COST
European Cooperation
on Scientific and
Technical Research

COST 826

Agriculture and biotechnology

Mycoplasmas of ruminants: pathogenicity, diagnostics, epidemiology and molecular genetics
Volume 3

Edited by L. Stipkovits, R. Rosengarten and J. Frey

Report

EUR 18756 EN

EUROPEAN COMMISSION

Édith CRESSON, Member of the Commission responsible for research, innovation, education, training and youth

DG XII/B.1 — RTD actions: Cooperation with non-member countries and international organisations — European Economic Area, COST, Eureka and international organisations

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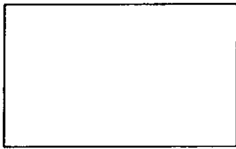

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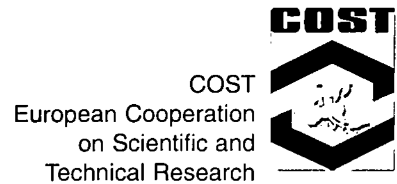

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Mycoplasmas of ruminants: pathogenicity, diagnostics, epidemiology and molecular genetics

Volume 3

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1999

EUR 18756 EN

A great deal of additional information on the European Union is available on the Internet.
It can be accessed through the Europa server (http://europa.eu.int).

Cataloguing data can be found at the end of this publication.

Luxembourg: Office for Official Publications of the European Communities, 1999

ISBN 92-828-5222-9

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Printed in Belgium

PRINTED ON WHITE CHLORINE-FREE PAPER

PREFACE

The industrialised countries suffered during the last decade from a marked increase of re-emerging and new emerging infectious diseases which affected humans and animals. Among those, mycoplasmal diseases of animals are of particular concern. They constitute a threat for animal health and livestock production. The control and elimination of these infectious diseases constitute a challenge to veterinarians, officials of veterinary services, researchers and industry. The aim of COST Action 826 „Ruminants' Mycoplasmoses" is to provide a network for the exchange of scientific and technical knowledge, and for the promotion and co-ordination of research and development in the field of mycoplasmal diseases of ruminants. This Action is expected to rapidly provide the necessary elements for the elaboration of strategies for the prevention and eradication of ruminants' mycoplasmoses. Recent discoveries on the phylogenetic relationships among the different *Mycoplasma* species involved in ruminants' mycoplasmoses revealed the difficulty to clearly differentiate by genetic methods some of these species. It manifested the urge for more basic research to be done with this important group of mycoplasmas. This report is the result of the „Third Workshop on Mycoplasmas of Ruminants: Pathogenicity, Epidemiology, and Molecular Genetics" of COST Action 826 held in Budapest, Hungary, from May 5 - 8 1998. It resumes the recent outcomes in basic and applied research in the field of mycoplasmas of ruminants and the newest results on the prevalence of ruminants' mycoplasmoses in the different European regions.

The Workshop was organised by the Veterinary Medical Research Institute of Hungarian Academy of Sciences at Hotel Agro located on the Sváb Hill, overlooking the Danube and the city of Budapest. It gave to all participants the unique chance to attend a most dynamic workshop who brought together 80 veterinarians, officials, researchers and industrials from many European countries and from other continents working in the different disciplines of ruminants' mycoplasmoses. The workshop showed which directions the development of diagnostic methods and preventive concepts will take in the future.

I would like to thank Prof. László Stipkovits and his collaborators for the excellent organisation of the Workshop and for providing the participants not only an outstanding scientific programme but also giving them the opportunity to get an insight in Hungarian live and culture. I would like to thank Prof. Renate Rosengarten, Vienna for her precious input during the preparations and our COST Secretary Dr. Françoise Coudert, Brussels for her help in the realisation of this Workshop. Particular thanks to the sponsors: The National Committee for Technological Development, Budapest; The Hungarian Academy of Sciences, Budapest; Pfizer A.H.S.A., Budapest; Bayer A.G., Leverkusen, Germany; Novartis Animal Health GmbH, Kundl, Austria; Laboratorios Calier S.A., Barcelona, Spain; and Elanco Animal Science Research, Eli Lilly and Company Ltd, Basingstoke, UK. The sponsors manifested with their financial support their interest in our research activities and made possible the organisation of this most productive Workshop.

Berne, August 4 1998

Joachim Frey
Chairman, COST 826

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ANTIGENIC VARIATION IN MYCOPLASMAS: PAST, PRESENT AND FUTURE.

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Institute of Bacteriology, Mycology and Hygiene[1], University of Veterinary Medicine, 1210 Vienna, Austria. CNEVA-Lyon, Laboratoire de Pathologie Bovine[2], 69342 Lyon cedex 07, France. Ecole Nationale Vétérinaire de Lyon, Pathologie du Bétail[3], 69280 Marcy-l'Etoile, France. Ecole Nationale Vétérinaire, Département Elevage et Produits[4], 31076 Toulouse Cedex, France.

Over the past 10 years, an increasing number of studies have reported the presence of genetic systems generating high frequency antigenic variation in mycoplasma. The term "antigenic variation" defines here the ability of a single organism to generate sub-populations expressing an alternate form of a surface component recognized and distinguished by antibodies. In fact, all the mycoplasma species examined so far possess surface proteins that vary in expression and/or in structure. In most cases, these systems are composed of a set of related, but distinct single-copy genes encoding components which are abundantly expressed at the surface of the cell and highly immunogenic in the host.

Clues to the existence of systems generating antigenic variation in mycoplasmas have first emerged while studying *M. hyorhinis* surface lipoproteins (11). In this swine pathogen, a set of single-copy genes encoding variable lipoproteins (Vlps) is subjected to discrete, high-frequency mutations that independently affect the expression and the size of each Vlp product (6, 12, 15). Variation in expression is governed by spontaneous insertion/deletion mutations in a polyA tract located in the promoter region of each *vlp* gene that drastically alter *vlp* gene transcription. Changes in Vlp sizes are the result of spontaneous mutations that modify the length of the 3' end of each *vlp* gene by in-frame insertion or deletion of tandem repeated sequences. Thus, each *vlp* gene represents a highly mutable module encoding a distinct product sharing similar structures with other *vlp* genes. Similar mutational events are responsible for the variation in size and expression of the Vaa adhesin of *M. hominis* , a human pathogen that causes arthritis. Oscillation in Vaa expression occurs by insertion/deletion of a single adenine nucleotide in a polyA tract located at the 5' end of the corresponding gene. In contrast to the Vlp system of *M. hyorhinis*, this switch mechanism alters the *vaa* reading frame by creating frameshift mutations. In Vaa$^-$ variants, this results in inserting a stop codon that considerably truncates the product encoded by the *vaa* open reading frame (17). Vaa size variants arise by gain or loss of identical tandem repeated blocks of amino acids located in the central region of the protein (16). Mechanisms involving DNA rearrangements as a means of high-frequency phenotypic variations have also been reported for two mycoplasma species. The first example refers to *M. pulmonis,* a this murine pathogen in which the *vsa* locus that encodes the variable V-1 surface lipoprotein antigens undergoes DNA rearrangements at a high frequency (3). This locus is composed by a cluster of 7 *vsa* genes, with only a single *vsa* gene being expressed in a given cell. V-1 antigen variation is generated by site-specific DNA inversions which occur between a 34-bp sequence present at the beginning of each *vsa* gene and re-allocate the 5' end (promoter and ribosome binding site) from an

expressed *vsa* gene to a silent one. This 5' region, the so-called expression site, is a single chromosomal copy containing a promoter region that controls ON and OFF transcription of *vsa* genes (4). The second example of phenotypic switching by DNA rearrangements has recently been described for the Vsp lipoprotein family of the bovine pathogen *M. bovis* (9, 10, 11). Whether the reported oscillation in Vsp expression is due to DNA inversion or DNA insertion is currently being investigated. The V-1 antigens of *M. pulmonis* and the Vsps of *M. bovis*, both undergo size variation through gain or loss of tandem repeated units in their corresponding genes. Systems generating high-frequency surface antigenic variation have also been reported for many other pathogenic mycoplasmas for which the underlying genetic mechanisms remain to be elucidated. Nevertheless, the mutational processes described here might be a common trait of mycoplasmas inherited from a natural long evolution within immunocompetent hosts.

The wide spread distribution of multigene families encoding variable components associated with the fact that they have been maintained throughout the regressive evolution of the mycoplasma genome reflect their importance, though very little is known about their specific function. Overall, these sophisticated systems may provide the organism with the ability to rapidly vary its membrane architecture, allowing the pathogen to adapt to diverse host niches as well as to escape the host immune defense during infection (7). In *M. hyorhinis*, the role of Vlp structural variation has recently emerged as a means to escape hyperimmune host antibody damage (5). More specifically, variants expressing Vlp with extended length were resistant to complement-independent growth inhibition by host antibodies while their counterparts expressing short versions of the same Vlp were susceptible. Detailed analysis showed that Vlps are not the target of the inhibitory activity but, by extending their length, provide a shield that prevents host antibody binding to vital epitopes that are surface exposed. In contrast, *M. bovis* escapes host antibody inhibition by switching off the expressed Vsp (8). This indicates that different mutational strategies can be used by mycoplasmas to face the host immune system. However, evasion of the host immune defence is only one aspect of the function of these complex variable systems. For instance, variations of the *M. pulmonis* V-1 antigen has been correlated with lung lesion severity in the mice model representing respiratory mycoplasmosis , as well as with haemadsorption, which is also observed in the avian pathogen *M. gallisepticum* (1).

Antigenic variation can severely complicate the final outcome of routine identification of mycoplasma isolated from clinical material based on immunocolony blot using monoclonal or poplyclonal antibodies (13). For instance, heterogeneous colony blot patterns can be observed when *M. bovis* PG45 colonies are immunostained with a conventional diagnostic hyperimmune antisera. This heterogeneous pattern that revealed populations displaying weak or strong stainings is the result of antigenic variation of surface components expressed by the strain and did not indicate the presence of another mycoplasma species cross reacting with the antisera. As well, propagation by sub-cultivation of randomly selected agar grown population of established mycoplasma strains may select for a particular sub-population presenting a different antigenic makeup than that predominately expressed by the original parent strain. This can result in using strains with a same

designation but dissimilar virulence-related surface properties in different laboratories (13).

As we learn more about systems generating antigenic variation in mycoplasmas the question of their specific contribution in establishing diseases remains to be answer. Nevertheless, variable surface proteins of mycoplamas may offer valuable tools for epidemiological studies and the design of new serodiagnostic assays as they are often highly immunogenic in the host.

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CONTAGIOUS BOVINE PLEUROPNEUMONIA AND CONTAGIOUS CAPRINE PLEUROPNEUMONIA HISTORICAL REVIEW AND ACTUAL THREAT FOR EUROPEAN CATTLE

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„I know that most scientific discoveries can be described in few words. However searching for their origin and studying exactly how they developed, show the slowness needed for their emergence. Hence their description can be done in two different ways: the first one consists in enouncing them and proving their rightness promptly, the second one based on historical facts consists in recalling all individual efforts that were needed for their emergence. [....] The second one highlights human intelligence [...] and shows that nothing durable can be done without much efforts." L. Pasteur

Contagious bovine pleuropneumonia (CBPP) and contagious caprine pleuropneumonia (CCPP) are two infectious diseases of ruminants that share a number of characteristics but differ in some aspects. They are both caused by members of the „mycoides cluster", that induce pleuropneumonia lesions in their respective hosts, cattle or goats. *Mycoplasma mycoides* subsp. *mycoides* SC (MmmSC) and *M. capricolum* subsp. *capripneumoniae* (Mccp) which are the causative agents of CBPP and CCPP are subspecies that have the peculiarity of being very homogeneous, at least from a phenotypic point of view. Compared to them, the other members of the „mycoides cluster" are very variable. Although MmmSC and Mccp have theoretically a strict host specificity, it has been shown quite recently for both of them that they could be recovered from other hosts, particularly from sheep.

However, CBPP and CCPP differ in other points. From an historical point of view it is noteworthy that MmmSC was the first pathogenic mycoplasma to be isolated and characterized in 1898 (Nocard and Roux) as Mccp was one of the last one, in 1976, almost a century later (MacOwan and Minette). This reflects both the difficulty of growing Mccp in vitro but also the variety of pathogenic mycoplasmas of the „mycoides cluster" that may exist in goats and mask the presence of the CCPP agent. The lesions caused by these two mycoplasmas differ also in their mode of extension. In the case of CBPP there is an enlargement of the interlobular septa and extension of the lesion within the lobules as in the case of CCPP there is, first, a localized lesion that progressively extends to the periphery. The subcutaneous injection of MmmSC to cattle is inducing an invading oedema after 10 days, the so called „Willems reaction" when there is strictly no reaction after the subcutaneous injection of Mccp to goats. This difference might be due to the mycoplasma itself as the injection of MmmSC to goats is reported to lead also to an inflamatory reaction. Finally CCPP has apparently never occurred in Europe but is still present in North Africa as CBPP was once widespread in Europe but never existed in North Africa.

The history of CBPP and CCPP might give us clues for the reasons of their actual distribution.

Contagious bovine pleuropneumonia seems to have existed in Europe many centuries ago, the first descriptions fitting this disease could be found in writings from Gallo in Italy (1550) but also from C.Testienne in France (1554) (Curasson 1942). However it is only during the 18th century that the disease was clearly described, in Switzerland in 1732 by J. Scheuchzer and in France in 1765 by C. Bourgelat, the founder of the veterinary schools. At this time the disease seemed to be restricted to mountainous regions in various countries: in Switzerland in the Jura, in France in the monts d'Auvergne and Vosges, in Italy the Piemont and in Germanic states in Bavaria and Wuertenberg. The spread of CBPP throughout Europe started at the end of the 18th century and culminated in the middle of the 19th century. All European countries became infected: Northern France 1822, Belgium 1828, Holland and Prussia 1830, Schleswig Holstein 1841, Sweden 1847, Norway (hence a Swedish territory) 1860, the first cases were seen in Spain in 1846 in the Barcelona region and later on in 1864 in central Spain. The Napoleonic wars were incriminated in the spread of the disease in Europe (Turner 1959). However, it seems that the contamination of the Paris region occurred in 1790 shortly after the French revolution and that the spread of CBPP to the other European states took place after the collapse of the Napoleonic empire. Trade might have played a more important role than wars, the United Kingdom, for example, becoming infected in 1841 once it had lifted a ban on the import of cattle (Dujardin Beaumetz, 1900).

The control of CBPP was initially hampered, at the early stage of it's dissemination in Europe around 1820-1830, by the ignorance of its mode of spread. At this time scientists were divided in two categories, those believing in „spontaneous generation" and others believing in infectious agents. Concerning CBPP much uncertainty remained until 1840 when Delafond published a comprehensive paper describing the disease and its mode of spread. A little later, in 1852, Willems established the ways to „inoculate" relatively safely infectious material in cattle in order to protect them. More than thirty years later this prophylactic measure was still not unanimously accepted and the fourth international veterinary congress held in Brussels rejected it . It was finally recommended, during the fifth international veterinary congress in 1889, as an auxiliary measure to reduce significantly the number of outbreaks before stamping out policies can be put into force and achieve complete eradication (Galtier 1897). This knowledge was the basis for the establishment of efficient control strategies and eventually lead to eradication of the disease from many countries: 1888 Holland, 1895 Switzerland, 1896 Great Britain, 1900 Belgium, 1902 France, 1919 Austria, 1924 Baltic states and 1934 Poland and Soviet Union. It is noteworthy that eradication was achieved in most European countries before anything was known concerning the etiological agent as it was first identified in 1898 by Nocard and Roux. Initial strategies that focused on the slaughter of sick animals only were met with failure as in Austria from 1881 to 1891 (Tabl. 1). Association of slaughter of the diseased animals and the „inoculation" of all the others in the region permitted a dramatic reduction of number of cases in the Spoeling district in the Netherlands. Later on, strict stamping out policies enabled complete eradication. These stamping out policies were also applied with success in United Kingdom and United States without the help of „inoculations".

In Europe, a major exception is the Iberian peninsula as it seems that CBPP has never been eradicated there. According to Leclainche in 1936 and Curasson in 1942, the disease was still prevailing in Spain. Sporadic outbreaks occurred in

Portugal in 1953 and 1954 (Regalla et al. 1996) and CBPP vaccines were produced in Spain in the sixties. Further evidence of the persistence of CBPP in this region has been found in molecular epidemiology studies based on Insertion Sequence profiles. These studies showed that all recently isolated European strains had profiles that were easily distinguishable from strains of African origin, excluding the possibility of a reintroduction of CBPP through importation from Africa (Cheng et al. 1995).

The history of CCPP is completely different as it apparently never existed in Europe and was discovered only very lately. The first description was made in Algeria in 1873 by P. Thomas but this author failed to prove the infectious nature of the disease. He could not reproduce the disease by any means of inoculation and concluded that it should be caused by variations of temperature. A little later, the infectious nature of the disease was clearly demonstrated by Hutcheon in 1881 in the „Province of the good hope“ when CCPP was introduced by a shipment of angora goats from Turkey. The implementation of preventive „inoculations“ and slaughtering of contaminated animals enabled the eradication of the disease from the southern part of the African continent by 1889 and is still free up to now. As with CBPP in Europe, this eradication was possible without having any knowledge concerning the etiological agent but depended on: 1) the knowledge of the infectious nature of the disease, 2) the presence of conspicuous lesions that permit a rapid diagnosis, 3) the possibility to protect animals by „inoculations“, 4) the strict implementation of sanitary rules that combine compulsory slaughtering measures and compensations. Paradoxically, the progress in bacteriology could have been the reasons for a setback in the knowledge of CCPP. The success in isolating the CBPP agent boosted the search for the agent of CCPP but lead to isolations of contaminants or other infectious agents. This was particularly the case in Turkey where CCPP seems to have been present for centuries and exported in 1881 to southern Africa but also more recently to the Sultanate of Oman. Early studies by M. Nicolle, a student of L. Pasteur, lead to the isolation of contaminants that were possibly was is now known as *Pasteurella* (Tabl. 2). Other trials lead to the isolation of other mycoplasmas of the „mycoides cluster“ such as strain PG3 by Chu and Beveridge in 1953, the reference strain of *M. mycoides* subsp. *capri* (Mmc). This latter strain was since then considered as the etiological agent of CCPP and is still considered as such by many authors in spite of the enlighting works of Mac Owan and Minette and coworkers in Kenya in 1976. Contrarily to CBPP there is only one example of eradication of the disease in a country, possibly because the fight against CCPP relied for many years on the use of vaccines prepared with the wrong mycoplasma strain (Mmc) or later relied on the use of antibiotics that cannot lead to the eradication of the disease when they are not combined with other measures such as vaccination and slaughter of affected animals.

What are the risks of reintroduction of CBPP and CCPP in Europe?

