Persistence in Livestock Mycoplasmas—a Key Role in Infection and Pathogenesis

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Abstract

Purpose of Review *Mycoplasma*, economically important pathogens in livestock, often establishes immunologically complex persistent infections that drive their pathogenesis and complicate prophylaxis and therapy of the caused diseases. In this review, we summarize some of the recent findings concerning cellular and molecular persistence mechanisms related to the pathogenesis of mycoplasma infections in livestock.

Recent Findings Data from recent studies prove several mechanisms including intracellular lifestyle, immune dysregulation, and autoimmunity as well as microcolony and biofilm formation and apoptosis of different host cell types as important persistence mechanisms in several clinically significant *Mycoplasma* species, i.e., *M. bovis*, *M. gallisepticum*, *M. hyopneumoniae*, and *M. suis*.

Summary Evasion of the immune system and the establishment of persistent infections are key features in the pathogenesis of livestock mycoplasmas. In-depth knowledge of the underlying mechanisms will provide the basis for the development of therapy and prophylaxis strategies against mycoplasma infections.

Keywords Livestock mycoplasma · Persistent infection · Immune dysregulation · Apoptosis · Cell invasion · Biofilm

Introduction

Immune dysregulation, immune evasion, cell invasion, or biofilm formation is part of a sophisticated strategy, which enables mycoplasmas to establish persistent and chronic infections.

Mycoplasmas are characterized by the lack of a cell wall, resistance to β -lactam antibiotics, low G + C content, and extremely small genome sizes connected with a highly limited metabolic capacity. Striking features of pathogenic *Mycoplasma* species include a strict parasitic and host-specific lifestyle (either extracellularly on epithelial cells or erythrocytes or intracellularly in different host cells), a high

metabolic adaption to the host's metabolism, and their fastidious nature (high demands on culture conditions) or even a found unculturability in vitro in hemotrophic mycoplasmas [1, 2].

Mycoplasma infections in livestock are known as a significant cause of economic loss and welfare concern in the agricultural sector worldwide. Both, primary pathogenic and facultatively pathogenic *Mycoplasma* species are involved. The economic impact is on the one hand the result of the clinical signs caused by the mycoplasmas themselves and on the other hand due to the increased susceptibility to other infectious agents caused by the infection-induced immune dysregulation [3]. Therapy and prophylaxis are not easy due to the common persistence of *Mycoplasma* infections and their complex pathogenesis. As a result, endemic infectious diseases are combated by the metaphylactic use of antibiotics posing a significant problem in the livestock sector concerning the high amount of used antimicrobial substances and the development of antibiotic resistance [4–7].

In livestock, the most relevant pathogenic species are *M. bovis* and *M. mycoides* subspecies *mycoides* in cattle; *M. agalactiae*, *M. capricolum*, and *M. mycoides* ssp. *capri* in small ruminants; *M. hyopneumoniae* in pigs; and

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M. gallisepticum in poultry. In addition, facultative pathogens like *M. hyorhinis* and *M. hyosynoviae* in pigs or *M. synoviae* in poultry are also connected to large economic losses in farms [8–10]. Clinical manifestations range from respiratory diseases to mastitis, arthritis, polyserositis, agalactia, and reproductive disorders (Table 1) [8, 10, 34, 35]. Furthermore, a highly specialized group of bacteria with a unique cell tropism to red blood cells (RBCs) was reclassified to the genus *Mycoplasma* as so-called hemotropic mycoplasmas (trivially named hemoplasmas) at the beginning of the twentieth century [25, 36]. Hemotropic mycoplasmas in livestock comprise *M. suis*, *M. parvum*, and ‘*Candidatus M. haemosuis*’ in pigs, *M. wenyonii* and ‘*Ca. M. haemobos*’ in cattle as well as *M. ovis* and ‘*Ca. M. haemovis*’ in sheep and goats (Table 1) [1, 13, 27–30, 37, 38]. The clinical outcome of hemoplasma infections is highly variable ranging from life-threatening anemia to various chronic syndromes (e.g., mild anemia, poor performance, reproductive disorders) or even asymptomatic courses [1, 26]. Economic significance was particularly described for *M. suis*, the causative agent of infectious anemia in pigs (IAP) due to its high prevalence and its chronic immunosuppressive nature [1, 39].

