Mycoplasma bovis and bacterial pathogens in the bovine respiratory tract

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ABSTRACT: Bovine respiratory disease caused by *Mycoplasma bovis* is a major health problem of cattle worldwide. It inflicts considerable financial losses on beef herds and is the most common cause of mortality in dairy cattle. Bacteriological examination of 35 nasal cavity samples from calves younger than three months of age identified *Mycoplasma bovis* in eight (22.9%) samples. These cattle were followed until 17 months of age, and repeated examination of nasal cavity samples before necropsy identified *Mycoplasma bovis* in four (11.4%) samples. At necropsy and lung samples for bacteriological and histological examination were collected. To identify microorganisms from the *Mollicutes* class isolated from the nasal cavities of cattle we used the PCR method. Furthermore, *Mycoplasma bovis* was identified on the grounds of biochemical characteristics and by the disk growth inhibition test. The organism was found in 5.7% of calves younger than three months of age in combination with *Pasteurella* spp. *Mycoplasma bovis* in combination with *Pasteurella multocida* and *Mannheimia haemolytica* was isolated from 5.7% and 2.9% of cattle at 17 months. However, *Pasteurella multocida* was common in cattle at 17 months and *Mannheimia haemolytica* was isolated from both age groups of cattle. Histopathological examination of lung samples revealed broncho-interstitial pneumonia in 14.3% of samples. *Mycoplasma bovis* was isolated from 60.0% of broncho-insterstitial pneumonia cases. The organism was isolated more frequently from the group of calves rather than from the cattle group (*P* < 0.05). The most common bacterial agents were *Pasteurella multocida* and *Mannheimia haemolytica.*

Keywords: cattle; bacteria; mycoplasma; respiratory tract

Bovine respiratory disease (BRD) is a major health problem of calves and adult cattle and has great economic impact on the cattle industry (Arcangioli et al., 2008). The cause is multifactorial and disease appears to result from the interaction of infectious microorganisms and such predisposing factors as host defence, environment and stress (Hartel et al., 2004). Viral pathogens usually play a role and may cause primary respiratory disease and mild respiratory disorder in calves (Baule et al., 2001; Callan and Garry, 2002). *Mannheimia haemolytica*, *Pasteurella multocida*, *Histophilus somni*, *Arcanobacterium pyogenes*, or the *Mollicutes Mycoplasma dispar* and *Mycoplasma bovis* are usually associated with concurrent virus infection (Arcangioli et al., 2008). A number of studies have demonstrated a synergistic role of viruses in bovine respiratory disease in increasing the pathogenicity of both viral and bacterial concomitant infections (Farshid et al., 2002).

Mycoplasma bovis is a common inhabitant of the upper and lower respiratory tract of healthy cattle. This mycoplasma species increases the severity of respiratory disease in calves and can also act as a primary pathogen (Gagea et al., 2006). In Europe, *Mycoplasma bovis* is believed to be responsible for 25% to 33% of outbreaks of calf pneumonia (Nicholas et al., 2002). In a study of calves in the Netherlands *Mycoplasma bovis* was isolated from 20% of pneumonic lungs from fattening herds but only in a small number of apparently healthy calves. In 1994 in the North and South of Ireland *Mycoplasma bovis* was isolated from 13% to 23% of pneumonic lungs. In France (2001) *Mycoplasma bovis* was isolated from 30% of calf herds with pneumonia. In the same year in Britain about 20% to 25% of pneumonic herds contained animals with antibodies against *Mycoplasma bovis* (Nicholas and Ayling, 2003). Nicholas and Ayling (2003) estimated

that *Mycoplasma bovis* is responsible for at least a quarter to a third of respiratory disease losses in the United Kingdom (Nicholas and Ayling, 2003; Arcangioli et al., 2008).