The surveillance of an animal disease requires not only a way of diagnosing the disease but also the knowledge of the conditions for its transmission and also an efficient system for notification and for sounding the alert (Blancou 1996). As with any other infectious disease, the risk is associated with the importation of an animal from a CBPP or CCPP infected zone. When considering an official import, the risk lies in the inability of the current serological tests, the official CFT or the more recent cELISA, to detect all infected animals. In that case, testing solely the exported

animals seems inadequate and a control of the complete herd of origin seems more appropriate. A possible risk could also lie in the importation of animals that are normally not known to be infected by CCPP or CBPP, such as sheep, in which MmmSC and Mccp have been isolated on various occasions. Their possible role in the transmission of these diseases, certainly of minor importance in normal conditions, needs further elucidation. Finally importation of these diseases could also remain unnoticed if it involves hypovirulent strains that do not induce important lesions nor any seroconversion. For all these reasons, the presence of CBPP in some European countries is a permanent threat to the others as it was recently evidenced in Italy in 1990. All efforts should therefore be implemented to gain a free status in the European Union. An example of successful eradication campaign was given by Australia quite recently (1961-1967), such a campaign should be easier and less expensive now in Europe thanks to the implementation of newly developed diagnostic tests such as cELISA, western blots and PCR. Paradoxically the risk of importing CBPP or CCPP from Africa may be limited. The reason is that the presence of these two diseases is well documented there and the import of live animals is strictly regulated. Countries like Botswana or Namibia that do export meat to Europe are making all the necessary efforts to maintain a free status, sometimes at an enormous cost. The risk might be much greater from countries where the exact infection status is not well monitored and where civil unrest might lead to uncontrolled animal movements. From that point of view the actual situation of pleuropneumonia in the Middle East and Asia certainly needs to be investigated. Historical accounts seem to indicate that both diseases are present in Asia. CBPP has been introduced in Japan in 1924 with Chineese cattle, Japan eventually eradicated the disease in 1932 but there is no information concerning China. India also seems to have been contaminated at some time but the accuracy of old reports might suffer from the difficulty to distinguish MmmSC from MmmLC. More recent reports indicate that CBPP might still be present. More closely to Europe, Koweit has once been infected by CBPP but recent events might have transported the disease to Irak where it could spread to neighbouring countries such as Turkey which is at the door of Europe. Similar uncertainties also exist for CCPP at the sole difference that its presence in Turkey is well established. Contagious caprine pleuropneumonia could well have been introduced in Greece at a time, during the war between Greece and Turkey in 1921 when Greek army seized Turkish goats (Melanidi and Stylanopoulo 1928) but apparently no outbreaks occurred after 1931 (Stylanopoulos 1933). Once again the imported disease may have been due to other mycoplasmas than Mccp but the possibility of spread of CCPP through troubled areas such as Macedonia, Albania and the former Yugoslavia certainly merit attention.

Due to the widespread use of antibiotics, the direct economical consequences of the reintroduction of CBPP to disease free areas would certainly be less severe today than it was during the last century. However the indirect consequences would be disastrous as it should be followed by massive cattle slaughters, a tragedy for the owners, and prohibition of cattle movements which would slow down European integration. Dr Provost declared in 1987 that „CBPP is an original disease, full of paradox which history is parallel with the history of veterinary medicine and microbiology", this assertion is still valid fifteen years later and CBPP and CCPP are still challenging the scientists and the veterinary services in Europe.

Table 1: Selected types of control strategies and their success, according to Nocard 1898.

1) Slaughter of affected and surveillance of conaminated

AUSTRIA

Year	1881	1882	1883	1884	1885	1886	1887	1890	1891
N° sick	1633	1060	901	1358	1740	1502	1686	2028	1794

This policy did not enable a decrease in the number of sick animals. The slaughter of all sick and contaminated, after 1892, lead to a rapid eradication. Sporadic outbreaks occured in 1919 (11 herds)

Official freedom 1919

2) Slaughter of sick animals, „inoculation" of all others

Spoeling district in the **NETHERLANDS**

Year	1878	1879	1880	1881	1882	1883	1884	1885
inoculated	34784	24396	22407	24594	22172	14563	4769	286
sick	1208	475	177	267	184	153	134	59

The slaughter of all animals in an outbreak became compulsory in 1884 in all the Netherlands which became free of CBPP by 1888

Official freedom 1888

3) Slaughter of all animals in an outbreak („stamping out")

UNITED KINGDOM

From 1888 to 1890, more than 30.000 animals were slaughtered without any decrease in the number of new outbreaks. This could have been caused by a loose implementation of the policy by the local authorities. Implementation at a national level lead to rapid success

Year	1890	1891	1892	1893
sick	2057	778	130	30

Official freedom 1896

UNITED STATES

Up to 1887 the slaughter of the sick animals only was compulsory without noticeable impact. Stamping out became compulsory afterwards (March 3rd 1887)

Year	1887	1888	1889	1890	1891
Slaughtered	2918	7743	6486	3709	683

Official freedom 1892

Table2 : From first description of CCPP to the isolation of the causative agent.
This table lists the main descriptions of CCPP from 1873 to 1976. The etiology of the outbreaks is infered from the description of the lesions and the inoculations to various animals.

			Possible etiological agent		
Year	Author	Country	Mccp	MmmLC	Other
1873	THOMAS	Algeria	X		
1881	HUTCHEON	South Africa	X		
1888	DUQUESNOY	France		?	
1889	STEEL	India		X	
1894	PUSCH	Germany		?	
1896	NICOLE	Turkey		X	X
1897	LECLAINCHE	France		?	
1905	CASTELET	Algeria	X		
1911	CADEAC				
1912	SCHELLHASE	Tanganyka		X	
1914	WALKER	India	X		
1921	VAN SACEGHEM	Rwanda			X
1928	MELANIDI	Greece	X		
1929	METTAM	Kenya	X		
1931	BEATON	Nigeria		X	
1932	PIRANI	Erythrea		X	
1933	STYLANOPOULO	Greece	X		
1935	S	Turkey	X		
1937	KOLAYLI	Palestinia		X	
1940	GILBERT	India		X	
1943	LONGLEY	Turkey			X
1949	AYGÜN	Turkey			X
1950	CHU	Spain		X	
1951	LOIZELIER	India,Nigeria			X
1955	LONGLEY	Angola		X	
1957	GERLACH	Greece		X	
1967	CHRISTODOULO	Iran		?	
1967	U	Sudan	X		
1973	ENTESSAR	USA		X	
1976	ABDULLA	Kenya		X	
1976	PEARSON	Kenya	X		
	MacOWAN				
	MacOWAN				

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THE ROLE OF RUMINANT MYCOPLASMAS IN SYSTEMIC INFECTIONS

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INTRODUCTION

Despite increasing information indicating that some species of mycoplasmas can disseminate systemically, mycoplasmas are still believed to be surface parasites which live on the epithelium of the respiratory and urogenital tracts and of the mammary gland and establish localized sites of infections. Indeed, like the majority of other bacterial pathogens, mycoplasmas colonize their hosts via their mucosal surfaces, and the resulting local infections manifest themselves usually in relatively mild clinical disease forms, such as pneumonia, various genital tract diseases and mastitis, which are rarely of the fulminant type, but rather show chronicity. In some pathogenic mycoplasmas, for instance in the human pathogens *Mycoplasma pneumoniae* and *Mycoplasma genitalium*, the close contact to the mucosal epithelium, is mediated via a specific „attachment organelle", the so-called „tip structure" which shows clustered surface material involved in attachment. While the molecular biology of adhesion is well-known in these two human mycoplasma pathogens, there are only few examples of animal mycoplasmas for which evidence of adhesion structures has been established. One important animal mycoplasma species that causes infections with clinical signs which are usually or exclusively restricted to the respiratory system is *Mycoplasma hyopneumoniae*. In enzootic pneumonia of pigs caused by this organism, gross lung lesions occur most frequently in the anterior lobes, and the mycoplasmas can be visualized in large numbers covering the respiratory epithelium in immunofluorescent-stained lung sections (8).

While it is true that most pathogenic mycoplasmas are surface parasites and cause localized infections which are typically chronic in nature, there are certain mycoplasma species which have the potential to become invasive, i.e., they are able to cross the mucosal barriers and spread through the infected host after getting access to the bloodstream (6). This ability to disseminate systemically accounts also for their isolation from multiple body sites and their association with a variety of clinical manifestations (11).

Among such invasive mycoplasmas which disseminate and progress to systemic infections some are involved in important diseases of ruminants that are economically damaging for the dairy and meat industry in the affected countries. One prominent example is *Mycoplasma bovis*, that is not only causing mastitis (2), enzootic pneumonia (4) and reproductive tract disease (12) in cattle, but also arthritis (5), meningitis (14) and subcutaneaus abscesses (7) which develop during the systemic stage of infection. A second example is *Mycoplasma agalactiae*, that causes contagious agalactia in sheep and goats, which is characterized by clinical

signs which are markedly similar to those of *M. bovis* infections in cattle, and may be manifested as mastitis, arthritis and keratoconjunctivitis (3), and occasionally genital tract infections (13). In this brief overview *M. bovis* is presented as one representative ruminant mycoplasma that is able to produce systemic infections. It is focussing mainly on the diverse clinical disease forms and their economic impact, and will also address new insights into possible mechanisms by which mycoplasmas may first colonize or target mucosal epithelial cells and then spread after entering the bloodstream to cause systemic infections.

ECONOMIC IMPACT OF *MYCOPLASMA BOVIS* INFECTIONS

M. bovis infections occur worldwide and are a major problem in the US and Canada and certain parts of Europe with an increasing prevalence in Germany (10), Northern Ireland (1), Switzerland (9) and The Netherlands (15), as well as in Austria (T. Beier, J. Spergser, and R. Rosengarten, unpublished), and Hungary (L. Stipkovits, personal communication). Bovine mastitis is clearly the most serious disease caused by *M. bovis* with infection rates in the US of up to 70% of a herd (2) and a major economic impact. A survey of bulk milk tank samples done in the US state New York over nine years reported that about 2.3% of tank samples were positive for *M. bovis* by culture (Ricardo Rosenbusch, personal communication). Surveys done in Iowa yielded very close to the same percentages (Ricardo Rosenbusch, personal communication). In Iowa, tank-positive herds experienced culling rates of 30-70% of cows. Extrapolating, one could calculate that losses of at least 0.6% of milk cows (namely 30% of 2%) in production occur worldwide due to *M. bovis* mastitis. For the US, losses due to *M. bovis* mastitis have been estimated at 108 million dollars per year (Ricardo Rosenbusch, personal communication). Some other estimates are available for eastern Canada, where 52% of dairy herds are affected with *M. bovis* mastitis, resulting in culling rates of close to 33% and average milk production losses of 6.4 kg milk per cow each day (Ricardo Rosenbusch, personal communication).

M. bovis is also considered an important respiratory pathogen causing enzootic pneumonia in young veal calves and feedlot cattle (4). The losses are associated with loss of weight gain and with loss of carcass and carcass value and are estimated for the US at about 32 million dollars per year (Ricardo Rosenbusch, personal communication).

M. bovis is infrequently isolated from the genital tract and has been occasionally isolated from aborted fetuses and natural cases of seminovesiculitis (12). Under experimental conditions, *M. bovis* has been shown to cause endometritis, salpingitis, oophoritis, as well as abortion and seminovesiculitis. Contaminated bovine semen for artificial insemination is considered an important vehicle for international transmission of *M. bovis* and other mycoplasmas involved in bovine reproductive failure which results in considerable losses in production.

MYCOPLASMA BOVIS AS INVASIVE PATHOGEN

Chris Howard et al. (6) reported already several years ago that *M. bovis* can become invasive and translocate across the respiratory mucosa. This ability may account for its involvement in meningitis and arthritis of suckling calves, as reported by Stipkovits et al. (14), as well as in feedlot arthritis (5) and skin abscesses (7). There are no estimates on economic losses due to *M. bovis* arthritis, but arthritis losses are usually expressed as losses of animals due to untreatable lameless, whereby the carcass is sold at much lower value/kg. Animals with *M. bovis* polyarthritis show acute severe lameness, pyrexia, recumbency and therapy resistance to antibiotics. In many cases *M. bovis* can be found in the affected joints and in the respiratory tract, from which it might have spread hematogenically to the synovial membranes of the large joints. Necropsy can also reveal changes in the joint cartilage. Arthritis caused by *M. bovis* is mainly characterized by accumulations of fibrin and neutrophilic leukocytes in the joint cavity, coagulative necrosis of the joint capsule, proliferation of synovial lining cells and infiltration of the synovial membrane with mononuclear inflammatory cells. In a recent experimental *M. bovis* arthritis study two calves were inoculated in the right carpal joint with an arthritogenic *M. bovis* strain, while two additional calves were vaccinated before intraarticular infection (H. Linkner, F. Poumarat, P. Belli, D. Le Grand, C. Citti, R. Rosengarten, and M. Hewicker-Trautwein, unpublished). After necropsy, *M. bovis* was recovered from all joints into which it was inoculated and, in addition, was recovered from up to seven non-inoculated joints of three calves. In samples from both carpal joints from one of the vaccinated animals, organisms were still present for more than eight weeks post inoculation. This indicates the spread of *M. bovis* organisms to non-inoculated joints and, in spite of previous vaccination, their persistence for several weeks within joint fluids and tissues. Histologically, all calves showed coagulative necrosis of the joint capsule of the inoculated joint. Furthermore, inflammatory changes of the synovium were found in some of the non-inoculated joints. By immunoperoxidase labelling, *M. bovis* antigen was found closely associated with the periphery of necrotic tissue lesions and could be demonstrated extracellularly and within the cytoplasm of neutrophilic leukocytes and macrophages. How the spread of the mycoplasmas from the inoculated joint to the non-inoculated joints occurs is unknown. But it is worth speculating that the internalization of the mycoplasmas by host cells may be a strategy by which they gain access to various locations in the host and cause systemic infections. Recent findings (F. Winner, C. Citti, and R. Rosengarten, unpublished) indicating that pathogenic mycoplasmas are in fact capable of active invasion into cells will certainly open new ways for explaining many aspects of mycoplasma local and systemic infections.

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THE IMPACT OF MYCOPLASMA INFECTIONS IN RESPIRATORY DISEASES OF CATTLE IN EUROPE

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INTRODUCTION

In calves, infectious pneumonias caused by viruses, mycoplasmas and bacteria constitute a major problem in countries all over the world. Economic loss attributable to infectious respiratory disease in young cattle production are substantial. Thomas et al. (1978) using data from the Milk Marketing Board's beef progeny tests, concluded that enzootic pneumonia, pneumonic consolidation and pleurisy together account for more than half of the losses and for the majority of losses from fatal disease. Thomas (1978) estimated the morbidity rate of bovine respiratory disease in the United Kingdom to be 32.6% and the mortality rate to be 2.7% and Thomas et al. (1978) calculated the depression in performance to be 4.5%. In UK in 1995 the population of cattle aged two years or less was 5.8 million, out of a total cattle population of 11.7 million (Anon 1996) with a mean liveweight at sale of 530kg which sold at an average price of £1.20 (Anon 1996). The implication is that based upon 1995 figures, 1.9 million cattle may have been affected by respiratory disease with a consequent loss in performance of £54 million to the UK cattle industry. This is consistent with estimates made in 1989 by Gourlay et al. who estimated losses of £50 million annually in weaned calves (Gourlay et al. 1989). In addition, respiratory disease may have been responsible for up to 157 000 fatalities which would have had a potential market value of £99 million. The 1995 European cattle population was 84.5 million animals with a mean carcass value of 800ECU, of which approximately 50 million were young cattle (Anon 1996). The above data implies that as many as 16 million cattle in Europe may be affected annually with respiratory disease, and that non- fatal production losses may cost the industry as much as 576 million ECU. Virtala et al. (1996) using linear regression models demonstrated the additional consequence that each week of pneumonia can reduce bodyweight gain of female dairy calves by 800g during the first three months of life, this typically lengthens the period until first calving for these animals. *Mycoplasma bovis* is isolated from about 25% of all fatal cases of calf pneumonia (Ball et al. 1995), the numbers isolated at the Central Veterinary Laboratories in 1996 was twice that of 1995 and has been isolated from several outbreaks of respiratory disease where the calf mortality exceeded 10 percent, in other incidents *M.bovis* was apparently the only significant finding (Nicholas 1997). It is not possible to make a precise estimate of the economic impact of mycoplasmas as a cause of respiratory disease, but it does seem that mycoplasmas are an important cause of financial loss due to the European cattle industry.

The aetiology of calf bronchopneumonia is complex and can involve viruses, mycoplasmas and bacteria (Dyer 1982; Bryson 1985). It has been stated that the impact of the bovine respiratory disease complex can be correlated with the escalating industrialisation of cattle production, the intensive operations, commingling of animals from multiple sources, exposure to many organisms, stress

and management factors (Andrews 1997). It is also true that some mycoplasma infections including Contagious Bovine Pleuropneumonia (CBPP) infections can be severe problems in the extensive pastoral systems of Africa. Apart from occasional outbreaks, CBPP is not a major problem in Western Europe and is not discussed in this paper. Mycoplasma infections in Europe are typically attributed to intensive housing of animals, and in the experience of the author large scale epidemics which occur in the extensive and nomadic cattle populations of Africa, may also be attributed to the practice of grouping animals tightly overnight in protective 'bomas'.

The interaction of mycoplasmas with the host can vary from commensal in nature to one fatal in outcome. Even mycoplasma species considered to be pathogenic, can apparently exist as commensals within the host (Jasper 1982; Walker 1995). The predisposition of cattle to respiratory disease has been attributed to the relatively small volume and large dead space of the lung, leaving little dead physiological reserve (Andrews 1997). Compared to other species, such as the pig, cattle have a small lung volume per kilogram of body mass and therefore are less able to tolerate compromise of their lung function due to respiratory infections.

THE ORGANISM AND GEOGRAPHIC DISTRIBUTION

Mycoplasma infections have been recognised since Nocard and Roux first described the agent causing CBPP in 1898. Mycoplasmas are part of the class of micro-organisms called the *Mollicutes*, which include the smallest organisms capable of autonomous replication. Although over 120 species have been described, only some of the genera *Mycoplasma, Ureaplasma* and *Acholeplasma* are recognised as being pathogenic.

Mycoplasmas can affect all vertebrates and some species also occur in plants and arthropods. In vertebrates mycoplasmas exist as extracellular parasites with a special affinity for mucous membranes. Over 20 species have been isolated from cattle, with a predilection for infection of the respiratory tract, mammary gland, urogenital tract, serous membranes or nervous system. *M.bovis* is exceptional from most mycoplasmas in that it has been demonstrated to be actively invasive of lung tissue (Thomas et al. 1986, 1987).

Since 1967, mycoplasma have been isolated from the respiratory tract of bovines throughout Europe (Gourlay et al. 1970). There are differences in geographic distribution, although it is possible that this may be accounted for by the difficulty in culturing in some organisms e.g. *M.dispar.* Other organisms such as *M.bovis* are easy to cultivate, but seem to have true differences in geographical occurrence, in this case the detection has often been stimulated by the appearance of typical clinico-pathologic symptoms. *M. arginini, M.bovigenitalium, M.bovirhinus, A.laidlawii* and *Ureaplasma diversum* appear to be ubiquitous and have been isolated in laboratories throughout the world. *M.bovis* has been widely isolated in Europe, North America and Japan, whilst *M.dispar* has been isolated in some European countries, the USA and Australia. *M.canis* has been isolated in calves in the Netherlands and UK, *M.canadense* in UK and Northern America, *M.alkalescens* in Eastern Europe, USA, Australia and New Zealand whilst *A.axanthum* and *A.modicum* have been reported most in Eastern Europe. The increased freedom of movement of animals in Europe, particularly of young calves to veal units which may move from Poland to Spain, or Ireland to Italy at one or two weeks of age, facilitates the rapid transfer of micro-organisms across the European continent, generally into

the major veal producing countries which are currently The Netherlands, Italy, France, Spain and Belgium. *M.mycoides* has been isolated in most countries with dry tropical zones, including outbreaks in Southern European countries.

PATHOGENICITY

Most of the literature supports the view that, of the mycoplasmas implicated in respiratory disease in European cattle, *M.bovis* is pathogenic and *M.dispar* and ureaplasmas are potentially pathogenic. This has been supported furthermore by reproduction of disease by experimental inoculation of *M.dispar, Ureaplasma spp., M.bovis,* and in addition *M.bovigenitalium* singly and in combination into the lower respiratory tract of experimental calves (Gourlay et al. 1976; Howard et al. 1976; Gourlay et al. 1979). A number of other mycoplasmas have been proposed as pathogenic, although to date, disease has not been experimentally reproduced. These organisms are *M.bovirhinus, M.arginini, M. bovigenitalium* (Jurmanova et al. 1975) *A.modicum* (Stipkovits et al. 1973) and *M.canis* (ter Laak et al. 1993a; Nicholas et al. 1995). Studies on explant cultures of the bovine trachea (Thomas & Howard 1974; Thomas et al. 1987) revealed that *M.dispar* produced cytopathic effects on the ciliated epithelial cells, progressive sloughing of cells and patchy flattening of epithelial cells over a 6 day period. The cytopathic effect was associated with large numbers of mycoplasmas, but no toxic factor was found in organ culture fluid. *M.dispar* and *Ureaplasma diversum* were shown to be capable of causing a mild sub-clinical bronchiolitis with lymphoid cuffing in gnotobiotic calves (Howard et al. 1976) , whereas *M.bovirhinus* and *A.laidlawii* were non-pathogenic (Gourlay et al. 1979). *M.dispar* and *M.bovirhinus* were shown to exist in the non-diseased respiratory tract, and this called into question the role of *M.dispar* as a primary pathogen (Thomas & Smith 1972). It appears that while *M.dispar* and *Ureaplasma diversum* can be isolated from both acute and chronically diseased respiratory tracts, and are capable of producing mild subclinical pneumonias, their role as primary pathogens in the field is uncertain and their role in the cuffing pneumonia syndrome of calves is not as clear cut as with *M.hyopneumoniae* in the pig. The major disease significance with *M.dispar* and *Ureaplasma diversum* is therefore likely to be in rendering the respiratory tract more susceptible to complicating bacterial infection.