Mycoplasma-related infectious diseases are as aforementioned multifactorial and often associated with other viral or bacterial infections [1, 40, 41]. But also, abiotic factors (e.g., stress, crowding, housing condition, climate) influence the susceptibility of the hosts and the clinical outcome of the diseases. This complexity is also reflected on the level of the

molecular pathogenesis since clinical signs and pathomorphological changes are not primarily caused by the direct damage of the host by the bacterium itself (e.g., by classic virulence factors such as toxins) but also by the host’s defense against the mycoplasmas [2, 42]. It is well known that *Mycoplasma*-infected animals develop both extensive innate and adaptive immune response to fight against infection. However, this immune response often fails to eliminate the pathogens leading to persistence and chronicity. Moreover, the induction of persistent lifelong infections in humans and animals allows the mycoplasmas to live in balance with the host system and, thus, to assure the survival of their species. To establish persistent infections and to ensure their survival despite immune response and antibiotic treatment, mycoplasma has evolved highly specialized mechanisms against adverse conditions [43], a fact that is particularly remarkable in the light of the small genomes and the limited biosynthetic capabilities of mycoplasmas. Long been recognized immune modulation and immune evasion mechanisms include molecular mimicry and antigenic variation which is comprehensively reviewed by Citti and co-workers in 2010 [44]. The present review summarizes the recent findings on novel strategies of livestock mycoplasmas to realize persistence by autoimmunity, apoptosis of different host cells, cell invasion, and biofilm formation. Finally, based on the presented findings, attempts should be made to provide approaches and strategies for future research work and, thus, to combat these bacterial infectious agents.

Table 1 Overview of livestock *Mycoplasma* species, main host animal species, and clinical significance

Main host animal species	<i>Mycoplasma</i> species	Clinical significance	References
Cattle	<i>M. mycoides</i> spp. <i>mycoides</i>	Contagious bovine pleuropneumonia (CBPP)	[11]
	<i>M. bovis</i>	Mastitis, arthritis, part of bovine respiratory tract disease (BRTD)	[12]
	<i>M. wenyonii</i>	Hemolytic anemia, asymptomatic infections	[13, 14]
	‘ <i>Ca. M. haemobos</i> ’	Hemolytic anemia, asymptomatic infections	[13, 15]
Sheep and goat	<i>M. agalactiae</i>	Contagious agalactia	[9, 16]
	<i>M. mycoides</i> ssp. <i>capri</i>	Contagious agalactia	[9, 16]
	<i>M. capricolum</i> spp. <i>capricolum</i>	Contagious agalactia	[9, 16]
	<i>M. capricolum</i> spp. <i>capripneumoniae</i>	Contagious caprine pleuropneumonia (CCPP)	[17]
	<i>M. ovis</i>	Hemolytic anemia	[18]
	‘ <i>Ca. M. haemovis</i> ’	Hemolytic anemia	[19]
Pig	<i>M. hyopneumoniae</i>	Enzootic pneumonia	[6, 20]
	<i>M. hyorhinis</i>	Arthritis, polyserositis, conjunctivitis	[21–23]
	<i>M. hyosynoviae</i>	Arthritis	[10, 24]
	<i>M. suis</i>	Infectious anemia of pigs (IAP)	[1, 25, 26]
	<i>M. parvum</i>	Non-pathogenic	[27]
	‘ <i>Ca. M. haemosuis</i> ’	Infectious anemia of pigs and asymptomatic infections	[28–30]
Poultry	<i>M. gallisepticum</i>	Chronic respiratory disease, arthritis, reduced performance	[8, 31–33]
	<i>M. synoviae</i>	Respiratory disease, synovitis, eggshell apex abnormalities and egg drops	[8, 31, 32]

