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POSTGRADUATE SCHOOL OF VETERINARY SCIENCE

Examination of Mycoplasma bovis infection in cattle

Doctoral Thesis

Miklós Tenk

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SZENT ISTVÁN UNIVERSITY
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Head:

Professor Péter Rudas, DSc
Szent István University, Faculty of Veterinary Science
Department of Physiology and Biochemistry

Supervisor:

……..
Professor László Stipkovits, DSc.
Veterinary Medical Research Institute
Hungarian Academy of Sciences

Co-Supervisors:

Professor János Varga, academician
Szent István University, Faculty of Veterinary Science
Department of Microbiology and Infectious Diseases

Róbert Glávits, DVM, PhD
Central Veterinary Institute
Department of Patho-histology

…… ……
Dr. Péter Rudas Dr. Miklós Tenk

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1.
Abbreviations

ATCC	American Type Culture Collection
DNA	Deoxy-Ribonucleic-Acid
CFU	Colony Forming Unit
bp	Base pair(s)
BVD	Bovine Virus Diarrhea
BRSV	Bovine Respiratory Syncitial Virus
CBPP	Contagious Bovine Pleuropneumonia
ELISA	Enzyme Linked Immunosorbent Assay
HRPO	Horse Radish Peroxidase
IF	Immunofluorescence
IH	Immunohistochemistry
IP	Immunoperoxydase
IBR	Infectious Bovine Rhinotracheitis
kDa	kiloDalton(s)
LPS	Lipopolysaccharide
Mab	Monoclonal antibody
µl	Microliter
mM	Millimole
OD	Optical Density
OIE	Office Internationale des Epizooties
nm	Nanometer
PI3	Parainfluenza-3 virus
PBS	Phosphate Buffered Saline Solution
PBS-Tween	Phosphate Buffered Saline Solution with 0.5% Tween-20
PCR	Polymerase Chain Reaction
RNA	Ribonucleic-Acid
SC	Small Colony Type
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrilamid Gel Electrophoresis
Vsp	Variable Surface Protein
WB	Western Blot

2.
Summary

In Chapter 1 the usage of capture ELISA test and a selective differentiating medium in the diagnostics of the *Mycoplasma bovis* infection is presented. Out of 52 strains isolated from 510 various clinical specimens 43 were proven to be *M. bovis* by both culturing and capture ELISA. Out of 92 lung specimens 15 mycoplasma strains were isolated. All of them were identified as *M. bovis* by the above mentioned diagnostic methods. Samples from *M. bovis* challenged animals were examined by culturing, selective culturing and capture ELISA. The latter two methods have been proven to be rapid, highly specific and useful in the diagnostics of *Mycoplasma bovis* infection of cattle. Both capture ELISA and selective-differentiating medium give the same result as the time consuming conventional culturing of this organism.

In Chapter 2 the production and testing of monoclonal antibodies against M*ycoplasma bovis* is discussed. To produce monoclonal antibodies, Balb/c AnN Crl BR mice were inoculated with the cell suspension of a Hungarian *Mycoplasma bovis* strain designated 26034. Three days after the last immunisation the spleen of the immunised mouse was removed aseptically. The fusion of spleen cells with Sp2/0-Ag14 murine myeloma cells was performed in the presence of polyethylene glycol. The obtained hybrid cells were selected with HAT medium. Two weeks after the fusion the supernatants of the cells grown were tested by an own-developed indirect ELISA. The results showed that 63 antibody-producing hybridomas had been obtained. For accurate determination of the molecular weight of antigen determinants, the supernatants giving positive reaction in the ELISA were tested by Western blotting. According to the results, the obtained monoclonal antibodies recognise the antigen determinants of the following molecular weights: 1B11: 63 kDa, 1C7: 63 kDa, 2C5: 22, 25 and 27 kDa, 2C9: 69 kDa, 3G12: 67, 69 and 72 kDa, 4H9: 63 kDa, 5B8: 22, 25 and 27 kDa, 5D3: 22, 25 and 27 kDa, 5C11: 69 kDa, 5E5: 22, 25 and 27 kDa, 6F11: 63 kDa, and 6H10: 22, 25 and 27 kDa. The 12 cell groups selected on the basis of the Western blotting were cloned twice by end-point dilution method. The cloned cells were propagated, and with 5 cell lines antibodies were produced in the CELLine bioreactor. Cell line 3G12 showed the highest productivity with an average daily output of 1.5 mg immunoglobulin. Cell line 5E5 produced 1.1 mg, 6H10 0.8 mg, 2C9 0.47 mg and 6F11 0.4 mg antibody per day. The isotype of the antibodies was determined by ELISA. The antibodies produced by the 12 cell lines tested were assigned to the IgG_1 subclass according to the heavy chain. Ten cell lines produced κ and two produced λ light-chain antibody. Possible cross-reactions of the produced monoclonal anti-*M. bovis* antibodies with certain *Mycoplasma*, *Ureaplasma* and *Acholeplasma* species were tested by an indirect ELISA procedure. All of the 12 antibodies tested gave a reaction with the antigen of *M. bovis* strain designated 26034. Monoclonal antibodies 3G12 (67, 69, 72 kDa) and 5B8 (22, 25, 27 kDa) gave no cross-reaction with antigens other than strains of the homologous *Mycoplasma* species. The other antibodies reacted with the *M. bovigenitalium F7, M. sp. 8389, M. oculi* and *M. gallisepticum S6* antigens. Owing to its high specificity and affinity, primarily the antibody produced by cell line 3G12 is considered suitable for use in immunodiagnostic tests of *M. bovis* infections.

The produced antibodies were also tested with immunohystochemical method. These examinations demonstrated that the antibodies 6H10, 6F11 and 4H9 are suitable for the in situ detection of the *M. bovis* antigen.

In Chapter 3 the prevalence of *Mycoplasma bovis* and the evaluation of its pathogenic role is discussed by application of a mathematical model. Thirty four large cattle herds were screened for the presence of *Mycoplasma bovis* infection by examination of cattle slaughtered at slaughterhouse for pneumonic lesions in the lungs, culturing of *M. bovis* from lung lesions and testing sera for presence of antibodies against *M. bovis*. A statistical model was developed, which confirmed the relationship between these 3 parameters. Among 595 examined cattle, 33.9% had pneumonic lesions, Mycoplasmas were isolated from 59.9% of pneumonic lung samples, only 10.9% of sera from those animals contained *M. bovis* antibodies. In 25.2% of

cases mycoplasmas were isolated from lungs with no macroscopic lesions. The average seropositivity rate of individuals was 11.3%, however in certain herds it reached more than 50%. The proportion of seropositive herds was 64.7%.
Comprehensive associations were found between serological responses against *M. bovis* and the observed lung lesions with the help of statistical calculations.
In Chapter 4 the efficacy of valnemulin is presented in an experimental challenge trial. *Mycoplasma bovis* infection was experimentally induced in groups of 6 young calves. A further group was uninfected and served as a control. Ten days after infection, medication with either enrofloxacin (Baytril, Bayer) or valnemulin (Econor, Novartis) was instituted via the milk replacer for a further 10 days, after which all calves were killed. Infection resulted in depression, pyrexia, inappetance and prominent respiratory signs. Arthritis occurred in 2 animals, and 2 (unmedicated) animals died.
At post mortem examination extensive lesions were present in the lungs and *M. bovis* was re-isolated from infected unmedicated calves' lungs.
Medication with either enrofloxacin or valnemulin resulted in a rapid diminution of clinical signs, restoration of appetite and reversal of weight loss. Isolation of *Pasteurella multocida* from the calves' lungs was suppressed by both medicaments.
Valnemulin resulted in a more rapid reduction of clinical scores, and eliminated *M. bovis* from the lungs more effectively than enrofloxacin.
In Chapter 5 development and application of an improved PCR system is presented. This system was developed using the forward primer described by Ghadersohi et al (1997) and a new reverse primer Mbr2 based on the Vsp gene region of *Mycoplasma bovis*, since both the original system and its further developed semi-nested variant (Hayman et al., 2003) do not work. The PCR did not amplify the pathogenic and ubiquitous mycoplasmas as well as bacteria commonly occurring in bovine respiratory and mammary tract. The assay detected as low as 150 CFU/ml of *Mycoplasma bovis* in broth culture enabling the diagnostic use of it with high sensitivity.

3.
Review of the literature

3.1. Introduction

There are several mycoplasma species colonizing the bovine respiratory mucous membranes. Some of them are considered to be pathogenic whereas others are ubiquitous, part of the normal flora (ter Laak et al., 1992a,b).

The diseases caused by mycoplasmas in cattle -although their role is usually underestimated- are of major importance. Disregarding *Mycoplasma mycoides subsp. mycoides SC,* the causative agent of CBPP -which is by the way the only OIE List A bacterial disease- other Mycoplasma species can cause massive respiratory, venereal and other diseases as well. Among these *Mycoplasma bovis* is the most important and most pathogenic bovine mycoplasma in Europe and North America. This organism is a significant cause of bovine pneumonia (Pfützner and Sachse, 1996), mastitis (Byrne et al., 2000), arthritis (Stipkovits et al., 1993), genital disorders and abortion (Byrne et al., 1999, Langford, 1975, Ruhnke, 1994) and reduction of in vitro fertility (Kissi et al., 1985, Eaglesome et Garcia, 1990). Rarely it can be isolated from other diseases, such as otitis (Walz et al., 1997), meningeal abcesses (Stipkovits et al., 1993), decubital abscesses (Kinde et al., 1993) and keratoconjonctivitis of calves (Jack et al., 1977, Kirby and Nicholas, 1996). Its pathogenic role is often ignored although several experimental infections have proved it (Gourlay et al., 1985, Thomas et al., 1986, Rodriguez et al., 1996).

The harms caused by respiratory diseases in cattle cause approximately a sum of 576 million euros per year in Europe. *M. bovis* is estimated to be responsible for at least for the quarter or third of these losses (Nicholas and Ayling, 2003). In the USA this organism causes a loss of $ 32 million per year as a result of the loss of the weight gain and the diminished carcass value. The expenses due to *M. bovis* mastitis are estimated to be much higher ($ 108 million) there (Rosengarten and Citti, 1999)

3.2. History

M. bovis was first isolated in the USA from the milk of a mastitic cow in 1961 (Hale et al., 1962). First it got the name *Mycoplasma bovimastitidis* then *Mycoplasma agalactiae subsp. bovis*, because of the similar clinical picture to the contagious agalactia of sheep caused by *M. agalactiae*. Later following the examination of the 16S ribosomal RNA it was elevated to species rank and received the name *Mycoplasma bovis* (Askaa and Ernø, 1976). During the past decades it has spread to numerous countries with the global transport of animals and sperm.

3.3. Taxonomy

Mycoplasma bovis belongs to the class *Mollicutes* to the order *Mycoplasmatales* family *Mycoplasmataceae* and the genus *Mycoplasma* (Razin et al., 1998). This species is very similar to *M. agalactiae* in many aspects. The 16S RNA sequences show small differences -only in 8 nucleotides- between these species (Mattsson et al., 1994). The sequencing of the uvrC gene also revealed differences (Subramaniam et al., 1998). In comparaison of pulsed field gel electrophoresis profiles, Tola et al., (1999) estimated the genomic size of *M. bovis* to 961 ± 18,9 Kbp while *M. agalactiae* has 945 ± 84 Kbp.

The high number of common antigens in these two species often causes immunological cross-reactions (Boothby et al., 1981). However the differetiative laboratory diagnosis is possible since these species confined to their host animal species and can be distinguished from each other by whole-cell protein profiles, DNA-DNA hybridization and PCR-fingerprinting.

3.4. Biological properties of Mycoplasma bovis

Like all mollicutes, *M. bovis* is small and pleomorphic, lacks a cell wall and has a low G+C ratio of 27.8-32.9 mol% (Hermann, 1992). *M. bovis* is also similar to *M. agalactiae* in its biochemical properties, as it neither ferments glucose nor hydrolyses arginine but instead of these compounds

uses organic acids, lactate and pyruvate as energy sources for growth (Miles et al., 1988). The film and spot formation can also be seen on the surface of solid media indicating the possession of lipolytic activity. The biochemical properties of the most frequent bovine mycoplasmas compared to the ones of *M. bovis* can be seen in Table 3.1.

Table 3.1. Biochemical properties of the most frequent bovine mycoplasmas (Nicholas and Ayling, 2003)

Mycoplasma	Glucose	Arginine	Urease	Film	Casein	Phosphatase	Tetrazolium/ Aerobic	Tetrazolium/ Anaerobic
M. bovis	-	-	-	+	-	+	+	+
M. bovirhinis	+	-	-	-	+/-	+/-	+	+
M. bovigenitalium	-	-	-	+	-	+	-	+
M. bovoculi	+	-	-	+	-	+/-	+	+
M. canis	+	-	-	-	+/-	-	-	+
M. californicum	-	-	-	-	nk	+	-	+**
M. canadense	-	+	-	-	nk	+*	-	+
M. dispar	+	-	-	-	nk	-	+	+
M. mycoides SC	+	-	-	-	+	-	+	+
U. diversum	-	-	+	-	-	-	nk	nk

nk- not known
*- weak
**-most strains

Mycoplasmas are usually considered to be highly susceptible to various environmental factors such as high temperature, dryness, etc. Despite this *M. bovis* can survive outside the host environment at 4°C nearly for 2 months in sponges and milk, for 20 days on wood and for 17 days in water. At 20°C the survival periods drop to one-two weeks and at 37°C to one week. In deep-frozen semen the agent remains infective for years (Pfützner, 1984).
M. bovis is usually susceptible to the commonly used disinfectants although the biological materials (milk, discharges, etc) can dramatically reduce their efficacy. Formalin and peracetic acid are proved to be very effective for general disinfecting purposes. Iodofores are also efficient. This enables their use for teat dipping. Unfortunately disinfecting materials based on hypochlorites are unsuitable for this purpose, because of the high concentrations and long exposure periods needed to obtain suitable efficacy. This can be a problem, because these compounds are widely used in disinfection of milking machines (Jasper et al., 1976, Pfützner et al., 1983b).

3.5. Epidemiology of the Mycoplasma bovis infection

3.5.1. The reservoir and the source of infection

Clinically healthy young cattle can harbor *M. bovis* in the respiratory tract without clinical symptoms and shed it through their nasal discharge for month or years. The genital tract of both male and female animals can also be sources of the infection. The agent can also be introduced into *M. bovis*-free herds by artificial insemination with deep frozen bull semen, in which mycoplasmas can survive for several years. Clinically healthy cows can shed *M. bovis* in their milk. This represents one of the major sources of infection for suckling calves (Pfützner, 1990).
Mycoplasma bovis can also colonize sheep (Bocklisch et al., 1987) and goat (Egwu et al., 2001) and the pathogen can be transmitted to cattle with these animals. Rarely, it can be isolated from other animals such as rabbits (Boucher et al., 1999). Sometimes even humans can be affected with even severe respiratory disease (Madoff et al., 1979), which suggests people working with cattle can be active carriers too.
Taking into consideration the relative high resistance of mycoplasmas under some environmental conditions (Nagatomo et al., 2001) the role other factors such as the litter, tools and the hands and clothing of the staff cannot be ignored in spreading of the infection.

3.5.2. Transmission of the disease

M. bovis settles down predominantly in the broncho-alveolar region in the respiratory tract. The aerogen infection is caused by the small, inhaled droplets, which are set free by coughing of

infected animals. Contaminated dust particles can also be the source of infection. After the onset of the respiratory disease the infection spreads rapidly in the herd. After the contact with a diseased calf *M. bovis* appears in the nasal discharge of the animals within 24 hours. Seven days after the first detection *M. bovis* can be isolated from the most of the animals (Pfützner et al., 1983a, Pfützner and Schimmel, 1985, Stipkovits et al., 2000).

The joints of calves become affected by haematogenic spread of mycoplasmas. This usually occurs when the rate of Mycoplasma pneumonia is high among calves or the mastitis among cows is high, and the pathogen is shed at a high number (Romváry et al., 1977).

The udder is infected through the teat canal. In addition to the weakened protective barrier of the teat canal other factors usually contribute to the infection, such as high population density and poor hygienic conditions or feeding. The milking process makes possible the mycoplasmas to invade the teat canal. Because of the mentioned contributing factors *M. bovis* mastitis is more frequent in large dairy herds (Thomas et al., 1981).

After the onset of the mastitis other animals become infected rapidly during the milking process. Only small number of living organisms is enough to cause the infection of the teat canal (Bennett and Jasper, 1980). Cows affected by Mycoplasma bovis mastitis shed a large number of mycoplasmas (10^5-10^8 CFU/ml), but significant shedding usually occurs before the clinical signs appear (10^3-10^6 CFU/ml). This way the unidentified healthy shedders are the most dangerous in spreading the organism. Taking these facts into consideration surfaces of the milking machines, wiping cloths, hands of the workers and the milk reflux from the neighboring cows play an important role in the transmission of *M. bovis*. Rarely transmission of *M. bovis* also occurs by inadequate intracysternal application of anti-mastitic drugs (Pfützner and Sachse, 1996).

The male genital tract becomes infected from the contaminated environment or by direct contact of other animals shedding *M. bovis*. The organism can reach the inner genital organs intracanalicularly from the prepuce. This way it can cause orchitis, vesiculitis, decrease of the semen quality and consequently shedding it in the semen (Kreusel et al., 1989)

The female genital tract also becomes affected by an ascendant way of infection. Apart from the natural infections from the environment, the major problem is the artificial insemination of cows with infected semen (Eaglesome and Garcia, 1990, Richter et al., 1989). The association between the uterus infection and mastitis is still unclear, since mycoplasma metritis could not be reproduced by intravenous inoculation of cows by *M. bovis* although the organism can frequently be isolated from the uterus and aborted fetuses of mastitic cows (Bocklisch et al., 1986, Pfützner and Schimmel, 1985).

Field and experimental studies have proved a close transmission cycle from the infected cow to the fetuses and newborn calves (Pfützner and Schimmel, 1985). At the first time newborn calves can become infected vertically from the uterus. More frequent way of vertical infection for suckling calves is the milk of asymptomatic shedder or mastitic cows. The nasal mucus of other affected calves or cows is an important factor in horizontal spreading of *M. bovis*. The organism remains infective in the respiratory tract and can be transmitted to the next generation. A field survey demonstrated that after the appearance of *M. bovis* in the nasal fluid of young calves – which earlier had become infected from mastitic milk of their mothers– it had rapidly spread among the young cattle of different ages. The infection caused elevated antibody titers as well. Later, during pregnancy of heifers *M. bovis* "disappeared", it could not be isolated from clinical samples. Later, after parturition the infective agent could be recovered from different samples, such as amnionic fluid, endometrium of slaughtered animals and fetuses, etc., suggesting haematogenic dissemination and vertical spread of the infection to fetuses and calves through the genital organs. Seemingly *M. bovis* remains virulent throughout this transmission cycle, although a clinical disease can not always be seen (Horváth et al., 1983, Pfützner and Sachse, 1996, Stipkovits et al., 2000). Thus cows are most susceptible to *M. bovis* mastitis at parturition and maximum lactation. Respiratory disease and arthritis occurs in calves of very young age particularly if cows with clinical mycoplasma mastitis are present in the neighborhood.

3.5.3. Association with other pathogens

M. bovis is frequently associated with other pathogenic microorganisms such as BRSV, PI-3, bovine adenoviruses, BVDV, *Pasteurella multocida*, *Mannheimia haemolytica*, *Arcanobacterium pyogenes*, *Haemophilus somnus*, *Mycoplasma dispar*, *Mycoplasma canis* and *Ureaplasma diversum*. These infective agents usually change the clinical picture caused by *M. bovis*.

Some of these interactions have been studied experimentally. Gourlay and Houghton (1985) infected calves with *M. bovis* and *M. haemolytica*. They experienced that more severe pneumonia was seen when *M. bovis* was inoculated before *Mannheimia haemolytica* and not after it. Contrary to this Thomas and others (1986) experienced that BRSV did not enhance the lesions in combined infections with *M. bovis*.

3.6. Pathogenicity of Mycoplasma bovis

The mechanisms of the pathogenesis of *M. bovis* are still relatively unknown, although it is clear that it uses complex strategies to invade the host organism. Adhesion of the mycoplasmas to the host cells is the primary and key factor of pathogenesis. According to the experiments by Thomas et al. (1991) *M. bovis* adheres to the neutrophils and the macrophages in a dose-dependent way and there is a lack of phagocytotic activity to *M. bovis* by these cells. It had been previously proved that *M. bovis* is able to persist and multiply on the surface of these cells (Howard, 1984). In contrary to other bovine mycoplasmas -by the adhesion to non-phagocyte cells- *M. bovis* triggers the apoptosis of lymphocytes with the help of some still unknown proteins (Vanden Busch and Rosenbusch, 2001). The mechanism and the nature of the adhesion were studied by in vitro models with the type strain PG45 on embryonic bovine lung (EBL) cells (Sachse et al., 1993a, 1996, 2000). These experiments showed the specificity and kinetics of the adhesion and the involvement of specific proteins, particularly protein P26, and the participation of sialic acid residues and probably also sulfatide groups, as binding receptors in this process (Sachse et al., 1993a). It has been observed in both in vivo and in vitro on tracheal cell cultures that *M. bovis* does not adhere specifically to epithelial cilia of tracheal cells as *M. pneumoniae* or *M. dispar* does (Howard et al., 1987, Rodriguez et al., 1996). The importance of the P26 protein in the adhesion (Sachse et al., 1993a) is variable among the *M. bovis* strains, although this role has not been proved in vivo. Certain variable surface lipoproteins (Vsps, see later) have been also demonstrated to be involved in the adhesion of the reference strain PG45 on continuous lines of EBL cells (Sachse et al., 2000). Further oligopeptides derived from the repetitive sequences of Vsps A, B, E and F are able to partially inhibit this kind of adhesion. Certain sequences from the Vsp genes also encode immunogenic epitopes. This common feature proves the importance of the Vsps in the pathogenesis of *M. bovis*.

M. bovis has also an anti-phagocytotic capability, which helps to survive the attack of the host immune system (Thomas et al., 1991), although the killer feature of the macrophages is not impaired. If a specific anti-*M. bovis* hyperimmune serum is added to in vitro cultures of macrophages and neutrophils, they become capable to ingest and destruct the mycoplasmas (Howard et al., 1976, Howard, 1984). The inhibitory mechanisms of *M. bovis,* with which it prevents phagocytosis, are not known.

Unlike *Mycoplasma dispar* -which is localized on the surface of the epithelial cells- *M. bovis* is able to invade the tracheo-bronchial epithelium and multiply there. This has been proved by both in vitro studies on tracheal rings and in vivo on naturally and experimentally infected animals (Howard et al., 1987). *M. bovis* penetrates through the respiratory epithelium into the intracellular space (Howard et al., 1987, Rodriguez et al., 1996), which enables the mycoplasmas a long persistence, and to evade the host immune system in chronic infections (Rodriguez et al., 1996). *M. bovis* can also enter the circulatory system. This systemic infection (mycoplasmaemia) is the cause of arthritis. At this time the pathogen can be isolated from different organs, such as liver, kidneys, etc. (Poumarat et al., 1996).

In addition *M. bovis* has a 73-kDa thermo-stable polysaccharide complex consisting of glucose, glucosamine or galactosamine, and a heptose, localized in the cytoplasm membrane tightly associated there with proteins. This polysaccharide compound can be extracted with 75% ethanol and has toxic properties. This toxin does not act like an exotoxin, but it has an effect on increasing capillary permeability and activating the complement cascade system triggering inflammatory response this way. This effect is similar to the mechanism of the LPS of the Gram-negative bacteria (Geary et al., 1988). Others did not confirm the presence of this toxin. According to the findings of Bryson et al. (1999) –with *M. bovis* vaccinated and then re-infected animals– the microorganism triggered a heavy exacerbation of the lesions in the vaccinated group comparing to the control animals suggesting the role of the cellular immunity and type IV hypersensitivity in the pathogenesis of *M. bovis*.
M. bovis is capable to change rapidly its antigenic structure by switching on and off of certain surface lipoproteins. This dynamic antigenic hypervariability (Behrens et al., 1994) causes major problems in applications of certain diagnostic tests and vaccine production. This kind of extreme variability can be observed among isolates from different pathological processes or locations (Behrens et al., 1994, Poumarat et al., 1994). These dynamic variations in the expression can be observed in different clones of the same strain by electron microscopy (Behrens et al., 1996a).
These hypervariable surface antigens are mainly lipoproteins (Vsp, signed from A to O). These lipoproteins have strong immunogenic properties. Their amino-terminal is hydrophilic, whereas the central region is hydrophobic. These proteins consist mainly of periodically repetitive polypeptides variable in size as well (Lysnyansky et al., 1999, 2001). The N-terminal of these Vsps is conservative to the first 29 amino acids.
Lysnyansky and others (1996) first described the on-off switching and the variability in size of the Vsps. An insertion of a sequence into the promoter region is responsible for the non-expression (off status) of the protein. The inversion of the sequences can provoke the juxtaposition of a promoter so the protein will be expressed (on status) (Lysnyansky et al., 2001). The insertion or deletion of a repetitive sequence into another, triggers a variation in size (Yogev et al., 2002). These DNA rearrangements occur spontaneously and at a high frequency. The intergenic recombinations -in which according to the present knowledge 13 genes and 13 sites of recombination are involved in the Vsp locus of the type strain PG45- obviously generate numerous variations. (Yogev et al., 2002).
The Vsps have multiple functions in *M. bovis*. They help in the adhesion to the host cells, to evade the immune system by frequent variations and to adapt to different conditions in the host and the environment (Poumarat et al., 1996). Sachse and others (1996, 2000) examined the role of the Vsps in the adhesion to the host cells. Le Grand and others (1996b) proved by in vitro studies that application of certain anti-Vsp Mabs modifies the expression of these Vsps. On the removal of the Mabs the original expression form can be observed but new variants also emerge. This phenomenon explains the extreme variability in protein structure among field strains, which can also make some Mabs difficult to use in the routine diagnosis (Rosengarten and Yogev, 1996).

