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Short communication

# Long-term survival of *Mycoplasma bovis* in necrotic lesions and in phagocytic cells as demonstrated by transmission and immunogold electron microscopy in lung tissue from experimentally infected calves

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#### ABSTRACT

In the lungs of cattle infected with *Mycoplasma bovis* persistence of the agent for several weeks after infection has been demonstrated by different methods, e.g. isolation of the organism, immunohistochemistry for antigens, and in situ hybridization. The presence of macrophages and neutrophils with cytoplasmic M. bovis antigen suggests that phagocytosis occurs in vivo. It is, however, unknown whether this intracellular immunolabeling detected residual antigen after phagocytosis of M. bovis or surviving organisms in macrophages that use the intracellular survival as a strategy for evasion of the immune response. The aim of this electron microscopic investigation was to study the distribution of *M. bovis* within caseonecrotic lung lesions and to examine the phagocytes for intracellular presence of the agent. In lung tissue sections from 9 experimentally infected calves originating from two different infection experiments large numbers of mycoplasmas were detected by transmission electron microscopy and by immunogold electron microscopy using M. bovis-specific polyclonal antibodies. M. bovis were found throughout caseonecrotic foci and within the lumen of bronchi containing exudate. The majority of mycoplasmas were located extracellularly within necrotic lung lesions and around neutrophilic granulocytes and macrophages, while fewer organisms were found within the cytoplasm of phagocytes. The results of this study show that there is long-time survival of numerous intact M. bovis in necrotic lung lesions even though large numbers of neutrophils and macrophages are present. These findings show that the phagocytes are not able to eliminate M. bovis from the lungs from necrotic and inflamed lung tissue and indicate that persistence of the agent is possibly due to its capacity to avoid phagocytosis. © 2012 Published by Elsevier B.V.

## 1. Introduction

Infection with *Mycoplasma bovis* is an important cause of pneumonia in cattle (Caswell and Archambault, 2008). In the lungs of infected animals *M. bovis* persists for several

weeks after infection, as demonstrated by isolation of the organism, by immunohistochemistry for *M. bovis* antigens, and by *in situ* hybridization for *M. bovis* DNA (Thomas et al., 1986; Howard et al., 1987; Gourlay et al., 1989; Rodríguez et al., 1996; Jacobsen et al., 2010; Hermeyer et al., 2011, 2012). Immunohistochemically, *M. bovis* antigen is detected in caseonecrotic lung lesions and in the cytoplasm of numerous macrophages and, less frequently, in neutrophilic granulocytes (Rodríguez et al., 1996; Khoda-karam-Tafti and López, 2004; Hermeyer et al., 2011, 2012). In the lung, as in other organs, macrophages and

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neutrophils constitute the first line of defence against microorganisms. The presence of macrophages and neutrophils with cytoplasmic *M. bovis* antigen in lungs of infected calves suggests that phagocytosis, possibly following opsonization, occurs *in vivo*. It is, however, unknown whether this intracellular immunolabeling detected residual antigen after phagocytosis of *M. bovis* and killing by macrophages that use intracellular survival as a strategy for evasion of the immune response (Caswell and Archambault, 2008).

In this investigation, tissue sections from areas of caseonecrotic pneumonia in calves experimentally infected with *M. bovis* were examined by transmission electron microscopy (TEM) and immunogold electron microscopy with *M. bovis*-specific antibodies. We demonstrated at the ultrastructural level that, in the lungs of calves with chronic pneumonia, in spite of the presence of numerous phagocytes there is long-term survival of intact *M. bovis*, indicating that the elimination of this pathogen by this these phagocytic cells is not effective and that the agent possibly is avoiding phagocytosis.