Dysregulation of the Immune System

Studies using molecular high-throughput technologies confirmed that *Mycoplasma* infections are accompanied by massive changes in the gene expression profiles of the host. Whole blood transcriptome profiling studies of *M. suis*-infected pigs and *M. mycoides* ssp. *mycoides*-infected cattle revealed a downregulation of the gene expression leading to a general immune suppression [45, 46]. It must be considered that this global approach reflects the sum of all local and systemic inflammatory and immune relevant host responses during the acute phase of infection. Detailed analysis of the immune response and underlying pathways either in organs, epithelial cells, or different immune cell subpopulations in vivo as well as in vitro revealed a more discriminating picture of the interference of the immune system due to *Mycoplasma* infections. Both immune stimulation and immune suppression were reported indicating a complex and dynamic interplay between activation and inhibition, depending on the phase of infection, the host's immune status, the cell types or subset of peripheral blood mononuclear cells (PBMCs), and the virulence of the pathogen [47•, 48–51]. Ex vivo and in vitro analyses of tracheal epithelial cells after *M. gallisepticum* infection delivered a macrophage activation and upregulation of several inflammatory cytokines including IL-1 β , IL-6, IL-8, IL-12p40, CCL-20, and NOS-2 [52, 53]. Moreover, *M. hyopneumoniae* and *M. bovis* were shown to stimulate the immune response through macrophages by elevating the release of pro-inflammatory cytokines such as IL-2, IL-6, and IL-1 β [47•, 50, 51]. Other studies revealed a general downregulation of innate immune response-related genes in mammary lymph nodes in *M. bovis*-induced mastitis or an immune suppression through a significant reduction of PBMC proliferation as well as a shift of the cytokine profile from pro-inflammatory to anti-inflammatory [48–50]. Furthermore, *M. gallisepticum* induces a decrease in the expression of inflammatory cytokines such as TNF α , IL-1 β , and IL-6 in infected embryonic chicken fibroblast and lung cells [54]. Decreased levels of the pro-inflammatory cytokines, e.g., TNF α and IFN γ , with concomitant elevation of anti-inflammatory cytokines like IL-10 were also reported in *M. bovis*-infected cattle and monocyte cultures [49, 55•]. However, the effect of *M. bovis* on the cells depends on the corresponding PBMC subpopulation since the expression of IFN γ was upregulated in the different types of T cells and NK cells and downregulated in monocytes, dendritic cells, and B cells [50].

Several pathogenic *Mycoplasma* species such as *M. hyopneumoniae*, *M. pneumoniae*, and *M. ovipneumoniae* are capable to evade the phagocytic uptake by host macrophages representing the major

component of the innate immunity [56, 57, 58•]. Recently, a detailed look on the gene expression profile of *M. hyopneumoniae*-infected porcine alveolar macrophages expectedly showed a very complex and dynamic process involving massive alterations in the expression profiles mainly of the inflammatory and innate immune response, apoptosis, signal transduction, and cell adhesion [59]. Interestingly, phagocytic uptake of *M. hyopneumoniae* was not enhanced by convalescent antibodies indicating a possible opsonization prevention mechanism in *M. hyopneumoniae*. Indeed, an IgG capture and cleavage system was described in *M. mycoides* subspecies *capri* which may inhibit IgG-mediated immune defense including opsonization [60•]. This two-protein system MIB-MIP consists of an Ig-binding protein (MIB) and an Ig serine protease (MIP), which synergistically cleave the IgGs, thus enabling the evasion of humoral effector functions (e.g., complement activation, opsonization). Homologs of the MIB-MIP system were also identified in the majority of pathogenic livestock *Mycoplasma* species including *M. hyopneumoniae*, *M. gallisepticum*, *M. bovis*, and *M. suis* [60•].

Autoimmunity

Autoimmunity is a further immune-related mechanism that is used by mycoplasmas to manipulate the host immune system leading to evasion of a protective immune response. Thereby, autoreactive processes allow the pathogen to mask the pathogen-specific immune response. Cold reactive IgM autoantibodies (cold agglutinins) produced 1 to 2 weeks after infection are well recognized in *M. pneumoniae* and *M. suis* infections [1, 61]. The target structures of cold agglutinins are not fully elucidated, but it is assumed that cold agglutinins are directed against altered antigens or carbohydrate I antigen on the RBC surface [25, 61]. During *M. suis* infections, cold agglutinins contribute to the development of hemolytic anemia but also RBC agglutination at low temperatures leading to a blockage of the capillaries in the terminal vascular bed, e.g., at the edge of the ear, and thus to typical aural cyanosis [1, 62, 63].

