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Review

Virulence, persistence and dissemination of *Mycoplasma bovis*

Sibylle Bürki [a,b], Joachim Frey [a], Paola Pilo [a,*]

[a] Institute of Veterinary Bacteriology, Vetsuisse Faculty, University of Bern, Bern, Switzerland
[b] Graduate School for Cellular and Biomedical Sciences, University of Bern, Bern, Switzerland

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ABSTRACT

Bovine mycoplasmosis due to *Mycoplasma bovis* causes several important bovine diseases such as pneumonia, mastitis, arthritis, otitis, genital disorders or keratoconjunctivitis. Variable surface lipoproteins, adhesion, invasion of host cells, modulation of the host immune system, biofilm formation and the release of secondary metabolites like hydrogen peroxide, as well as synergistic infections with other bacterial or viral pathogens are among the more significantly studied characteristics of the bacterium.

The aim of this review is to summarize the current knowledge regarding the virulence of *M. bovis* and additionally, factors contributing to the dissemination and persistence of this pathogen in the bovine host will be discussed.

Contents

1. Introduction

Mycoplasma bovis was first isolated in 1961 in the United States (Hale et al., 1962). This wall-less bacterium belongs to the class of the *Mollicutes* and causes bovine mycoplasmosis, an infection that leads to a variety of

* Corresponding author at: Institute of Veterinary Bacteriology, University of Bern, Länggassstrasse 122, 3012 Bern, Switzerland. Tel.: +41 31 631 2369; fax: +41 31 631 2634.
E-mail address: paola.pilo@vetsuisse.unibe.ch (P. Pilo).

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clinical manifestations, mostly of chronic nature, including bronchopneumonia (Caswell and Archambault, 2007; Gagea et al., 2006), otitis (Maeda et al., 2003), mastitis (Jasper, 1994), genital disorders (Doig, 1981; Hermeyer et al., 2012b), arthritis (Gagea et al., 2006), meningitis (Ayling et al., 2005) or keratoconjunctivitis (Alberti et al., 2006). *M. bovis* can affect a large variety of tissues and organs and is also isolated from healthy cattle. This bacterium is considered to be one of the major emerging pathogens of cattle in industrialized countries threatening livestock production (Nicholas, 2011). To date, there is no effective vaccine to prevent *M. bovis* infections (Mulongo et al., 2013), therapy with antibiotics is scarcely efficient and increasing antimicrobial resistance is reported (Gautier-Bouchardon et al., 2014).

Currently, there is limited understanding of the molecular mechanisms of pathogenicity of mycoplasmas and they appear to be multifactorial. Pathogenic *Mycoplasma* species, in contrast to other bacteria, do not distinguish themselves by the production of potent toxins, with few exceptions like an ADP-ribosyl-transferase described in *Mycoplasma pneumoniae* (Kannan and Baseman, 2006).

Until recently, the lack of genetic tools and limited research techniques hampered efforts to investigate the pathogenesis and virulence factors of *M. bovis*, which is essential to develop therapeutic and prophylactic measures to combat bovine mycoplasmosis. Random transposon mutagenesis was recently used to generate mutants of *M. bovis* (Chopra-Dewasthaly et al., 2005; Sharma et al., 2012) and a new plasmid, replicating extrachromosomally in *M. bovis*, was published (Li et al., 2014). With the availability and further improvement of these techniques, it should be possible to obtain detailed information about the interactions of *M. bovis* with its host in the near future.

This review presents an overview of the current knowledge about the pathogenic mechanisms of *M. bovis*. The following aspects are discussed: (1) co-infections with other bacterial or viral organisms, (2) antigenic variation, (3) adhesion, (4) bovine cells invasion, (5) modulation of the host immune system and (6) secondary metabolites and biofilm formation.

