately 10^8 CFUs of bacteria. Bacterial colonization observed after challenge with mutant strains Δ0215 and Δ0690 was significantly reduced (scatter plot in panel A), with each data point representing a single gland, and the horizontal bars indicating the median of data from three or more mice. Bacterial counts of Δ0690 mutant were rescued following co-challenge with WT *M. bovis* PG45 (A). Disease manifestation was characterized by neutrophil recruitment into alveolar milk spaces and demonstrated in representative microscopic images of H&E and anti Ly6G (neutrophil marker) immunohistochemical staining (round insets in B) of paraffin section from WT, Δ0215 and Δ0690 challenged glands (B; top, middle, and bottom panels, respectively). Using RT-qPCR, the relative expression of the neutrophil marker Ly6G (C) and inflammatory marker genes MIP2, KC, Nos2, TNFα, IL1β, and IL6 (D), was quantified relative to RNA samples extracted from the mammary tissues of normal non-challenged lactating control mice. Data are presented as box plots showing higher neutrophil recruitment and expression of inflammatory markers following challenge with WT bacteria compared to the mutant strains. Statistical significance was determined by non-parametric Mann–Whitney two-independent-samples test, with a *P* value of 0.05 or less considered significant. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Scale bars; 200 μm and 500 μm are shown (B).


A
B
C
D
Log CFU/g
WT
Δ0215
Δ0690
Co-challenge
Control
Relative expression (ΔΔCt)
Ly6G
Kc / Cxcl1
Mip2 / Cxcl2
Nos2
TNF-α
IL-1β
IL-6

were identified in glands, challenged with the WT and Δ0215 mutant, the relative expression of the LY6G as well as the genes MIP2, KC, IL1β, TNFα, and IL6 was significantly decreased in glands challenged with Δ0690 mutant (Fig. 4.4C and Fig. 4.4D). These findings support the conclusion that Δ0215, but especially Δ0690 mutants exhibited reduced bacterial fitness and attenuation in the murine mammary glands.

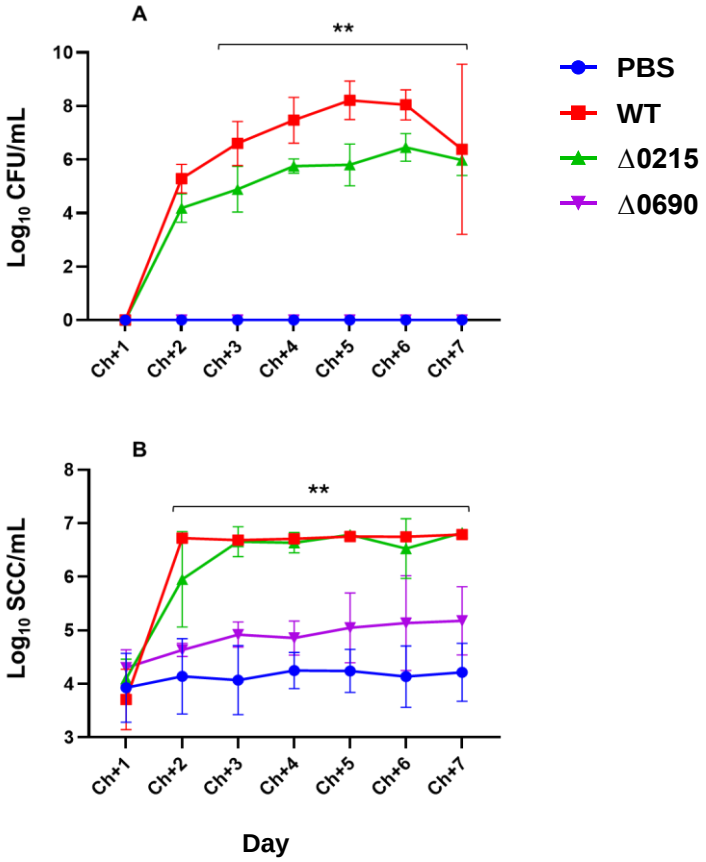
The inactivation of 5'-NT led to loss of fitness and attenuated virulence of *M. bovis* PG45 in bovine mammary glands

To strengthen our observations in the murine model and further validate the results, we conducted a complementary *in vivo* challenge in cows using *M. bovis* WT and its mutants. Normal lactating cows were challenged by intramammary (IMM) infusion of *M. bovis* WT, and Δ0215 and Δ0690 mutants. Two diagonal quarters in 3 cows were challenged with each strain or PBS as controls, while the complementary diagonal quarters were served as non-challenged controls. Clinical evaluations of cows and individual quarters were conducted using the clinical mastitis score (CMS) of milk and udder, and CMT scores and milk samples were collected for bacterial counts daily over a 7-day period following challenge. At the end of the study, all animals were sacrificed, and mammary tissues were sampled for histological analysis.

Based on the bacterial counts (Fig. 4.5A), SCC (Fig. 4.5B), combined CMS_CMT scores (Fig. 4.5C) and histological analysis (Fig. 4.6), our results demonstrated a loss of mammary fitness and attenuation by the Δ0690 mutant. Indeed, Δ0690 mutant was unable to colonize the lactating bovine mammary gland, whereas the WT and Δ0215 mutant successfully established colonization of the challenged glands (Fig. 4.5A) and elicited inflammatory response, characterized by high SCC, clinical mastitis (Fig. 4.5B and Fig. 4.5C) and neutrophil recruitment into the alveolar and milk spaces (Fig. 4.6).

More specifically, the CMS_CMT scores in cows challenged with Δ0215 did not show a significant difference (P=0.103) compared to WT-challenged cows. In contrast, cows challenged with Δ0690 demonstrated significantly lower scores ($P<0.001$) compared to the WT-challenged group (Fig. 4.5C). Furthermore, in comparison to the PBS-challenged group, a significant

Figure 4.5. Loss of mammary fitness and virulence by *M. bovis* Δ0690 mutant. Lactating cows were treated by IMM infusion with WT, Δ0215, and Δ0690 bacterial strains or PBS as non-challenged controls. Scatter plots show daily (days 1-7 after challenge) bacterial counts (CFU/ml; A), somatic cell counts (SCC; B), and individual quarter level of clinical mastitis (CMS; C) for each treatment in challenged glands. Plots were constructed using SuperPlotOfData (Goedhart, 2021). ** $P<0.001$.


