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Examination of Mycoplasma bovis infection in cattle

Brief Summary of Doctoral Thesis

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INTRODUCTION

Mycoplasma bovis was isolated by Hale from a mastitic cow in 1961. The clinical picture was similar to the one of the contagious agalactia of goats and sheep, caused by *M. agalactiae*. Thus it was believed to be a subspecies of *M. agalactiae* (*M. agalactiae subsp. bovis*) but later it was elevated to species rank. Since that time *M. bovis* has become the bovine pathogenic mycoplasma species causing supposedly the biggest economic losses in Europe and North America. The loss of the weight gain, diminished carcass value and mastitis caused by *M. bovis* is estimated to be some hundred million dollars per year.

This organism is a significant cause of pneumonia and arthritis in young animals, mastitis, abortion and fertility problems in cows and genital disorders and reduction of *in vitro* fertility in bulls.

The mortality rate in calves affected by pneumo-arthritis can be high. The body weight gain is usually reduced and the time needed to reach the maturity for breeding takes longer in animals got through the infection.

Although *M. bovis* infection is known in Hungary from 1975, it is rarely diagnosed in the field. The reason for that is that the respiratory disease in calves is usually assigned to respiratory viruses due to the similar clinical and pathological picture. The bacteria of secondary infections (usually *Pasteurella multocida*, *Mannheimia haemolytica* or *Haemophilus somnus*) similarly change the clinical and pathological picture in both cases.

The disease due to *M. bovis* is difficult to treat since antibiotic resistance is common and the treatment with drugs, which have been proved to be effective *in vitro*, often fails under field conditions.

The individual diagnosis is of high importance, in order to maintain the herds' status or find the animals shedding virulent strains.

Non-pathogenic mycoplasma species, colonizing bovine mucous membranes and the overgrowing bacteria of secondary infections usually cause problems in the diagnosis of *M. bovis* infection.

Further troubles are caused by the rapidly changing antigenic structure of *M. bovis*, which require careful application of immune-diagnostic tests.

Taking into consideration these facts I have dealt with clinical and complex diagnostic aspects of *M. bovis* infection in cattle throughout my research work.

AIMS OF THE STUDY

My aims in this study were the following:

To evaluate the use of two rapid culture methods for *M. bovis*, the selective-differentiating culture with a special medium and the capture ELISA

To produce of monoclonal antibodies against *M. bovis,* determine their properties, such as type, specificity and their use in the diagnostic work

To evaluate the prevalence of *M. bovis* in Hungary by the detection of specific antibodies with ELISA test. Look for the relationship between the ELISA, culture and the results of pathological examination of lungs by a mathematical model

To evaluate the efficacy of the antibiotic valnemulin in a challenge trial

To set up a working PCR system for the detection of *M. bovis*

MATERIALS AND METHODS

1.
RAPID DIAGNOSIS OF *MYCOPLASMA BOVIS* INFECTION IN CATTLE WITH CAPTURE ELISA AND A SELECTIVE DIFFERENTIATING MEDIUM

From four Hungarian dairy herds infected with Mycoplasma bovis nasal and tracheal swabs and milk samples were collected. Samples from lungs showing pathological changes were also collected from slaughterhouses as well. Further nasal swab and lung samples were obtained from an animal challenge. The samples were inoculated into Medium-B broth. The growth of mycoplasmas was checked with inoculation onto solid Medium-B.

If growth of mollicutes was observed on the agar plates a small amount from the broth cultures were applied into the wells of capture-ELISA plates sensitized with specific M. bovis polyclonal rabbit hyper-immune serum. After a 3-day incubation period, biotinilated, anti-*M. bovis* Mab 5A10 was applied onto the plates. The reactions were evaluated by the absorbance values read at 450 nm wavelength. Besides this the samples from the challenge experiment were also inoculated onto Mycoplasma bovis diagnostic medium agar plates (Mycoplasma Experience Ltd, UK). These plates were incubated in anaerobic conditions for 7 days. The colonies and their color reactions were examined under a stereomicroscope.