The ability to undergo antigenic variation by phenotypic alteration of immunodominant surface lipoproteins and the resulting modulation of the host immune response can facilitate the persistence of *Mycoplasma bovis* and the development of chronic infection in the face of an immune response and prolonged antibiotic therapy (Gagea et al., 2006). Infection is usually introduced to *Mycoplasma bovis*-free herds by clinically healthy calves or young cattle shedding mycoplasma. Its appearance on some farms suffering low grade respiratory disease may lead to increased morbidity and mortality. Infected cattle shed the mycoplasma via the respiratory tract for many months and even years acting as reservoirs of infection. Animals become infected via the respiratory tract, the teat canal or genital tract; artificial insemination with infected semen is another common route (Nicholas and Ayling, 2003).

Although the presence of *Mycoplasma bovis* role in the cattle respiratory tract is generally recognised, little is known regarding *Mycoplasma bovis* in Lithuania. Therefore, the aim of the present study was a more detailed investigation of the prevalence of *Mycoplasma bovis* in the respiratory tract of cattle of different ages and the association of mycoplasma with other bacteria.

MATERIAL AND METHODS

In this study 35 male cattle were investigated according to the requirements of the Law of Republic of Lithuania on animal care, keeping and using No 8–500 ("Valstybės Žinios", 1997 11 27, No. 108). The investigated cattle were kept in barns throughout the year. Clinical assessment of each animal was performed before samples were taken from the nasal cavity. General appearance and respiratory signs were scored on a 3-point scale proposed by Tenk (2005) where 0 = normal, 1 = subdued, slightly depressed, 2 = depressed, reluctant to rise and 3 = very depressed, unresponsive to external stimuli. Respiratory signs: 0 = normal, 1 = hyperpnoea or slight dyspnoea, 2 = moderate hyperpnoea or obvious dyspnoea, 3 = respiratory distress. Nasal discharge was scored in the following manner: 0 = absent, 1 = mild, 2 = significant and purulent. Coughing was scored as 0 = absent, 1 = mild,

2 = severe and frequent – several in one minute (Tenk, 2005). The rectal temperature of each animal was also measured. Nasal swabs samples were collected for bacteriological investigation from calves after arrival on the farm (at an age of less than three months) in June 2006. These cattle were followed until 17 months of age and repeated examination of nasal cavity samples was performed before necropsy in August 2007.

Macroscopic lesions of the lungs were investigated at the time of slaughter. Lungs lesions were scored by percentage according to Dungworth (1993): − = no detectable gross lung lesion, + = total lesion extension less than 10 cm^2, ++ = total lesion extension greater than 10 cm^2, +++ = total lesion extension involving at least 75% of an affected lung lobe. Thirty five samples of lung tissue were collected for histopathological examination. The lung samples were taken from areas with inflammation and from normal areas.

Mycoplasma cultivation procedures were performed according to the method described by Friis (1975). For the isolation of mycoplasma from nasal swab samples selective liquid Friis media (NHS20 broth and SB broth) and solid Friis medium were used. Mycoplasma isolation from lung samples was performed according to the recommendations of Gourlay and Howard (Friis, 1975; Gourlay and Howard, 1983). Inoculated broth media were cultivated under aerobic conditions. Solid media were incubated in microaerophillic conditions for 7 to 14 days. All media were incubated at 37 °C. The growth of mycoplasma was observed every 48 to 96 h by enlarging the microscope view 40 times. Isolation of *Mycoplasma bovis* was accomplished in broth media by carrying out 10-fold dilutions from 10^{-1} to 10^{-4} and inoculating the last dilution onto solid media agar.

To confirm the presence of the *Mollicutes* class the polymerase chain reaction (PCR) was used. DNA from isolated microorganisms was extracted with a 5% solution of Chelex (Sigma, USA).

Mycoplasma cultures that grew in liquid medium were centrifuged for 2 min at 10 000 rpm. After centrifugation the medium was removed and 500 μl of 5% Chelex solution (Sigma, USA) was added to every test tube. The suspension was heated at 56 °C for 30 min and 95 °C for 10 min. The heated solution was then centrifuged twice for 2.5 min at 10 000 rpm. The top layer was then transferred to new test tubes. Extracted DNA was immediately used for PCR amplification.

