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SCREENING OF HUNGARIAN CATTLE HERDS FOR SEROPOSITIVITY TO *MYCOPLASMA BOVIS*

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A total of 860 serum samples collected at 86 cattle farms in different parts of Hungary were screened for the presence of antibodies to *Mycoplasma bovis* using an ELISA test with a recombinant *M. bovis* membrane protein as antigen. Antibodies to *M. bovis* were detected in sera collected on all farms, and no farms negative for *M. bovis* were found. In 88.38% of the herds more than 50% of the sampled animals were infected by *M. bovis*. A total of 82.91% of the animals had antibodies to *M. bovis*. The proportion of seropositive animals was higher in the older age groups, and a significant difference was seen in the level of seropositivity between young and older age groups. The results show that *M. bovis* infection is widespread on Hungarian dairy farms, and its prevalence has increased in the recent decade. The high infection rate of Hungarian cattle herds with *M. bovis* shows that special attention should be paid to evaluating the aetiological role of *M. bovis* in bovine respiratory disease complex (BRDC) cases because *M. bovis* has an immunosuppressive effect and can predispose cattle to other respiratory infections, too.

Key words: *Mycoplasma bovis*, antibody, ELISA, seropositivity

The bovine respiratory disease complex (BRDC) is one of the most important diseases of cattle causing considerable losses all over the world, and *Mycoplasma bovis* is an important agent in its aetiology (Cusack et al., 2003; Nicholas and Ayling, 2003). It is responsible for a wide range of clinical diseases including bronchopneumonia, arthritis, genital diseases, mastitis (Gagea et al., 2006*b*; Arcangioli et al., 2008; Bürki et al., 2015), otitis (Arcangioli et al., 2012) and keratoconjunctivitis (Kirby and Nicholas, 1996). By replicating on the mucous membranes of the respiratory tract and interacting with other pathogenic bacteria, it compromises the defence mechanism of the host and has a synergistic effect with the pathogenic activity of other bacteria (Gagea et al., 2006*a*). It is an

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important agent of pneumonia at the beginning of the feeding period and in young dairy calves (Gagea et al., 2006*a*; Giovannini et al., 2013). According to Nicholas and Ayling (2003), *M. bovis* is responsible for one quarter to one third of the losses caused by bovine respiratory diseases.

Several methods are used for the detection of *M. bovis* or the antibodies produced to it. Besides isolation of the agent, capture ELISA and different PCR methods are available (Tenk et al., 2002; Nicholas and Ayling, 2003; Tenk et al., 2006). Antibodies to *M. bovis* can be detected with ELISA tests using whole cell or chemically treated antigens. To prevent cross-reactions with other mycoplasmas, different membrane proteins are frequently used as antigens in ELISA tests (Adamu et al., 2013; Fu et al., 2014). Some new ELISA tests can even differentiate between vaccinated and naturally infected cattle (Han et al., 2015). The serological detection of *M. bovis* infection is regarded more reliable in the diagnostic work, since antibody levels remain high for many months and the presence of antibodies indicates an invasive infection in contrast to nasal colonisation (Nicholas and Ayling, 2003).

Mycoplasma bovis is widespread in the cattle population: it can be found on the mucous membranes of the respiratory and genital tracts as well as in the udder. This agent could be detected in 11.6% of lung tissue samples from dairy calves younger than one month (Giovannini et al., 2013), and antibodies to *M. bovis* were found in all veal calves and 76% of adult cattle at slaughter in Italy (Radaelli et al., 2008). *Mycoplasma bovis* could be found in 34.4% of nasal samples from three-month-old calves (Siugzdaite et al., 2012), while it was present in 53.3% of the respiratory samples of calves in 4 veal herds in Pennsylvania (Soehnlen et al., 2012). A high level of seropositivity (60–100%) to *M. bovis* was seen in the majority of veal calves in France, too (Arcangioli et al., 2008). On the other hand, *M. bovis* was detected in only 2% of the calves in backgrounding and stocker cattle operations in Georgia in the US (Wiggins et al., 2007), and no *M. bovis* positive sera could be found in 135 randomly selected Norwegian dairy herds (Gulliksen et al., 2009). *Mycoplasma bovis* had first been detected in Hungarian cattle herds several decades ago (Romváry et al., 1977), and its pathologic importance was confirmed later (Stipkovits et al., 2000; Bashiruddin et al., 2001; Tenk et al., 2004). The latter authors could detect antibodies to *M. bovis* in 11.3% of the animals tested; however the agent could be cultured from 37.0% of the animals.

