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Original article

# Laboratory diagnosis of mycoplasma infection in young cattle

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## Abstract

The aim of this study was to detect *Mycoplasma* species in the respiratory tract of 110, 310 and 510 day-old groups of cattle by serological, bacteriological and histopathological investigations. Antibodies against *M. bovis* were found in 75% of the 110 day-old, in 50% – of the 310 day-old and in 55% – of the 510 day-old groups of cattle.

Bacteriological examination of the samples from nasal cavities revealed that *Mycoplasma* carriers were found in 60% of the 110 day-old group of cattle, 40% of the 310 day-old and 40% of the 510 day-old group of cattle. Using the PCR method *Mycoplasma* was isolated from 25% of lung samples of the 510 day-old group of cattle. *Mycoplasma bovis* and *Mycoplasma dispar* were confirmed by serological investigations. Foci of bronchointerstitial pneumonia were determined by histopathological examination in 27.5% of lung samples. *Mycoplasma bovis* was isolated in 72.7% of bronchointerstitial pneumonia cases. Data processing with an SPSS 13.0 statistical package led to the conclusion that *Mycoplasma bovis* was found more frequently in the 110 day-old group of cattle (the youngest age group in this study) rather than in the 310 and 510 day-old groups of cattle ( $\chi^2 = 6.531$ ;  $p = 0.038$ ). The results obtained led to the conclusion that serological, bacteriological and histopathological examinations are important in detecting particular animal – carriers of *Mycoplasma*.

**Key words:** cattle, carriers, *Mycoplasma*, *Mycoplasma bovis*

## Introduction

Several *Mycoplasma* species colonizing the bovine respiratory mucous membranes have been found. Some of them are considered to be pathogenic, whereas others are ubiquitous, part of the normal flora (Tenk 2005). *Mycoplasma bovis* (*M. bovis*) is the most frequent *Mycoplasma* species involved in cattle mycoplasma infections in North America and Europe (Francoz et al. 2005). In the past decade, *M. bovis* has

emerged as an increasingly important cause of respiratory disease, otitis media and arthritis in younger than 3 month calves (Foster et al. 2009). In Europe, *M. bovis* is believed to be responsible for 25-33% of outbreaks of calf pneumonia. This *Mycoplasma* species is also associated with the diseases of bulls' genital tract (seminal vesiculitis, epididymitis, orchitis) and reproductive disorders of cows as well (mastitis, metritis, salpingitis and abortion) (Nicholas and Ayling 2003, Justice-Allen et al. 2010, Punyapornwithaya et al. 2010).

The mechanisms by which this organism causes damages are thought to occur principally through attachment to the host cells. Cells are damaged under the effect of toxic factors such as complex polysaccharide production, nutrient deprivation or metabolic changes in the host cells (Vanden Bush and Rosenbusch 2004). Clinical disease caused by *M. bovis* tends to be chronic, debilitating and unresponsive to antimicrobial therapy (Maunsell et al. 2009).

Diseases in young cattle may also be provoked by *Mycoplasma dispar* (*M. dispar*), *Mycoplasma californicum*, *Mycoplasma canis*, *Mycoplasma alkalescens*, *Mycoplasma arginini*, *Mycoplasma bovirhinis*, *Mycoplasma bovis genitalium* and *Mycoplasma bovoculi*. The most important seems to be *M. dispar*. This species causes disruption of normal ciliary function in the tracheal epithelium and predisposes the lower respiratory tract to infection with primary lung pathogens. *M. dispar* is frequently isolated from 3-4 month-old calves with respiratory diseases; however, it may also be detected in healthy animals (Hirose et al. 2003, Marques et al. 2007, Maunsell and Donovan 2009). Some of these species are often found as a part of the microbial flora of the upper respiratory tract in healthy calves, and in most reports they have been isolated in mixed infections with other known pathogens (Maunsell and Donovan 2009).

