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In vitro and in vivo cell invasion and systemic spreading of *Mycoplasma agalactiae* in the sheep infection model

Shivanand Hegde[a,1], Shrilakshmi Hegde[a,1], Joachim Spergser[a], René Brunthaler[b], Renate Rosengarten[a], Rohini Chopra-Dewasthaly[a,*]

[a] *Division of Clinical Microbiology and Infection Biology, Institute of Bacteriology, Mycology and Hygiene, Department of Pathobiology, University of Veterinary Medicine, Veterinaerplatz 1, A-1210 Vienna, Austria*
[b] *Institute of Pathology and Forensic Veterinary Medicine, Department of Pathobiology; University of Veterinary Medicine, Veterinaerplatz 1, A-1210 Vienna, Austria*

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ABSTRACT

Generally regarded as extracellular pathogens, molecular mechanisms of mycoplasma persistence, chronicity and disease spread are largely unknown. *Mycoplasma agalactiae*, an economically important pathogen of small ruminants, causes chronic infections that are difficult to eradicate. Animals continue to shed the agent for several months and even years after the initial infection, in spite of long antibiotic treatment. However, little is known about the strategies that *M. agalactiae* employs to survive and spread within an immunocompetent host to cause chronic disease. Here, we demonstrate for the first time its ability to invade cultured human (HeLa) and ruminant (BEND and BLF) host cells. Presence of intracellular mycoplasmas is clearly substantiated using differential immunofluorescence technique and quantitative gentamicin invasion assays. Internalized *M. agalactiae* could survive and exit the cells in a viable state to repopulate the extracellular environment after complete removal of extracellular bacteria with gentamicin. Furthermore, an experimental sheep intramammary infection was carried out to evaluate its systemic spread to organs and host niches distant from the site of initial infection. Positive results obtained via PCR, culture and immunohistochemistry, especially the latter depicting the presence of *M. agalactiae* in the cytoplasm of mammary duct epithelium and macrophages, clearly provide the first formal proof of *M. agalactiae's* capability to translocate across the mammary epithelium and systemically disseminate to distant inner organs. Altogether, the findings of these in vitro and in vivo studies indicate that *M. agalactiae* is capable of entering host cells and this might be the strategy that it employs at a population level to ward off the host immune response and antibiotic action, and to disseminate to new and safer niches to later egress and once again proliferate upon the return of favorable conditions to cause persistent chronic infections.

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Introduction

Commonly described as the smallest and simplest bacteria, mycoplasmas are important pathogens of humans and animals with rather complex and sophisticated pathogenic attributes (Rosengarten et al., 2000). Having lost many of their metabolic pathways during a so-called degenerative evolution from a Gram-positive ancestor, these wall-less prokaryotes readily obtain their nutrition from host cells by mostly colonizing epithelial surfaces and thereby cause slow-progressing chronic diseases that are difficult to cure (Razin et al., 1998). Well-known for their antigenic variation systems, they have adapted sophisticated mechanisms to evade immune clearance, survive in the host and have evolved to infect new host niches (Rottem and Barile, 1993; Chopra-Dewasthaly et al., 2012; Citti and Blanchard, 2013). Mycoplasmas lack typical bacterial virulence factors like toxins, and the molecular determinants of their pathogenicity are largely unknown. This can be partially attributed to their fastidious and slow growth, relative recalcitrance to genetic manipulations, and also to their strict host-specificity that hinders the development of convenient small animal models (Razin et al., 1998; Citti and Blanchard, 2013).

Mycoplasma agalactiae is an economically important pathogen and the main etiological agent of contagious agalactia (CA) syndrome in sheep and goats, mainly characterized by mastitis,

* Corresponding author. Tel.: +43 1 25077 2104; fax: +43 1 25077 2190.
E-mail addresses: Rohini.Chopra-Dewasthaly@vetmeduni.ac.at, rohinicd@gmail.com (R. Chopra-Dewasthaly).
[1] Contributed equally to this work; to be considered as joint first authors.

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conjunctivitis and arthritis as predominant symptoms of a localized infection. Sporadically septicaemia, arthritis, pneumonia and reproductive disorders have also been reported (Bergonier et al., 1997; Gomez-Martin et al., 2013), indicating that the pathogen is capable of crossing the epithelial barrier to reach distant host niches, as also evidenced in a recent report where naturally infected asymptomatic male goats were shown to harbour *M. agalactiae* in atypical inner organs like brain and heart (Gomez-Martin et al., 2012). Nevertheless, *M. agalactiae* has so far been regarded as an extracellular parasite and it is unknown how it transforms local infections into systemic ones.

Persistence and chronicity are other hallmarks of *M. agalactiae* infections. Both diseased and asymptomatic animals continue to shed the pathogen for long periods of time, sometimes lasting up to several years (Bergonier et al., 1997). Antibiotic treatments are often unsuccessful as they can only reduce clinical symptoms but tend to promote the carriers that stay unaffected (Nicholas, 2002). Chronically infected and serologically negative herds with no signs of disease are a common clinical-epidemiological situation in endemic areas. Such animals easily escape disease control and eradication measures, and are capable of flaring up frequent CA outbreaks under stress conditions (Gomez-Martin et al., 2013) leading to huge economic losses. Despite such agronomical significance, *M. agalactiae's* pathogenic determinants and mechanisms of infection and persistence are largely unknown, a fact that can be attributed to its long resistance to genetic manipulation until 2005 (Chopra-Dewasthaly et al., 2005a, b), and also because it does not exhibit the more practical phenotypes associated with mycoplasma pathogenicity, such as hemadsorption and the presence of terminal tip structure as attachment organelle, and lacks convenient small animal models or cell lines for appropriate studies.

