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Comparison of serological, molecular and cultural diagnostic methods for the detection of *Mycoplasma bovis* infections in cattle*

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Previous studies on *Mycoplasma bovis* in Poland have used serological methods and culture to isolate the causative organism. PCR and PCR/DGGE molecular tests, antigen ELISA, culture, and antibody ELISA methods were evaluated on 713 animals from 73 Holstein-Friesian herds in Poland with suspected *M. bovis* infections. The prevalence of *M. bovis* antibodies in cattle sera was 10.8%. Antigen detection from nasopharyngeal swabs was lowest with the specific PCR having the lowest number of positive samples 39 (5.5%), followed by culture 49 (6.9%), antigen ELISA 52 (7.3%) and PCR/DGGE 66 (9.3%). Statistical analysis showed that all of the methods had high correlation but the antibody ELISA had the lowest level of comparability with each of the other methods. The correlation between clinical signs indicating possible *M. bovis* infection and test positivity confirmed that clinical signs are not pathognomonic. Statistical analysis revealed that the stronger the positive result in the antibody ELISA the more likely it was to detect *M. bovis* by PCR and PCR/DGGE.

KEY WORDS: cattle / ELISA / *Mycoplasma bovis* / PCR/DGGE / prevalence

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Calf pneumonia is a significant economic and animal welfare problem in cattle and one of the major causes of this bovine respiratory disease (BRD) is *Mycoplasma bovis* [Nicholas and Ayling 2003, Maunsell *et al.* 2011]. In Europe, *M. bovis* is believed to be responsible for 25% to 33% of calf pneumonia cases [Nicholas *et al.* 2002]. In Poland *M. bovis* seroprevalence was reported as 76.7% of all Polish cattle population [Bednarek *et al.* 2012] and 64.3% of animals with bovine respiratory disease (BRD) [Dudek and Bednarek 2012].

The infection is usually introduced to herds by clinically healthy calves or young cattle shedding the mycoplasma and once established it is difficult to eradicate [Kirby and Nicholas 1996, Nicholas and Ayling 2003, Ball and Nicholas 2010, Nicholas 2010]. Infection with *M. bovis* can cause other clinical conditions, including: arthritis; mastitis; keratoconjunctivitis; infertility and abortion. It adversely affects growth rates resulting in increased costs of production and additional treatment costs, resulting in large economic losses to the cattle industry. Early detection of disease, improved husbandry conditions, and treatment with an effective antimicrobial are currently the only approaches to try and control the disease.

Various laboratory tests are currently used for the screening, detection and confirmation of the pathogen in cattle. Detection of the *M. bovis* organism is generally carried out either by a capture enzyme-linked immunosorbent assay (ELISA), or culture isolation using special media, or molecular tests. Serological methods are useful screening tests but of limited use at the early stage of infection as sero-conversion is usually at least two weeks post infection. The isolation and culture of *Mycoplasma* species requires specialist skills and is not always successful [Sachse *et al.* 1993] due to multiple mycoplasma infections [Ayling *et al.* 2015], or presence of other bacteria. It has been previously reported that the specificity of serological, culture and some molecular tests have limitations that may result in misidentification or inconclusive results [Sachse *et al.* 1993].

These studies aim to compare and evaluate the significance of differences and the correlation indices between results obtained using different mycoplasma diagnostic methods (ELISA tests, culture, PCR and PCR/DGGE).

Material and methods

Materials. From different regions of Poland, 713 serum and 713 swab samples were taken from 73 cattle herds suspected of being infected with *M. bovis*. The blood samples were collected from the *vena jugularis externa* and then centrifuged at 1500 x *g* for 10 min to obtain sera and stored at 5±3°C for a maximum of 48 h before testing. Nasopharyngeal swabs were placed in transport medium (Universal Transport Medium, Copan, USA) and kept at 5±3°C until they were tested. The swabs from the same animals were taken in duplicates, the first for DNA extraction and the second for culture, which were initiated within 24 h.