*Mycoplasma* infection is usually introduced into *M. bovis*-free herds by clinically healthy calves or young cattle. These animals may harbor *M. bovis* and shed the microorganism through their nasal discharge for months or years. The aerogen infection is caused by the small, inhaled droplets, which are set free by coughing from the infected animals. After the onset of the respiratory disease the infection rapidly spreads in the herd (Tenk 2005, Maunsell and Donovan 2009). Since *M. bovis* is often found as a resident of the bovine respiratory tract of apparently normal cows, transfer from the lungs to the mammary gland by haematogenous or other ways has been postulated. Alternatively, transmission of *M. bovis* infection can occur by contact with contaminated mastitis treatment solutions and milking equipment and milkers hands. Calves may become infected by suckling of cows with *M. bovis* mammary infections (Byrne et al. 2005, Tenk 2005, Punyapornwithaya et al. 2010). The genital tract of both male and female animals can be a source of infection. Furthermore, the agent may also be introduced into *M. bovis*-free herds by artificial insemination with deep frozen bull semen, in which *Mycoplasma* can survive for several years. In addition, mycoplasma-infected people may suffer from severe respiratory disease, which suggests that people working with cattle may become active carriers (Tenk 2005).

*M. bovis* infection is diagnosed by a variety of methods including isolation of the agent, immunohistochemical staining, use of a specific PCR probe, and

detection of specific antibodies in the serum (Tenk 2005).

*M. bovis* has lipid as well as protein antigens, both eliciting antibody responses, and antibody levels remain high for several months or even years after *M. bovis* infection (Nicholas and Ayling 2003). Maternal antibody may also result in high antibody levels in young calves, although with a half-life of 12 to 16 days, and the level typically decreases until a few months of age. In order to assess the seroprevalence of *M. bovis* infection and to select *M. bovis*-free animals for the creation of disease-free herds the ELISA method has been used (Maunsell and Donovan 2009).

Prevalence of *Mycoplasma* in cases of cow mastitis was investigated for the first time in Lithuania in 1995 (Siugzdaite 1995). Therefore, data about the role of *Mycoplasma* in the upper and lower part of the respiratory tract in Lithuania were quite limited. The aim of this study was to detect *Mycoplasma* species in the respiratory tract of cattle of different ages by serological, bacteriological and histopathological investigations.

## Materials and Methods

**Animals.** The study was performed in the Lithuanian cattle breeding station, a total number of 40 dairy, beef breed male cattle was studied. Cattle were brought to the control breeding farm from various regions of Lithuania at the age of 3 months. New animals from other herds were introduced to the station continuously. The investigated cattle were kept on a farm throughout the year. The animals were fed twice daily. In winter the young cattle were fed herb hay, silage and concentrate forage. In summer the cattle were given green grass mass and concentrate forage. 12 Lithuanian Black-and-White, 8 Lithuanian Rufouse (dairy breed), 9 Limousin and 11 Charolais (beef breed) animals of pure breeds were selected for the investigation. Cattle clinical status, general appearance, respiratory signs, presence of nasal discharge, severity of cough and rectal temperature were examined before taking samples from the nasal cavity. The experimental cattle were not tested with regard to *Mycoplasma* from the respiratory tract before our investigations. Samples for serological and bacteriological investigation from cattle nasal cavities were taken three times (40 samples at a time): from the 110, 310 and 510 day-old groups according to the requirements of the Law of the Republic of Lithuania on animal care, keeping and using No 8-500 (Valstybes Zinios, 1997 11 27, No 108).

At the slaughtering time macroscopic lesions were evaluated by visual examination of the lungs in the 510 day-old group of cattle. Forty samples from lungs were taken for bacteriological and histological investigations.

Samples from the nasal cavity were taken with sterile cotton tampons and put into sterile test tubes with transportation medium (Liofilchem, Italy). Lungs samples were put into sterile Petri plates and transported in a container to the Laboratory of Microbiology at the Department of Infectious Diseases, Lithuanian Veterinary Academy, for further examination.

### Blood collection and determination of antibodies against *Mycoplasma bovis*

Blood samples were collected from the tail vein of each age group of cattle. Samples were collected into sterile test tubes without anticoagulant and kept for 24 hours at room temperature. On the same day the samples were presented to the Laboratory of Microbiology at the Department of Infectious Diseases. Samples were centrifuged for 15 min. at a speed of 1500 rpm. in order to separate serum.

