Mycoplasma dispar prevalence in the upper respiratory tract of cattle and the antimicrobial susceptibility of the isolates

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The aim of this study was to make a survey of the presence of Mycoplasma dispar on a cattle breeding farm and to determine antimicrobial susceptibility of the isolates.

The study was carried out at a farm in Lithuania. Nasal swabs for bacteriological investigation were collected from ninety dairy, beef and mixed type of cattle from 90 to 300 days of age. Mycoplasma cultivation procedures were carried out using Friis selective media. To confirm the presence of Mollicutes class the polymerase chain reaction (PCR) was used. Isolates were identified according to biochemical and antigenic characteristics.

The minimum inhibitory concentration of twenty field isolates of Mycoplasma dispar to tulathromycin, tylosin, lincomycin, enrofloxacin, florfenicol, and oxytetracycline was determined by using a micro-broth dilution method.

Mycoplasma dispar was detected in the nasal cavity of 15 out of 84 clinically healthy animals (17.9 %), and in 5 out of 6 animals with respiratory disorders (83.3 %). The isolates were most susceptible to tulathromycin, lincomycin, enrofloxacin and florfenicol. Three (15 %) isolates were resistant to oxytetracycline.

The susceptibility to oxytetracycline significantly differed between Mycoplasma dispar isolates compared to the susceptibility of tulathromycin ($P < 0.001$), lincomycin ($P < 0.001$) tylosin ($P < 0.001$), enrofloxacin ($P < 0.001$), and florfenicol ($P < 0.001$).

Keywords: antimicrobial agents; cattle; Mycoplasma dispar

Introduction

Cattle respiratory diseases are one of the major health problems in feedlots. The most important of these diseases is the bovine respiratory disease complex (BRDC). The clinical entity of BRDC most often manifested is bronchopneumonia. It is usually associated with the assembly of large numbers of weaned calves into a feedlot environment. BRDC has a multifactorial etiology and develops as a result of complex interactions between environmental factors, host or animal factors and pathogens [1]. The role of Mycoplasma dispar (M. dispar) in BRDC is uncertain, primarily due to culturing difficulties, which means that many laboratories that test for mycoplasmas, overlook this species, leading to a considerable underestimation of the prevalence [2].

Mycoplasma dispar is a common inhabitant of the upper and lower respiratory tract of healthy cattle. Several studies has shown that Mycoplasma dispar currently was present in 50 % of the examined herds, and bacterial agents of the syndrome, i.e. Pasteurella multocida, Arcanobacterium pyogenes or Mannheimia haemolytica coexisted with these cases [3]. In the development of respiratory disease in dairy calves, M. dispar may play an initiating role for leading to subclinical and clinical pneumonia. Dutch studies [4, 5] showed that M. dispar was present in 92 % of pneumonic lungs from 148 calves and only in 40 % of healthy lungs from 270 calves. In Denmark, Tegtmeier et al. [6] isolated M. dispar from 13 of 31 lungs showing fibrin-necrotizing bronchopneumonia, from 15 of 31 lungs with suppurrate bronchopneumonia and from 3 of 31 lungs with embolic bronchopneumonia. In Canada, M. dispar was isolated from more than half of 300 pneumonic calf lungs [7].

Mycoplasma dispar appears less immunogenic in calves, and vaccine given either intramuscularly or intratracheally or both do not produce a detectable protection from colonization, which may at least partly form its pathogenicity [8].

Often the cost of respiratory disease estimated on data concerning morbidity and mortality, usually based on the clinical appearance of animals. The assumption is that clinically normal animals are not affected by the disease, and do not contribute to the economic impact of disease outbreak. However, it is shown that many animals that are exposed to respiratory pathogens will seroconvert and may be sub-clinically infected, which has a negative effect on the growth rate, fattening period and carcass
grades. These costs are likely to be underestimated in studies of the impact of respiratory disease [9].

