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Mycoplasma bovis infections and co-infections with other *Mycoplasma* spp. with different clinical manifestations in affected cattle herds in eastern region of Poland

Ewelina Szacawa[1], Krzysztof Niemczuk[1], Katarzyna Dudek[1], Dariusz Bednarek[1], Ruben Rosales[2], Roger Ayling[2]

[1]Department of Cattle and Sheep Diseases,
National Veterinary Research Institute, 24-100 Pulawy, Poland
[2]Department of Bacteriology, Animal and Plant Health Agency (APHA),
Woodham Lane, Addlestone, Surrey KT15 3NB, UK
ewelina.szacawa@piwet.pulawy.pl

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Abstract

The aim of the study was to evaluate the presence of *Mycoplasma bovis* infection and co-infections with other *Mycoplasma* spp. infections in cattle. The tested population was one in the eastern region of Poland containing 66 dairy cows and 23 calves showing different clinical signs and suffering from pneumonia, mastitis, and arthritis. The incidence of *M. bovis* in co-infections with other *Mycoplasma* spp. was examined using serological traditional mycoplasma culture methods, and the molecular methods - PCR and polymerase chain reaction/denaturing gradient gel electrophoresis (PCR/DGGE). The PCR/DGGE method for detecting *Mycoplasma* spp. in cattle was used for the first time in Poland. The seroprevalence of *M. bovis* in the affected cattle herds in the eastern region of Poland was 47.8% in calves and 19.7% in dairy cows. The direct detection and identification of *M. bovis* from nasopharyngeal swabs by PCR revealed that 56.5% of calves were positive, but all of the dairy cows were negative. The PCR/DGGE identified eight (34.8%) instances of *M. arginini* and eight (26.1%) instances of *M. bovirhinis* co-infecting with *M. bovis* in ten calves. The seroprevalence of *M. bovis* in the tested population was 33.7%. Any future attempts to control mycoplasma infections require an insight into the current epidemiological situation of *M. bovis* infection and its relationship to other mycoplasmas in causing clinical disease in cattle. Using these diagnostic methods we have demonstrated that mycoplasmal infections are often caused by multiple species of *Mycoplasma* and not just the primary *M. bovis* pathogen.

Keywords: cattle, bovine mycoplasmosis, *Mycoplasma bovis*, PCR, PCR/DGGE.

Introduction

Bovine respiratory infectious diseases frequently have multifactorial aetiology. Bovine respiratory viruses (bovine respiratory syncytial virus, bovine herpesvirus type 1, bovine viral diarrhoea virus, and parainfluenza virus type 3) as well as conventional bacteria (*Mannheimia haemolytica*, *Pasteurella multocida*, and *Histophilus somni*) may all be contributing factors. Bovine respiratory disease (BRD) may also be caused by mycoplasma, and related species including *M. bovis*, *M. dispar*, *Ureaplasma diversum*, *M. bovigenitalium*, *M. bovirhinis*, *M. alkalescens*, *M. arginini*, and *M. canis* (2, 6 - 8, 10, 13, 16). In Europe, *M. bovis* is believed to be responsible for 25% to 33% of calf pneumonia cases (12). *M. bovis*-associated pneumonia may occur in cattle at different ages, including dairy cows, beef cattle, feedlot animals, and adults, especially after transport stress. Pneumonic clinical signs are nonspecific and include fever, cough, tachypnea, dyspnea, and decreased

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appetite, with or without nasal discharge (1, 15). The co-infection of *M. bovis* with other causative organisms can increase the severity of respiratory disease in calves, as well as morbidity and mortality rates (9), leading to a severe form of calf pneumonia, BRD, which is one of the most important problems in young cattle and inflicts high losses on the cattle industry (17). In addition to respiratory disease, *M. bovis* can also cause clinical disease with pathological changes in the mammary gland, joints, conjunctival sac, and ears (5, 13, 20). *M. bovis* infection is problematic to resolve, as a vaccine against the infection is currently not commercially available in Europe. In addition, more and more strains are acquiring antibiotic resistance (4, 5, 13).