PATHOLOGY

M. bovis is the most pathogenic of the respiratory mycoplasmas found commonly in Europe and the predominant pathology associated with pneumonia is a bronchitis, bronchiolitis and bronchopneumonia. In advanced cases localised consolidation and necrosis may occur. Infection with mycoplasma on its own is rare, the fatal outcome in cases of bronchopneumonia is in the majority of cases due to complicating bacterial infections. The histological appearance of *M.bovis* reveals a distinct cellular response, with peri-bronchial and peri-bronchiolar infiltration, which may involve extensive lymphoid hyperplasia (Bryson 1985) and lobular necrosis not unlike that seen in pneumonic pasteurellosis (Thomas et al. 1986). Large numbers of alveolar macrophages, oedema and thickening of the alveolar walls may all be observed. Electron-microscopy reveals damage to the bronchial epithelium,

including loss of cilia and distension of mitochondria with loss of cristae (Pirie & Allan 1975, Allan et al 1976, Allan et al 1977).
Otits media and spontaneous lameness has been noted in association with the pneumonic symptoms, and a pneumonia-arthritis syndrome has been described (Galassi 1974, Romvary et al. 1975, Thomas et al. 1975, Pignatelli 1977) The most common bacterial pathogens associated with mycoplasma infections in decreasing order of occurrence are *Pasteurella haemolytica, P. multocida, Actinomyces pyogenes, Haemophilus somnus* and on occasions *Salmonella* spp.

CLINICAL PRESENTATION

Calves may therefore present with a combination of increased respiratory rates, abdomino-costal breathing with rales evident upon auscultation, raised rectal temperatures, inappetance, conjunctivitis, lethargy and 'starry' coats, primarily due to the respiratory component. Head tilt, drooped ears, swollen painful joints and lameness may also be apparent.

Table 1: Mycoplasma spp associated with respiratory disease and isolated from cattle in Europe

Species	Respiratory tract	Joints	Eyes
Mycoplasma			
M.alkalescens	*	*	
M.arginini	*		*
M.bovigenitalium	*		
M.bovirhinus	*	*	*
M.bovis	P	*	*
M.canadense	*	*	
M.canis	*		
M.dispar	P		
M.mycoides.mycoides			
SC	P	*	
LC	*	*	
Acholeplasma			
A.axanthum	*		
A.laidlawii	*		*
A.modicum	*		
Ureaplasma			
Ureaplasma diversum.	P		*
Unnamed			
*M.*species Group 7	*	*	

P= proven pathogenicity in respiratory tract

EPIDEMIOLOGY

In 1993, forty-eight countries responded to a questionnaire from the Office International des Epizooties. It was found that, it was mainly the industrialised countries which recognised Mycoplasmoses of the respiratory tract to be a significant production problem and in the majority of cases, these involved calves in

intensive production facilities (Nicolet 1994). The various mycoplasmas involved in order of importance are quoted as *M.bovis, M.dispar, Ureaplasma diversum, Acholeplasma modicum, M. bovigenitalium* and *M.bovirhinus*. These agents were often found concomitantly with viral and bacterial infections. Analysing lung tissue samples submitted to the Central Veterinary Laboratories in UK, in 1995 and 1996, 23% and 22% respectively of samples submitted for ELISA were positive for *M.bovis* (Nicholas & Ayling - personal communication). Thomas & Smith (1972) in a study of mycoplasma distribution in the non-pneumonic bovine respiratory tract demonstrated that *A.laidlawii* and *M.bovirhinus* predominately inhabited the upper respiratory tract, *M.dispar* was isolated throughout, but predominated in the lower tract. Calves of 3-4 months appeared to carry a much higher burden of mycoplasmas throughout the tract than the very young calf or the yearling or adult, which had very few mycoplasmas, and were confined mainly to the upper respiratory tract.

Mycoplasmas are generally spread in herds or densely housed/poorly ventilated conditions. One group investigating the isolation rate from nasal swabs stated that in the course of a 3 week episode the isolation rate rose from 20% to 95% of calves (Jurmanova et al 1975). Romvary et al. (1975) stated that *M.bovis* persists in the joints and respiratory tract and that infection may therefore persist in a closed herd for years. It would seem that once established in the upper respiratory tract, mycoplasma infection may persist in older animals and be transmitted to younger stock by direct contact. These younger animals are more susceptible to developing clinical symptoms. In herds where *mycoplasma* have been established as a causative agent of mastitis, clinically healthy cows shedding the pathogen in their milk may represent a permanent reservoir (Pfutzner 1990). *M.bovis* is spread during milking directly from cow to cow by infected milk and indirectly via milking machines and other equipment and the milker's hands. The organism is also spread through the air, since it is commonly detected in the throat and nose of cows in infected herds, consequently it is advisable to separate purchased veal calves and beef cattle from dairy cattle (ter Laak et al 1992) .Sheep have also been proposed as vectors of *M.bovis* (Bocklisch et al. 1987).

DIAGNOSIS

Clinical symptoms are often the first indication to most farmers or field veterinarians of pneumonic infection. When conducting a pathological investigation into the aetiology of an outbreak of respiratory disease, staining of tissue sections is of little value for the detection of *mycoplasma*, because they stain poorly with Gram's stain, however, immunoperoxidase staining has led to the detection of large numbers of *M.bovis* at the periphery of necrotic tissue. This typical histopathology and labelling of organisms can be a definitive and relatively convenient method of diagnosis (Thomas et al. 1986). Isolation remains the most common means of diagnosing mycoplasma infections. To maintain any degree of success, samples must be fresh. Bacterial contamination even in the presence of inhibitors (e.g. thallium acetate or penicillin) can make cultivation impossible in liquid medium. Protection of swabs from drying during refrigerated transport with immediate inoculation of liquid broth from tissue swabs (solid medium cultures are also advisable) have been identified as a means of maximising success (Pfutzner & Sachse 1996).

Most bovine mycoplasmas grow readily in conventional mycoplasma media, however, particularly on primary isolation, some species may require special media and methods. Species identification is therefore essential. A useful initial means of provisional identification for bovine mycoplasmas after primary isolation was published by Gourlay and Howard in 1983 (Table 2).

Penicillin or other antibiotics that affect peptidoglycan synthesis are often incorporated into the media to reduce bacterial contamination. Colonies usually appear within 3-4 days, however some may take a week or longer to become evident. Where small numbers of organism are present pre-enrichment culturing in mycoplasma broth can be beneficial to improve recovery rate. Isolation of *Ureaplasma diversum* requires special urea containing media at pH 6.5. These micro-organisms rapidly break down urea causing a sharp rise in pH, giving vivid colour changes in liquid medium, but severely limiting colony size development on solid medium. However, the inclusion of phosphate buffers and phenol red in agar medium allows the growth of ureaplasma colonies to a size equivalent to that of mycoplasma colonies. The medium is diagnostic for ureaplasmas as a precipitate forms in the colonies and the agar medium changes colour from yellow to red (Windsor et al. 1975).

Colonies are small and best visualised with the aid of a stereo-microscope. The typical fried -egg or 'mammilated' appearance is suggestive, however not all mycoplasmas present with this morphology (e.g. *M.dispar*).

Biochemical tests are used in identifying individual species but final identification of a specific species requires indirect fluorescent antibody, immunoperoxidase or growth inhibition tests.

Alternative methods such as cellular fatty acid analysis and polymerase chain reaction (PCR) are currently being developed as ways of differentiating mycoplasma species. In the case of *M.bovis*, it has been reported (Behren et al. 1996) that a series of prominent surface proteins (Vsps) which are immunogenic and undergo dynamic and spontaneous changes in size and expression are present on the cell surface. These may also play a role as mediators of adherence to host cells and in escaping immune destruction. These Vsps may also have an effect on the reduced ability of the diagnostic laboratory to identify some *M.bovis* isolates by conventional serological methods such as the film inhibition test (FIT) and immunofluorescent antibody tests (IFAT). The use of species-specific PCR provides an alternative approach to identifying *M.bovis* which overcomes potential problems associated with conventional test methods (Ayling et al. 1997). Due to the small sample sizes employed the sensitivity of a PCR test cannot equal that of a properly validated medium but low numbers can be detected and the technique is rapid (Hotzel et al. 1993; Sachse 1993; Hotzel 1996). Presently however, the technical complexities of the PCR test (which include contamination control and electrophoretic detection of amplified products) confine its use to the research laboratory. Providing the PCR technique can be simplified and the cost reduced, it has the potential to replace cultural methods of diagnosis accepting the limitation that presently used protocols detect viable and non-viable organisms.

Although the biochemical properties of mycoplasmas are not particularly diverse, metabolic activities such as glucose fermentation, arginine hydrolysis, triphenyltetrazolium chloride (TTC) reduction and the presence of phosphatase can

be useful for the preliminary characteristation of isolates. Utilisation of such activities may allow the development of diagnostic media for the differentiation of mycoplasma isolates. The inclusion of TTC in solid medium can result in the formation of red *M.bovis* colonies and colourless other *mycoplasma* species (Windsor, unpublished observations) could result in a diagnostic medium for *M.bovis*. In addition, the inclusion of selective inhibitors, such as nisin, may results in the suppression of contaminating *mycoplasmas* (Abu-Amero et al 1996). The use of such media could indicate the presence of *M.bovis* after incubation for three days, require little labour or technical skill and allow immediate progression to *in vitro* antimicrobial testing.

Table 2:
Guide to provisional identification of bovine mycoplasmas on primary isolation based upon cultural characteristics (adapted from Gourlay and Howard 1983)

Broth	pH change	Colonies on solid medium		Likely species
Glucose (GS broth)	No change or slightly acidic	Typical		*M.verecundum*
	Moderately	Typical		*M.bovigenitalium*
	acidic			*M.bovis*
	Strongly acidic	Typical	Digitonin/SPS sensitive	*M.bovirhinis* *M.bovicoli* *M. canis* *M.mycoides* *M.sp Gp 7*
			Digitonin/SPS resistant	*A.axanthum* *A.laidlawii* *A.modicum* *M.dispar*
		Atypical		*M.dispar*
Urea (U4 broth)	Strongly alkaline	Small		*Ureaplasma spp.*
	Slightly acidic	Typical	Digitonin/SPS sensitive	*M.bovigenitalium* *M.bovirhinis* *M.bovis* *M.verecundum*
			Digitonin/SPS resistant	*A.laidlawii*
	Strongly acidic	Typical	Digitonin/SPS sensitive	*M.bovicoli* *M.mycoides* *M.sp Group 7*
			Digitonin/SPS resistant	*A.axanthum*
Arginine (A broth)	Strongly alkaline	Typical		*M.alkalescens* *M.arginini* *M.canadense*
	Acidic	Typical		*M.mycoides* *M.sp.Group 7*

SEROLOGICAL EXAMINATION

Antibodies can be detected in the blood by indirect haemagglutination (IHA), film inhibition and enzyme-linked immunosorbent assay (ELISA). However neither the antigen for haemagluttination nor the ELISA test kits are commercially available, which makes comparison between laboratories and standardisation impossible. There are however two major limitations to the application of the highly sensitive ELISA techniques. These are firstly, that it may two to three weeks for antibodies to develop after the onset of disease, which prevents infection being detected during the incubation period and secondly, the sensitivity of this method is insufficient to identify shedders (Pfutzner & Sachse 1996).

Recent advances in mono-clonal antibody techniques targeting adhesion factors on the *mycoplasma* membrane has allowed increased sensitivity, and detection of infection during the incubation phase should now be possible (Pfutzner & Sachse 1996).

Western-blotted protein patterns have been used to demonstrate inter-strain antigen variations, which could be useful for epidemiological surveys in which the source of the outbreak must be identified (Poumarat et al.1994).

Apart from areas where CBPP is present, serology is not routinely used as a diagnostic tool for bovine mycoplasmosis, although ELISA and IHA have been used widely for surveillance purposes.

CONTROL AND TREATMENT

Under experimental conditions, vaccination has been shown to reduces losses due to pneumonia and arthritis following infection with *M.bovis* and *M.dispar* (Howard et al 1977; Chima et al 1978). A quadrivalent vaccine adjuvated with Quil A, containing the antigens of bovine respiratory syncytial virus, parainfluenza type 3, *M.bovis* and *M.dispar* was shown to reduce the incidence and severity of *M.bovis* infection in the field (Howard 1987). However, to date, no commercial production of *M.bovis* vaccine has been realised in Europe.

Control of calf pneumonia should include measures to reduce environmental stress, ensure adequate housing with good circulation of air and where possible 'all-in, all-out' stocking management to prevent cross contamination between batches of calves. Field investigators have demonstrated that seven days after the first detection of *M.bovis* in a herd, it could be isolated from nasal swabs of most other calves (Pfutzner 1983,1985) the speed at which respiratory infections spread is a generally a reflection of the stocking density and the adequacy of ventilation in the housing. Cleaning and disinfection between batches is also important. Whilst it is often suggested that *mycoplasmas* cannot survive outside of the host for prolonged periods, it has been reported that *M.bovis* survived on sponges for 57 days, in milk for 54 days, on straw for 20 days and on wood and in water for 17 days at 4°C, the survival period at 17°C was one to two weeks and at 37°C it was one week (Pfutzner & Sachse 1996).

Colostrum feeding is very important in boosting the overall immunity of calves. Many outbreaks of respiratory disease occur at a time when maternal antibodies are waning or have disappeared. In one study calves with a total serum immunoglobulin levels in excess of 20 zinc sulphate turbidity test units (ZSTU) at 10 days of age were found to be significantly less prone to fatal and non-fatal respiratory disease

during the first 5 months of life than calves with levels below 20 ZSTU (Thomas & Swann 1973).

Therapy is aimed at both the mycoplasmal and bacterial components of a typically mixed respiratory infection. Mycoplasmas are not sensitive to antibiotics which act on the cell walls of bacteria (penicillins, cephalosporins, sulphonamides). In order to attain the appropriate spectrum of antibiotic activity, macrolides, lincosamides, tetracyclines and quinolones are most commonly used.

Recently strains were collected from field sites in UK, France, Germany, Belgium and The Netherlands, and MIC's were determined by broth dilution, using sensititre plates (Accumed Ltd) (Reeve-Johnson unpublished data) (Table 3).

Table 3: MIC_{50} for mycoplasma isolates from cases of bovine respiratory disease (μg/ml)

				Strain				
Antibiotic	*M.bovis*	*M.alkalescens*	*M.bovirhinus*	*M.bovicoli*	*M.dispar*	*A.laidlawii*	*A.modicum*	*U.diversum*
Tilmicosin	0.024	0.048	0.024	0.048	0.125	0.024	0.048	0.048
Tylosin	0.48	0.48	0.125	0.125	0.125	0.048	0.048	0.2
Oxytetracycline	8				64			16
Enrofloxacin	1				0.25			8

The above data is consistent with earlier data. ter Laak (1993b) determined the MIC_{50} for *M.bovis*, *M.dispar* and *U.diversum* against a variety of commonly used antibiotics and Ball et al. (1995) determined the MIC's for *M.bovis* for four antibiotics, commonly used in veterinary practice (Table 4).

Table 4: MIC data from European field isolates of pathogenic mycoplasmas from cattle

	ter Laak et al (1993b)			Ball et al (1995)
Number of strains	16	19	17	23
	M.bovis	*M.dispar*	*U.diversum*	*M.bovis*
	MIC_{50}	MIC_{50}	MIC_{50}	Range
Tilmicosin				<0.06-32
Tylosin	0.25	0.06	0.5	
Oxytetracycline	16	16	4	
Enrofloxacin	1	0.25	4	1-2
Doxycycline	1	0.5	0.25	
Spiramycin	0.5	0.5	16	
Spectinomycin	2	1	8	4-16
Lincomycin	0.5	0.5	>64	0.125-2
Streptomycin	16	64	>64	
Penicillin	>64	>64	>64	

MIC values only give an indication of inhibition of growth *in vitro*. The clinical effectiveness of a compound in the living animal depends upon a number of other factors. Most important of these are:

1. The pharmacological properties of the therapeutic compound (i.e. how well it is absorbed into the body, where it concentrates in the body and how long the antibiotic remains present at a suitable concentration in the zone that it comes into contact with the infecting organism)

2. An effective immune response by the body

Some antimicrobial compounds have been shown to have a preferential distribution for the lungs (Thomson & Peloso 1989) and it has also been demonstrated that in the pneumonic lung the distribution pattern may be altered. Thomson & Laudert (1992) demonstrated 3 times the concentration of the macrolide antibiotic tilmicosin in diseased lungs than had been previously observed in non-pneumonic lungs in feed-lot cattle.

Studies investigating the intra-cellular levels of antimicrobials in rats have indicated selective concentration of some compounds in immuno-competent cells (Carlier et al. 1987). Table 5 summarises results considering the ratio of different antibiotics from the three major classes of antibiotics used for their anti-mycoplasma action. The results consider the ratio of concentration of antibiotics in the polymorphonucleocytes and alveolar macrophages compared to the extra-cellular levels.

Table 5: The accumulation of antibiotics in immunocompetent cells

Antimicrobial family	Polymorphonuclear neutrophil C/E ratio	Alveolar Macrophage C/E ratio
Tetracyclines	0.75-3.1	2.8-4.4
Quinolones	2.0-8.0	2.0-8.0
Macrolides	4.4-18	38.0

C/E ratio = Cellular to Extracellular ratio
N.B. ranges are given where a number of studies have been conducted

The macrolide family of antibiotics have been the group of compounds most used, and are generally well suited to the control and prevention of mycoplasma associated respiratory diseases. In addition to favourable *in vitro* MIC and pharmacokinetic distribution patterns in the body, work in pigs and chickens has provided further evidence of concentration in the immunologically active phagocytic cells which are the principal defence mounted by the body against infection (Shryock & Scorneaux 1997a,1997b).

A number of researchers have demonstrated that many of the antibiotics in the macrolide family have a number of potentially beneficial effects on the action of the immune system, such as enhancing phagocytosis, enhancing the rate of killing of bacteria (*in & ex vivo*), altering chemotactic responses and even alteration of bacterial virulence (Labro 1993).

DISCUSSION

Mycoplasma species are isolated from cases of pneumonia in all parts of Europe, however the prevalence is under-estimated because few laboratories are attempting to isolate them compared to the number attempting bacteriological isolations as part of their routine investigations into disease outbreaks. Observation for suggestive gross pathology and subsequent confirmation by culture or PCR are techniques that can be practically applied as a means of surveillance.

Respiratory mycoplasmosis is a particular problem in young cattle and the losses in growth and production may be large for both clinical and sub-clinical infections. Mycoplasma may be asymptomatically present in the upper respiratory tract of older animals, where they form a constant source of reinfection for young

animals and may therefore be a principle reason for the persistence of mycoplasma infections for periods of years in closed herds. Experimental and field studies have shown there to be a closed cycle of infection with *M.bovis* from the infected cow to foetus or post-partum via the new-born calf to young cattle. During mastitis *M.bovis* is transmitted through the uterus, and newborn calves may be infected either through the milk or the environment (Pfutzner & Sachse 1996). The agent is capable of persisting in the respiratory tract, where it remains infectious and may be passed to the next generation, as well as between individuals at any stage.