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2.
PRODUCTION OF MONOCLONAL ANTIBODIES RECOGNIZING MULTIPLE *MYCOPLASMA BOVIS* ANTIGENS AND THEIR TESTING

Balb/c AnN Crl BR mice were immunized with the antigen from the Hungarian *M. bovis* strain designated 26034. After the immunizations blood samples were taken from the animals. The antibody levels were checked with our own-developed ELISA test. The spleen the mouse giving the best immune response was removed aseptically. The spleen cells were taken into fusion with Sp2/0-Ag14 murine myeloma cells. The obtained hybrid cells were selected with HAT medium. Two weeks after the fusion, the supernatants of the grown cells were tested by indirect ELISA. To determine the molecular weights of the antigen determinants, the supernatants giving positive reaction in the ELISA, were tested by Western blot. The 12 cell groups selected on the basis of the Western blots were cloned twice by end-point dilution method. The cloned cells were propagated, then with 5 cell lines, antibodies were produced in a bioreactor. The isotypes of the antibodies were determined with indirect ELISA assay. The cross-reactions of them with mycoplasma, ureaplasma and acholeplasma species were also determined with ELISA. The specificity of the antibodies was tested with immuno-histochemistry test performed on formalin-fixed, paraffin-embedded tissue samples.

3.
EXAMINATION OF THE ROLE OF *MYCOPLASMA BOVIS* IN BOVINE PNEUMONIA AND A MATHEMATICAL MODEL FOR ITS EVALUATION

Blood and lung samples were collected from 595 cattle. The carcasses were subjected to normal meat inspection and any pathological changes of the respiratory tract were recorded. Sera were examined with CHECKIT Mycoplasma Bovis Sero ELISA kit (Bommeli AG, Liebefeld-Bern, Switzerland). Lung samples were cultured in liquid Medium 'B'. Mycoplasmas were identified by their colony morphology, biochemical characteristics and immuno-fluorescence. The data were evaluated with statistical methods. The results of occurrence of pneumonia, *M. bovis* culture and ELISA test were analyzed with a probability model, which was solved with a method of linear optimization.

4.
THE EFFICACY OF VALNEMULIN (ECONOR®) IN THE CONTROL OF DISEASE CAUSED BY EXPERIMENTAL INFECTION WITH *MYCOPLASMA BOVIS*

In our experiments 24 calves aged 10-35 days were divided into the following groups:

1. Group: Non-infected, non-treated
2. Group: *M. bovis*-infected, non-treated
3. Group: *M. bovis*-infected treated with enrofloxacin
4. Group: *M. bovis*-infected treated with valnemulin

The animals were infected into the nasal cavity with the broth culture of virulent *M. bovis* strain 5063. The clinical values and the body weight gain of the calves were recorded throughout the challenge. The medication was performed from the 10^{th} day of the challenge for 8 days in milk substitute. The animals were exterminated on the 21^{st} day of the challenge. Both macroscopical and microscopical lung alterations were recorded. Mycoplasmas and bacteria were cultured from the organs of the animals. The sera were examined with ELISA test.

5.
CRITICAL EVALUATION OF SOME DIAGNOSTIC PCR SYSTEMS SPECIFIC TO *MYCOPLASMA BOVIS* USING AN IMPROVED ASSAY

The PCR systems previously described by Ghadersohi et al., 1997 and Hayman and Hirst, 2003 were improved. These systems produced no results with specific DNA template. A new reverse primer (MbR2, 5'-aatgaagctactgatccaag-3') was made and used with the original forward primer (MB1). Template DNA was prepared from the most frequently occurring bovine mycoplasma and bacteria strains with phenol-chloroform method. The samples were reacted in 50 µl volumes with 1.5 mM $MgCl_2$ and 20 pM RedTaq polymerase (Sigma) concentrations. The PCR conditions were the following: 35 cycles; 94ºC 20 sec, 52ºC 20 sec, 72ºC 1 min; final extension 72˚C 5 min.