2. Interactions with other microorganisms

In naturally infected cattle, *M. bovis* is often detected in association with other microorganisms leading to the hypothesis that synergism could be involved in the severe lung lesions observed in necropsy material (Booker et al., 1999, 2008; Martin et al., 1989; Shahriar et al., 2002). The most common identified microorganisms in association with *M. bovis* are *Pasteurella multocida*, *Mannheimia haemolytica*, *Histophilus somni*, bovine respiratory syncytial virus (BRSV), bovine herpes virus 1 (BHV-1), bovine viral diarrhea virus (BVDV) and parainfluenza virus type 3 but as described below some studies resulted in contradictory findings (Booker et al., 1999, 2008; Martin et al., 1989; Shahriar et al., 2002).

Natural infection with *M. bovis* can result in exudative bronchopneumonia with occasional extensive foci of coagulative necrosis, whereas experimental infections lead to subclinical pneumonia and less severe lung lesions (Rodriguez et al., 1996). Hence, severe pulmonary disease symptoms caused by *M. bovis* are suspected to be the consequence of synergistic interactions between mycoplasmas and other microorganisms causing significant issues for farm management. Additionally, the age and the immune status of individual animals are probably also important for the development of disease, as *M. bovis* alone was described to be able to cause pneumonia in very young calves, while synergistic viral or bacterial infections may be necessary to cause the typical extensive caseonecrotic lesions in the lungs of adult animals in feedlots (Hermeyer et al., 2012a; Prysliak et al., 2011).

More specifically, *M. bovis* seems to interplay with the bacterial pathogens *H. somni*, *M. haemolytica* and *P. multocida*. This was shown in experimentally induced bovine respiratory disease where *M. bovis* acted as a predisposing factor for severe symptoms in calves after secondary infection with *P. multocida* (Gourlay and Houghton, 1985). Other investigations considered an onset of *M. bovis*-mediated symptoms in tissue pre-damaged through lesions caused by *P. multocida*, *M. haemolytica* or *H. somni*. In these cases, *M. bovis* was supposed to withstand the curing of primary infections caused by the *Pasteurellaceae* by antibiotics or by the host immune system (Gagea et al., 2006). In another study, a significant association of *H. somni* and *M. bovis* was detected in feedlot calves, where 80% of *H. somni* cases were positive for *M. bovis*, whereas no significant association was seen between *M. bovis* and BVDV or *M. haemolytica* (Booker et al., 2008).

Co-infections with BVDV were shown to result in more severe respiratory disease in cattle through the immunosuppressive effects of the virus (Martin et al., 1990; Potgieter, 1995). However, in feedlot cattle contradictory data are available regarding the association between *M. bovis* and BVDV (Booker et al., 2008; Gagea et al., 2006; Prysliak et al., 2011; Shahriar et al., 2002). Concerning co-infection of *M. bovis* with BRSV, no significant increase in the severity of clinical signs was detected when compared to single infections with *M. bovis* (Thomas et al., 1986). Interestingly, experimental co-infection with BHV-1 and *M. bovis* were performed to induce classical *M. bovis* lesions in organs (Prysliak et al., 2011). Indeed, co-infection of 6–8-month-old feedlot calves resulted in caseonecrotic bronchopneumonia typical of *M. bovis* infection, while infection with *M. bovis* alone caused only small consolidations in the lung. Moreover the mortality rate was high, revealing the severe synergistic effect of this co-infection between *M. bovis* and BHV-1 (Prysliak et al., 2011).

3. Antigenic variation

Highly variable antigenic profiles of *M. bovis*, which are strain independent, have been observed (Poumarat et al., 1994). This finding suggested that high frequency phase (ON–OFF) and size variation of membrane surface lipoproteins occur as have been demonstrated for several other *Mycoplasma* species (Razin et al., 1998). The antigenic heterogeneity of *M. bovis* strains is unrelated to the geographical origin, organ of isolation or type of disease

induced by single strains but is variable among different subclones of the same strain. This variability was shown to be based on several prominent amphiphilic, integral, membrane proteins, containing cross-reactive epitopes acting as major immunogens (Rosengarten et al., 1994). High frequency antigenic switching was demonstrated to be affected by the presence of cognate *M. bovis* antibodies *in vitro* (Le Grand et al., 1996). These points supported previous assumptions indicating that this system serves as a successful strategy to maintain diversity in strain subpopulations allowing *M. bovis* to evade the host immune system. Thereby, the host's attempts to eliminate this bacterium are massively compromised contributing to the chronic manifestation of diseases caused by *M. bovis* (Buchenau et al., 2010).