For the identification of antibodies against *M. bovis* the sera were examined using a commercial diagnostic test Bio-X *Mycoplasma bovis* ELISA kit (BIO K 162, Belgium). The test was carried out according to the instruction manual.

### Isolation and identification of *Mycoplasma*

Bacteriological examination of the samples from nasal cavities of cattle for *Mycoplasma* species was carried out using Friis selective media (NHS20 broth and SB broth) and solid Friis medium (Friis 1975). The nasal swabs were placed in the broth by carrying out dilutions from  $10^{-1}$  to  $10^{-4}$ , and inoculating the last dilution onto the solid media with selective additions. Selective media (NHS20 and SB) were cultivated in aerobic conditions. Solid media were incubated in microaerophilic conditions for 7-14 days. Growth of *Mycoplasma* was observed every 48-96 hours by enlarging the view with a microscope at 40 magnification. All media were incubated at 37°C. Isolation of *Mycoplasma* from the lung samples was performed according to the recommendations of Gourlay and Howard (Friis 1975, Gourlay and Howard 1983). Pure *Mycoplasma* culture was received according to the recommendations of Goll (Goll 1994).

For the identification of *Mollicutes* class polymerase chain reaction (PCR) was used. DNA from isolated microorganisms was extracted with 5% solution of Chelex (Sigma, USA). Isolated microorganisms were analyzed by PCR using forward primer, MW28 (5'- CCAGACTCCTACGGGAGGCA - 3') and reverse oligonucleotide primer MW29 (5'- TGCGAGCATACTACTCAGGC - 3') (Grida Lab, Lithuania) that are specific for *Mollicutes* class

(Bashiruddin et al. 2005). This primer pair generates a 560 bp product.

The PCR amplification was performed on a PTC - 100 Programmable thermal controller (MJ Research Inc., USA) at 94°C for 5 min, 35 cycles at 95°C for 30 s, 56°C for 30 s, 72°C for 30 s and final amplification at 72°C for 5 min. Electrophoresis of PCR products was performed in TAE buffer (40 mM Tris, 20 mM acetic acid 1mM EDTA), at 100V power flow for 60 min. Products of PCR amplification were analysed in 1.2% Top Vision LE GQ Agarose gel (MBI, Fermentas) with 1.3% ethidium bromide in UV lamp rays. GeneRuler TM 100 bp DNA Ladder (MBI, Fermentas) marker was used in this study. Reference *Mycoplasma bovis* Donetta PG45 strain was chosen as a positive control and sterile bidistilled water was used as a negative control.

In order to identify *Mycoplasma* strains by species, isolates were tested for biochemical properties: glucose fermentation, arginine hydrolysis, phosphatase activity, tetrazolium reduction, and production of spots and films (Aluotto et al. 1970). To determine antigens of *Mycoplasmas* a disk growth inhibition (DGI) test was carried out based on Clyde (1964) recommendations, and paper disks with antiserums were used: 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 bovis* PG11 strain against *Mycoplasma bovis* (Clyde 1964, Aluotto et al. 1970).

### Histopathological evaluation of lung samples

Having performed a slaughter house examination of 40 cattle, samples for histopathological study were taken from anterior (*cranialis*), cardiac (*cardiacus*) and auxiliary (*accessorius*) lobes. Samples were taken with sterile tools, placed in sterile Petri plates and transported in a container. Histopathological investigation of lung samples was performed in the Pathology centre of the Department of Infectious Diseases, Lithuanian Health Science University, Veterinary Academy. Samples were fixed with a 10% neutral solution of formalin buffer, washed with running tap water and soaked using a processor of tissue soaking from Shandon; samples were then poured into paraffin and cut using a rotational microtome. 4 µm thickness tissues were cut from a paraffin block of the sample, stained with hematoxylin and eosin, and examined with an optical microscope (Olympus BX41, Olympus). Tissues were photographed using an Olympus camera (Japan).

### Statistical analysis

An SPSS 13.0 statistical package was used for data analysis. Differences in Mycoplasma presence frequency in different age groups and in different seasons were calculated using the  $\chi^2$  criteria. The data were considered to be statistically significant when  $P < 0.05$ .