It is presumed that *M. bovis* as an immunosuppressive agent has an important role in the pathogenesis of BRDC in Hungary but we do not know how widespread the infection is at present. A few years ago 34 large cattle herds were screened (Tenk et al., 2004), but the increased trade of recent years could have changed the figures. This perception is supported by the results of a recent phylogenetic examination of Hungarian *M. bovis* strains, which has confirmed the heterogeneity of the strains by detecting two clades and five subclades in one of

the clades (Sulyok et al., 2014). The aim of the present work was to obtain information on the current seroprevalence of *M. bovis* in cattle herds in Hungary in order to assess its possible role in BRDC.

Materials and methods

Blood samples were collected in 86 large cattle herds from different parts of Hungary between February and October 2008. The samples were collected during routine diagnostic examinations, thus an ethical approval was not required for the study. Altogether 82 dairy herds were included in the screening, together with one beef herd and three herds of mixed use. The size of the herds ranged between 216 and 2600 head of cattle. Ten blood samples were randomly collected in each herd from animals preferably older than three months; however, there were 26 samples which had been collected from calves younger than three months. The age of the animals ranged between 37 days and 7 years. The samples were transported to the laboratory in a cooler box within 4 h after collection, and the sera were separated and stored at –18 °C until tested.

The Bio K 162 Bio-X *Mycoplasma bovis* ELISA Kit (Bio-X Diagnostics, Jemelle, Belgium) sensitised with a recombinant protein from *M. bovis* was used. The tests were carried out according to the manufacturer's instructions. Briefly, 100 µl each of the 1:100 diluted sera was put into two wells of the microtitration plate: one well was sensitised with *M. bovis* protein, while the other served as negative control. The plates were incubated at 37 °C for 1 h. After washing, the conjugate (a peroxidase-labelled anti-bovine IgG1 monoclonal antibody) was added to the wells and incubated at 37 °C for 1 h. The plates were washed, hydrogen peroxide substrate and tetramethylbenzidine chromogen were added and incubated at room temperature for 10 min, then the reaction was stopped. The intensity of the blue colour was read at 450 nm using an ELISA reader (Multiskan EX, Labsystems, MTX Lab Systems Inc.). The test was validated using the positive serum provided by the manufacturer; it was regarded valid if the optical density of the positive serum exceeded the value stated by the producer.

The test was evaluated according to the manufacturer's instructions. To define the exact optical density (OD) value of the sample, the signal of the negative control well was subtracted from the measured value of the sample. This OD was compared with that of the positive serum and expressed as the percentage of the latter (sample coefficient, ODC%). The ODC% values were scored; below 10.5% the test was considered negative, and the positive results were scored between + and +++++. The average ODC% of the sera of four age groups (< 3, 4–6, 7–9, 10–12, > 12 months) were calculated using the Unscrambler 10.3 programme (CAMO Software AS., Oslo, Norway) and evaluated by Student's *t*-test. The statistical unit was the individual animal.

Results and discussion

Antibodies to *M. bovis* were detected in all herds, no *M. bovis* free herds were found. In most herds (88.38%) more than 50% of the animals were seropositive to *M. bovis* (Table 1).

Table 1

Seropositivity of cattle herds to *Mycoplasma bovis*

Number of positive samples out of 10 collected in a herd	Number of herds	% of herds
0	0	0
1	0	0
2	2	2.32
3	1	1.16
4	1	1.16
5	6	6.98
6	6	6.98
7	9	10.47
8	10	11.63
9	17	19.77
10	34	39.53

Regarding the seropositivity of the individual animals, only 17.09% of the cattle were seronegative but most of the seropositive animals did not have high levels of antibodies (Table 2). The level of seropositivity tended to increase with age (Table 3).