3.7. Clinical signs and pathological changes associated with *M. bovis* infection

3.7.1. Respiratory symptoms and pathology
The pathogenicity of *M. bovis* has been already proved with several respiratory challenges. *M. bovis* triggered clinical symptoms and characteristic pathological changes by its own without the presence of any other infective agents and *M. bovis* was re-isolable from the infected animals. The intra-tracheal inoculation of *M. bovis* induced respiratory clinical signs and pulmonary lesions in gnotobiotic calves (Gourlay et al., 1979).
The symptoms of the infection are non-specific. According to the observations of Stipkovits and others (2000) the signs of infection, such as fever, depression, loss of appetite, hyperventilation, dyspnoea, nasal discharge and coughing, were recognizable as early as on the 5th day of age by

newborn calves. The disease spreads rapidly throughout the herd, which can be seen by the rapid elevation of the number of animals with clinical signs. These signs do not abate with the administration of classic antibacterial compounds, such as penicillin or gentamycin.
Lopez and others (1986) described the characteristic lung lesions for *M. bovis* infection as severe peribronchial lymphoid hyperplasia with mild exudation of neutrophils and macrophages into the cranioventral parts of the lungs. Rodriguez and others (1996, 2000) found exudative bronchopneumonia and extensive foci of coagulative necrosis surrounded by inflammatory cells in the lungs of naturally infected animals, whereas the challenged animals in addition to it showed suppurative bronchiolitis and varying degrees of peribronchiolar mononuclear cell cuffing. According to their IH investigations the M. bovis antigen was situated at the periphery of the areas of coagulative necrosis, in necrotic exudates, and in close association with infiltrating macrophages and neutrophils.

3.7.2. Arthritis

The intra-articular (Pfützner et al., 1983a), but also the intravenous or intra-bronchial inoculation of *M. bovis* causes heavy polyarthritis in calves. Sometimes arthritis can also be observed in adult cattle (Henderson and Ball, 1999). In natural infections usually the carpal and the tarsal joints are affected. As it was seen by Chima and others (1981), the synovia of the artificially infected animals was infiltrated with lymphocytes, macrophages and neutrophils. The immunoglobulin levels in the synovia were high in these cases. By macroscopic examination, fibrinopurulent exudate was seen the joint spaces. Ryan and others (1983) observed massive fibrinosuppurative synovitis and tenosynovitis, erosion of cartilage, and its replacement by polypoid granulation tissue. Microscopically extensive ulceration of synovial membranes, leukocytic infiltration of the subsynovium, congestion, hyperemia, and thrombosis of the subsynovial vessels were seen.

3.7.3. Mastitis

The low-dose intramammal inoculation of *M. bovis* causes heavy mastitis in cows (Illing, 1979, Horváth et al., 1980, Bocklisch et al., 1991), but also in mice. In the latter experiment systemic symptoms followed by the inoculation were heavier in low-passage strains (Thorns et Boughton, 1980), suggesting the loss of virulence by the passages. The mastitis could be artificially produced by different strains isolated from different sites (respiratory- and urogenital tract, conjunctiva and mammary gland), and observed udder edema and its infiltration with neutrophils, lymphocytes and macrophages (Horváth et al., 1983, Pfützner et al., 1983a). In an experimental intramammal infection of ewes by Bocklisch and others (1991) the infected animals had febrile clinical mastitis, and the transmission of the infective agent to other udder halves and later to other animals could be observed. Among mastitic herds *M. bovis* is the most frequently identified mycoplasma (Bennett and Jasper, 1978). These infections are usually sporadic at large dairy herds but the transmission is rapid (Kunkel, 1985). Mastitis due to *M. bovis* is most frequent in winter (Brown et al., 1990, Feenstra et al., 1991, Gonzalez et al., 1992, Brice et al., 2000, Byrne et al., 2001).
In most of the cases more quarters are affected, and the milk is usually seropurulent. Usually there are no systemic clinical signs of mastitis except for the diminished milk production (Kunkel, 1985, Poumarat et al., 1996). The resistance to the antibiotic treatment is also pathognostic (Gunning et Shepherd, 1996).

3.7.4. Infections of genital organs

LaFaunce and McEntee (1982) inoculated *M. bovis* into the seminal vesicle producing seminal vesiculitis with persistent shedding of *M. bovis* in the vesicular gland for more than 8 months. Other ways of infection did not produce this. In natural cases it can occasionally be associated with genital infections and abortions (Pfützner and Sachse 1996, Byrne et al., 2000). *M. bovis* contamination of semen affects quality and fertility (Ibrahim et al., 1984, Kissi et al., 1985).

3.8. Diagnosis of the *M. bovis* infection

3.8.1. Sampling

Since the symptoms and lesions due to *M. bovis* are not characteristic a sufficient laboratory diagnosis must be obtained. The sites of sampling should be chosen according to the observed alterations. Nasal, genital and conjunctival swab samples should be taken into adequate liquid growth medium. Milk, synovia, semen and other liquid samples can be cultured in the laboratory. All of the samples must be kept at around 4 °C and transported to the lab within a few hours. It must be taken into consideration with respiratory sampling that *M. bovis* can be better recovered from broncho-alveolar lavages than nasal swabs, although this method is much more difficult (Thomas et al., 2002).

3.8.2. Culture and identification

Although *M. bovis* belongs to the well growing mycoplasmas its isolation requires a specially equipped laboratory. There are more variants of suitable culture media for the isolation of *M. bovis* (Medium B, Hayflick's medium, Friis medium, etc.). These media usually contain a rich protein base (e.g. heart infusion), serum (horse and/or swine), yeast extract, glucose and/or pyruvate and selective agents (ampicillin, thallous acetate and amphotericin-B). After 3-5 days of incubation this organism forms typical fried-egg shape colonies. Films and spots production can also be observed. Shimizu (1983) described a selective agar medium with Tween-80, which detects colonies of *M. bovis* based on the lipase reaction. Another commercially available selective agar medium visualizes the colonies by a red color reaction (Windsor and Bashiruddin, 1999).

The first step in the identification of the strains is to determine their basic biochemical properties (Ernø and Stipkovits, 1973a,b). *Mycoplasma bovis* seems to be unique by the use of pyruvate (Megid et al., 2001). Before doing biochemical tests one should make sure that the isolate contains only one species. Therefore isolates must be filtrated through 200 nm filters and cloned 3 times until a pure culture is reached. The species-specific identification require anti-*M. bovis* hyperimmune serum, which can be used in growth inhibition, film inhibition, metabolic inhibition and indirect IF tests (Lauerman, 1994). The IF or IP tests can be used in mixed cultures too. The use of Mabs make these tests more specific, although their sensitivity may drop.

The cultures (also mixed ones) can also be identified by specific Mab-based sandwich or capture ELISA (Ball and Findlay, 1998) or dot immunobinding test using polyclonal sera (MF dot, Poumarat et al., 1991).

3.8.3. Immunological tests

Antibodies to *M. bovis* persist for several months and can be detected with various methods. The use of these tests are particularly useful, when the isolation of the agent is difficult due to chronic infection or regular treatment with antibiotics at a high dosage (Nicholas and Ayling, 2003).

It should be taken into consideration that *M. bovis* can be present only in the nasal cavity and in this case it is usually not immunogenic. If it gets invasive the antibodies become detectable. The serological tests are unusable during the first 10-14 days of outbreaks before the specific antibodies show up.

3.8.3.1. ELISA

The ELISA tests are useful diagnostic methods for diagnosing *M. bovis* outbreaks but also for screening purposes or checking the status of herds for e.g. trade reasons (Uhaa et al., 1990a, Pfützner and Sachse, 1996). There are commercially available tests with mixed antigens in order to minimize the false negative reactions due to the antigenic variability of *M. bovis* (Le Grand et al., 2002). There are also non-commercial ELISA tests developed by local laboratories used to detect *M. bovis* infection (Boothby et al., 1981, Uhaa et al., 1990a, Byrne et al., 2000). The

ELISA tests performed from milk can also be applied in the examination of mastitis outbreaks (Byrne et al., 2000).

3.8.3.2. SDS-PAGE and Western blot

This technique can be used to compare the antigenic structure of the strains or for the examination of the humoral immune response patterns of the host animal. For these tests the whole cell lysate must be subjected to SDS-PAGE, then the cleaved proteins can be transferred onto nitrocellulose membranes. These membranes can be examined either with Mabs or polyclonal antisera. The immunological profiles obtained from Western blots can be compared to whole protein profile of the bacteria on the silver or Coomassie-blue stained acrylamide gels (Beier et al., 1998, Poumarat et al., 1999).

3.8.3.3. Immunohistochemistry

The use of specific antibodies either by IF (Knudtson et al., 1986) or IH (Adegboye et al., 1995) can be used for the in situ detection of *M. bovis.* The latter method enables the visualization of the antigen together with the specific lesions (Rodriguez et al., 1996).

3.8.4. DNA-based methods

The relatively difficult culturing and the cross-reactions in the serological methods have focused the attention on the DNA-based techniques.

Plasmid probes containing random genomic fragments were used in dot blot hybridization tests to identify *M. bovis*. Cross-reactions often occurred with *M. agalactiae* or *M. arginini* (Hötzel et al., 1993, McCully and Brock, 1992). Mattsson and others (1994) used synthetic oligonucleotide probes from the 16S RNA gene for this purpose. These probes are laborious and not specific and sensitive enough for the routine diagnosis.

The use of PCR makes the identification of *M. bovis* much shorter comparing to the conventional culture methods. In addition the mycoplasmas can be detected even if the organs or the broth cultures are contaminated with bacteria. For these tests specific and conservative gene sequences must be targeted. A good target is for example the 16S ribosomal RNA gene (Chavez Gonzalez et al., 1995). More than one bovine respiratory mycoplasma species can be detected simultaneously using PCR tests based on the 16S RNA gene sequences (*U. diversum, M. bovis, Mycoplasma bovirhinis, Mycoplasma alkalescens* and *Mycoplasma bovigenitalium*– Vasconcellos Cardoso et al., 2000, Hirose et al., 2001). Although these systems are highly specific and sensitive, they cross-react with *M. agalactiae*.

Ghadersohi et al. (1997) designed PCR primers from sequences obtained from a *M. bovis* specific dot blot hybridization probe. Haymann et al (2003) improved this system to a semi-nested setup. Using the specific sequences of the UvrC gene Subramaniam and others (1998) could distinguish between *M. bovis* and *M. agalactiae*. Pinnow and others (2001) developed a specific nested PCR test, with which the preservative-treated milk samples can also be examined.

3.9. Disease prevention and control

3.9.1. Antibiotic therapy

The efficacy of the treatment of the respiratory diseases due to *M. bovis* depends on the right choice of the compound, its distribution in the tissues and last but not least the simultaneous anti-bacterial effect in secondary and mixed infections of e.g. *Pasteurella multocida*, *Mannheimia haemolytica* or *Haemophilus somnus* (Poumarat et al., 1996).

The antibiotic susceptibility of the isolates can only be examined by the determination of the minimal inhibitory concentration (MIC) values instead the disc diffusion test (Hannan, 2000). All of the mycoplasmas are resistant to beta-lactames because of the lack of the cell wall. The same resistance can be observed to nalidixic acid, polymyxin, rifamycin, trimethoprim and to

sulfonamides (Poumarat et al., 1996). *M. bovis* as other mycoplasmas is sensitive to antibiotics, which inhibit the protein or nucleic acid synthesis. The most effective antibiotics are the pleuromutilins (tiamulin, valnemulin) and the fluoroquinolones (Taylor-Robinson and Bébéar, 1997, Thomas et al., 2003). The tetracyclines, the macrolids and the aminoglycosides used to be good against *M. bovis*, but lately there are elevated numbers of resistant strains (Ball et al., 1995, Ayling et al., 2000).
Tiamulin has excellent activity against *M. bovis* (ter Laak et al., 1993, Friis and Szancer, 1994, Hannan et al., 1997). An analog compound of tiamulin is valnemulin, which has proven to be effective in the control *M. bovis* infection under field conditions (Stipkovits et al., 2001).
The fluoroquinolones (enrofloxacin and danofloxacin) are found to be also effective against the most *M. bovis* strains in vitro (Ball et al., 1995, Hannan et al., 1997, Ayling et al., 2000), but some authors do not recommend their use in the practice, because of their failure in diminishing respiratory losses (Nicholas and Ayling, 2003).
It must be taken into consideration by choosing the compound that in vitro results not always cover the in vivo efficacy (Ayling et al., 2000), moreover the chemotherapy fails in many cases (Nicholas et al., 2003, Haines et al., 2001).
A crucial factor in the spread of the disease due to *M. bovis* is the contamination of the deep-frozen semen. Visser and others (1999) described spectinomycin -in a combination with gentamycin, tylosin and lincomycin- to be effective in the elimination of *M. bovis* from artificially infected semen.

3.9.2. Prophylaxis

The problems with chemotherapy induced a strong need for vaccine development. Although Howard and others (1987) were successful in preventing respiratory disease by an inactivated preparation of RS, PI3, *M. dispar* and *M. bovis*, there is no potent vaccine till present day. This is a major problem in the prophylaxis of the infection (Le Grand et al., 1996). Urbaneck and others (2000) applied a formalin inactivated herd-specific vaccine against *Mannheimia haemolytica* and *M. bovis*, which reduced the losses due to pneumonia and the costs of the chemotherapy. Nicholas and others (2002) found a saponized-inactivated *M. bovis* vaccine to be effective by reducing respiratory signs, pathological changes and loss of body weight in a challenge trial in calves. The vaccination trial to prevent *M. bovis* mastitis failed and had made the situation even worse (Nicholas and Ayling, 2003).
The prophylactic therapy is recommended when new calves are introduced into a heavily infected local herd (Nagatomo et al., 1996).
It is very important to examine the animals arriving from other herds to detect the asymptomatic shedders. In the herds where mastitis is present it is advisory to cull the diseased animals, which are massive sources of re-infection. This process must be followed by adequate hygienic procedures to prevent re-infection (Pfützner, 1990).
The disease can be controlled by slaughtering the carrier animals, which had been done for more than 30 years in Denmark (Feenstra et al., 1991). This system has dramatically reduced the disease due to *M. bovis.* Since it was discontinued the prevalence got higher again (Kusiluka et al., 2000). These measures require reliable, specific and sensitive diagnostic methods (Pfützner and Sachse, 1996).
As general practice the rules of the good animal housing must be followed to prevent calf diseases due to *M. bovis*. The overcrowding of the houses leads to stress and elevated levels of ammonia, which increases the chances of the respiratory diseases.
It is preferred to apply the “all-in, all-out” practice by moving the animals or if it is not possible the direct contact between the calves and the older animals should be postponed to the latest time possible to prevent early infection.
The regular control of deep-frozen semen is an essential measure to prevent the venereal transmission of the disease. This system is successfully working in Canada (Garcia et al., 1986).

3.10. Aims of the study

Taking into consideration the spread of *M. bovis* infection all over the world, including Hungary, and the significant economical losses due to it as well as the difficulties in its diagnosis our aims of the study were the following:

1. Evaluate the use of two rapid culture methods for *Mycoplasma bovis*, the selective-differentiating culture with a special medium and the capture ELISA.
2. Production of monoclonal antibodies against *Mycoplasma bovis,* determine their properties, such as type, specificity and their use in the diagnostic work.
3. Evaluate the prevalence of *Mycoplasma 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.
4. Evaluate the efficacy of the antibiotic valnemulin in a challenge trial.
5. Set up a working PCR system for the detection of *Mycoplasma bovis.*

4.
Chapter 1. Rapid diagnosis of *Mycoplasma bovis* infection in cattle with capture ELISA and a selective differentiating medium

4.1. Introduction

The disease caused by M. bovis has been known for several years in Hungary (Romváry et al., 1975) it is very rarely diagnosed. The reason for it is that the respiratory diseases of calves are usually ascribed to respiratory viruses because of the similar clinical and pathological picture. This is confirmed by the fact that the same secondary infective agents (*Pasteurella multocida*, *Mannheimia haemolytica*, *Haemophilus somnus*) change rapidly the clinical picture in both cases.
Numerous mycoplasma (*M. bovis, M. dispar, M. bovirhinis, M. bovigenitalium*) and acholeplasma (*Acholeplasma axanthum, A. modicum, A. laidlawii*) species colonize the bovine respiratory tract (Lauerman, 1994). The presence of these species causes problems in the routine diagnosis of *M. bovis* infection, since they cannot be differentiated by their colony morphology. In addition there can be the cells of more than one species within a single colony. The isolates should be filter-cloned and than identified by their biochemical properties (fermentation of glucose, arginine-hydrolysis, phosphatase test) and than -with the help of specific hyperimmune sera raised in rabbit- the final diagnosis can be stated by growth inhibition, IF or IP tests. This process is usually time-consuming and delays the time of the correct diagnosis.
In this work the application of the „capture" ELISA and the selective isolation of *M. bovis* is presented.

4.2. Materials and methods

4.2.1. Sample collection and handling

From four Hungarian dairy herds infected with Mycoplasma bovis nasal and tracheal swabs and milk samples were collected. Swabs were homogenized in 1ml PBS then 100 µl of this solution was transferred into Medium-B broth containing phenol red and glucose (Ernø and Stipkovits, 1973a). An amount of 100 µl of the milk samples was treated the same way.
Bovine lung samples were collected from slaughterhouses as well. Small pieces from altered lungs were homogenized in PBS and 10% cell suspension was cultured as previously mentioned. Further nasal swab and lung samples were obtained from an animal challenge. These samples were cultured the same way as mentioned before.

4.2.2. Mycoplasma culture

Cultures were incubated at 37 ^{o}C. The slight color change of broth media due to pH shift was examined daily. The cultures were inoculated onto solid Medium-B using the running drop technique on days 2 and 5 (Lauerman, 1994). The agar plates were incubated at 37 ^{o}C in 5% CO_2 atmosphere for 14 days. The plates were examined under stereomicroscope for the detection of mycoplasma growth. Identification of *M. bovis* was performed by IF test (Bradbury, 1998).

4.2.3. Capture ELISA

If growth of mollicutes was observed on the agar plates 20 µl of the corresponding broth cultures were applied to the capture-ELISA plates sensitized with specific M. bovis polyclonal rabbit hyperimmune serum. The first dilution was 1:10 then the samples were diluted to 1:1000 with serial tenfold dilution. All samples were measured in duplicate rows. Fresh broth culture of strain M. bovis „5063", which was isolated from a pneumonic calf lung served as positive control. The negative control was sterile Medium-B. After a 3-day incubation the plates were washed three times with PBS-Tween. The biotinilated Mab 5A10 (obtained from dr. Ball) served as specific antibody. After 1h incubation at 37^{o}C the plates were washed and streptavidin-peroxidase

(Sigma, St. Louis, USA) was pipetted onto them. The incubation was the same as mentioned before. TMB/E (Chemicon International, Temecula, USA) was used as substrate, and the reactions were stopped with 2.5 M H_2SO_4. The OD values were read with a Labsystems Multiskan MS ELISA plate reader at a wavelength of 450 nm. If all dilutions of the sample exceeded 0.4 OD value it was considered as positive. The absorbance of the negative control was always under 0,06 OD value (Ball et al., 1994).

4.2.4. Culturing on selective differentiation medium

Besides this the samples from the challenge experiment were also inoculated onto Mycoplasma bovis diagnostic medium agar plates (Mycoplasma Experience Ltd, UK).
The selective plates were incubated in anaerobe jars (AnaeroGen, Oxoid Ltd, Basingstoke, UK) at 37 ^{o}C for 7 days. The colonies and their color reactions were examined under a stereomicroscope.

4.3. Results

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 IF test. The other isolates belonged to other species. The results of the IF test had been confirmed by the „capture-ELISA" in 100% (Table 4.1.).

Table 4.1. Detection of *Mycoplasma bovis* from clinical samples

	Capture ELISA	Conventional culture
Sample No.	52	52
M. bovis	43	43
Other species	0	9

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. In this case capture ELISA fortified the presence of *M. bovis* too (Table 4.2.).

Table 4.2. Detection of *Mycoplasma bovis* from lung samples of slaughtered animals

	Capture ELISA	Conventional culture
Sample No.	92	92
M. bovis	15	15
Other species	0	0

The results of the samples from the experimental infection are presented in Table 4.3.

Table 4.3. Detection of *Mycoplasma bovis* from challenged animals

	Control group		Infected group	
Method	Before challenge	After challenge	Before challenge	After challenge
Capture-ELISA	0/6	0/6	0/12	10/12*
M. bovis selective culture	0/6	0/6	0/12	12/12
„Mycoplasma positive" with conventional culture	5/6	6/6	8/12	12/12
M. bovis identified with conventional culture, biochemical and serological probes	0/6	0/6	0/12	12/12

*2 samples were contaminated with bacteria

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 (Table 4.4.).

Table 4.4. Specificity of the capture ELISA test

	M. bovis capture ELISA positive
39 other Mycoplasma and Acholeplasma species	0
8 different Mycoplasma bovis strains	8

4.4. Discussion

The *Mycoplasma bovis* infection of cattle can be diagnosed by various methods besides the conventional culture, which requires specially equipped laboratories (Sachse et al., 1993).
Among the immunological methods ELISA test has been used for a long time for herd diagnostics (Uhaa et al., 1994.). This method is also suitable for the antibody detection from milk (Byrne et al., 2000). Contrary to this capture ELISA does not visualize antibodies circulating in the blood, but directly and specifically detects live *Mycoplasma bovis* cells. This method does not cross-react with other Mycoplasma or Acholeplasma species. The bacterial contaminants of the broth cultures mostly do not show false positive reaction except for Staphylococcus aureus where in some cases a slight color reaction can be observed (Ball et al., 1994). Otherwise the contaminated broth cultures can be recognized by their turbidity and the intensive color reaction to the naked eye, and the characteristic bacterium colonies can be observed after streaking onto 5% sheep blood agar. However rarely the bacterial contamination leads to sudden and large-scale change in pH, which can suppress the growth of *M. bovis*. This can be the explanation for the 2 negative results in the infected group.
With the help of the selective media (Shimizu, 1983, Windsor and Bashiruddin, 1999) the infective agent can be detected in one step from the organ samples.
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 within the colonies characteristic red crystals are formed (Figures 4.1., 4.2.). 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 examined *M. bovis* isolates. The inhibitory additives of 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, IF, etc) can be omitted. Both methods are rapid and specific so they can help to set up the rapid diagnosis of *Mycoplasma bovis* infection.

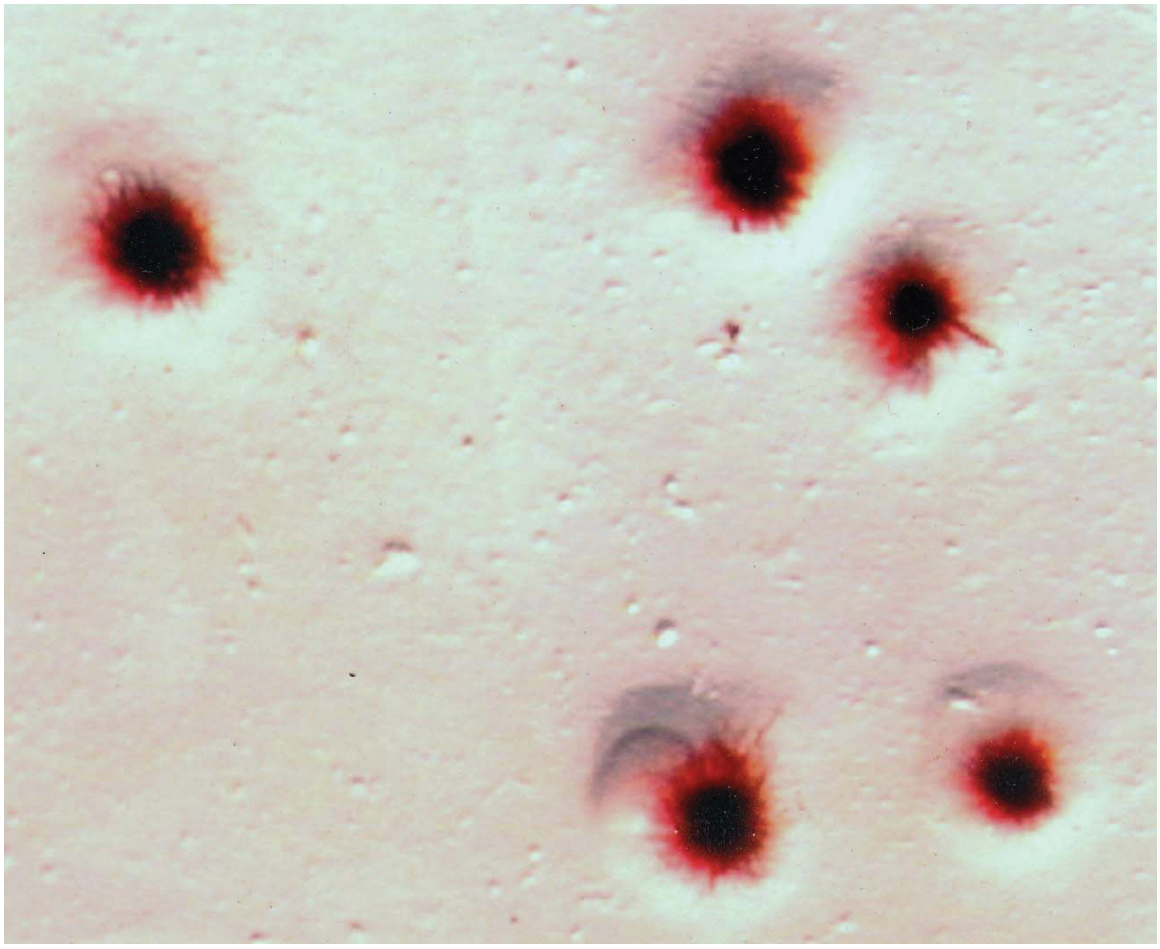

Figure 4.1. *Mycoplasma bovis* colonies on *Mycoplasma bovis* diagnostic medium (10×)

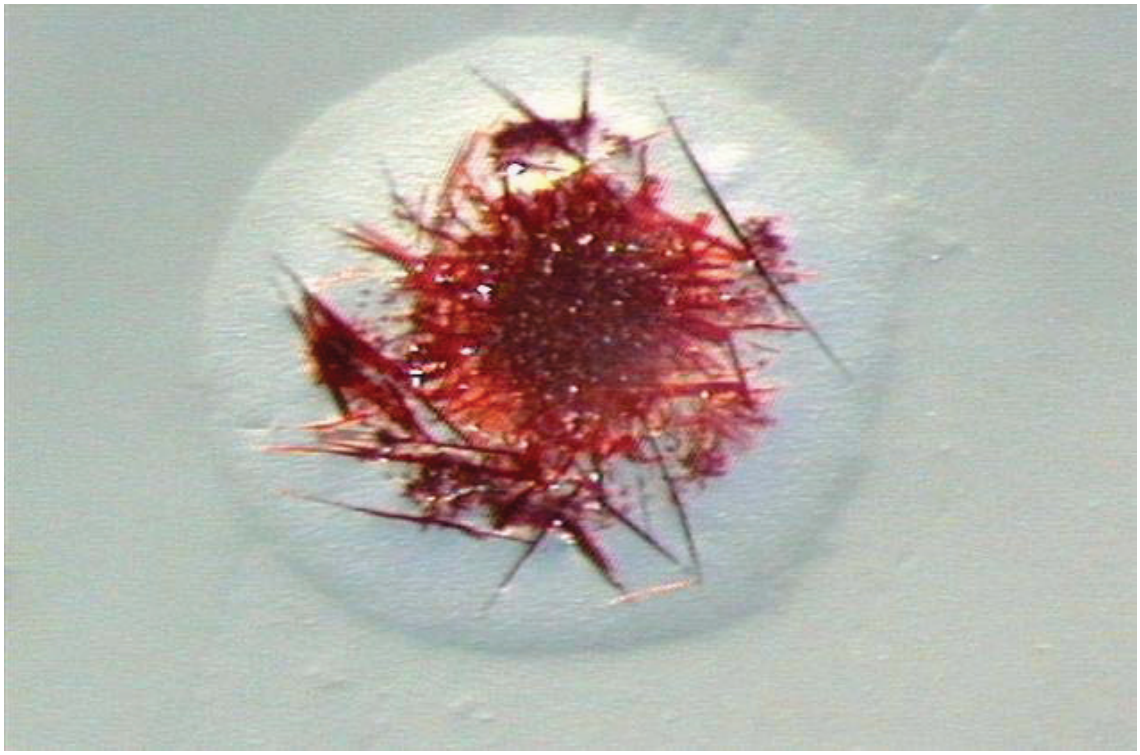

Figure 4.2. Characteristic crystals in a close-up *Mycoplasma bovis* colony (*Mycoplasma bovis* diagnostic medium, 50×)

5.
Chapter 2. Production of monoclonal antibodies recognizing multiple *Mycoplasma bovis* antigens and their testing

5.1. Introduction

Mycoplasma bovis infection is very common in the cattle stocks of Europe and North America (Pfützner, 1990). It causes mastitis (Brown et al., 1990., Horváth et al., 1983, Mészáros et al., 1986) and metritis (Langford et al., 1978), impaired spermatozoan motility (Jurmanova and Sterbova, 1977, Kissi et al., 1985) and meningitis (Stipkovits et al., 1993) in adult bovine animals and pneumoarthritis in calves (Langford, 1976, Romváry et al., 1975). Although the pathogen is easier to culture than other *Mycoplasma* species, its species identification is hampered by several factors. Differentiation from other *Mycoplasma* and *Acholeplasma* species occurring in the upper respiratory tract and genital organs of cattle is one of these difficulties. In view of these facts, development of a diagnostic method based on monoclonal antibodies was set as an objective. As a first step, suitable monoclonal antibodies had to be obtained.