## 2. Materials and methods

For this study, formalin-fixed, paraffin-embedded lung tissue samples from 9 calves with caseonecrotic pulmonary lesions were selected. The samples had been collected from calves of two different M. bovis infection experiments (Table 1). Details of histopathological findings and of immunohistochemical results of detection of M. bovis antigen within the lung tissue samples and further data about 7 of these calves have been described elsewhere (Hermever et al., 2011, 2012). Two of the 9 calves had been inoculated with another M. bovis field strain. This experiment had been carried out at the same time and under similar conditions as the infection of one of the calves infected with M. bovis strain 1067 (calf no. 2/3 in Table 1). On hematoxylin and eosin (H&E) stained paraffin sections (5 µm thick) of lung tissue areas of bronchi and necrotic foci to be examined were selected using light microscopy. Thereafter, the paraffin sections were reembedded in epoxy resin for TEM using a modification of the technique described by Ngai et al. (1985). Ultrathin

sections were cut at 60-90 nm and stained with uranyl acetate and lead citrate and viewed under a Zeiss EM 10 A transmission electron microscope (Zeiss, Oberkochen, Germany). Immunogold electron microscopy was performed on paraffin sections of lung tissue prior to embedding using a polyclonal rabbit antibody with specificity for *M. bovis* (Rosengarten et al., 1994). Sections were deparaffinized, treated for 20 min with 0.05 M glycine diluted in 0.1 M phosphate-buffered saline (PBS), and then washed in PBS. Sections were then treated for 30 min at 95 °C with demasking solution  $G^{\mathbb{R}}$  (BioLogo. Kronshagen, Germany) for antigen retrieval. After washing in PBS, the sections were blocked for 30 min in PBS containing 0.5% bovine serum albumin (BSA), 0.1% liquid gelatine, and 5% normal goat serum. Incubation with the primary antibody was performed at 4 °C overnight and followed by several washes in 0.5% BSA and 0.1% liquid gelatine in PBS. Thereafter, incubation with goat antirabbit IgG antibodies conjugated to 10 nm gold particles (British Biocell International, Cardiff, UK), diluted 1:40 in PBS, was performed for 2 h at room temperature. After several washing steps carried out as described above, sections were postfixed for 5 min in PBS containing 2% glutaraldehvde. After final rinses in distilled water, the sections were stained with Mayer's hematoxylin for 1 min and re-embedded in epoxy resin using a modification of the technique described by Ngai et al. (1985) and examined with a Zeiss EM 10 A transmission electron microscope. Pellets of a log phase broth culture of *M. bovis* obtained by centrifugation were fixed in 4% formalin, embedded in paraffin, and paraffin sections  $(5 \,\mu m)$  were processed for TEM and immunogold electron microscopy with the primary *M. bovis*-specific rabbit antibodies as described above. In negative control sections of lung tissue and embedded broth culture the primary antibody was replaced by normal rabbit serum.

### 3. Results and discussion

There are only a few studies describing the ultrastructure of *M. bovis in vitro* and/or *in vivo*, *i.e.* in broth cultures, bovine fetal tracheal organ cultures, and within tissue samples from cows with *M. bovis* induced mastitis

Table 1

Experimental data of 9 calves with caseonecrotic lung lesions originating from two different inoculation experiments with M. bovis.

Numbers of calves <sup>b,c</sup>	Age at inoculation	M. bovis strain	Inoculation	CFU <sup>a</sup> of <i>M. bovis</i>	Necropsy (days post infection)
2 <sup>b</sup> /3 <sup>c</sup>	4 weeks	1067	Intratracheally	$1  imes 10^8$	21
10 <sup>b</sup> /5 <sup>c</sup>	8 weeks	1067	Endobronchially	$7.4  imes 10^9$	21
13 <sup>c</sup>	8 weeks	1067	Endobronchially	$7.4  imes 10^9$	21
14 <sup>c</sup>	8 weeks	1067	Endobronchially	$7.4  imes 10^9$	21
15 <sup>c</sup>	8 weeks	1067	Endobronchially	$7.4  imes 10^9$	21
16 <sup>c</sup>	8 weeks	1067	Endobronchially	$7.4  imes 10^9$	21
17 <sup>c</sup>	8 weeks	1067	Endobronchially	$7.4  imes 10^9$	21
7393 <sup>d</sup>	4 weeks	Field strain	Intratracheally	$1  imes 10^{10}$	21
7672 <sup>d</sup>	4 weeks	Field strain	Intratracheally	$1\times 10^{10}$	21

<sup>a</sup> Colony forming units per ml culture medium.