Antigenic profiles of subclones of *M. bovis* type strain PG45 revealed highly dynamic variations at the level of expression and molecular masses of the antigens (Rosengarten et al., 1994). These antigens were shown to belong to a family of phase and size variant variable membrane surface lipoproteins (Vsps). In the type strain PG45, this family comprises 13 different, single-copy *vsp* genes, organized in a chromosomal cluster, the *vsp*-locus. This locus of approximately 23 kb contains two additional ORFs with high homology to the mobile genetic elements IS*4* and IS*30*. ORFs encoding *vspA*, *vspB*, *vspC*, *vspE*, *vspF*, *vspG*, *vpsH*, *vspI*, *vspJ*, *vspK*, *vspL*, *vspM*, *vspN* and *vspO* (Fig. 1) were identified as well as their deduced proteins, VspA, VspB, VspC, VspE, VspF, VspG, VspH, VspI, VspJ, VspK, VspL, VspM, VspN, and VspO (Behrens et al., 1994; Lysnyansky et al., 1996, 1999). The members of the *vsp*-locus were designated as putative lipoproteins, having amphiphilic characteristics as well as fatty acids and cysteine-residues. The *vsp* genes were shown to contain a conserved 5′ noncoding sequence, divided in 2 cassettes, the first one (cassette I) is 99% homologous in all *vsp* genes and encodes a putative ribosomal binding site and the second one (cassette II), located upstream of cassette I, is more variable (Fig. 2) (Lysnyansky et al., 1999). This sequence is followed by an open reading frame with an N-terminal region exhibiting 98–99% identity among strains and encompassing a prolipoprotein signal peptide (Fig. 2) (Lysnyansky et al., 1999; Nussbaum et al., 2002), while the C-terminal domain of Vsps is surface-exposed (Behrens et al., 1994; Lysnyansky et al., 1996, 1999; Rosengarten et al., 1994).

The co-expression of several Vsps leads to *M. bovis* surface mosaics with different specific structural and antigenic features. Co-expression of *vsp* genes was observed to be restricted to two genes per isolate, while the rest of the *vsp* genes remain transcriptionally silent (Lysnyansky et al., 1999). It has to be noted that an intergenic recombination event between *vspA* and *vspO* results in *vspC* (Lysnyansky et al., 2001a) explaining the absence of co-expression of the *vspA* and *vspC* genes, the lack of *vspC* in strains expressing the *vspA* and *vspB* genes and the structural similarity between VspA and VspC proteins (Lysnyansky et al., 1999). The production of the chimeric product *vspC* with the highly conserved first cassette (cassette I) of the N-terminus acquired from *vspO* and the second cassette (cassette II) as well as the variable C-terminus obtained from *vspA* further increases the possibilities of antigenic variation, since *vspC* is also capable of phase and size variations. There is, however, a loss of genetic information in VspC expressing isolates since *vspA*, *vspM*, *vspN* and *vspO* are deleted. Therefore, the *in vivo* survival of VspC variants harboring the truncated *vsp*-locus is questionable (Lysnyansky et al., 2001a).

Antigenic variability includes shifts of banding patterns through insertions and deletions in specific repetitive blocks (high-frequency size variation) resulting in 10-, 9-, and 5-different size variants for VspA, VspB and VspC, respectively (Behrens et al., 1994). Eighteen diverse repetitive blocks were discovered among all *vsp* genes of strain PG45, extending from their N-, to their C-terminus, differing in size and amino acid structure. Some of these blocks are present in only one gene, while others exist in several distinct Vsps and are arranged as tandem domains varying in repetitions and encompassing up to 80% of the protein (Fig. 2) (Lysnyansky et al., 1999). These repetitive sequences together with the specific *vsp* inversion sequences (*vis*) within the highly conserved cassette I (Fig. 2) are perfect targets for recombination events leading to duplications, inversions or deletions of those domains (Lysnyansky et al., 1996, 2001a). Furthermore, highly conserved cassettes II serve as general active promoters for several Vsps and regulate the expression of genes situated downstream (Fig. 2) (Lysnyansky et al., 2001b). High-frequency phase variation in antigen expression results in gain or loss of surface antigens due to chromosomal recombination events (Behrens et al., 1994). These events occur *in vitro* at a frequency of 10^{-2} to 10^{-3} per cell per generation (Lysnyansky et al., 1996). At the genetic level, rearrangements were visualized through *Hind*III-digestion of chromosomal DNA and resulted in different lengths of fragments comparing VspA and VspC ON and OFF variants respectively (Lysnyansky et al., 1996, 2001b).