5.2. Materials and methods

5.2.1. Production of *Mycoplasma bovis* antigen

The broth culture of the Hungarian *M. bovis* strain designated 26034 was centrifuged with 20,000 *g*, then the sediment was washed three times in a tenfold volume of PBS and centrifuged. The antigen thus produced was stored at -20 °C until used.

5.2.2. Monoclonal antibodies

Five 8-week-old female Balb/c AnN Crl BR mice (Charles River, Wilmington, Massachusetts, USA) were used for immunization. The mice were inoculated intraperitoneally (i.p.) with the cell suspension (1×109 CFU/ml) of *M. bovis* strain 26034. The mice received 10 µl, 25 µl, 50 µl, 100 µl and 150 µl cell suspension per mouse, respectively. The antigen was used after 1:2 dilution in physiological saline and emulsification with 100 µl complete Freund's adjuvant (Sigma Aldrich Co., St. Louis, MO, USA). On days 33 and 64 after the first injection the mice were inoculated i.p. again with the same doses using incomplete Freund's adjuvant (Sigma Aldrich Co., St. Louis, MO, USA). On day 14 and on day 10 after the two immunizations, respectively, blood samples were taken from the lateral caudal vein of the mice. The antibody level of the blood was determined by an own-developed indirect ELISA procedure. On day 137, after the first immunization, the mouse that gave the best immune response was inoculated intravenously (i.v.) with the antigen diluted 1:2 in physiological saline. Three days after the last immunization the spleen of the mouse was removed aseptically. The fusion of spleen cells with Sp2/0-Ag14 murine myeloma cells (Shulman et al., 1978) was performed in the presence of polyethylene glycol (Sigma Aldrich Co., St. Louis, MO, USA). The obtained hybrid cells were selected with HAT medium (Sigma Aldrich Co., St. Louis, MO, USA). Two weeks after the fusion the supernatants of the grown cells were tested by indirect ELISA. The 12 cell groups selected on the basis of the Western blotting were cloned twice by end-point dilution method. The cloned cells were propagated, then with the cell lines designated 2C9, 3G12, 5E5, 6F11 and 6H10 antibodies were produced in the CELLine bioreactor (Integra Biosciences, Zurich, Switzerland).

5.2.3. SDS-PAGE

To determine the molecular weights of the antigen determinants, the supernatants giving positive reaction in the ELISA were tested by WB as described by Laemmli (1970). For that purpose, the broth culture of *M. bovis* strain designated 26034 was centrifuged with 20,000 *g* and then the sediment was washed three times in a tenfold volume of PBS and centrifuged. The *M. bovis* cell

suspension was lysed with an 1:1 mixture of 125 mM Tris (Reanal, Budapest, Hungary), 10% 2-beta-mercaptoethanol (Serva, Heidelberg, Germany), 20% glycerol (Reanal, Budapest, Hungary), pH 6.8 and 4% SDS (Reanal, Budapest, Hungary). The samples were denaturated in a water-bath of +96 °C temperature for 4 min. For electrophoresis, a 12.5% polyacrylamide gel was prepared from acrylamide:bisacrylamide solution (Bio-Rad Laboratories, Hercules, CA, USA) of 37.5:1 ratio. The denaturated samples were stained with 0.1% bromophenol blue (Reanal, Budapest, Hungary). Sigmamarker Wide Range marker (Sigma Aldrich Co., St. Louis, MO, USA) was used as molecular weight standard. The amount of protein applied on the gel corresponded to the protein content of approximately 10^8 cells.
The samples were electrophoretized at 150 V constant voltage for 3.5 hours in a Protean II apparatus (Bio-Rad Laboratories, Hercules, CA, USA).

5.2.4. Immunoblot

The separated proteins were transferred onto a 0.45 μm nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany) with the help of a Semi-dry Transfer Cell (Bio-Rad Laboratories, Hercules, CA, USA) at adjusted values of 20 V and 380 A in 30 min. Subsequently the nitrocellulose membrane was cut into 3 mm thick strips and the free binding sites were blocked with 2 ml 5% fat-free milk powder solution (1 hour at +37 °C). The cell supernatants were added to the blocking solution in 1:3 dilution. The samples were incubated at +37 °C for 2 hours and subsequently washed twice with PBS, twice with PBS containing 0.05% Tween-20 (Serva, Heidelberg, Germany) and then again twice with PBS. Subsequently, 1 ml of the rabbit anti-mouse IgG (H+L) conjugate (Jackson Immuno Research Labs, Inc., West Grove, Pennsylvania, USA), labelled with peroxidase and diluted 1:500 in PBS, was measured onto the strips. After incubation at +37 °C for 2 hours, the strips were washed again as described previously, then stained with Zymogram solution that contained 4-chloronaphthol (Sigma Aldrich Co., St. Louis, USA) and H_2O_2 as substrate. Finally the molecular weight of the strips giving positive reactions was determined.

5.2.5. Testing of cell supernatants by indirect ELISA

The same antigen of the *M. bovis* strain designated 26034 was used in the ELISA. The working dilution of the antigen was determined by titration in carbonate buffer (pH 9.6) and PBS (pH 7.2). The antigen was diluted with PBS (pH 7.2), then 100-μl aliquots were measured into the wells of the ELISA plate (Analyzer Ltd., Budapest, Hungary). After incubation at +4 °C overnight, the plates were washed three times with PBS washing-diluting buffer containing 0.05% Tween-20 (Sigma Aldrich Co., St. Louis, MO, USA), then 100-μl volumes of the cell supernatants diluted 1:2 with PBS-Tween buffer were measured into the wells. The plates were incubated at +37 °C for 90 min, then washed five times with PBS-Tween buffer. Subsequently 200 μl rabbit anti-mouse IgG (H+L)-HRP conjugate (Jackson Immuno Research Labs Inc., West Grove, Pennsylvania, USA) diluted 1:5000 in PBS-Tween buffer was measured into the wells. After 90-minute incubation at +37 °C, the plates were washed as described above, then the enzyme activity was visualized by the addition of 100 μl of tetramethylene benzidine (TMB; Diavet Ltd., Budapest, Hungary). After incubation for 10 min the reaction was stopped by the addition of 50 μl of 4N H_2SO_4 solution. Evaluation was done with the help of a 450 nm optical filter and a Multiscan Ms reader (Labsystems Oy, Helsinki, Finland). Each plate contained a negative control (RPMI containing 10% fetal serum). During the evaluation of results the samples showing OD values 1.5 times higher than the negative control were considered positive.

5.2.6. Determination of the isotype of antibodies

The antigen made from the *M. bovis* strain designated 26034 (1×10^9 CFU/ml) was diluted 1:300 in carbonate buffer (pH 9.6), then measured into the wells of the ELISA plates Analyzer Ltd., Budapest, Hungary) in 100-μl volumes. After incubation at +4 °C overnight, the plates were washed with PBS-Tween washing-diluting buffer (0.05%) three times. Subsequently, 100-μl

volumes of FCS-free cell supernatants diluted 1:2 with PBS-Tween buffer were measured into the wells. The plates were incubated at +37 °C for 120 min. This was followed by five cycles of washing with PBS-Tween buffer, then the plates were incubated at room temperature for 30 min with 100-µl volumes of 1:800 diluted goat anti-mouse IgG1, IgG2a, IgG2b, IgG3, IgA, IgM (Sigma Aldrich Co., St. Louis, MO, USA) heavy-chain-specific antibodies. After the washing cycles, 100 µl 1:6000 diluted, HRPO-labelled rabbit anti-goat IgG conjugate (Sigma Aldrich Co., St. Louis, MO, USA) was measured into the wells. After 15-min incubation at room temperature the plates were again washed as described above, and then the enzyme activity was visualized by the addition of 100 µl TMB (Diavet Ltd., Budapest, Hungary). After 8-min incubation the reaction was stopped by the addition of 50 µl 4N H_2SO_4 solution. Evaluation was done with the help of a 450 nm optical filter and a Multiscan Ms reader (Labsystems Oy, Helsinki, Finland).

The light chain was determined by indirect ELISA as described above, using HRPO-labeled κ and λ light-chain-specific antibodies raised in rabbits (Agricultural Biotechnology Research Center, Gödöllő, Hungary).

5.2.7. Study of cross-reactions

5.2.7.1. Antigen production

Possible cross-reactions of the produced monoclonal anti-*M. bovis* antibodies with certain *Mycoplasma*, *Ureaplasma* and *Acholeplasma* species were studied by indirect ELISA. To produce antigens for this purpose, the broth culture of *Mycoplasma* strains was centrifuged with 20,000 *g*, washed with PBS three times, and then lysed with the mixture of 10% 2-beta-mercaptoethanol (Serva, Heidelberg, Germany), 20% glycerol (Reanal, Budapest, Hungary) and 4% SDS (Reanal, Budapest, Hungary). The samples were denaturated in 96 °C water-bath for 4 min, and then the protein content of the antigens was determined at a wavelength of UV 280 nm.

5.2.7.2. ELISA

The working dilution of the antigens was determined by titration in different buffers. The working dilution of the monoclonal antibodies under test was also determined in a similar manner. The antigens were diluted with PBS (pH 7.2) in 2.5 µg/ml concentration, then measured into the wells in two parallel rows in 100-µl volumes. After incubation at +4 °C overnight, the plates were washed three times with PBS-Tween washing-diluting buffer. Subsequently, 100-µl volumes of cell supernatant diluted 1:15 in PBS-Tween buffer and, as negative control, RPMI containing 10% fetal serum were measured into the wells in parallel. The plates were incubated at +37 °C for 90 min. After five cycles of washing in PBS-Tween buffer, 200 µl rabbit anti-mouse IgG (H+L)-HRPO conjugate (Jackson Immuno Research Labs, Inc., West Grove, Pennsylvania, USA) was measured into the wells in 1:5000 dilution. The plates were incubated at +37 °C for 90 min. Further on the steps of the indirect ELISA procedure, discussed under the testing of cell supernatants, were followed.

5.2.7.3. Evaluation of the ELISA results

During the evaluation of results the intensity of the cross-reactions was expressed as a ratio of the OD values given by the antibody under test and the negative control. If the $OD_{supernatant}$ / $OD_{negative\ control}$ ratio was between 2 and 3, the cross-reaction was considered weak (+). Ratios between 3 and 5 indicated a moderate (++) while those above 5 a strong (+++) cross-reaction (Table 5.1).

Table 5.1. Cross-reactions of monoclonal anti-*Mycoplasma bovis* antibodies tested by indirect ELISA (2C9, 3G12, 5E5, 6F11, 6H10, 4H9, 1C7, 5B8, 2C5, 5D3, 5C11, 1B11) with certain *Mycoplasma* (M.), *Ureaplasma* (U.) and *Acholeplasma* (A.) species

	2C9 69 kDa	3G12 67,69 72 kDa	5E5 22, 25, 27 kDa	6F11 63 kDa	6H10 22, 25, 27 kDa	4H9 63kDa	1C7 63 kDa	5B8 22, 25, 27 kDa	2C5 22, 25, 27 kDa	5D3 22, 25, 27 kDa	5C11 69 kDa	1B11 63 kDa
M. bovis 26034	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
M. bovis 5063	+++	+	+++	+++	+++	++	–	+++	+++	+++	–	–
M. bovis Donetta	+++	++	+++	+	+++	–	–	+	+	+++	–	–
M. mycoides ssp. capri PG3	–	–	–	–	–	–	–	–	–	–	–	–
M. dispar 10125	–	–	–	–	–	–	–	–	–	–	–	–
M. bovirhinis PG 43 (F3)	–	–	–	–	–	–	–	–	–	–	–	–
M. californicum	–	–	–	–	–	–	–	–	–	–	–	–
A. laidlawii 10116	–	–	–	–	+	–	–	–	–	+	–	–
M. bovigenitalium F7	+	–	+++	+	+++	–	–	–	–	+++	–	–
M. gallinarum B 142 P	–	–	–	–	–	–	–	–	–	–	–	–
M. modicum 292	–	–	–	–	+	–	–	–	–	–	–	–
M. oculi	++	–	++	+++	+	–	–	–	–	–	–	–
M. ovipneumoniae	–	–	–	–	–	–	–	–	–	–	–	–
M. arginini	–	–	–	–	–	–	–	–	–	–	–	–
M. oculosi	–	–	–	+	+	–	–	–	–	–	–	–
A. axanthum	–	–	–	–	+	–	–	–	–	–	–	–
M.gateae B 139 P	–	–	–	–	+	–	–	–	–	–	–	–
M. sp. 7 Leach	–	–	–	+	+	–	–	–	–	–	–	–
M. alcalescens PG 51	–	–	–	–	+	–	–	–	–	–	–	–
M. anseris 8389	++	–	+	+	+++	–	–	–	–	++	–	–
M. glycophilum	–	–	–	–	++	–	–	–	–	–	–	–
M. hominis PG 21	–	–	–	–	–	–	–	–	–	–	–	–
M. fermentans PG 18	–	–	–	+++	+++	–	–	–	–	–	–	–
M. orale	–	–	–	–	–	–	–	–	–	–	–	–
M. salivarum	–	–	–	–	–	–	–	–	–	–	–	–
M. arthritidis	–	–	–	–	++	–	–	–	–	–	–	–
U. sp. T 917	–	–	–	–	–	–	–	–	–	–	–	–
U. sp. 1004	–	–	–	–	–	–	–	–	–	–	–	–
U. sp. 5	–	–	–	++	+	–	–	–	–	–	–	–
M. equifetale 868	+	–	–	+	+	–	–	–	–	–	–	–
M. neurolyticum	–	–	–	–	–	–	–	–	–	–	–	–
M. leonis	–	–	–	–	–	–	–	–	–	–	–	–
M .sp. N 3	–	–	–	–	–	–	–	–	–	–	–	–
M. conjunctivae	+	–	–	–	+	–	–	–	–	–	–	–
M. molare	–	–	–	+	+	–	–	–	–	–	–	–
M. maculosum	–	–	–	–	++	–	–	–	–	–	–	–
M. gallisepticum S6 Holland	+	–	+++	+	+	–	–	–	–	–	–	–
M. canadense	–	–	++	–	+++	–	–	–	–	++	–	–
M. spumans	–	–	–	–	–	–	–	–	–	–	–	–
M. agalactiae	+	–	–	–	–	–	–	–	–	–	–	–

Intensity of the reaction:
+++ - strong
++ - medium
+ - weak
– - no reaction

5.2.8. Immunhistochemistry

For these investigations an udder sample from cow experimentally challenged with *M. bovis* (obtained from dr. Stipkovits) was used, which showed marked alterations. For examining the sections plates prepared with 2% silane solution (Sigma Aldrich Co., Hungary) were used. The reaction was performed in a capillary system (Sequenza Immunostaining Center, Shandon Co., UK). From the formalin-fixed, paraffin-embedded tissue 4 μm-thick sections were made. After deparaffinization the antigen digestion was performed with 0.1% Protease XIV solution (Sigma Aldrich Co., Hungary) at room temperature for 5 min. The samples were incubated with 3%

H_2O_2 at room temperature for 10 min, then after washing with PBS the next incubation step was in 2% skim milk powder solution for 20 min at ambient temperature. Then the sections were incubated with 1:100, 1:1000, 1:10 000 diluted anti-*M. bovis* Mabs overnight at room temperature. A section incubated in PBS served as negative control. The antigen-antibody attachment was detected with a HRPO-labeled streptavidin-biotin kit (Universal LSAB2 Kit-HRP, Dako Co., Denmark), whereas 3-amino-9-ethylcarbazole (Sigma Aldrich Co., Hungary) served as chromogen. The sections were counter-stained with Mayer hematoxilin for 20 seconds and covered with glycerol-gelatin and viewed at 100-400× magnifications.

5.3. Results

5.3.1. Produced Cell lines and the type of the antibodies

Among 1658 hybridomas produced as a result of a fusion, a total of 63 cell lines producing anti-*Mycoplasma bovis* antibodies were found. Of them, the antibodies produced by the tested 1B11, 1C7, 2C5, 2C9, 3G12, 4H9, 5B8, 5C11, 5D3, 5E5, 6F11 and 6H10 cell lines were assigned to the heavy-chain subclass IgG1. Cell lines 1B11, 1C7, 2C5, 4H9, 5B8, 5C11, 5D3, 5E5, 6F11 and 6H10 produce a κ while cell lines 2C9 and 3G12 a λ light-chain antibody.

5.3.2. Recognized antigen determinants

According to WB studies, the monoclonal antibodies recognize the antigen determinants of the following molecular weights: 1B11: 63 kDa, 1C7: 63 kDa, 2C5: 22, 25 and 27 kDa, 2C9: 69 kDa, 3G12: 67, 69 and 72 kDa, 4H9: 63 kDa, 5B8: 22, 25 and 27 kDa, 5D3: 22, 25 and 27 kDa, 5C11: 69 kDa, 5E5: 22, 25 and 27 kDa, 6F11: 63 kDa, and 6H10: 22, 25 and 27 kDa (Figure. 5.1.).

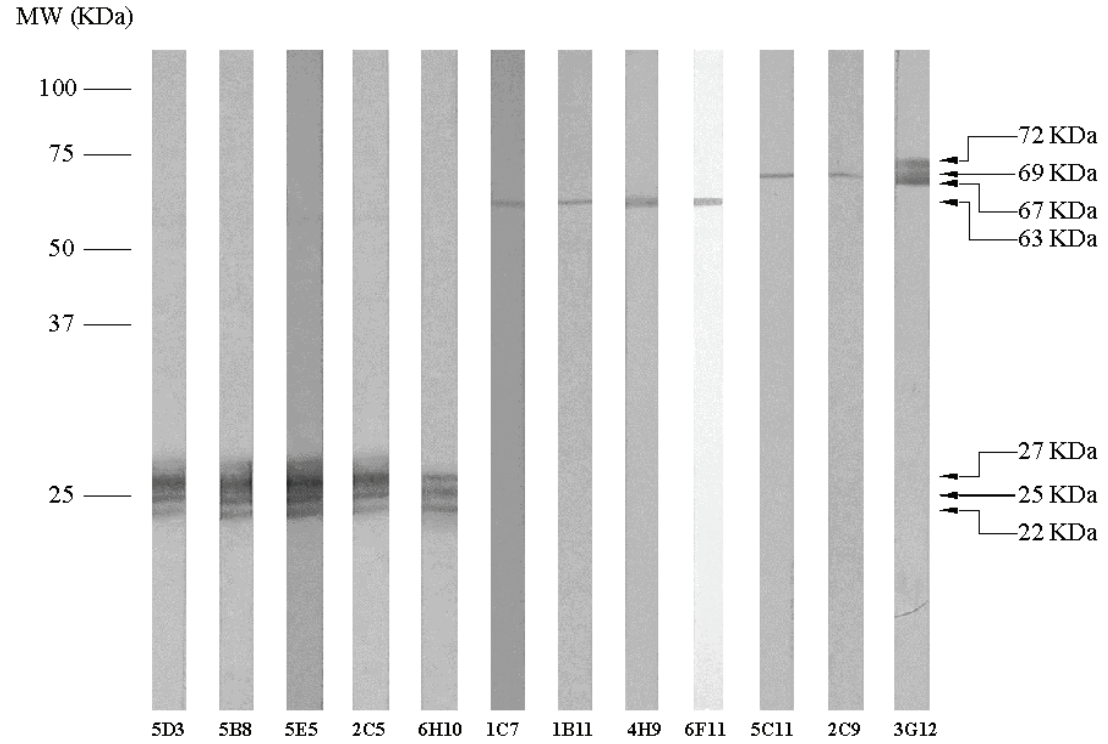

Figure 5.1. Immunoblotting analysis of *Mycoplasma bovis* field strain 26034 with different monoclonal antibodies.

5.3.3. Productivity of cell lines

Of the 5 hybridomas produced in the bioreactor, cell line 3G12 showed the highest productivity, with an average immunoglobulin output of 1.5 mg per day. Cell line 5E5 produced 1.1 mg, 6H10 0.8 mg, 2C9 0.47 mg and 6F11 0.4 mg antibody per day.

5.3.4. Cross-reactions

During the study of cross-reactions (Table 5.1.), all of the 12 antibodies tested reacted with the antigen of *M. bovis* strain designated 26034. Monoclonal antibodies 1C7, 1B11 and 5C11 recognized only the homologous antigen and did not recognize other strains belonging to the *M. bovis* species. Monoclonal antibodies 3G12 (67, 69, 72 kDa) and 5B8 (22, 25, 27 kDa) did not give cross-reaction with antigens other than those of strains belonging to the *M. bovis* species. In contrast, monoclonal antibody 6H10 reacted with 20 strains belonging to other species, in addition to the *M. bovis* strains. The other monoclonal antibodies most frequently reacted with the *M. bovigenitalium F7, M. sp 8389, M. oculi* and *M. gallisepticum S6* and less often with the *M. canadense, M. conjunctivae, M. oculosi, A. laidlawii 10116, M. fermentans PG 18, M. equifetale 868* and *M. molare* antigens. None of the monoclonal antibodies gave a cross-reaction with the *M. agalactiae* antigen except for Mab 2C9.

5.3.5. IH testing

The results are presented in Table 5.2. The antigen could be observed as small dots situated intra- or extracellularly. The marginal zone of the extensive necrotic areas contained particularly big amounts of antigen (Figure 5.2. and 5.3.). The infective agent could be detected in the inflammated acini (Figure 5.4.) and rarely in the macrophages (Figure 5.5.).