RESULTS

1.
RAPID DIAGNOSIS OF *MYCOPLASMA BOVIS* INFECTION IN CATTLE WITH CAPTURE ELISA AND A SELECTIVE DIFFERENTIATING MEDIUM

From the 510 nasal and tracheal swabs, organs and milk samples collected from the herds, mycoplasmas could be isolated in 52 cases. Out of these 43 (83%) was *M. bovis* by immuno-flurescence test.
From the lung specimens collected from the slaughterhouses, 92 mycoplasma isolates were obtained. Out of these 15 was proven to be *M. bovis* with conventional culture. The capture ELISA fortified the presence of *M. bovis* with 100% identity in both cases. From the non-infected group, *M. bovis* could be detected neither with culturing nor with capture ELISA. At the same time other species could be isolated. The same can be stated about the pre-challenge samples of the infected group. On day 14 of challenge *M. bovis* either could be detected with culture or capture ELISA (except for 2 animals).
The selective medium detected *M. bovis* from all of these samples.
The specificity of the capture ELISA was examined with reference strains of 39 mycoplasma and acholeplasma species, other than *M. bovis*, and with 8 different *M. bovis* strains. In these tests only the *M. bovis* strains showed positive reaction. None of the other examined species caused cross-reaction
The selective medium used by us helps the development of *M. bovis* colonies and in anaerobic conditions. Their growth causes a red colorization in the medium; meanwhile characteristic red crystals are formed within the colonies. Other bovine mycoplasmas do not or weakly grow on it. Except for *M. verecundum,* the red colorization is absent too. The color reaction was marked and characteristic to all of the examined *M. bovis* isolates. The inhibitory additives to the agar medium suppressed the growth of the bacterial contaminants.
Either the capture ELISA test or the *M. bovis* selective differentiating agar was equally suitable for the rapid processing of large amount of samples. With the use of them, the time consuming and expensive filter cloning, biochemical and immunological tests (growth inhibition, immuno-fluorescence, etc) can be omitted. Both methods are rapid and specific, so they can help to set up the rapid diagnosis of *Mycoplasma bovis* infection.

2.
PRODUCTION OF MONOCLONAL ANTIBODIES RECOGNIZING MULTIPLE *MYCOPLASMA BOVIS* ANTIGENS AND THEIR TESTING

A total of 63 cell lines producing anti-*Mycoplasma bovis* antibodies were found. From the produced monoclonal antibodies the 1B11, 1C7, 4H9 and 6F11 63 kDa, the 2C5, 5B8, 5D3, 5E5 and the 6H10 22, 25 and 27 kDa, the 2C9 and 5C11 69 kDa, whereas the 3G12 67, 69 and 72 kDa molecular weight of antigen determinants recognized. The antibodies produced by the tested 12 cell lines were of heavy-chain subclass IgG1. Ten of them produced κ, while the other 2 lines were of λ light-chain antibodies.
All of the 12 antibodies reacted with *M. bovis* antigens. The 3G12 and the 5B8 gave no reaction except for the strains of the homolog *Mycoplasma* species. The other examined antibodies cross-reacted with *M. bovigenitalium* and *M. hyopneumoniae.* There was no reaction with *M. agalactiae*. Particularly the antibodies raised against 22, 25 and 27 kDa determinants gave cross-reactions, which refers to the close relationship of the epitopes of the mycoplasmas examined by us. Predominantly the antibodies of 3G12 and 5C11 cell lines seem to be suitable

for immuno-diagnostic purposes e.g. immuno-fluorescence or capture ELISA) due to their high specificity and affinity.
In situ detection of *M. bovis* antigen was successful with immuno-histochemistry method with the antibodies 6H10, 6F11 and 4H9.