Ethics statement. The Local Ethical Committee on Animal Testing at University of Life Sciences in Lublin (Poland) were informed of the study; but formal ethical approval was not required [Anon 2006]. The samples were collected from animals by authorized veterinarians during clinical examination following standard procedures. The samples were collected specifically for this study with the agreement of the farmers.

Clinical signs. The veterinarians examining the cattle recorded clinical information that included: respiratory signs (coughs, dyspnea, and nasal discharge); osteo-articular signs (swollen joints, and abscesses); mastitis including swollen udder, and decreased milk production; and any other clinical symptoms such as increased rectal temperature; apathy; abortion; and keratoconjunctivitis.

ELISA tests. For detection of *M. bovis* antibodies in serum the indirect sandwich ELISA (Bio-X Diagnostics, Jemelle, Belgium) was used. Positive or negative results with an evaluation of the degree of positivity were calculated according to the manufacturer's instruction. The detection of *M. bovis* antigen from nasopharyngeal swabs was by culture and by using the antigenic direct ELISA (Bio-X Diagnostics, Jemelle, Belgium) following the manufacturer's instructions.

Isolation. *Mycoplasma* species were isolated by culturing in Eaton's broth and on Eaton's agar plates, and incubated in a humidified atmosphere at 37±2°C with 5% CO_2 [Nicholas and Baker 1998]. The broths and agar plates were observed for typical mycoplasma growth for up to 21 d. Observations included swirls of growth, film formation on the surface of the broth, and for any colour change which may be from red to orange for the non-glucose fermenting *Mycoplasma* species such as *M. bovis*, or to yellow for glucose fermenters such as *M. bovirhinis*.

DNA isolation. DNA extraction from nasopharyngeal swabs in transport medium was performed using the QIAmp DNA Mini Kit (QIAGEN, Hilden, Germany) following the manufacturer's procedure. The extracted DNA was then stored at ≤-70°C until used for further analysis. If mycoplasma-like colonies were observed during the culture procedure, DNA extraction was carried out on cells harvested from 1 ml broth culture. The cells were centrifuged for 5 min at 13000 x *g*, then washed three times in 1 ml PBS (pH=7.4) and centrifuged using the same conditions. The pellet was resuspended in 180 µl lysosyme (20 mg/ml) and incubated in 37±1°C for 30 min [Akwuobu *et al.* 2014] before extracting the DNA as before using the QIAmp DNA Mini Kit.

Polymerase chain reaction (PCR) for uvrC gene. The *M. bovis* specific PCR was carried out in a T-personal thermocycler (Biometra, Göttingen, Germany) according to Subramaniam *et al*. [1998] with modifications to the number of cycles, from 35 to 40, temperature of primer annealing, from 52°C to 60°C, and elongation time, from 60 s to 90 s. *M. bovis* PG45 (ATCC 25523) DNA was used as the positive control and water as a negative control. The Perfect Plus 2 kb DNA Ladder (EurX, Gdańsk, Poland) molecular marker was electrophoresed with the PCR amplicons on 2% agarose gels and then stained with ethidium bromide (0.5 µg/ml). The expected product size was 1626 bp.