The role of Mycoplasma dispar as an active agent in cases of bovine respiratory disease is poorly elucidated in Lithuania. The aim of this study was to investigate the presence of Mycoplasma dispar on a breeding farm and to determine antimicrobial susceptibility of the isolates.

Materials and methods

Animals. M. dispar was studied in a cattle breeding farm in Lithuania from 90 bulls (30 dairy cattle, 30 beef cattle, and 30 dairy-beef cattle breeds). Male cattle were investigated according to the requirements of the Law of Republic of Lithuania on animal care, keeping and using No B1-639 (“Valstybės Žinios”, 2009 01 22, No. 8-287). The herd included 300 male cattle. The age of the investigated cattle ranged from 90 to 300 days. The 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 farm continuously. The investigated cattle were kept in barns until time of slaughter, which were at 510 days of age.

The cattle were fed herb hay; silage and concentrate forage in winter. In summer the cattle were given green grass mass and concentrate forage. No vaccines and antibiotics were used as preventive measures.

A clinical assessment of each animal was performed before samples from nasal cavity were collected. General appearance and respiratory symptoms were scored on a 4 point scale [10] (Table 1). The rectal temperature of each animal was measured.

Table 1. Evaluation rates of the cattle health status and respiratory symptoms

<table>
<thead>
<tr>
<th>4 point scale</th>
<th>General appearance</th>
<th>Respiratory signs</th>
<th>Nasal discharge</th>
<th>Coughing</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>normal</td>
<td>absent</td>
<td>absent</td>
<td>absent</td>
</tr>
<tr>
<td>1</td>
<td>subdued, slightly depressed</td>
<td>hyperpnoea or slight dyspnea</td>
<td>mild secretion</td>
<td>mild</td>
</tr>
<tr>
<td>2</td>
<td>depressed, reluctant to rise</td>
<td>moderate hyperpnoea or obvious dyspnea</td>
<td>catarrhal secretion</td>
<td>rare</td>
</tr>
<tr>
<td>3</td>
<td>very depressed, unresponsive to external stimuli</td>
<td>respiratory distress</td>
<td>suppurative secretion</td>
<td>Frequent – several in one minute</td>
</tr>
</tbody>
</table>

Isolation and identification of Mycoplasma dispar.

Twenty M. dispar isolates were obtained by the Microbiology Laboratory of Department of Infectious Diseases in the Veterinary Academy of Lithuanian University of Health Sciences.

The bacteriological examination of samples from nasal cavities of cattle for Mycoplasma dispar species was carried out using Friis selective media [11]. The nasal swabs were placed in the Friis NHS-20 broth by making dilutions from $10^{-1}$ to $10^{4}$ and were incubated in microaerophilic conditions for 3–4 days. After the colour of the broth culture changed indicating mycoplasma growth, broth was inoculated on to Friis NHS-20 agar plates. The solid media were incubated in microaerophilic conditions for 14–21 days [11]. The cultures of M. dispar were purified three times by conventional filtration cloning techniques with a 450 nm pore size membrane filter [12].

A polymerase chain reaction (PCR) was used for the identification of the Mollicutes class [13].

DNA from isolated mycoplasma was extracted with 5 % solution of Chelex (Sigma, USA). Isolated microorganisms were analyzed by PCR using forward primer, MW28 (5’– CCAGACTCCTACGGAGGCA – 3’) and reverse oligonucleotide primer MW29 (5’– T GCGAGCATACTCTAGGC – 3’) (Grinda Lab, Lithuania) that are specific for the Mollicutes class [13]. This pair of primer generates a 560 bp product.

For the identification of M. dispar species, isolates were tested for biochemical properties: glucose fermentation, arginine hydrolysis, phosphatase activity, tetrazolium reduction and production of spots and films [14]. To determine Mycoplasma dispar discs growth inhibition (DGI) test was used [15]. All isolates were stored in NHS-20 broth at –70 °C (N-medium - 320 ml, horse serum - 40 ml, swine serum - 40 ml, cycloserine - 60 mg).