<sup>b</sup> Calf no. in Table 2 (see Hermeyer et al., 2011).

<sup>c</sup> Calf no. in Table 2 (see Hermeyer et al., 2012).

<sup>d</sup> Spergser, J., Rosengarten, R., 1998 (unpublished results).

(Jasper et al., 1969; Hirth et al., 1970; Stanarius et al., 1981; Thomas et al., 1987). In the pelleted broth culture, which was used in this study as a positive control for immunogold electron microscopy, the *M. bovis* organisms were round to oval with an approximate diameter of 0.2–0.45 µm and a varying electron density in the cytoplasm. In sections of the culture examined by immunogold electron microscopy the 10 nm gold particles were intimately associated with the surface of the organisms (Fig. 1). Mycoplasmas grown in broth or organ cultures, depending on their viability and growth phase, can have a uniform or pleomorphic morphology (Boatman and Kenny, 1970; Hirth et al., 1970; Thomas et al., 1987). The log phase broth culture used in this study, after harvesting, had been kept for approximately 24 h at room temperature before processing for TEM was completed. The use of a log phase culture during which the organisms undergo binary fission at maximal physiological activity probably explains the rather uniform morphology as well as the rather uniform electron dense loose cytoplasm containing ribosomes among which are interspersed lucent and irregular areas containing fine strands that represent the genomic DNA (Boatman, 1979).

In tissue sections from all calves re-embedded for TEM large numbers of mycoplasmas were seen throughout the caseonecrotic foci and within the lumina of bronchi containing exudate. Mycoplasmas were not seen within the cytoplasm of bronchial epithelial cells. In the center and at the periphery of necrotic foci numerous mycoplasma cells were present in small aggregates or larger clusters suggesting replication (Fig. 2a and b). The shape of the organisms was round to oval or pleomorphic and they had an approximate diameter of  $0.45-0.80 \mu$ m. In the immunogold electron microscopic studies specific labeling of the mycoplasma cells by gold particles was seen (Fig. 3). The cytoplasm of the mycoplasmas was of varying density and



**Fig. 1.** Immunogold electron microscopy of pelleted broth culture of *M. bovis* with gold particles on the surface of uniform organisms. Preembedding technique with polyclonal *M. bovis*-specific antibodies,  $128,000 \times$ .

was surrounded by a mostly intact cell membrane. In the compact cytoplasm circular electron lucent spaces were sometimes seen (Fig. 2b), as described in *in vitro* and *in vivo* studies by other investigators (Hirth et al., 1970; Stanarius et al., 1981), indicating that the organisms were in different stages of physiological activity as seen *in vitro* in stationary phase mycoplasma cultures (Boatman, 1979). The organisms in lung tissue largely resembled the *M. bovis* cells described by Stanarius et al. (1981) in the mammary gland tissue of experimentally infected cows. In sections from necrotic lesions from two calves many mycoplasmas had deposits of electron dense, granular material on their outer surface (Fig. 2b). These deposits possibly represent adsorbed antibodies, as discussed by Stanarius et al. (1981). Mycoplasmas were often present around neutrophilic



Fig. 2. (a) Transmission electron micrograph of the outer rim of macrophages surrounding a necrotic lung lesion. There are numerous extracellular *M*. *bovis* organisms and also intracellular organisms within the cytoplasm of degenerate macrophages,  $8000\times$ . (b) Mycoplasmas surrounded by a thin limiting cell membrane (open arrowheads), often with intracellular electron lucent areas (asterisks). Granular material was present on the surface of several organisms (closed arrowheads), 50,400×.