The aim of this study was to evaluate the epidemiological situation of *M. bovis* and concurrent mycoplasma infections in the affected cattle from the eastern region of Poland using complex diagnostic analysis consisting of serology, culture, and molecular testing. The PCR/DGGE was used for the first time in Poland for the detection of concurrent mycoplasma infections in cattle (11).

Material and Methods

Animals. This study was performed on 89 cattle which included 23 two-month-old Black and White breed calves derived from three herds having 35-60 calves and 66 adult Holstein-Friesian cows from a herd of 120 in the Lubelskie Province (eastern Poland) with an average milk yield of 6500 kg. Nasopharyngeal swabs and sera were collected from all animals and 54 milk samples pooled from each quarter were collected from the dairy cows during 2014.

Serological and antigenic testing. The blood samples were collected from the *vena jugularis externa* and then centrifuged to obtain sera and stored at 4ºC for a maximum 48 h before testing for *M. bovis* antibodies using the indirect sandwich ELISA (Bio-X Diagnostics, Belgium). Positive or negative results with an evaluation of positivity degree (from 0 to 4+) were calculated according to the manufacturer's instructions.

Nasopharyngeal swabs were taken from all animals from the affected herds: the *M. bovis* seropositive cattle and asymptomatic animals in the herds. The swabs were placed in transport medium and kept at 4°C until the analysis, which was done within 24 h. The initial detection and identification of *M. bovis* was performed using the antigenic direct ELISA (Pulmotest, Bio-X Diagnostics, Belgium). Positive or negative results in this test were calculated according to the manufacturer's instructions, using the quality control sheet provided with the kit. *Mycoplasma* spp. were isolated by culturing in Eaton's broth and on Eaton's agar plates, and incubated at 37ºC with 5% CO_2 (14). Plates and broths were incubated for up to 21 d. The broths were observed for typical mycoplasma swirls of growth, for film formation on the surface of broth, and for any colour change, which may be from red to orange for the non-glucose fermenting *Mycoplasma* spp. such as *M. bovis*, or to yellow for glucose fermenters such as *M. bovirhinis*. If mycoplasma-like colonies appeared, DNA was extracted using the QIAmp DNA Mini Kit (Qiagen, Germany) and an *M. bovis* specific PCR was performed (18). In addition the PCR/DGGE method following Eaton's broth enrichment (11) was used to detect mixed *Mycoplasma* spp. infections.

Milk samples were collected from all quarters from cows with clinical signs of mastitis or arthritis and were stored at 4ºC for a maximum of 24 h until being tested. A 100 µL sediment from 1 mL of centrifuged milk was cultured for mycoplasmas in Eaton's medium (14). Antibody testing of milk samples used the indirect sandwich ELISA (Bio-X Diagnostics, Belgium). DNA extraction and molecular testing was as described for the nasopharyngeal swabs.

Molecular testing. The method used was an *M. bovis* specific PCR with the use of primers for the *uvrC* gene in a total reaction volume of 50 µL (18). Using a T-personal thermocycler (Biometra, Germany), the conditions for the PCR were as follows: initial denaturation at 95ºC for 5 min, followed by 33 cycles of denaturation at 94ºC for 30 s, annealing of primers at 60ºC for 30 s, extension at 72ºC for 90 s, and a final extension step of 72ºC for 7 min. Samples were kept at 4ºC until analysis. PCR products were analysed on 2% agarose gels stained with ethidium bromide and visualised under UV light by digital camera. The procedure was modified from that described by Subramaniam *et al.* (18) because slightly different annealing and extension temperatures and times were used. The procedure was validated for the use at the NVRI laboratory using 16 control samples of different *Mycoplasma* spp. and other bacteria species for specificity testing and *M. bovis* strain ATCC 25523™ for sensitivity testing. Sequencing of the *uvr*C gene fragment was performed on a GS FLX/Titanum sequencer (Roche, USA), supplied by GENOMED (Poland).