Table 5.1. Results of the IH testing of the produced monoclonal antibodies

Mab	1:100	1:1000	1:10 000
6H10	±	±	++
6F11	±	±	+++
4H9	±	–	++
1C7	±	+	–
5C11	–	±	–
3G12	±	±	–
1B11	±	–	–
2C5	–	–	±
5E5	–	–	–
5D3	–	–	–
2C9	–	–	–

–: no reaction, ±: some weak reaction,
+: some moderate reaction sporadically
++: moderate reactions in numerous sites
+++: strong reactions in numerous sites

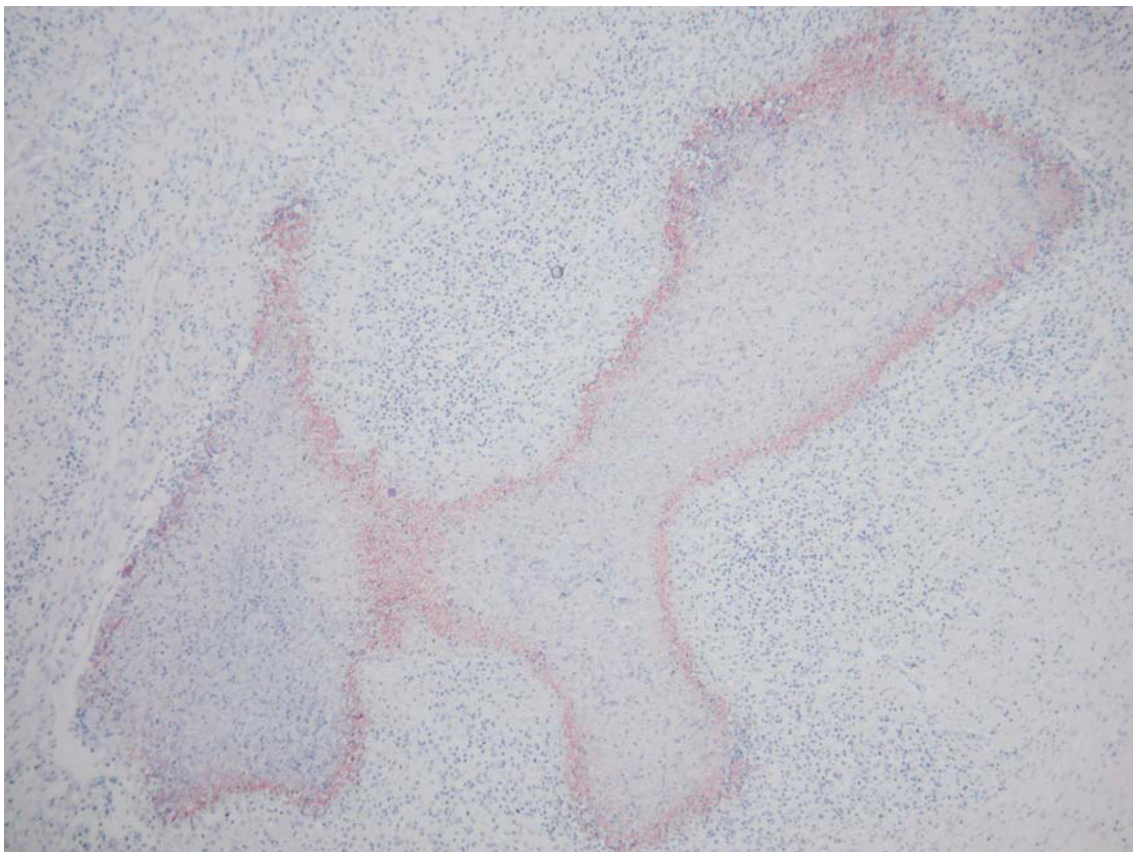

Figure 5.2. IH reaction with Mab 4H9: Large necrosis. 40×

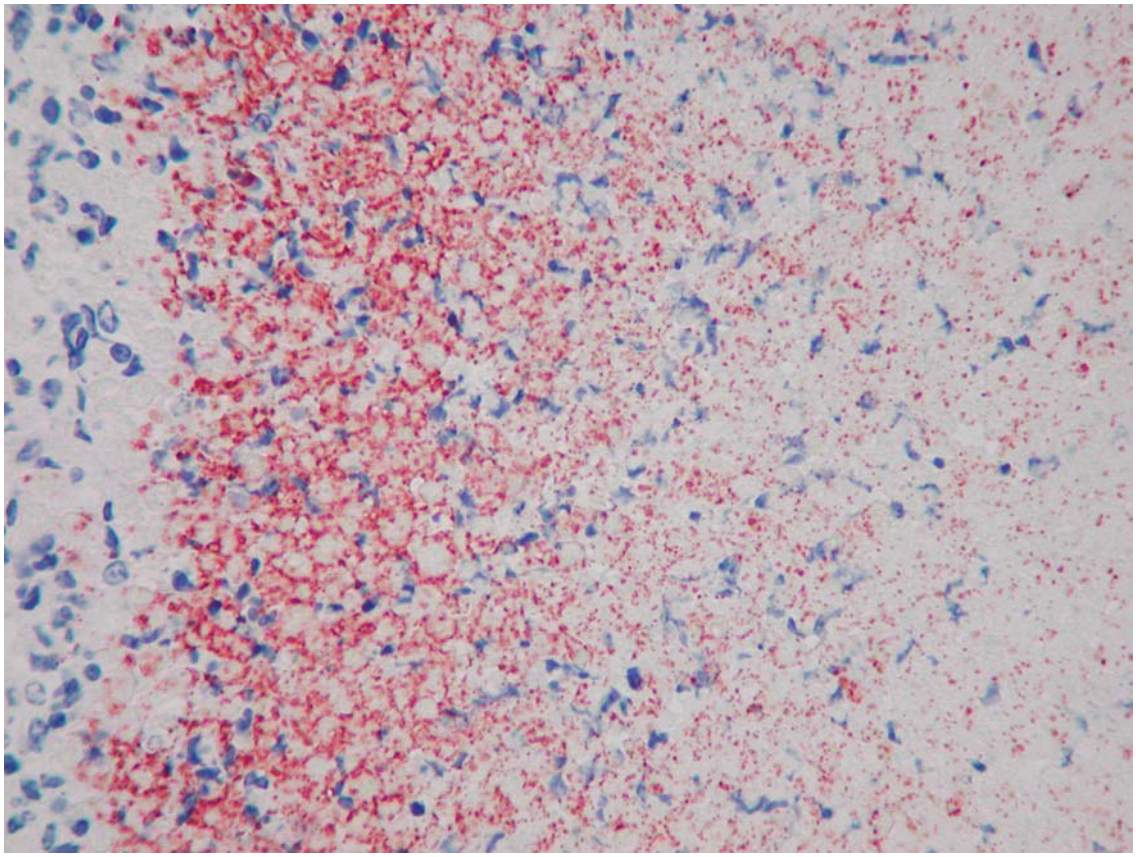

Figure 5.3. IH reaction with Mab 4H9: The edge of the necrosis. 400×

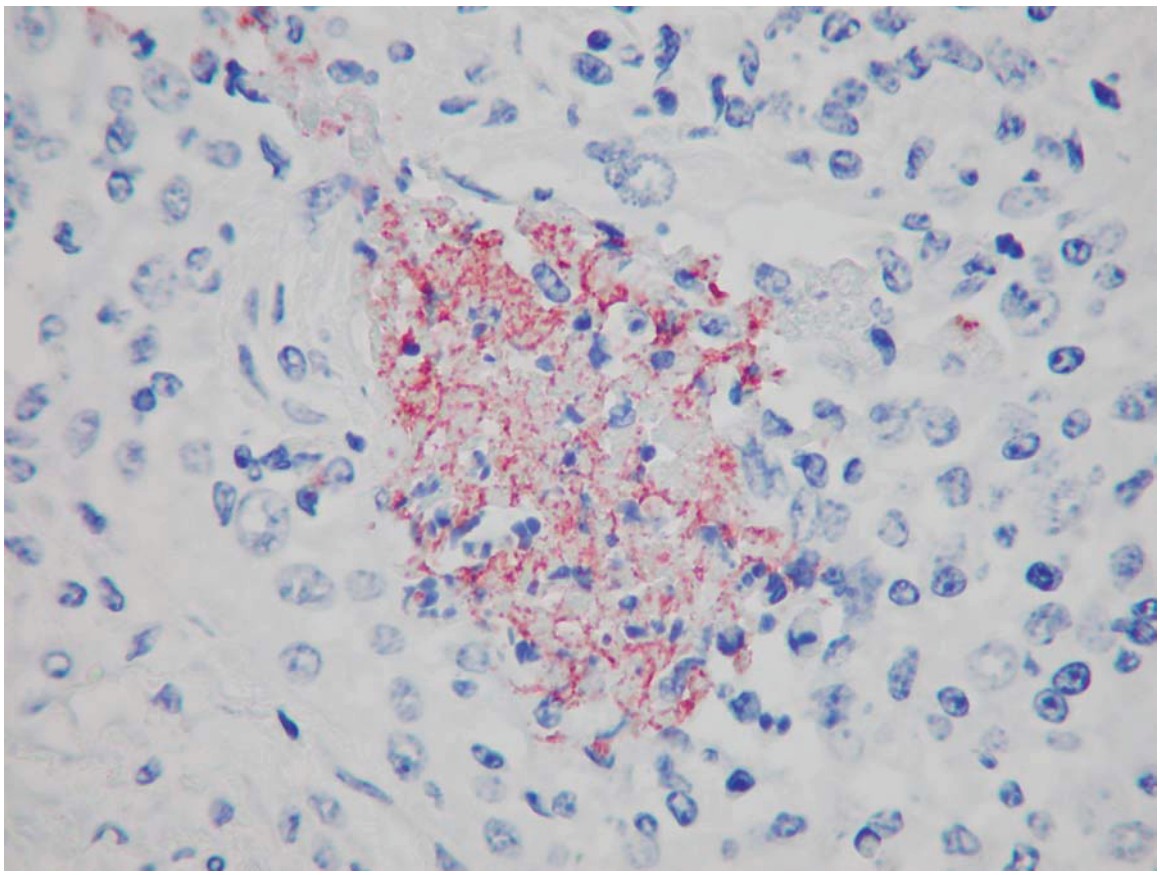

Figure 5.4. IH reaction with Mab 4H9: The antigen in the acynus. 400×

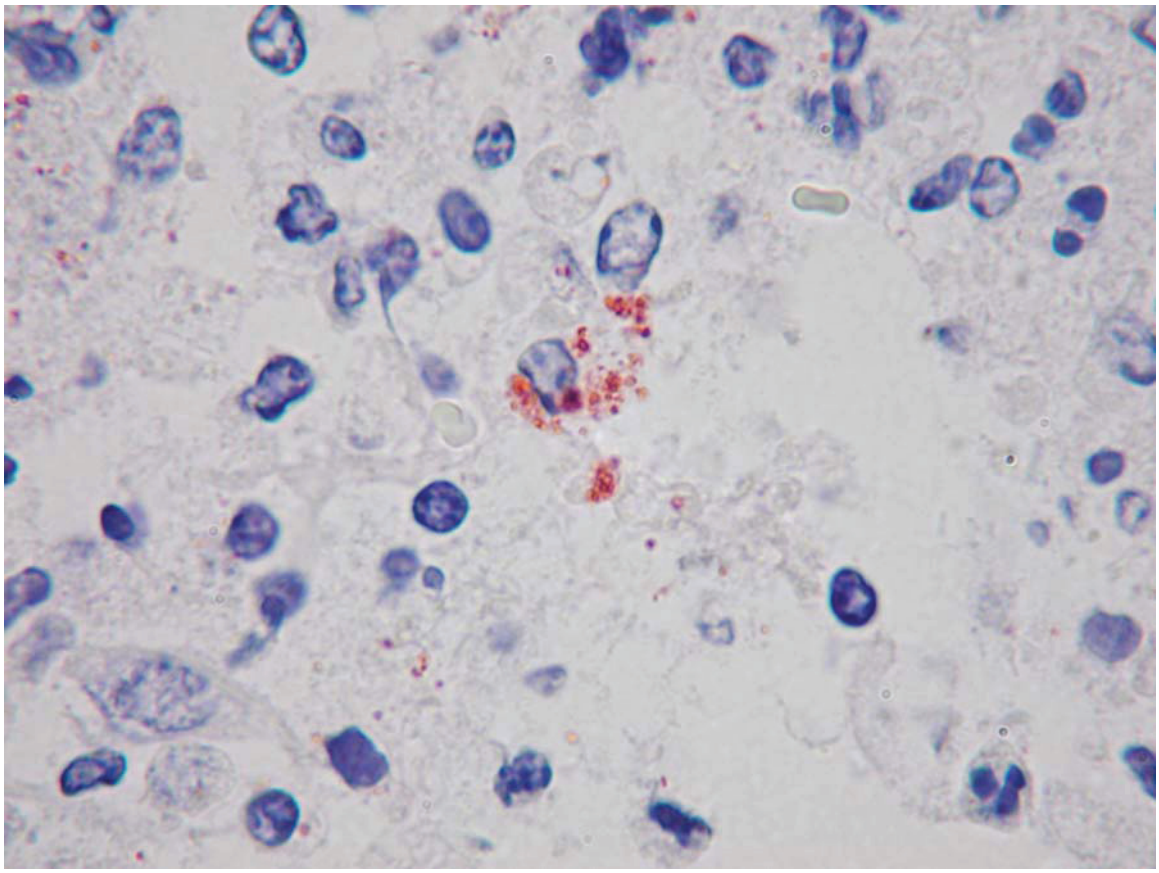

Figure 5.5. IH reaction with Mab 4H9: The antigen in the macrophage. 1000×

5.4. Discussion

The present studies have confirmed the data published in the international literature in numerous respects. Namely, it has been found that a certain proportion of monoclonal antibodies produced against the *M. bovis* antigen react with antigens prepared from other *Mycoplasma* species (Berthold et al., 1992, Rasberry and Rosenbusch, 1995). It has been confirmed that certain epitopes of *M. bovis* are related to the antigen determinants of *M. gallisepticum.* According to Yogev et al. (1994), there is a genetic similarity between the so-called PvpA membrane protein of *M. gallisepticum* and the variable surface lipoproteins (Vsps) of *M. bovis*, which can explain the cross-reaction observed.

At variance with the earlier findings (Boothby et al., 1981, Berthold et al., 1992, Rasberry and Rosenbusch, 1995), during the ELISA analysis of monoclonal and polyclonal antibodies produced against *M. bovis* the monoclonal antibodies produced in this study, with the exception of 2C9, did not react with the antigen of *M. agalactiae* otherwise biologically and biochemically closely related to *M. bovis* (Berthold et al., 1992, Rasberry and Rosenbusch, 1995). This is not surprising, as by DNA-DNA hybridization Razin and Freundt (1984) found only 40% similarity between *M. agalactiae* and *M. bovis*.

Furthermore, the results of the present study also confirm the earlier data (Poumarat et al, 1994) indicating that monoclonal antibodies can differentiate between strains belonging to the same species, in the present case to the species *M. bovis*. These results could be obtained as a result of the successful production of antibodies against a wide scale of *M. bovis* antigen determinants (p22, p25, p27, p63, p67, p69, p72).

Amongst monoclonal antibodies in our possession the Mab against the 27kDa antigen determinant of *M. bovis* responsible for adherence to cells (Sachse et al, 1993). This surface glycoprotein enables *M. bovis* bacteria to adhere to alveolar phagocytes (macrophages) (Howard et al. 1976) and to neutrophilic granulocytes (Thomas et al., 1991).

Our cell lines 1B11, 1C7, 4H9 and 6F11 are also considered valuable. These hybridomas recognise the antigen determinant of 63 kDa molecular weight. According to data of the special literature (Behrens et al., 1994 Rosengarten et al, 1994, Beier et al., 1998) the above-mentioned antigen - belonging to the so-called variable surface lipoproteins (Vsps) - is closely related to the polymorphism of *M. bovis*. The family of the Vsp antigens comprises three members: VspA, VspB and VspC (Behrens et al., 1994 Rosengarten et al, 1994, Beier et al., 1998). The size variability of Vsps antigens is related to the appearance of new epitopes and to the reduction of the number of epitopes. These antigens play a role in adhesion to cells and influence pathogenicity and immunogenicity (Sachse et al., 2000). The repeat sequences consist of 6-87 amino acids and possess active units, each of which has specific biological functions (Lysnyansky et al., 1996, 2000). The produced Vsps-specific antibodies allow us to study the host cell - *M. bovis* relationship, the differences between the isolated strains and their association with virulence.

In addition to the Vsp lipoproteins occurring in the membrane of *M. bovis,* the membrane protein of 67 kDa molecular weight (pMB67) also has an important role: it induces antibody response during natural infection or disease and may be suitable for the development of vaccines of diagnostic preparations (Behrens et al., 1996). It should be mentioned here that the present work yielded a cell line (3G12), which produces antibody against the pMB67 protein in addition to the p69 and p72 antigen determinants, and that antibody probably recognizes a common epitope. It has high specificity and affinity, and thus may be suitable for immunodiagnostic tests in infections caused by *M. bovis.*

With the IH studies it had been proved that the antibodies 6H10, 6F11 and 4H9 are suitable for the in situ recognition of the *M. bovis* antigen so they can be adapted for the confirmation of the presence of this Mycoplasma in the tissues.

Further studies are needed whether some of the produced antibodies are suitable for other immuno-diagnostic tests, such as IF or capture ELISA.

6.
Chapter 3. Examination of the role of *Mycoplasma bovis* in bovine pneumonia and a mathematical model for its evaluation

6.1. Introduction

Mycoplasma bovis is widely spread and causes major economic losses in cattle herds in Europe and North America (Boothby et al., 1983, Rebhun, 1995, Burnens et al., 1999). This organism can causes severe respiratory disease polyarthritis in calves (Pfützner and Sachse, 1996). It was isolated from other alterations such as subcutaneous abscesses, otitis and meningitis being associated frequently with respiratory disease (Kinde et al., 1993, Stipkovits et al., 1993; Walz et al., 1997). *M. bovis* infection can be diagnosed by a variety of methods including isolation of the agent, immunohistochemical staining, use of a specific PCR probe in lung samples and detection of specific antibodies in the serum (Boothby et al., 1981, Brys et al., 1992, Sachse et al., 1993, Uhaa et al., 1990*a, b, c*, LeGrand et al., 2002). Awareness of the serological status of the herd is important for preventing economic losses by control measures (Brys et al., 1992, Pfützner and Sachse, 1996). The aim of this study was to examine the occurrence of pneumonia, the isolation of *M. bovis* from the lungs and the presence of its specific antibodies in sera of cattle originating from different farms of Hungary and to study the relationship among them.

6.2. Materials and methods

6.2.1. Sample collection and handling

Blood and lung samples were collected from 595 cattle originating from 34 large dairy herds with approximately 300 animals per herd. Sample collection was done at random. Most of the animals were adult cows. The carcasses were subjected to normal meat inspection and any pathological changes of the respiratory tract were recorded. From the clotted blood samples sera were separated from the blood cells by centrifugation at 500 *g* for 5 min and stored at -20 °C until used. Tissue samples were collected from the altered parts of the lungs. The lung samples were transported to the laboratory at 4 °C and cultured within 4 h.

6.2.2. ELISA testing

Sera were examined with CHECKIT Mycoplasma Bovis Sero ELISA kit (Bommeli AG, Liebefeld-Bern, Switzerland). The test was carried out according to the instruction manual. Serum samples as well as positive and negative control sera were diluted 1:100 with CHECKIT diluting solution. Two hundred µl of the diluted sera were distributed into the wells of sensitized plates and incubated at room temperature for 90 min. After the incubation period the plates were washed 3 times with CHECKIT washing solution. Two hundred µl of the included peroxidase labelled anti-ruminant IgG conjugate (Bommeli AG, Liebefeld-Bern, Switzerland) was added to the wells in a dilution of 1:600 and incubated as described above. After washing off the excessive conjugate the reaction was visualized with 200 µl of CHECKIT chromogen solution. The reaction was stopped after 15 min with 50 µl of CHECKIT stop solution. The absorbance values were read at 405 nm with a Titertek Multiscan MS plate reader. The OD% value was calculated from the measured OD values of the samples and the negative and positive sera as follows:

$$[(OD_{sample} - OD_{negative\ serum}) / (OD_{positive\ serum} - OD_{negative\ serum})] \times 100 = OD\%$$

According to the description of the original kit the animals having OD% values between 60 and 80% are suspicious of *M. bovis* infection and a value above 80% is a clear sign of infection. Since our aim was to detect any trace of *M. bovis* antibodies, OD% values above 60% were regarded as positive.

6.2.3. Mycoplasma culturing

Lung samples were cultured in liquid Medium 'B' (Ernø and Stipkovits, 1973*a, b*) according to the method described by ter Laak et al. (1992*c*). The inoculated tubes were incubated at 37 °C and plated on days 3 and 7. The plates were incubated at 37 °C in 5% CO2 atmosphere and were examined under a stereomicroscope every 2 days. Mycoplasmas were identified by their colony morphology, biochemical characteristics and immunofluorescence as described by Bradbury (1998).

6.2.4. Statistical evaluation of data

The animals examined were divided into five groups based on the ranges of OD% values (< 20, 20-40, 40-60, 60-80 and 80% <) of their sera. Based on the data of presence or absence of pneumonia (P) and the *M. bovis* culture (C) the animals were divided into four groups (P+C+, P+C-, P-C+ and P-C-). A total of 20 groups were formed on this basis. Inside the groups formed on the basis of pneumonia and *M. bovis* culture the average, empirical and relative deviations of OD% values were calculated. The values of empirical deviation by all pairs of groups were compared by F-test and the averages were compared by Student's *t*-test in all possible combinations. Within the four groups mentioned above the relative frequencies of animals in each OD% range were calculated. The frequency distributions in all cases were checked by chi-squared tests. The rates of pneumonic animals were also examined in all OD% ranges.

6.2.5. Application of the probability model

Probability modelling was used to examine the association among pneumonia and the results of *M. bovis* culture and serology. The approach of modelling resembles the ones described by Fahim et al. (2003), Fodor and Kovács (2003), Ladányi et al. (2003) and Máthé-Gáspár and Kovács (2003). During the interpretation of data methods of Tamás (2003) were followed. We used a method based on optimization with the solver algorithm of Microsoft Excel for fitting the model (i.e. solving the described system of equations).

6.3. Results

6.3.1. Observed data

Out of the 595 animals, 202 (33.9%) had pneumonic lesions, 220 (37.0%) were positive by *M. bovis* culture, and serum samples of 67 cattle (11.3%) were positive for *M. bovis* antibodies (Table 6.1.). 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% (22 out of 34).

Table 6.1. Distribution of the animals according to examined parameters (ELISA, pneumonia and culture; observed and predicted values)

ELISA/ Culture/ Pneumonia	Observed	Predicted by model
+/+/+	18	17,81
+/-/+	4	4,04
+/+/-	21	20,96
+/-/-	24	24,27
-/+/+	103	103,05
-/-/+	77	77,4
-/+/-	78	78,28
-/-/-	270	270,55
Number of positive animals		
ELISA	67	67,11
CULTURE	220	220,13
PNEUMONIA	202	202,16

6.3.2. Statistical evaluation

The relative frequencies of animals divided into 20 groups based on the ranges of OD% values and the results of observed pathological lung changes and *M. bovis* culture are shown in Table 6.2. It can be seen that the relative frequencies of animals (54.73; 49.75 and 47.58) are the highest in the 0-20, 20-40 and 40- 60 OD% ranges in the group where there is no pneumonia and the mycoplasma culture is negative. These OD% values are not signs of *M. bovis* infection. At the same time, in the 60-80 OD% range and above the 80 OD% range - which point to *M. bovis* infection - the relative frequencies of animals were higher in those groups where the result of mycoplasma culture was positive (P-C+ and P+C+), as compared to the groups where the isolation was negative. The average of the OD% values was the lowest where there was no pneumonia and *M. bovis* culture was also negative, whereas in the other groups the average value was higher (Table 6.3). None of the group pairs (e.g. P+C+ and P+C-, etc.) had equal empirical deviations of OD% values (p = 95%) by the F-test, and the examination of the averages of OD% values of all groups led to similar results by Student's *t*-test.

Table 6.2. Relative frequencies (%) of individuals in certain case groups within the OD% ranges

OD%	P+ C+	P+ C-	P- C+	P- C-	Total
0-20	21,39	9,45	14,43	**54,73**	100
20-40	17,73	16,75	15,76	49,75	100
40-60	19,35	**19,35**	13,71	47,58	100
60-80	20,00	5,00	30,00	45,00	100
80-	**37,04**	7,41	**33,33**	22,22	100

P=pneumonia
C=mycoplasma culture
+= presence
-= absence

Table 6.3. Base statistical values of OD% in groups of animals

	P+ C+	P+ C-	P- C+	P- C-
Average	33	33	40	29
Empirical deviation	26	20	29	21
Relative deviation	1,27	1,62	1,38	1,40

P=pneumonia
C=mycoplasma culture
+= presence
-= absence

The empirical deviations in group pairs P+C- and P+C+, P-C- and P+C+, P-C+ and P+C-, as well as P-C- and P-C+ were significantly different (F values < 5%), in P-C+ and P+C+ they were non-significantly different (F value: 33%), while in group pairs P-C- and P+C- they were non-significantly equal (F value: 69%). As regards the group averages, only the P-C- and P-C+ pairs showed a significant difference by Student's *t*-test. The P+C- and P+C+ pair showed nonsignificant equality (*t* value: 90%), whereas the other pairs exhibited nonsignificant difference (*t* values between 5 and 50%) at this level of probability. The rate of pneumonic animals in the different OD% ranges can be seen in Figure 6.1. In the 'negative' OD% ranges (< 60%) this rate is between 0.3 and 0.4, while in the 'positive' range between 60 and 80 OD% this rate is only 0.25 but in the range above 80% it is 0.44. The explanation of this apparent discrepancy will be given later (hypothesis and modeling). For a deeper analysis of this phenomenon we examined the relative frequencies of individuals in OD% ranges by groups based on pathological lung changes and *M. bovis* culture (Figure 6.2.). None of the frequency distributions of the different group pairs were found to be equal by chi-squared test (p = 95%).

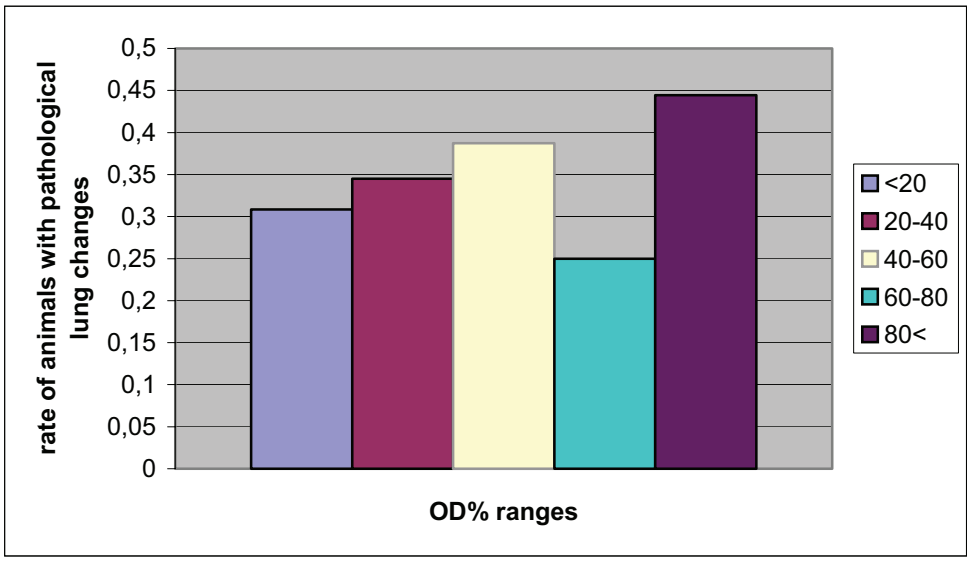

Figure 6.1. The rate of animals with pneumonia within the ranges of OD%

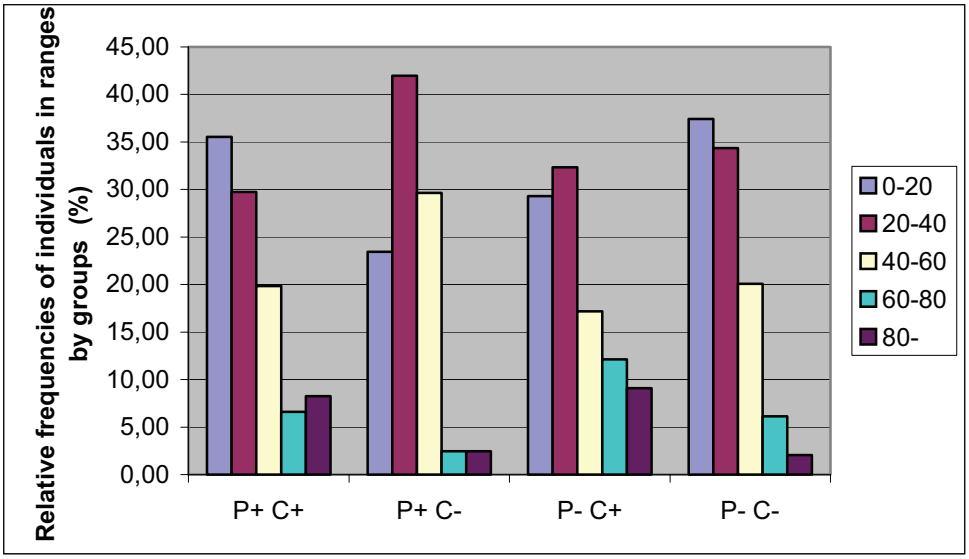

Figure 6.2. Relative frequencies (%) of individuals in ranges of OD% by groups based on pathological lung changes and Mycoplasma culture

P=pneumonia
C=mycoplasma culture
+= presence
-= absence

6.3.3. Hypothesis

As it can be seen in Table 6.1., we found positive animals with all of the three parameters (pneumonia, culturing of *M. bovis* and ELISA test) but there were other cases in high numbers where there was no pneumonia and the animals were negative by ELISA but they were positive by culture. In other cases the ELISA test was positive and in spite of this mycoplasma culture lead to negative results. Sometimes both parameters were positive. These data obviously need further explanation.
For one of the possible interpretations of the relationship among pneumonia, *M. bovis* culturing and the results of *M. bovis* serology using modeling we set the following hypotheses for testing:

1. To our hypothesis in the background of the observed distribution of the examined parameters (pneumonia, culture and ELISA) the following causes were involved:

> **Group α:** *Mycoplasma bovis* generating antibodies, which can be detected with the *M. bovis* specific ELISA test used and can be detected by culturing
> **Group β:** *M. bovis*, which cannot be detected with specific ELISA test
> **Group γ:** Other pathogen bacteria (e.g. Pasteurella multocida or Haemophilus somnus) or viruses (e.g. Bovine adenoviruses, Parainfluenza-3) causing pneumonia, which cannot be detected by *M. bovis* specific ELISA or by mycoplasma culturing methods.