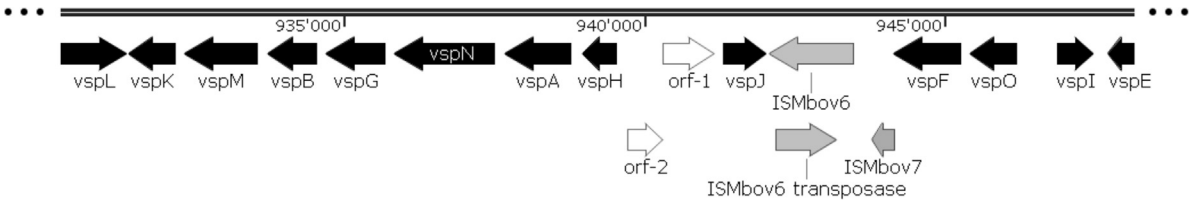

Fig. 1. *vsp*-locus of *M. bovis* type strain PG45. Image produced using SnapGene® Viewer 2.3.2 Software from the published whole genome sequence of strain PG45 (CP002188.1; phenotype VspO ON) (sequence published by Wise et al. (2011)).

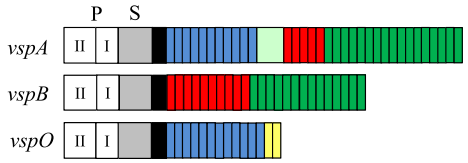

Fig. 2. Simplified structural features of the *vspA vspB* and *vspO* genes of *M. bovis* type strain PG45. The white block (P) represents a highly conserved 150 bp non-coding 5′ region upstream from the ATG codon, divided in 2 cassettes. The first (cassette I), includes the putative ribosomal binding site, whereas the second (cassette II) is a general active promoter in *vspA*. The 75 bp highly homologous DNA sequence coding for the *vsp* signal peptide is displayed by the gray block (S), followed by a third block in black, conserved in all *vsp* genes. Colored blocks indicate the reiterated coding sequences, extending from the N- toward the C-terminus. Those repetitive domains can be shared among different *vsp* genes (blue-, red-, and dark green blocks) or are *vsp*-specific (bright green-, and yellow blocks).

Variation in expression and size of Vsps was demonstrated *in vivo* in the respiratory tract of experimentally infected calves (Buchenau et al., 2010). Moreover, Vsps were shown to be expressed in 98.5% of 250 *M. bovis* field isolates tested using the monoclonal antibody 1E5, which was suggested to bind to a repetitive domain present in the VspA, VspB and VspC proteins (blocks shown in dark green in Fig. 2) (Poumarat et al., 1999; Rosengarten et al., 1994). The different genomic *vsp* patterns and phenotypic Vsps antigenic profiles seem to be extremely complex (Poumarat et al., 1999) although a study investigating polymorphisms of *vsp* genes and the associated expression profiles demonstrated a certain relatedness among field isolates sharing a common history (Beier et al., 1998). Comparing type strain PG45 with *M. bovis* field strains reveals a massive variation in *vsp* reiterated sequences as well as strain-specific repetitive sequences. This may define the individual antigenic features of particular strains, resulting in an extended *vsp* repertoire (Nussbaum et al., 2002). Particular examples are *M. bovis* field strain 2610 encoding a completely new member of the Vsp family acting as an adhesin and the Chinese strain Hubei-1 expressing the variable surface lipoprotein A (VpmaX), lacking the N-terminal protein region and upstream DNA sequence conserved in PG45 but showing adhesion properties (Thomas et al., 2005; Zou et al., 2013). The difference in the number of *vsp* genes in the *vsp*-loci of PG45 (13 *vsp*-related ORFs) and of the Chinese strain HB0801 (6 *vsp*-related ORFs) is striking, despite the conserved structure of the N-, and C-termini (Qi et al., 2012). This high variation in *vsp* genes and consequently in their repetitive units may arise from different selective pressures from the host on individual strains and therefore increases the number of possible variations in the *M. bovis* population (Nussbaum et al., 2002).