In addition, autoantibodies of the IgG class (warm-reactive autoantibodies) were induced during *M. suis* infections which target the β -actin of RBCs. B cell clones specific for cytoskeletal actin or band 3 are known as physiological components, and it was suggested that *M. suis* leads to a massive and unspecific B cell proliferation of these B cell clones. Other theories include the alteration of the RBC due to the adhesion of *M. suis* resulting in the exposure of so far hidden cytoskeletal antigens or molecular mimicry due to autologous epitopes between the adhesion protein MSG1 and β -actin [64].

Programmed Cell Death (Apoptosis, Eryptosis) as Modulating Mechanism

Modulating programmed cell death pathways is another mechanism used by mycoplasmas to manipulate the immune system during the pathogenesis in farm animals [65–69]. Apoptosis enhancement of immune cells could negatively regulate the host's defense system. On the other hand, downregulation of apoptosis is a crucial factor to achieve persistence and ensure bacterial survival. For instance, triggering apoptosis in thymocytes was described for *M. gallisepticum*, thus interfering profoundly both with the development and the effectiveness of the adaptive immune response [66]. In addition, *M. hyopneumoniae* is reported to significantly provoke apoptosis in PBMCs (i.e., lymphocytes and monocytes) as well as in alveolar macrophages, the essential cell type for local innate immunity in the lungs [70]. Modulation of apoptosis was also found for *M. bovis* for PBMC and RBCs [50]. Interestingly, in vitro studies determined both apoptosis induction and delay in PBMCs suggesting that *M. bovis* is capable to regulate the cellular apoptosis machinery depending on the infection phase [49–51]. Programmed cell death of RBCs (so-called eryptosis) was also found during infection with the hemotrophic *M. suis* [65].

The severity of the clinical disease outcome could be proportional to the degree of apoptosis induced by mycoplasmas [65, 71]. Moreover, host- and strain-specific differences in apoptosis of alveolar macrophages were also found for *M. bovis*. For example, *M. bovis* isolates from bison showed less modulation of apoptosis in vitro than *M. bovis* isolates from cattle [72]. Felder and co-workers described similar observations in connection with eryptosis in *M. suis* infections in pigs. In these investigations, a clear correlation between the eryptosis ratio and the virulence of the *M. suis* isolates in connection with the severity of the course of the infection was found [65].

Despite these reports, the molecular mechanisms responsible for apoptosis modulation are not fully elucidated. Beyond the induction of oxidative stress, mitochondrial dysfunctions or membrane changes due to direct interactions of the mycoplasmas with the host cell membrane are discussed as the cause of apoptosis [66, 71]. Thereby, lipid-associated membrane proteins function as an apoptosis inducer in *M. hyopneumoniae* infections in vitro through p38 MAPK and Bax/Bcl-2 signaling pathways as well as caspase activation [71, 73]. Moreover, the involvement of the calcium homeostasis, as well as ER stress, was observed in the apoptosis events induced in infected tracheal cells [74]. In *M. gallisepticum*, the GroEL protein (heat shock protein 60) was detected as an inducer of apoptosis in PBMCs. The interaction of the GroEL with annexin A2 obviously plays a key role in this process [69].

In *M. bovis* infections, this apoptosis process is associated with an increased expression of PD-1 (programmed cell death protein 1) and thus the involvement of the so-called PD-1/PD-L1 pathway. Prostaglandin E2 (PGE2) plays a role as an

inducer of the PD-L1 expression and could thus be involved in immunosuppression during an *M. bovis* infection [55•, 75].

Cell Invasion

Previously, mycoplasmas were considered to be extracellular bacteria that attach to the surface of epithelial cells of the respiratory tract, the joints, and the mammary glands (*M. bovis*, *M. hyopneumoniae*, *M. gallisepticum*) or to RBCs (hemotrophic *Mycoplasma* species). However, by now, it is more and more recognized that several mycoplasmas can invade and persist within host cells. *Mycoplasma penetrans* originally isolated from the urogenital tract of HIV patients was the first described cell invader [76]. During the last few years, exhaustive research using modern imaging technologies proved the capability to enter non-phagocytic and/or phagocytic cells also for several livestock-infecting *Mycoplasma* species.