The DGGE method was used as described by McAuliffe *et al.* (11) with modifications. The method is based on the prevention of migration of DNA fragments following strand separation caused by chemical denaturants. Each *Mycoplasma* spp. forms characteristic "fingerprinting" in the gel lane. This enables the detection and differentiation of 67 *Mycoplasma* spp. of veterinary importance. Amplification was performed with a GC-341 primer specific for bacteria and MYC-543 as the second primer, which is mollicute-specific; both primers are based on the V3 region of 16S rDNA gene. Samples

were kept at 4ºC until analysis. PCR products were checked for correct amplification by electrophoresis on a 2% agarose gel stained with ethidium bromide under UV illumination. The PCR/DGGE was performed using the DCode Universal Mutation Detection System (Bio-Rad, USA). The PCR products were separated by loading onto 10% polyacrylamide/bis (37.5:1) gels with denaturing gradients from 25% to 60% (where 100% is 7 M urea and 40% (v/v) deionised formamide) in 1 × Tris, acetic acid, and EDTA (TAE) electrophoresis buffer (EurX, Poland). Electrophoresis was performed for 19 h at 100 V at 60ºC. For the product visualisation, gels were stained with SYBR Gold (Life Technologies, USA) in 1 × TAE for 30 min and restained in distilled water for 30 min at room temperature, visualised under UV, and stored as a digital image (11).

Statistical analysis. Statistical analyses of various parameters were performed using Statistica 8.0 (Statsoft, USA) software. The correlation between the observed clinical signs of *M. bovis* infection and the results of serological, cultivation, and PCR testing was determined by Spearman's rank correlation coefficient (nonparametric). The differences with $\alpha < 0.05$ were considered as significant.

Results

Eighty-nine cattle from four farms situated in the Lubelskie Province (eastern part of Poland), showing clinical signs suggestive of mycoplasma infection, were included in the study. The clinical signs were characteristic of pneumonia, mastitis, and arthritis. Nasal discharge, fever, cough, dyspnea, apathy, lack of appetite, high somatic cell counts, swollen udder and joints, and decreased level of milk production were observed. As many as 87% of the examined calves had clinical signs indicating the possibility of *M. bovis* infection, whilst the percentage was lower (21.2) in the adult cattle.

The results of serological examinations using an indirect ELISA, irrespective of cattle age, indicated widespread exposure to *M. bovis* infection with 33.7% being seropositive by either milk or sera. Calves had more seropositive results. Eleven (47.8%) calves were positive, with seven (30.4%) animals determined as highly positive (≥ 2+) (Fig. 1). In the dairy cows only seven (10.6%) serum samples and 16 (29.6%) milk samples were seropositive for *M. bovis* antibodies (Fig. 2).

In the calves, *M. bovis* antigen was directly detected in 11 nasopharyngeal swabs from 23 (47.8%) animals by the antigenic direct ELISA, whereas the direct isolation and identification of *M. bovis* by culturing in Eaton's medium and classical PCR testing gave 13 (56.5%) positive results (Table 1). *M. bovis* antigen or DNA was not detected in the nasopharyngeal swabs and milk samples from adult cattle, even though by serological testing seven cows were positive. Sixteen milk samples were positive by serological ELISA. The 11 *M. bovis* specific PCR products from calves were confirmed by the sequencing of the *uvr*C gene fragment. Application of the PCR/DGGE method facilitated the detection and differentiation of *M. bovis* and co-infecting *Mycoplasma* spp. in the swabs collected from the nasopharyngeal cavities of calves. *M. bovis* was detected in eleven calves, four of them were co-infected with *M. bovis*, *M. bovirhinis*, and *M. arginini*, three animals with *M. bovis* and *M. bovirhinis*, and three with *M. bovis* and *M. arginini*. Four animals were infected with *M. bovirhinis* and one with *M. bovirhinis* and *M. arginini*. Only one animal was infected solely with *M. bovis* and seven calves were free from *Mycoplasma* spp. infection (Table 2).