4. Adhesion

Adhesion is one of the first steps during mycoplasma infection (Rottem, 2003). Therefore adhesins expressed on the mycoplasmal membrane are of great importance because of their direct contact with host cells (Sachse et al., 1996). The intimate contact of mycoplasmas with host cells is crucial for mycoplasmal survival. Because of their small genomes, mycoplasmas lack a battery of genes involved in essential biosynthetic pathways and have to acquire essential substances such as amino acids, nucleotides and lipids from the host. For this purpose, a fusion between the mycoplasmal and the host membranes was proposed to allow the exchange of membrane and intracellular components (Razin and Jacobs, 1992). Interestingly, there is no evidence of the presence of a tip organelle in *M. bovis* (Behrens et al., 1996) serving as a structure for a polar accumulation of major adhesins as for *M. pneumoniae* (Razin and Jacobs, 1992). Putative adhesins of *M. bovis* are probably spread over the surface in the form of membrane proteins (Behrens et al., 1996; Rottem, 2003).

In *in vitro* assays, *M. bovis* strain PG45 adhesion to embryonic bovine lung cells (EBL) was shown to be temperature dependent with maximal adhesion at 37 °C. The cell receptor binding capacity was limited with saturation reached at a multiplicity of infection (MOI) of 225:1 in EBL cells (Sachse et al., 1996) and 100:1 in bovine bronchial epithelial (BBE) cells (Thomas et al., 2003b).

Large variation in cytoadherence rates (3.4–19.1%) were recorded among various *M. bovis* strains independently from the organ of isolation but determined by the type of host cells tested (Sachse et al., 1996; Thomas et al., 2003a). Significantly lower adherence was observed in less or non-pathogenic strains compared to virulent strains (Thomas et al., 2003a,b). Moreover, cytoadherence rates were lower using a fibroblast cell line and primary BBE cells compared to rates in epithelial cell lines (Thomas et al., 2003a,b). Additionally, *M. bovis* strains seem to loose adherence ability after continuous *in vitro* passaging (Thomas et al., 2003a,b).

M. bovis adhesion is driven by protein interactions since trypsin treatment of *M. bovis* leads to a partial decrease in adherence (Sachse et al., 1996; Thomas et al., 2003b). Moreover, sialic acid residues in *M. bovis* proteins were shown to play a role in cytoadhesion (Sachse et al., 1993, 1996).

At the level of specific proteins, the 32 kDa membrane surface-exposed protein P26 was shown to be a major adhesin of *M. bovis* in the EBL cell model (Sachse et al., 1993, 1996). However, the monoclonal antibody 4F6 directed against P26 did not significantly reduce adhesion to BBE cells (Thomas et al., 2003b). Another adhesion-related factor of *M. bovis* is the membrane-associated glycolytic enzyme α-enolase, since it induces mycoplasma-adherence to EBL cells by binding plasminogen (Song et al., 2012). In fact, pretreating EBL cells with plasminogen augmented *M. bovis* adhesion by 11.9% (Song et al., 2012). Pre-treatment of *M. bovis* with low concentrations of trypsin increased their proteolytic activity and adhesion rate to EBL cells, indicating that other proteolytic enzymes activated by partial digestion with minimal amounts of trypsin are also involved in adhesion (Song et al., 2012).