An intracellular location of *M. bovis* was shown in situ in degenerated macrophages in lung tissue of infected animals and in inflammatory cells, hepatocytes, renal tubule epithelial cells, and facial nerve bundles of necrotic tissue samples [77, 78]. In addition, the ability of *M. bovis* to invade bovine mononuclear blood cells (PBMC), embryonic calf turbinate cells, embryonic bovine tracheal cells, and epithelial cells from bovine kidney and the mammary gland was demonstrated in several studies in vitro [34, 50, 79, 80]. Recently, in *M. hyopneumoniae* which was considered as a strict extracellular pathogen for a long time, the capacity to enter porcine epithelial cells in vitro was shown [81•]. Moreover, invasiveness is demonstrated in further livestock mycoplasmas, i.e., *M. gallisepticum* in RBCs, HeLa cells, and chicken embryo fibroblasts; hemotrophic *M. suis* in RBCs; and *M. agalactiae* in epithelial cells and fibroblasts [82–85].

Several advantages such as protection from the host's immune defense, resistance against antibiotic therapy, and the supply with sufficient nutrients are associated with the intracellular lifestyle [82, 84]. Thus, the intracellular niche offers a protected environment to establish a chronic persistent infection that allows the egress and propagation upon the return to favorable extracellular conditions in the host. Moreover, internalization and, therefore, cellular masking of the pathogen may also contribute to a better translocation through cell barriers and/or effective dissemination by the bloodstream. Actually, *M. hyopneumoniae* could be isolated outside the respiratory tract in pericardial and synovial joint fluids in slaughter pigs with fibrinous pericarditis, and *M. gallisepticum* was found in various inner organs (e.g., kidney, liver, brain) of chicken experimentally infected via aerosol [81•, 86, 87]. It seems clear that mycoplasmas can overcome typical body barriers like the blood-brain barrier (BBB) due to their intracellular lifestyle [83, 88•]. For instance, various *Mycoplasma* species normally found as colonizers in organ systems, e.g., the udder or respiratory tract, were also detected in the

brain of the infected animals even associated with encephalopathies and ataxias; e.g., *M. agalactiae* was found in large amounts in the brain associated with non-purulent encephalitis as well as ataxia in young animals [89]. Moreover, *M. gallisepticum* and *M. synoviae* were also isolated from the brain of animals with meningeal vasculitis and encephalitis [88•]. So, pathogenic mycoplasmas may pass the BBB and cause neuropathological effects. How they cross this barrier is currently unknown. The involvement of toxins (e.g., CARDS toxins) that could lead to an increase in BBB permeability through inflammatory reactions is discussed [88•, 90].

Invasiveness may also be associated with an increase in virulence, as described for some livestock *Mycoplasma* species. For example, cell-invasive *M. gallisepticum* strain R_low was shown to be more virulent than the marginally invasive strain R_high [91], and the RBC invasive *M. suis* strain 08/07 also exhibited a considerably enhanced virulence in experimentally infected pigs [82]. Comparative analyses of the genome sequences revealed no correlates for the differences in virulence between invasive and noninvasive strains. In addition, no hints for classically known bacterial invasins were found in the *Mycoplasma* genomes. Although invasiveness seems to be a very important pathogenesis determinant in livestock *Mycoplasma* infections, our knowledge on the underlying molecular mechanisms are rather fragmentary.

Initially, adhesion is the first essential step toward internalization. Obviously, the interaction of bacterial proteins and extracellular matrix proteins such as fibrinogen plays an important role as an initial trigger for cell entry. Binding of *M. gallisepticum* to plasminogen and fibronectin and of *M. hyopneumoniae* to fibronectin was described by several research groups [81••, 91, 92]. Various fibronectin-binding proteins were also identified for *M. bovis* [93–96]. It is supposed that binding to fibronectin acts as a mediating bridge between the pathogen and host cell receptors of the integrin family initiating the uptake in non-phagocytic cells. Recently, it was shown by Raymond and co-workers that the interaction of *M. hyopneumoniae* with surface-associated fibronectin mediates the association with the integrin $\beta 1$ on the surface of epithelial cells, thus initiating signaling events and stimulating the cellular uptake [81••]. Binding to cell surfaces via plasminogen, on the other side, could also contribute to cell invasion via the formation of hydrogen bonds [97]. In addition, it is also supposed that extracellular β -actin could be an important receptor for *M. hyopneumoniae* on pig lung cells and *M. suis* on epithelial cells [98, 99].

