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An overview of the Pathogenesis and Virulence of *Mycoplasma gallisepticum* infection – a review

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ABSTRACT

Mycoplasma gallisepticum (Mg) belongs to the class *Mollicutes*, the smallest and simplest microorganisms which lack a cell wall and are capable of self-replication outside a host. Mg is the primary etiological agent of chronic respiratory diseases in poultry, and causes major economic losses. Recently, owing to advances in scientific knowledge, large data sets have become available for Mg, providing sequencing data, new typing strategies, diagnostic methods, and means for comprehensive studies. The aim of this review is to summarize the current knowledge regarding the virulence, variable surface lipoproteins, invasion of host cells, adhesion, antigenic variation, biofilm formation, and modulation of the host immune system. Moreover, the broader picture includes the emergence of Mg successfully combating host immunity and adapting to the new host or niches, having evolved a number of strategies and mechanisms, contributing to the pathogenesis and dissemination of Mg infection.

Key words: *Mycoplasma Gallisepticum*; virulence; pathogenesis; dissemination

Introduction

Mycoplasmas belong to the class *mollicutes*, the smallest prokaryotic microorganism capable of self-replication (LU et al., 2017; RAZIN et al., 1998). Previous reports showed that *Mycoplasma* species are found in a variety of hosts, including insects, plants, animals and humans (ROTTEM and NAOT, 1998). Among them, *Mycoplasma gallisepticum* (Mg) causes chronic respiratory disease in chickens, and infectious sinusitis in turkeys (LEY et al., 1996; LEY, 2003). The disease develops slowly, causing severe inflammation of the respiratory tract, and results in immune dysregulation in avian species (PFLAUM et al., 2015). The pathogen often persists in a flock for a long time and most adult chickens have latent infections (EVAN et al., 2005; DHONDT et al., 2014). Favorable conditions, such as heat and cold stresses, high feeding density, fouling of the chicken house, accumulation of feces, excessive ammonia, sudden changes in climate, and excessive temperature differences, can contribute to the outbreak and spread of the disease (LEY, 2003). Previous studies reported that Mg infection

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causes major economic losses in terms of reduced egg production, weight gain and hatchability, low carcass quality, and infected flocks become susceptible to other diseases (LI et al., 2019). Currently, the molecular mechanisms underlying the pathogenicity of *Mycoplasmas* appear to be multifactorial. In contrast to other bacteria, Mg do not produce potent toxins such as *Mycoplasma pneumoniae* that contribute to the pathogenicity of infection (KANNAN and BASEMAN, 2006). Until recently, limited research techniques and a lack of genetic tools impeded efforts to study in detail the virulence and pathogenesis of Mg, which is of prime importance for development of prophylactic and therapeutic measures to combat Mg infection. Recently, the development and improvement of various molecular detection methods, including whole genome sequencing (WGS), and PCR detection of field isolates, have made it possible to study the molecular mechanisms and interactions of MG with its host (BALL et al., 2020; FUJISAWA et al., 2019). Genomic studies have revealed considerable diversity among *Mycoplasma* species (CALDERON-COPETE et al., 2009; CHAMBAUD et al., 2001; GLASS et al., 2000; JAFFE et al., 2004; PAPAZISI et al., 2003). However, there are common and linked characteristics among *Mycoplasma* species which include phase variation, cell surface lipoproteins, evolution of virulence, and complex interactions with the cellular immune response (BROWNING et al., 2011). Therefore, this review gives an overview of the current literature on the pathogenic mechanisms of Mg from the point of view of lipoproteins, cell adhesion, invasion, antigenic variations due to phase variation of surface proteins, biofilm formation, and modulation of the host immune system.