Vsps were also shown to play a role in *M. bovis* cell adhesion (Sachse et al., 1996) since host cells bound Vsps in Western blots (Sachse et al., 1996). Moreover, addition of purified Vsps decreased *M. bovis* cytoadhesion and Vsps were retained on host cell layers during cell adhesion

experiments (Sachse et al., 2000). This was evidenced through the partial cytoadherence inhibition of *M. bovis* PG45 toward EBL cells, using oligopeptides from repetitive domains of Vsps (Sachse et al., 2000). The *M. bovis* specific monoclonal antibodies 1E5 and 4D7 (both against common epitopes in VspA, VspB and VspC), 2A8 (against VspC) and 9F1 (against VspF) were used to investigate if mycoplasmal Vsps contact sites are accessible to antibodies. Results showed a partial blocking of adherence of *M. bovis* strains PG45 and 0435 by all specific monoclonal antibodies due to their binding to adherence sites of Vsps. However, the inhibition of adherence was dependent on the cell line used (Sachse et al., 1996, 2000; Thomas et al., 2003b). Variation of surface antigens through insertion or deletion of the repetitive units in Vsps generates additional epitopes or removes some epitopes, resulting in an increase or decrease in cytoadhesion also leading to a wider or smaller range of ligands to bind to (Sachse et al., 2000).

5. Cell invasion

In calves infected with *M. bovis*, mycoplasmas were found in the cytoplasm of various cell types such as macrophages, neutrophils, hepatocytes, bile duct epithelial cells, renal tubular cells or axons of facial nerves. *M. bovis* antigen was additionally detected in monocytes, lymph nodes and also occasionally in bronchiolar epithelial cells (Adegboye et al., 1995; Kleinschmidt et al., 2013; Maeda et al., 2003; Rodriguez et al., 1996). The survival of *M. bovis* in phagocytes indicates intracellular persistence of the bacteria presumably through an alteration of one of the steps following engulfment during the process of phagocytosis (Kleinschmidt et al., 2013).

In an *in vitro* assay, *M. bovis* strain Mb1 was found to persist in various bovine peripheral blood mononuclear cells (PBMC) subpopulations such as T cells, T helper cells, B cells, monocytes, γδ T cells, cytotoxic T cells, natural killer (NK) cells and dendritic cells, as well as in bovine erythrocytes (van der Merwe et al., 2010). Dependent on the cell type used and the time of infection, different intracellular localizations of *M. bovis* were identified. *M. bovis* associated with the cytosolic side of the cell membrane, present in vacuole-like structures as well as a diffuse distribution were observed. Different extent and rate of invasion between cell types and PBMC subpopulations may occur due to distinct receptors required for *M. bovis* adhesion and invasion, or to differential signaling induced by *M. bovis* reliant on the cell type (van der Merwe et al., 2010).

Interestingly, *M. bovis* was not found inside alveolar epithelial cells of the udder (Stanarius et al., 1981). However, our recent *in vitro* investigations using fluorescence and transmission electron microscopy confirmed an intracellular stage of virulent *M. bovis* in primary embryonic calf turbinate cells (Fig. 3).

Overall, *M. bovis* invasion of epithelial cells, as well as immune cells could contribute to the dissemination of the pathogen to different infection sites in the host and impair control of *M. bovis* infection through antibiotic treatment. However, further investigations are necessary to dissect the molecular mechanisms involved in persistence of *M. bovis* in different bovine cell types.

6. Modulation of the host's immune system

Mycoplasmal membrane proteins are important because of their direct interaction with the host immune system. *M. bovis*–host cell interactions seem to be dependent on the cell type, or the subset of PBMCs. Van der Merwe and collaborators observed induction of IFN-γ