In 55 of the 89 animals examined, there were no clinical signs or evidence of mycoplasma infection, as confirmed by a range of methods used in this study. The rest of the animals (38%) showed clinical signs including pneumonia, mastitis, or arthritis, which may be characteristic of *M. bovis* infection. Among them the most severely affected calves displayed pneumonia, often with nasal discharge, cough, dyspnea, apathy, and lack of appetite. Some calves also had arthritis. The most severely affected calves were found to have mixed infections of *M. bovis*, *M. bovirhinis*, and *M. arginini*. In the dairy cattle affected with mastitis and arthritis, swollen udders and joints, fever (average temperature about 39.9ºC), dramatically decreased yield and quality of milk, and in some severe cases atrophy of affected quarters were observed. Sporadically, a decrease in milk production in some affected cows was also connected with warm, painful, and swollen carpal and tarsal joints (Tables 1 and 3). The clinical forms of mycoplasmal mastitis were often non-responsive to routinely used antibiotic therapy. Positive correlation was found between the observed clinical signs of *M. bovis* infection and the serological results in the group of dairy cows (R-Spearman = 0.65, $P < 0.05$). In the group of calves, positive correlation between the clinical signs of the infection and PCR results (R-Spearman = 0.44, $P < 0.05$), as well as between serological and cultivation results (R-Spearman = 0.37, $P > 0.05$) was found.

Table 1. The comparison of the results of serological, cultivation, and molecular examinations of swabs and serum samples from calves with clinical signs indicating the possibility of *M. bovis* infection

No. of calf	PCR (swab)	PCR/DGGE (swab)	*M. bovis* antigen (swab)	Cultivation (swab)	*M. bovis* antibodies (+: positivity level) (serum)	Major clinical signs
1	+	+	-	+	3+	respiratory signs, fever
2	+	+	+	+	1+	respiratory signs, polyarthritis
3	-	-	-	-	3+	respiratory signs, fever
4	+	+	-	+	2+	respiratory signs, fever
5	-	-	-	-	3+	respiratory signs
6	+	+	-	+	4+	respiratory signs, fever, polyarthritis
7	+	+	+	+	4+	respiratory signs, fever, polyarthritis
8	-	-	+	-	-	respiratory signs
9	+	+	+	+	-	respiratory signs
10	+	+	-	+	-	respiratory signs
11	-	-	-	-	-	respiratory signs
12	+	+	+	+	2+	respiratory signs, fever
13	-	-	+	-	-	respiratory signs
14	+	+	+	+	1+	respiratory signs, polyarthritis
15	+	+	+	+	-	respiratory signs
16	+	+	+	+	-	respiratory signs, arthritis
17	+	+	+	+	-	respiratory signs, arthritis
18	+	+	+	+	-	respiratory signs
19, 23	-	-	-	-	1+	respiratory signs, fever
20, 21, 22	-	-	-	-	-	-

Table 2. The detection of *M. bovis* DNA and DNA of other co-infecting mycoplasmas in nasopharyngeal swabs of calves by PCR/DGGE in comparison with the results of serological examination for *M. bovis* antibodies in calf serum

No. of calf	*M. bovis*	*M. bovirhinis*	*M. arginini*	*M. bovis* antibodies (+: positivity level)
1	+	-	-	3+
2	+	-	+	1+
3	-	+	-	3+
4	+	+	-	2+
5	-	-	-	3+
6	+	-	+	4+
7	+	+	+	4+
8	-	-	-	-
9	-	+	-	-
10	+	-	+	-
11	-	+	-	-
12	+	+	-	2+
13	-	+	-	-
14	+	-	+	1+
15	+	+	+	-
16	+	+	+	-
17	+	+	+	-
18	-	+	+	-
19	-	-	-	1+
20	-	-	-	-
21	-	-	-	-
22	-	-	-	-
23	-	-	-	1+

Table 3. The comparison of the results of serological, cultivation, and molecular examinations of swab, milk, and serum samples from dairy cows with clinical signs indicating the possibility of *M. bovis* infection (na – not available, SCC – somatic cell counts)