Adhesion. Mg adhere primarily to the surface of the respiratory epithelium during the early stages of infection, which is called adhesion (ROTTEM, 2003). Adhesion plays a central role in the attachment of Mg to its host's cell surface, and relies on a special terminal bleb-like structure on the cell surface of Mg, which firmly adheres to the surface of the host's respiratory mucosa through adhesion proteins on the surface, causing cell degeneration and damage to the cilia. In addition, the protective layer of epithelial cells is damaged, allowing other pathogens to enter the body with ease, leading to various secondary diseases (HENDERSON and JENSON, 2006). Previous studies best characterized the key proteins involved in adhesion as GapA and CrmA (MUDAHI-ORENSTEIN et al., 2003; PAPAZISI et al., 2002), PvpA (BOGUSLAVSKY et al., 2000), OsmC-like protein MG1142 (JENKINS et al., 2007) and MGC2 (HNATOW et al., 1998), which are essential for firm attachment of Mg, and their virulence and colonization in chickens. In addition, studies reported that metabolic enzymes, such as Eno (CHEN et al., 2011), triosephosphate isomerase (BAO et al., 2015), and pyruvate kinase (HE et al., 2015), are also cytoadherence proteins in Mg. It has been shown previously that adhesion and attachment are necessary for the exchange of essential lipids, nucleotides, and amino acids during Mg survival (RAZIN and JACOBS, 1992). Importantly, mutants of Mg which lack cytoadhesion proteins were found to be less pathogenic than mutants which possess cytoadhesion proteins (PAPAZISI et al., 2000). The factors that affect the binding of Mg to a cell surface receptor include sialyl receptors, for instance a glycophorin found on the cell surface of red blood cells (CHAMBAUD et al., 1999; KEELER et al., 1996). It is clear from these studies that adhesion plays a key role in Mg-induced inflammation and host-elicited immune responses. Future studies are needed in the identification and characterization of cytoadhesion proteins associated with Mg pathogenicity, as these proteins could be the Achilles' heel of the Mg.

Lipoproteins. Lipoproteins from mycoplasmas are often only diacylated and lack the amide-linked lipid chain (SUTCLIFFE et al., 2012). The acyl groups of lipoproteins anchor in the cytoplasmic membrane, with exposure to the extracellular milieu. Several lipoproteins are targets of growth inhibitory antibodies or act as virulence factors (SCHMIDT et al., 2004; WASHBURN et al., 2003). The genes of lipoproteins appeared within operons associated with ABC transporters. Therefore, lipoproteins are believed to be involved in the transport of nutrients into the cell (ATALLA et al., 2015). In addition, it has been reported that lipoproteins are also associated with the induction of apoptosis (HOPFE and HENRICH, 2008), ATPase activity, and cytadherence (HOPFE and

HENRICH, 2004). Several lipoproteins have been identified and characterized in Mg. Among them, an immunodominant 64kDa lipoprotein (LP 64), hydrophobic in nature, was isolated from the membrane of Mg, and its role in cytadherence was confirmed in the study (FORSYTH et al., 1992). The haemagglutinins of Mg, such as pMGA, are lipoproteins that appear to have arisen from horizontal gene transfer and encoded by a multi-gene family, contain 30-70 genes (BENCINA, 2002). Recently, comparative genomic analysis identified a homologue of second lipoprotein gene associated with virulence in attenuated strains of Mg (SZCZEPANEK et al., 2010). Numerous studies have reported the crucial role of membrane surface lipoproteins in the induction of inflammation, eliciting host immune responses, and its association with virulence (ATALLA et al., 2015; HU et al., 2016). Extensive variation has been found in these lipoproteins, such as phase variation, which contributes to the successful evasion of Mg from the host immune system, and results in the establishment of chronic infection (TULMAN et al., 2012). Furthermore, it is necessary to undertake further research on lipoproteins and their role in Mg infection to better assess the structural variations, mutations, and mechanisms by which lipoproteins interact with host cells.

Cell Invasion. Mg invade a wide variety of cells and tissues, including brain cells, erythrocytes, epithelial cells, and immune cells (ISHFAQ et al., 2019a; ROSALES et al., 2017; MOHAMMED et al., 2007; ISHFAQ et al., 2020; WINNER et al., 2000). A previous study reported that Mg invasion caused mucosal thickening of the respiratory epithelium and resulted in the release of catarrhal exudate and mucus goblet from the cells (MOHAMMED et al., 2007). The pathogen invades, survives and replicates in chicken fibroblasts, erythrocytes and HeLa-229 cells. Previous studies have shown that Mg parasitizes non-phagocytic cells from the cell membrane to the perinuclear region (WINNER et al., 2000). The invasive ability of Mg depends on the strain, passage and the infected dose. It is worthy to mention here that some species of *Mycoplasmas* have the ability of cytadherence but lack the ability of invasion (BASEMAN et al., 1995). Mg host cell invasion also occurs in vivo along with its capability to escape and enter eukaryotic cells and disseminate to various organs. These findings support the invasive properties of this devastating organism (CHIN et al., 1991). Another study reported that an experimental Mg infection can be established through successful invasion of host cells by different strains of Mg (MUCH et al., 2002). In addition, invasion of chicken erythrocytes was studied in detail both in vivo and in vitro, and Mg were found to have penetrated erythrocytes under a scanning electron microscope. In addition, gentamicin invasion assay showed that Mg resisted gentamicin-treatment and survived in erythrocytes after 30 min of infection (VOGL et al., 2008). Overall, Mg invasion of various immune and epithelial cells contributes to its dissemination to various distant sites in the host, and impedes the control of Mg infection through chemopreventive agents. However, further research is needed to investigate the persistence and invasion of Mg infection in various cell types.