Table 2

Seropositivity of individual cattle to *Mycoplasma bovis*

Antibodies to *M. bovis*		Number of animals	% of animals
Negative		147	17.09
Positive	+	525	61.05
	++	132	15.35
	+++	42	4.88
	++++	14	1.63
	+++++	0	0
	Total	713	82.91
Total		860	100

Nowadays the aetiological role of *M. bovis* in BRDC is widely accepted, and there are more and more data on its occurrence in cattle herds in different countries. There are several diagnostic methods for evaluating the level of infec-

tion in herds. Detection of antibodies to *M. bovis* is frequently used, as this test shows the infection for a longer time even if antibiotic treatment is performed, and it indicates not only carriage of *M. bovis* on mucous membranes but also invasiveness (Nicholas and Ayling, 2003). When using serological tests, it is difficult to rule out false seropositivity caused by related antigens of other mycoplasmas. The ELISA test applied in the present project is widely used; thanks to its recombinant *M. bovis* membrane protein antigen the reaction is considered specific (Gulliksen et al., 2009; Gabinaitiene et al., 2011; Arcangioli et al., 2012; Fu et al., 2014). Examination of the sera using further ELISA tests or paired samples could refine the results but our aim was only to obtain information on the current seroprevalence of *M. bovis* in order to assess its possible role in BRDC.

Table 3

Seropositivity of the different age groups of cattle to *Mycoplasma bovis*

Age	Number of animals	Antibodies to *M. bovis*				Average ODC%
		Positive		Negative		
		Number	%	Number	%	
< 90 days	26	15	57.69	11	42.31	18.28[a]
91–180 days	678	552	81.42	126	18.58	28.26[b]
181–270 days	113	106	93.81	7	6.19	34.38[c]
271–365 days	10	10	100	0	0	37.08[bcd]
> 365 days	22	20	90.91	2	9.09	28.33[abcd]
No data on age	11	10	90.91	1	9.09	n. d.
Total	860	713	82.91	147	17.09	n. d.

Means within the column with different superscripts differ ($P < 0.05$); n. d.: not done; ODC: optical density coefficient

The high rate of *M. bovis* infection in the cattle herds examined was rather surprising. A similar evaluation detected 11.3% seropositivity in Hungary when examining 595 animals in 34 cattle herds using a different type of ELISA; however in some herds the rate of the seropositivity reached 57.2% (Tenk et al., 2004). According to our data the proportion of seropositive herds increased from 64.7% to 100%. The majority of animals were weakly positive, which can be due to asymptomatic colonisation of the respiratory mucous membranes by *M. bovis*. The elevated rate of infection may result from the increased trade of cattle in the recent decade as it is seen in several other countries. According to a survey in Italy, 76% of beef and 100% of veal calves were seropositive to *M. bovis* (Radaelli et al., 2008). A high infection rate was found at a Lithuanian cattle breeding station, where 75% of 110 day-old calves were seropositive (Gabinaitiene et al., 2011). In 8 out of 9 French feedlots 60 to 100% of the animals had seroconverted (Arcangioli et al., 2008). *Mycoplasma bovis* was detected more frequently from

pneumonic calves (Gagea et al., 2006*a*; Soehnlen et al., 2012). The proportion of seropositive animals increased with age and a significant difference was seen in the level of seropositivity between young and older animals.

The high infection rate of Hungarian cattle herds with *M. bovis* shows that special attention should be paid to evaluating the aetiological role of *M. bovis* in BRDC cases. The pathogenic impact of *M. bovis* is highly underestimated. As a primary pathogen it can cause various diseases in cattle and due to its immunosuppressive nature it can predispose cattle to other respiratory diseases. The identification of *M. bovis* infection is a precondition of the effective treatment, prevention and control of BRDC.

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