Isolated microorganisms were analyzed by PCR using the forward primer, MW28 (5'-CCGACTCCTACGGGAGGCA-3') and reverse oligonucleotide primer MW29 (5'-TGCGAGCA-TACTACTCAGGC-3') (Grida Lab, Lithuania) that are specific for the *Mollicutes* class (Bashiruddin et al., 2005). The expected product size is 560 bp in length. The PCR amplifications were performed in a final volume of 50 µl containing 10 × PCR buffer (100mM Tris-HCH, pH 8.8, 500mM KCl, 0.8% no-nidet P40; MBI, Fermentas), 25mM $MgCl_2$ (MBI, Fermentas), 2mM dNTP mix (MBI, Fermentas), 500 IU Taq DNA polymerase (MBI, Fermentas), and 0.5 µl of each of the oligonucleotides.

The amplifications were performed on a PTC-100 Programmable thermal controller (MJ. Research Inc., USA). An initial denaturation step of 94 °C for 5 min was followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 56 °C for 30 s and extension at 72 °C for 30 s. Final amplification was carried out at 72 °C for 5 min. Electrophoresis of PCR products was performed in TAE buffer (40mM Tris, 20mM acetic acid 1mM EDTA), at 100 V for 60 min. Products of PCR amplification were analysed in 1.2% Top Vision LE GQ Agarose gels (MBI, Fermentas) with 1.3% ethidium bromide under a UV lamp (Siugzdaite, 2002). The GeneRuler TM 100 bp DNA Ladder (MBI, Fermentas) was used to estimate the sizes of PCR products. The reference *Mycoplasma bovis* Donetta PG45 strain was used as a positive control and sterile distilled water was used as a negative control.

To identify the species of mycoplasma strains isolates were subjected to biochemical tests: glucose fermentation, arginine hydrolysis, activity of phosphatase, tetrazolium reduction, and production of spots and films (Aluotto et al., 1970). To determine mycoplasma antigens disk growth inhibition (DGI) tests based on Clyde's (1964) recommendations were carried out with the following antiserums: reference *Mycoplasma bovis* Donetta PG45 strain against *Mycoplasma bovis*, reference *Mycoplasma dispar* 462/2 strain – against *Mycoplasma dispar*, reference *Mycoplasma bovirhinis* PG43 – against *Mycoplasma bovirhinis*, and reference *Mycoplasma bovigenitalium* PG11 strain against *Mycoplasma bovigenitalium* (Clyde, 1964, Aluotto et al., 1970).

For investigation of secondary bacterial flora of nasal cavities and cattle lungs blood agar containing 5% sterile horse blood and Columbian agar (Liofilchem, Italy), Heart and brain infusion agar (Mast laboratories ltd, England), Mannitol salt agar, Pseudomona (Cetrimide) agar, Drigalsky lactose agar and Kanamycin – aesculine azide agar (Liofilchem, Italy) were used. Petri dishes were cultivated under aerobic conditions for 24 to 48 h at 37 °C.

A single colony was then subcultured and tested by Gram staining (Diagnostica Marck, Germany) and for catalase and oxidase activities. Differentiation between *Pasteurella multocida* and *Mannheimia haemolytica* was achieved using haemolysis on blood agar and on Drigalsky lactose agar (fermentation of lactose) (Liofilchem, Italy). A pure culture of these microorganisms was identified according to biochemical characteristics: glucose, lactose and manitol fermentation. *Enterococcus* spp. identification was based on the growth of colonies in Kanamycin – aesculine azide agar (Liofilchem, Italy).

Histopathological examination of lung samples was performed at the Pathology Centre of the Department of Infectious Diseases, Lithuanian Veterinary Academy. Lung samples were fixed in a 10% neutral solution of formalin, washed with tap water and soaked using a tissue soaking processor from Shandon. Samples were then poured into paraffin and cut using a rotational microtome. Four µm thick tissue sections were cut from the paraffin block of each sample, stained with hematoxylin and eosin, and examined with an optical microscope (Olympus BX41, Japan). Tissues were photographed using an Olympus camera (Japan).