A
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Log10 CFU/mL
10
8
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Ch+3
Ch+4
Ch+5
Ch+6
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PBS
WT
Δ0215
Δ0690
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Log10 SCC/mL
8
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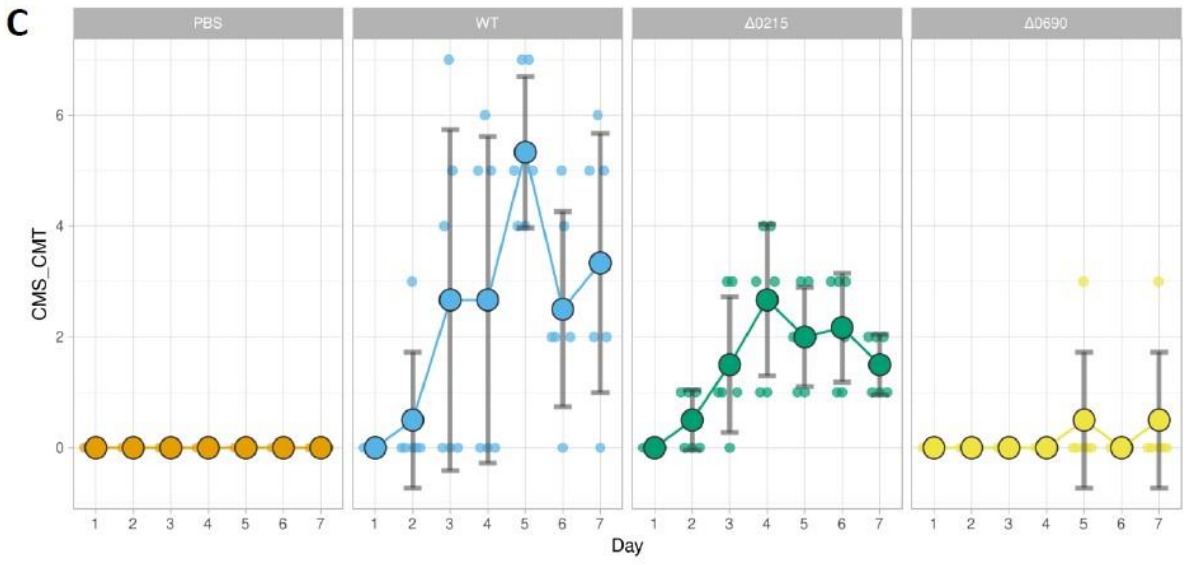
C
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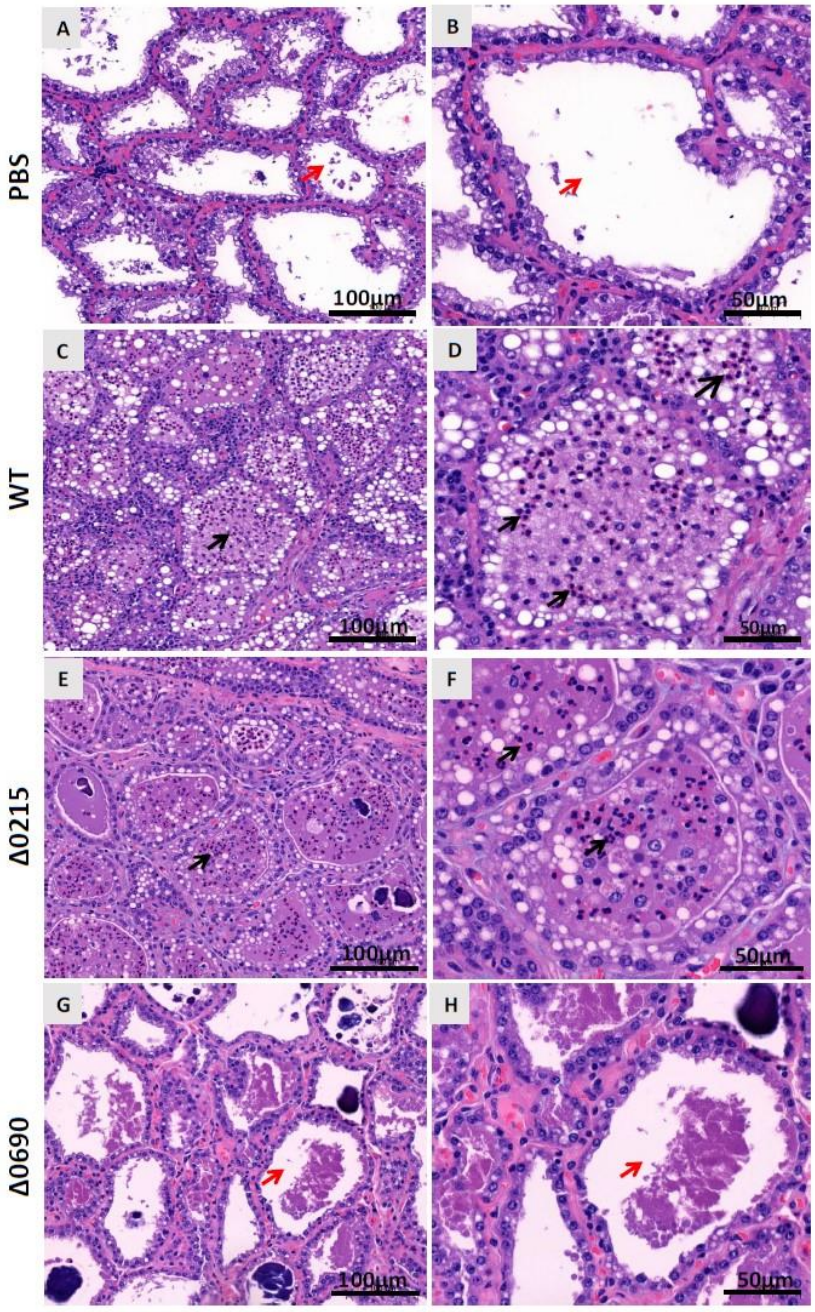

Figure 4.6. *M. bovis* Δ0690 mutant failed to elicit the recruitment of neutrophils into milk spaces. H&E images of bovine mammary glands challenged with PBS (A&B), *M. bovis* PG45 (C&D), Δ0215 (E&F) and Δ0690 (G&H) mutants. Challenge with the WT (C&D) and the Δ0215 mutant (E&F) elicited the recruitment of neutrophils (black arrows) into milk spaces, while immune cells populations were absent in milk spaces of mammary glands challenged with PBS (A&B) and the Δ0690 mutant (G&H; red arrows).

increase (P<0.001) in SCC over the sampling period was observed in both the WT and Δ0215 challenged groups. Additionally, while the SCC of cows challenged with Δ0215 did not exhibit a significant difference compared to the WT challenged group throughout the study period, the SCC of cows challenged with Δ0690 was significantly lower (P<0.001) (Fig. 4.5B). These results were also supported by similar bacterial counts in daily milk samples of WT and Δ0215, with no growth observed for the Δ0690 mutant strain (Fig. 4.5A).