Fig. 3. Immunogold electron microscopy of extracellularly located *M. bovis* organisms at the periphery of a necrotic lung lesion. Pre-embedding technique with polyclonal *M. bovis*-specific antibodies,  $10,000 \times$ .

granulocytes and macrophages, and some of them were also seen within the cytoplasm of these phagocytes (Figs. 2 and 4a,b). In some cells, mycoplasmas were apparently located within cytoplasmic vacuoles, but most phagocytes were degenerate, especially those located within the center of areas of necrotic tissue, and were not well preserved enough to determine whether the mycoplasmas were located within phagosomes and/or phagolysosomes.

Previous studies of the lung tissue from the calves examined in this investigation revealed that persistence of M. bovis antigens and M. bovis DNA is associated with signs of both innate and adaptive immune responses, such as infiltration with numerous neutrophils and macrophages, and proliferation of bronchus-associated lymphoid tissue (Hermeyer et al., 2011, 2012). The present investigation showed that, in spite of these immune responses, numerous, most likely viable, M. bovis cells persist within necrotic tissue lesions and are also present within the cytoplasm of phagocytes. There are only a few studies in which the interactions of phagocytic cells with M. bovis have been studied in vitro (Howard et al., 1976; Van der Merwe et al., 2010). One study, in which the in vitro interaction of bovine alveolar macrophages and bovine polymorphonuclear leukocytes with M. bovis was investigated (Howard et al., 1976), indicated that opsonization with specific sera promoted phagocytosis and killing of M. bovis by these phagocytes. In another study, after incubation of bovine peripheral blood mononuclear cells with M. bovis the organisms were found intracellularly in different cell types, including monocytes, suggesting that phagocytosis and/or possibly attachment and invasion play a role in the interaction of host cells with the pathogen (Van der Merwe et al., 2010). It is not clear from the results of this study whether the whole M. bovis organisms seen in the cytoplasm of phagocytes had been phagocytosed and/ or if they had actively invaded. In the lung, neutrophils and macrophages play a key role in innate immunity as they



**Fig. 4.** (a) Transmission electron micrograph of a degenerate neutrophilic granulocyte at the periphery of a necrotic lung lesion,  $32,000 \times$ . (b) *M. bovis* organisms surrounded by a cell membrane (arrows) and located within the cytoplasm and also close to the cell surface of the neutrophil,  $64,000 \times$ .

can recognize, ingest and destroy bacteria, a process which is a pre-requisite for microbial antigen processing and antigen presentation (Srikumaran et al., 2008). Immunohistochemical studies of the lungs of calves with spontaneous and experimental M. bovis infection have shown that, in spite of the innate and specific immune responses of the host, *M. bovis* antigen is present in the cytoplasm of neutrophils and macrophages both at early and chronic stages (Rodríguez et al., 1996; Khodakaram-Tafti and López, 2004; Buchenau et al., 2010; Hermeyer et al., 2011, 2012). A previous investigation in which the same necrotic lung foci of the same calves examined in this study were immunolabeled for M. bovis antigen (Hermeyer et al., 2011, 2012) showed that large amounts of extracellular antigen are present adjacent to phagocytes. In the present study, the number of extracellular organisms clearly exceeded the number of those within the cytoplasm of neutrophils