3.
EXAMINATION OF THE ROLE OF *MYCOPLASMA BOVIS* IN BOVINE PNEUMONIA AND A MATHEMATICAL MODEL FOR ITS EVALUATION

Out of the 595 animals, 33.9% had pneumonic lesions, 37.0% were positive by *M. bovis* culture, and serum samples of 11.3% of cattle were positive for *M. bovis* antibodies. The overall proportion of seropositive animals was 11.3%, which varied between 0 and 57.2% in various herds. The percentage of seropositive herds was 64.7%.
Our system of hypotheses and the model formed from it can be used for the quantitative interpretation of the associations among pneumonia and the ability to detect *M. bovis* by isolation and its antibodies by ELISA. Our data suggest that the culture of *M. bovis* and the detection of its antibodies by ELISA are equally important methods complementary to each other in the diagnosis of *M. bovis* infection.
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4.
THE EFFICACY OF VALNEMULIN (ECONOR®) IN THE CONTROL OF DISEASE CAUSED BY EXPERIMENTAL INFECTION WITH *MYCOPLASMA BOVIS*

This study showed that *M. bovis* infection of calves produces pronounced clinical signs; depression, dyspnoea, respiratory distress, nasal discharge, coughing, and pyrexia. The signs started to develop 5-7 days after challenge and progressively worsened. Infected animals started to refuse milk about 10 days after challenge and body weight gain was reduced significantly in comparison with uninfected controls. By three weeks after challenge the signs were severe and resulted in the death of some of the unmedicated animals.
M bovis infection resulted in macroscopical and microscopical lung lesions usually involving the apical and cardiac, but sometimes the diaphragmatic lobes to the extent of 10-50% of the total lung area.
M. bovis was readily reisolated from the lungs of infected animals, but also from internal organs such as the liver, spleen, and kidney of infected non-medicated calves. *M. bovis* was also isolated from joints and induced a serological response which could be detected by ELISA testing.
The disease induced by *M. bovis* was successfully treated with enrofloxacin and valnemulin. Significant improvement of clinical signs was observed within 4-6 days after the start of medication, and clinical scores were reduced significantly in medicated groups compared with those of the infected non-medicated group or their pre-challenge status. Medication restored the animals' appetite and weight gain approached that of the non-challenged control animals.
Treatment also resulted in a reduction of macroscopical and histological lung lesions as well as in a decrease in the reisolation rate of M. *bovis* from lungs and, particularly, from kidney, spleen and liver.
Both valnemulin and enrofloxacin performed well in this study. However the reduction in days with clinical signs, and the reduction in the rate of mycoplasma reisolation from liver, spleen and kidney were superior in the valnemulin.

5.

CRITICAL EVALUATION OF SOME DIAGNOSTIC PCR SYSTEMS SPECIFIC TO *MYCOPLASMA BOVIS* USING AN IMPROVED ASSAY

Our PCR system yielded a 319 base-pair *M. bovis* specific product. It could detect 150 CFU/ml of cells in broth cultures. It did not give cross-reactions with bacteria and mycoplasmas other than *M. bovis,* including *M. agalactiae*.

SCIENTIFIC PUBLICATIONS OF THE THESIS

In domestic journals, in Hungarian language

Tenk M., Ball, H. and Stipkovits L. (2002). [Szarvasmarhák *Mycoplasma bovis* fertõzöttségének gyors diagnosztikája capture-ELISA-próbával és szelektív differenciáló táptalaj segítségével.] *Magy. Áo. Lapja* **124,** 333-336.

In domestic journals, in English

Tenk, M., Stipkovits, L. and Hufnágel, L. (2004). Examination of the role of *Mycoplasma bovis* in bovine pneumonia and a mathematical model for its evaluation. *Acta Vet. Hung.* **52,** 445-456.