4.4. Discussion

In this study, we employed genome-wide transposon mutagenesis to pinpoint and characterize mutants within MBOVPG45_0215 and MBOVPG45_0690, which encode the major membrane nuclease MnuA and 5'-NT, respectively. The MnuA and 5'-NT are lipoproteins and as such are exposed on the mycoplasma's cell-surface, providing access to the extracellular space. The MnuA efficiently degrades nucleic acids (Fig. 4.3B; (Sharma et al., 2015)), while 5'-NT acts subsequently to hydrolyze nucleotides (Fig. 4.3A). This enzymatic cascade generates substrates that can be transported through the cell membrane. Interestingly, the Δ0690 mutant displayed a significant reduction in hydrolytic activities towards 5'-3'AMPs and ATP and, thereby combining 5'-/3'-nucleosidase and nucleoside triphosphate diphosphohydrolase (NTPDase) activities, albeit the latter to a lesser extent (Fig. 4.3A). This mode of action aligns with observations in various bacteria, both Gram-positive and Gram-negative (Fan et al., 2012; Itami et al., 1989; Liu et al., 2014; Ma et al., 2017; Rittmann et al., 2005; Thammavongsa et al., 2013). In contrast in mammals, the conversion of ATP to adenosine (Ado) requires sequential activity of two CD39 (NTPDase) and CD73 (5'-nucleotidase) enzymes (Alcedo et al., 2021; Zimmermann, 1992). It can be hypothesized that the ability of *M. bovis* 5'-NT to hydrolyze ATP and 5'/3'AMPs provides the pathogen with a mechanism for rapid and efficient nutrient acquisition. This adaptive strategy likely contributes to the pathogen's ability to thrive and effectively counteract the host defenses.

The significant reduction in 5'-NT activity towards ATP and 5'-3' AMPs in Δ0690 (Fig. 4.3A), underscores the important role of the inter-domain region where mTn integration took place (Fig. 4.1A) for both the NTPDase and 5'-NT activities. Indeed, extensive research on *E. coli* UshA has revealed that the mechanical hinge governing the rotation and bringing the substrate binding site of the C-terminal domain to the catalytic site of the N-terminal domain is located within the

inter-domain linker helix (Knofel & Strater, 2001). Expressing the UshA N-terminal domain *in vitro* without this linker helix leads to protein misfolding and an approximate 80% decrease in activity (Krug et al., 2013). Notably, the linker helix in UshA is relatively short (19 amino acids) compared to the significantly longer inter-domain region in *M. bovis* 5'-NT, which spans 109 amino acids (Fig. 4.1A). This discrepancy highlights the need for further investigation into the extended region's role and significance in the structure and functionality of *M. bovis* 5'-NT.

Under axenic conditions, the Δ0690 mutant displayed a slow log-phase growth and a reduced colony size, whereas the Δ0215 mutant exhibited growth comparable to the isogenic WT (Fig. 4.2). At stationary phase, there was no discernible difference in bacterial counts between the WT and Δ0690, indicating the presence of alternative mechanisms for nucleotide breakdown and recycling. In a recent study, Zhu et al. (Zhu et al., 2020) characterized three *M. bovis* proteins belonging to the DHH phosphodiesterases superfamily. These proteins demonstrated the ability to convert their substrates, either cyclic dinucleotides or nanoRNAs, into mononucleotides. Additionally, Singh et al., (Singh et al., 2011) reported the existence of an atypical class C acid phosphatase (CAPs; MBOVPG45_0528) in *M. bovis*. CAPs are known for their broad substrate specificity, with some acting as 5′ or 5'-3' nucleotidases or as nicotinamide mononucleotide (NMN) 5′-NTs (Kemmer et al., 2001; Passariello et al., 2003; Reidl et al., 2000; Reilly & Calcutt, 2004). Notably, MBOVPG45_0528 is located adjacent to two DHH phosphodiesterases identified by Zhu et al., (Zhu et al., 2020) suggesting their co-transcription. Given the absence of *de novo* nucleotide biosynthesis, the presence of multiple pathways to regulate nucleotide levels is vital for mycoplasmas.

The assessment of fitness and pathogenesis of Δ0215 and Δ0690 mutants, using both murine and cow mastitis models, yielded complementary insights and mutually reinforced our findings (Figs 4-6). Disruption of MBOVPG45_0690 significantly attenuated *M. bovis* resulting in compromised colonization (Fig. 4.4A and Fig. 4.5A), decreased abilities to activate inflammatory marker genes in murine mammary glands (Fig. 4.4B and 4.4C), significantly lower SSC (Fig. 4.5B), and reduced ability to elicit inflammatory changes in milk and udder tissues in bovine mammary glands (Fig. 4.5C and Fig. 4.6). The overall contribution of MnuA mutant in the pathogenesis of bovine mastitis was similar to the WT (Fig. 4.5 and Fig. 4.6), and it is also

consistent with the observations in the murine model (Fig. 4.4). Circulation of *M. bovis* field strains, with truncated *mnu*A, isolated from cases of mastitis and pneumonia, supports our experimental data regarding the ability of Δ0215 to cause mastitis.

Exonucleases and 5'-NT have been implicated in the fitness, virulence, and pathogenicity of certain bacteria (Soh et al., 2020; Zakataeva, 2021). In *Staphylococcus aureus*, these proteins act synergistically. The nuclease degrades the DNA backbone of NETs into dNMPs. Concurrently, AdsA (5'-NT) hydrolyzes dAMP to deoxyadenosine (dAdo), triggering caspase-3-dependent apoptosis in macrophages. This process affects phagocytic activity and facilitates the establishment of infection (Thammavongsa et al., 2013). In *Streptococcus equi* subsp. *zooepidemicus*, the enzymes act in tandem possessing both nuclease and 5'-NT activities. This dual functionality enables efficient nutritional acquisition and immune evasion (Ma et al., 2017). Moreover, surface-exposed 5'-NTs impact inflammatory and cell immune responses by regulating the ATP/Ado ratio (Antonioli et al., 2013; Cekic & Linden, 2016). Indeed, extracellular ATP and Ado are important controllers of immune cell functions acting through binding to their specific receptors present on different types of cells, including macrophages, neutrophils and dendritic cells. While extracellular ATP stimulates pro-inflammatory response, Ado induces anti-inflammatory effects on immune cells. It has been shown that e-ATP enhances mycoplasma lipoproteins –induced cytotoxicity towards different cells (Gabridge & Polisky, 1977; Hopfe & Henrich, 2004; Into, Fujita, et al., 2002; Into, Okada, et al., 2002), while the effect of Ado remains largely unexplored. Ultimately, any disruption to the delicate ATP/Ado balance can alter the dynamics of the host-pathogen interactions, influencing the inflammatory status, disease progression, and overall outcome.

In conclusion, our study has shown that *M. bovis* 5'-NT, rather than MnuA, plays a pivotal role in pathogen survival, fitness, and virulence in bovine mastitis. Further research is warranted to explore whether targeting this gene could be a promising strategy for vaccine development and therapeutic interventions.

Ethical consideration

The IACUC approvals were obtained prospectively from the Ethics Committee for Animal Experimentation, the Hebrew University of Jerusalem (IACUC MD-18-15686-3), and the University of Tennessee Knoxville (Registration # 2870-0322).