Antimicrobial agents. Antimicrobials tested in this study were tulathromycin (Pfizer, United States), tylosin (Chemifarma, Italy), lincomycin (Pfizer, United States), enrofloxacin (Vetoquinol, Austria), florfenicol (KRKA, Slovenia) and oxytetracycline (Chemifarma, Italy). Serial twofold dilution of drugs was performed in test tubes with NHS-20 broth. Final concentrations of 100 µg/ml for tylosin, lincomycin, enrofloxacin, florfenicol, oxytetracycline and 32 µg/ml for tulathromycin were prepared with some modifications as described by Hannan [16]. Tylosin, oxytetracycline and enrofloxacin were obtained in pure form. Tylosin and oxytetracycline were first dissolved in 10 % methanol, enrofloxacin in 2 % potassium hydroxide, and then the solution was completed to final volume using distilled water. Liquid form of lincomycin, florfenicol and tulathromycin were diluted in distilled water to prepare stock solutions. The stock solutions were sterilized by filtering through a 200 nm pore size membrane filter and used immediately.

Micro-broth dilution test. Minimal inhibitory concentration (MIC) of each antimicrobial agent was determined by applying micro-broth dilution test according to the recommendations of Hannan [16] and Ter Laak et al. [17]. Each antimicrobial agent was serially diluted into twofold dilution in Friis NHS-20 broth (containing no selective supplements) from the highest concentration (100 µg/ml and 32 µg/ml) in wells of micro titer plates; each well containing 100 µl. The last columns of micro titer plates were filled with 100 µl of broth medium without antimicrobials (growth control). One hundred micro liters’ of mycoplasma inoculums ($10^4$– $10^6$cu/ml of each strain freshly cultured for 2 h at 37 °C) were added to columns. Plates were sealed and incubated...
aerobically at 37 °C for 3 to 4 days. The susceptibility of \( M. \) dispers isolates to an antimicrobial agent was indicated by a growth-induced pH shift in the medium. MIC for each isolate and each antimicrobial was defined as the lowest concentration of antimicrobials to completely inhibit visible growth (pH change) in the broth. MIC\(_{50}\) and MIC\(_{90}\) values were defined as the lowest concentrations capable of inhibiting the growth of 50 % and 90 % of isolates, respectively [16–18].

The number of mycoplasmas for inoculum in MICs test was determined by micro-broth dilution method described by Hannan [16]. Broth culture of each isolate was diluted to tenfold dilution in Friis NHS-20 broth and dispensed in to microtiter plates. The plates were sealed and incubated aerobically at 37 °C. The lowest dilution showed a change of color of the broth medium from red to yellow was determined as the number of color changing units (ccu) of mycoplasma culture.

To ensure micro-broth dilution test reproducibility and validity, strain 462/2 of \( M. \) dispers was used as a reference. MIC test was repeated three times.

**Interpretation of MIC results.** Since there are no accepted breakpoints for the \( M. \) dispers susceptibilities determination, criteria described by Hannan [16] were used for interpretation of MIC results. Values are shown in Table 2.

<table>
<thead>
<tr>
<th>Antimicrobial material groups</th>
<th>Tetracyclines</th>
<th>Lincosamides</th>
<th>Quinolones</th>
<th>Amphenicols</th>
<th>Macrolides</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitive</td>
<td>≤ 4 µg/ml</td>
<td>≤ 2 µg/ml</td>
<td>≤ 0.5 µg/ml</td>
<td>≤ 4 µg/ml</td>
<td>≤ 1 µg/ml</td>
</tr>
<tr>
<td>Intermediate</td>
<td>≤ 8 µg/ml</td>
<td>≤ 8 µg/ml</td>
<td>≤ 1 µg/ml</td>
<td>≤ 8 µg/ml</td>
<td>≤ 2 µg/ml</td>
</tr>
<tr>
<td>Resistant</td>
<td>&gt; 8 µg/ml</td>
<td>&gt; 8 µg/ml</td>
<td>≥ 2 µg/ml</td>
<td>≥ 16 µg/ml</td>
<td>≥ 4 µg/ml</td>
</tr>
</tbody>
</table>