The SPSS 13.0 statistical packet was used to analyse the data. The frequency of mycoplasma and other microorganisms and evaluated intervals of prognosis were established by calculating the probability of error according to the formula (p = empirical probability of sign, n = number of cattle in sample). The frequency of microorganism isolation was estimated in different age groups of animals and in different seasons by calculating the classic χ^2 criteria. Data with a $P < 0.05$ were considered as significant.

RESULTS

At the time of clinical examination no abnormal clinical signs were recorded in any of the animals. Bacteriological investigation resulted in the isolation of mycoplasma from 12 (34.3%) nasal swabs taken from 35 calves at the age of less than three months. Seven (20%) of these animal had mycoplas-
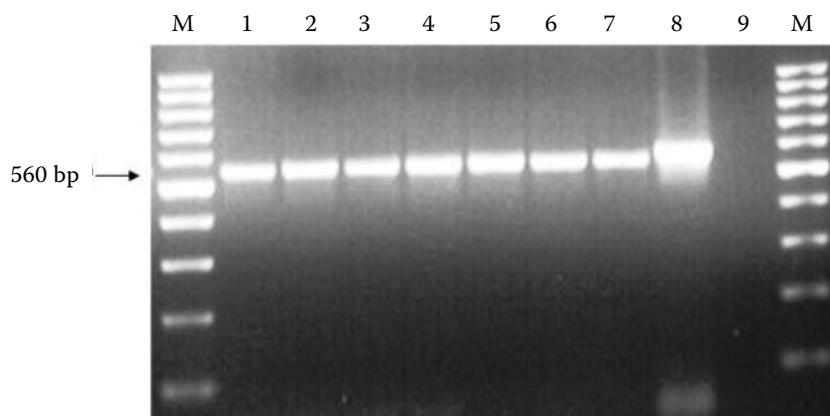
Figure 1. *Mollicute* class confirmation using the PCR method. Lane M = GeneRuler TM 100 bp DNA Ladder (MBI, Fermentas) marker; lanes 1–7 = *Mollicute* class isolates; lane 8 = positive control; lane 9 = negative control

ma in their nasal cavities at the next examination before the animals turned 17 months of age.

Twenty-six mycoplasma strains were isolated from the nasal cavities of cattle of various ages. The sizes of all examined PCR amplicons in an agarose gel matched the 560 bp product that is typical of the *Mollicutes* class (Figure 1).

The results of *Mycoplasma bovis* isolation from cattle of different ages are shown in Figure 2. Mycoplasma growth in liquid media was observed after three to four days. *Mycoplasma bovis* colonies on solid media were identified after seven to nine days and had the "fried egg" morphology typical for this mycoplasma species. Identification of *Mycoplasma bovis* species was based on partial glucose fermentation that is typical to *Mycoplasma bovis*, as well as on significant phosphatase activity and reduction of tetrazolium salts. The growth of these mycoplasmas was inhibited by the *Mycoplasma bovis* Donetta PG 45 antiserum.

After bacteriological examination of cattle lung samples mycoplasmas were isolated from 6 (17.1%) out of 35 lungs. To confirm that these belonged to the *Mollicutes* class isolated microorganisms were tested using the PCR method. It was determined that all isolated microorganisms from lungs samples belonging to the *Mollicutes* class. Examination of the biochemical and antigenic characteristics of these mycoplasmas resulted in the identification of one mycoplasma species – *Mycoplasma bovis* (100%).

The results of bacterial isolation from the nasal cavities of cattle of different ages are shown in Figure 2. The common bacterial respiratory pathogens such as *Pasteurella multocida*, *Mannheimia haemolytica* and *Enterococcus* spp. were isolated

from the nasal cavities of 12 (34.3%) calves at the age of less than three months. The same bacterial species were isolated from 14 (40%) cattle at the next sampling time (at the age of 17 months).