### Results

No disorders of the respiratory system (short breath, respiratory exhaustion, depression, nasal discharge or cough) were noted in the examined cattle during the whole experiment. Rectal temperature of younger cattle (younger than a year) was 38.5-40°C, and rectal temperature of cattle older than a year was 37.5-39.5°C. All the cattle examined were clinically healthy.

The number of seropositive and seronegative cattle that were determined by ELISA test are presented in Fig. 1. Mycoplasma was isolated bacteriologically from nasal cavities of 24 (60%) animals in the 110 day-old group, of 16 (40%) animals in the 310 day-old group and of 16 (40%) animals in the 510 day-old group. No Mycoplasma was detected from nasal swabs of 16 (40%) animals in the 110 day-old group, of 24 (60%) animals in the 310 day-old group and of 24 (60%) animals in the 510 day-old cattle group.

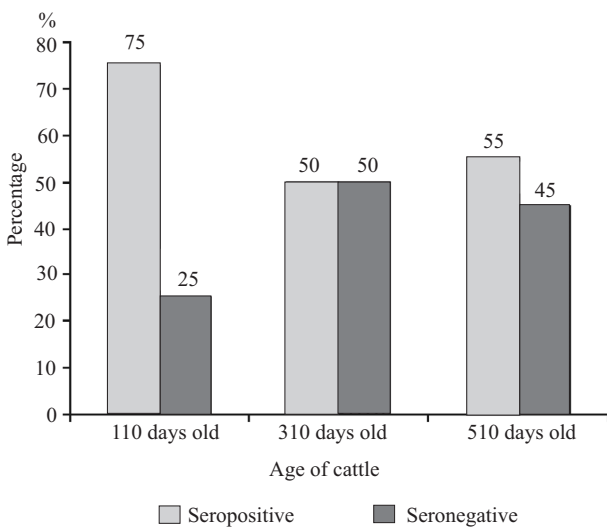


Fig. 1. Percentage of seropositive and seronegative cattle.

Fifty nine strains were isolated from nasal cavities of various age cattle groups. Line lengths of all examined PCR products in agarose gel corresponded to 560 bp number, which is typical in *Mollicutes* class (Fig. 2).

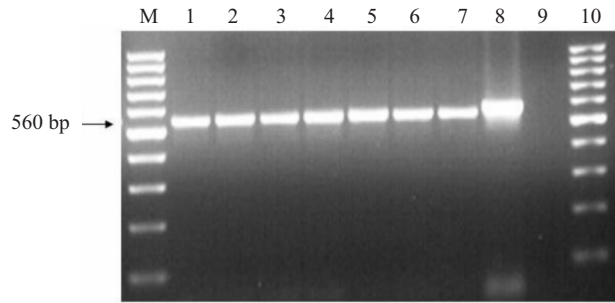


Fig. 2. Confirmation of *Mollicutes* class using PCR method. Lane M: the GeneRuler™ 100 bp DNA Ladder (MBI, Fermentas) marker; lanes 1-7: *Mollicutes* class isolates; lane 8: positive control; lane 9: negative control.

Forty four (74.6%) *Mycoplasma* strains were distinguished by partial glucose fermentation, which is typical for *M. bovis*, as well as high phosphatase activity and reduction of tetrazolium salts. Growth of the Mycoplasma was inhibited by *Mycoplasma bovis* Donetta PG 45 antiserum. Biochemical characteristics typical for *Mycoplasma dispar* (*M. dispar*) species were typical for 5 (8.5%) *Mycoplasma* strains. These organisms fermented glucose, reduced tetrazolium salts, and were not active in phosphatase, yet they remained sensitive to *Mycoplasma dispar* 462/2 antiserum. We attributed ten (16.9%) impossible to identify *Mycoplasma* strains to the nearest, according to biochemical qualities, species of *Mycoplasma* spp. These *Mycoplasma* strains showed negative DGI reaction to all Mycoplasma antisera used in this study. The identified *Mycoplasma* species which were isolated from nasal cavities of cattle groups of different age are shown in Fig. 3.