**Statistical analysis.** Descriptive statistical analyses were performed with SPSS 13.0 Windows statistical packet (2004). Kruskal–Wallis test was used to examine the equality between the isolates medians for each MIC value. \( P < 0.05 \) was considered as significant.

**Results and discussion**

Six (6.7 %) cattle of the investigated group had respiratory system disorders: animals were slightly depressed; there were abnormal sounds on auscultation of the respiratory tract, some of them suffered from cough or nasal discharge. The rectal temperature of these cattle was > 39.5 °C. No respiratory system disorders were noticed in 84 (93.3 %) of the investigated cattle – these cattle were clinically healthy.

The bacteriological examination of samples from the nasal cavity of 90 investigated cattle identified Mycoplasma dispers in twenty (22.2 %) samples. PCR confirmed that all (100 %) \( M. \) dispers isolates belong to Mollicutes class. Fifteen (17.9 %) clinically healthy and five (83.3 %) cattle with respiratory system disorders shed Mycoplasma dispers in their nasal cavity.

The antimicrobial susceptibility test to six different antimicrobial agents revealed that all (100 %) \( M. \) dispers isolated from clinically healthy cattle was susceptible to tulathromycin. The range of MICs for tulathromycin was 0.06 to 0.5 µg/ml. All (100 %) isolates of \( M. \) dispers were susceptible to tylosin. The range of MICs for tylosin was 0.39–1.56 µg/ml. According to the breakpoints of MICs to quinolone, 100 % of the isolates were susceptible to enrofloxacin. The range of MIC for enrofloxacin was 0.39–0.78 µg/ml. All (100 %) isolates of \( M. \) dispers from cattle of different ages were susceptible to florfenicol and lincomycin. The range of MICs for florfenicol and lincomycin were 0.39–3.12 µg/ml and 0.39–0.78 µg/ml, respectively.

In the present study, 17 (85 %) isolates of \( M. \) dispers were found to be sensitive to oxytetracycline. Three (15 %) isolates were resistant to oxytetracycline. The range of MICs for oxytetracycline was from 1.56 to 25 µg/ml.

All (100 %) the isolates of \( M. \) dispers isolated from cattle with respiratory disease were susceptible to tulathromycin, tylosin, enrofloxacin, florfenicol, and lincomycin. There were two types of isolates with respect to their susceptibility to oxytetracycline. Three (60 %) of five isolates were susceptible to oxytetracycline, two (40 %) – were resistant.

The statistical data analysis revealed that MIC results obtained during the study did not contradict each other \( (P < 0.001) \). MIC data were evaluated applying SPSS 13.0 statistical package, and it was determined that \( M. \) dispers isolates had a similar susceptibility pattern with respect to tulathromycin, enrofloxacin, florfenicol, and lincomycin. Statistically significant differences of susceptibility of \( M. \) dispers to florfenicol compared with tulathromycin \( (P < 0.001) \), tylosin \( (P < 0.001) \), lincomycin \( (P < 0.001) \), and enrofloxacin \( (P < 0.001) \) were found. Susceptibility of \( M. \) dispers isolates to oxytetracycline significantly differed compared to tulathromycin \( (P < 0.001) \), lincomycin \( (P < 0.001) \), tylosin \( (P < 0.001) \), enrofloxacin \( (P < 0.001) \), and florfenicol \( (P < 0.001) \).