Bought in stock are a risk of introducing new infections into a herd. Whilst avoidance of buying infected stock is a theoretical measure of control, this is difficult to ensure in practice for most commercial operations. Appropriate environmental management and husbandry are important practical control measures which can be taken. 'All-in all-out' systems and cleaning of sheds between batches are important in light of the survivability of the organism under appropriate conditions.

Appropriate use of antibiotics is effective in preventing new infections from bought in stock and limiting the economic impact of infections in young stock. In choosing the therapy, antibiotics which work by a mechanism other than by acting on the cell wall are appropriate. Macrolides, quinolones, lincosamides and tetracyclines are appropriate antibiotic choices. MIC's may be used to gain an impression of efficacy *in vitro*, however this may not reflect the *in vivo* results, which depend upon the pharmacokinetics of the drug (i.e. how the antibiotic distributes through the body) and whether effective concentrations come into contact with the infecting organism. The alveolar macrophages and neutrophils are the body's major mechanism against an infection in the lung. Recent studies have shown that some classes of antibiotic actively concentrate in the phagolysosomes of these cells, which may make them particularly appropriate therapeutic choices.

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IMPACT OF MYCOPLASMA MASTITIS IN CATTLE

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The first incident of mastitis associated with a *Mycoplasma* spp. was in England in 1960, and the causal agent was *M. bovigenitalium* (1). In 1962, *M. bovis* was implicated in mastitis in the USA (2), and over the following 10 -15 years, the importance of mycoplasma mastitis, particularly in association with *M. bovis*, became evident in the American dairy industry. Other *Mycoplasma* spp. have subsequently been shown to be causes of bovine mastitis. Of particular importance have been *M. californicum*, first recorded in USA in 1972 (3), and *M. canadense*, first recorded in Canada in 1975 (4).

Mycoplasma mastitis in cattle was probably of relatively minor importance in Europe until the mid 1970's. However, from this period onwards, first *M. bovis* and later *M. californicum* was introduced, probably from the importation of cattle or semen from America. This importation of these more pathogenic *Mycoplasma* spp., which are capable of causing severe clinical udder damage and persistent infection, resulted in cases of mycoplasma mastitis all over Europe. As was the case in the USA, the problems were more persistent in the larger herds, in particular those of some eastern Europen countries.

Bovine mycoplasma mastitis is probably considerably under diagnosed world-wide, simply because of a lack of awareness of the condition in the industry, and the limited application of mycoplasma detection methods in diagnostic laboratories. Diagnosis is often only made after the problem has become chronic, following a lack of response to antibiotic therapy, which is largely designed for the control of the more common bacterial mastitic pathogens. Another factor contributing to under diagnosis is good management; it is quite conceivable that mycoplasma mastitis within a herd can be effectively controlled in some instances by good management, even in the absence of effective therapy. This was apparent in the first *M. californicum* outbreak in Northern Ireland, where, by the time the organism was diagnosed, the condition was chronic but confined to a small number of cows in a herd of 120 milking animals (5). In this case, following diagnosis, the problem was eliminated with limited financial loss. Generally speaking, the larger the herd, the more difficult it is to control mastitis purely by management, and this is particularly so for the less common forms of mastitis which do not respond to the available antibiotic treatments. Such factors probably contribute to the apparent persistence of mycoplasma mastitis in some of the larger dairy herds.

Even allowing for under diagnosis, mycoplasma mastitis is still rare in Europe, and in comparison to both clinical and sub-clinical bacterial mastitis, it is of minor overall economic importance. However for individual farmers who experience an outbreak of *M. bovis* or *M californicum* mastitis, it can, of course, have a major economic impact. In Northern Ireland over the past twenty years, milk samples submitted to the Stormont laboratory in Belfast have been routinely cultured for mycoplasma. During this period, the number of mycoplasma mastitis incidents has been low. They have been in total, two herd outbreaks of *M. californicum* (5,6),

a single cow case of *M. canadense* (7), four of *M. bovigenitalium* (8), and two subclinical *M. bovis* herd problems associated with arthritis (9). More recently, two herd outbreaks of *M. bovis* mastitis have occurred in the Republic of Ireland (10).

The most likely route of many mycoplasma infection of the udder is via the teat canal. All the four main *Mycoplasma* spp associated with mastitis have been isolated from the vagina and semen of cattle. *M. bovigenitalium* and *M. canadense*, in particular, are common genital isolates. It can be postulated that contaminated urine splashing on the udder can bring the organism to the teat aperture, allowing access during milking. Once an infected animal is in a milking herd, the opportunity exists for spread amongst the herd, and this is dependent on many management factors, similar to the spread of bacterial mastitis. An example of the widespread dissemination of *M. californicum* within a herd due to poor husbandry practice, was the second outbreak that occurred in Northern Ireland (6). The most likely cause of the spread in this herd was due to unhygienic use of dry cow therapy tubes. The mastitis outbreak resulted in cows calving with little or no milk. A third of the cows on the farm, 101 animals, were eventually culled, and a number of calves died from neonatal enteritis having received insufficient colostrum. Apart from infection originating within a herd as described above, it can also commonly be introduced by the ipurchase of an infected animal.

Although variation in both persistence and severity has been shown with both *M. bovigenitalium* and *M. canadense*, infection with these mycoplasmas is generally less serious than infection with *M. bovis* and *M. californicum.* Initial infection generally produces clinical milk, but returns to the production of apparently normal milk within a few days. The length of infection is variable, but elimination of the organism generally occurs, leaving a gland with a greatly reduced milk yield. *M. bovis* and *M. californicum* cause a much more persistent udder infection, which frequently spreads to neighbouring glands and often causes complete cessation of milk production and atrophy of the infected gland. *M. bovis* has been shown to be intermittently excreted over the two subsequent lactation periods following recovery from initial infection, ensuring prolonged exposure of the herd to infection (11). The ultimate effect of clinical mycoplasma mastitis, whether the organism is eventually eliminated or not, is permanent damage to the gland resulting in a significant reduction in milk yield, with minimal recovery during the next lactation

Because of the non-availability of effective antibiotic treatment for mycoplasma mastitis, control of infection is dependant on identification of infected animals and segregation. This has been effective in the elimination of the problem from many dairy herds (6,10,12). Several repeat tests of the milk from each individual animal are neccessary, as is the strict segregation of infected animals, which are then milked after the healthy animals. Thorough regular cleansing and disinfection of milking machinery is essential. Culling of infected animals, because of the drastic reduction of milk yield, is the usual consequence of an outbreak, but it is also important to identify and cull any carrier animals which have apparently recovered but are still shedding the organism.

The general findings have been that antibiotic therapy in field cases of *M. bovis* is ineffective (reviewed in 13). However, a recent preliminary study has demonstrated the ellimination of the organism from naturally infected cattle using enrofloxacin (14). In addition, a small experimental study demonstrated effective elimination of *M. californicum* using a mixture of tylosin and oxytetracycline over a three day period (15). Such studies probably merit further investigation, in particular

with recently developed antibiotics which have been shown to have anti-mycoplasmal activity. Because of the rapid and permanent drop in the milk yield following mycoplasma infection, antibiotic therapy would have to be administered early in infection to be economically effective; but it would be an usful addition to a control strategy in herds where mycoplasma mastitis has been diagnosed, and new infections are recognised at an early stage.

Disease caused by *M. bovis* is not limited to mastitis. This organism has an invasive capacity which is poorly understood. *M. bovis* has been brought into herds by the introduction of heifers which have mastitis immediately after calving. This appeared to be the most likely source of a herd outbreak of *M. bovis* mastitis in the Republic of Ireland (10). The implication of this source of infection is that the animal is infected intrauterine, and this has also been indicated by the presence of *M. bovis* arthritis and/or pneumonia in new born calves (16). The invasive capacity of *M. bovis* and its contribution to mastitis requires further investigation to determine such factors as the invasive variation of strains, route of invasion and organs affected, and identification of body regions where the organism can become latent. Associated with possible strain variation is the unknown potential of calf respiratory carriage as a source of udder infection. Since the introduction of *M. bovis* into Ireland, north and south, its contribution to calf pneumonia has become widespread. However, there is little evidence of the organism spreading to dairy herds on the same farms. Does this imply that the route of udder infection via the teat canal is less common than by other more systemic invasive routes ? In addition, the ability of *M. bovis* mastitis to become endemic in a herd, with some cows becoming carriers secreting low levels of the organism, invites investigation into the relationship between the host defenses and the clinical manifestation of infection.

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DIAGNOSIS OF CBPP BY PCR AND LASER INDUCED FLUORESCENCE

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INTRODUCTION

Contagious bovine pleuropneumonia (CBPP) is today spreading very fast in many African countries. At the same pace, there is an increasing demand for rapid and sensitive diagnostic methods that can be used to screen for the disease in herds or to confirm the initial diagnosis based on clinical symptoms and/or pathology.

The infectious agent of CBPP, *Mycoplasma mycoides* subsp. *mycoides* SC (referred to as *Mmymy*SC), is a member of the *Mycoplasma mycoides* cluster, together with six closely related mycoplasmas; *M. capricolum* subsp. *capricolum*, *M. capricolum* subsp. *capripneumoniae (Mcacp)*, *M. mycoides* subsp. *capri, M. mycoides* subsp. *mycoides* LC, *Mycoplasma* sp. bovine group 7 and *M. putrefaciens* (10, 12). The latter species is not included in the classical *M. mycoides* cluster.

The species within the *M. mycoides* cluster are difficult to distinguish by morphological and biochemical analyses. Many of the diagnostic methods for CBPP that are used today are based on serological reactions like ELISA, complement fixation tests, immunocytochemical tests and protein immunoblotting, methods which can be time consuming to perform and some of them are not sufficiently specific or sensitive (3, 5, 7). Most of the diagnostic PCR assays that have been described (1, 4, 6, 8, 11) are based on the CAP-21 gene whose gene product still has unknown function. Alternative diagnostic PCR systems which are based on other parts of the genome can be useful in some situations.

The 16S rRNA genes provide well examined sequences with segments of different evolutionary variability, which are ideal target regions for primers in group specific or species specific amplification. All mycoplasmas belonging to the *M. mycoides* cluster have two rRNA operons (9). In a particular poly(A) region, evolution has imposed an insertion in the 16S *rrnA* gene and a deletion in the 16S *rrnB* gene for the *Mmymy*SC while the other members of the cluster have two genes of intermediate sequence length (10). We used this sequence length difference to design a PCR which was specific for the *M. mycoides* cluster and which distinguished *Mmymy*SC directly upon electrophoresis, without any preceding manipulations of the PCR product. A Cy5 dye in the 5'-end of the forward primer enabled us to use the DNA sequencer for electrophoresis to achieve the required resolution for separating fragments that differ two bp in size. The detection of laser induced fluorescence also resulted in a very high sensitivity.

MATERIALS AND METHODS

This study comprised 16 *Mmymy*SC strains from Europe, Africa and Australia, the *Mcacp* strain 7/19 and the type strains of the *M. mycoides* cluster

PG1^T, California kid^T, F38^T, PG3^T, Y-goat^T, PG50^T, KS-1^T and of *M. bovis* Donetta^T. All strains were grown in F medium (2), except strains L2, Gladysdale, KH3J, 94111, Filfili, 9050-529/I, V5 and 6479 which were kindly provided by Dr. J. Frey as DNA samples. Cells from 1 ml culture were washed once in phosphate buffered saline, resuspended in 1 ml water and lysed by heating the suspension to 100°C for five minutes. The suspension was chilled on ice and stored at -20°C until use.

Oligonucleotide primers (Table 1) that were designed to amplify a segment of the 16S *rrnA* and *rrnB* were ordered from Amersham Pharmacia Biotech. The forward primer was labelled with Cy5 in the 5'- end and both primers were FPLC purified by the manufacturer. Amplification was carried out in a mixture of 10 mM Tris-HCl buffer (pH 8.3), 2 mM $MgCl_2$, 50 mM KCl, 0.1% Tween, 0.8 mM dNTP, 5 pmol forward primer, 5 pmol reverse primer and 0.6 U AmpliTaq® DNA Polymerase (Roche Molecular Systems) in a total volume of 50 µl. The reactions were performed in a Techne Thermocycler using denaturation for 5 min at 96°C followed by 28 cycles of: 30 s denaturation at 96°C, 30 s annealing at 60°C and 2 min extension at 72°C. Extension was prolonged for 5 min at 72°C in the last cycle. Analysis of the amplicons was done by electrophoresis with the ALFexpress™ DNA Sequencer and the results were evaluated in the Fragment Manager software (Amersham Pharmacia Biotech). A denaturing polyacrylamide gel (8% acrylamide and 7M urea) was prepared by adding 1.6 ml stock solution of 40% acrylamide to 25 ml ALF™ grade Ready Mix Gel (Amersham Pharmacia Biotech) and the gel was cast in a short gel cassette of 15 cm vertical length and 3 mm thickness. The PCR product was diluted in sterile water when required and 4 µl was mixed with 5 fmol Cy5 Size Marker in formamide-containing loading dye. Samples were loaded in every second well of the gel and the electrophoresis was run in 0.6 X TBE buffer at 1500 V, 60 mA, 25 W and 55° C with a sampling interval of 2 s. The instrument was paused after four min to fill the gel with the remaining samples. External size marker with 50 bp spacing was loaded in two lanes of the gel.

Table1. Oligonucleotide sequences.

Primer	Sequence	Position in the 16S rRNA genes*
forward	5'-Cy5-GTACAAAGAGTTGCAATCCTGTGAA-3'	1225-1249
reverse	5'-ACCGCGACATAGCTGATTCGCGA-3'	1353-1330

* according to the consensus sequence in (10).

RESULTS

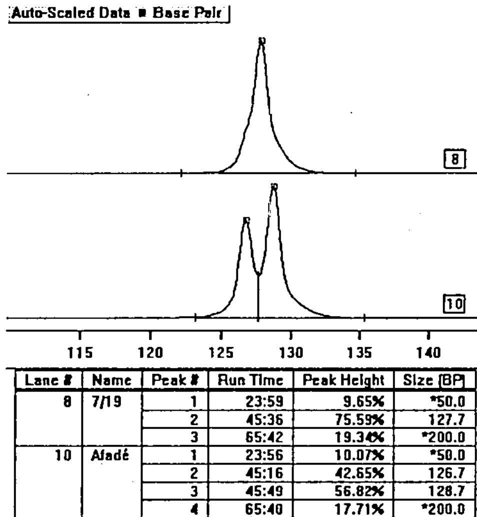
All tested mycoplasma strains of the *Mmymy*SC species generated two PCR products of sizes 127 bp and 129 bp. PCR of the control strains belonging to the classical *M. mycoides* cluster resulted in equal-sized amplicons of 128 bp. No products were obtained from the negative controls *M. putrefaciens* and *M. bovis*.

The resolution of the gel was high enough to separate the amplicons of two bp size difference and the products were clearly displayed as two peaks, while cluster members other than *Mmymy*SC appeared as single peaks (Fig. 1). When appropriate size standards were co-run with the PCR product and the FM™ 1.2

software was used for analyses, the sizes of the fragments were accurately determined.

The total time for electrophoresis was approximately 80 min when the 200 bp internal size standard was used, while the PCR products appeared already after 45 min.

Two independent dilution series of PG1 culture were used to assess the sensitivity of the system. The lowest level for detection was obtained in reactions where the amount of template was equivalent to 0.3 and 0.4 CFU, respectively.



Lane #	Name	Peak #	Run Time	Peak Height	Size (BP)
8	7/19	1	23:59	9.65%	*50.0
		2	45:36	75.59%	127.7
		3	65:42	19.34%	*200.0
10	Afadé	1	23:56	10.07%	*50.0
		2	45:16	42.65%	126.7
		3	45:49	56.82%	128.7
		4	65:40	17.71%	*200.0

Fig. 1. Fluoregram curves of amplicons from *M. capricolum* subsp. *capripneumoniae*, strain 7/19 (lane 8) and *M. mycoides* subsp. *mycoides* SC strain Afadé (lane 10) after electrophoresis with the DNA sequencer and processing in the FM™ software. The elapsed time, the peak height and the calculated size in basepairs, are specified in the table. The asterisk (*) denotes the internal size standards that were co-run with the PCR product.

CONCLUSIONS

The possibility to identify an organism due to sequence length differences between genes that occur in two operons, was demonstrated. By labelling the forward primer with a Cy5 dye, we could use the DNA sequencer for high resolution electrophoresis and detection of laser induced fluorescence. A sensitive diagnostic system for CBPP, based on direct PCR, was thereby obtained.

The primers of the diagnostic system proved to be group specific for members of the classical *M. mycoides* cluster and did not amplify any of the other tested species. After electrophoresis with the DNA sequencer, the *Mmymy*SC was distinguished from the other members due to the length difference between the two 16S rRNA genes.

ACKNOWLEDGEMENTS

This work has been financially supported by grants from the commission of the EU (DG VI) for "Development of new and improved diagnostic tests for CBPP in Europe" (contract FAIR1-CT95-0711), the Swedish Council for Forestry and Agricultural Research and the Swedish International Development Cooperation Agency.

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DNA REPAIR GENES *uvrC* AS GENETIC TARGETS FOR DISCRIMINATION OF CLOSELY RELATED MYCOPLASMAS

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INTRODUCTION

Molecular genetic techniques, in particular *in vitro* amplification of DNA fragments by PCR show a high potential for accurate and rapid identification of *Mycoplasma* species and subspecies. These techniques become particularly important for the differentiation of Mycoplasmas which are phenotypically closely related, but which show big differences in virulence and in epidemiological significance. Molecular genetic differentiation of *Mycoplasma* species, however, requests a strategy based on stable genes which are not involved in genetic variability and which differ enough between the species. Generally, 'housekeeping' or constitutive genes, which are expressed in all living organisms because they provide basic functions such as replication, transcription and translation, are good candidates for genetic differentiation of species. One of these essential genes, *uvr*C, encodes, deoxyribodipyrimidine photolyase (EC 4.1.99.3). Deoxyribodipyrimidine photolyase is an enzyme of the excision DNA-repair system, Uvr ABC, which removes damaged DNA segments via concerted dual incisions by an ATP-dependent enzyme system (2). This system has been found in all free living species tested, from the smallest known self replicating organism *M. genitalium* to humans cells (3). We describe in this paper the cloning and sequencing of the replication repair gene *uvr*C from *Mycoplasma bovis* and from *Mycoplasma agalactiae* and the analysis of the conservation of this gene in these two *Mycoplasma* species. The *uvr*C gene sequences were used to design PCR assays for the specific identification of *Mycoplasma bovis* and of *Mycoplasma agalactiae*.

MATERIALS AND METHODS

Type strains *M. agalactiae* PG2^T and *M. bovis* PG45 ^{T} and about 20 field strains of each, isolated from milk, bulk tank milk, lung and nasal swab samples were used in this study. They include all currently isolated serotypes of *M. agalactiae* as well as *M. bovis* strains representing different antigenic profiles. The Mycoplasmas were cultured in standard Mycoplasma medium. Genomic DNA of Mycoplasmas was extracted as described (1). The Mycoplasma species *M. capricolum, M. bovigenitalium, M. arginini, M. canadense, M. conjunctivae, M .alcalescens, M. dispar, M .californicum, M. bovoculi, M. bovirhinis, M. ovipneumoniae, A. oculi, M. verecundum, M. mycoides mycoides SC, M. mycoides mycoides LC, M. mycoides capri, M. capricolum capripneum.*, *Mycoplasma* bovine gr. 7, *M. putrefaciens, Mycoplasma* sp. serotype 11 were used as controls.

