SHORT COMMUNICATION

Sheep primary cells as *in vitro* models to investigate *Mycoplasma agalactiae* host cell interactions

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One sentence summary: The study is an important step forward in developing *in vitro* models that will facilitate analyses of *Mycoplasma agalactiae* and related ruminant mycoplasmas' host–pathogen interactions at the molecular level.

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ABSTRACT

Appropriate infection models are imperative for the understanding of pathogens like mycoplasmas that are known for their strict host and tissue specificity, and lack of suitable cell and small animal models has hindered pathogenicity studies. This is particularly true for the economically important group of ruminant mycoplasmas whose virulence factors need to be elucidated for designing effective intervention strategies. *Mycoplasma agalactiae* serves as a useful role model especially because it is phylogenetically very close to *M. bovis* and causes similar symptoms by as yet unknown mechanisms. Here, we successfully prepared and characterized four different primary sheep cell lines, namely the epithelial and stromal cells from the mammary gland and uterus, respectively. Using immunohistochemistry, we identified vimentin and cytokeratin as specific markers to confirm the typical cell phenotypes of these primary cells. Furthermore, *M. agalactiae*'s consistent adhesion and invasion into these primary cells proves the reliability of these cell models. Mimicking natural infections, mammary epithelial and stromal cells showed higher invasion and adhesion rates compared to the uterine cells as also seen via double immunofluorescence staining. Altogether, we have generated promising *in vitro* cell models to study host–pathogen interactions of *M. agalactiae* and related ruminant pathogens in a more authentic manner.

Keywords: invasion; adhesion; primary cell culture; immunohistochemistry; ruminant mycoplasmosis; host cell interactions

Mycoplasmas are one of the smallest and simplest self-replicating wall-less bacteria (Razin, Yogev and Naot 1998). Despite small genomes, they have evolved as one of the most successful pathogens of humans and animals using complex molecular and cellular strategies (Baseman and Tully 1997; Rosengarten *et al.* 2001). *Mycoplasma agalactiae* is the main etiological agent of contagious agalactia syndrome in small ruminants, which is mainly characterized by mastitis in lactating ewes. Additional symptoms include arthritis, keratoconjunctivitis, septicemia and sporadic genital infections (Bergonier, Berthelot and Poumarat 1997; Corrales *et al.* 2007). These infections are highly chronic and persistent even after prolonged antibiotic treatments leading to significant economic losses (Nicholas 2002).

Adhesion to host cells is a prerequisite for mycoplasma colonization, and adherence deficient mutants are mostly avirulent

(Razin and Jacobs 1992). Mycoplasma adhesion is complex and often involves many adhesins, accessory proteins and host receptors (Rottem 2003). However, for *M. agalactiae*, except for P40 no other adhesin has been identified and the host–pathogen interface is hardly understood (Fleury *et al.* 2002). Recent studies have also revealed *M. agalactiae's* capability to go intracellular and to spread from a local site of infection to distant body parts including lungs, brain and uterus (Gomez-Martin *et al.* 2012; Hegde *et al.* 2014).

Identification of the molecular players involved in *M. agalactaie's* pathogenicity is vital to the development of the much needed successful vaccines. Detailed studies to understand the pathogenicity determinants are largely hampered by the unavailability of suitable infection models. This is because mycoplasmas are known for their strict host and tissue specificity (Rottem and Yogev 2000). Although immortalized cell lines are commonly used in mycoplasma studies, including those employing human HeLa or HEp-2 cells for pathogens of pigs, poultry and ruminants (Dusanic *et al.* 2009; Kornspan, Tarshis and Rottem 2010; Buim *et al.* 2011; Hegde *et al.* 2014), the genetic and chemical modifications of these cell lines pose questions on the reliability of the obtained data, which if not totally misleading, might not correlate well with *in vivo* results (Struve and Krogfelt 2003; Lawlor *et al.* 2005; Landry *et al.* 2013). For instance, HeLa being a cancerous cell line contains numerous deleterious chromosomal rearrangements and exhibits significantly different gene expression patterns compared to normal cells and this can significantly affect experimental interpretations (Landry *et al.* 2013). All this, combined with the complete lack of an appropriate cell or small animal model for *M. agalactiae*, instigated us to isolate and develop primary sheep cells as ideal models that closely mimic *in vivo* conditions. We generated four different sheep primary cell lines: mammary epithelial (MECs) and stromal cells (MSCs) from sheep udder, which is the most common site of infection, and uterine epithelial (UECs) and stromal cells (USCs) from sheep endometrium, a less frequent infection site (Hegde *et al.* 2014). We also demonstrated the reliability of these cell models in host–pathogen interaction studies using *in vitro* cell adhesion and invasion assays.