PCR/Denaturing gradient gel electrophoresis (PCR/DGGE). PCR was performed using the primers, specific for V3 16S rDNA [McAuliffe *et al.* 2005] in a T-personal thermocycler (Biometra, Göttingen, Germany). The PCR amplicons were checked for correct amplification in 2% agarose gels stained with ethidium bromide (0.5 μg/ml). The DGGE method [McAuliffe *et al.* 2005] with modifications [Dudek *et al*. 2016] was then used for analysis. PCR amplicons were loaded onto a 10% polyacrylamide/bis (37.5 : 1) gels with denaturing gradients of urea and deionized formamide from 25-60% and electrophoresed at 100 V at 60˚C for 19 h using DCode Universal Mutation Detection System (Bio-Rad, USA). Following electrophoresis gels were stained with SYBR Gold (Life Technologies, Vilnius, Lithuania) and visualized under UV and images recorded digitally. The following controls were used: DNA from the reference strain of *M. bovis* (ATCC 25523), from type strains of *M. bovigenitalium* (ATCC 19852), *M. bovoculi* (ATCC 29104) and *Acholeplasma laidlawii* (ATCC 23206) and NCTC type strains of: *M. canadense*, *M. canis*, *M. arginini*, *M. bovirhinis*, *M. dispar*, *M. alkalescens*, *M. mycoides* subsp. *mycoides* SC and *Ureaplasma diversum*, which were obtained from the Animal Plant and Health Agency, Weybridge, United Kingdom.

Statistical analysis. Pearson's chi-square test was used to compare the results obtained using the five methods. To assess the association between the presence of co-infection *M. bovis* with other species (*M. bovoculi*, *M. canadense*, *M. canis*, *M. arginini*, *M. bovirhinis*, *M. dispar*, *M. alkalescens* and *Ureaplasma diversum*); and occurrence of clinical signs, the test of significance chi-square with Yates adjustment was applied. The level of correlation between the occurrence of clinical signs; and the results obtained using particular test methods, χ^2 (chi-square) was used. To evaluate the influence of a positive result in ELISA for anti-*M. bovis* antibodies detection, the occurrence of clinical signs and the results obtained with the use of particular methods Mann-Whitney test was used. All analyses were conducted using the program STATISTICA version 10 (Software StatSoft, Inc.). For the purpose of this study, the following guidelines for interpreting the degree of correlations were used: r = 0-0.09: no or negligible relationship; r = 0.1-0.29: weak relationship; r = 0.3-0.49: moderate relationship; r = 0.5-0.69: strong relationship; r = 0.7-0.99: very strong relationship; r = 1: full relationship [Stanisz 2006].

Results and discussion

The samples all came from herds that had clinical signs of respiratory disease that may have been caused by *M. bovis*. Details of the clinical signs are given in Table 1, which shows 329 of the 713 (46.1%) individual cattle had some clinical signs of infection and in Table 3, which shows number of herds with different clinical signs. Serological testing of the 713 cattle showed that just 77 (10.8%) were seropositive for *M. bovis*. However, detection of the antigen from nasopharyngeal swabs was lower with the culture method just detecting 49 (6.9%) positive samples; and molecular tests PCR/DGGE 66 (9.3%) positives; and specific PCR 39 (5.5%) positive samples.

These results are given as comparative analysis in Table 1. Table 2 shows the values of the correlation coefficient between the results obtained. Statistical analysis shows that all of the methods have a high degree of correlation but the antibody ELISA had the lowest level of comparability with the other methods. Regarding the clinical signs indicating mycoplasma infection, the highest value of correlation coefficient was noted between prevalence of clinical signs and PCR/DGGE (0.34) and ELISA (0.32) results. Slightly lower values of correlation coefficient between clinical signs and isolation (0.27) or PCR (0.26) was noted. The lowest correlation (0.25) occurred when the clinical signs were compared with ELISA for antibody detection. Moreover, the PCR/DGGE confirmed the presence of other pathogens belonging to *Mollicutes* class including: *M. bovirhinis*, *M. dispar*, *M. canis*, *M. arginini, M. canadense*, *M. bovoculi*, *M. alkalescens* and *Ureaplasma diversum* (data not published). The statistical analysis revealed that there is no dependence between *M. bovis* in co-infection with different mycoplasma species and/or *U. diversum* and the presence of clinical signs of possible mycoplasmal infection.