Gene libraries were made by cloning total genomic DNA partially digested with *Sau*3A into the *Bam*H1 site of λZAP-express™ vector (Stratagene, La Jolla, CA) using the suitable *Escherichia coli* K-12 host strains and the protocol provided by the producer. *In vivo* excisions of selected clones on plasmid vector pBK-CMV from the phage plaques were made by selection from Km^R colonies after infection with the helper phage M13 according to the supplier's protocol. Sequences were determined with an ABI Prism model 310 genetic analyzer. DNA sequences were assembled and edited by using the Sequencher 3.0 program (GeneCodes, Ann Arbor, MI) to obtain contiguous sequences

PCR reactions were carried out in 50 µl reaction mixtures which contained 2 ng genomic template DNA (or 1 µl washed *Mycoplasma* culture of 10^5 - 10^6 cells/ml for routine determinations), 20 pmol of each primer, 1 mM each dNTP, 10 mM Tris/HCl pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl and 1.25 U Taq DNA polymerase and were subjected to 35 cycles of amplification with the parameters 30 s at 94 °C, 30 s at the corresponding annealing temperature (Table 1) and 1 min at 72 °C. To determine if the *uvr*C genes of *M. bovis* and *M. agalactiae* are conserved within the respective species, aliquots (10 µl) of PCR products were digested with *Dde*I and analyzed by polyacrylamide gels electrophoresis on 8% gels.

Table 1
Oligonucleotide primers for PCR reactions

Primer	Nucleotide position	Sequence	Annealing Tem.	Fragment size [bp]
Primers used for species identification				
Mycoplasma bovis				
MBOUVRC2-L	362-381 [a]	5'- TTACGCAAGAGAATGCTTCA -3'	52 °C	1626
MBOUVRC2-R	1988-1969 [a]	5'- TAGGAAAGCACCCTATTGAT -3'		
Mycoplasma agalactiae				
MAGAUVRC1-L	263-283 [b]	5'- CTCAAAAATACATCAACAAGC -3'	50 °C	1624
MAGAUVRC1-R	1889-1870 [b]	5'- CTTCAACTGATGCATCATAA -3'		

RESULTS

A clone from the *M. bovis* strain PG45 gene bank which contained a 3.5 kb insert comprising the entire *uvr*C gene was selected and sequenced. It contained an ORF of 1716 bp encoding a protein of 571 amino acids which showed significant similarity to UvrC of many Gram-positive and Gram-negative prokaryotes. The *uvr*C gene of *M. agalactiae* type strain PG2 was cloned on two separate plasmids from the *M. agalactiae* gene bank. The plasmids contained overlapping fragments covering the entire *uvr*C gene. The *uvr*C gene of *M. agalactiae* is 1716 bp in length and corresponds to a UvrC protein of 571 amino acids. Both *uvr*C genes are preceded by a consensus sequence of a ribosome binding site (RBS) three bp upstream the ATG_{Met}-start codon and a strong stem-loop structure representing a potential transcription stop signal is found downstream *uvr*C encompassing the stop codon. The GenBank EMBL accession numbers: $uvrC_{M.\ bovis}$ AF003959, $uvrC_{M.\ agalactiae}$ AF003960.

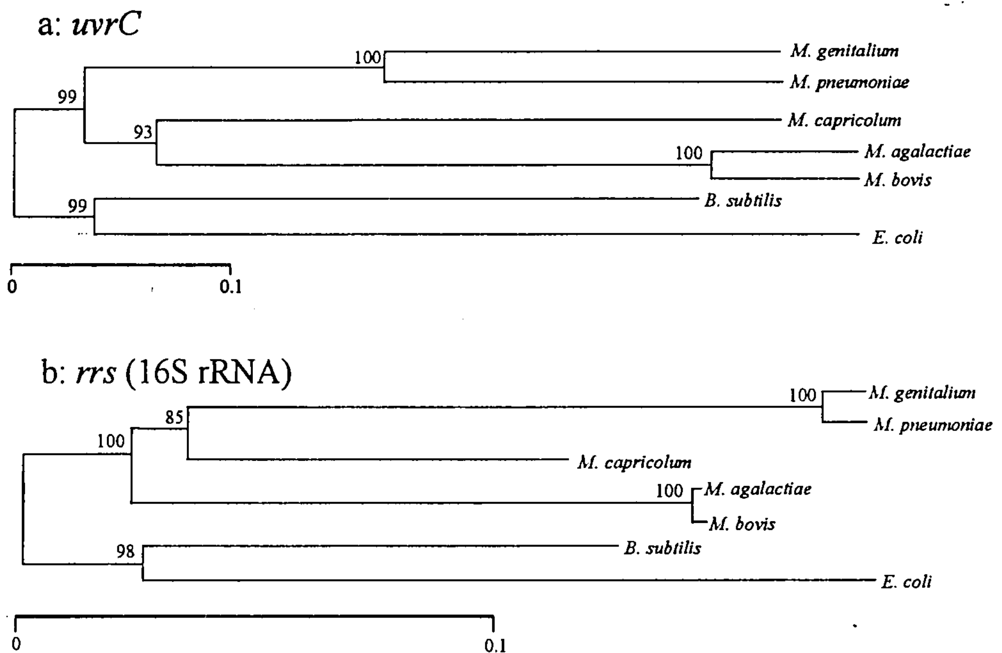

Figure 1
Phylogenetic relationship based on a) the *uvrC* gene sequences and b) the 16S rRNA gene sequences (*rrs*) of *M. bovis, M. agalactiae, M. capricolum, M. genitalium, M. pneumoniae. B. subtilis* and *E. coli* were used as outgroups. The trees were derived by using the neighbor joining method. Bootstrap values given at the junctions were calculated from 1000 trees. Scale bar indicates 0.1% sequence divergence.

Alignment of the *M. bovis* and *M. agalactiae uvrC* gene sequences showed 82.6 % identity at the nucleic acid level respectively 87.7 % identity at the amino acid level. Hence, the *uvrC* genes differed significantly more than the 16S rRNA genes of these two species. (Fig. 1) Phylogenetic trees constructed from a few *Mycoplasma* species of which both uvrC and 16S rRNA sequence data were available, showed stronger differences between the species and significant branching when they were based on the *uvrC* compared to 16S rRNA gene sequences (Fig. 1).

A pair of oligonucleotide primers MBOUVRC2-L/R (Table 1) was designed from the *uvrC* gene sequence of *M. bovis* based on segments that showed high heterogeneity with *uvrC* of *M. agalactiae.* PCR with this primer pair amplified a 1.6 kb fragment from *M. bovis* type strain PG45 and from 13 *M. bovis* field strains originating from various countries and different clinical samples. Analysis of the amplification products by the frequently cutting restriction enzyme *Dde*I showed identical restriction fragment profiles for all strains matching the fragments from computerized restriction mapping with *Dde*I based on the *M. bovis uvrC* gene DNA sequence. No amplification was found with this primer pair when template DNA from various *M. agalactiae* strains and the other *Mycoplasma* species were used.

For amplification of the *M. agalactiae uvrC* gene, a primer pair MAGAUVRC1-L/R (Table 1) matching segments which differ strongly in nt from *uvrC* of *M. bovis* was designed. PCR with this primer pair amplified a 1.6 kb fragment from *M. agalactiae* type strain PG2 and from 29 field strains from different countries isolated

during the last four decades. All amplification products showed identical *Dde*I restriction patterns corresponding those calculated from the DNA sequence of the *uvrC* gene of *M. agalactiae*. No amplification products were seen with these primers with other *Mycoplasma* species.

CONCLUSIONS

In view of the close phylogenetic relatedness and inherent intra-species genetic variabilities of *M. bovis* and *M. agalactiae,* differentiation of these *Mycoplasma* species must be based on a target gene which is conserved within each and yet species specific. Analysis of *uvr*C gene segments amplified in PCR reactions demonstrated identical *Dde*I restriction patterns for type and field strains of each species *M. bovis and M. agalactiae*. Therefore, the gene *uvr*C seems to be conserved within each of the two species even though substantial intra-species genetic variabilities have been observed for both *M. bovis* and *M. agalactiae*. The primer pairs MBOUVRC2-L/R and MAGAUVRC1-L/R designed from the *uvr*C genes of *M. bovis* and *M. agalactiae*, respectively, were demonstrated to be species specific due to the lack of amplification in heterologous reactions. Also, no amplification of *uvr*C gene fragments with either primer pair was observed in other related *Mycoplasma* species denoted in Materials and Methods.

The use of *uvr*C for species identification was demonstrated further with Mycoplasma strains, isolated from the lungs of pneumonic goats in India. The sequences of the 16S rRNA genes (*rrs*) of these particular strains differed only by 3 nucleotides (nt) from that of *M. bovis* and 4 nt from that of *M. agalactiae* thus rendering a differentation impossible. However, amplification of the *uvr*C gene occurred only with the *M. agalactiae* specific *uvr*C primers but not with the *M. bovis* *uvr*C primers. Both strains therefore could be identified as *M. agalactiae* using the *uvr*C PCR.

In summary, the *uvr*C gene is species specific and well conserved within each *M. bovis* and *M. agalactiae*. It is sufficiently different between the two species in order to facilitate a good resolution of these two mycoplasmas which are difficult to be identified by other genetic methods such as using their 16S rRNA gene sequences. On the other hand, *uvr*C seems to be sufficiently conserved within each species inspite of the high genetic and antigenic heterogeneity which is found amongst *M. bovis* and *M. agalactiae* strains. Therefore, it is an ideal target gene for PCR based identification of *M. bovis* and *M. agalactiae*.

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DETERMINATION OF GENOME SIZE AND RESTRICTION PATTERN POLYMORPHISM OF *MYCOPLASMA AGALACTIAE* AND *MYCOPLASMA BOVIS* BY PULSED FIELD GEL ELECTROPHORESIS

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INTRODUCTION

Mycoplasma agalactiae and *Mycoplasma bovis* have a determinant role in many ovine and bovine diseases respectively. M. agalactiae is the etiological agent of contagious agalactia in sheep and goats (1). M. bovis is the causative agent of mastitis, arthritis, pneumonia and fertility disorders in cattle (2). This species is very closely related with *M. agalactiae*, in fact until 1976 this species was classified as *M. agalactiae* subsp. bovis because it has the same biological and biochemical characteristics of M. agalactiae (3). Furthermore, comparative analysis of 16S rRNA sequence has shown 99.47% similarity between M. bovis and M. agalactiae (4).

On the other hand, these two species are clearly distinguishable using growth inibition (GI) and immunofluorescence (IMF) tests. In addition, DNA-DNA hybridization has revealed only 40% homology between *M. agalactiae* and *M. bovis* (5). Recently, many reports have described the use of pulsed field gel electrophoresis (PFGE) for evaluating intraspecies and interspecies genetic variation (6, 7). Furthermore, PFGE analysis of the chromosome DNA digested with rarely cutting enzymes allows accurate determination of genome size (8).

The aim of this study was the comparison of restriction fragment length polymorphism and genome size estimation both for M. agalactiae and for M. bovis using the contour clamped homogeneous electric field (CHEF) system of PFGE.

MATERIALS ANS METHODS

Strains and growth conditions:

M. agalactiae type strain (PG2, Bga) and *M. bovis* type strain (PG45, NCTC 10131) were grown in modified Hayflick medium with 10% (v/v) equine serum at 37°C until the exponential growth phase. Mycoplasmas were pelletted at 20,000 x g for 30 min, washed and resuspended in phosphate saline buffer (PBS, 0.1 M phosphate, 0.33 M NaCl, pH 7.4).

DNA preparation and PFGE:

High-molecular weight genomic DNAs were prepared as previously described (7). Block slices from both *M. agalactiae* and *M. bovis* cultures were digested with twenty-eight restriction endonucleases. For restriction endonuclease treatment, agar slices were equilibrated for 15 min. at 4°C with 500 µl of restriction buffer provided by the respective manufacturers. The buffer was replaced with 150 µl of fresh buffer containing 10 to 40 enzyme units and 500 µg/ml of acetylated bovine serum albumin

(BSA, Promega). After 20 hours of incubation, blocks were washed for 30 min in TE buffer (10 mM Tris-HCl, 1mM EDTA, pH 8.0).

PFGE was performed at 14° C with a CHEF Mapper System (Bio-Rad, Richmond, CA) in 0.5 x TBE buffer (45 mM Tris, 45 mM boric acid, 2 mM EDTA, pH 7.6). A 1% ultrapure agarose (Bio-Rad) running gel was prepared in 0.5 x TBE buffer. After electrophoresis, gels were stained in ethidium bromide (1 µg/ml) for 30 min and then destained in 1 mM $MgSO_4$ for at least 3 hrs before being photographed under UV light. DNA fragments sizes were determined by comparing bands with DNA standards: *Saccharomyces cerevisiae* strain YNN295 chromosome marker (Bio-Rad) and Pulse marker (0.1-200 Kb, Sigma).

RESULTS AND DISCUSSION

Twenty-eight endonucleases were tested for their ability to digest the chromosomal DNA of *M. agalactiae* and M. bovis. *Sma* I, *Ecl*XI and *Bsi*WI enzymes digested DNA of both micro-organisms producing a few well-separated bands. *Mlu*I, *Bss*HII, *Sal*I, *Xho*I and *Nru*I endonucleases cleaved well only *M. agalactiae* DNA. In *M. bovis* instead they always produced irregular patterns because of incomplete digestions which caused variable fluorescent intensity in consecutive DNA bands. *Apa*I enzyme digested *M. bovis* DNA exclusively producing 3 fragments.

To visualize all fragments in the range from 3 to 710 Kb we used several different migration conditions. Detection of all fragments obtained with group A enzymes was made using 20.18 migration hours with pulse times of 0.47-12.91 sec. *Bsi*WI gave 3 fragments between 121 and 515 Kb in *M. agalactiae* and 3 fragments between 90 and 700 in M. bovis. *Sma*I produced 7 fragments between 4 and 459 Kb in PG2 and 8 fragments between 5 and 335 Kb in PG45. Finally, *Ecl*XI produced, for both *M. agalactiae* (PG2) and *M. bovis* (PG45), 7 fragments between 4 and 490 Kb and 5 and 291 Kb respectively.

Under the same conditions, *Sal*I, *Nru*I, *Xho*I, *Mlu*I and *Bss*HII digested solely *M. agalactiae* DNA giving fragments between 4 and 410 Kb. *Apa*I digested solely *M. bovis* giving 3 fragments in the range of 160-485 Kb.

Genome sizes were calculated on the basis of eight specific digestions for *M. agalactiae* and four specific digestions for *M. bovis*. Calculations based on these digests indicate that the genome size of *M. agalactiae* is approximately 945 ± 8.4 Kb whereas that of *M. bovis* is 961 ± 18.9 Kb. Comparing these sizes to those of other Mycoplasmas determined by PFGE, we find that *M. agalactiae* and *M. bovis* fall between M. *genitalium* [600 Kb (9)] and *M. mycoides* subsp. *mycoides* [1,200 Kb (10)].

In conclusion, restriction enzyme digestion with infrequently cutting enzymes and subsequent analysis by PFGE showed a marked polymorphism in *M. agalactiae* and *M. bovis* chromosomes. It is our opinion that further study is needed to place the genetic markers within their respective physical maps.

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CHARACTERIZATION OF *hsp60* AND *hsp70* GENES OF MYCOPLASMAS

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INTRODUCTION

Heat shock proteins (Hsp) are among the most conserved molecules known. These proteins are produced by prokaryotic and eukaryotic cells in response to heat shock and also a variety of other environmental stresses. Hsp are classified into four families according to their molecular weight (Hsp 90, Hsp70, Hsp60, and small Hsp). Hsp are important for the maintenance of normal cellular functions during stress conditions by playing a crucial role in unfolding denatured proteins, preventing unproductive protein-protein interactions and others. Hsp fulfil also important physiological functions during normal cell metabolism by supporting the translocation of precursor-proteins through membranes (1, 2).

Although highly conserved proteins are commonly weak immunogens in several studies Hsp of a broad spectrum of pathogens have been shown to be immunodominant antigens during the course of infection. On one hand, owing to their extreme conservation, Hsp could also provide a clue to certain autoimmune diseases, but on the other hand Hsp might be useful as vaccines against microbial infections because of their immunodominant properties (3).

Mycoplasmas arose by degenerative evolution from a branch of the eubacterial phylogenetic tree containing gram-positive eubacteria with DNA having low G+C contents. The size of the genome of mycoplasmas ranges from 600 to 2000 kilobase pairs (kb) approaching the theoretical limit for the minimal amount of genetic information in a free living organism. Only little is known about Hsp and Hsp-encoding genes of mycoplasmas (4). In previous work we detected different Hsp in several mycoplasma species and characterized Hsp of the Hsp60- and Hsp70-family (5). The aim of this study is, (i) to identify and sequence Hsp-encoding genes of different mycoplasma species, (ii) to compare these nucleotide sequences to the nucleotide sequences of Hsp-encoding genes of other bacteria, and (iii) to obtain recombinant Hsp to investigate the influence of these molecules on mycoplasmoses.

MATERIALS AND METHODS

Mycoplasma (M.) arthritidis ISR1, *M. agalactiae* PG2, *M. bovis* PG45, and *M. hyopneumoniae* J were cultured in modified Hayflick and Friis medium according to standard conditions. DNA was extracted by the NUCLEON-II-DNA-Kit (Scotlab, Wiesloch, Germany). Oligonucleotides used as primers in PCR for the amplification of the *hsp60* gene were deduced by multiple sequence alignment (MULTIALIGN, Heidelberg Unix Sequence Analysis Resources, HUSAR) of the *hsp60* gene of *M. pneumoniae, M. genitalium, Clostridium (Cl.) perfringens,* and *Bacillus (B.) subtilis.*

Ten regions of very high sequence homolgies were chosen. Sequences of primers (degenerated primers) for the amplification of the *hsp*70 gene were deduced from highly conserved regions of the *hsp*70 gene of *Cl. perfringens* (6).

The PCR was performed according to standard protocols with an annealing temperature of 60°C (*hsp*60), or 62°C (*hsp*70). Following isolation and purification (Geneclean Kit II, Dianova, Hamburg, Germany) the PCR products were sequenced (ABI Prism7, Perkin Elmer Cetus) and analysed (GAP and MULTIALIGN, HUSAR). Fragments of the *hsp*60 and *hsp*70 genes were cloned and expressed in *E. coli* strain JM 105 using the vector pGEX-5X-3 according to standard protocols. Recombinant fusion proteins with glutathione-S-transferase (GST) were obtained by IPTG-induction. Analysis of these proteins was performed by SDS-PAGE in Coomassie staining and immunoblotting using antisera directed against Hsp60 and Hsp70. Purification of the recombinant fusion proteins was carried out by aggregate preparation.

RESULTS and CONCLUSIONS

Twenty-five fragments of 200 to 1400 bp of the *hsp*60 gene *of M. arthritidis, M. agalactiae, M. bovis* and *M. hyopneumoniae* representing different parts of the molecule were obtained by PCR. Only samples showing distinct bands with the size expected by comparison with other *hsp* genes were purified from the agarose gel and sequenced. These amplicons were identified as fragments of the *hsp*60 gene by alignment of their nucleotide sequences to *hsp*60 sequences of other bacteria already known. PCR generated fragments of about 600 bp of the *hsp*70 gene of *M. arthritidis* and *M. bovis* could be separated by agarose gel electrophoresis, purified and aligned as indicated above.

Sequence analyses in order to obtain information about homologies were carried out with *M. pneumoniae, M. genitalium, Cl. perfringens, B. subtilis, E. coli* (*hsp*60, *hsp*70) and *M. capricolum, Erysipelothrix (Er.) rhusiopathiae* (*hsp*70). The nucleotide sequence of the *hsp*60 genes of *M. arthritidis, M. agalactiae, M. bovis* and *M. hyopneumoniae* obtained in our investigations and the *hsp*60 sequence of *M. pneumoniae* already known showed a homology of about 96%. Compared to *hsp*60 nucleotide sequences of other bacteria (*Cl. perfringens*, *B. subtilis* and *E. coli)* a decreasing percentage of homology (60-52%) could be detected corresponding to their degree of phylogenetic relationship to mycoplasmas. Interestingly, the sequence comparison of the *hsp*60 gene of the mycoplasma species investigated in this study and *M. pneumoniae* revealed a homology of only 38 % to the *hsp*60 gene of *M. genitalium*.