Mycoplasma dispers is occasionally isolated from the respiratory tract of diseased cattle, and in most reports it has been isolated in mixed infections with other known pathogens [3]. Frequently, these organisms are related with chronic or subclinical respiratory disease in cattle [19]. Several studies revealed that \( M. \) dispers was found in upper respiratory tract of healthy animals [4, 20].

The cattle breeding farm involved in this study houses calves brought from different regions of the country. Healthy cattle may be infected by carriers of mycoplasmas during direct contact or by aerosols. Stress
experienced by changes in food, temperature, humidity, and ventilation and by mixing of animals from different sources is risk factors that maintain stationary mycoplasma infection. Our bacteriological studies of samples from nasal cavities showed that *M. dispar* was isolated from 17.6 % of clinically healthy cattle and from 83.3 % of cattle with respiratory disease symptoms. In a Dutch study, *M. dispar* was detected in 40 % of clinically healthy calves [5]. Marques et al. [20] found *M. dispar* in the nasal cavity of 6.16 % of healthy calves and in 34.84 % of animals with respiratory disease. Clinically healthy calves may serve as a reservoir for *Mycoplasma dispar*, because they harbor *M. dispar* in the nasal cavity and shed the microorganism through their nasal discharge [4, 20]. Our bacteriological studies revealed that a majority of respiratory symptoms in calves may be caused by *M. dispar*. These findings are confirmed by the study of calves in England, where *M. dispers* was isolated from the nasal cavity of 93 % of healthy calves. Further studies on animals from the same source have indicated that many of these calves suffer from subclinical pneumonia, and *M. dispar* was isolated from the lungs of 97 % of the animals [19]. It is necessary to take mycoplasmas in to consideration when examining for the microbiological cause of respiratory infection in cattle.

This is the first report on the *in vitro* resistance of *M. dispar* against six different antimicrobial agents in Lithuania. Since there are no universally accepted standards to determine antimicrobial susceptibility of mycoplasma, we have chosen the micro-broth dilution method. The inhibition of *M. dispar* growth by antibiotics was established by the organism's inability to ferment glucose and thus produce a colour change of the phenol red indicator in the medium from red to orange-yellow.

Macrolides are classified according to the number of carbon atoms which comprise the lactone ring, reaching from 12 to 16 members. It has been suggested that the 14 membered macrolides (erythromycin) were very effective against human mycoplasma pneumonia caused by *M. pneumonia* [21]. However, in the past decade, *in vitro* susceptibility studies on mycoplasma have shown an increased resistance to the erythromycin [18, 22, 23]. Several studies have shown that a mixture of a 13 and 15-membered ring macrolide (tulathromycin) and 16-membered macrolides (tylosin) are known to be more effective against mycoplasma than the 14-membered macrolides [23, 24]. We found that isolates of *M. dispar* isolated from clinically healthy cattle and cattle with respiratory system disorders were sensitive to tulathromycin and tylosin. The MICs of tylosin for *M. dispar* isolates in the present study was higher (0.39–1.56 μg/ml) than the MICs of tylosin reported by Ter Laak et al. [17] (0.03–0.25μg/ml). In both cases the MIC values of *M. dispar* to tylosin was lower than the breakpoint of the MIC value for macrolides (≤1 μg/ml). However, tylosin has been used for many years to control bacterial diseases in Lithuanian cattle farms, and despite this, tylosin still has good *in vitro* activity against isolates of *M. dispar*. High susceptibility of *M. dispar* isolates to tulathromycin makes it an attractive drug for treating mycoplasma infections in the field. Furthermore, *in vivo* investigations of tulathromycin have demonstrated a broad spectrum of efficiency in the therapy of bovine respiratory disease [13].

Lincomycin and enrofloxacin had a similar effect against isolates of *M. dispar*. All isolates were susceptible to both agents. The MICs for lincomycin and enrofloxacin ranged from 0.39 to 0.78 μg/ml. Ter Laak et al. [17] reported a similar effect of lincomamides and quinolones against isolates of *M. dispar*. The MIC values of *M. dispar* to lincomycin were 0.5–1 μg/ml and to enrofloxacin – 0.25–0.5 μg/ml.