Antibodies against microorganisms in Group α can be detected by the specific *M. bovis* ELISA test, whereas Group β and Group γ are not detectable this way.
Group α and Group β can be detected by *Mycoplasma bovis* culture if they are present at a detectable amount -above the threshold of sampling and examination- whereas Group γ not.
Group α, Group β and Group γ can be involved in pneumonia if they are present above the threshold of pathogenicity.

2. The frequency of distributions of the mentioned microbe groups in the examined population are marked with the signs “a”, “b”, “c”, “d”, “e” and “f” in Table 6.4. The signs 0, I, II and III are quantitative categories meaning: 0<I< II<III.

The quantity I of Group α is marked with "a", which means ELISA positive cases. The quantity II of Group α is marked with "b", which means ELISA and culture positive cases. The quantity III of Group α is marked with "c", which contains ELISA, culture and pneumonia positive cases. If Group α is not there or it is below the threshold of detection then this rate is "1-(a+b+c)" because there is no other possibility. Since Group β cannot be detected by ELISA test so here the 0 and I quantitative categories cannot be present. The category II of Group β is marked with "d", the III with "e". Consequently the summarized frequencies of Group β 0 and I are "1-(d+e)". Since Group γ cannot be detected with any of the mentioned mycoplasma-specific methods the only sign of their presence is the pneumonia. Category III of Group γ is marked with "f"-el, below this with "1-f".
Microorganisms of Groups α, β and γ in category III are capable to cause pneumonia per se.
Either microorganisms of Groups α and β in category II are present at that quantity in which they can be detected by *Mycoplasma bovis* culture, but they do not trigger pneumonia alone.
In category I Group α can be detected by specific ELISA test but not with *Mycoplasma bovis* culture. It does not cause pneumonia alone.
In category 0 none of these groups can be detected by the mentioned methods so they were not present by our aspect.

3. Signs "a", "b", "c", "d", "e", and "f" within the groups mean the rate of animals (between 0 and 1) in each category. The sum of them in each row is 1 so they form a complete event in each group.
4. As it can be seen in Figure 6.1. in the ranges above 60 OD% (which are considered positive) the rate of pneumonic animals in the relatively lower and higher OD% ranges shows significant differences (60-80%: 0.25; above 80%: 0.44). For the explanation of this anomaly we introduced the following supplementary conditions:
Group α below the quantity of I has a weak aspecific effect, which can weaken the effect of other pathogens causing pneumonia. Since if the presence of *M. bovis* in low quantity (Group α I) only did not cause pneumonia only, then in this range the rate of pneumonic animals would be similar to the 40-60 and below OD% ranges. Contradictory the rate of pneumonic animals is much lower instead of 0.3-0.4 it is only 0.25). Similar phenomenon is well known from the literature (Tschernig et al., 2002). If this effect does not exist, then equal phenomenon can be observed if the detection of *Mycoplasma bovis* by ELISA fails or hindered in animals diseased by other pathogens. We did not make distinction between these two phenomena during the testing our hypothesis. This effect is marked with "i", (scalar value between 0 and 1).
Group α above the quantity of II has a stronger pathogenic effect, which can make the animals more sensible to other microbes (Bennett and Jasper, 1977). This effect is marked with "g", (also a scalar value between 0 and 1). (The possible opposite case can cause equal phenomenon i.e. in the animals had been infected and diseased by other pathogens the spread and multiplication of *Mycoplasma bovis* increases or its detection by ELISA test improves for other reasons.

6.3.4.Description of the model

We created a probability model for the mathematical description of the hypothesis, which is represented by the following equations:

p (E+C+P+)= a(df+e)(1-i) + b(e+(1-e) (f(1-i) + (1-f)g)) + c [Equ 1.]
p (E+C+P-)= ad(1-f) + b(1-e)(1-f)(1-g) + a(df + e)i + b(e + (1-e)f)i [Equ 2.]
p (E+C-P+)= a(1-(d+e))f(1-i) [Equ 3.]
p (E+C-P-)= a(1-(d+e))((1-f) + fi) [Equ 4.]
p (E-C+P+)= (1-(a+b+c))(df+e) [Equ 5.]
p (E-C+P-)= (1-(a+b+c))(d(1-f)) [Equ 6.]
p (E-C-P+)= (1-(a+b+c))(1-(d+e))f [Equ 7.]
p (E-C-P-)= (1-(a+b+c))(1-(d+e))(1-f) [Equ 8.]

The number of animals within the observed category is n×p, where n is the total number of the examined animals. The meaning of signs representing the rates of animals within each category and the fitted values are presented in Table 6.4.

Table 6.4. Supposed frequency of distribution of microbe groups α, β and γ in the examined population (parameters of the model)

Microbe Groups	*Quantitative Categories of Microbe levels*			
	0	I	II	III.
Group γ	**1-f /77.79%/**			**f /22.21%/**
Group β	**1-(d+e) /65.73%/**		**d /19,02%/**	**e /15.25%/**
Group α	**1- (a+b+c) /88.9%/**	**a /7.24%/**	**b /3,83%/**	**c /0%/**
Parameters of interaction: i= 0.3573; g=0.4128 i- weak positive (or alternative) effect of Group α in I g- negative (or alternative) effect of Group α in II				

The numeric values of the table are the fitting parameters as results of fitting the model. Essentially, the linear system of eight-degree equations with eight unknowns was solved, which resulted in these values.
The formation of these equations was done by summarizing the elemental events, which can cause the observed phenomenon. The values in the equations represent values of frequency so multiplication represents “and” logical operation which means that both conditions should be fulfilled between the parts of the expression, meanwhile addition means “or” logical operation where either one or the other condition can be fulfilled.
Based on the data of Table 6.1. it can be seen that the model can be fitted to the observed data, so the system of hypotheses is suitable for the explanation of the observed numerical relationships.

6.4. Discussion

Several pathogens can cause respiratory disease in cattle. *Mycoplasma bovis* infection can cause severe lung changes, too (Boothby et al., 1983, Bashiruddin et al., 2001), which can severely affect herd productivity.
Our data call attention to the fact that pneumonia resulting from *M. bovis* infection occurs frequently in adult cattle as well. These results are in accordance with data obtained in the Netherlands, Switzerland and France (ter Laak et al., 1992*a, b*, Burnens et al., 1999; Le Grand et al., 2001), taking into consideration that pneumonia seems to be more frequent in domestic herds (33.9%). At the same time, *M. bovis* can more frequently be isolated from pneumonic lungs in Hungarian herds (59.9%) than in herds in the Netherlands (20%) or Ireland (13-23%; Byrne et al., 2001). These data confirm the role of *M. bovis* infection in triggering pneumonia. In 39.1% of the cases the lung alterations could not be associated with mycoplasma infection, showing that other pathogens and further factors also contribute to the development of pneumonia in adult cattle at high rates.
Mycoplasmas could frequently be isolated from lung samples (25.2%) without macroscopic lung lesions. It can be supposed that in these cases the infection and thus mycoplasma colonization were at an early stage at the time of isolation. In 9.2% of the cases antibodies to *M. bovis* were detectable in animals without pneumonia. This indicates that *M. bovis* can be involved in

pathological processes other than pneumonia, e.g. mastitis, metritis or other rare diseases (Uhaa et al., 1990*b, c*).

According to some authors (Boothby et al., 1981, Boothby et al., 1983; Burnens et al., 1999) ELISA testing of blood samples is a useful method for diagnosing *M. bovis* infection. We have come to the same conclusion too.

Although the average seropositivity rate of individuals was relatively low, in certain herds it was rather high. The rate of seropositive herds was also high. Nicholas et al. (2001) obtained similar results during a monitoring study in England, where 20-25% of cattle herds affected with pneumonia contained animals seropositive to *M. bovis*, while only in a few herds were there animals with high antibody titers against respiratory viruses.

Mycoplasma bovis increases the chances of development of pneumonia in animals with an OD% value higher than 80%, helping other agents exert their pathogenic effect.

Our model 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. The application of this model has proved that our hypothesis is suitable for explaining the effect of *M. bovis* in inducing pneumonia and its influence on the effect of other pathogens. 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.

7.
Chapter 4. The efficacy of valnemulin (Econor®) in the control of disease caused by experimental infection with Mycoplasma bovis

7.1. Introduction

A syndrome represented by pneumonia and arthritis (Langford, 1977) and caused by *Mycoplasma bovis* infection occurs throughout the world. It has also been associated with meningitis in calves (Stipkovits and others, 1993), decubital abscesses (Kinde and others, 1993, keratoconjunctivitis (Kirby and Nicholas, 1996, Pfützner and Sachse, 1996) and otitis media (Walz and others, 1997). In a survey conducted by the Office Internationale d'Epizooties (OIE) in over 48 countries, *M. bovis* was a major component of the calf pneumonia complex, with isolation rates of 23 to 35 % (Nicholas and others, 2000). In Britain alone losses in calves are estimated to be as much as £50 million per year from mortality and costs of treatment (Rebhun and others, 1995).
Disease due to *M. bovis* is difficult to treat (Romváry and others, 1975, Stalheim, 1976). *M. bovis* strains are often resistant to antibiotics (Ball and others, 1995, Thomas and others, 2001). According to recent data (Ayling and others, 2000, Nicholas and others, 2000) all strains of *M. bovis* have developed resistance to tilmicosin, most were found to be resistant to oxytetracycline, and about 20 % were resistant to spectinomycin and florfenicol. Only danofloxacin was effective *in vitro* against all strains.
The newly-developed antimicrobial valnemulin (Econor, Novartis Animal Health), is highly active against mycoplasma (Windsor and others, 1996; Aitken and others, 1996). It proved to be effective in the control of *M. bovis* infection under field conditions (Stipkovits and others, 2001), and it was of interest to test its efficacy in the treatment of the disease in calves induced by *M. bovis* under experimental conditions.

7.2. Materials and methods

7.2.1 Animals
Male calves, 10-35 days of age were obtained from a commercial dairy herd (in Hungary). The herd consisted of 1200 cows and had been closed for five years. The herd was infected with infectious bovine rhinotracheitis (IBR) virus, bovine virus diarrhoea (BVD) virus, adenovirus (Types I and II) as well as Parainfluenza-3 (PI3) virus. Bovilis BVD (Intervet) and Bovilis IBR marker vaccines (Intervet) were used according to the manufacturer's recommendation. Animals were also infected with *P. multocida* and *M. bovirhinis,* but *M. bovis* had never been detected. Over the previous 4 years, 60 calves, 2-6 weeks of age, had been tested twice yearly by culture and PCR testing of nasal swabs, as well as serologically for the presence of specific *M. bovis* antibodies. Serological testing and culture from nasal swabs also confirmed the absence of *Pasteurella* and *Mannheimia.* The calves selected for this study were similarly tested with negative results.
All new-born calves received colostrum from their dams within 2 hours of birth. During the first 4 days of life, animals were fed twice daily with milk from their mothers, then with milk replacer. From 15 days of age, the animals were also offered calf concentrate and lucerne hay to appetite. At four days of age calves were identified by individually numbered plastic ear tags and were placed into individual open-air outdoor boxes.

7.2.2. Animal management and experimental design
Twenty-four male calves, aged 10-35 days were transported to the Hungarian Veterinary Medical Research Institute. They were placed in 4 pens, with 6 calves in each, providing 4 treatment groups. Milk replacer, premix and hay were fed as on the source farm. During the experiment, 3L milk replacer was offered to calves in the morning, and 4L in the evening. After

5 days acclimatization, the calves were weighed and regrouped so that mean bodyweights and average age of calves in all groups/pens did not differ significantly ('Student's "t" test). This was Day 0 of the trial. Group 1 served as a negative control for *M. bovis* infection. The animals were non-medicated and placed in a pen situated 20 meters apart from the other pens. The other groups were infected and housed in separate but adjacent pens. Group 2 was infected but received placebo Premix, Group 3 received enrofloxacin (Baytril; Bayer) as a 10% Solution, and Group 4 received valnemulin as a 10% Premix (Econor; Novartis) (Table 7.1.). Placebo and valnemulin Premix were prepared by dissolving 30g in 6L milk replacer. Calves were given 10ml of this mixture/kg bodyweight at the morning and evening feed to provide a dose of 10mg/kg bodyweight per day. Enrofloxacin solution was prepared by mixing 15ml in 6L milk replacer. Calves were given 10ml of this solution/kg bodyweight at the morning and evening feed to provide a dose of 5mg/kg bodyweight/day. The solutions prepared as above were added to 1L milk replacer, stirring to ensure adequate distribution of the drug and offered to the calves at each feed, ensuring that the whole quantity was consumed. Following another 2 liters of milk replacer was given in the morning and 3 liters in the evening to provide a normal intake. Any milk replacer unconsumed was recorded after each feeding. Medication began on day 10 of the experiment and continued for a further 9 consecutive days to Day 18.

Table 7.1. Experimental plan

Experimental group	Day 0-1	Days 0 & 1	Daily (Days -3 to 18)	Day 10	Days 10 - 18	Day 21
1 Non-challenged, non-medicated	Bacterial culture from nasal swabs. Blood-sampling for *M. bovis* antibody. Clinical Scoring Measurement of rectal temperature Weighing		Clinical Scoring (General Appearance, Respiratory signs (inc. nasal discharge, cough), presence of arthritis) Recording of milk replacer offered/refused Measurement of rectal temperature	Bacterial culture from nasal swabs. Blood-sampling for *M. bovis* antibody Weighing		Bacterial culture from nasal swabs. Blood-sampling for *M. bovis* antibody. Calves killed; macroscopic and histopathological post-mortem examination. Culture for mycoplasma from lungs and tissues. Weighing (Day 18)
2 Challenged, non-medicated		*M. bovis* challenge			Placebo medication	
3 Challenged, enrofloxacin-medicated		*M. bovis* challenge			Enrofloxacin medication	
4 Challenged, valnemulin-medicated		*M. bovis* challenge			Valnemulin medication	

7.2.3. Preparation of the challenge culture and the method of challenge

M. bovis strain designated 5063, cultured from the lung of a 3 week-old calf suffering from pneumonia and arthritis was cloned and identified according to biochemical characteristics and serological tests (growth inhibition, epifluorescence and PCR) (Erno and Stipkovits, 1973b, Frey et al., 1999). The strain was freeze-dried and stored at -20^{o}C. Before infection the strain was propagated in fluid medium B (Erno and Stipkovits, 1973a) and the concentration of colony-forming units (CFU) determined as 2.2×10^{8}/ml. Challenge was done by spraying 10ml of fresh broth culture for one minute from a distance of 10 cm in front of nares of the calves on day 0 and 1 of the experiment. Calves in the non-infected control group were sprayed with 10 ml of sterile broth.

7.2.4. Clinical assessment

Clinical assessment was performed twice daily before feeding. General appearance and respiratory signs were scored on a 3-point scale. General Appearance: 0=normal, 1=subdued, slightly depressed, 2=depressed, reluctant to rise and 3=very depressed, unresponsive to external stimuli. Respiratory signs: 0=normal, 1=hyperpnoea or slight dyspnoea, 2=moderate hyperpnoea or obvious dyspnoea, 3=respiratory distress.

Nasal discharge was scored: 0=absent, 1=mild, 2=significant and purulent.

Coughing was scored as 0 = absent, 1=mild, 2 =severe and frequent - several in one minute.

Arthritis was also scored: 0=absent, 1=enlargement of 1 or more joints, 2=significant exudate in and noticeable lameness in 1 or more joints.

The rectal temperature of each animal was measured every morning at 08 00 hours throughout the study (from day -3 to 18). The number of animals showing a temperature above 39.5^{o}C was counted in each group.

The amount of milk offered and consumed was recorded in the morning and in the evening throughout the study (from day -3 to 18).

Animals were weighed before infection (Day -1), and on Days 10 and 18 of the study.

7.2.5. Microbiological examination

Before challenge (Day -1), before starting medication (Day 10) and on Day 21, nasal swabs were taken from each calf for isolation of *Mannheimia haemolytica, Pasteurella multocida and Haemophilus somnus,* and culture and PCR testing (Frey and others, 1999) for *Mycoplasma bovis.* Culture and identification were performed according to the methods described by Barrow and Felthan (1993).

Animals were killed by exsanguination after electrical stunning on Day 21 of the experiment. The lungs, liver, spleen and kidneys of the animals were cultured for *M bovis* (ter Laak, 1992), and the lungs also for *M. haemolytica, P. multocida* and *H. somnus.*

7.2.6. Serology

Blood samples were taken before challenge (Day -1), before starting medication (Day 10) and on Day 21, and examined for antibodies against *M. bovis* using the CHEKIT *M. bovis* SERO ELISA kit. Sera taken at the end of experiment were also tested for antibodies to BVD, IBR, Adenoviruses, PI3 and Respiratory Syncytial virus (RSV).

7.2.7. Pathological examination

Animals that died during the experiment as well as those killed on Day 21 at the end of the study were examined for the presence of gross pathological lesions in the nasal cavity, trachea, and internal organs (lung, spleen, liver, kidney). Macroscopical lung lesions were scored as 0=no lesions, 1=small areas of catarrhal inflammation in 1 lobe, 2 = small areas of catarrhal inflammation in 2 or more lobes, 3 = about 50% lung area affected, 4 = more than 50% lung area affected. Lungs were also examined histopathologically. Lesions comprised interstitial pneumonia accompanied by perivascular and peribronchial lymphoid cell infiltration, catarrhal pneumonia accompanied by accumulation of desquamated cells and serous exudate in bronchioles and alveoli and the presence of inflammatory foci encapsulated by connective/fibrous tissues) and were scored as 0=normal, 1=mild, 2=diffuse.

7.2.8. Evaluation of the efficacy of treatment

The number of days with abnormal general clinical appearance, respiratory signs, nasal discharge and coughing and their scores before and after treatment were compared between groups by means of χ^2 testing.

The number of animals showing a temperature above 39.5^{o}C, and the number of occasions when animals refused to take part or all of the quantity of milk offered, were recorded for all groups and compared between groups by χ^2 testing.

Mean ELISA Optical Density before challenge and on Day 21, and the mean body weight gains for the periods Days 0-10, Days 10-18 and Days 0-18 of the experiment in all groups were calculated and compared using "Student" 's "t" test.
Total lung lesion scores and histological lesion scores were compared between groups using χ^2 testing and this test was also used to compare the total number of *M. bovis* isolations from tissues at slaughter.

7.3. Results

7.3.1. Microbiology

Before infection *M. bovis* could not be cultured from the nasal cavity of any calf. However 9 days after challenge, *M. bovis* was detected by culture and PCR in nasal samples of all challenged animals, although not in the control groups. After slaughter, *M. bovis* was detected by culture in 87.5% of samples taken from lungs, spleens, livers and kidneys of the challenged unmedicated Group 2. A significant ($p<0.05$ to <0.001) reduction in the isolation rate from these organs was found in the valnemulin Group (8.3%) and enrofloxacin Group (33.3%). The isolation rate in the valnemulin group was significantly lower ($p<0.05\%$) than that in the enrofloxacin group (Table 7.2.).
Before *M. bovis* challenge *M. haemolytica* and *H. somnus* could not be isolated from the experimental calves.
P. multocida was present in the nasal cavity of a high proportion of calves both before and after infection. However at the end of the experiment, when animals were killed, *P. multocida* was isolated from 4 out of 6 infected non-medicated animals but from none of the uninfected or medicated calves' lungs.

Table 7.2. Lesions and microbiological isolation from calves at slaughter

Group	**M. bovis re-isolated from**						**No. of calves with affected lungs**	**Total lung lesion score**	**Total histological lesion score**
	Lung	**Spleen**	**Liver**	**Kidney**	**Total**	**Joint**			
1	0/6	0/6	0/6	0/6	0/24***	Nd	3	4	14
2	6/6	6/6	5/6	4/6	21/24**	2/2	6	15***	45***
3	4/6	0/6	1/6	3/6	8/24	Nd	2	4	15
4	1/6	0/6	0/6	1/6	2/24*	Nd	3	6	14
Statistical significance N.d. = not done					* different from enrofloxacin group ($p<0.05$) ** different from enrofloxacin group ($p<0.05$); valnemulin group ($p<0.001$) *** different from all infected groups ($p<0.05$ - <0.001)			*** different from all other groups ($p<0.001$)	*** different from all other groups ($p<0.001$)

7.3.2. Serology

There was no statistical difference in mean *M. bovis* ELISA optical density **(**OD) values of the groups before challenge. After challenge values increased in all infected groups, and mean values were significantly higher than in the non-infected group. There was no difference between the enrofloxacin and non-medicated groups, but significantly lower OD values were recorded in the valnemulin, than in the enrofloxacin group (Table 7.3.).

Table 7.3. Body weight gains and serological responses of calves to experimental M. bovis challenge.

Group	Day 0	Day 0 to 10	Day 0 to 18	Day -1	Day 21
	Weight (kg)	Weight Gain (kg)	Weight Gain (kg)	Mean *M. bovis* ELISA OD	Mean *M. bovis* ELISA OD
1	46.0	3.8***	9.9	0.34	0.25*
2	46.0	-1.7	3.7***	0.22	0.61
3	46.3	-1.0	9.0	0.34	0.78
4	46.7	0.5	11.0	0.25	0.46***
Statistical significance	All NS	*** different from all challenged groups ($p<0.001$)	*** different from un- challenged and both medicated groups ($p<0.001$)	All NS	*** different from enrofloxacin group ($p<0.001$) * different from all challenged groups ($p<0.05$ - 0.001)

7.3.3. Clinical assessment

Before challenge abnormal clinical signs were recorded in none of the animals and only very mild signs subsequently noted occasionally in the non-challenged group. After challenge with *M. bovis,* high clinical scores for depression, dyspnoea, respiratory distress and various degrees of nasal discharge (Figures 7.2. and 7.3.) and coughing were recorded. Two animals died (on Days 15 and 17) in the challenged non-medicated group Up to the initiation of medication on Day 10, no differences were noticed between the challenged groups (Figure 7.1.). After the start of treatment a statistically significant ($p<0.05$-0.001) reduction in the number of days with clinical signs were observed by day 4-5 and in clinical scores by days 3-5 in the medicated compared with the unmedicated groups. The total scores in the enrofloxacin group were higher than those in the valnemulin-medicated group. Between Days 1 to 3 and Days 4 - 6 of medication, clinical scores decreased only very slightly in the enrofloxacin-medicated group but markedly in the valnemulin group, the difference being statistically significant with respect to some of the clinical parameters.

Lameness was seen frequently, but typical arthritis, with local pain and accumulation of fluid in the joints was observed in only 2 calves (Figure 7.6.), in the non-medicated group. At post mortem, *M. bovis* was isolated from the joints of these 2 animals.

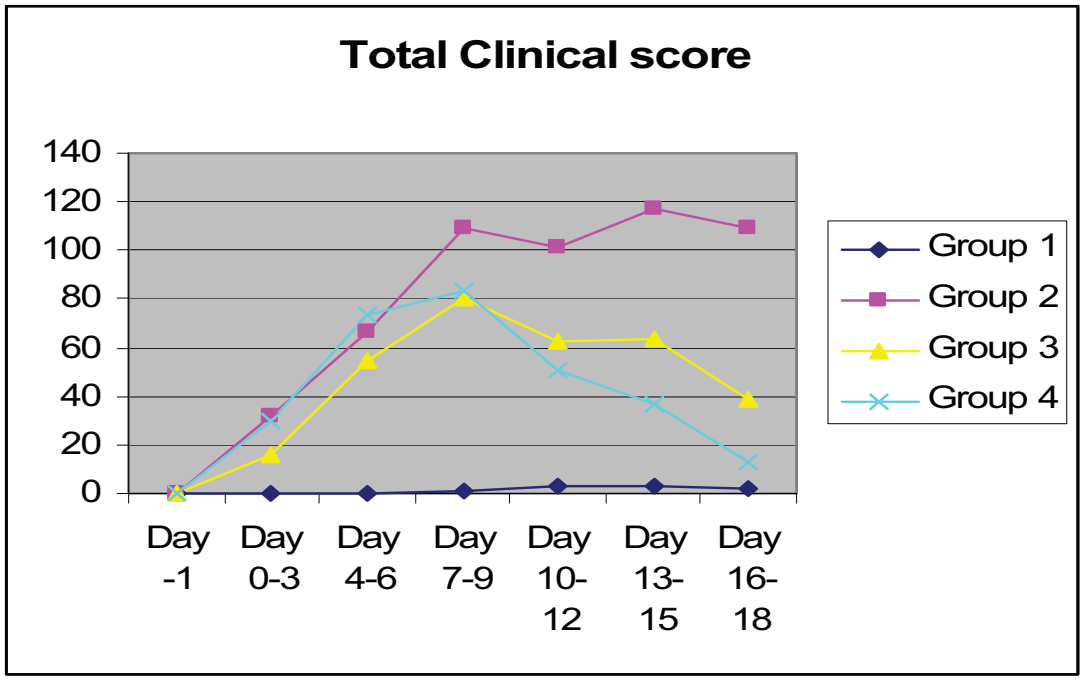

Figure 7.1. Clinical scores during challenge of calves with *Mycoplasma bovis* (medication from Day10)

7.3.3.1. Milk refusal

Over the first 9 days of the study only a few calves left some of the milk on a few occasions. However as the disease progressed feed refusal increased progressively, reaching 50-75% of the milk offered shortly before the calves were slaughtered. Over the medication period the incidence of milk refusal remained low in the treated groups, while it increased significantly in the challenged unmedicated group ($p<0.001$). The incidence of milk refusal was significantly ($p<0.05$) lower in the valnemulin than in the enrofloxacin-medicated group (Table 7.4.).