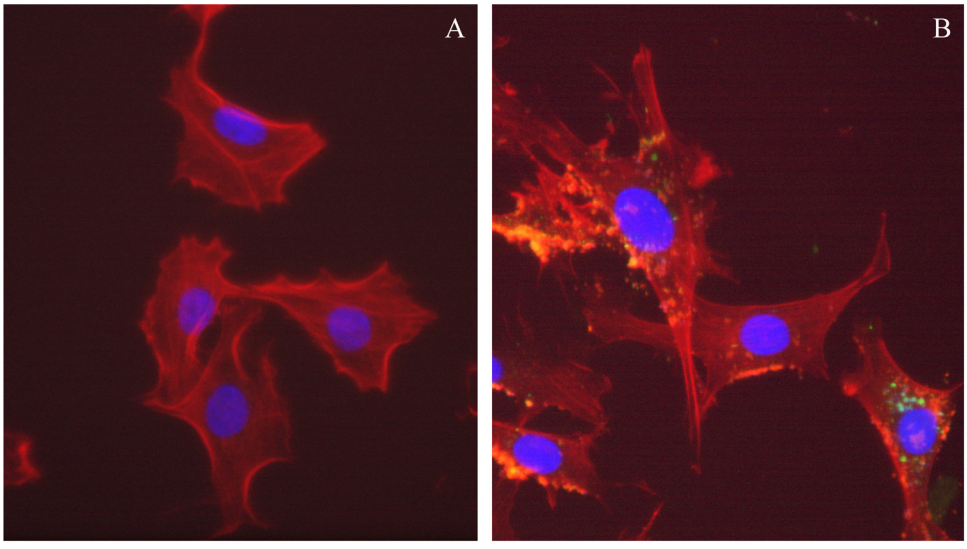

Fig. 3. Differential fluorescence microscopy image of primary embryonic calf turbinate cells infected with *M. bovis* strain JF4278 for 6 h, multiplicity of infection (MOI) 3400:1. Cell nuclei appear in blue and F-actin in red. Panel A: Uninfected turbinate cells. Panel B: intracellular *M. bovis* are stained in green and extracellular as well as cell-attached mycoplasmas are marked in dark yellow to orange. Merged images, 600× magnification.

in T cells, T helper cells, cytotoxic T cells, NK cells and γδ T cells, whereas no IFN-γ induction was measured in monocytes, dendritic cells or B cells (van der Merwe et al., 2010). Similarly, there are controversial studies concerning *M. bovis* induced apoptosis of PBMCs (Mulongo et al., 2014; Vanden Bush and Rosenbusch, 2002). Under *in vitro* conditions, the induction of apoptosis in lymphocytes by *M. bovis* was reported (Vanden Bush and Rosenbusch, 2002), whereas a delay of the apoptosis process in bovine monocytes infected with *M. bovis* was observed in another study (Mulongo et al., 2014). Moreover, there are reports suggesting suppression as well as stimulation of the host immune system by *M. bovis*. Immune stimulation seems to occur through macrophages, T cells, or complement activation as well as by the expression of cytokines up-regulating the immune response (Jungi et al., 1996; Kauf et al., 2007; Razin et al., 1998; Robino et al., 2005; Rosati et al., 1999; van der Merwe et al., 2010; Vanden Bush and Rosenbusch, 2003). Immune suppression is suspected to occur by the expression of anti-inflammatory cytokines or chemokines like IL-10 and by the suppression of expression of pro-inflammatory cytokines such as IFN-γ and TNF-α (Mulongo et al., 2014). IL-10 then shifts the adaptive immune response to express T helper cells type 2 (Th2), resulting in strong expression of IgG1, leading to poor opsonization and immunity. Alternatively, suppression of the host immune system could be accomplished by the down-regulation of lymphocyte proliferation, by a putative mycoplasmal lympho-inhibitory protein, or through interference with the lympho-proliferative response to phytoagglutinin (Thomas et al., 1990; van der Merwe et al., 2010; Vanden Bush and Rosenbusch, 2003). Thereby the proliferation of lymphocytes is down-regulated, whereas their cytokine expression is not altered. *M. bovis* seems to suppress the lymphocyte-mediated immune response *via* a decrease of their population (van der Merwe et al., 2010). Another strategy of *M. bovis* to restrain the host immune response is to bind neutrophils and thereby inhibit their oxidative burst (Thomas et al., 1991). The modulation of the host immune response is compatible with a prolonged survival and systemic dissemination of *M. bovis* in the host as observed in infected cattle (Mulongo et al., 2014).