No. of dairy cow	PCR (swab/milk)	PCR/DGGE (swab/milk)	Cultivation (swab/milk)	*M. bovis* antibodies (+: positivity level) (serum/milk)	Major clinical signs
1-8, 11-20, 23,25, 31, 35-37, 50, 51, 55, 59-61,63-66	-/-	-/-	-/-	-/-	-
9,10	-/na	-/na	-/na	-/na	-
21	-/-	-/-	-/-	1+/1+	mastitis, arthritis
24	-/-	-/-	-/-	1+/1+	mastitis, decreased milk yield, high SCC
22, 27, 33	-/-	-/-	-/-	-/1+	mastitis, decreased milk yield, high SCC
26, 28, 30, 39, 56	-/-	-/-	-/-	-/1+	-
29, 49	-/-	-/-	-/-	-/-	mastitis
34	-/-	-/-	-/-	1+/3+	mastitis, decreased milk yield , arthritis
38	-/-	-/-	-/-	1+/1+	-
40-48	-/na	-/na	-/na	-/na	-
53	-/-	-/-	-/-	-/3+	mastitis, decreased milk yield , high SCC
54, 57	-/-	-/-	-/-	1+/-	arthritis
58	-/-	-/-	-/-	-/2+	mastitis, decreased milk yield , high SCC
62	-/na	-/na	-/na	2+/na	-
52	-/-	-/-	-/-	-/1+	decreased milk yield , high SCC
32	-/-	-/-	-/-	-/1+	infertility, high SCC

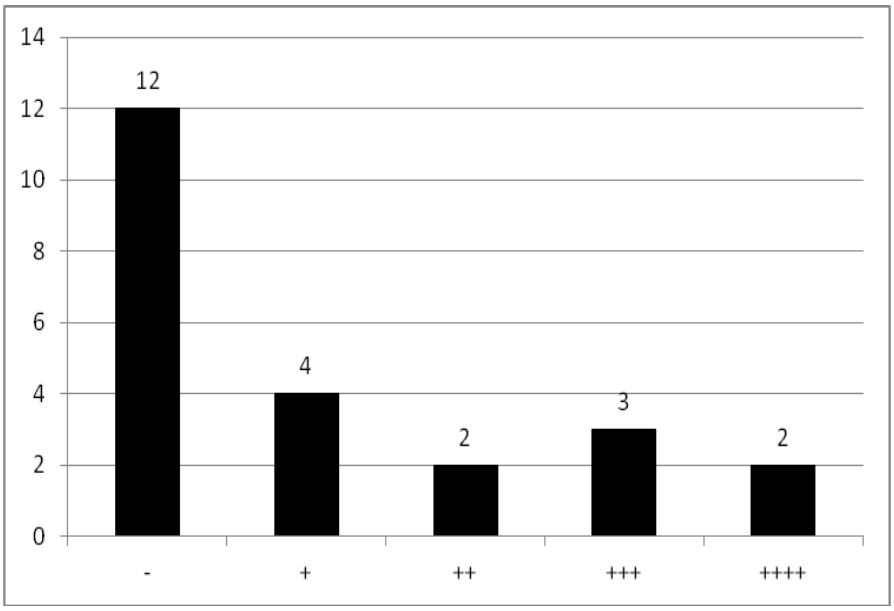


Fig. 1. *M. bovis* antibodies in calf sera; of 23 serum samples examined, two had 4 + positivity, three had 3 +, two had 2+, four had 1+, and 12 were negative