and macrophages. These findings demonstrate that the organisms cannot be eliminated by phagocytosis. These results indicate that the persistence of *M. bovis* may be due to its capacity to avoid phagocytosis by so far unknown mechanisms. Possibly, evasion of the immune response and persistence of the mycoplasmas, at least in part, results from variable expression of variable surface membrane proteins (Vsps) of *M. bovis*, as discussed by Hermeyer et al. (2012). During chronic stages of *M. bovis* induced pneumonia more IgG1 than IgG2 is present in the sera of infected animals and more IgG1 producing plasma cells can be seen by immunohistochemical staining in pneumonic lung tissue (Vanden Bush and Rosenbusch, 2003; Hermeyer et al., 2012). Because IgG2 is a superior opsonin, the low amounts of IgG2 could lead to less efficient opsonization, resulting in reduced phagocytosis. Previous examination of lung tissue of the same calves examined in the present study (Hermeyer et al., 2011, 2012) showed that, beside macrophages located at the periphery of necrotic foci, many alveolar macrophages (AM) in the surrounding lung parenchyma contain cytoplasmic M. bovis antigen. Whether AM in the lungs of infected calves, in addition to antigen, also contain whole M. bovis organisms remains to be determined.

In summary, our findings on lung tissue of calves experimentally infected with M. bovis show at the ultrastructural level that there is long-term survival of numerous whole *M. bovis* in necrotic lung lesions. The majority of organisms were located extracellularly within necrotic lung lesions, while fewer numbers of organisms were present in the cytoplasm of phagocytic cells. The findings show that, although numerous neutrophils and macrophages are present, these phagocytes are not able to eliminate M. bovis from necrotic and inflamed lung tissue and indicate that persistence of the agent is possibly due to its capacity to avoid phagocytosis by unknown mechanisms. Further investigations are required to study these interactions, e.g. the mode of ingestion of the pathogen, the subsequent events within the cytoplasm of bovine phagocytic cells, and possible factors involved in avoidance of phagocytosis.

### **Conflict of interest**

The authors have no conflict of interest.

### Ethics

The animal experiments were conducted with the approval of the Austrian Bundesministerium für Wissenschaft und Verkehr. The registration number for the experiment with 3 calves (nos. 2/3, 7393, 7672) (see Table 1) was 68.205/78-Pr/4/1998. The registration number for the experiment with 6 calves (nos. 10/5, 13–17) (see Table 1) was 68.205/29-Pr/4/2000.