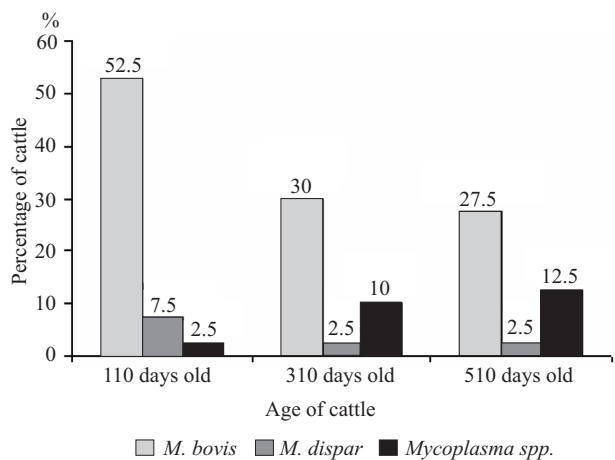


Fig. 3. *Mycoplasma* species isolated from nasal cavity of 110, 310 and 510 day-old cattle groups.

Visual examination after slaughtering did not reveal macroscopic lesions typical for mycoplasmal infections in lungs in the 510 day-old cattle group. Emphysema was detected at the edges of eight (20%) lungs; these changes could have appeared at the time of slaughter.

During histological examination signs of pneumonia typical for chronic bronchointerstitial pneumonia were detected in 11 (27.5%) samples. Only *M. bovis* was isolated from the lungs (Fig. 4).

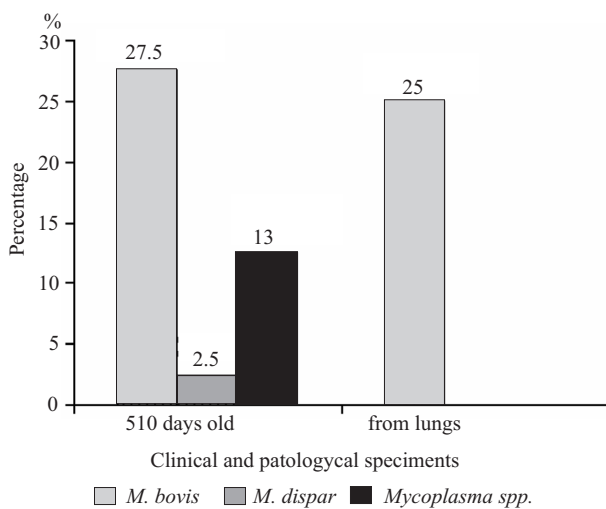


Fig. 4. *Mycoplasma* species isolated from 510 day-old cattle nasal cavity and lungs.

In the case of chronic bronchointerstitial pneumonia, infiltration of lymphocytes, and macrophages, as well as single eosinophiles and neutrophils was observed, not only around small bronchioles and blood vessels, but also in the walls of bronchioles. Walls of bronchi tended to be thickened in some parts. Atelectasis was observed in partitions of alveoles, and the cavity of some bronchioles was filled with exudation or mucus (Fig. 5).

*Mycoplasma bovis* was isolated in 8 (72.7%) cases of bronchointerstitial pneumonia. No other microorganisms were isolated from the samples with diagnosed bronchointerstitial pneumonia.

Evaluation of the data obtained using the SPSS 13.0 statistical package revealed that only frequency of *Mycoplasma bovis* presence differed reliably according to age. *M. bovis* ( $\chi^2 = 6.531$ ;  $p = 0.038$ ) was more frequently isolated from the 110 day-old cattle group (the youngest examined age group) than from the 310 and 510 day-old cattle groups. Evaluation of seasonal influence on *Mycoplasma* isolation frequency was not statistically significant ( $\chi^2 = 0.908$ ;  $p = 0.341$ ).