Sequence analysis of the 600 bp gene-fragments of the *hsp*70 gene of *M. arthritidis* and *M. bovis* showed a homology of nearly 100 %. After comparison to the *hsp*70 gene of *M. pneumoniae, M. capricolum* and *M. genitalium* homologies of 65% could be detected. According to their phylogenetic relationship homologies of the *hsp70* gene-fragment of *M. arthritidis* and *M. bovis* to the *hsp*70 gene of *Cl. perfringens, Er: rhusiopathiae, B. subtilis* and *E. coli* ranged from 55 to 63%.

After cloning and expression two recombinant *M. arthritidis*-Hsp60 fusion proteins of about 57 and 64 kDa (with a GST portion of 29 kDa) were obtained. In addition, *a M. arthritidis* and a *M. bovis* Hsp70 fusion protein with an Hsp portion of 3 kDa were obtained (Fig. 1, 2).

In futher investigations (i) a TGA codon of the *M. arthritidis hsp70* fragment will be altered by site-directed mutagenesis into TGG (encoding for tryptophan) to obtain a recombinant protein with an Hsp70 portion of 14 kDa, and (ii) the influence of the recombinant Hsp on mycoplasmoses will the be investigated

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ACKNOWLEDGEMENT

This study was supported by the Deutsche Forschungsgemeinschaft (Ru 459/2-1). Barbara Scherm is a fellow of the „Graduiertenkolleg: Cell and Molecular Biology in Veterinary Medicine (547 73-4)" funded by the Deutsche Forschungsgemeinschaft

Fig.1: Analysis of recombinant Hsp60 fusion proteins of *M. arthritidis* after separation by SDS-PAGE in (A) Coomassie staining and (B) Westernblot using antisera against Hsp60 of *Synechococcus* sp.. M:Molecular weight marker; 1:*E.coli* JM105 transformed with pGEX-5X-3; 2:*E.coli* JM105 transformed with pGEX-5X-3 after IPTG-induction (expression of glutathione-S-transferase; GST); 3:*E.coli* JM105 transformed with pMa26*hsp*60; 4:*E.coli* JM105 transformed with pMa26*hsp*60 after IPTG-induction; 5:Aggregate preparation of the recombinant fusion protein Ma26Hsp60; 6:*E.coli* JM105 transformed with pMa410*hsp*60; 7:*E.coli* JM105 transformed with pMa410*hsp*60 after IPTG-induction; 8:Aggregate preparation of the recombinant fusion protein Ma410Hsp60

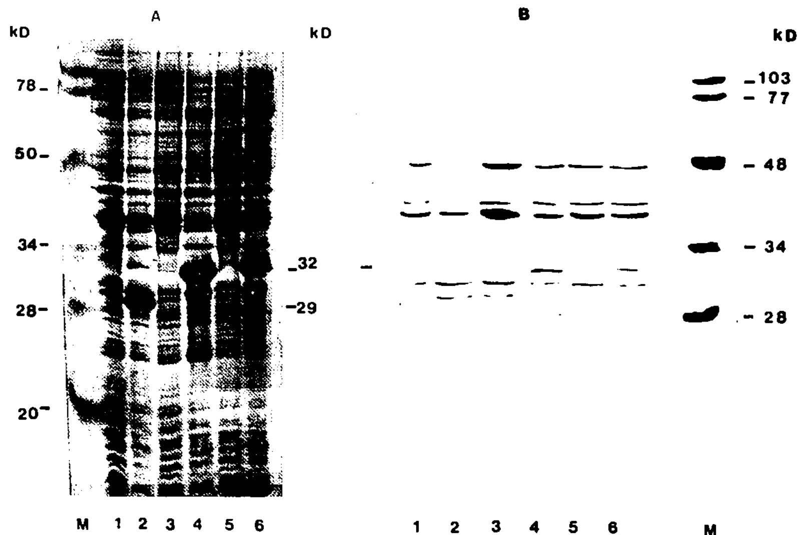

Fig.2: Analysis of recombinant Hsp70 fusion proteins of *M. arthritidis* und *M. bovis* after separation by SDS-PAGE in (A)Coomassie staining and (B)Westernblot using antiserum directed against Hsp70.
M: Molecular weight marker; 1:*E.coli* JM105 transformed with pGEX-5X-3; 2:*E.coli* JM105 transformed with pGEX-5X-3 after IPTG-induction (expression of glutathione-S-transferase; GST); 3:*E.coli* JM105 transformed with pMa*lhsp*70; 4:*E.coli* JM105 transformed with pMa*lhsp*70 after IPTG-induction; 5:*E.coli* JM105 transformed with pMb*hsp*70; 6:*E.coli* JM105 transformed with pMb*hsp*70 after IPTG-induction

OCCURRENCE OF *vspA* SEQUENCES IN *Mycoplasma bovis* FIELD ISOLATES

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Mycoplasma bovis displays a family of antigenically and structurally related, but distinct surface lipoproteins (Vsps) that undergo high-frequency variation in expression and size, from one clone to its progeny. In type strain PG45, three members of the Vsp family, VspA, VspB and VspC have been characterized so far and shown to react with the monoclonal antibody (MAb) 1E5. Sequencing of a DNA fragment containing the *vspA* gene revealed the presence of two additional *vsp*-like genes, *vspE* and *vspF* for which the corresponding translation products have not yet been identified. However, the full extend of the *vsp* repertoire in *M. bovis* PG45 has yet to be defined.

To evaluate the occurrence of Vsps in natural populations, the presence of *vsp* sequences or Vsp epitopes was investigated in *M. bovis* field isolates. First, 250 isolates originating from diverse geographical locations (France, Italy, Switzerland, Spain and Germany) were screened by dot immunobinding (DB), Western blotting(WB) and colony blotting (CB) using MAb 1E5. Results revealed that among the 250 isolates, 20 were negative by DB,10 were negative by DB and WB, and only 4 were negative by DB, WB and CB. Furthermore, the 20 isolates shown to be negative by DB were cloned and the genomic DNA of these cloned isolates was digested by *Hind*III and hybridized with oligonucleotide probes corresponding (i) to a sequence located in a region encoding the signal peptide (S) of *vspA* and suspected to be highly conserved among the *vsp* genes, or (ii) to *vspA*- repeated motifs, RA. A similar experiment was also performed on isolates that reacted with Mab 1E5. Results showed that all the isolates tested hybridized with both, the S and RA probes, suggesting the presence of *vspA* gene sequences even in the four isolates shown not to react with MAb 1E5 in DB, WB, and CB. The presence of multiple fragments hybridizing with the S oligonucleotide probe suggested that the isolates examined in this study, all contain additional *vsp* genes. Whether these sequences belong to functional *vsp* genes, expressed by the organism, is not yet defined. In a concomitant study, Vsps were shown to strongly react with hyperimmune sera collected from animals naturally or experimentally infected with *M. bovis*, even with sera from an early stage of infection. Taken together, these data indicate that despite their variability, the Vsps and in particular VspA, represent excellent candidates in designing serodiagnostic assays.

INTRASPECIFIC VARIATION IN THE 16S rRNA GENE SEQUENCES OF *MYCOPLASMA AGALACTIAE* STRAINS

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INTRODUCTION

Mycoplasma agalactiae is the causative agent of contagious agalactia, a disease syndrome of goats and sheep which occurs mainly in Europe, Western Asia, and North Africa (6). In cattle similar disease symptoms are caused by *Mycoplasma bovis* (9), and the two species have been shown to be very closely related according to as well biochemical and immunological methods as 16S rRNA sequence comparison. Different detection systems have been developed for the identification of *M. agalactiae* and many of them are based on PCR of the 16S rRNA gene. Both species have been found to have two rRNA operons, as do many mycoplasmas (2, 7). Sequence differences often exist between the two rRNA operons and the corresponding nucleotide positions are referred to as polymorphisms (7). Furthermore, sequence differences may also exist between for instance the 16S rRNA genes of the same operon, but from different strains of the same species. Polymorphisms and sequence differences between strains of the same species are referred to as intraspecific variations (3). Results from analyses by 16S rRNA based PCR indicate that intraspecific variations may occur in *M. agalactiae*. It has recently been shown that intraspecific variations between strains exist in *M. agalactiae* (10). The 16S rRNA genes from 9 field strains of *M. agalactiae* of different geographical origins (The Canary Islands, Egypt, France, Greece, Hungary, Spain, Swedish, quarantine, Switzerland, and Tanzania) were therefore sequenced to investigate the extent of intraspecific variations within the species.

MATERIALS AND METHODS

All strains and their origins are listed in Table 1. As judged from biochemical and serological data, all these strains were *M. agalactiae*. The strains were grown in F-medium (1) and cloned four times if this was not previously done. DNA was prepared from one millilitre of outgrown culture suspension by conventional phenol/chloroform extraction. The 16S rRNA genes were amplified with a set of primers (7) complementary to the universal regions U1 and U8 as defined by Gray et al (5) The PCR reactions were performed in 50 µl volumes according to standard PCR protocols with 10 pmol of each primer and 0.75 µl Advantage cDNA Polymerase Mix (Clontech, USA). The PCR products were diluted 6-fold and cycle sequencing reactions with Thermo sequenase (Amersham Pharmacia Biotech, Sweden) and fluorescently labelled primers (7) were carried out according to the manufacturers recommendations. The samples were then loaded onto a sequencing gel of the ALFexpress™ automated laser fluorescent DNA sequencer (Amersham Pharmacia Biotech, Sweden) followed by electrophoretic separation, on-line detection, and computerised sequence evaluation.

Table 1. Strain designations, countries of origin and polymorphic positions of the *Mycoplasma agalactiae* strains examined in this work and the type strain of the species (PG2, accession no U44763 [7]). The nucleotide positions are numbered according to the sequence of strain 396/12. The polymorphisms are designated according to the IUB nucleotide ambiguity code.

Strain	PG2^T	396/12	CC4b	5725	41	17	L9	5G	M133/87	847.121
Origin	Spain	Hungary	Spain	France	Switzerland	Greece	Canary Islands	Egypt	Tanzania	Quarantine
Position										
126	**M**	**M**	A	A	**M**	**M**	C	C	C	C
127	G	G	G	G	G	G	G	G	**R**	G
170	**Y**	**Y**	T	T	**Y**	**Y**	T	T	T	T
182	G	G	G	G	G	G	G	G	**R**	G
188	C	C	**Y**	**Y**	C	C	C	C	C	C
374	**K**	G	G	G	G	**K**	G	G	G	G
375	G	**R**	G	G	G	G	G	G	G	G
419	C	C	C	C	C	C	**Y**	**Y**	C	C
420	G	G	G	G	G	G	G	G	**R**	G
431	C	C	C	C	C	C	C	C	**Y**	C
611	A	A	A	A	A	A	A	A	**R**	A
665	**W**	T	T	T	**W**	**W**	T	T	T	T
708	C	C	C	C	C	C	C	C	**Y**	C
722	G	G	G	G	G	G	G	G	**R**	G
798	-	**G**	-	-	-	-	-	-	-	-
1032	**Y**	**Y**	**Y**	**Y**	**Y**	**Y**	T	T	T	T
1048	**R**	**R**	**R**	**R**	G	**R**	G	G	G	G
1118	**Y**	C	C	C	C	C	C	C	C	C

RESULTS

All variable positions found in the examined strains are listed in Table 1. The number of differences between the strains (including PG2^T) varied from 1 to 14 (mean and median value 6). Considering that there are only a few sequence differences between some closely related mycoplasma species this is a very high number. It has to be pointed out, however, that all except one of these differences are polymorphic positions, i.e. it is only one of the operons in one of the strains that differ in each position. All these strains are, therefore, likely to represent the species *M. agalactiae*.

All of the European strains, including the type strain PG2 that was originally isolated in Spain (4), had a similar polymorphic pattern. There was one polymorphism (1032_Y) that was shared by all the European strains and the type strain. Two other polymorphic positions (126_M, and 1048_R) were shared by 4 and 5 strains, including the type strain, respectively. Among the European strains, the Hungarian strain had a polymorphism (375_R) that was not found in the type strain.Furthermore, the Spanish and the French strains shared a polymorphism (188_Y) that was not included in the type strain.The strain 17 (Greece) was almost identical to the type strain. The two strains shared 6 out of the 7 polymorphisms

found in the type strain. Strain CC4b (Spain) and strain 5725 (France) were identical. In position 126 where all other strains had either a C or a C/A polymorphism they had an A. Strains CC4b and 5725 had two polymorphisms that they shared with other European strains and one that they only shared with each other. The Hungarian strain 396/12 had one unique polymorphism and one unique sequence length difference in a poly(G) region. Sequence length variations have been observed earlier in the 16S rRNA genes of members of the *Mycoplasma mycoides* cluster, but only in poly(A) regions (8). The strain 396/12 also had 4 other polymorphic positions which were all in common with one or more European strains.

Four of the analysed strains, 5G, M133/96, 847.121, and L9, did not contain any of the polymorphisms found in the type strain. Two of the strains, 5G (Egypt) and L9 (Canary Islands), were identical and shared a single polymorphism that was not found in any other isolate. Interestingly, the strain M133/87 (Tanzania) contained 7 polymorphic positions, all of them unique for this strain.

CONCLUSIONS

This study of 9 different *M. agalactiae* strains show that there is substantial variability between the 16S rRNA gene sequences of this species. Among the nine analysed strains, 18 polymorphic positions were identified. This fact may cause problems in the detection systems for the identification of *M. agalactiae* that are based on the 16S rRNA gene sequences. Variations that create false results may eventually appear in restriction sites or in primer target regions. If the same kind of intraspecific variation occurs in *M. bovis* there is a risk that strains which cannot be differentiated on the 16S rRNA level will be found.

Despite the large variability found in *M. agalactiae* there is no obvious evolutionary pattern as the one found in the *M. capricolum* subsp. *capripneumoniae* strains sequenced by Petterson et al (8). It may be possible, however, to find such a pattern among European *M. agalactiae* strains, but this will require sequencing of more strains and also sequencing of the two rRNA operons from all strains.

ACKNOWLEDGEMENTS

We wish to thank Stefan Jernberg and Karin von Schantz for rapid and skilful technical support. We also owe our gratitude to Dr Soraya Deniz, Dr Al El Ebeedy, Dr Èva Kaszanyitzky, Dr Jaques Nicolet, Dr François Poumarat, and Professor Kriton Sarris, who has provided us with some of the strains. This work has been financially supported by the Scientific Advisory Committee of the National Veterinary Institute.

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PRODUCTION AND SEQUENCE ANALYSIS OF A *MYCOPLASMA CAPRICOLUM* SUBSP. *CAPRIPNEUMONIAE* (F38-BIOTYPE) RECOMBINANT EXPRESSION LIBRARY

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INTRODUCTION

Mycoplasma capricolum subsp. *capripneumoniae* (*Mcc*) is the causal agent of contageous caprine pleuropneumonia (CCPP), a major disease of goats throughout Asia and Africa. There is little knowledge on the pathogenic mechanisms and host immune responses during mycoplasmal infections and the lack of knowledge on the molecular biology of these organisms has hindered vaccine research. Currently, most mycoplasma vaccines involve the use of the whole organism, inactivated or attenuated. These vaccines often give only low level, short term protection and immunisation itself can result in severe reactions and even death.

The aim of this work is to produce cost-effective diagnostic reagents and a vaccine against the causal agent of CCPP using recombinant DNA technology. The ideal vaccine would be safe and readily administered, would confer high levels of protection and would not revert to a virulent form. This role would be best filled by a recombinant protein subunit vaccine incorporating only the most protective antigens of the organism.

Recombinant *Mcc* expression libraries have been constructed in both plasmid and bacteriophage lambda vectors and work is ongoing to assess their vaccine potential.

MATERIALS AND METHODS

Strains and media: *Escherchia coli* strains JM109 (Promega), opal suppressor strain ISM612 (obtained from F. C. Minion). JM109 was grown in LB broth or on LB agar. ISM612 was grown in super broth plus 0.2 % glucose and 100ug/ml chloramphenicol, or the agar equivalent and with the additional supplementation of 0.2% maltose and 10mM $MgSO_4$ for phage work.

Plasmid library: the AT-rich mycoplasmal DNA was partially digested with the restriction endonuclease *Tsp* 509 I (which has a 4 bp (AATT) recognition sequence) to create a semi-random fragment library (as per Minion *et al.* [1]). The fragments were ligated into the *Eco* RI site of the vector pGEM-3Z (Promega) to enable identification of recombinants using blue/white selection. The ligation reaction was transformed into *E. coli* JM109 and plated on LB agar with ampicillin at 100 ug/ml for selection of recombinants [2]. The cloned fragment sizes ranged from less than 100bp to approximately 2 kb, as determined by restriction analysis with enzymes *Sma* I and *Nar* I (Boehringer Mannheim). The 118 recombinants obtained were sequenced (ABI Prism dRhodamine Terminator Cycle Sequencing Ready Reaction

kit, Perkin-Elmer, automated sequencer) and homology searches of the Swissprot database were carried out. The clones were screened by Western blotting (standard procedure) using rabbit hyperimmune antisera raised against *Mcc* (F38-biotype, obtained from G. E. Jones) pre-absorbed against *E. coli*.

Phage library: the phage library was constructed using the lambda SCREEN-1 *Eco* RI Arms kit (Novagen) as per the kit instructions. The amplified library was screened with both rabbit hyperimmune serum and with goat convalescent serum obtained from a natural CCPP outbreak (Tesfaalem Tekleghiorghis, CVL, Eritrea).

RESULTS

Plasmid library.

Sequence data: Analysis of 118 clones revealed that *Mcc* DNA is particularly AT-rich (75%). This is approximately the same percentage found in the related *M. capricolum* and greater than that in the more distantly related *M. genitalium* (68%) and *M. pneumoniae* (60%). Homology searches: Some of the more significant homologies found in the database searches are listed in Table 1, with the amino acid sequences of some of these homologs shown in Table 2. The first insert homology listed, in *Mcc* itself, confirms part of the already established sequence of its methionine aminopeptidase.

Table 1. Swissprot database homology searches of selected clones

INSERT LENGTH (a.a.)	SPECIES SHOWING HOMOLOGY	HOMOLOGOUS SEQUENCE	IDENTITIES (a.a.)
48	*M.capripneumoniae*	methionine aminopeptidase (peptidase M)	48/48 (100%)
	M. genitalium	methionine aminopeptidase (peptidase M)	19/43 (44%)
	M. pneumoniae	methionine aminopeptidase (peptidase M)	15/47 (31%)
45	*M. gallisepticum*	ATP synthase alpha chain	30/38 (78%)
	M.genitalium	ATP synthase alpha chain	28/45 (62%)
	M. pneumoniae	ATP synthase alpha chain	26/37 (70%)
32	*M. mycoides*	signal recognition particle (protein 54 homolog)	28/31 (90%)
101	*M. capricolum*	phosphocarrier protein (HPr)	99/101 (98%)
149	*M. genitalium*	probable helicase MG308 homolog	33/85 (38%)
	M. pneumoniae	probable helicase MG308 homolog	31/85 (36%)
44	*M. genitalium*	ATP-dependent protease binding subunit (CLPB)	21/44 (47%)
	M. pneumoniae	ATP-dependent protease binding subunit (CLPB)	20/42 (47%)

Immunoscreening: Western blotting (using rabbit hyperimmune serum) of 118 clones, in both *E. coli* JM109 and the opal suppressor strain ISM612, revealed no positive clones. This suggests that none of the cloned inserts were expressed, possibly as a result of their insertion into the incorrect reading frame. Poor expression of cloned mycoplasmal DNA can occur in *E. coli,* as many mycoplasmal species use the opal stop codon to encode tryptophan. Here, the *E. coli* opal suppressor strain ISM612 was used in an attempt to circumvent this problem. It is also possible that expression may be below a detectable level or that the protein folding may not be appropriate for antigenic epitopes to be displayed and hence recognised by the primary antibody. However, given that 118 recombinants were obtained, the failure of any clones to give detectable protein expression (as measured by immunoblotting) is an unusually poor yield.