7. Biofilm formation and secondary metabolites

Biofilm production contributes to bacterial persistence in the environment and inside the host leading to the chronicity of a disease. Additionally, biofilms can also increase damage in host tissues since phagocytes are attracted, releasing lysosomal enzymes, reactive oxygen, and nitrogen species (ROS and RNS), whereas phagocytosis is rather inefficient under these conditions (Hermeyer et al., 2011; McAuliffe et al., 2006). Several *Mycoplasma* species including *M. bovis* produce biofilms apparently independently of their pathogenicity (McAuliffe et al., 2006). The capacity of several *Mycoplasma* species to adhere to host cells appears to be essential since adherence to coverslips was observed as the initial step of biofilm formation (McAuliffe et al., 2006). The extent of biofilm formation under *in vitro* growth is very diverse among *M. bovis* strains and correlates with the different molecular types or Vsp profiles (McAuliffe et al., 2006). Since Vsps are supposed to be involved in adhesion (Sachse et al., 1996, 2000), certain Vsp patterns may display different adhesion capabilities, resulting in a variable ability to form biofilms (McAuliffe et al., 2006). Biofilm production increases bacterial resistance toward environmental stressors and host defenses (Mah and O'Toole, 2001). Indeed, biofilm forming *M. bovis* strains were more resistant to heat and desiccation enabling their survival in the environment, whereas no change in minimal inhibitory concentrations (MIC) of fluoroquinolones and tetracyclines was detected (McAuliffe et al., 2006).

Secondary metabolites were shown to be involved in the pathogenesis of *Mycoplasma* sp. (Hames et al., 2009; Pilo et al., 2005). Hydrogen peroxide (H_2O_2) production is a major virulence factor of several *Mycoplasma* species, leading to cell death, inhibition of ciliary action or peroxidation of lipids (Hames et al., 2009; Pilo et al., 2005; Tryon and Baseman, 1992). In necropsy material, H_2O_2 production was identified in all *M. bovis* isolates (Schott et al., 2013). Additionally, ROS and RNS were detected following the recruitment and stimulation of macrophages and neutrophils. The combination of mycoplasmal H_2O_2 and ROS/RNS from white blood cells may result in the severe typical caseonecrotic lung lesions (Hermeyer et al., 2011; Schott et al., 2013). H_2O_2 formation by *M. bovis* type and field strains was also measured *in vitro* showing strain variability concerning the amount of H_2O_2 produced (Khan et al., 2005). Amounts of H_2O_2 generated from NADH oxidation varied from 0 to 1.1 mol H_2O_2/mol O_2 depending on the strains. This is proposed to be due to NADH-oxidase mainly producing either water or H_2O_2 (Khan et al., 2005). *In vitro* passage of strains led to a reduction of H_2O_2 levels, whereas oxidation-rates of other substrates did not change. This was demonstrated by the loss of a 32 kDa protein after extensive passaging of a *M. bovis* strain in axenic medium that correlated with a 50% reduction of H_2O_2 production (Khan et al., 2005). However, L-α-glycerophosphate (GP) was never oxidized by any strain tested, indicating the absence of glycerophosphate oxidase (GlpO) in all non-fermentative, non-arginine-hydrolyzing *M. bovis* strains (Khan et al., 2005).

8. Conclusion

Among the multiple virulence attributes of *M. bovis* described in this review, variable surface lipoproteins have been intensively investigated. They play an important role to circumvent the host immune recognition system and in adaptive evolution, compensating for the limited genome size of *Mycoplasma* species. Furthermore, some of these surface antigens are involved in mycoplasma adhesion to host cells, which is a requirement for the establishment of infection. Other virulence attributes were less studied but the advent of new molecular tools should lead to a better understanding of the mechanisms involved in the pathogenesis of this bacterium. Biofilm formation is likely to be essential for persistence of *M. bovis* in the host and in its close environment. Moreover,

host cell damage due to secreted metabolites of *M. bovis* such as H_2O_2 deserves more investigation to understand the molecular processes involved as well as the role of co-infecting microorganisms and the modulation of the host immune response. Finally, invasion and intracellular survival of *M. bovis* in bovine cells could contribute to the persistence and dissemination in the host and may lead to the resistance of *M. bovis* infections to antibiotic treatments and vaccination trials against bovine mycoplasmosis.

Conflicts of interest

None.

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Due to restrictions concerning the number of quoted references in reviews, the authors apologize for not having been able to include all publications.

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