Pasteurella multocida in association with *Mycoplasma bovis* was isolated from two (5.7%) nasal swabs of calves younger than three months old. *Mannheimia haemolytica* in association with *Mycoplasma bovis* was isolated from two (5.7%) of these calves. *Pasteurella multocida* in association with *Mycoplasma bovis* was isolated from two (5.7%) nasal swabs from these cattle at the age of seventeen months. *Mycoplasma bovis* in association with *Mannheimia haemolytica* was isolated from one (2.9%) nasal swab of the same cattle.

From the lung samples of these cattle the same species of microorganisms were isolated: *Pasteurella multocida* from two (5.7%), *Mannheimia haemolytica* from one (2.9%) and *Enterococcus* spp. from five
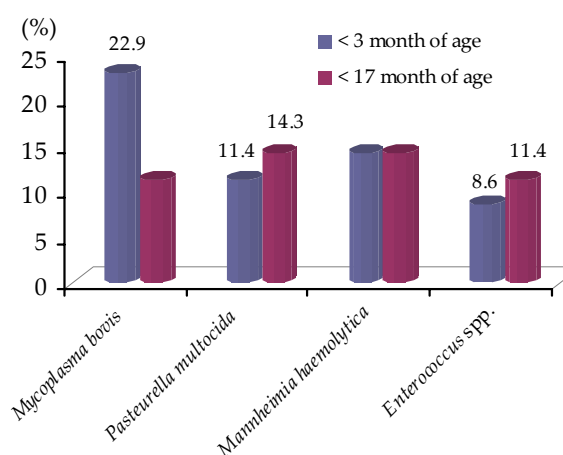

Figure 2. Isolation of *Mycoplasma bovis* from 35 nasal cavities of cattle of three and seventeen month of age

(14.3%) lungs. One bacterial species – *Pasteurella multocida* in association with *Mycoplasma bovis* – was isolated from one (2.9%) cattle lung.

Investigation at the time of slaughter revealed no macroscopic lesions typical for mycoplasma infections in the lungs of the cattle. Histological examination showed signs of pneumonia that are typical of chronic broncho-intersitial pneumonia in five (14.3%) samples. In thirty (85.7%) samples, changes typical of pneumonia were not registered. In the case of chronic broncho-insterstitial pneumonia, infiltration of lymphocytes, macrophages, and single eosinophiles and neutrophils were observed in small bronchioles and blood vessels. The walls of the bronchus were thickened in some places. The cavity of some bronchioles was filled with exudation. *Mycoplasma bovis* was isolated from three (60%) cases of bronchointerstitial pneumonia. *Pasteurella multocida* was isolated from one (20%) sample with diagnosed bronchointerstitial pneumonia.

It was determined that the frequency of finding *Mycoplasma bovis* differed reliably only according to age. It was isolated from cattle at the age of less than three months more frequently ($P < 0.05$) than from cattle at the age of seventeen months. There was no statistically important influence of season on the frequency of isolation of microorganisms ($P < 0.05$).

DISCUSSION

Mycoplasmas are one of the pathogens which cause bovine respiratory disease. By themselves they can cause a mild respiratory disease, but are more often isolated from pneumonic lungs in association with other pathogens (Hartel et al., 2004). The most common isolate from the upper respiratory tract of the cattle in our study was *Mycoplasma bovis*. According to the scientific literature, infection is usually introduced to *Mycoplasma bovis*-free herds by clinically healthy calves or young cattle shedding mycoplasma (Gourlay et al., 1989). A clinical study of endemic pneumonia, from which *Mycoplasma bovis* was isolated from the calf herd, showed that nearly half of dairy calves were shedding mycoplasmas at five days of age and this figure was over 90% at the age of four weeks (Stipkovits et al., 2001). Calves can become infected with mycoplasma by drinking milk from cows that have mastitis (Tenk, 2005). Examination of the microflora from nasal