Table 2. Amino acid sequences and homologies of selected clones

Clone	Sequence alignment
Clone 59 *M. mycoides* signal recognition: particle protein 54 homolog) (447 a.a.)	1 IXNNVKQKALGGYIFEGANAHQQMIKIVHEE 31 I NNVKQKALGGYI EGA+AHQQMIKIVHEE 53 IINNVKQKALGGYISEGASAHQQMIKIVHEE 83 Identities = 28/31 (90%) Positives = 29/31 (93%)
Clone 109 *M. Capricolum* phosphocarrier protein (HPr) (336 a.a.)	1 LNITDERLGLTFQLSSILFTLVGAIIFWSRNPMSFWKSGVGILFGFPIFL 50 LNITDERLGL FQLSSILFTLVGAIIFWSRNPMSFWKSGVGILFGFPIFL 66 LNITDERLGLIFQLSSILFTLVGAIIFWSRNPMSFWKSGVGILFGFPIFL 115 QLFGVAFGLLANLVGVFNNNNNNNAWSDIYNLLVQSVAEILVIIFAFSKINN 101 QLFG+AFGLLANLVGVFNNNNNNNAWSDIYNLLVQSVAEILVIIFAFSKINN QLFGLAFGLLANLVGVFNNNNNNNAWSDIYNLLVQSVAEILVIIFAFSKINN 166 Identities = 99/101 (98%) Positives = 100/101 (99%)
Clone 116 *M. genitalium* probable helicase MG308 homolog (410 a.a.)	1 ISKIVEILHKNNIKQVAELHGNLQPRLRLSMLKKIQNNEFKYLVATDVA 50 + ++ ++L NNI ++G+L + R + K NN+ K LV +D 229 LKQLTQLLSNNNIS-FGSIYGSLTYQERKNNFTKATNNKLKLLVVSDLF 278 SRGVDIKGVSHIISINLPSDLTYYIHRSGRTGRNNS 85 SRG+D+ S +IS +LP ++YIHRSGR R NS SRGIDLNYFSVVISWDLPKIDSFYIHRSGRVARLNS 313 Identities = 33/85 (38%) Positives = 52/85 (61%)

Lambda phage library

Immunoscreening: Four lambda phage libraries were prepared, one with a primary titre of 3×10^3 and the remaining three with primary titres of 2×10^4. Initial screening of the libraries (plated on *E. coli* ISM 612) revealed 13 clones with a positive immunogenic response to the hyperimmune rabbit serum (a yield of approximately 1/1000). Re-screening of these 13 clones with goat convalescent serum yielded one strongly positive clone. This may have potential as a vaccine candidate, and further studies are underway to investigate this.

CONCLUSIONS

Although the sequence analysis of the plasmid library inserts did not reveal any clones of obvious vaccine potential, it did allow a comparative determination of the AT content of *Mcc* DNA, and revealed some interesting homologies: the ATP synthase alpha chain of *M. gallisepticum* (78% homology) [3], *M genitalium* (62% homology) [4] and *M. pneumoniae* (70% homology) [5]; the signal recognition particle (protein 54 homolog) of *M mycoides* at 90% homology [6]; the phosphocarrier protein (Hpr) of *M. Capricolum* at 98% homology [7]; a probable helicase MG308 homolog in *M. genitalium* [4] and *M. pneumoniae* at 38 and 36% homology respectively, and the ATP-dependent protease binding subunit (CLPB) protein of *M. genitalium* [4] and *M. pneumoniae* [5], both at 47% homology.

Immunoscreening of the phage library with rabbit hyperimmune serum yielded 13 positive clones, one of which also screened positive with the goat convalescent serum. This clone has possible potential as a vaccine or serological reagent, and will be investigated further by Western blot analysis and sequencing.

The low observed yield of immunopositive clones is problematic (only 1/1000 recombinants detectable by immunoscreening) and it is unclear at this stage whether this is due to poor levels of expression or inadequacies in the detection systems. Future work will involve further screening of the libraries to identify more positive clones and optimisation of immunoscreening protocols.

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VARIABLE SURFACE PROTEINS OF *MYCOPLASMA BOVIS*. A ROLE IN CYTOADHESION

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Although variable surface proteins (Vsps) of *M. bovis* are known to be major immunogens their function in the pathogenesis of the respective bovine diseases has yet to be elucidated. Besides their suggested role in the subversion of the host immune system there were indications that these antigens could be involved in mycoplasma attachment to host cells. In the present study, a number of different in vitro assays mimicking interactions between pathogen and host cells were conducted in order to examine the potential of Vsps to participate in attachment processes.

The first indication of the involvement of certain Vsps in cytoadhesion was obtained using the "reverse adherence assay", where Western blots of *M. bovis* proteins were incubated with a suspension of ^{35}S-labelled EBL cells (model host cells). On the resulting autoradiography images Vsps were among the major adhesion bands. When a *M. bovis* membrane preparation was examined, host cell attachment was particlarly intense to three bands, all of which were identified as Vsps. Using an adheence assay in tissue culture plates, we were able to demonstrate a reduction of *M. bovis* adherence rates to EBL cells caused by the presence of Vsp-specific MAbs 1E5, 2A8, or 4D7, respectively. The addition of defined amounts of affinity-purified Vsps to adherence assays led to significant increases in *M. bovis* adherence rates. To demonstrate the affinity between Vsps and host cell receptors a binding assay was conducted in which lysed *M. bovis* cells were allowed to interact with a lawn of EBL cells. The protein fraction retained on the host cell layer was shown to contain major amounts of Vsps. Adherence trials involving oligopeptides whose amino acid sequence was derived from internal segments of VspA, VspB, VspE, and VspF, rspectively, clearly showed that some of these oligopeptides were capable of reduing adherence rates.

Altogether, the data obtained from these experiments provide extensive evidence of involvement of Vsp antigens in attachment processes.

VARIABLE SURFACE COMPONENTS IN RUMINANT AND AVIAN MYCOPLASMAS: A COMPARATIVE ANALYSIS

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INTRODUCTION

Mycoplasma pathogens of birds and ruminants, in particular *Mycoplasma gallisepticum*, *M. bovis* and *M. agalactiae*, can cause one or more of several clinical conditions depending on tissue location in the host, such as respiratory disease, mastitis, arthritis and conjunctivitis. In many cases the causative organism causes a chronic disease in which the organism can be reisolated from the host from months up to several years after the initial infection. With respect to contagious agalactia in sheep and goats caused by *M. agalactiae*, asymptomatic carriers are a major source of disease spread (1). Since mycoplasmas lack a cell wall and are therefore vulnerable to immune lysis, what then aids the survival and dissemination of these organisms within and between hosts, with or without clinical symptoms? Many researchers believe that antigenic variation of the membrane surface proteins/lipoproteins of these bacteria play a critical role in the way these organisms interact with the host. Many of these surface proteins are lipid modified and are covalently attached to the membrane via peptidase II cleavage and acylation processing. They are encoded by genes that usually belong to multigene families, which is a significant investment of DNA coding space considering they have undergone a degenerative evolution to reduce their genomes to a minimum size. In this paper three major surface proteins will be compared: pMGA1.1 of the avian pathogen *M.* gallisepticum (2); VspA of the bovine pathogen *M. bovis* (3) and limited comparison using data obtained in our laboratory on Vpma (recognised by monoclonal antibodies (mAbs) 3B3 and 4G12 (4)) of the ovine/caprine pathogen *M. agalactiae*.

SURFACE PROTEIN FEATURES

The mycoplasma proteins, pMGA1.1, VspA and Vpma, are all surface exposed, abundant and immunogenic in their respective hosts (2, 3, 4, this laboratory). The most interesting feature of pMGA1.1 and VspA is that each have been shown to belong to multigene families (5, 6). There have been estimated to be between 32 to 70 pMGA genes, occupying between 8-17% of the *M. gallisepticum* genome, depending on the strain, which represents the largest known family of translated genes in prokaryotes. This is a remarkable feature considering the fact that mollicutes have evolved and maintained a genome of minimal size (8). The variation in gene number between strains suggests that expansions and contractions in the number of pMGA genes have occurred during the evolution of these strains. These pMGA genes are clustered in tandem and in the same orientation. Since all but one of the defined genes do not contain premature stop codons, there appears to have been an evolutionary constraint to preserve the integrity of their open reading frames. In contrast, the Vsp gene family of *M. bovis* is much smaller,

consisting of one defined (*vspA*) and 5 undefined genes (6). Like pMGA genes, the *vsp* genes are also clustered but are not in the same orientation. Both pMGA1.1 and VspA have been shown to be lipid modified and indeed, their gene sequences contain lipoprotein signal sequences. For pMGA genes this is an 'AASC' signal motif at the end of a 25 amino acid hydrophobic leader sequence which is highly conserved between pMGA genes. However, the *vspA* gene contains an atypical lipoprotein signal of 'AAKC' but which is also following a highly conserved leader sequence (6).

Both pMGA and Vsp are most probably involved in the process of attachment of mycoplasmas to host cells. The pMGA1.1 molecule of strain S6 has been shown to be a haemagglutinin and hence proposed to play a role in adhesion (2). A monoclonal antibody (1E5) recognising Vsps had the ability to reduce attachment of *M.* bovis cells to a bovine cell line implicating the involvement of Vsps in cytadhesion (7).

An important feature that pMGA, Vsp and Vpma proteins share is that they can all undergo antigenic variation. For pMGA, this variation results from gene switching where the expression of one pMGA gene is switched OFF and another is switched ON. The determining factor that governs whether a particular pMGA gene is ON or OFF lies in the precise number of GAA repeats contained in a motif located in the vicinity of all pMGA promoters. *In vitro, M. gallisepticum* cells grown in the presence of pMGA1.1 antibodies caused pMGA1.1 expression to be turned OFF in the resulting population while another pMGA gene, pMGA1.9, was turned ON. A proteolytic cleavage product of pMGA1.9 was found to co-partition with pMGA1.9 into the TX-114 detergent phase implying that pMGA1.9 and perhaps other pMGA gene products possess a homotypic binding property. It is possible that pMGA is involved in binding to self and that pMGA switching alters the extent to which mycoplasma cells adhere to each other which may be necessary during the various stages of colonisation and further migration throughout the host. Following *in vivo* expression of pMGA1.1 and variant forms of pMGA (uncharacterised pMGA gene products) during experimental infection of chickens with *M. gallisepticum*, it appeared that pMGA switching occurred in a stage dependent manner in the host. By two days, approximately 50% of the expanding *M. gallisepticum* population had ceased to express pMGA1.1 and at later intervals $pMGA^-$ cells accounted for almost all of the bacterial population recovered. Most reisolated $pMGA^-$ cells reverted to sectored $pMGA1.1^+$ colonies. As the infection progressed, $pMGA^-$ cells which could revert to both $pMGA1.1^+$ and variant form $pMGA^+$ sectors were observed, finally followed by the emergence of $pMGA^-$ cells which reverted to only variant form $pMGA^+$ sectors or entirely variant form $pMGA^+$ cells. Antibodies specific for pMGA1.1 in both chicken serum and tracheal washes were not detectable until 6 days after infection. Therefore chicken pMGA1.1-specific antibodies could not account for the down modulation of pMGA1.1 expression in *M. gallisepticum* cells recovered from the host. This implied that the pMGA switching mechanism in *M. gallisepticum* may have a host receptor mediated basis. It is therefore likely that the transition of infecting *M. gallisepticum* cells from a predominantly $pMGA^+$ to a predominantly $pMGA^-$ phenotype plays an obligate early role in the pathogenesis of this organism and that switching from one pMGA gene to another may be important in the chronic phase of infection.

The Vsps of *M. bovis* not only undergo ON-OFF phase variation but also size variation. The molecular mechanism of *vsp* gene switching is not entirely understood

but is associated with major DNA rearrangements within the *vsp* locus (6), and therefore quite an elaborate recombination mechanism has been developed by *M. bovis* for this purpose. Size variation in VspA is due to expansions and contractions in the numerous reiterated coding repeats occupying 80% of its amino acid sequence. In contrast, pMGA does not undergo size variation and this may account for the requirement of such a large gene repertoire in this organism to maintain competitiveness in the hostile environment of the host. There is only approximately 15-16% identity between the protein sequences of VspA and pMGA1.1/pMGA1.9 but since *M. bovis* and *M. gallisepticum* infect different host species it may not be expected for two protein homologues involved in host cell interaction to share a high identity level. Interestingly, both VspA and pMGA genes share a proline rich amino-terminus which is a characteristic shared at the carboxyl-terminal regions of the adhesin proteins P1 and P30 of *M. pneumoniae* (9). Following this proline rich segment in pMGA1.1 and VspA there is a small region of high identity, that is also present in all pMGA genes, which may be significant since the amino acid identity level between pMGA1.1 and other pMGA genes can be as low as 21%.

Vpma is an abundant 39 kDa protein of *M. agalactiae* strain PG2 and is recognised by two non-overlapping mAbs, 3B3 and 4G12 (4). This protein has also been shown to undergo antigenic variation in our laboratory. Clonal isolates have been obtained which have spontaneously undergone phase switching between $Vpma^-$ and $Vpma^+$ phenotypes. When there is an overloading of Vpma in Western Blot analysis there is no observable laddering effect from minor populations that have switched in size as can be seen for Vsps which suggests that Vpma does not contain reiterated coding repeats and that these two proteins are not structurally similar.

CONCLUSION

Although pMGA and Vsp are not structurally similar and have different molecular mechanisms of antigenic variation, these systems, including Vpma, provide the organism with a rapid means to diversify their surface architecture. This may represent an adaptation response to improve or maintain colonisation at the initial site of infection even when cytopathological changes are taking place during the natural course of the infection. Other important consequences of antigenic variation include the ability to evade the host immune system and/or to alter tissue tropism.

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ANTIGENIC VARIABILITY of *Mycoplasma agalactiae* SURFACE PROTEINS

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INTRODUCTION

The 'Contagious Agalactia' syndrome, caused particularly by *Mycoplasma agalactiae*, is clinically characterized by a quite rapid disappearance of symptoms (mainly in adults and in sheep), whereas infection and seropositivity can persist for several months (1). These features - particularly chronicity - are quite frequent in animal and human mycoplasmoses, which are considered as a kind of parasitism. Sophisticated genetic mechanisms permitting these wall-less organisms to diversify their surface attributes have been described in several mycoplasma species and are thought to play a key role for evasion to the host system (2).

In a previous study (3), nine specific monoclonal antibodies (MAbs) were generated against surface components of *M. agalactiae* type strain PG2 and french field strain P89. Eight out of these nine MAbs reacted with a 39 kDa band. These epitopes were subsequently shown to vary in expression among a collection of 245 field isolates originating from ten countries. On the other hand, the majority of the recognized surface componants showed spontaneous phase variations within clonal lineages after colony immunoblot assays.

The present study was set up to investigate the *in vitro* effect of host immune response on variable antigen systems. In this short paper, we focused on the induced variations of two specific epitopes recognized by MAbs 3B3 and 4G12.

MATERIALS AND METHODS

1. Cultures, clones and sub-clones

Organisms were grown in modified PPLO broth. All experiments were performed with PG2 type strain. Obtention of clonal lineages of PG2 was performed by plating on standard agar medium an initial culture representing an heterogeneous population, then by transferring colonies to nitrocellulose for colony immunoblot. Individual colonies from plated populations were picked under microscope according to the results of colony immunoblot with MAbs 3B3 and 4G12 and propagated at 37°C in 1ml of modified PPLO broth. These sub-clones were then assessed by Western immunoblot assay as described below.

2. Polyclonal antibodies

Results of immunological pressure tests in terms of antigenic profiles were analysed by Western blotting either with experimental rabbit hyperimmune sera or positive and ovine natural sera. The titers of these sera were determined with an ELISA test.

'Immunogenicity' of the epitopes recognized by specific MAbs was assessed by Western blot analysis with 18 ovine or caprine natural sera from 3 different countries and with sequential sera from an experimental model (ewes challenged with a french field strain) (4).

3. Colony and Western immunoblot assays

Expression and size of surface-exposed proteins were assessed by Western blotting, and selection of clonal variants by colony immunoblots as previously described (3).

4. In vitro immunological pressure tests (IP)

This test was performed in sterile 96 well plates. Series of ten-fold dilutions were prepared from PG2 culture in late log phase. Each dilution was pipeted in 160 µl aliquots per well and, for each dilution, 2 wells (one for assay and one for control) were filled. Positive sera or MAb positive ascites were dispensed undiluted in 40 µl aliquots in the assay well ; negative sera or ascites were dispensed in the control wells. Then various dilutions of sera were tested. Plates were sealed with plastic films and incubated at 37°C for 3 to 7 days. Sera were previously heated at 56°C for 30 minutes.

Whole cell lysates of the resulting populations were then tested by Western blot for their capacity to express the 3B3 and 4G12 target epitopes.

RESULTS

1. Intraclonal and intraspecific antigenic variability of M. agalactiae

A Western blot analysis of intra-clonal and intra-specific antigenic variability of *M. agalactiae* was performed using several MAbs, particularly 3B3 and 4G12 which detected ON-OFF switching proteins. Within PG2 clonal lineages selected by colony immunoblots, we observed typical phase and size variations with these two Mabs. Western blot revealed with 3B3 showed size variation ranging approximatively from 15 to 40 kDa.

When comparing Western blot patterns within a collection of 60 field isolates from 10 countries, the same kind of variations appeared, affecting the number and/or the size of epitope-bearing proteins. Interestingly, we showed that a partial link existed between certain Western blot patterns and the geographical origin of isolates, even for variably expressed epitopes as 3B3 and 4G12. In light of the intraclonal variation occuring in strain PG2, these results would suggest that certain field isolates expressed a predominant phenotype depending on their geographical origin.

2. 'Immunogenicity' of the specific and variable surface componants

Western blot analysis of strain PG2 showed that hyperimmune sera obtained from naturally infected animals strongly recognized *M. agalactiae* products of molecular weights ranging between 66-70 kDa and 38-39 kDa, as well as a 35 kDa protein.

Although originating from different geographic areas, these sera seemed to uniformly react with the same set of proteins. Specific surface componants detected by MAbs are recognized by animals eventhough bands intensity varies. The two MAbs 3B3 and 4G12 were shown to react with the 39 kDa product.

With sera collected from the experimental model, we could define specific kinetics of antibodies appearance from challenge to 104 days post-inoculation.

3. Modulation of surface antigens expression by host immune response

In this preliminary study, IP were mainly performed with initial heterogeneous PG2 populations resulting from decimal dilutions of late log phase cultures. A current experiment deals with the effect of different sera on clonal variants of PG2.

• A first experiment of IP was conducted by propagating PG2 in liquid media in presence of sera collected from a rabbit experimentally inoculated with PG2 (plus control animal sera). In contrast to the control culture, populations propagated with *M. agalactiae* hyperimmune sera did not express the 39 kDa protein that contains the two epitopes.

These results were independent on the capacity of the serum to inhibit *M. agalactiae* growth, since no growth inhibition was observed with the rabbit hyperimmune sera.

• A second experiment was performed by propagating PG2 in presence of 3B3 or 4G12 MAbs at different dilutions, with 3 successive IP steps. For this purpose, for each MAb, after a first IP performed in the same conditions as described above (IP1), a second IP was done by sub-culturing the last positive culture dilution whether in presence of the same MAb or of a negative ascite (IP2). The same protocol was done a third time (IP3).

A clear growth inhibition appeared at IP1, but no difference with the control cultures at IP2 and IP3. With 3B3, a phase variation appeared at IP2 (39 kDa), but was reversible (IP3).

• A third experiment is currently under progress. Our aim was to perfom the same kind of IP test on clonal variants regarding to expression and/or size of surface epitopes 3B3 and 4G12. Clonal isolation of various ON or OFF 3B3 variants and subsequent Western blot analysis using 3B3 and 4G12 allowed us to identify two clones : the first one (55-5) expressing a major 39 kDa and minor 21 kDa proteins which bound both with 3B3 and 4G12, and the second one (55-7) missing the 39 kDa protein and showing an abundant 34 kDa protein.