The procedure to isolate glandular epithelial cells (ECs) and stromal cells (SCs) from the tissues was adapted from an earlier study (Bartel *et al.* 2013). Briefly, pieces of the mammary gland and uterine tract were obtained separately in PBS with 0.5% gentamicin and 1.5% nystatin (all solutions from Sigma Aldrich, Austria unless mentioned) from an adult lactating sheep in accordance with the institutional ethics committee (BMWFW-68.205/0106-WF/II/3b/2014) during necropsy. Minced tissues were transferred to complete culture medium (CCM) (88% DMEM high glucose medium with 1% L-glutamine, 10% fetal calf serum, 1% gentamicin–nystatin solution) containing 1 mg mL^{-1} collagenase I for tissue disintegration. Subsequently, SCs were separated from epithelial structures using two filtration steps (Bartel *et al.* 2013). After washing with PBS and centrifugation, the SC pellets were resuspended in CCM to achieve single-cell suspension. For EC culture, glandular epithelial structures harvested from the second filtration step were trypsinized to achieve single-cell suspension. Any further SC contamination in EC culture was removed based on slower attachment behavior (Arnold *et al.* 2001) and repeated selective trypsinization of SCs (Owens 1976). All cells were grown for 10–14 days at 37°C with 5% CO_2. Medium was changed every second day, and cells were stored between passages two and four in liquid nitrogen until further use. These ovine primary cells were characterized via immunohistochemistry as described earlier (Bartel *et al.*

2013) except that anti-cytokeratin (Cell Marque, Rocklin, USA) and anti-vimentin (Dako, Glostrup, Denmark) Abs were used at 1:250 and 1:200 dilutions after boiling for 10–15 min in Tris-EDTA, pH 9.0 and citrate buffer, pH 6.0, respectively. Primary antibody incubations were performed overnight at 4°C and detected via Alexa 488 secondary antibody (1:100), while DAPI was used for nuclear counterstaining (both from Molecular Probes, Invitrogen, Austria). Sections were evaluated using LSM510 Meta confocal laser scanning microscope using ZEN2000 software (Zeiss, Austria).

For *in vitro* adhesion and invasion assays, sheep primary cells were cultured in CCM without antibiotics and HeLa-229 cells (CCL-2.1, ATCC, USA) as described earlier (Hegde *et al.* 2014). All cells were tested to be free of mycoplasma contamination by culture and PCR (Chavez Gonzalez *et al.* 1995). *Mycoplasma agalactiae* pathogenic type strain PG2 was grown in SP4 medium as described before (Chopra-Dewasthaly *et al.* 2005) for 48 h and diluted serially in respective cell culture media to get an MOI of 5–200 before infecting cultured cell monolayers. Infected cells were incubated at 37°C with 5% CO_2 for 4 and 24 h during adhesion and invasion assays, respectively. Serial dilutions of grown culture were plated on SP4 plates containing 1% (wt/vol) noble agar (Difco, Austria) to calculate the colony-forming units (CFU) at the time of infection. Colonies were counted under BMS 74955 stereomicroscope after 4–5 days of incubation at 37°C.