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References

Aboklaish, A. F., Dordet-Frisoni, E., Citti, C., Toleman, M. A., Glass, J. I., & Spiller, O. B. (2014). Random insertion and gene disruption via transposon mutagenesis of *Ureaplasma parvum* using a mini-transposon plasmid. *Int J Med Microbiol*, *304*(8), 1218-1225. https://doi.org/10.1016/j.ijmm.2014.09.003

Adkins, P.R.F., Middleton, J.R., Fox, L.K., Pighetti, G.M., Petersson-Wolfe, C., National Mastitis C. Laboratory handbook on bovine mastitis. Third edition ed. New Prague, Minnesota: National Mastitis Council, Inc. New Prague, Minnesota; 2017

Alcedo, K. P., Bowser, J. L., & Snider, N. T. (2021). The elegant complexity of mammalian ecto-5'-nucleotidase (CD73). *Trends Cell Biol*, *31*(10), 829-842. https://doi.org/10.1016/j.tcb.2021.05.008

Ambroset, C., Peticca, A., Tricot, A., & Tardy, F. (2022). Genomic features of *Mycoplasma bovis* subtypes currently circulating in France. *BMC Genomics*, *23*(1), 603. https://doi.org/10.1186/s12864-022-08818-9

Antonioli, L., Pacher, P., Vizi, E. S., & Hasko, G. (2013). CD39 and CD73 in immunity and inflammation. *Trends Mol Med*, *19*(6), 355-367. https://doi.org/10.1016/j.molmed.2013.03.005

Baranowski, E., Guiral, S., Sagne, E., Skapski, A., & Citti, C. (2010). *Mycoplasma agalactiae* interaction with mammalian cells: the critical role of dispensable genes. *Infect. Immun.*, *78*, 1542-1551. https://doi.org/10.1128/iai.01195-09

Beiter, K., Wartha, F., Albiger, B., Normark, S., Zychlinsky, A., & Henriques-Normark, B. (2006). An endonuclease allows *Streptococcus pneumoniae* to escape from neutrophil extracellular traps. *Curr Biol*, *16*(4), 401-407. https://doi.org/10.1016/j.cub.2006.01.056

Bizarro, C. V., & Schuck, D. C. (2007). Purine and pyrimidine nucleotide metabolism in Mollicutes. *Genetics and Molecular Biology*, *30*(1), 190-201. https://doi.org/doi.org/10.1590/S1415-47572007000200005

Bokma, J., Vereecke, N., De Bleecker, K., Callens, J., Ribbens, S., Nauwynck, H., Haesebrouck, F., Theuns, S., Boyen, F., & Pardon, B. (2020). Phylogenomic analysis of *Mycoplasma bovis* from Belgian veal, dairy and beef herds. *Vet Res*, *51*(1), 121. https://doi.org/10.1186/s13567-020-00848-z

Buchanan, J. T., Simpson, A. J., Aziz, R. K., Liu, G. Y., Kristian, S. A., Kotb, M., Feramisco, J., & Nizet, V. (2006). DNase expression allows the pathogen group A *Streptococcus* to escape killing in neutrophil extracellular traps. *Curr Biol*, *16*(4), 396-400. https://doi.org/10.1016/j.cub.2005.12.039

Cacciotto, C., & Alberti, A. (2022). Eating the enemy: mycoplasma strategies to evade neutrophil extracellular traps (NETs) promoting bacterial nucleotides uptake and inflammatory damage. *Int J Mol Sci*, *23*(23). https://doi.org/10.3390/ijms232315030

Cacciotto, C., Dessì, D., Cubeddu, T., Cocco, A. R., Pisano, A., Tore, G., Fiori, P. L., Rappelli, P., Pittau, M., & Alberti, A. (2019). MHO_0730 as a Surface-Exposed Calcium-Dependent Nuclease of *Mycoplasma hominis* Promoting Neutrophil Extracellular Trap Formation and Escape. *J Infect Dis*, *220*(12), 1999-2008. https://doi.org/10.1093/infdis/jiz406

Calcutt, M. J., Lysnyansky, I., Sachse, K., Fox, L. K., Nicholas, R. A. J., & Ayling, R. D. (2018). Gap analysis of *Mycoplasma bovis* disease, diagnosis and control: An aid to identify future development requirements. *Transbound Emerg Dis*, *65 Suppl 1*, 91-109. https://doi.org/10.1111/tbed.12860

Cekic, C., & Linden, J. (2016). Purinergic regulation of the immune system. *Nat Rev Immunol*, *16*(3), 177-192. https://doi.org/10.1038/nri.2016.4

Chang, A., Khemlani, A., Kang, H., & Proft, T. (2011). Functional analysis of *Streptococcus pyogenes* nuclease A (SpnA), a novel group A streptococcal virulence factor. *Mol Microbiol*, *79*(6), 1629-1642. https://doi.org/10.1111/j.1365-2958.2011.07550.x

Chopra-Dewasthaly, R., Marenda, M., Rosengarten, R., Jechlinger, W., & Citti, C. (2005). Construction of the first shuttle vectors for gene cloning and homologous recombination in *Mycoplasma agalactiae*. *FEMS Microbiol. Lett.*, *253*, 89-94.

Dordet Frisoni, E., Marenda, M. S., Sagne, E., Nouvel, L. X., Guerillot, R., Glaser, P., Blanchard, A., Tardy, F., Sirand-Pugnet, P., Baranowski, E., & Citti, C. (2013). ICEA of *Mycoplasma agalactiae*: a new family of self-transmissible integrative elements that confers conjugative properties to the recipient strain. *Mol. Microbiol., Online*. https://doi.org/10.1111/mmi.12341 [doi]

Eaves, G. N., & Jeffries, C. D. (1963). Isolation and properties of an exocellular nuclease of *Serratia marcescens*. *J Bacteriol*, *85*(2), 273-278. https://doi.org/10.1128/jb.85.2.273-278.1963

Fan, J., Zhang, Y., Chuang-Smith, O. N., Frank, K. L., Guenther, B. D., Kern, M., Schlievert, P. M., & Herzberg, M. C. (2012). Ecto-5'-nucleotidase: a candidate virulence factor in *Streptococcus sanguinis* experimental endocarditis. *PLoS ONE*, *7*(6), e38059. https://doi.org/10.1371/journal.pone.0038059

Friis, N. F. (1975). Some recommendations concerning primary isolation of *Mycoplasma suipneumoniae* and *Mycoplasma flocculare*. *Nordisk veterinaermedicin*, *27*, 333-339.