In the present study MICs values of isolates of *M. dispar* to amphenicols were lower than the breakpoint (4, 8 and 16 μg/ml). All isolates were susceptible to florfenicol. This is in accordance with Ter Laak et al. [17] who reported MIC values of 2–4 μg/ml to amphenicols.

Fifteen per cent of *M. dispers* isolated from clinically healthy cattle and forty per cent isolates from cattle with respiratory disease were resistant to oxytetracycline in our study. The remaining isolates were susceptible to this antimicrobial agent. Ter Laak et al. [17] also isolated oxytetracycline-resistant isolates from bovines. In the present study, the MIC values of oxytetracycline for all *M. dispers* field isolates were lower (1.56 to 25 μg/ml) than the MICs to oxytetracycline reported by Ter Laak et al. [17]. In the past decade, *in vitro* susceptibility investigations of bovine mycoplasma have shown an increasing resistance to the antimicrobials of the tetracycline group [25]. Supposedly, high mutation rates of mycoplasma can rapidly develop resistance to antimicrobials as a result of gene mutation, or acquisition of new genetic material [26].

We believe that oxytetracycline-resistant *M. dispar* isolates could have accumulated mutations in genes through a wide use of tetracycline and oxytetracycline to control cattle respiratory disease and *E. coli* infections in calves.

**Conclusion**

In the present study, *Mycoplasma dispar* was isolated from the nasal cavity of 17.9 % of clinically healthy and 83.3 % of cattle with respiratory disease. The isolates were susceptible to tulathromycin, lincomycin, enrofloxacin and florfenicol, while 15.0 % of the isolates were resistant to oxytetracycline.

**References**


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MYCOPLASMA DISPAR PAPLITIMAS GALVIJŲ VIRŠUTINIUOSE KVĖPAVIMO TAKUOSE IR ANTIMIKROBINIS ATSPARUMAS IŠSKIRTOMAS PADERMĖMS

Santrauka

Tyrimo tikslas – nustatyti galvijus Mycoplasma dispar nešiojus ir ištirti išskirtų mikoplazmų padermių jautrumą antimikrobinėms medžiagoms.

Mėginti bakteriologiniams tyrimams buvo surinkti iš 90 pieninio, mėsinio ir mišraus tipo galvijų nosies ertmės, kurių amžius buvo nuo 90 iki 300 dienų. Mikoplazmoms išskirti iš tiriamosios medžiagos naudota Friis selektyvi terpė. Mollicutes klasei nustatyti panaudota polimerazės grardinė reakcija (PGR). Mikoplazmų padermės iki rūšies nustatytos tiriant jų biochemines ir antigenines savybes. Minimali antimikrobinės medžiagos slopinimo koncentracija (MSK) nustatyta taikant serijinį paskiedimų metodą.

Ištyrus 84 kliniškai sveikus galvijus, Mycoplasma dispar buvo išskirti iš 15 (17,9 %) galvijų nosies ertmės. Iš 6 respiratorinėmis ligomis sergančių galvijų 5 (83,3 %) buvo Mycoplasma dispar nešiojai. Mažiausios antimikrobinės medžiagų koncentracijos, slopinančios Mycoplasma dispar padermių augimą, buvo tulatromicina, lincomicina, enrofloksacino ir florfenikolio.

Nustatėme, kad Mycoplasma dispar padermių jautumas oksitetraciklinui statistiškai reikšmingai skyrėsi nuo šių padermių jautrumo tulatromicinui ($P < 0,001$), lincomicinui ($P < 0,001$), tilozinui ($P < 0,001$), enrofloksaciniui ($P < 0,001$) ir florfenicolui ($P < 0,001$).

Reikšminiai žodžiai: antimikrobinės medžiagos, galvijai, Mycoplasma dispar.