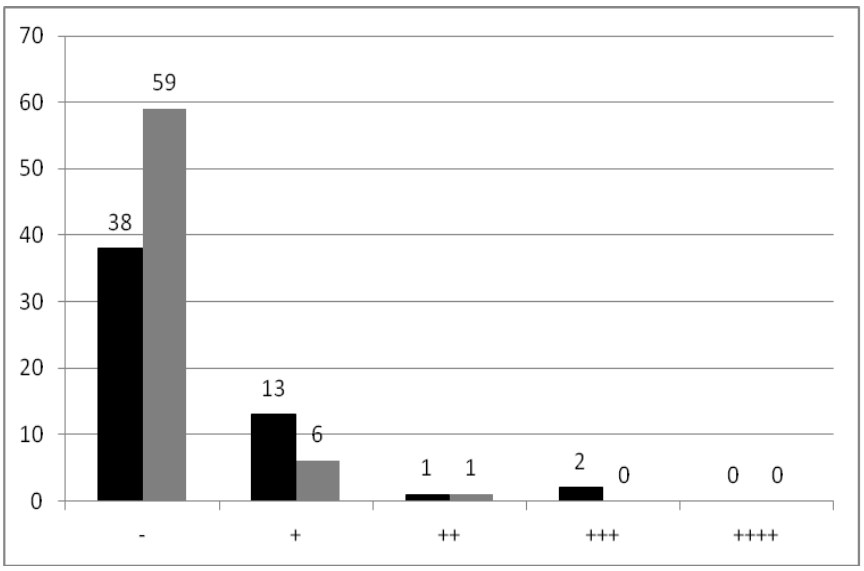


Fig. 2. *M. bovis* antibodies in dairy cow serum (grey colour) and milk samples (black colour). Of 66 serum samples examined one had 2+ positivity, six had 1+, and the rest were negative. Of 54 milk samples examined two had 3+ positivity, one had 2+, 13 had 1+, and 38 were negative

Discussion

Many of the cattle studied had clinical signs which were specific for mycoplasma infections, and in the current study we either detected antibodies to *M. bovis* or DNA to a number of *Mycoplasma* species. These results unequivocally confirmed *M. bovis* infection in these cattle herds situated in the Lubelskie Province.

Generally, serological investigation demonstrates previous exposure to infection, in this case mycoplasma infection, but it does not confirm current infection. *Mycoplasma* culture is a difficult and time-consuming process, as the mycoplasmas can be very fastidious, whereas the use of molecular testing enables the DNA detection of live and inactivated microorganisms. Clinical observation is not sufficient to diagnose *Mycoplasma* infections so infection or disease needs to be confirmed using laboratory methods. Here we have demonstrated that a combination of serological, molecular, and microbiological techniques are the best to diagnose *Mycoplasma* infections. However, in several cases the serological result does not match with the detection of the organism. For example, calf no. 3 had a 3+ *M. bovis* serological result, but only *M. bovirhinis* was detected; in contrast, calf no. 10 had *M. bovis* and *M. arginini* detected but was serologically negative. This may be due to the sensitivity and specificity of the serological test, the stage of infection and the antibody response, or the nasopharyngeal swab sampling, where organisms are only intermittently shed. It may be that bronchoalveolar lavage sampling would improve the detection rate of *Mycoplasma* species when compared with testing nasopharyngeal swabs (19).

The results obtained with the use of PCR/DGGE method, which is not widely available, were compared to those previously presented by Josefová *et al.* (10) in the Czech Republic, by Ayling *et al.* (3) in England and Wales, and also by Chazel *et al.* (6) in France. In the present study, 47.8% of calves were infected with *M. bovis*, in comparison with 8% in the Czech Republic, 40.2% in England and Wales, and 55.0% in France. In our study there were co-infections of *M. bovis* cases with other *Mycoplasma* spp., specifically with *M. bovirhinis* and/or *M. arginini*. In France the most frequent co-infections of *M. bovis* were mainly reported with *M. bovirhinis* and *M. arginini*. In England and Wales, *M. bovis* was the most identified pathogen in co-infection with *M. alkalescens* (42.3%), *M. bovirhinis* (29.6%), and *M. arginini* (11.8%). In the Czech Republic there was only 8% *M. bovis*, 45% *M. bovirhinis*, and 2% *M. arginini* in clinical cases. Chazel *et al.* (7) presented data showing that *M. bovirhinis* is a strictly opportunistic pathogen and *M. arginini* - a putative one. Josefová *et al.* (10) claim that according to their research the most mycoplasmas were effectively isolated from nasal swabs and not from milk samples. Therefore it can suggest that results of our study are more compatible with those obtained in other European countries using the same methods for *M. bovis* detection. It has been reported that some mycoplasmas can occur in different countries with different prevalence according to local epidemiological situation (3, 6, 10).

In conclusion, *M. bovis* seroprevalence in the tested cattle population was 33.7%. We gleaned additional information that mycoplasma infection is often caused by multiple species of *Mycoplasma* and not just as the primary *M. bovis* pathogen. There is

a need to assess mycoplasma infections and the current epidemiological situation of *M. bovis* infection and its relationship to other mycoplasmas causing clinical disease in cattle.

Conflict of Interests Statement: The authors declare that they have no conflict of interests regarding the publication of this article.

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