Antigenic variation and biofilm formation. Studies have demonstrated the high antigenic variation (ON-OFF) and size variation of the membrane surface in different *Mycoplasmas* species (RAZIN et al., 1998). In Mg, *vlhA* gene family lipoproteins have been identified as involved in the evasion of the host immune system and mediate the phase variations of Mg's surface architecture (BEARSON et al., 2003; GLEW et al., 2000). A previous study demonstrated that SpxA protein in Mg strain S6 contributed to the transition of the state, and the mechanisms of adaptation and bacterial evolution during invasion of eukaryotic cells (MAZIN et al., 2014; MATYUSHKINA et al., 2016). In another study, the *pvpA* gene was identified as a phase variable and cytoadherence protein in Mg. The *pvpA* gene has been found to undergo variations in expression per cell per generation at a frequency range from 10^{-3} to 10^{-4} (YOGEV et al., 1994). These findings suggest that antigenic phase variation serves as a successful strategy for Mg to escape/evade the host immune system. Consequently, Mg leads to chronic infections inside the host (BROWNING et al., 2011). High frequency phase variations contributed to immunodominant antigens, encoded by multigene families, that are a common feature of mycoplasmas genomes.

These multigene families range covers over 40 paralogous genes, most appearing functional, from gene duplication, and some members contain frame shift mutations (PAPAZISI et al., 2003). These genes undergo large scale expansion and are often closely related in *Mycoplasma* species, infecting a similar range of host species. Generally, strain-to-strain genetic variations are caused by successive rounds of expansion, as seen in the VPMA gene family (NOUVEL et al., 2010). Recently, a study compared sequences of *vlhA* promoters and Mg genes by bioinformatics analysis. *vlhA* promoters showed conserved sequences downstream and upstream to GAA repeats. Variations were found in the number of GAA units among orthologous groups and strains (ORLOV et al., 2018). So far, various mechanisms have been described for antigenic and phase variations (CITTI et al., 2010), but it would be interesting in future studies to investigate how many repeats alter at a time, whether the change depends on the sequences surrounding the GAA repeats, or on the number of repeats of a given gene and their physicochemical properties. In addition, the study of biofilm formation is ubiquitous in anti-mycoplasmal studies. Biofilm provides protection to bacterial species in unfavorable environmental conditions and contributes to persistent infections (WANG et al., 2012). Biofilm effectively resists antibiotic treatment, both chemically and mechanically, and results in antimicrobial resistance (HU et al., 2010). A previous study identified biofilm formation in Mg and unraveled the interaction between the pathogen and host, which is probably associated with the persistence of Mg infections. Strains such as NCL, CG_5, YL_4, R_{low} (P_{10} and P_{100}) and F were weak biofilm producers, and strains S_6 (P_5 and P_{20}), D_{9604}, SU_{15} and Nobilis MG 6/85, S_6 (P_5 and P_{20}) were strong biofilm producers. While crystal violet staining assay confirmed that strains F_{36} and Vaxsafe MG ts-11 did not produce a biofilm (CHEN et al., 2012). CHEN et al., (2010) reported the genes associated with biofilm formation in Mg by using mini-Tn4001-SGM transposon mutagenesis (CHEN et al., 2010). Transposon mutagenesis is an effective tool for studying and creating mutant libraries associated with biofilm formation (WANG et al., 2017). In order to understand the formation of biofilm by Mg better, and to combat Mg infection in avian species, particularly in commercial flocks (MCAULIFFE et al., 2006), further investigation will be needed to scrutinize the factors and genes involved in variations in biofilm formation in Mg strains.