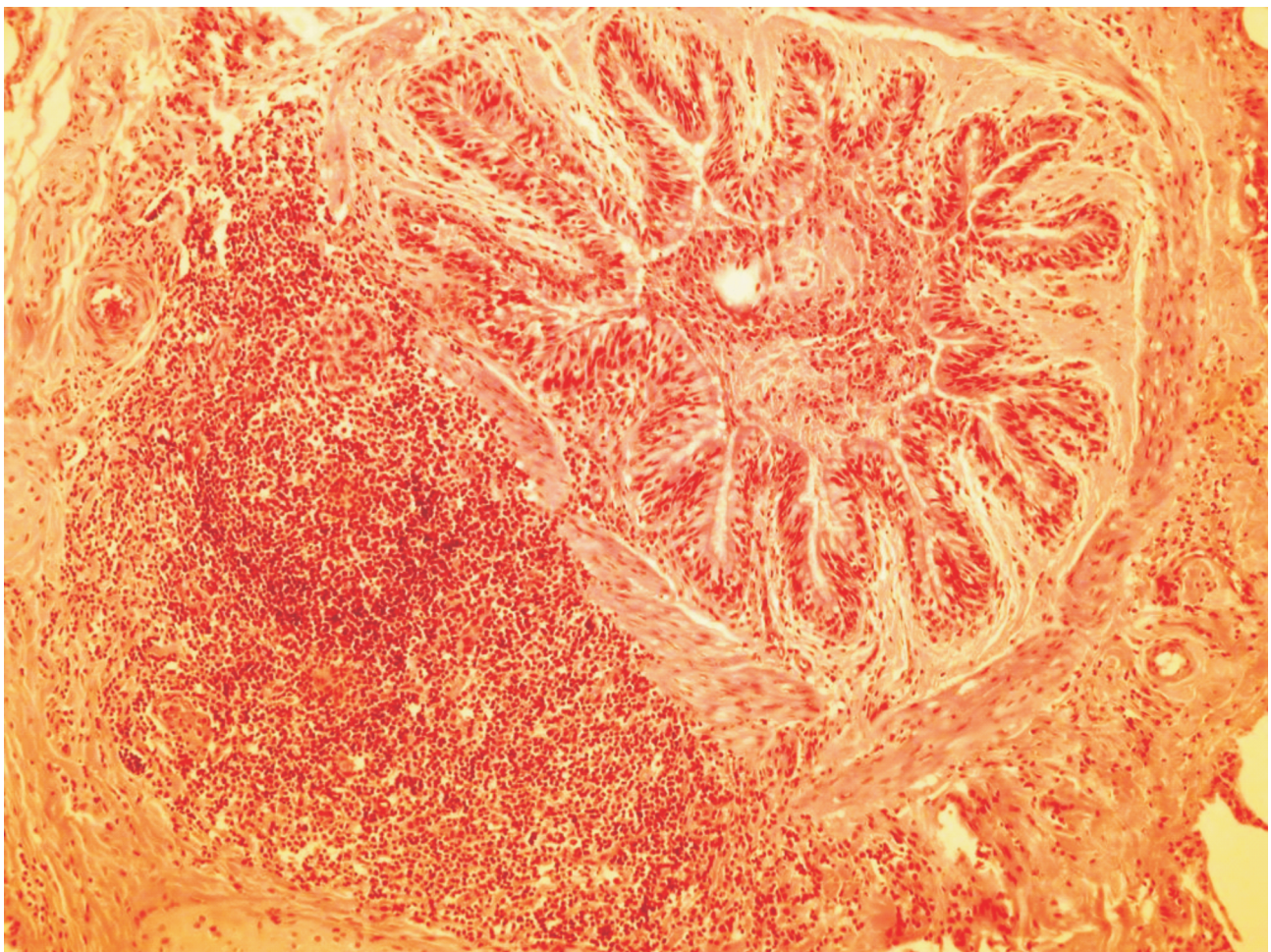


Fig. 5. Chronic bronchointerstitial pneumonia. Peribronchial infiltration of lymphocytes, macrophages and neutrophils; cavity of bronchiole is filled with mucus. Hematoxylin and eosin. Magnification x200.

## Discussion

Detection of *Mycoplasma* antibodies may have a significant epidemiological and clinical application, as it can help identify animals which have been infected within a large herd even in the absence of shedding pathogens. The results of the present study reveal that 75% of 110 day-old, 50% of 310 day-old and 55% of 510 day-old cattle groups have antibody against *M. bovis*. Radaelli (2008) obtained similar results during a monitoring study of cattle in Italy, where 76% of beef cattle and 100% of veal calves were found positive using a *M. bovis* – specific ELISA test (Radaelli et al. 2008). The *Mycoplasma bovis* seropositivity rate in British cattle (22%), and in Hungarian cattle (11%), where a serological survey was carried out, is lower than in our study (Ayling et al. 2004, Tenk 2005).