To quantify mycoplasma adhesion, unbound mycoplasmas were washed thrice with PBS and serial dilutions of mammalian cell suspensions plated on SP4 agar to calculate the CFU of adhered mycoplasmas. Adhesion was calculated as percentage ratio of CFU of adhered mycoplasmas after 4 h of infection to the total CFU of mycoplasmas measured in parallel wells that lacked mammalian cells. Qualitative double immunofluorescence (DIF) staining and quantitative gentamicin invasion assays were performed as described earlier (Hegde *et al.* 2014). Invasion frequency was calculated as percentage ratio of CFU of mycoplasmas recovered after 3 h of gentamicin treatment to the total CFU of mycoplasmas added initially. Viability of mammalian cells was checked using Trypan blue (Life Technologies, Austria) staining before and after gentamicin treatment and no adverse effects were found. All experiments were performed at least three times in duplicates and results represented as mean values ± standard deviation.

UECs had lower doubling rates than MECs and showed polygonal to cobblestone morphology, whereas MECs had typical epithelial morphology (Fig. S1, Supporting Information). At higher passages, the doubling rate of UECs declined and they exhibited an enlarged flattened phenotype. Both MSCs and USCs were fast growing and showed the typical spindle-shaped fibroblast phenotype (Fig. S1, Supporting Information). To differentiate primary cell populations from each other, the expression of key cell markers was assessed via immunohistochemistry, whereby antibodies against cytokeratin, an epithelial-specific intermediate filament protein, identified ECs, whereas SCs were characterized using the mesenchymal marker vimentin. MECs were detected positive for cytokeratin (Fig. 1A) and negative for vimentin (Fig. 1A inset), whereas MSCs were positive for vimentin (Fig. 1B). Negative controls for the secondary staining system were without any signals (Fig. 1C and D). Both UECs and USCs resulted in immunohistochemistry patterns similar to MECs and MSCs, respectively (data not shown).

Quantitative *in vitro* adhesion assay revealed a comparable adhesion of *M. agalactiae* to HeLa (33.6% ± 5.6) and MECs (and 32% ± 11.5) but interestingly, highest adhesion was observed with MSCs (45% ± 3.4). So far, *M. agalactiae* was known for its
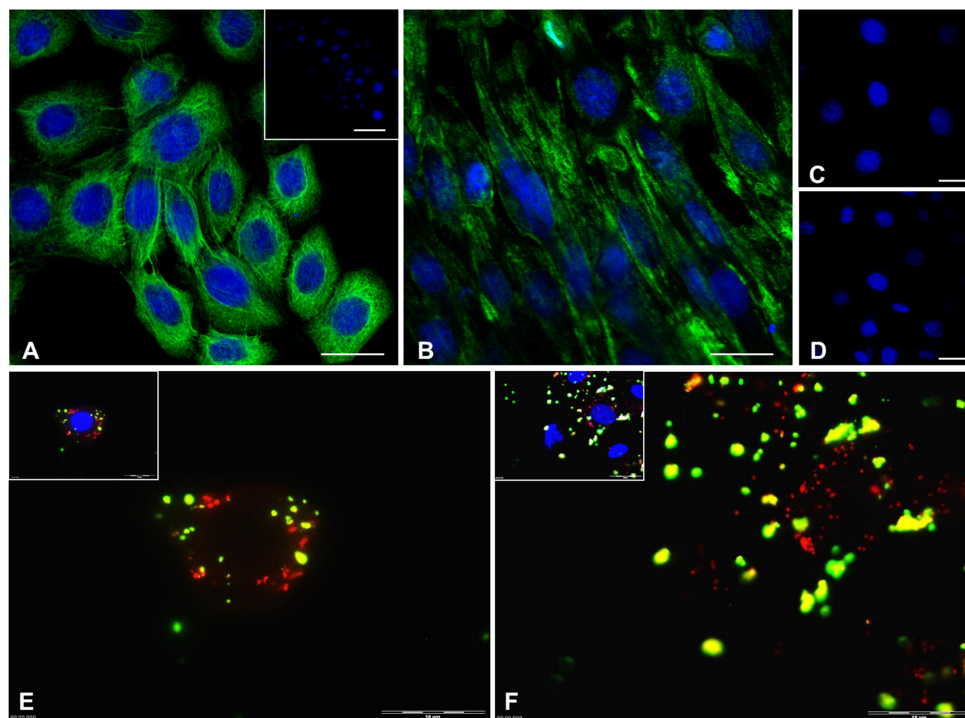
Figure 1. Immunohistochemical characterization and *M. agalactiae* invasion of sheep primary cell lines. **(A–D)**, immunohistochemical staining of sheep MECs (A, C) and MSCs (B, D) using mouse monoclonal anti-cytokeratin antibody (A) and mouse monoclonal anti-vimentin antibody (B, inset in A). The negative controls for the secondary staining system gave no reactions in both ECs (C) and SCs (D). Nuclear counterstaining was performed using DAPI. Scale bars, 10 μm (A–D) and 20 μm (inset in A). **(E–F)**, DIF images showing the invasion of *M. agalactiae* into sheep MECs (E) and MSCs (F). Extracellular mycoplasmas were labeled using FITC-tagged secondary antibody, whereas both intracellular and extracellular mycoplasmas were labeled with Texas Red-tagged secondary antibody. Merged images depicted here show extracellular mycoplasmas in yellow and intracellular mycoplasmas in red color. Insets within E and F represent the corresponding DAPI counterstained images, respectively. Scale bars, 10 μm.