Gabridge, M. G., & Polisky, R. B. (1977). Intracellular levels of adenosine triphosphate in hamster trachea organ cultures exposed to *Mycoplasma pneumoniae* cells or membranes. *In vitro*, *13*(8), 510-516. https://doi.org/10.1007/BF02615144

Goedhart, J. (2021). SuperPlotsOfData-a web app for the transparent display and quantitative comparison of continuous data from different conditions. *Mol Biol Cell*, *32*(6), 470-474. https://doi.org/10.1091/mbc.E20-09-0583

Goncheva, M. I., Chin, D., & Heinrichs, D. E. (2022). Nucleotide biosynthesis: the base of bacterial pathogenesis. *Trends Microbiol*, *30*(8), 793-804. https://doi.org/10.1016/j.tim.2021.12.007

Gondaira, S., Nishi, K., Tanaka, T., Yamamoto, T., Nebu, T., Watanabe, R., Konnai, S., Hayashi, T., Kiku, Y., Okamoto, M., Matsuda, K., Koiwa, M., Iwano, H., Nagahata, H., & Higuchi, H. (2020). Immunosuppression in Cows following Intramammary Infusion of *Mycoplasma bovis*. *Infect Immun*, *88*(3). https://doi.org/10.1128/iai.00521-19

Hale, H. H., Helmboldt, C. F., Plastridge, W. N., & Stula, E. F. (1962). Bovine mastitis caused by a *Mycoplasma* species. *Cornell Vet*, *52*, 582-591.

Hengge, R., Gründling, A., Jenal, U., Ryan, R., & Yildiz, F. (2016). Bacterial Signal Transduction by Cyclic Di-GMP and Other Nucleotide Second Messengers. *J Bacteriol*, *198*(1), 15-26. https://doi.org/10.1128/jb.00331-15

Hopfe, M., & Henrich, B. (2004). OppA, the substrate-binding subunit of the oligopeptide permease, is the major ecto-ATPase of *Mycoplasma hominis*. *J. Bacteriol.*, *186*(4), 1021-1028. http://jb.asm.org/cgi/content/abstract/186/4/1021

Huerta-Cepas, J., Serra, F., & Bork, P. (2016). ETE 3: reconstruction, analysis, and visualization of phylogenomic data. *Mol. Biol. Evol.*, *33*(6), 1635-1638. https://doi.org/10.1093/molbev/msw046

Idzko, M., Ferrari, D., & Eltzschig, H. K. (2014). Nucleotide signalling during inflammation. *Nature*, *509*(7500), 310-317. https://doi.org/10.1038/nature13085

Into, T., Fujita, M., Okusawa, T., Hasebe, A., Morita, M., & Shibata, K. (2002). Synergic effects of mycoplasmal lipopeptides and extracellular ATP on activation of macrophages. *Infect Immun*, *70*(7), 3586-3591. https://doi.org/10.1128/IAI.70.7.3586-3591.2002

Into, T., Okada, K., Inoue, N., Yasuda, M., & Shibata, K. (2002). Extracellular ATP regulates cell death of lymphocytes and monocytes induced by membrane-bound lipoproteins of Mycoplasma fermentans and *Mycoplasma salivarium*. *Microbiol Immunol*, *46*(10), 667-675.

Itami, H., Sakai, Y., Shimamoto, T., Hama, H., Tsuda, M., & Tsuchiya, T. (1989). Purification and characterization of membrane-bound 5'-nucleotidase of *Vibrio parahaemolyticus*. *J Biochem*, *105*(5), 785-789. https://doi.org/10.1093/oxfordjournals.jbchem.a122745

Kauf, A. C. W., Rosenbusch, R. F., Paape, M. J., & Bannerman, D. D. (2007). Innate immune response to intramammary *Mycoplasma bovis* infection. *J. Dairy Sci.*, *90*(7), 3336-3348. http://jds.fass.org/cgi/content/abstract/90/7/3336

Kemmer, G., Reilly, T. J., Schmidt-Brauns, J., Zlotnik, G. W., Green, B. A., Fiske, M. J., Herbert, M., Kraiss, A., Schlor, S., Smith, A., & Reidl, J. (2001). NadN and e (P4) are essential for utilization of NAD and nicotinamide mononucleotide but not nicotinamide riboside in *Haemophilus influenzae*. *J Bacteriol*, *183*(13), 3974-3981. https://doi.org/10.1128/JB.183.13.3974-3981.2001

Kiedrowski, M. R., Kavanaugh, J. S., Malone, C. L., Mootz, J. M., Voyich, J. M., Smeltzer, M. S., Bayles, K. W., & Horswill, A. R. (2011). Nuclease modulates biofilm formation in community-associated methicillin-resistant *Staphylococcus aureus*. *PLoS One*, *6*(11), e26714. https://doi.org/10.1371/journal.pone.0026714

Knofel, T., & Strater, N. (2001). *E. coli* 5'-nucleotidase undergoes a hinge-bending domain rotation resembling a ball-and-socket motion. *J Mol Biol*, *309*(1), 255-266. https://doi.org/10.1006/jmbi.2001.4657

Knudtson, W. U., Reed, D. E., & Daniels, G. (1986). Identification of Mycoplasmatales in pneumonic calf lungs. *Vet Microbiol*, *11*(1-2), 79-91.

Kornspan, J. D., Tarshis, M., & Rottem, S. (2010). Invasion of melanoma cells by *Mycoplasma hyorhinis*: Enhancement by protease treatment. *Infect. Immun.*, *78*(2), 611-617. https://doi.org/10.1128/iai.01017-09

Krug, U., Patzschke, R., Zebisch, M., Balbach, J., & Strater, N. (2013). Contribution of the two domains of *E. coli* 5'-nucleotidase to substrate specificity and catalysis. *FEBS Lett*, *587*(5), 460-466. https://doi.org/10.1016/j.febslet.2013.01.010

Leiva, L. E., Zegarra, V., Bange, G., & Ibba, M. (2023). At the Crossroad of Nucleotide Dynamics and Protein Synthesis in Bacteria. *Microbiol Mol Biol Rev*, *87*(1), e0004422. https://doi.org/10.1128/mmbr.00044-22

Li, L., Abdelhady, W., Donegan, N. P., Seidl, K., Cheung, A., Zhou, Y. F., Yeaman, M. R., Bayer, A. S., & Xiong, Y. Q. (2018). Role of Purine Biosynthesis in Persistent Methicillin-Resistant *Staphylococcus aureus* Infection. *J Infect Dis*, *218*(9), 1367-1377. https://doi.org/10.1093/infdis/jiy340

Li, P., Zhang, Y., Li, X., Zhou, W., Li, X., Jiang, F., & Wu, W. (2019). *Mycoplasma hyopneumoniae* Mhp597 is a cytotoxicity, inflammation and immunosuppression associated nuclease. *Vet Microbiol*, *235*, 53-62. https://doi.org/10.1016/j.vetmic.2019.05.011

Liu, P., Pian, Y., Li, X., Liu, R., Xie, W., Zhang, C., Zheng, Y., Jiang, Y., & Yuan, Y. (2014). *Streptococcus suis* adenosine synthase functions as an effector in evasion of PMN-mediated innate immunit. *J Infect Dis*, *210*(1), 35-45. https://doi.org/10.1093/infdis/jiu050

Livak, K. J., & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods*, *25*(4), 402-408. https://doi.org/10.1006/meth.2001.1262

Ma, F., Guo, X., & Fan, H. (2017). Extracellular nucleases of *Streptococcus equi* subsp. *zooepidemicus* degrade neutrophil extracellular traps and impair macrophage activity of the host. *Appl Environ Microbiol*, *83*(2). https://doi.org/10.1128/AEM.02468-16

Masukagami, Y., De Souza, D. P., Dayalan, S., Bowen, C., O'Callaghan, S., Kouremenos, K., Nijagal, B., Tull, D., Tivendale, K. A., Markham, P. F., McConville, M. J., Browning, G.