Modulation of the host immune system. The possible interaction of Mg with host cells and modulation of the host immune system is shown in Figure 1. The interaction of *Mycoplasmas* with the host mucosal epithelium results in a strong lymph proliferative response, and this response is a primary pathogenetic mechanism in many *Mycoplasmoses*. The diacylated lipoproteins in *Mycoplasma* membranes play a major role in eliciting immune responses (HIMMELREICH et al., 1996), and are potent stimulators of macrophages (MUHLRADT et al., 1998). These lipoproteins could activate toll like receptors (TLRs) such as TLR-2/TLR-4/TLR-6, the NF-κB pathway, and result in the release of various inflammatory cytokines (FUJITA et al., 2003; TAKEDA et al., 2002). $CD8^+$ lymphocytes were seen initially after Mg infection in chickens after 7 days, $CD4^+$ and B lymphocytes predominated in the tracheal mucosa. These findings revealed an initial inflammatory response, and subsequent development of a chronic lymphocytic response to Mg infection (GAUNSON et al., 2006). In another study, after 24 h of infection, CXCL13 and lymphotactin genes were significantly upregulated, and also on days 4 and 8 after Mg infection, transcription of CXCL13, CXCL14, lymphotactin, RANTES, IL-1β, MIP-1β, and IFN-γ genes was dramatically upregulated in the tracheal mucosa. The increase in these chemokines and cytokines is correlated with the degree and severity of inflammation (MOHAMMED et al., 2007). Furthermore, Mg infection suppressed mitogenic activity in splenic lymphocytes, which led to a systemic immunosuppressive effect (MUNETA et al., 2008). BEAUDET et al. (2017) studied the detailed transcriptional profiling using RNA sequencing of chicken's tracheal response after virulent strain R_{low} Mg infection over a 7-day time period. The study gives a new insight into the inflammatory response produced by Mg interaction with the chicken's respiratory mucosal surface and elucidated a complex inflammatory response, contributing to the development of chronic

respiratory disease. The expression of many cytokines and chemokines changed differentially over the course of the infection. Immune-related signaling pathways, including Toll-like receptors (TLR), Jak-STAT, mitogen-activated protein kinase and the nucleotide oligomerization domain-like receptor pathways were found to be associated with these inflammatory responses (BEAUDET et al., 2017). In addition, several studies reported that Mg interacts with various cells and tissues both in vivo and in vitro, and results in a profound inflammatory response, induction of apoptosis and triggers autophagy (ISHFAQ et al., 2019b; MAJUMDER and SILBART, 2015; TIAN et al., 2016; LI et al., 2019; WU et al., 2019; BAO et al., 2020). The modulation of the host's immune response leads to the successful dissemination and prolonged survival of Mg inside the host. It is of prime importance to address Mg-host interaction in future studies, to pave the way for the successful prevention and control of this devastating pathogen, while minimizing diseased pathogenesis via immune dysregulation and inflammation (MAJUMDER and SILBART, 2015).