Table 7.4. Milk refusal (number of occasions on which milk refused out of total feeds (2 feeds per day)), and rectal temperatures (number of days with temperature >39.5°C)

Group	Elevated rectal temperature Day 0-9 (max. 54)	Elevated rectal temperature Day 10-18 (max. 54)	Milk refusals Day 0-9 (total feeds 108)	Milk refusals Day 10-18 (total feeds 108)
1	0	0	0	0
2	27	32***	7	53***
3	18	5	13	5
4	19	10	0**	0*
Statistical significance (uninfected group different from infected unmedicated group throughout)		*** different from medicated groups ($p<0.001$)	** different from enrofloxacin and non-medicated groups ($p<0.01$)	*** different from medicated groups ($p<0.001$) * different from enrofloxacin group ($p<0.05$)

7.3.3.2. Rectal temperature

After *M. bovis* challenge rectal temperature was elevated in all challenged groups but not in the non-infected control group. The highest rectal temperature recorded was 40.7°C. The number of days with elevated temperature reached 31.5-59.2% of the total in challenged groups. No

statistically significant differences were noticed between the incidence of elevated temperature in the challenged groups before treatment. However after challenge the incidence of raised temperatures decreased significantly ($p<0.05$) in medicated groups in comparison with previously or with the non-infected group. There was no significant difference between the 2 medicated groups (Table 7.4.).

7.3.3.3. Body weight gain

Body weights of calves before the start of the experiment ranged from 35.4 to 55.6 kg and there were no statistically significant differences between groups. Mean body weight gains of all groups 9 days after *M. bovis* challenge were significantly lower than in the non-infected control group. By Day 18 of the study mean weight gain of the infected unmedicated group was lower than that of the non-challenged controls or the medicated groups. There were no differences between the two medicated groups before challenge or over the whole study period (Table 7.3.).

7.3.3.4. Pathology

Macroscopic and histological lesions developed after challenge of calves with *M. bovis* and are summarised in Table 7.2. In the non-infected control group, three animals had mild lesions in the lung with a total lung lesion score of 4. In Group 2 (challenged with *M. bovis* but not treated) all 6 calves had lesions (Figure 7.4.) and the total lung lesion score was 15 ($p<0.05$). Four animals in the challenged, non-medicated group had pleurisy (Figure 7.5.) but none in the non-challenged or medicated groups. Histopathological changes comprised interstitial pneumonia, perivascular and peribronchial infiltration of lymphoid cells, catarrhal pneumonia with desquamation of epithelial cells and accumulation of serous exudate in alveoli and bronchi, and the presence of inflammatory foci encapsulated by connective/fibrous tissue. The total histological lesion score was 45 in the challenged unmedicated group, compared with 14 in the unchallenged group and 15 and 14 in the groups medicated with enrofloxacin and valnemulin respectively. The difference between the two medication groups was not statistically significant.

7.4. Discussion

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. There was considerable variation between animals with respect to the severity of clinical signs.

These clinical signs were similar to those observed in field conditions (Stipkovits et al., 2001), including the development of occasional arthritis (Pfützner and Sachse, 1996, Adegboye et al., 1996).

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. These results confirm data published by Poumarat and others, (1988) and Rodriguez and others, (1995).

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 (Le Grand and others, 2001).

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. There was considerable variability in weight gains in the challenged unmedicated group whereas the coefficient of variation of weight gain in both medicated groups approached that of the uninfected controls. 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.

The positive effect is in agreement with results obtained by the treatment with valnemulin of calves suffering natural *M. bovis* infection (Stipkovits et al., 2001) as well as with results from *in vitro* antibiotic sensitivity testing of the *M. bovis* challenge strain, in which values of 0.5µg/ml for enrofloxacin, and 0.0625µg/ml for valnemulin were obtained. These MIC values were similar for enrofloxacin to those obtained with danofloxacin (Nicholas et al., 2000), but somewhat higher than values reported for valnemulin with M. *hyopneumoniae* (0.0024µg/ml (Aitken et al, 1996) and 0.005µg/ml (Windsor et al., 1996)) and *M. synoviae* (0.0001-0.0005µg/ml) (Windsor et al., 1996). They are much lower than those obtained for the macrolides tylosin tartarate and erythromycin by Stalheim (1976) and Romváry et al. (1975).

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.

Medication of the animals prevented the colonization of the lungs with *P. multocida*.

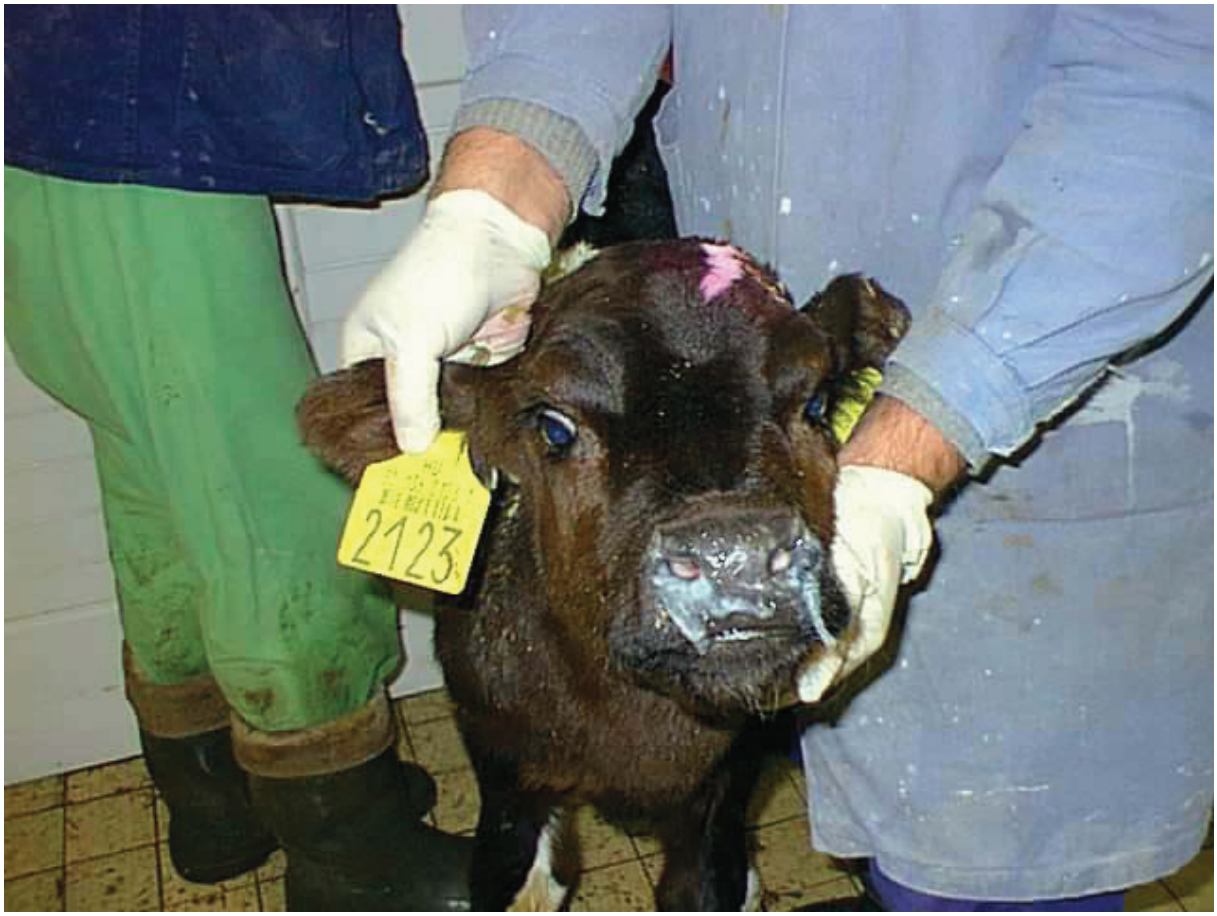

Figure 7.2. Nasal discharge induced by experimental challenge of the calve with M. bovis

Figure 7.3. Disease of the calve 17 days post challenge by M. bovis

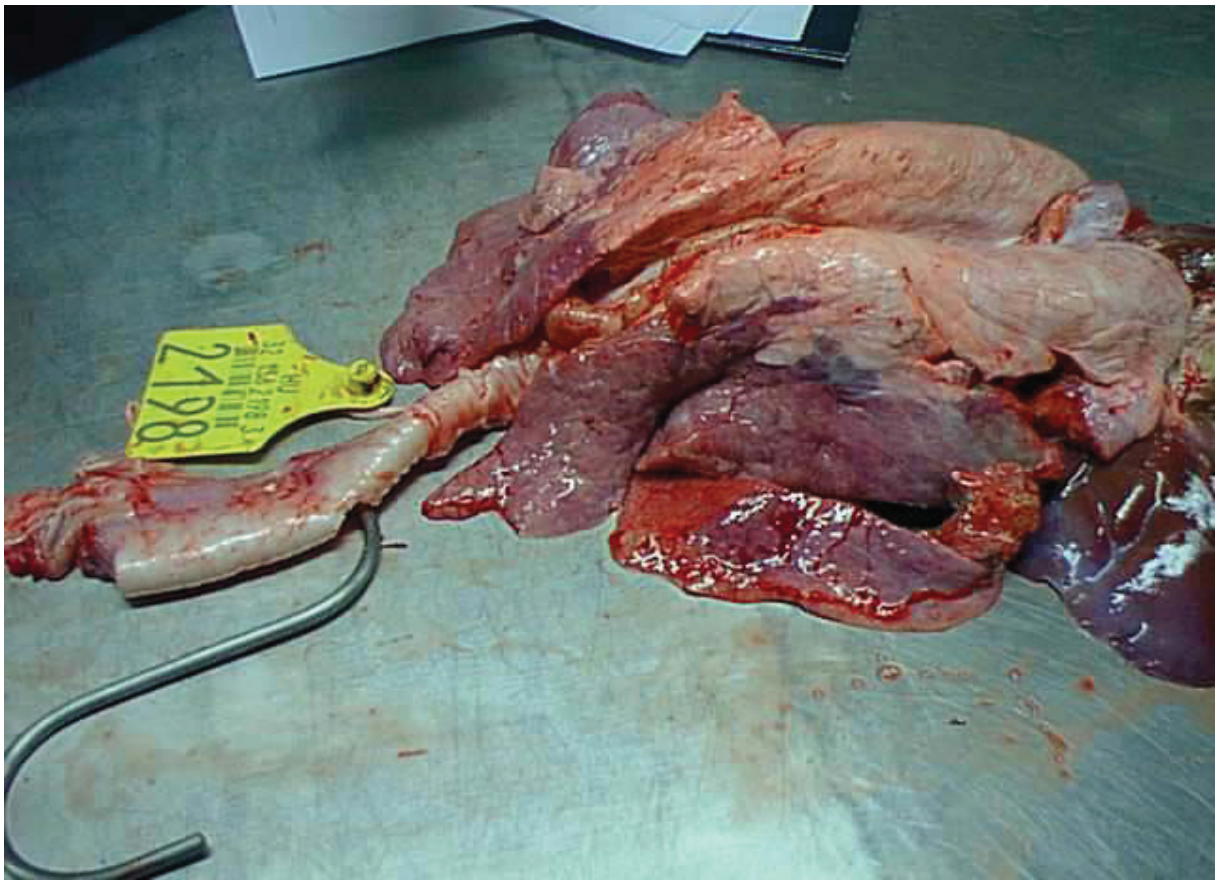

Figure 7.4. Lung lesions of the calve challenged by M. bovis

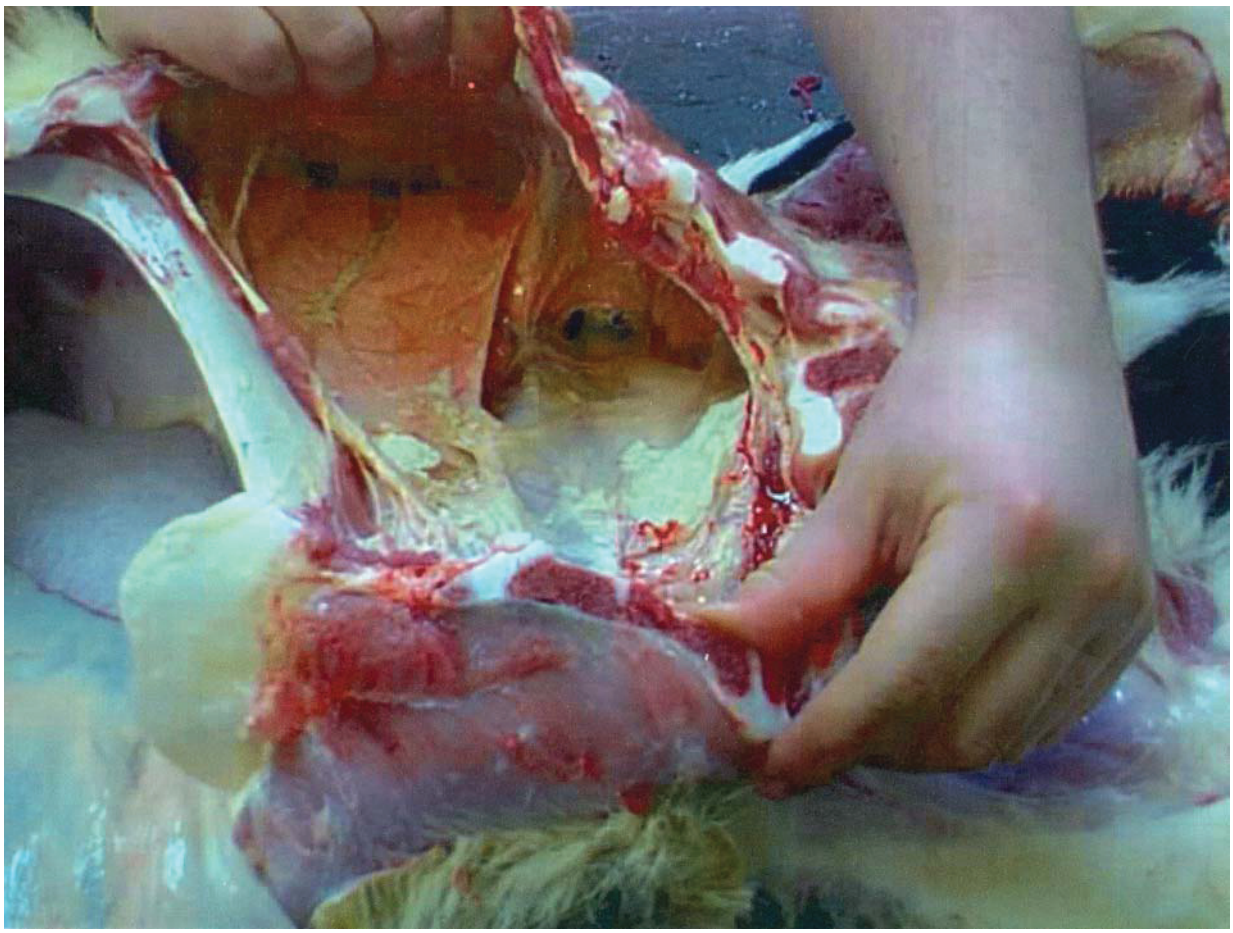

Figure 7.5. Massive fibrinous pleurisy in a calve challenged by M. bovis

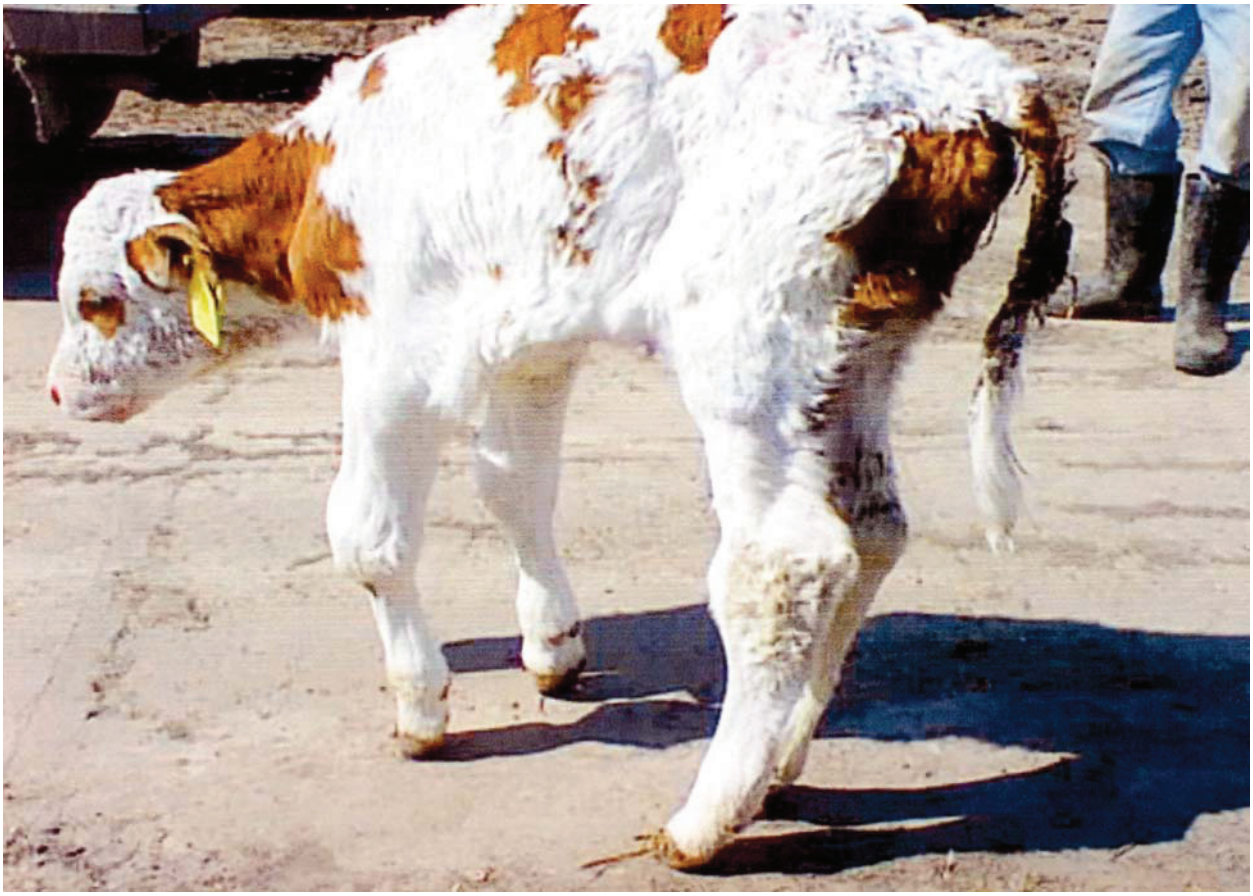

Figure 7.6. Arthritis in an infected calve

8.
Chapter 5.
Critical evaluation of some diagnostic PCR systems specific to *Mycoplasma bovis* using an improved assay

8.1. Introduction

Mycoplasma (M.) bovis is an important, but sometimes overlooked pathogenic mycoplasma in cattle. It causes major economic losses mainly by pneumonia, arthritis and loss of weight gain in calves, mastitis in cows and reproductive problems of both cows and bulls (Romvary et al.1977-79, Kreusel et al., 1989, terLaak et al., 1992, Pfützner and Sachse, 1996, Nicholas, 2003). It can rarely be involved in other diseases too such as meningitis (Stipkovits et al., 1993), otitis media (Walz et al., 1997) and abortion (Byrne et al., 1999). *M. bovis* infection can be diagnosed in several ways (Sachse et al., 1993). Isolation and identification of this organism by metabolic and immunological assays is time-consuming and requires specially equipped laboratories (Pfützner and Sachse, 1996, Poveda and Nicholas, 1998). The isolation from chronically ill animals is sometimes difficult, because of the overgrowing of bacteria of secondary infections or the inhibitory effect of the administered antibiotics. Even the normal mycoplasmal flora of the bovine respiratory tract can delay the proper identification (terLaak et al., 1992). The serological tests - particularly enzyme-linked-immunosorbent-assay (ELISA) tests - have been also demonstrated as useful tools for the herd diagnosis of *M. bovis* infection (Boothby et al., 1981, Uhaa et al., 1990a, Burnens et al., 1999, LeGrand et al., 2002). Cross-reactions with other mycoplasmas or frequently occurring intra-species antigenic variations (Rosengarten et al., 1994) can make the results of serological tests doubtful (Ayling et al., 1997). Moreover, the individual shedders are sometimes difficult to find by ELISA test, because of the late antibody response, which can make control measures ineffective (Sachse et al., 1993). With the use of specific polymerase chain reaction (PCR) the chance of the detection of the organism is increasing in case of both early and chronic infections, compared to culture and serological methods (Hirose et al., 2001).

The PCR systems targeting the 16S RNA gene (Johansson, 1996, Chávez González et al., 1995) amplifies *M. agalactiae* as well, which is a closely related species and differs only in 8 nucleotide positions (Mattsson et al., 1994). Ghadersohi et al. (1997) designed PCR primers from the sequences obtained from a *M. bovis* specific dot blot hybridization probe. An additional forward primer was designed by Haymann et al (2003) to improve the specificity and sensitivity of this assay by using the three different primers in seminested setup. Clear discrimination of *M. bovis* from *M. agalactiae* can be obtained by the assay of Subramaniam et al., 1998, targeting the UvrC gene.

M. bovis expresses a set of variable cell surface lipoproteins (Vsp). These Vsps vary spontaneously in size and expression with high frequency. The N-terminal part of these lipoproteins contains a conservative prokaryotic lipoprotein signal sequence and their surface exposed C-terminal sections have widely repetitive parts (Behrens et al., 1994, Rosengarten et al., 1994). The presence of the host antibodies can modulate the on-and-off switching and the size of these structures (Le Grand et al., 1996). The nucleotide sequence of the Vsp genomic locus is characteristic to the M. bovis species, therefore can be an ideal region to be targeted with PCR assays.

Present paper describes a PCR assay designed to a Vsp gene with high specificity and sensitivity to detect *M. bovis*.

8.2. Materials and methods

8.2.1. Mycoplasma and bacteria strains, culture conditions

Table 8.1. shows the list of mycoplasma and bacteria strains and their sources. Mycoplasmas were grown in Hayflick's liquid medium at 37 ^{o}C in 5% CO_2 atmosphere. Bacteria were streaked onto Columbia agar (Merck) and incubated in aerobic conditions at the same temperature.

Table 8.1. Mycoplasma and bacteria strains examined with the PCR system

Species name	Strain	Source
Mycoplasma bovis	Donetta	ATCC
Mycoplasma dispar	NCTC 10125	PHLS
Mycoplasma agalactiae	PG2	ATCC
Mycoplasma verecundum	NCTC 10145	PHLS
Mycoplasma californicum	ST-6	PHLS
Mycoplasma bovirhinis	PG43	PHLS
Mycoplasma canadense	NCTC 10152	PHLS
Mycoplasma canis	PG 14	ATCC
Mycoplasma arginine	G230	ATCC
Mycoplasma bovigenitalium	PG11	PHLS
Acholeplasma modicum	PG 49	ATCC
Acholeplasma laidlawii	PG8	PHLS
Mycoplasma mycoides sp mycoides Small Colony type	PG1	PHLS
Streptococcus bovis	mastitic isolate	CVI
Staphylococcus epidermidis	ATCC 12228	NCAIM
Pseudomonas aeruginosa	ATCC 9027	NCAIM
Staphylococcus aureus	ATCC 25923	NCAIM
Escherichia coli	mastitic isolate	CVI
Yersinia enterocolica	isolate	CVI
Enterococcus faecalis	ATCC 11700	NCAIM
Arcanobacter pyogenes	mastitic isolate	CVI
Streptococcus uberis	mastitic isolate	CVI
Pasteurella multocida	mastitic isolate	CVI

ATCC-American Type Cultures Collection, USA
PHLS-Public Health Laboratory, UK
CVI-Central Veterinary Institute, Hungary
NCAIM-National Collection of Agricultural and Industrial Microorganisms, Hungary

8.2.2. Extraction of genomic DNA

500 µl of the log phase broth culture of mycoplasma strains and a 0.5 McFarland dense suspension of bacteria prepared in sterile phosphate buffered saline solution (PBS) was subjected to DNA extraction. These solutions were mixed with 100 µl of 6X Proteinase K buffer with a final concentration of 0.1 M Tris-Cl pH 8.0, 0.06 M EDTA pH 8.0, 0.5% sodium dodecyl sulfate (SDS), and were incubated overnight at 56^{o}C with 100 µg of proteinase K (Sigma). DNA was extracted once with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1, pH8) solution followed by an equal volume of chloroform-isoamyl alcohol (24:1, pH8) extraction. DNA from the aqueous phase was precipitated with 0.1 volume of 3 M sodium acetate pH 5.2 and 2 volumes of 96% ethanol. The precipitated DNA was washed once with 1ml 70% ethanol. The dried pellet was suspended in 50 µl nuclease-free water.

8.2.3. Primer determination

As forward primer, Mb1 (5'-aaggtacaccagctaacccag-3'), described by Ghadersohi et al., (1997) was used.
The Mbr2 reverse primer (5'-aatgaagctactgatccaag-3') was designed based on M. bovis variable surface lipoprotein (Vsp) genomic region (Lysnyansky et al., 1999) using the Primer select program of the Winstar software package (Lasergene).

8.2.4. PCR conditions

The PCR reactions were prepared in a 50 µl total volume, containing 1X buffer for a final 10 mM Tris-Cl pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl concentration, 200 µM of dNTP, 20 picomole of each primer, 5 µl of DNA and 2.5 U of RedTaq polymerase. After a 5 min initial denaturation at 94ºC, 35 cycles were performed with the following parameters: 20 sec at 94˚C, 20 sec at 52ºC, 1 min at 72˚C. After cycling, a final extension was applied for 5 min at 72˚C.

8.2.5. Detection of amplicons

Five microliters of each PCR product were subjected to electrophoresis in 2% agarose (Serva) gels containing 0.5 µg/ml ethidium bromide. The bands were visualized under UV light (UVP), followed by photographing and editing using the GrabIt software (UVP).