M. agalactiae demonstrates surface antigenic diversity via high-frequency switching of six immunodominant surface lipoproteins (Vpmas) caused by Xer1 recombinase encoded on the same pathogenicity island-like locus (Glew et al., 2002; Chopra-Dewasthaly et al., 2008; Czurda et al., 2010). Though lacking in concrete proof, such variable systems are often believed to play important roles in pathogenicity via host immune evasion and adaptation. Our data from a recent experimental infection study with Xer1-disrupted Vpma 'phase-locked' mutants (Chopra-Dewasthaly et al., 2008) clearly demonstrated that Xer1 is not a virulence factor of *M. agalactiae* and Vpma phase variation is not necessary for establishing infection though it might critically influence the survival and persistence of the pathogen under natural field conditions (Chopra-Dewasthaly et al., 2012). P40, a cytadhesin, and P48 with macrophage stimulatory activity, are two other lipoproteins, which seem to have important pathogenicity related attributes (Rosati et al., 1999; Fleury et al., 2002). Besides, production of hydrogen peroxide (Khan et al., 2005), biofilm formation (McAuliffe et al., 2006) and identification of genes involved in indirect host cell interactions (Baranowski et al., 2010) are also implicated in *M. agalactiae's* pathogenicity.

In view of the prevailing scenario, we tried to investigate whether *M. agalactiae* has the capacity to enter, survive and exit the eukaryotic host cells in a viable state, as this could explain the chronic, persistent and difficult-to-eradicate nature of its infections in spite of long antibiotic therapies. This phenomenon may also allow it to reach more favorable host niches by crossing the epithelial barrier as cell invasion is often considered a major factor for systemic spread (Cieri et al., 2002; Much et al., 2002). Here, we provide evidence for the first time that *M. agalactiae* is able to invade eukaryotic host cells whereby quantitative results are supported by the qualitative double immunofluorescence assay. Intracellular mycoplasmas were detected not only after in vitro infection but also in vivo in various tissue samples from experimentally infected sheep using immunohistochemistry. Also, by the isolation of mycoplasmas from various internal organs of sheep experimentally infected via the intramammary route we formally demonstrate that *M. agalactiae* has the capability to cross local epithelial barriers and to disseminate to distant body sites. The findings of this study, together with the sophisticated antigenic variation system, could explain the persistence and chronicity of *M. agalactiae* infections.

Materials and methods

Mycoplasma growth

M. agalactiae pathogenic type strain PG2 (Sirand-Pugnet et al., 2007) was used in this study and was previously isolated from an infected goat in Spain (Fleury et al., 2002). It was grown in Aluotto or SP4 medium supplemented with penicillin, pyruvate, and phenol red as indicator as described before (Chopra-Dewasthaly et al., 2005b). Mycoplasma cultures were grown for 48 h and diluted serially in minimal essential medium (MEM) supplemented with non-essential amino acids and 10% heat inactivated fetal bovine serum (FBS) (Gibco BRL, Life Technologies) prior to infection of cultured mammalian cells. Number of viable mycoplasmas at the time of infection was determined by plating serial dilutions on SP4 plates containing 1% (wt/vol) Difco Noble agar and counting colonies under BMS 74955 stereomicroscope after 4–5 days of incubation at 37 °C.

Cell culture

HeLa-229 (ATCC CCL-2.1), Bovine endometrium cell line BEND (ATCC CRL-2398) and Buffalo lung fibroblasts (BLF; ATCC IMR-31) were the cell lines used in this study and were purchased from the American Type Culture Collection (ATCC; Manassas, USA) and certified to be free of mycoplasmas. HeLa-229 was maintained in MEM, BLF in McCoy's 5a medium (Sigma) with 10% heat inactivated FBS, and BEND cells in 1:1 mixture of Hams F12 and Eagle's MEM with Earle's BSS (Sigma-Aldrich) as per the instructions of ATCC. Trypsin and PBS were purchased from PAA Laboratories GmbH, Pasching, Austria or Sigma-Aldrich. 1×10^4 cells/well were seeded into Lab-Tek II Chamber Slides (Nunc International, Naperville, IL) for immunofluorescence staining and 5×10^4 cells/well were seeded into 24-well plates (CELLSTAR® Greiner Bio-One GmbH, Germany) for the gentamicin invasion assay 48 h prior to infection to attain confluence. Cell cultures were regularly checked for mycoplasma contamination by culture and PCR.

Mycoplasma infection and gentamicin invasion assay

Gentamicin invasion assay was carried out as described before with some modifications (Elsinghorst, 1994; Winner et al., 2000). *M. agalactiae* was grown for about 48 h indicated by metabolic color change before pelleting at $10{,}000 \times g$ at 4 °C for 10 min and resuspending in MEM. The cells were passed through 27-gauge needle for three to four times to disrupt any cell aggregates. Eukaryotic cells were infected with diluted cultures of mycoplasmas at a multiplicity of infection (MOI) of about 10–30 and incubated at 37 °C with 5% CO_2 for 4, 8, 16 and 24 h. Thereafter, extracellular bacteria were killed by incubation in MEM containing 400 μg/ml of gentamicin for an additional 3 h period. Although a concentration of 50 μg/ml gentamicin is known to be completely inhibitory for *M. agalactiae* growth (Chopra-Dewasthaly et al., 2005b), a higher concentration of 400 μg/ml was used to ensure the reliability of the assay and was experimentally determined to be sufficient to kill 100% of *M. agalactiae* in 3 h duration. After gentamicin treatment, supernatants were checked for the presence of any viable mycoplasmas by plating on SP4 agar. Subsequently, the cells were washed two to three times

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