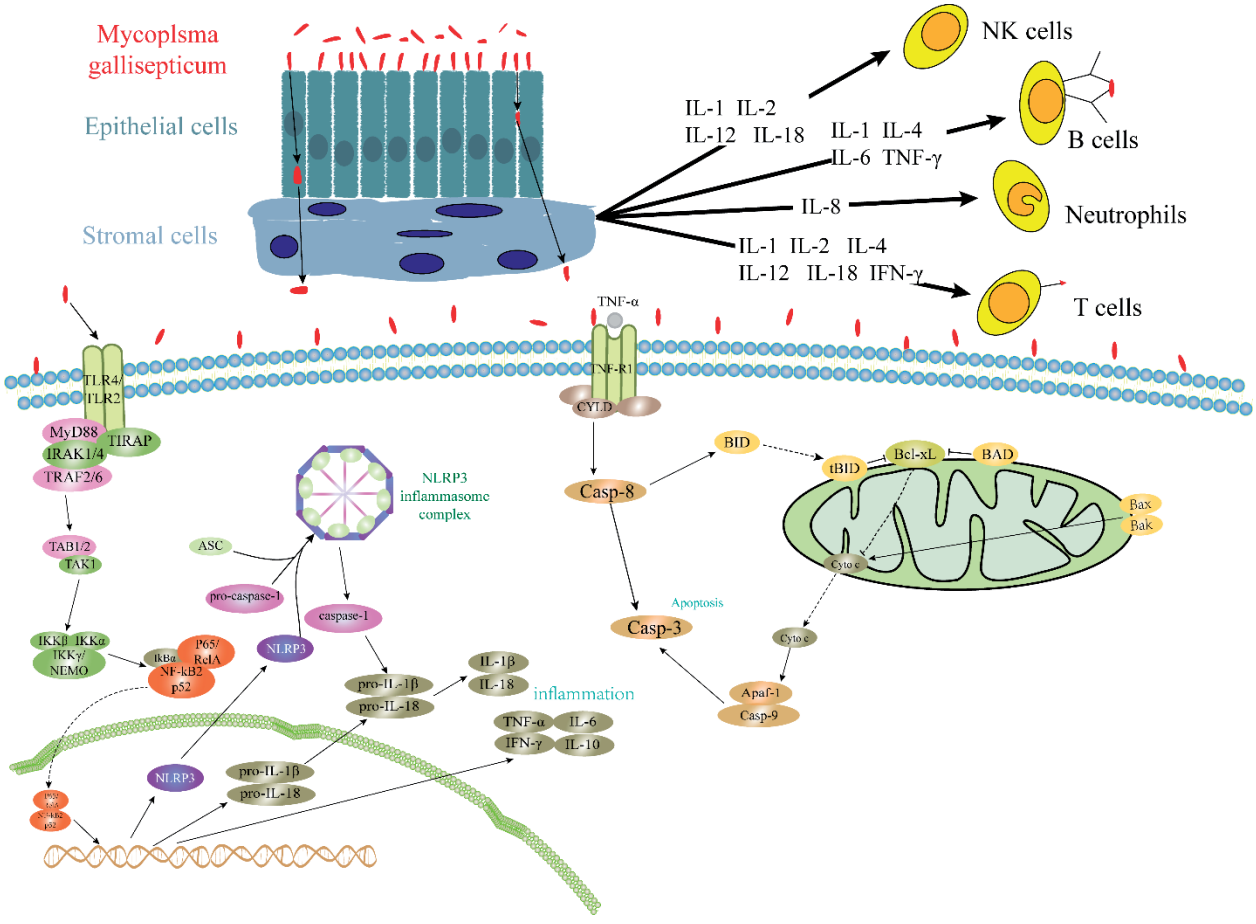

Fig. 1. Interaction and attachment of Mg cells with the host cell surface

The proteins and accessory molecules located on the membrane of Mg cells mediate adhesion to host cell surface receptors. Possible mediators and cytokine induced by Mg infection of host cells. Several cytokines were released by host cells when exposed to Mg. These cytokines further stimulate a wide range of immune cells.

Conclusions

The membrane surface lipoproteins are extensively investigated and explained in this review, among the other virulence characteristics of Mg. Lipoproteins play an important role in the induction of inflammatory response and escape from the host immune system. Antigenic phase variation is another phenomenon in Mg, likely involved in avoidance of host immune recognition. Moreover, some lipoproteins are involved in adhesion to host cells or surface epithelium. Biofilm formation is probably indispensable for the persistence and survival of Mg. The survival, adherence and invasion of Mg into host cells could contribute to the dissemination and persistence of Mg infection, and could lead to antibiotic resistance and failure of vaccinations against Mg infections. Nevertheless, current means for preventing and controlling Mg infection are still limited, and therefore novel vaccines and new antibiotics, with the benefits of avoiding resistance and rapid detection methods, still need to be developed in order to eradicate and fully prevent Mg infection.

Conflicts of Interest

None

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SAŽETAK

Mycoplasma gallisepticum (Mg) pripada razredu *Mollicutes*, najmanjem i najjednostavnijem mikroorganizmu koji nema staničnu stijenku i sposoban je za samoreplikaciju izvan domaćina. *M. gallisepticum* primarni je etiološki uzročnik kroničnih respiratornih bolesti u peradi, koji uzrokuje goleme gospodarske gubitke. Zahvaljujući napretku u znanstvenim spoznajama, u posljednje su vrijeme postali dostupni veliki skupovi podataka o ovoj bakteriji, iz kojih se može doznati o sekvenciranju, novim strategijama tipizacije, dijagnostičkim postupcima i sredstvima koji mogu poslužiti u sveobuhvatnom istraživanju. Cilj je ovog preglednog rada bio rezimirati aktualne spoznaje o virulentnosti, varijabilnim površinskim lipoproteinima, invaziji stanica domaćina, adheziji, antigenskim varijacijama, formiranju biofilma i modulaciji imunosnog sustava domaćina. Obuhvaćena je i šira slika koja uključuje slučajeve da se *M. gallisepticum* uspješno bori protiv imunosti domaćina i prilagođuje novim domaćinima ili nišama čime je razvijen niz strategija i mehanizama koji pridonose patogenezi i širenju infekcije ovom bakterijom.

Ključne riječi: *Mycoplasma Gallisepticum*; virulencija; patogeneza; širenje uzročnika