Different approaches to the diagnosis of *M. bovis* infection are used. For disease surveillance or as a herd screening test serological approaches are often used as that is a cheaper and more rapid approach to diagnosis. However, that does have limitations, in detecting previous exposure to infection and does not detect the very early stages of infection [Sachse *et al.* 1993, Sachse and Frey 2003, Dudek *et al.* 2013]. The data obtained in the serological test had the highest number of positive results, but many of those results were not confirmed using antigen detection methods. This could be due to lack of specificity by the serological test, or that the antigen detection tests lacked sensitivity or were limited by the sampling method, or that *M. bovis* is known to shed intermittently. Other factors that may affect the sensitivity of the antibody ELISA tests includes the ability of *M. bovis* to vary its surface antigens altering the immunological properties [Razin *et al.* 1998]. In addition recent *in vitro* data has demonstrated the ability of *M. bovis* to invade and persist in host cells [Bürki *et al.* 2015], which is not surprising, considering the organism must be able to circulate in the host's body to infect so many sites within its host. This intracellular life could help *M. bovis* escape the host's immune defense and the development of a specific antibody response.

Definitive confirmation of infection is by isolation of the causative organism, but culture methods may take weeks, so either the capture ELISA method or molecular methods are used more routinely. The specific PCR had the lowest number of positive samples – 39, followed by culture – 49, antigen ELISA – 52 and PCR/DGGE – 66, suggesting that the PCR/DGGE may be the more sensitive antigen detection test. Statistical analysis showed a positive correlation with statistically significant differences between compared methods. The highest correlation coefficient (0.75) was observed in the case of culture method and PCR/DGGE, slightly lower value was noted between culture and PCR (0.74). High levels of correlation were obtained between the PCR and PCR/DGGE and the ELISA (antigen detection); and between the culture methods and the PCR.

There are a few reasons why differences are seen between the serological, molecular and culturing tests. It should be noted that the serological ELISA test has recently changed, with different interpretive points. This may account for the lower level of sero-prevalence detected in this study than reported previously [Bednarek *et*

Table 1. Comparison of the serological ELISA results; and PCR, PCR/DGGE; and isolation for detection of *Mycoplasma bovis* from nasopharyngeal swabs from cattle

Method	Result	ELISA (antibody)		ELISA (antigen)		PCR		PCR/DGGE		Culture		Clinical signs	
		+	-	+	-	+	-	+	-	+	-	+	-
ELISA (antibody)	+77	-	-	22	55	20	57	23	54	21	56	70	7
	-636	-	-	30	606	19	617	43	593	28	608	259	377
ELISA (antigen)	+52	22	30	-	-	31	21	35	17	36	16	47	5
	-661	55	606	-	-	8	653	31	630	13	648	282	379
PCR	+39	20	19	31	8	-	-	36	3	33	6	39	0
	-674	57	617	21	653	-	-	30	644	16	658	290	384
PCR/DGGE	+66	23	43	35	31	36	30	-	-	44	22	65	1
	-647	54	593	17	630	3	644	-	-	5	642	264	383
Culture	+49	21	28	36	13	33	16	44	5	-	-	46	3
	-666	56	608	16	648	6	658	22	642	-	-	283	381
Clinical signs	+329	70	259	47	282	39	290	65	264	46	283	-	-
	-384	7	377	5	379	0	384	1	383	3	381	-	-

Table 2. Results of statistical analysis of correlations between ELISA methods, PCR, PCR/DGGE and isolation used for detection of *Mycoplasma bovis*

Method	ELISA (antibody)	ELISA (antigen)	PCR	PCR/DGGE
ELISA (antibody)	-	-	-	-
ELISA (antigen)	0.28*	-	-	-
PCR	0.31*	0.67*	-	-
PCR/DGGE	0.25*	0.56*	0.69*	-
Culture	0.28*	0.69*	0.74*	0.75*

The degree of correlations: r = 0-0.09: no or negligible relationship; r = 0.1-0.29: weak relationship; r = 0.3-0.49: moderate relationship; r = 0.5-0.69: strong relationship; r = 0.7-0.99: very strong relationship; r = 1: full relationship [Stanisz 2006].
*p<0.05.