In international journals, in English

Dénes, B., Tenk, M., Tekes, L., Varga, I., Ferenczné, I. P., and Stipkovits, L. (2003). Recognition of multiple *Mycoplasma bovis* antigens by monoclonal antibodies. *Hybrid.Hybridomics.* **22**, 11-16.

Stipkovits, L., Ripley, P. H., Tenk, M., Glávits, R., Molnár, T. and Fodor L. (in press) The efficacy of valnemulin (Econor®) in the control of disease caused by experimental infection of calves with *Mycoplasma bovis* . *Res. Vet. Sci.*

Tenk, M., Bálint, A., and Dencső, L. (submitted for publication). Critical evaluation of some diagnostic PCR systems specific to *Mycoplasma bovis* using an improved assay. *J. Vet. Med. B*

In national journals, in other languages

Dénes, B., Tenk, M., Tekes, L and Stipkovits, L. (2002): [Monoklonalnie antitela protiv *Mycoplasma bovis* (predvarityelnoe coobcsenyie).] *Vet Medicina* **80,** 1993-1995.

Congress papers published in proceeding books

Fábián, K., Tenk, M., and Stipkovits, L. (1999) Examination of antibody response of cows and their calves to *Mycoplasma bovis* by Western blot.
in: COST 826 – Mycoplasmas of ruminants: pathogenicity, diagnostics, epidemiology and molecular genetics. (Eds.: Stipkovits, L., Rosengarten, R., and Frey, J.). European Commission, Directorale-General XII, Science, Research and Development, Brussels, 3. pp. 141-143.

Stipkovits, L., Glávits, R., Ripley, P., Molnár, T., Tenk, M. and Szeredi, L. (2000) Pathological and immunohistochemical studies of pneumonia in calves experimentally induced by Mycoplasma bovis.
in: COST 826 – Mycoplasmas of ruminants: pathogenicity, diagnostics, epidemiology and molecular genetics. . (Eds.: Bergonier, D., Berthelot, X., and Frey, J.). European Commission, Directorale-General XII, Science, Research and Development, Brussels, 4. pp. 27-30.

Tenk, M., and Stipkovits, L. (2001) Examination of *Mycoplasma bovis* infection in cattle with indirect ELISA. Annual meeting of the 50-year-old Hungarian Society for Microbiology. Proceedings. Balatonfüred, october, 2001.

Tenk, M., Dénes, B., and Stipkovits, L. (2001) Detection of *Mycoplasma bovis* from broth culture with dot immunobiding technique with monoclonal antibody. Annual meeting of the 50-year-old Hungarian Society for Microbiology. Proceedings. Balatonfüred, october, 2001.

Tenk, M., Ball, H., and Stipkovits, L. (2002). Capture ELISA test and a selective-differenciating medium for the detection of *Mycoplasma bovis* infection in cattle. Annual meeting of the Hungarian Society for Microbiology. Proceedings. Balatonfüred, october, 2002.

Other scientific lectures

Tenk, M. Fábián, K., and Stipkovits, L. (1999) Examination of the sera of cattle infected with *Mycoplasma bovis*-with Western Blot. Academic reports of the Veterinary Scientific Committee of HAS

Stipkovits, L., Glávits, R., Ripley, P., Molnár, T., Tenk, M., and Szeredi, L. (1999) Pathological and immunohistochemical studies of pneumonia in calves experimentally induced by *Mycoplasma bovis*. Int. Symp. Mycoplasma of ruminants. June 2-4, 1999, Toulouse, France

Stipkovits, L. and Tenk, M. (2000) Results of the serological screening of *Mycoplasma bovis* Academic reports of the Veterinary Scientific Committee of HAS

Tenk, M., Dénes, B., and Stipkovits, L. (2001) Detection of *Mycoplasma bovis* from respiratory swab samples with BIO-DOT method using, specific monoclonal antibody. Academic reports of the Veterinary Scientific Committee of HAS

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