F., & Sansom, F. M. (2017). Comparative metabolomics of *Mycoplasma bovis* and *Mycoplasma gallisepticum* reveals fundamental differences in active metabolic pathways and suggests novel gene annotations. *mSystems*, *2*(5). https://doi.org/10.1128/mSystems.00055-17

Merrill, C., Ensermu, D. B., Abdi, R. D., Gillespie, B. E., Vaughn, J., Headrick, S. I., Hash, K., Walker, T. B., Stone, E., & Kerro Dego, O. (2019). Immunological responses and evaluation of the protection in dairy cows vaccinated with staphylococcal surface proteins. *Vet Immunol Immunopathol*, *214*, 109890. https://doi.org/10.1016/j.vetimm.2019.109890

Mikkola, S. (2020). Nucleotide Sugars in Chemistry and Biology. *Molecules*, *25*(23). https://doi.org/10.3390/molecules25235755

Minion, F. C., Jarvill-Taylor, K. J., Billings, D. E., & Tigges, E. (1993). Membrane-associated nuclease activities in mycoplasmas. *J. Bacteriol.*, *175*(24), 7842-7847. http://jb.asm.org/content/175/24/7842.abstract

Mitiku, F., Hartley, C. A., Sansom, F. M., Coombe, J. E., Mansell, P. D., Beggs, D. S., & Browning, G. F. (2018). The major membrane nuclease MnuA degrades neutrophil extracellular traps induced by *Mycoplasma bovis*. *Vet Microbiol*, *218*, 13-19. https://doi.org/10.1016/j.vetmic.2018.03.002

Oliver, S. P. (2004). *Microbiological Procedures for the Diagnosis of Bovine Udder Infection and Determination of Milk Quality National Mastitis Council (U.S.)* (Fourth ed.). National Mastitis Council (U.S.) NMC, Verona, WI.

Passariello, C., Schippa, S., Iori, P., Berlutti, F., Thaller, M. C., & Rossolini, G. M. (2003). The molecular class C acid phosphatase of *Chryseobacterium meningosepticum* (OlpA) is a broad-spectrum nucleotidase with preferential activity on 5'-nucleotides. *Biochim Biophys Acta*, *1648*(1-2), 203-209. https://doi.org/10.1016/s1570-9639(03)00147-x

Pollack, J. D., Williams, M. V., & McElhaney, R. N. (1997). The comparative metabolism of the mollicutes (Mycoplasmas): the utility for taxonomic classification and the relationship of putative gene annotation and phylogeny to enzymatic function in the smallest free-living cells. *Crit Rev Microbiol*, *23*(4), 269-354. https://doi.org/10.3109/10408419709115140

Pressler, K., Mitterer, F., Vorkapic, D., Reidl, J., Oberer, M., & Schild, S. (2019). Characterization of Vibrio cholerae's Extracellular Nuclease Xds. *Front Microbiol*, *10*, 2057. https://doi.org/10.3389/fmicb.2019.02057

Price, M. N., Dehal, P. S., & Arkin, A. P. (2010). FastTree 2-approximately maximum-likelihood trees for large alignments. *PLoS ONE*, *5*(3), e9490. https://doi.org/10.1371/journal.pone.0009490

Razin, S., Yogev, D., & Naot, Y. (1998). Molecular biology and pathogenesis of mycoplasmas. *Microbiol. Mol. Biol. Rev.*, *62*(4), 1094-1156.

Reidl, J., Schlor, S., Kraiss, A., Schmidt-Brauns, J., Kemmer, G., & Soleva, E. (2000). NADP and NAD utilization in *Haemophilus influenzae*. *Mol Microbiol*, *35*(6), 1573-1581. https://doi.org/10.1046/j.1365-2958.2000.01829.x

Reilly, T. J., & Calcutt, M. J. (2004). The class C acid phosphatase of *Helicobacter pylori* is a 5' nucleotidase. *Protein Expr Purif*, *33*(1), 48-56. https://doi.org/10.1016/j.pep.2003.08.020

Rittmann, D., Sorger-Herrmann, U., & Wendisch, V. F. (2005). Phosphate starvation-inducible gene *ushA* encodes a 5' nucleotidase required for growth of *Corynebacterium glutamicum* on media with nucleotides as the phosphorus source. *Appl Environ Microbiol*, *71*(8), 4339-4344. https://doi.org/10.1128/AEM.71.8.4339-4344.2005

Rodwell, A. W., & Whitcomb, R. F. (1983). Methods for direct and indirect measurement of mycoplasma growth. In S. Razin & J. G. Tully (Eds.), *Methods in Mycoplasmology* (Vol. 1, pp. 185-196). Academic Press.

Salamon, H., Nissim-Eliraz, E., Ardronai, O., Nissan, I., & Shpigel, N. Y. (2020). The role of O-polysaccharide chain and complement resistance of *Escherichia coli* in mammary virulence. *Vet Res*, *51*(1), 77. https://doi.org/10.1186/s13567-020-00804-x

Samant, S., Lee, H., Ghassemi, M., Chen, J., Cook, J. L., Mankin, A. S., & Neyfakh, A. A. (2008). Nucleotide biosynthesis is critical for growth of bacteria in human blood. *PLoS Pathog*, *4*(2), e37. https://doi.org/10.1371/journal.ppat.0040037

Schneider, P., Brill, R., Schouten, I., Nissim-Eliraz, E., Lysnyansky, I., & Shpigel, N. Y. (2022). Lipoproteins Are Potent Activators of Nuclear Factor Kappa B in Mammary Epithelial Cells and Virulence Factors in *Mycoplasma bovis* Mastitis. *Microorganisms*, *10*(11). https://doi.org/10.3390/microorganisms10112209

Schneider, P., Salamon, H., Weizmann, N., Nissim-Eliraz, E., Lysnyansky, I., & Shpigel, N. Y. (2023). Immune profiling of experimental murine mastitis reveals conserved response to mammary pathogenic *Escherichia coli*, *Mycoplasma bovis*, and *Streptococcus uberis*. *Front Microbiol, 14*, 1126896. https://doi.org/10.3389/fmicb.2023.1126896

Sharma, S., Tivendale, K. A., Markham, P. F., & Browning, G. F. (2015). Disruption of the membrane nuclease gene (MBOVPG45_0215) of *Mycoplasma bovis* greatly reduces cellular nuclease activity. *J Bacteriol, Online*. https://doi.org/JB.00034-15

Singh, H., Reilly, T. J., Calcutt, M. J., & Tanner, J. J. (2011). Expression, purification and crystallization of an atypical class C acid phosphatase from *Mycoplasma bovis*. *Acta Crystallogr Sect F Struct Biol Cryst Commun, 67*(Pt 10), 1296-1299. https://doi.org/S1744309111031551