cavities of cattle revealed that the largest percentage (22.9%) of carriers of *Mycoplasma bovis* was in the group of three-month old cattle. Siugzdaite (2002) carried out investigations in order to detect *Mycoplasma bovis* from calves aged less than three months of age without clinical symptoms of pneumonia. *Mycoplasma bovis* was isolated from nasal swabs of 24.4% of calves (Siugzdaite, 2002). Ter Laak (1992) found *Mycoplasma bovis* in 3% of clinically healthy calves. Statistical analysis of our study data demonstrated that the majority of *Mycoplasma bovis* isolations were from the group of cattle less than three months old (Ter Laak et al., 1992). In our study, the first samples for bacteriological examination from calf nasal cavities were taken after arrival on the experimental farm. The stress experienced during transportation and contact of clinically healthy cattle with seropositive cattle could predispose to a stationary mycoplasma infection (Tschopp et al., 2001). This was confirmed by the study of Allen et al. (1991), in which clinically healthy calves that were newly brought to a nursing station were examined for the presence of mycoplasma. *Mycoplasma bovis* was isolated from 40% to 60% of clinically healthy cattle (Allen et al., 1991).

Chronic broncho-intersitial pneumonia was detected in 14.3% of lung samples. *Mycoplasma bovis* was isolated from 60% cases of bronchointerstitial pneumonia. This is in concert with data demonstrating that *Mycoplasma bovis* caused acute pulmonary infection in calves during the first month of life. After the acute phase calves often develop chronic respiratory disease characterized by inconspicuous clinical signs and poor weight gain (Nicholas and Ayling, 2003; Radaelli et al., 2008). Radaelli et al. (2008) investigated *Mycoplasma bovis* infection in veal calves and adult cattle and concluded that pneumonic lung lesions are more numerous in healthy adult cattle that show no clinical sings (27%) than in veal calves (8%). In calves, a large number of lung samples (81%) that were evaluated grossly as normal, microscopically revealed the presence of mild to moderate pneumonic pathology (Radaelli et al., 2008).

Pneumonia caused by *Mycoplasma bovis* can be complicated by such pathogenic agents as *Pasteurella multocida*, *Mannheimia haemolytica*, *Acranobacterium pyogenes* (formerly *Actinomyces pyogenes*) or *Mycoplasma arginini*, *Histophilus somni* and by viruses (Thomas et al., 2002; Gagea et al., 2006). Secondary microflora from nasal cavities was also isolated from our investigated cattle

(Figure 2). The same microorganisms isolated from cattle nasal cavities were isolated in association with *Mycoplasma bovis*.

Mycoplasma bovis adhesins affect neutrophils and lymphocytes, resulting in oxidation, chemiluminescence and degranulation in these cells (Finch and Howard, 1990). Due to these effects the host's immune response weakens, and favourable conditions for secondary respiratory disease infections caused by *Pasteurella* spp. develop (Umemory et al., 2005). In our study *Pasteurella multocida* in association with *Mycoplasma bovis* was isolated from 5.7% of nasal swabs from cattle less than three months of age and at 17 months. *Pasteurella multocida* is a common pathogen reported to be involved in bovine respiratory disease (Hartel et al., 2004).

Mannheimia haemolytica in association with *Mycoplasma bovis* was isolated from 5.7% of cattle at the age of less than three months and from 2.9% of nasal swabs at the age of 17 months. According to Houghton and Gourlay (1983), *Mycoplasma bovis* in association with *Mannheimia haemolytica* aggravates clinical disease and causes more extensive changes in lungs than *Mycoplasma bovis* acting together with *Pasteurella multocida* (Houghton and Gourlay, 1983). To prevent the spread of mycoplasma control measures should be implemented on the investigated farm.

CONCLUSION

This study demonstrated that the highest number of *Mycoplasma bovis* carriers was in the group of calves younger than three months old. The most common bacterial agents were *Pasteurella multocida* and *Mannheimia haemolytica*. *Mycoplasma bovis* was detected in 14.3% of lungs with lesions atypical for mycoplasma infections.

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