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#### References

- Boatman, E.S., 1979. Morphology and ultrastructure of the *mycoplasma-tales*. In: Barile, M.F., Razin, S. (Eds.), The Mycoplasmas, vol. I Cell Biology. Academic Press, New York, pp. 63–102.
- Boatman, E.S., Kenny, G.E., 1970. Three-dimensional morphology, ultrastructure, and replication of *Mycoplasma felis*. J. Bacteriol. 101, 262– 277.
- Buchenau, I., Poumarat, F., Le Grand, D., Linkner, H., Rosengarten, R., Hewicker-Trautwein, M., 2010. Expression of Mycoplasma bovis variable surface membrane proteins in the respiratory tract of calves after experimental infection with a clonal variant of Mycoplasma bovis type strain PG45. Res. Vet. Sci. 89, 223–229.
- Gourlay, R.N., Thomas, L.H., Wyld, S.G., 1989. Increased severity of calf penumonia associated with the appearance of *Mycoplasma bovis* in a rearing herd. Vet. Rec. 124, 420–422.
- Caswell, J.L., Archambault, M., 2008. Mycoplasma bovis pneumonia in cattle. Anim. Health Res. Rev. 8, 161–186.
- Hermeyer, K., Jacobsen, B., Spergser, J., Rosengarten, R., Rosengarten, R., Hewicker-Trautwein, M., 2011. Detection of *Mycoplasma bovis* by insitu hybridization and expression of inducible nitric oxide synthase, nitrotyrosine and manganese superoxide dismutase in the lungs of experimentally-infected calves. J. Comp. Pathol. 145, 240–250.
- Hermeyer, K., Jacobsen, B., Spergser, J., Rosengarten, R., Hewicker-Trautwein, M., 2012. Chronic pneumonia in calves after experimental infection with *Mycoplasma bovis* strain 1067: characterization of lung pathology, persistence of variable surface protein antigens and local immune response. Acta Vet. Scand. 54, 9.
- Hirth, R.S., Tourtelotte, M.E., Nielsen, S.W., 1970. Cytopathic effects and ultrastructure of *Mycoplasma agalactiae* var. *bovis* (Donetta strain). Infect. Immun. 2, 105–111.
- Howard, C.J., Taylor, G., Collins, J., Gourlay, R.N., 1976. Interaction of *Mycoplasma dispar* and *Mycoplasma agalactiae* subsp. *bovis* with bovine alveolar macrophages and bovine lacteal polymorphonuclear leukocytes. Infect. Immun. 14, 11–17.
- Howard, C.J., Thomas, L.H., Parsons, K.R., 1987. Immune response of cattle to respiratory mycoplasmas. Vet. Immunol. Immunopathol. 17, 401– 412.
- Jacobsen, B., Hermeyer, K., Jechlinger, W., Zimmermann, M., Spergser, J., Rosengarten, R., Hewicker-Trautwein, M., 2010. In situ hybridization for the detection of *Mycoplasma bovis* in paraffin-embedded lung tissue from experimentally infected calves. J. Vet. Diagn. Invest. 22, 90–93.
- Jasper, D.E., Sekhri, K.K., Eroschenko, V.P., Jain, N.C., Dellinger, J.D., 1969. Ultrastructure of mycoplasma from bovine mastitis as observed *in vitro* and *in vivo*. Am. J. Vet. Res. 30, 521–531.
- Khodakaram-Tafti, A., López, A., 2004. Immunohistopathological findings in the lungs of calves naturally infected with *Mycoplasma bovis*. J. Vet. Med. A 51, 10–14.
- Ngai, H.K., Chan, K.W., Or, S.B., Yau, W.L., 1985. A rapid method for reprocessing paraffin sections for diagnostic electron microscopy. J. Pathol. 145, 59–62.
- Rodríguez, F., Bryson, D.G., Ball, H.J., Forster, F., 1996. Pathological and immunohistochemical studies of natural and experimental *Mycoplasma bovis* pneumonia in calves. J. Comp. Pathol. 115, 151–162.
- Rosengarten, R., Behrens, A., Stetefeld, A., Heller, M., Ahrens, M., Sachse, K., Yogev, D., Kirchhoff, H., 1994. Antigen heterogeneity among isolates of *Mycoplasma bovis* is generated by high-frequency variation of diverse membrane surface proteins. Infect. Immun. 62, 5066–5074.
- Srikumaran, S., Kelling, C.L., Ambagala, A., 2008. Immune evasion by pathogens of bovine respiratory disease complex. Anim. Health Res. Rev. 8, 215–229.
- Stanarius, A., Seffner, W., Pfützner, H., 1981. Studies into mycoplasmic mastitis in cattle Ninth communication: electron-microscopic findings from experimental *Mycoplasma bovis* mastitis. Arch. Exper. Vet. Med., Leipzig 35, 511–524 (in German).
- Thomas, L.H., Howard, C.J., Stott, E.J., Parsons, K.R., 1986. Mycoplasma bovis infection in gnotobiotic calves and combined infection with respiratory syncytial virus. Vet. Pathol. 23, 571–578.
- Thomas, L.H., Howard, C.J., Parsons, K.R., Anger, H.S., 1987. Growth of *Mycoplasma bovis* in organ cultures of bovine foetal trachea and comparison with *Mycoplasma dispar*. Vet. Microbiol. 13, 189–200.
- Vanden Bush, T.J., Rosenbusch, R.F., 2003. Characterization of the immune response to *Mycoplasma bovis* lung infection. Vet. Immunol. Immunopathol. 94, 23–33.
- Van der Merwe, J., Prysliak, T., Perez-Casal, J., 2010. Invasion of bovine peripheral blood mononuclear cells and erythrocytes by *Mycoplasma bovis*. Infect. Immun. 78, 4570–4578.