8.2.6. DNA sequencing

To prove the identity of the amplified products, DNA sequencing was carried out: the PCR products were purified with micro columns (QIAquick Gel Extraction Kit, Qiagen) according to the manufacturer's instructions. The pure DNA products were sequenced from both directions with the same primers as for amplification reactions with an ABI Prism sequencer (Applied Biosystems, Model 377), using the Big Dye Terminator V3.0 sequencing kit (Applied Biosystems).

8.3. Results

8.3.1. Specificity and sensitivity of the PCR probe

Our PCR system was specific to *M. bovis* yielding a 319 bp product but did not amplify DNA from most frequently occurring pathogenic or ubiquitous mycoplasmas and bacteria of bovine origin (Figure 8.1.).

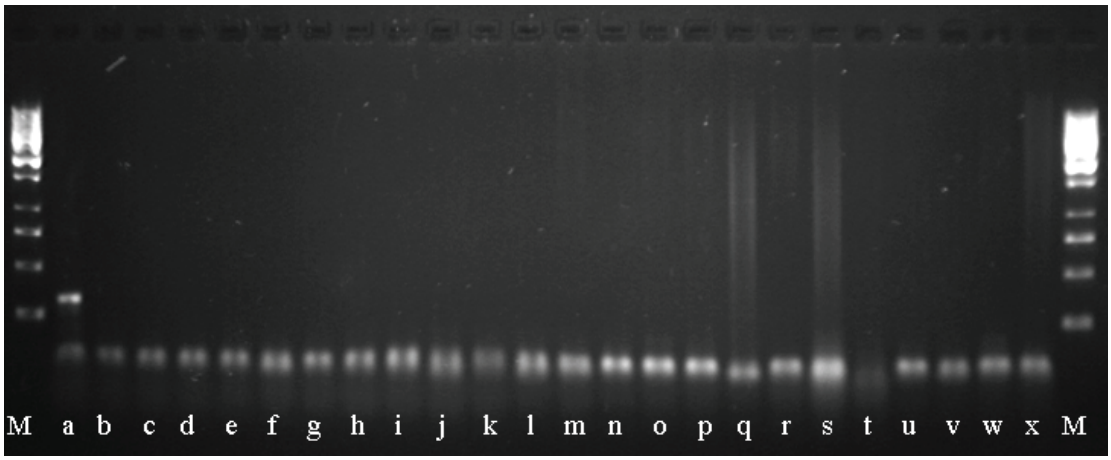

Figure 8.1. Specificity of the PCR assay, agarose gel electrophoresis photo.
Lane M: Molecular weight marker (123 bp DNA ladder, Sigma). Lane a: Mycoplasma bovis, Lane b: Mycoplasma dispar, Lane c: Mycoplasma agalactiae, Lane d: Mycoplasma verecundum, Lane e: Mycoplasma californicum, Lane f: Mycoplasma californicum, Lane g: Mycoplasma bovirhinis, Lane h: Mycoplasma canadense, Lane i: Mycoplasma canis, Lane j: Mycoplasma arginini, Lane k: Mycoplasma bovigenitalium, Lane l: Acholeplasma modicum, Lane m: Acholeplasma laidlawii, Lane n: Mycoplasma mycoides sp mycoides Small Colony type, Lane o: Streptococcus bovis, Lane p: Staphylococcus epidermidis, Lane q: Pseudomonas aeruginosa, Lane r: Staphylococcus aureus, Lane s: Escherichia coli, Lane t: Yersinia enterocolica, Lane u: Enterococcus faecalis, Lane v: Arcanobacter pyogenes, Lane w: Streptococcus uberis and Lane x: Pasteurella multocida

Cross-amplification did not occur with *M. agalactiae*. The sequence analysis confirmed that the amplified PCR products are *M. bovis* specific.
The PCR products could be detected up to 10^{-9} dilution with simple agarose gel electrophoresis and ethidium bromide staining. Since the original culture contained 3×10^{11} organism (determined by agar plate dilution) the system can detect approximately 150 CFU/ml in broth culture (Figure 8.2.).

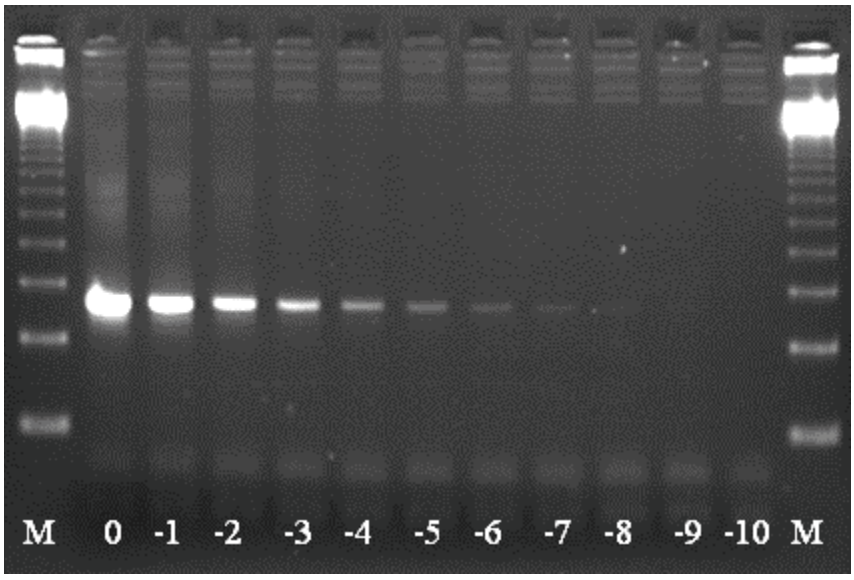

Figure 8.2. Sensitivity of the PCR probe, agarose gel electrophoresis photo.
Lane M: Molecular weight marker (GeneRuler 1 Kb DNA ladder (Fermentas)).
Lanes 0 to -10 PCR reactions with 10^n diluted DNA

8.4. Discussion

DNA-based techniques, especially PCR can yield rapid and specific diagnosis of infections caused by *M. bovis* (Hirose et al., 2001). Even though, *M. agalactiae* is a rare inhabitant in cattle, PCR systems should differentiate between the two species. The use of assays based on the 16S RNA gene frequently show cross-amplification between the two species or the product needs to be subjected to additional restriction enzyme analysis to obtain different restriction patterns (Johansson et al., 1996). Therefore these tests are dubious in such diagnostic work where the herds or individual animals' (e.g. breeding bulls) *M. bovis* status is questionable.
The PCR system designed for UvrC gene sequences (Subramaniam et al., 1998) provides high specificity and clear distinction from *M. agalactiae*. However, the 1.6 kb PCR product is of relatively high molecular weight, which sometimes may result in low sensitivity in the detection of the agent. This decrease in sensitivity by 10^2-10^3 times was observed in experiments with serially diluted *M. bovis* DNA compared to our system.
To circumvent the above-mentioned difficulties, different PCR assays targeted the Vsp genomic locus, because this genomic region is unique among mycoplasma species, and characteristic to the *M. bovis* species, allowing the development of a species-specific PCR assay. The drawback of choosing this region for PCR detection is that it contains several inverted repeat regions, and even between different *M. bovis* isolates, the sequence patterns significantly differ, which require a very careful selection of primers. Therefore the development of a new, reliable PCR assay has been targeted. The Vsp gene based system described by Ghadersohi et al. (1997) –improved by Hayman and Hirst, (2003) by adding a new forward primer (MbF) to the system–provided rapid detection of this organism according to the authors.
In spite of this, our experiments showed that the PCR assay by Ghadersohi could not detect *M. bovis* at all even when several different PCR conditions (various primer, template, magnesium

concentrations and temperature profiles) were applied (Hayman, 2003). The semi-nested system by Hayman et al. (2003) with a new forward primer –which is actually a reverse primer– yielded only artifacts (primer dimers) in our hands, even stronger without specific *M. bovis* DNA template (Figure 8.3.).

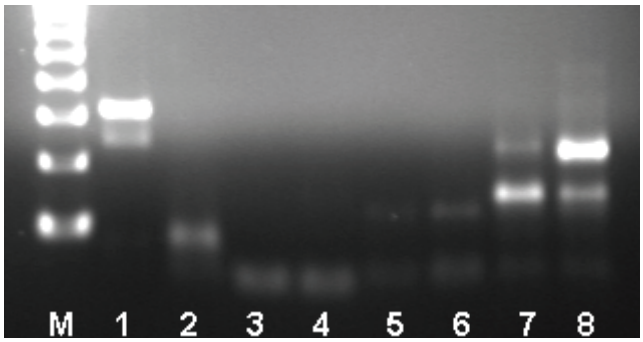

Figure 8.3. Comparison of the PCR assay with the new reverse primer (Lanes 1 and 2) with the one of Ghadersohi et al. (1997) (Lanes 3 and 4) and Hayman and Hirst (2003) (Lanes 5,6,7 and 8) M.-molecular weight marker (GeneRuler 100 bp DNA ladder (Fermentas)., *M. bovis* PG45 type strain DNA (1,3,5 and 7) and negative control (Lanes 2,4,6 and 8). Lanes 7 and 8 represent the second reaction of the semi-nested assay from the products of 5 and 6 respectively.

Since these results indicated that the reverse primer is not working properly, our aim was to produce a functional assay partly based on the previously described systems, and compare their sensitivity and specificity.

Our Vsp sequences based system detects different isolates of *M. bovis* consequently with high sensitivity meanwhile no other frequently occurring bovine mycoplasma or bacteria strains are amplified. No further tests (e.g. enzyme restriction) needed to identify the specific product.

Although this assay has not been validated to test clinical samples without pre-enrichment, according to our experiments it can successfully detect *M. bovis* from “simulated milk samples” (Hirose et al., 2001).

9.
Overview of the new results

1. The selective-differentiating culture with a special medium, and the capture ELISA have been proved to be useful diagnostic tools in the diagnosis of *M. bovis* infection. Both of these tests were suitable for examining field clinical and experimental challenge samples. The expensive and time-consuming filter-cloning and immunological identifying techniques could be omitted. Both methods were specific and sensitive however contaminating bacteria can sometimes disturb the results of the capture ELISA.

2. Monoclonal antibodies were successfully produced against *Mycoplasma bovis*. Their properties were extensively investigated including the recognized epitopes, their isotypes and cross-reactions. As it has been examined by IH studies, some of the produced hybridomas are suitable for the in situ visualization of the *M. bovis* antigen in formalin-fixed paraffin-embedded sections of the affected organs.

3. For the examination of the prevalence of *Mycoplasma bovis* in Hungary, a complex diagnostic work was carried out by the detection of specific antibodies with ELISA test, examining of the lung changes in slaughtered animals and culturing *M. bovis*. A new method – a mathematical model– was formed to evaluate the relationship between these 3 parameters. The model has been proved to be applicable for that purpose and it could reveal more comprehensive relationship by *M. bovis* in inducing pneumonia and its influence on the effect of other pathogens.

4. In the challenge trial extensive experiences could be gained about the clinical and pathological aspects of *M. bovis* pneumonia. As it is described in the scientific literature, the treatment of the pneumonia caused by *M. bovis* is fairly difficult. Valnemulin, which had been already proved to be effective in the treatment of *M. bovis* infection under field conditions, was valuable in this challenge trial too. This antibiotic compound accomplished slightly better than enrofloxacin and also prevented the colonization of secondary infective bacteria.

5. After many failures on attempting the diagnostic work with a previously described *Mycoplasma bovis* specific PCR system, the formation of an improved, working PCR assay was decided. The original, functional forward primer was used with a newly developed reverse one. This PCR assay was specific to *M. bovis* yielding a 319 bp product. It neither amplified other bovine mycoplasmas –including *M. agalactiae*– nor the most frequent bacteria species. This assay was able to detect 150 CFU/ml of organisms in broth culture and could also detect *M. bovis* in milk.

10.
References

Adegboye, D. S., Halbur, P. G., Nutsch, R. G., Kadlec, R. G., and Rosenbusch, R. F. (1996). Mycoplasma bovis-associated pneumonia and arthritis complicated with pyogranulomatous tenosynovitis in calves. *J. Am. Vet. Med. Assoc.* **209**, 647-649.

Adegboye, D. S., Hallbur, P. G., Cavanaugh, D. L., Werdin, R. E., Chase, C. C., Miskimins, D. W., and Rosenbusch, R. F. (1995). Immunohistochemical and pathological study of Mycoplasma bovis-associated lung abscesses in calves. *J. Vet. Diagn. Invest* **7**, 333-337.

Adegboye, D. S., Rasberry, U., Halbur, P. G., Andrews, J. J., and Rosenbusch, R. F. (1995). Monoclonal antibody-based immunohistochemical technique for the detection of Mycoplasma bovis in formalin-fixed, paraffin-embedded calf lung tissues. *J. Vet. Diagn. Invest* **7**, 261-265.

Aitken, I. A., Morgan, J. H., Dalziel, R., Burch, D. G. S., and Ripley, P. H. (1996). Antimicrobial susceptibility testing for SDZ PMD 296 (Econor- Sandoz Ltd) and six other antimicrobial agents against porcine mycoplasmas. *Proc. 14th IPVS Congress,* Bologna, Italy, p. 318.

Askaa, G. and Ernø, H. (1976) Elevation of Mycoplasma agalactiae subsp. Bovis to species rank Mycoplasma bovis. *Int. J. Syst. Bacteriol.* **26,** 323-325

Ayling, R. D., Baker, S. E., Peek, M. L., Simon, A. J., and Nicholas, R. A. (2000). Comparison of in vitro activity of danofloxacin, florfenicol, oxytetracycline, spectinomycin and tilmicosin against recent field isolates of Mycoplasma bovis. *Vet. Rec.* **146**, 745-747.

Ayling, R. D., Nicholas, R. A., and Johansson, K. E. (1997). Application of the polymerase chain reaction for the routine identification of Mycoplasma bovis. *Vet. Rec.* **141**, 307-308.

Ball, H. J., Finlay, D., and Reilly, G. A. (1994). Sandwich ELISA detection of Mycoplasma bovis in pneumonic calf lungs and nasal swabs. *Vet. Rec.* **135**, 531-532.

Ball, H. J., Reilly, C., and Bryson, D. G. (1995). Antibiotic susceptibility of *Mycoplasma bovis* strains isolated in Northern Ireland. *Ir. Vet. J.* **48**, 316-318.

Barrow, G. I., and Felthan, R. K. A. (1993). In: Cowan and Steel's Manual for the identification of medical bacteria. Cambridge, Cambridge University Press.

Bashiruddin, J. B., De Santis, P., Varga, E., and Stipkovits, L. (2001). Confirmation of the presence of Mycoplasma bovis in Hungarian cattle with pneumonia resembling pleuropneumonia. *Vet. Rec.* **148**, 743-746.

Behrens, A., Heller, M., Kirchhoff, H., Yogev, D., and Rosengarten, R. (1994). A family of phase- and size-variant membrane surface lipoprotein antigens (Vsps) of Mycoplasma bovis. *Infect. Immun.* **62**, 5075-5084.

Behrens, A., Heller, M., Rosenbusch, R., and Kirchhoff, H. (1996a). Immunoelectron microscopic localization of variable proteins on the surface of Mycoplasma bovis. *Microbiology* **142 (Pt 7)**, 1863-1871.

Behrens, A., Poumarat, F., Le Grand, D., Heller, M., and Rosengarten, R. (1996b). A newly identified immunodominant membrane protein (pMB67) involved in Mycoplasma bovis surface antigenic variation. *Microbiology* **142 (Pt 9)**, 2463-2470.

Beier, T., Hötzel, H., Lysnyansky, I., Grajetzki, C., Heller, M., Rabeling, B., Yogev, D., and Sachse, K. (1998). Intraspecies polymorphism of vsp genes and expression profiles of variable surface protein antigens (Vsps) in field isolates of Mycoplasma bovis. *Vet. Microbiol.* **63**, 189-203.

Bennett, R. H. and Jasper, D. E. (1977). Immunosuppression of humoral and cell-mediated responses in calves associated wtih inoculation of Mycoplasma bovis. *Am. J. Vet. Res.* **38**, 1731-1738.

Bennett, R. H. and Jasper, D. E. (1980). Bovine mycoplasmal mastitis from intramammary inoculations of small numbers of Mycoplasma bovis: local and systemic antibody response. *Am. J. Vet. Res.* **41**, 889-892.

Berthold, E., Heller, M., Pfützner, H., Leirer, R., and Sachse, K. (1992). Preparation and characterization of monoclonal antibodies against Mycoplasma bovis. *Zentralbl. Veterinärmed. B* **39**, 353-361.

Bocklisch, H., Kreusel, S., Brys, A., and Pfützner, H. (1991). Experimental infection of the udder of ewes due to Mycoplasma bovis. *Zentralbl. Veterinärmed. B* **38**, 385-390.

Bocklisch, H., Pfützner, H., Martin, J., Templin, G., and Kreusel, S. (1986). [Mycoplasma bovis abortion of cows following experimental infection]. *Arch. Exp. Veterinärmed.* **40**, 48-55.

Bocklisch, H., Zepezauer, V., Pfützner, H., and Kreusel, S. (1987). [Demonstration of Mycoplasma in sheep pneumonia and experimental pneumonia produced by Mycoplasma arginini]. *Arch. Exp. Veterinärmed.* **41**, 249-257.

Boothby, J. T., Jasper, D. E., Rollins, M. H., and Thomas, C. B. (1981). Detection of Mycoplasma bovis specific IgG in bovine serum by enzyme-linked immunosorbent assay. *Am. J. Vet. Res.* **42**, 1242-1247.

Boothby, J. T., Jasper, D. E., Zinkl, J. G., Thomas, C. B., and Dellinger, J. D. (1983). Prevalence of mycoplasmas and immune responses to Mycoplasma bovis in feedlot calves. *Am. J. Vet. Res.* **44**, 831-838.

Bradbury, J. M. (1998). Identification of mycoplasmas by immunofluorescence. In Miles, R., and Nicholas, R. (eds): Methods in Molecular Biology. (Vol. 104) Mycoplasma protocols. Humana Press. Totowa, New Jersey, USA, pp.119-125.

Brice, N., Finlay, D., Bryson, D. G., Henderson, J., McConnell, W., and Ball, H. J. (2000). Isolation of Mycoplasma bovis from cattle in Northern Ireland, 1993 to 1998. *Vet. Rec.* **146**, 643-644.

Brown, M. B., Shearer, J. K., and Elvinger, F. (1990). Mycoplasmal mastitis in a dairy herd. *J. Am. Vet. Med. Assoc.* **196**, 1097-1101.

Brys, A., Pfützner, H., Bocklisch, H., and Weigel, H. (1992). [Mycoplasma bovis-free breeding of cattle]. *Berl Munch. Tierarztl. Wochenschr.* **105**, 230-232.

Bryson, D. G., Ball H. J., Brice, N., Forser, F., and Pollock, D. S. (1999). Pathology of induced *Mycoplasma* bovis calf pneumonia in experimentally vaccinated animals. In: Stipkovits, L., Rosengarten, R., and Frey, J. (eds.) Mycoplasmas of ruminants: pathogenicity, diagnostics, epidemiology and molecular genetics. European Communities, Belgium, Vol. 3. pp. 128-132.

Burnens, A. P., Bonnemain, P., Bruderer, U., Schalch, L., Audige, L., Le Grand, D., Poumarat, F., and Nicolet, J. (1999). [The seroprevalence of Mycoplasma bovis in lactating cows in Switzerland, particularly in the republic and canton of Jura]. *Schweiz. Arch. Tierheilkd.* **141**, 455-460.

Byrne, W. J., Ball, H. J., Brice, N., McCormack, R., Baker, S. E., Ayling, R. D., and Nicholas, R. A. (2000). Application of an indirect ELISA to milk samples to identify cows with Mycoplasma bovis mastitis. *Vet. Rec.* **146**, 368-369.

Byrne, W. J., Brennan, P., McCormack, R., and Ball, H. J. (1999). Isolation of Mycoplasma bovis from the abomasal contents of an aborted bovine fetus. *Vet. Rec.* **144**, 211-212.

Byrne, W. J., McCormack, R., Brice, N., Egan, J., Markey, B., and Ball, H. J. (2001). Isolation of Mycoplasma bovis from bovine clinical samples in the Republic of Ireland. *Vet. Rec.* **148**, 331-333.

Chavez Gonzalez, Y. R., Ros, B. C., Bolske, G., Mattsson, J. G., Fernandez, M. C., and Johansson, K. E. (1995). In vitro amplification of the 16S rRNA genes from Mycoplasma bovis and Mycoplasma agalactiae by PCR. *Vet. Microbiol.* **47**, 183-190.

Chima, J. C., Wilkie, B. N., Nielsen, K. H., Ruhnke, H. L., Truscott, R. B., Maxie, G., and Chick, B. (1981). Synovial immunoglobulin and antibody in vaccinated and nonvaccinated calves challenged with Mycoplasma bovis. *Can. J. Comp. Med.* **45**, 92-96.

Eaglesome, M. D. and Garcia, M. M. (1990). The effect of Mycoplasma bovis on fertilization processes in vitro with bull spermatozoa and zona-free hamster oocytes. *Vet. Microbiol.* **21**, 329-337.

Egwu, G. O., Ameh, J. A., Aliyu, M. M., and Mohammed, F. D. (2001). Caprine mycoplasmal mastitis in Nigeria. *Small Rumin. Res.* **39**, 87-91.

Ernø, H. and Stipkovits, L. (1973a). Bovine mycoplasmas: cultural and biochemical studies. I. *Acta Vet. Scand.* **14**, 436-449.

Ernø, H. and Stipkovits, L. (1973b). Bovine mycoplasmas: cultural and biochemical studies. II. *Acta Vet. Scand.* **14**, 450-463.

Fahim, M. A., Hassainen, M. K. and Mostafa, M. H. (2003). Relationships between climatic conditions and Potato Late Blight epidemic in Egypt during winter seasons 1999-2001. *Appl. Ecol. Env. Res.* **1(1-2),** 159-172.

Feenstra, A., Bisgaard, M. E., Friis, N. F., Meyling, A., and Ahrens, P. (1991). A field study of Mycoplasma bovis infection in cattle. *Zentralbl. Veterinärmed. B* **38**, 195-202.

Friis, N. F. and Szancer, J. (1994). Sensitivity of certain porcine and bovine mycoplasmas to antimicrobial agents in a liquid medium test compared to a disc assay. *Acta Vet. Scand.* **35**, 389-394.

Fodor, N., and Kovács, G. J. (2003). Sensitivity of 4M maize model to the inaccuracy of weather and soil input data. *Appl. Ecol. Env. Res.* **1(1-2)**, 75-85.

Frey J., Subramaniam, S., Bergonier, D., and Nicolet, J. (1999). DNA repair genes *uvr*C as genetic targets for discrimination of closely related mycoplasmas. In Stipkovits, L., Rosengarten,

R., and Frey, J. (Eds.). Mycoplasmas of ruminants: Pathogenicity, diagnostics, epidemiology and molecular genetics, Vol. 3. Brussels: European Commission. pp 40-43.

Garcia, M. M., Truscott, R. B., McLaren, J., Stewart, R. B., Kingscote, B., and Burchak, J. (1986). Absence of Mycoplasma bovis in unprocessed frozen bull semen from Canadian artificial insemination centres. *Vet. Rec.* **119**, 11-12.

Geary, S. J., Tourtellotte, M. E., and Cameron, J. A. (1981). Inflammatory toxin from Mycoplasma bovis: isolation and characterization. *Science* **212**, 1032-1033.

Ghadersohi, A., Coelen, R. J., and Hirst, R. G. (1997). Development of a specific DNA probe and PCR for the detection of Mycoplasma bovis. *Vet. Microbiol.* **56**, 87-98.

Gonzalez, R. N., Sears, P. M., Merrill, R. A., and Hayes, G. L. (1992). Mastitis due to Mycoplasma in the state of New York during the period 1972-1990. *Cornell Vet.* **82**, 29-40.

Gourlay, R. N. and Houghton, S. B. (1985). Experimental pneumonia in conventionally reared and gnotobiotic calves by dual infection with Mycoplasma bovis and Pasteurella haemolytica. *Res. Vet. Sci.* **38**, 377-382.

Gourlay, R. N., Howard, C. J., Thomas, L. H., and Wyld, S. G. (1979). Pathogenicity of some Mycoplasma and Acholeplasma species in the lungs of gnotobiotic calves. *Res. Vet. Sci.* **27**, 233-237.

Gunning, R. F. and Shepherd, P. A. (1996). Outbreak of bovine Mycoplasma bovis mastitis. *Vet. Rec.* **139**, 23-24.

Haines, D. M., Martin, K. M., Clark, E. G., Jim, G. K., and Janzen, E. D. (2001). The immunohistochemical detection of Mycoplasma bovis and bovine viral diarrhea virus in tissues of feedlot cattle with chronic, unresponsive respiratory disease and/or arthritis. *Can. Vet. J.* **42**, 857-860.

Hale, H. H., Helmboldt, C. F., Plastridge, W. N., and Stula, E. F. (1962). Bovine mastitis caused by a Mycoplasma species. *Cornell Vet.* **52**, 582-591.

Hannan, P. C., Windsor, G. D., De Jong, A., Scmeer, N., and Stegemann, M. (1997). Comparative susceptibilities of various animal pathogenic mycoplasmas to fluoroquinolones. *Antimicrob. Agents Chemother*. **41**, 2037-2040.

Hannan, P. C. (2000). Guidelines and recommendations for antimicrobial minimum inhibitory concentration (MIC) testing against veterinary mycoplasma species. International Research Programme on Comparative Mycoplasmology. *Vet. Res.* **31**, 373-395.

Hannan, P. C., Windsor, H. M., and Ripley, P. H. (1997). In vitro susceptibilities of recent field isolates of Mycoplasma hyopneumoniae and Mycoplasma hyosynoviae to valnemulin (Econor), tiamulin and enrofloxacin and the in vitro development of resistance to certain antimicrobial agents in Mycoplasma hyopneumoniae. *Res. Vet. Sci.* **63**, 157-160.

Hayman, B. (2003). Development of a PCR for specific detection of Mycoplasma bovis from bovine milk and mucosal samples: a critique. *Vet. Microbiol.* author reply **97**, 167-168.

Hayman, B. and Hirst, R. (2003). Development of a semi-nested PCR for the improved detection of Mycoplasma bovis from bovine milk and mucosal samples. *Vet. Microbiol.* **91**, 91-100.

Henderson, J. P. and Ball, H. J. (1999). Polyarthritis due to Mycoplasma bovis infection in adult dairy cattle in Northern Ireland. *Vet. Rec.* **145**, 374-376.

Hermann, R. (1992). Genome structure and organization. In: Maniloff, J. (ed.). Mycoplasmas: molecular biology and pathogenesis. American Society of Microbiology, Washington DC. USA, pp. 157-168.