Soh, K. Y., Loh, J. M. S., & Proft, T. (2020). Cell wall-anchored 5'-nucleotidases in Gram-positive cocci. *Mol Microbiol, 113*(4), 691-698. https://doi.org/10.1111/mmi.14442

Thammavongsa, V., Missiakas, D. M., & Schneewind, O. (2013). *Staphylococcus aureus* degrades neutrophil extracellular traps to promote immune cell death. *Science, 342*(6160), 863-866. https://doi.org/10.1126/science.1242255

Thomas, G. A., Varney, N. F., & Burton, K. (1970). Nucleic acid synthesis and nucleotide pools in purine-deficient Escherichia coli. *Biochem J, 120*(1), 117-124. https://doi.org/10.1042/bj1200117

Triebel, S., Sachse, K., Weber, M., Heller, M., Diezel, C., Hölzer, M., Schnee, C., & Marz, M. (2023). De novo genome assembly resolving repetitive structures enables genomic analysis of 35 European *Mycoplasmopsis bovis* strains. *BMC Genomics, 24*(1), 548. https://doi.org/10.1186/s12864-023-09618-5

Wise, K. S., Calcutt, M. J., Foecking, M. F., Roske, K., Madupu, R., & Methe, B. A. (2011). Complete genome sequence of *Mycoplasma bovis* type strain PG45 (ATCC 25523). *Infect. Immun., 79*(2), 982-983. https://doi.org/10.1128/iai.00726-10

Yair, Y., Borovok, I., Mikula, I., Falk, R., Fox, L. K., Gophna, U., & Lysnyansky, I. (2020). Genomics-based epidemiology of bovine *Mycoplasma bovis* strains in Israel. *BMC Genomics, 21*(1), 70. https://doi.org/10.1186/s12864-020-6460-0

Yamamoto, T., Kida, Y., Sakamoto, Y., & Kuwano, K. (2017). Mpn491, a secreted nuclease of *Mycoplasma pneumoniae*, plays a critical role in evading killing by neutrophil extracellular traps. *Cell Microbiol*, *19*(3). https://doi.org/10.1111/cmi.12666

Yang, J. H., Bhargava, P., McCloskey, D., Mao, N., Palsson, B. O., & Collins, J. J. (2017). Antibiotic-induced changes to the host metabolic environment inhibit drug efficacy and alter immune function. *Cell Host Microbe*, *22*(6), 757-765 e753. https://doi.org/10.1016/j.chom.2017.10.020

Zakataeva, N. P. (2021). Microbial 5'-nucleotidases: their characteristics, roles in cellular metabolism, and possible practical applications. *Appl Microbiol Biotechnol*, *105*(20), 7661-7681. https://doi.org/10.1007/s00253-021-11547-w

Zheng, L., Khemlani, A., Lorenz, N., Loh, J. M., Langley, R. J., & Proft, T. (2015). Streptococcal 5'-Nucleotidase A (S5nA), a Novel *Streptococcus pyogenes* Virulence Factor That Facilitates Immune Evasion. *J Biol Chem*, *290*(52), 31126-31137. https://doi.org/10.1074/jbc.M115.677443

Zhu, X., Baranowski, E., Dong, Y., Li, X., Hao, Z., Zhao, G., Zhang, H., Lu, D., M, A. R., Chen, Y., Hu, C., Chen, H., Sagne, E., Citti, C., & Guo, A. (2020). An emerging role for cyclic dinucleotide phosphodiesterase and nanoRNase activities in *Mycoplasma bovis*: Securing survival in cell culture. *PLoS Pathog*, *16*(6), e1008661. https://doi.org/10.1371/journal.ppat.1008661

Zhu, X., Dordet-Frisoni, E., Gillard, L., Ba, A., Hygonenq, M. C., Sagné, E., Nouvel, L. X., Maillard, R., Assié, S., Guo, A., Citti, C., & Baranowski, E. (2019). Extracellular DNA: A Nutritional Trigger of *Mycoplasma bovis* Cytotoxicity. *Front Microbiol*, *10*, 2753. https://doi.org/10.3389/fmicb.2019.02753

Zimmerman, C. U., & Herrmann, R. (2005). Synthesis of a small, cysteine-rich, 29 amino acids long peptide in *Mycoplasma pneumoniae*. *FEMS Microbiol. Lett., Online*.

Zimmermann, H. (1992). 5'-Nucleotidase: molecular structure and functional aspects. *Biochem J*, *285 (Pt 2)*(Pt 2), 345-365. https://doi.org/10.1042/bj2850345

CHAPTER 5: GENERAL DISCUSSION AND FUTURE DIRECTIONS

M. bovis mastitis (MBM) is an emerging dairy problem worldwide as countries previously free of the disease are reporting mastitis outbreaks (Jordan et al., 2021; Tardy et al., 2020). Its prevalence and molecular epidemiology have been understudied in the U.S. Furthermore, there is no effective prevention and control strategy against this important cattle disease. The main constraints to developing effective control tools is limited knowledge of *M. bovis* virulence factors and the underlying pathogenesis during *M. bovis* intramammary infection. The work presented in this dissertation is an effort to contribute to filling these research gaps.

In chapter 1, we extensively explored the literature to understand the prevalence of MBM in the U.S., existing virulence factors and pathogenesis, host immune responses, and diagnostic tools used in the isolation and identification of *M. bovis.* The search terms such as '*Mycoplasma bovis'*, '*Mycoplasma bovis* mastitis', *'M. bovis* prevalence', '*M. bovis* virulence' and '*M. bovis* immune response' were used to search mainly the PubMed (https://pubmed.ncbi.nlm.nih.gov/) and other research databases. Relevant original research articles published in peer reviewed journals were reviewed and information was compiled for the main topics mentioned above. I found that reports on the prevalence of *M. bovis* mastitis in the U.S. and the State of Tennessee are limited. Virulence factors associated with adhesion, invasion, immune modulation, and substrate acquisition have previously been identified. However, intervention measures developed using some of these targets were shown to be less effective (Dudek et al., 2021) which signifies a need to identify other immunogenic and protective *M. bovis* antigens.

In Chapter 2, *M. bovis* prevalence in the bulk tank milk samples of Tennessee dairy farms was tested using microbial culture and PCR. We conducted whole genome sequencing of the isolates to determine genetic relatedness among the isolates as well as their relationship to other *M. bovis* isolates from the global perspective. We found 5.3% and 76.7% prevalence from 56 dairy farms using culture and PCR, respectively. The prevalence obtained using microbial culture is in agreement with previous few studies conducted by USDA in various U.S. States (USDA-APHIS, 2003, 2008). The difference in prevalence between culture and PCR was expected since culture has relatively poor sensitivity which is also further impaired by the fastidious growth nature of *M. bovis* (Parker et al., 2018). Pangenome analysis showed that the isolates identified in this study are genetically close to one another and to the reference strain *M. bovis* PG45

originally isolated from mastitis outbreak in the U.S. in 1962 (Hale et al., 1962). Interestingly, this is the first report of *M. bovis* detection in bulk tank milk specifically focusing on Tennessee dairy farms. *M. bovis* positive results from bulk tank milk indicates that there is at least one cow in the herd that sheds mycoplasma. However, here we strongly underscore the importance of future studies involving individual cow sampling using combination of tests such as ELISA, culture, and PCR. Overall, these findings can be an important resource for the Tennessee and the U.S. to set priorities for *M. bovis* prevention and control in the dairy industry.