The rate of seropositive animals in our study was rather high in the 110 day-old cattle group. One of the reasons why the rate of seropositive animals was high in the younger age group of experimental animals can be maternal antibodies. Maternal antibodies can result in high antibody levels in young calves; this typically decreases during the first few months of age (Maunsell and Donovan 2009). In addition, calves with severe chronic respiratory disease provoked by *M. bovis* generally have high serum IgG titers (Radaelli et al. 2008). Serum immunoglobulin concentration remains elevated from some months to years after *M. bovis* infection (Nicholas and Ayling 2003). In adult cattle high *M. bovis* seropositivity can be associated with mild to moderate chronic and unspecific pneumonic processes that may represent a long-term sequel of earlier *M. bovis* respiratory infections (Radaelli et al. 2008).

The primary diagnostic means is current culture with specialized media. *M. bovis* from the nasal cavity of cattle was isolated in 52.5% in the 110 day-old, in 30% in the 310 day-old and in 27.5% in the 510 day-old cattle group. Siugzdaite (2002) carried out the investigation for detection of *M. bovis* from calves younger than three months without clinical symptoms of pneumonia. *M. bovis* was isolated from the nasal swabs of 24.4% of calves (Siugzdaite 2002). In a study of calves in the Netherlands, *Mycoplasma bovis* was found in a small number (3%) of apparently healthy animals (Nicholas and Ayling 2003).

*M. dispar* was isolated mostly (7.5%) from the 110 day-old cattle group, and was rare (2.5%) in the 310 day-old and 510 day-old cattle groups. Healthy calves and cattle can be infected with *M. dispar* aerogenically from animals that have chronic or subclinical respiratory diseases. According to the scientific literature, *M. dispar* is often isolated from 3-4 month-old calves suffering from pneumonia (Hirose et al. 2003, Marques et al. 2007).

We isolated 27.5% *M. bovis* from the nasal cavity and 25% from lungs without macroscopic lesions of the 510 day-old cattle. Tenk (2005) reported that *M. bovis* can be isolated from 25.2% of lung samples, without macroscopic lung lesions (Tenk 2005). Gagea (2006) isolated *M. bovis* from 46% of histologically unchanged lungs (Gagea et al. 2006). During our histological study of the same samples from lungs taken at the time of slaughtering, foci of chronic bronchointerstitial pneumonia were detected in 27.5% of lungs. *Mycoplasma bovis* was isolated from 72.7% of bronchointerstitial pneumonia cases. Consequently, pre-existing viral infections should be also considered as a cause of chronic pneumonic lesions (Radaelli et al. 2008).

Herds involved in the present study consist of calves brought from different regions of the country. Healthy cattle are infected by carriers of *Mycoplasma* by direct contact and aerosolically. Regrouping of animals to form herds of different age, stress experienced during transportation, and contacts of clinically healthy cattle with seropositive cattle are risk factors that maintain stationary *Mycoplasma* infection. Our bacteriological studies of the nasal cavity, serological studies of blood serum, and statistical data analysis carried out during the experiment have revealed that 110 day-old cattle (the youngest examined age group) are more susceptible to being carriers of *Mycoplasma bovis*. This proposition is in agreement with the investigations of Arcangioli (2008), who found that 79% of clinically healthy calves were carriers of *Mycoplasma bovis* (Arcangioli et al. 2008). Cattle-carriers of *Mycoplasma bovis* excrete *Mycoplasma* to the environment with nasal secretion for months or even years. Control is implemented by management techniques and strict, lifetime separation of infected cattle from the noninfected herd.

## Conclusion

Serological, bacteriological and histopathological examinations are important for the detection of cattle-carriers of *Mycoplasma*. *Mycoplasma bovis* can be the cause of lesions, atypical for *Mycoplasma*, in lungs of young cattle.

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