Table 3. Clinical signs indicating possible *Mycoplasma bovis* infection observed in the study herds

Observed clinical signs of infection	No. of herds (positive/all)
Apathy	73/73 (100%)
Nasal discharge	43/73 (58.9%)
Cough, dyspnea	33/73 (45.2%)
Swollen joints, abscesses	8/73 (11%)
Increased rectal temperature	6/73 (8.2%)
Swollen udder, decreased milk production	5/73 (6.9%)
Abortions	1/73 (1.4%)
Keratoconjunctivitis	1/73 (1.4%)

al. 2012, Dudek and Bednarek 2012]. A change in interpretive values based on milk sample examination is still being considered. Nielsen *et al.* [2015] estimated that the ELISA would be more specific and have improved sensitivity compared to the PCR, if the cut-off was increased to at least 50 ODC%, rather than using the manufacturer's recommended (37 ODC%) value. Considering the limitations of individual tests, the use of at least two different laboratory tests e.g. combination of serological test and molecular methods may improve the diagnosis of *M. bovis*. Although in this study the serological ELISA had more positives results in ELISA and negative in molecular tests, but this could be false positives in the ELISA test, or showing previous exposure to infection rather than current infection.

It should be noted that conventional culture method for identification of *M. bovis* is time-consuming and often gives false-negative results caused by overgrowth with another, contaminating bacteria or fast-growing commensal mycoplasmas [Nicholas *et al.* 2008] but in our studies a high correlation between culture and molecular tests is shown. PCR is widely used now to confirm or exclude *M. bovis* infection [Ghadersohi *et al.* 1997, Subramaniam *et al.* 1998]. It is well known that multiple mycoplasma species are present in infected cattle [Ayling *et al.* 2015], therefore the PCR/DGGE has the advantage of being the most sensitive detection method used in this study and it detects and differentiates the different mycoplasma species in one test. However, this PCR/DGGE test can be used only by specialist laboratories because of the cost of equipment, it is labour intensive and requires skilled staff to run and interpret the test [McAuliffe *et al.* 2005]. Here the specific PCR had the lowest number of positive samples, which may be a reflection of the higher annealing temperature that was used than in the initial publication by Subramaniam *et al*. [1998], although we had tested the sensitivity of the test at that annealing temperature. The PCR targets the *uvr*C gene, a region of *M. bovis* genome that is considered highly conserved [Thomas *et al.* 2004]. However analysis of nucleotide sequences of the amplicons from this study showed the presence of point mutations in *uvr*C gene [Szacawa *et al.* 2015]. Therefore it may be possible that some strains had snp's in primer annealing regions resulting in false negative PCR results. The most sensitive confirmatory test is PCR/DGGE, which also allows detection of other bacteria belonging to *Mollicutes* class.

There is no data showing comparative statistical analysis of different diagnostic techniques for *M. bovis* detection. Only Sachse [Sachse *et al.* 1993] describes the advantages and limitations of using detection methods for routine *M. bovis* diagnosis. Its analysis showed that PCR is potentially superior to all other methods, due to its high sensitivity, specificity and speed. In this study, we compared the reliability of the commercially available ELISA kits (both for the antibody and antigen detection); and laboratory molecular PCR and PCR/DGGE tests and culture. Statistical analysis showed a positive correlation with statistically significant differences between compared methods. It shows also that the stronger the positive result is with the ELISA the more likely it is to obtain a positive result with the PCR and PCR/DGGE tests. It revealed also that there is no dependence between *M. bovis* in co-infection with different mycoplasma species and/or *U. diversum* and the presence of clinical signs of possible mycoplasmal infection. The combination of both serological and molecular results with clinical observation is currently the approach to obtain a reliable diagnosis for *M. bovis* infections in cattle.

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