preferential colonization of MECs. Here, for the first time we have checked and demonstrated a much higher *M. agalactiae* adhesion to inner SCs compared to outer ECs. Detailed study of mycoplasma adhesins and corresponding host receptors would explain the differential adhesion of *M. agalactiae* to these cells. Both UECs and USCs demonstrated relatively lower adhesion counts of 23.3% $\pm$ 4.2 and 17.5% $\pm$ 14.7, respectively. Furthermore, DIF staining after 24 h of infection demonstrated lower numbers of invaded mycoplasmas in MECs (Fig. 1E) compared to MSCs (Fig. 1F). We confirmed these results using quantitative gentamicin invasion assay where both HeLa (1.1% $\pm$ 0.39) and MECs (1.06% $\pm$ 0.57) showed similar invasion frequency. In agreement with the adhesion assay and the DIF staining, MSCs showed the highest invasion frequency (2.9% $\pm$ 0.62), whereas both UECs and USCs showed comparatively lower invasion frequency of 0.23% $\pm$ 0.18 and 0.5% $\pm$ 0.2, respectively. The results prove that the primary cell infection models used here reflect the natural host–pathogen behavior as witnessed during field infections where udders are the most common site for *M. agalactiae* colonization and uterine infections are rather rare. We had earlier demonstrated *M. agalactiae's* capability to exit invaded host cells in a viable state and had proposed that it might be crossing the mammary epithelial barrier to disseminate to internal organs distant to the site of experimental intramammary infection (Hegde *et al.* 2014). In the current study, *M. agalactiae's in vitro* invasion into inner SCs from sheep udders reveals the possible first step *M. agalactiae* employs for systemic spreading after initial colonization. The anticipated development of more sophisticated polarized coculture systems from these primary

cells would serve as invaluable tools to understand *M. agalactiae* host cell interactions and pathogenicity.

In conclusion, having demonstrated *M. agalactiae's* consistent ability to adhere and invade into these primary sheep cell lines, we propose them to be promising *in vitro* cell models. Examining *M. agalactiae* interactions with these primary cells *in vitro* would not only avoid the complexity of undefined *in vivo* environment, but also approximate the *in vivo* environment more accurately as the cells retain many important physiological properties of their original tissues.

SUPPLEMENTARY DATA

Supplementary data are available at FEMSPD online.

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