M. bovis is becoming increasingly resistant to antimicrobial treatment and perhaps the only viable option is developing effective prevention tools such as vaccines. This necessitates investigation of *M. bovis*-host interaction and identification of *M. bovis* targets that can be used for immune-based interventions. The advent of next generation sequencing and bioinformatic pipelines has made significant contribution to unravelling microbe-pathogen interactions. This is possible through the development of new quantitative methods for analysis of large transcriptomic datasets. In Chapter 3, we employed whole transcriptome analysis to identify virulence factors of *M. bovis* and to characterize host responses following *in vitro* co-infection with MAC-T cells and intramammary cow challenge. The driving enthusiasm behind this study was that genes which are upregulated in *M. bovis* during early-stage of interaction with the host are likely to play role in pathogenicity. I found that insertion sequence genes that are involved in transposase activity were significantly up-regulated in *M. bovis* whereas genes associated with protein translation were significantly downregulated. Genes involved in apoptosis pathways and pro-inflammatory cytokines were significantly upregulated in bovine mammary epithelial cells whereas genes involved in steroid biosynthesis and cell cycle were significantly down-regulated. Genes encoding formation of neutrophil extracellular traps and proinflammatory cytokines were significantly up-regulated in the mammary gland of *M. bovis* challenged cows whereas genes involved in steroid biosynthesis and metabolism were significantly down-regulated. Given the highly variable nature of insertion sequence elements in *M. bovis* (Lysnyansky et al., 2009), their role as an intervention target needs further investigation. Apoptosis was the major event during the early-stages of *M. bovis*-MAC-T cells interaction. *M. bovis* induces apoptosis in several other bovine cells including lymphocytes, embryonic turbinate cells and neutrophils (Liu et al., 2020; S. Jimbo et al., 2017; Vanden Bush & Rosenbusch, 2002). *M. bovis* might induce apoptosis in

immune cells such as neutrophils, lymphocytes, and mammary epithelial cells in favor of its replication and survival. Furthermore, the widespread occurrence of this phenomenon in the mammary epithelial cells, which are important cells in milk synthesis, requires further investigation.

Nucleases and nucleotidases are enzymes which play an important role in nucleotide salvage especially in organisms such as *M. bovis* which heavily relies on the host due to its limited metabolic capabilities (Bizarro & Schuck, 2007). In chapter 4, we validated that 5'-nucleotidase is an essential virulence factor of *M. bovis* through intramammary challenge of dairy cows. The parent strain *M. bovis* PG45 was able to cause mastitis in dairy cows whereas its isogenic 5' nucleotidase mutant failed to cause mastitis. Based on this, live attenuated or protein-based sub-unit vaccine can be developed, however further investigation is needed to establish immunogenicity of 5'-nucleotidase and the efficacy of the resulting immune response. Furthermore, therapeutic alternatives which target 5' nucleotidase can also be considered.

References

Bizarro, C. V., & Schuck, D. C. (2007). Purine and pyrimidine nucleotide metabolism in Mollicutes. *Genetics and Molecular Biology*, *30*, 190-201.

Dudek, K., Szacawa, E., & Nicholas, R. A. J. (2021). Recent Developments in Vaccines for Bovine Mycoplasmoses Caused by *Mycoplasma bovis* and *Mycoplasma mycoides* subsp. *mycoides*. *Vaccines (Basel)*, *9*(6). https://doi.org/10.3390/vaccines9060549

Hale, H. H., Helmboldt, C. F., Plastridge, W. N., & Stula, E. F. (1962). Bovine mastitis caused by a *Mycoplasma* species. *Cornell Vet*, *52*, 582-591. https://www.ncbi.nlm.nih.gov/pubmed/13952069

Jordan, A., Sadler, R. J., Sawford, K., van Andel, M., Ward, M., & Cowled, B. (2021). *Mycoplasma bovis* outbreak in New Zealand cattle: An assessment of transmission trends using surveillance data. *Transbound Emerg Dis*, *68*(6), 3381-3395. https://doi.org/10.1111/tbed.13941

Liu, Y., Zhou, M., Xu, S., Khan, M. A., Shi, Y., Qu, W., Gao, J., Liu, G., Kastelic, J. P., & Han, B. (2020). *Mycoplasma bovis*-generated reactive oxygen species and induced apoptosis in bovine mammary epithelial cell cultures. *J Dairy Sci*, *103*(11), 10429-10445. https://doi.org/10.3168/jds.2020-18599

Lysnyansky, I., Calcutt, M. J., Ben-Barak, I., Ron, Y., Levisohn, S., Methé, B. A., & Yogev, D. (2009). Molecular characterization of newly identified IS3, IS4 and IS30 insertion sequence-like elements in *Mycoplasma bovis* and their possible roles in genome plasticity. *FEMS Microbiol Lett*, *294*(2), 172-182. https://doi.org/10.1111/j.1574-6968.2009.01562.x

Parker, A. M., Sheehy, P. A., Hazelton, M. S., Bosward, K. L., & House, J. K. (2018). A review of mycoplasma diagnostics in cattle. *J Vet Intern Med*, *32*(3), 1241-1252. https://doi.org/10.1111/jvim.15135

S. Jimbo, Suleman, M., Maina, T., Prysliak, T., Mulongo, M., & Perez-Casal, J. (2017). Effect of *Mycoplasma bovis* on bovine neutrophils. *Vet Immunol Immunopathol*, *188*, 27-33. https://doi.org/10.1016/j.vetimm.2017.04.011

Tardy, F., Aspan, A., Autio, T., Ridley, A., Tricot, A., Colin, A., Pohjanvirta, T., Smid, B., Harders, F., Lindegaard, M., Tølbøll Lauritsen, K., Lyhs, U., Wisselink, H. J., & Strube,

M. L. (2020). *Mycoplasma bovis* in Nordic European Countries: Emergence and Dominance of a New Clone. *Pathogens*, *9*(11). https://doi.org/10.3390/pathogens9110875

USDA-APHIS. (2003). *Mycoplasma in Bulk Tank Milk on U.S. Dairies*. USA: Department of Agriculture

USDA-APHIS. (2008). *Prevalence of Contagious Mastitis Pathogens on U.S. Dairy Operations*. USA: Department of Agriculture

Vanden Bush, T. J., & Rosenbusch, R. F. (2002). *Mycoplasma bovis* induces apoptosis of bovine lymphocytes. *FEMS Immunology & Medical Microbiology*, *32*(2), 97-103.

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