Hirose, K., Kawasaki, Y., Kotani, K., Tanaka, A., Abiko, K., and Ogawa, H. (2001). Detection of mycoplasma in mastitic milk by PCR analysis and culture method. *J. Vet. Med. Sci.* **63**, 691-693.

Horváth, Gy., Stipkovits, L., , Zöldág, L., Varga, Zs. and Mészaros, J. (1980). Kísérletes Mycoplasma-mastitis szarvasmarhán. *Magy. Áo. Lapja.* **36,** 610-614.

Horváth, Gy., Stipkovits, L., Varga, Zs., Zöldág, L., and Mészaros, J. (1983). Infection of cows by Mycoplasma bovis. *Arch. Exp. Veterinärmed.* **37**, 401-403.

Hötzel, H., Demuth, B., Sachse, K., Pflitsch, A., and Pfützner, H. (1993). Detection of Mycoplasma bovis using in vitro deoxyribonucleic acid amplification. *Rev. Sci. Tech.* **12**, 581-591.

Howard, C. J. (1984). Comparison of bovine IgG1, IgG2 and IgM for ability to promote killing of Mycoplasma bovis by bovine alveolar macrophages and neutrophils. *Vet. Immunol. Immunopathol.* **6**, 321-326.

Howard, C. J., Stott, E. J., Thomas, L. H., Gourlay, R. N., and Taylor, G. (1987). Protection against respiratory disease in calves induced by vaccines containing respiratory syncytial virus, parainfluenza type 3 virus, Mycoplasma bovis and M dispar. *Vet. Rec.* **121**, 372-376.

Howard, C. J., Taylor, G., Collins, J., and Gourlay, R. N. (1976). Interaction of Mycoplasma dispar and Mycoplasma agalactiae subsp. bovis with bovine alveolar macrophages and bovine lacteal polymorphonuclear leukocytes. *Infect. Immun.* **14**, 11-17.

Howard, C. J., Thomas, L. H., and Parsons, K. R. (1987). Comparative pathogenicity of Mycoplasma bovis and Mycoplasma dispar for the respiratory tract of calves. *Isr. J. Med. Sci.* **23**, 621-624.

Ibrahim, M. A. R., Stipkovits, L., Boldizsár, H., and Varga, Zs. (1984). Amino acid composition and characteristic indices of bovine seminal plasma in case of infection of semen by mycoplasmas. *Acta Vet. Hung.* **32**, 15-21.

Illing, K. (1979). [Clinical picture of experimental Mycoplasma bovis mastitis in cattle]. *Arch. Exp. Veterinärmed.* **33**, 945-948.

Jack, E. J., Moring, J., and Boughton, E. (1977). Isolation of Mycoplasma bovis from an outbreak of infectious bovine kerato conjunctivitis. *Vet. Rec.* **101**, 287.

Jasper, D. E. (1977). Mycoplasma and mycoplasma mastitis. *J. Am. Vet. Med. Assoc.* **170**, 1167-1172.

Johansson, K. E., Berg, L-O., Bölske, G., Deniz, S., Mattsson, J., Persson, M., and Pettersson, B. (1996). Specific PCR systems based on the 16S rRNA genes of Mycoplasma agalactiae and Mycoplasma bovis. In: Frey, J., and Sarris, K. (eds): Mycoplasmas of ruminants: pathogenicity,

diagnostics, epidemiology and molecular genetics. COST 826 Agriculture and biotechnology EUR 16934 EN.

Jurmanova, K. and Sterbova, J. (1977). Correlation between impaired spermatozoan motility and mycoplasma findings in bull semen. *Vet. Rec.* **100**, 157-158.

Kinde, H., Daft, B. M., Walker, R. L., Charlton, B. R., and Petty, R. (1993). Mycoplasma bovis associated with decubital abscesses in Holstein calves. *J. Vet. Diagn. Invest* **5**, 194-197.

Kirby, F. D. and Nicholas, R. A. (1996). Isolation of Mycoplasma bovis from bullocks' eyes. *Vet. Rec.* **138**, 552.

Kissi, B., Juhász S. and Stipkovits, L. (1985). Effect of mycoplasma contamination of bull semen on fertilization. *Acta Vet. Hung.***33,** 107-117.

Knudtson, W. U., Reed, D. E., and Daniels, G. (1986). Identification of Mycoplasmatales in pneumonic calf lungs. *Vet. Microbiol.* **11**, 79-91.

Kreusel, S., Bocklisch, H., Pfützner, H., Brys, A., Leirer, R., and Ziegenhals, U. (1989). [Experimental infections of bulls with Mycoplasma (M.) bovis and M. bovigenitalium]. *Arch. Exp. Veterinärmed.* **43**, 705-712.

Kunkel, J. R. (1985). Isolation of Mycoplasma bovis from bulk milk. *Cornell Vet.* **75**, 398-400.

Kusiluka, L. J., Ojeniyi, B., and Friis, N. F. (2000). Increasing prevalence of Mycoplasma bovis in Danish cattle. *Acta Vet. Scand.* **41**, 139-146.

Ladányi, M., Horváth, l., Gaál, M., and Hufnagel, L. (2003). An agro-ecological simulation model system. *Appl. Ecol. Env. Res.* **1(1-2)**, 47-74.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.

LaFaunce, N. A. and McEntee, K. (1982). Experimental Mycoplasma bovis seminal vesiculitis in the bull. *Cornell Vet.* **72**, 150-167.

Langford, E. V. (1975). Mycoplasma species recovered from the reproductive tracts of western Canadian cows. *Can. .J. Comp. Med.* **39**, 133-138.

Langford, E. V. (1976). Mycoplasma agalactiae subsp. bovis in pneumonia and arthritis of the bovine. *Can. J. Comp. Med.* **41**, 89-94.

Langford, E. V., Jurmanova, K., and Stalheim, O. H. V. (1978).Mycoplasma infection of the bovine urogenital system (Abstract). *Zbl. Bakt. Hyg. I. Abt. Orig. A.* **241,** 264.

Lauerman, L. H. (1994). Mycoplasmas of the bovine respiratory tract. In: Whitford, H. W., Rosenbusch, R. F., and Lauerman, L. H. (eds.). Mycoplasmosis in Animals: Laboratory diagnosis. Iowa State University Press Ames, Iowa, pp. 50-56.

Le Grand, D., Calavas, D., Brank, M., Citti, C., Rosengarten, R., Bezille, P., and Poumarat, F. (2001). Serological prevalence of Mycoplasma bovis infection in suckling beef cattle in France. *Vet. Rec.* **150**, 268-273.

Le Grand, D., Solsona, M., Rosengarten, R., and Poumarat, F. (1996). Adaptive surface antigen variation in Mycoplasma bovis to the host immune response. *FEMS Microbiol. Lett.* **144**, 267-275.

Lopez, A., Maxie, M. G., Ruhnke, L., Savan, M., and Thomson, R. G. (1986). Cellular inflammatory response in the lungs of calves exposed to bovine viral diarrhea virus, Mycoplasma bovis, and Pasteurella haemolytica. *Am. J. Vet. Res.* **47**, 1283-1286.

Lysnyansky, I., Ron, Y., and Yogev, D. (2001). Juxtaposition of an active promoter to vsp genes via site-specific DNA inversions generates antigenic variation in Mycoplasma bovis. *J. Bacteriol.* **183**, 5698-5708.

Lysnyansky, I., Rosengarten, R., and Yogev, D. (1996). Phenotypic switching of variable surface lipoproteins in Mycoplasma bovis involves high-frequency chromosomal rearrangements. *J.Bacteriol.* **178**, 5395-5401.

Lysnyansky, I., Sachse, K., Rosenbusch, R., Levisohn, S., and Yogev, D. (1999). The vsp locus of Mycoplasma bovis: gene organization and structural features. *J. Bacteriol.* **181**, 5734-5741.

Madoff, S., Pixley, B. Q., DelGiudice, R. A., and Moellering, R. C., Jr. (1979). Isolation of Mycoplasma bovis from a patient with systemic illness. *J. Clin. Microbiol.* **9**, 709-711.

Máthé-Gáspár, G. and Kovács, G.J. (2003). Use of simulation technique to distinguish between the effect of soil and weather on crop development and growth. *Appl. Ecol. Env. Res.* **1(1-2),** 87-92.

Mattsson, J. G., Guss, B., and Johansson, K. E. (1994). The phylogeny of Mycoplasma bovis as determined by sequence analysis of the 16S rRNA gene. *FEMS Microbiol.Lett.* **115**, 325-328.

McCully, M. A. and Brock, K. V. (1992). Development of a DNA hybridization probe for detection of Mycoplasma bovis. *J. Vet. Diagn. Invest* **4**, 464-467.

Megid, R., Nicholas, R. A., and Miles, R. J. (2001). Biochemical characterization of Mycoplasma bovirhinis, Mycoplasma dispar and recent bovine isolates of Mycoplasma canis. *Vet. Res. Commun.* **25**, 1-12.

Mészáros, J., Horváth, G., Stipkovits, L., and Varga, Z. (1986). Gross and histopathological study of experimental Mycoplasma mastitis of cattle. *Acta Vet Hung.* **34**, 201-209.

Miles, R. J., Wadher, B. J., Henderson, C. L. and Mohan, K. (1988) Increased yields of Mycoplasma spp. in the presence of pyruvate . *Lett. Appl. Microbiol.* **7,** 149-151.

Nagatomo, H., Shimizu, T., Higashiyama, Y., Yano, Y., Kuroki, H., and Hamana, K. (1996). Antibody response to Mycoplasma bovis of calves introduced to a farm contaminated with the organism. *J. Vet. Med. Sci.* **58**, 919-920.

Nagatomo, H., Takegahara, Y., Sonoda, T., Yamaguchi, A., Uemura, R., Hagiwara, S., and Sueyoshi, M. (2001). Comparative studies of the persistence of animal mycoplasmas under different environmental conditions. *Vet. Microbiol.* **82**, 223-232.

Nicholas, R. A. J., Wood, E., Baker, S., and Ayling, R. D. (2001). Mycoplasmas isolated from ruminants in Britain 1995-2000. In: Poveda, J. B., Fernandez, A., Frey, J., and Johansson, K. E. (eds.). Mycoplasmas of Ruminants: pathogenicity, diagnostics, epidemiology and molecular genetics. Vol. 5. European Commission, Brussels. pp. 116-120.

Nicholas, R. A. and Ayling, R. D. (2003). Mycoplasma bovis: disease, diagnosis, and control. *Res. Vet. Sci.* **74**, 105-112.

Nicholas, R. A., Ayling, R. D., and Stipkovits, L. P. (2002). An experimental vaccine for calf pneumonia caused by Mycoplasma bovis: clinical, cultural, serological and pathological findings. *Vaccine* **20**, 3569-3575.

Nicholas, R. A., Baker, S. E., Ayling, R. D. and Stipkovits, L. (2000). Mycoplasma infection in growing cattle. *Cattle Practice* **8,** 115-118.

Pfützner, H. (1984). [The tenacity of Mycoplasma bovis]. *Zentralbl. Bakteriol. Mikrobiol. Hyg.[A]* **258**, 38-41.

Pfützner, H**.** (1990). Epizootiology of Mycoplasma bovis infection of cattle, *In* Stanek, G., Cassell, G. H., Tully, J. H. and Whitcomb, R. F. (eds.) Recent advances in mycoplasmology, Proceedings of the 7th Congress of the International Organization for Mycoplasmology, vol. 20. Gustav Fisher Verlag, Stuttgart, Germany pp. 394-399.

Pfützner, H. and Sachse, K. (1996). Mycoplasma bovis as an agent of mastitis, pneumonia, arthritis and genital disorders in cattle. *Rev. Sci. Tech.* **15**, 1477-1494.

Pfützner, H. and Schimmel, D. (1985). [Mycoplasma bovis isolation in the offspring of cows with M. bovis mastitis and its epizootiological significance]. *Zentralbl. Veterinärmed. B* **32**, 265-279.

Pfützner, H., Kielstein, P., Martin, J., and Schimmel, D. (1983a). [Mycoplasma infection of calves. 2. Experimental infection of calves with Mycoplasma bovis]. *Arch. Exp. Veterinärmed.* **37**, 445-451.

Pfützner, H., Scherwa, B., and Trubner, S. (1983b). [Sensitivity of Mycoplasma bovis to disinfection agents applied to the udder area]. *Arch. Exp. Veterinärmed.* **37**, 485-489.

Pinnow, C. C., Butler, J. A., Sachse, K., Hötzel, H., Timms, L. L., and Rosenbusch, R. F. (2001). Detection of Mycoplasma bovis in preservative-treated field milk samples. *J. Dairy Sci.* **84**, 1640-1645.

Poumarat, F., Le Grand, D., Solsona, M., Rosengarten, R., and Citti, C. (1999). Vsp antigens and vsp-related DNA sequences in field isolates of Mycoplasma bovis. *FEMS Microbiol.Lett.* **173**, 103-110.

Poumarat, F., Perrin, M., Gauthier, N., Lepage, D. and Martel, J. L. (1988). Pathologie respiratoire des veaux de nurserie et des taurillons. Prévalence de *Mycoplasma bovis* parmi les différentes étiologies infectieuses au travers d'enquetes réalisées en Région Rhone–Alpes. *Rev. Méd. Vét*. **164,** 625-632.

Poumarat, F., Perrin, B., and Longchambon, D. (1991). Identification of ruminant mycoplasmas by dot immunobinding on membrane filtration (MF dot). *Vet. Microbiol.* **29**, 329-338.

Poumarat, F., Solsona, M., and Boldini, M. (1994). Genomic, protein and antigenic variability of mycoplasma bovis. *Vet. Microbiol.* **40**, 305-321.

Poumarat, F., Le Grand, D., and Bergonier, D. (1996). Propriétés générales des mycoplasmes et hypervariabilité antigénique. *Point. Vet.* **28**, 761-767.

Poveda, J. B., and Nicholas, R. A. (1998). Serological Identification of Mycoplasmas by Growth and Metabolism Inhibition Tests. In: Methods in Molecular Biology. Vol. 104: Mycoplasma Protocols, Miles, R. J., and Nicholas, R. A. (eds.). Humana press Inc., Totowa, NJ. pp. 105-112.

Rasberry, U. and Rosenbusch, R. F. (1995). Membrane-associated and cytosolic species-specific antigens of Mycoplasma bovis recognized by monoclonal antibodies. *Hybridoma* **14**, 481-485.

Razin, S., and Freundt, E. A. (1984) The Mycoplasmas. In: Bergey's Manual of Systematic Bacteriology, Vol. I. Williams and Wilkins, Baltimore, pp. 740-770.

Razin, S., Yogev, D., and Naot, Y. (1998). Molecular biology and pathogenicity of mycoplasmas. *Microbiol. Mol. Biol. Rev.* **62**, 1094-1156.

Rebhun, W. C. (1995). Respiratory diseases. In: Diseases of cattle. Baltimore: Williams and Wilkins pp. 79-80.

Richter, A. and Pfützner, H. (1989). [Detection of mycoplasmas in experimentally-infected bull sperm]. *Arch. Exp. Veterinärmed.* **43**, 721-724.

Rodriguez, F., Bryson, D. G., Ball, H. J., and Forster, F. (1996). Pathological and immunohistochemical studies of natural and experimental Mycoplasma bovis pneumonia in calves. *J. Comp. Pathol.* **115**, 151-162.

Rodriguez, F., Sarradell, J., Poveda, J. B., Ball, H. J., and Fernandez, A. (2000). Immunohistochemical characterization of lung lesions induced experimentally by Mycoplasma agalactiae and Mycoplasma bovis in goats. *J. Comp. Pathol.* **123**, 285-293.

Romváry, J., Rózsa, J., Stipkovits, L., és Szabó, J. (1975). Szarvasmarhák mycoplasmás eredetű ízület- és tüdőgyulladása. *Magy. Áo. Lapja.* **30,** 695-698.

Romváry, J., Rózsa, J., Stipkovits, L., and Mészaros, J. (1977). Incidence of diseases due to Mycoplasma bovis in a cattle herd. I. Pneumo-arthritis syndrome in calves. *Acta Vet. Hung.* **27**, 29-37.

Rosengarten, R., Citti, C. (1999). The role of ruminant mycoplasmas in systemic infection. In Stipkovits, L., Rosengarten, R., and Frey, J. (Eds.). Mycoplasmas of ruminants: Pathogenicity, diagnostics, epidemiology and molecular genetics, Vol. 3. Brussels: European Commission. pp 14-17.

Rosengarten, R., and Yogev, D. (1996). Variant colony surface antigenic phenotypes within mycoplasma strain populations: implications for species identification and strain standardization. *J. Clin. Microbiol.* **34**, 149-158.

Rosengarten, R., Behrens, A., Stetefeld, A., Heller, M., Ahrens, M., Sachse, K., Yogev, D., and Kirchhoff, H. (1994). Antigen heterogeneity among isolates of Mycoplasma bovis is generated by high-frequency variation of diverse membrane surface proteins. *Infect. Immun.* **62**, 5066-5074.

Ruhnke, H. L. (1994) Mycoplasmas associated with bovine genital tract infections. In: Whitford, H. W., Rosenbusch, R. F. and Lauerman, L. H. (eds.): Mycoplasmosis in Animals: Laboratory diagnosis. Iowa State University Press Ames, Iowa, pp. 50-56.

Ryan, M. J., Wyand, D. S., Hill, D. L., Tourtellotte, M. E., and Yang, T. J. (1983). Morphologic changes following intraarticular inoculation of Mycoplasma bovis in calves. *Vet. Pathol.* **20**, 472-487.

Sachse, K., Grajetzki, C., Rosengarten, R., Hanel, I., Heller, M., and Pfützner, H. (1996). Mechanisms and factors involved in Mycoplasma bovis adhesion to host cells. *Zentralbl. Bakteriol.* **284**, 80-92.

Sachse, K., Helbig, J. H., Lysnyansky, I., Grajetzki, C., Muller, W., Jacobs, E., and Yogev, D. (2000). Epitope mapping of immunogenic and adhesive structures in repetitive domains of Mycoplasma bovis variable surface lipoproteins. *Infect. Immun.* **68**, 680-687.

Sachse, K., Pfützner, H., Heller, M., and Hanel, I. (1993a). Inhibition of Mycoplasma bovis cytadherence by a monoclonal antibody and various carbohydrate substances. *Vet. Microbiol.* **36**, 307-316.

Sachse, K., Pfützner, H., Hötzel, H., Demuth, B., Heller, M., and Berthold, E. (1993b). Comparison of various diagnostic methods for the detection of Mycoplasma bovis. *Rev. Sci. Tech.* **12**, 571-580.

Shimizu, T. (1983). Selective medium for the isolation of Mycoplasma bovis from nasal discharges of pneumonic calves. *Res. Vet. Sci.* **34**, 371-373.

Shulman, M., Wilde, C. D., and Kohler, G. (1978). A better cell line for making hybridomas secreting specific antibodies. *Nature* **276**, 269-270.

Stalheim, O. H. (1976). Failure of antibiotic therapy in calves with mycoplasmal arthritis and pneumonia. *J. Am. Vet. Med. Assoc.* **169**, 1096-1097.

Stipkovits, L., Rady, M., and Glavits, R. (1993). Mycoplasmal arthritis and meningitis in calves. *Acta Vet. Hung.* **41**, 73-88.

Stipkovits, L., Ripley, P. H., Varga, J., and Palfi, V. (2001). Use of valnemulin in the control of Mycoplasma bovis infection under field conditions. *Vet. Rec.* **148**, 399-402.

Stipkovits, L., Ripley, P., Varga, J., and Palfi, V. (2000). Clinical study of the disease of calves associated with Mycoplasma bovis infection. *Acta Vet. Hung.***48**, 387-395.

Subramaniam, S., Bergonier, D., Poumarat, F., Capaul, S., Schlatter, Y., Nicolet, J., and Frey, J. (1998). Species identification of Mycoplasma bovis and Mycoplasma agalactiae based on the uvrC genes by PCR. *Mol. Cell Probes* **12**, 161-169.

Tamas, J. (2003). Problems and solutions of field scale agro-ecological data acquisition and data interpretations in agroinformatical domain. *Appl. Ecol. Env. Res.* **1(1-2)**, 143-157.

Taylor-Robinson, D. and Bebear, C. (1997). Antibiotic susceptibilities of mycoplasmas and treatment of mycoplasmal infections. *J. Antimicrob. Chemother.* **40**, 622-630.

ter Laak, E. A., Noordergraaf, J. H., and Boomsluiter, E. (1992a). The nasal mycoplasmal flora of healthy calves and cows. *Zentralbl. Veterinarmed.B* **39**, 610-616.

ter Laak, E. A., Noordergraaf, J. H., and Dieltjes, R. P. (1992b). Prevalence of mycoplasmas in the respiratory tracts of pneumonic calves. *Zentralbl. Veterinärmed. B* **39**, 553-562.

ter Laak, E. A., Wentink, G. H., and Zimmer, G. M. (1992c). Increased prevalence of Mycoplasma bovis in the Netherlands. *Vet. Q.* **14**, 100-104.

ter Laak, E. A., Noordergraaf, J. H., and Verschure, M. H. (1993). Susceptibilities of Mycoplasma bovis, Mycoplasma dispar, and Ureaplasma diversum strains to antimicrobial agents in vitro. *Antimicrob.Agents Chemother.* **37**, 317-321.

Thomas, A., Dizier, I., Trolin, A., Mainil, J., and Linden, A. (2002). Comparison of sampling procedures for isolating pulmonary mycoplasmas in cattle. *Vet. Res. Commun.* **26**, 333-339.

Thomas, A., Nicolas, C., Dizier, I., Mainil, J. and Linden, A. (2003) Antibiotic susceptibilities of recent isolates of Mycoplasma bovis in Belgium. *Vet. Rec.* **153,** 428-431.

Thomas, C. B., Van Ess, P., Wolfgram, L. J., Riebe, J., Sharp, P., and Schultz, R. D. (1991). Adherence to bovine neutrophils and suppression of neutrophil chemiluminescence by Mycoplasma bovis. *Vet. Immunol. Immunopathol.* **27**, 365-381.

Thomas, C. B., Willeberg, P., and Jasper, D. E. (1981). Case-control study of bovine mycoplasmal mastitis in California. *Am. J. Vet. Res.* **42**, 511-515.

Thomas, L. H., Howard, C. J., Stott, E. J., and Parsons, K. R. (1986). Mycoplasma bovis infection in gnotobiotic calves and combined infection with respiratory syncytial virus. *Vet. Pathol.* **23**, 571-578.

Thorns, C. J. and Boughton, E. (1980). Studies on the effect of growth medium composition on the antigenicity of Mycoplasma bovis. *J. Hyg. (Lond)* **84**, 29-36.

Tola, S., Idini, G., Rocchigiani, A. M., Manunta, D., Angioi, P. P., Rocca, S., Cocco, M., and Leori, G. (1999). Comparison of restriction pattern polymorphism of Mycoplasma agalactiae and Mycoplasma bovis by pulsed field gel electrophoresis. *Zentralbl. Veterinärmed. B* **46**, 199-206.

Tschernig, T., Lührmann, A., and Pabst, R. (2002). Recruitment of Lymphocytes and DendriticCells from the Blood to the Bronchoalveolar Space and the Draining Lymph Nodes after a Single Intrabronchial Application of the Lipopeptide MALP-2T. *Pathobiology.* **70**, 256–261.

Uhaa, I. J., Riemann, H. P., Thurmond, M. C., and Franti, C. E. (1990a). The use of the enzyme-linked immunosorbent assay (ELISA) in serological diagnosis of Mycoplasma bovis in dairy cattle. *Vet. Res. Commun.* **14**, 279-285.

Uhaa, I. J., Riemann, H. P., Thurmond, M. C., and Franti, C. E. (1990b). A cross-sectional study of bluetongue virus and Mycoplasma bovis infections in dairy cattle: I. The association between a positive antibody response and production efficiency. *Vet. Res. Commun.* **14**, 461-470.

Uhaa, I. J., Riemann, H. P., Thurmond, M. C., and Franti, C. E. (1990c). A cross-sectional study of hluetongue virus and Mycoplasma bovis infections in dairy cattle: II. The association between a positive antibody response and reproduction performance. *Vet. Res. Commun.* **14**, 471-480.

Urbaneck, D., Liebig, F., Forbrig, T., and Stache, B. (2000) Experiences with the use of herd-specific vaccines against respiratory infections with *Mycoplasma bovis* in a large cattle herd. *Prakt. Tierarzt* **81,** 756-763.

Vanden Bush, T. J. and Rosenbusch, R. F. (2004). Characterization of a lympho-inhibitory peptide produced by Mycoplasma bovis. *Biochem. Biophys. Res. Commun.* **315**, 336-341.

Vasconcellos Cardoso, M., Blanchard, A., Ferris, S., Verlengia, R., Timenetsky, J., and Florio Da Cunha, R. A. (2000). Detection of Ureaplasma diversum in cattle using a newly developed PCR-based detection assay. *Vet. Microbiol.* **72**, 241-250.

Visser, I. J., ter Laak, E. A., and Jansen, H. B. (1999). Failure of antibiotics gentamycin, tylosin, lincomycin and spectinomycin to eliminate Mycoplasma bovis in artificially infected frozen bovine semen. *Theriogenology* **51**, 689-697.

Walz, P. H., Mullaney, T. P., Render, J. A., Walker, R. D., Mosser, T., and Baker, J. C. (1997). Otitis media in preweaned Holstein dairy calves in Michigan due to Mycoplasma bovis. *J. Vet. Diagn. Invest* **9**, 250-254.

Windsor, G. D., and Bashiruddin, J. B. (1999). Coloured colonies of Mycoplasmas Frequently Isolated from Ruminants on Solid Agar Medium. In: COST Action 826 "Ruminants Mycoplasmoses". Toulouse, France.

Windsor, H. M., Ripley, P. H., and Hannan, P. C. T. (1996). An *in vitro* investigation of the sensitivity of German field isolates *of M. hyopneumoniae* and *M. hyosynoviae* to SDZ PMD 296, a new pleuromulin derivate. *Proc. 14th IPVS Congress,* Bologna, Italy. p. 226.

Yogev, D., Menaker, D., Strutzberg, K., Levisohn, S., Kirchhoff, H., Hinz, K. H., and Rosengarten, R. (1994). A surface epitope undergoing high-frequency phase variation is shared by Mycoplasma gallisepticum and Mycoplasma bovis. *Infect. Immun.* **62**, 4962-4968.

Yogev, D., Browning, G. F., and Wise K. S. (2002). Genetic mechanisms of surface variation. In: Razin S., and Herrmann R. (eds.) Molecular Biology and Pathogenicity of Mycoplasmas. Kluwer Academic, New York, pp. 417-444.

11.
Scientific publications of the thesis

Refereed research papers published in scientific journals

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

12.
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