Mycoplasma moonlighting proteins were shown to be involved in adhesion and extracellular matrix-binding activities which could be due to fully exploit the limited capacity of their minimal genomes. Examples for this include the α -enolase in *M. bovis*, *M. hyopneumoniae*, and *M. suis*; the GAPDH in *M. suis*; EFTu in *M. hyopneumoniae*; and NAD oxidase and the tRNA methyltransferase TrmFO in *M. bovis* [94, 96, 97, 100–104].

Overall, as already mentioned, there is still very little knowledge about the mechanisms and receptors that are used by livestock *Mycoplasma* species to invade cells and, therefore, still a great need for further research on this topic.

Biofilm and Microcolony Formation

The formation of biofilms and microcolonies represents a further mechanism of bacterial persistence. Adherent biofilms or bacteria within the biofilms are resistant to antibiotics, antibodies, and phagocytosis. Biofilms usually contain a large proportion of persister cells, which are periodically and intermittently released into the surrounding tissue and can, therefore, lead to a recurrent active infection after antibiosis or when the immune response decays [105, 106]. Besides, biofilms can also increase host tissue damage by attracting phagocytes and releasing lysosomal enzymes and reactive oxygen and nitrogen species (ROS and RNS), whereas phagocytosis is rather inefficient under these conditions [107].

Up to now, it is well known that livestock mycoplasmas are able to form biofilms on both abiotic and biotic surfaces [107, 108]. These consist of either single cells or functionally heterogeneous microcolonies, which are held together by bacterial polymeric substances (polysaccharides, lipids, proteins, and extracellular DNA) [107]. In vitro studies on the biofilm formation of *M. bovis* on abiotic surfaces showed that the cell adherence capacity appears to be essential since the adherence to cover glasses as the first step in the formation of biofilm in *M. bovis* strains was very different due to the different expression of variable surface proteins [107]. Furthermore, in the case of *M. hyopneumoniae* biofilm formation on abiotic surfaces, extracellular DNA release seems to be essential for biofilm formation [108].

Moreover, biofilm-forming *M. bovis* strains were as expected more resistant to heat and dehydration which allow them to better survive in the environment. There are different data on the antibiotic resistance of mycoplasma in biofilms. While no change in the minimum inhibitory concentrations (MIC) of fluoroquinolones and tetracyclines was found for *M. bovis*, there was a significant increase in the MICs for *M. hyopneumoniae* for quinolones, colistin, gentamicin, and oxytetracycline [107, 109].

Biofilms or biofilm-like microcolonies could also be detected in vivo in experimentally infected animals. In one study, microcolonies of *M. suis* were electron microscopically observed on vascular endothelial cells [99]. Raymond and co-workers ultrastructurally found similar structures on the ciliated epithelium of the respiratory tract in *M. hyopneumoniae*-infected pigs [108]. In both reports, there was also evidence that these biofilm-associated mycoplasmas could have morphological changes leading to a pleomorphic shape [99, 108].

Conclusions

A resume of the current research studies addressing the biology of *Mycoplasma* species causing economic significant diseases in farm animals indicates that the induction of persistent infections is the central and critical attempt of the highly adapted and host-specific *Mycoplasma* species to principally survive.

Therefore, analysis of the molecular pathways of the host-pathogen balance leading to persistence is one of the most important topics in mycoplasmal research. Next-generation high-throughput technologies (genomics, proteomics, transcriptomics, metabolomics) are very valuable tools to elucidate the pathobiology and host response in mycoplasma-associated livestock diseases caused by mycoplasmas in the future. Extended insights on persistence mechanisms will be the basis to develop much needed novel vaccine strategies and therapy options leading to reduced usage of antibiotics in farm animals.

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Compliance with Ethical Standards

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