IP tests were performed with these 2 clones using natural positive and negative ovine sera or pure broth as control. Growth inhibition was assessed in solid and in broth media.

After 3 days of culture, preliminary results showed that a clear growth inhibition (GI) appeared when clones were propagated with positive ovine sera *versus* control. This GI was affected by initial cultures titers : sera effect was more

pronounced for low initial titers. An interesting difference was demonstrated between 55-5 and 55-7 clones, as the growth of the first one was significantly more inhibited by ovine sera, whatever initial culture titer.
Western blot analysis of resulting populations are in progress.

CONCLUSIONS

• Important differences in specific epitope conservation were demonstrated by colony immunoblot assays with MAbs, certain field strains lacking some surface components. Furthermore, this partial link with geographic origin of *M. agalactiae* strains seems to be confirmed when considering the different Western blot patterns obtained with MAbs 3B3 and 4G12.

• Large changes in growth and/or Western blot profiles of predominant surface proteins of *M. agalactiae* PG2 strain were induced after multiplication in liquid media containing different sera. These results showed that certain external highly immunogenic proteins, bearing specific epitopes, are responsible for important *in vitro* phenotypic diversification, either by phase variations whithin different proteins carrying the target epitopes, or by direct neutralisation of the phenotype expressing it. Whether differences in the induced modifications are related to clonal variants is currently under inverstigation.

Analysis of genetic mechanisms responsible for this putative host avoidance could help to understand how this wall-less organism can adopt such a 'parasite' strategy during infection.

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ANALYSIS OF THE CAPSULAR POLYSACCHARIDE OF *MYCOPLASMA MYCOIDES* SUBSP. *MYCOIDES* SC, THE CAUSAL AGENT OF CBPP: PURIFICATION, COMPOSITION AND ITS ROLE IN INFECTION AND IMMUNITY

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INTRODUCTION

The capsular polysaccharide (CPS) of *Mycoplasma mycoides* subsp. *mycoides* small colony variant (*Mmm*SC) has long been considered to play a part in the pathogenesis of contagious bovine pleuropneumonia (CBPP), possibly through an immunopathological component (Gourlay & Shifrine 1966, Kakoma *et al.* 1973). Infection studies in cattle have suggested that the more virulent strains of *Mmm*SC produce more CPS (Lloyd *et al.* 1971), while injection of CPS itself into cattle often produces pathological consequences, and can increase the severity of the disease condition (Hudson *et al.* 1967). We have purified CPS from *Mmm*SC and undertaken a compositional and structural analysis. We have investigated the yield of CPS from a variety of field, type and vaccinal isolates, and investigated the role of CPS in both virulence and protective immunity using growth inhibition tests.

MATERIALS AND METHODS

Mycoplasma strains. Afade, V5 and Gladysdale obtained from R. Nicholas. $T_1$44 and T_1SR from the Botswana Vaccine Industry. N6 and M375, Botswana field isolates (W. Amanfu). Tan1 and Tan8, Tanzanian field isolates (B. Lema). All mycoplasma strains were grown in Gourlay's medium (broth or agar, Gourlay 1964).

Antisera. Rabbit hyperimmune sera- two injections of inactivated mycoplasma in adjuvant were given, followed by one intravenous injection of an aqueous suspension. Two rabbits were used for each strain. Pre-absorption with capsular polysaccharide (CPS): 10ug of CPS was added to 0.1ml serum, clarified by centrifugation, then repeated until the CPS antibody titre dropped to background levels (measured using a CPS ELISA). CPS ELISA: 1ug per well of pure CPS was incubated overnight in PBS using an irradiated microtitre plate (Greiner). Wells were blocked with 5% dried skim milk in PBST, and primary and secondary antibody incubations were performed for 2 hours. Growth inhibition tests were performed by spotting 20ul of undiluted serum onto 5mm diameter filter paper discs, placing the discs onto suitable dilutions of mycoplasma cultures and incubating at 37°C for 2-4 days prior to measuring the zone of inhibition under a light microscope.

Purification of CPS. Mycoplasma were removed from the medium by centrifugation. The medium was adjusted to pH 5.0, heated to 100°C for 30 minutes, adjusted back to pH 7.5, filtered through Whatmann 3MM paper and then precipitated with 2 volumes of ethanol. The precipitate was resuspended in 0.1 volume of dH_2O, RNAase and DNAase were added, left for 24 hours at 37°C, then SDS was added to 0.5% followed by proteinase K. This was left for 24 hours at 45°C, then dialysed against running tap water for 3 days using a 15kD molecular weight cut off membrane. The dialysate was then extracted once with phenol/chloroform, once with chloroform, ethanol precipitated, rinsed in 80% ethanol, resuspended in dH_2O and dialysed overnight. Material at this stage of purity

was used for CPS comparative studies (ELISA and CPS yield measurements). No protein could be detected, although some low molecular weight material could be observed following silver staining of SDS-PAGE gels. This material absorbed strongly at OD_{240} but did not cross react with *Mmm*SC antisera. For structural and compositional analyses, CPS from Gladysdale strain was further purified by gel filtration twice through a Sephacryl S-300 column. CPS came of at the start of the run, immediately after the void volume. The amount of CPS present was measured using the phenol/sulphuric acid method.

Analysis of CPS. Purified CPS was hydrolysed in 1.0M methanolic HCl at 80°C for 16 hours. After removal of reagents, trimethylsilyation was carried out in 200ul Tri-sil 2 reagent (Pierce) at 20°C for 30 minutes. Trimethylsilyl methylglycosides were analysed by GC-MS which was performed on a Fisons MD800 instrument with a DB-5 fused silica capillary column (30 minutes x .32mm, J & W Scientific). The temperature program was 65°C for 1 min, then to 140°C at 25°C/min, to 200°C at 5°C/min, to 300°C at 10°C/min. The derivatised sugars were then detected by electron impact mass spectrometry.

RESULTS

Purification and structure of the capsular polysaccharide (CPS) of *Mmm*SC. A considerable quantity of CPS is shed into the medium during growth and provides a convenient source of raw material for purification. The apparent molecular weight of CPS is extremely large, with the majority of the material remaining in the well of a 5% stacking gel following SDS-PAGE (visualised with Schiff's stain). It is eluted in the primary fractions following chromatography on Sephacryl S-300, immediately after the void volume (data not shown). Absorption of pure CPS in solution is minimal at all wavelengths, although a small amount of scattering is seen between OD_{200}-OD_{220}. The material appears stable in the pH range 4.0-10.5, with no apparent degradation over a 48 hour period (as measured following SDS-PAGE), or loss of immunogenicity (signal following Western blotting or ELISA). Electron impact mass spectrometry indicated the presence of fucose, mannose, galactose, glucose, galactosamine and glucosamine in an approximate ratio of 1:2:4:1:4:8. Preliminary Fast Atom Bombardment mass spectrometric experiments on acetolysed material suggest the aminosugars are N-acetylated in the intact polymer.

Variation in amount of CPS between strains of *Mmm*SC and correlation with sensitivity to growth inhibition (GI). For comparative studies of CPS production cultures were grown to stationary phase in Gourlay's medium (Gourlay 1964) and simultaneous and identical CPS purification regimes were followed for each strain. This was performed three times, and the averaged yield figures (normalised to strain M375) are shown in Table 1.

Clear differences can be seen in the yield of medium-derived CPS between strains. Strain M375 produces considerably less CPS; this can also be seen following Schiff's staining of SDS-PAGE gels of whole cell extracts where an equal loading density between strains is observed (as measured using Pierce's BCA protein assay; data not shown). Interestingly, a reasonably good inverse correlation exists between the sensitivity of each strain to GI and the amount of CPS produced by that strain (row 3). This would suggest that protective antibodies (as measured using the GI test) are directed against CPS epitopes, with those strains producing the most CPS being the least sensitive to inhibition.

Table 1 Correlation between CPS production and GI sensitivity of 9 strains of *Mmm*SC

Strain	Tan8	$T_1$44	Gladys	T_1SR	V5	N6	Afade	Tan1	M375
CPS yield	5.8	4.8	4.8	4.1	3.9	3.8	2.7	2.3	1
GI zone	37.4	16.1	24.0	19.3	20.1	30.0	19.3	37.6	47.7

Legend. The 9 strains of *Mmm*SC are shown from left to right in decreasing order of CPS yield. The GI sensitivity of each strain is shown below. This figure represents the cumulative zone of clearance (mm) from the sum of 54 different GI tests performed on that strain; each of the 18 rabbit hyperimmune serum samples (2 for each of the 9 strains of *Mmm*SC) repeated 3 times with the zones of clearance summed. It can be seen that the GI susceptibilities generally increase as the CPS yield decreases (reading from left to right); figures for the two strains which are exceptions to this are shown highlighted. CPS was purified from 50ml of spent culture medium for each strain on three separate occasions, and the results averaged and normalised to M375 = 1.

Correlation between CPS antibody titre and GI activity of rabbit hyperimmune sera. Rabbit hyperimmune sera raised against different strains of *Mmm*SC varied considerably in their effectiveness in GI tests. This was sometimes apparent even when comparing 2 serum samples raised against the same strain (Table 2). When the CPS antibody titre of sera were compared, a strong correlation was observed between GI activity and CPS antibody titre, suggesting that protective antibodies in the serum were directed at CPS. This finding was confirmed by pre-absorption of rabbit hyperimmune serum with pure CPS; all GI activity was then lost. Pre-absorption of bovine convalescent serum with pure CPS also resulted in a reduction or a complete loss of GI activity of the serum (dependent upon the strain used in the GI test), suggesting that CPS-antibodies may have a role in humoral immunity during a natural infection (March *et al.*, this volume).

Table 2. Correlation between CPS antibody titre and GI activity of *Mmm*SC rabbit hyperimmune sera

	1	2	3	4	5	6	7	8	9
Sera against	N6	T_1SR	N6	Tan8	Tan8	$T_1$44	V5	Afade	$T_1$44
CPS Ab. Titre	2.8	2.7	2.7	2.7	1.2	1.1	0.84	0.74	0.67
GI activity	21.6	28.5	34.1	13.3	1	15.2	8.6	0.6	3.2

	10	11	12	13	14	15	16	17	
Sera against	T_1SR	Tan1	Gladys	Gladys	Afade	V5	Tan1	M375	M375
CPS Ab. Titre	0.66	0.48	0.40	0.38	0.26	0.23	0.19	0.18	0.18
GI activity	6	2	0	0.5	0	0	0	0	0

Legend. Table 2 is split into 2 levels. The 18 different rabbit antisera (2 per strain of *Mmm*SC) are shown in decreasing order (1-18) of CPS antibody titre (measured using a 9 strain CPS mix ELISA). The GI activity of each antisera is shown below. This figure represents the cumulative zone of clearance (mm) for each individual serum sample, tested against each of the 9 strains of *Mmm*SC on 3 different occasions. A good correlation between CPS antibody titre and GI activity of the serum is apparent, apart from the 2 highlighted samples.

Conservation of CPS between strains. From the data presented above it would appear that GI activity for rabbit hyperimmune sera is a factor of the CPS antibody titre of the serum set against the CPS yield of the strain under test. This is most clearly shown in Table 3. The same GI profile can be seen for antisera raised against two different strains. Those strains most sensitive to one serum (e.g the low CPS-producer M375) are also most sensitive to the other. Thus homologous antiserum is not more effective, as would be expected if CPS was strain-specific. This would suggest a degree of conservation between CPS of different strains. From

ELISA studies using polyclonal antisera and purified CPS of the 9 strains we have observed minimal differences in the signal between strains, in agreement with this premise (data not shown).

Table 3. Comparison of GI profiles of antisera raised against 2 different strains of *Mmm*SC

Sera against	CPS titre	Zone of GI (mm) against test strain								
		Afade	Gladys	V5	$T_1$44	T_1SR	N6	M375	Tan1	Tan8
V5	0.84	0.5	0.5	0.5	0	0	0.5	1.5	1	1
T_1SR	2.7	1	1.5	1.5	1.2	1	1.5	2.7	2	2

Legend. Antisera raised against T_1SR or V5 tested against 9 different strains of *Mmm*SC. The CPS antibody titre is also shown. Serum with high CPS-antibody titre shows the most GI activity. Low CPS-producing strain (M375) is most susceptible to GI with both serum samples, rather than the homologous strains (V5 or T_1SR; shaded). Note that the GI profile of each antiserum is similar across the 9 strains tested.

CONCLUSIONS

The capsular polysaccharide (CPS) of *Mmm*SC, causal agent of CBPP has been purified. CPS has an apparent molecular weight of several hundred thousand kilodaltons, and has a relatively complex composition for a polysaccharide, being comprised of mannose, glucose, galactose, fucose, glucosamine and galactosamine sugars. The aminosugars appear to be acetylated in the intact polymer.

The amount of extracellular CPS produced by different strains of *Mmm*SC varies over a sixfold range. The reason for this variation is unclear, but the stability of the trait suggests a variation in the CPS operon(s) copy number between strains as a likely cause. Strains producing the most CPS are the least sensitive to growth inhibition (GI) using both rabbit hyperimmune and bovine convalescent antisera, strongly implicating CPS as a virulence or potentiating factor. The effectiveness of rabbit hyperimmune sera in GI tests correlates strongly with the CPS antibody titre of the sera. Pre-absorption of protective sera with CPS either completely removes or severely attenuates its GI activity (both rabbit hyperimmune and bovine convalescent), suggesting a role for CPS in humoral immunity.

Polyclonal antisera raised against CPS is cross reactive across all strains of *Mmm*SC so far tested, suggesting a high degree of interstrain conservation. This conservation, in conjunction with the relatively complex composition of CPS and its importance in virulence and humoral immunity, suggests CPS may have potential as a diagnostic reagent or vaccine, while a CPS operon probe could be a useful marker for virulence.

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GENETIC AND IMMUNOGENIC CHARACTERIZATION OF A 62 KDA LIPOPROTEIN (P62) IN *MYCOPLASMA MYCOIDES* SUBSP. *MYCOIDES* LC AND *MYCOPLASMA MYCOIDES* SUBSP. *CAPRI*

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INTRODUCTION

Mycoplasma mycoides subsp. *mycoides* LC and *Mycoplasma mycoides* subsp. *capri* are 2 *Mycoplasmas* belonging to the *Mycoplasma mycoides* cluster, a taxonomic group of 6 phylogenetically and serologically closely related pathogenic *Mycoplasmas* (5). The *M. mycoides* cluster includes as highly virulent species *Mycoplasma mycoides* subsp. *mycoides* SC and *Mycoplasma capricolum* subsp. *capripneumoniae* which are the agents of contagious bovine pleuropneumonia (CBPP) and contagious caprine pleuropneumonia (CCPP), respectively, which are very important pathogens with epidemiological and economic significance. *Mycoplasma mycoides* subsp. *mycoides* LC and *Mycoplasma mycoides* subsp. *capri* are less pathogenic organisms which cause pleuropneumonia, arthritis and mastitis in goats (6;11). One- and two-dimensional SDS-PAGE protein patterns, DNA-DNA hybridization and phylogenetic studies based on sequence analysis of 16S rRNA genes have shown the very close relatedness between both species (1;4;8;9;12). A serological immunofluorescence test has been proposed for their differentiation, but it is hampered by cross-reactivity among several strains of both species (8).
In the present study, we identified and characterized the gene for a lipoprotein of 62 kDa, named P62, from *M. mycoides* subsp. *mycoides* LC and *M. mycoides* subsp. *capri*. P62 was found to be an analogue to the major surface lipoproteins P72 and P67 characterized in *M. mycoides* subsp. *mycoides* SC and *Mycoplasma* species bovine group 7, respectively (3;7). The cloned gene encoding P62 was expressed in *E. coli*. Antibodies to recombinant P62 were used to assess the presence and antigenic specificity of P62 to *M. mycoides* subsp. *mycoides* LC and *M. mycoides* subsp. *capri*.

MATERIALS AND METHODS

Mycoplasma strains used in this study were: *M. mycoides* subsp. *mycoides* SC (PG1, Afadé and L2), *M.* sp. bovine group7 (PG50), *M. capricolum* subsp. *capricolum* (California kid), *M. capricolum* subsp. *capripneumoniae* (F38), *M. mycoides* subsp. *mycoides* LC (Y-goat; Swiss strains D 2503, D 2482/91 and D 2083/91; Portugese strain B 671/93; Spanish strains 266/94, 6P, 2/93, 152/93, 80 X 3, 83/93, 153/93 received from J. B. Poveda, Las Palmas; CP271, LC8065, 9096-C9415, 8756-13 and 8794-Inde received from F. Thiaucourt) and *M. mycoides* subsp. *capri* (PG3; N 108, capri L and 9139/11/91 received from F. Thiaucourt).

subsp. *capri* (PG3; N 108, capri L and 9139/11/91 received from F. Thiaucourt). They were cultured as described (2). Genomic DNA extraction was done by lysis with guanidinium thiocyanate (10). Genes were cloned using pBK-CMV plasmid propagated in strain XLOLR. P62 gene of *M. mycoides* subsp. *mycoides* LC was expressed in *E. coli* UGA_{Trp} suppressor strain YN2980 (13), a system developed for the expression of mycoplasmal proteins. Production of polyclonal anti-P62 serum was done by immunizing mice with the recombinant P62. Immunoblots were performed with anti-P62 serum adsorbed with *E. coli* strain YN2980 carrying the empty cloning vector pBK-CMV. Whole-cell antigens were separated electrophoretically by SDS-PAGE in 5-15% polyacrylamide gradient gels. A PCR assay was developed using primers MMMLC2-L CAATCCAGATCATAAAAAACCT and MMMLC1-R CTCCTCATATTCCCCTAGAA (annealing temperature 49°C) matching the ends of P62 gene. Amplification products obtained were analyzed on 0.7 % agarose gel.

RESULTS

The genes encoding P62 lipoproteins were cloned from strains *M. mycoides* subsp. *mycoides* LC and *M. mycoides* subsp. *capri*. They were subsequently sequenced and analyzed by comparison. Results obtained showed similarities of 97% identical nucleotides and 95% identical amino acid residues between these two subspecies, indicating that the P62 proteins and their genes in the two species are virtually the same. Analysis of potential signal sequences on P62 indicated a consensus sequence for a potential recognition site of a prokaryotic signal peptidase II with a lipid attachment site at the N-terminal part of the lipoprotein. This site is preceded by a putative transmembrane region.

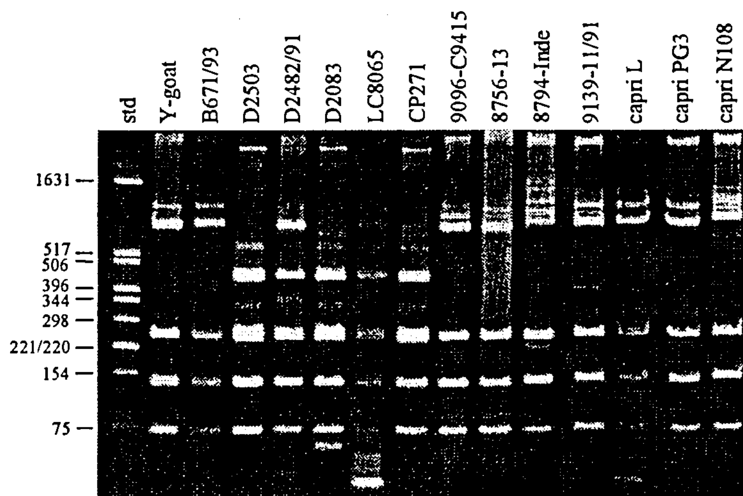

Figure 1
Restriction fragment analysis of *M. mycoides* subsp. *mycoides* LC and *M. mycoides* subsp. *capri* strains. P62 specific PCR products of DNA from type strains and field strains were digested with *AluI*. These digests were electrophoresed on an 8% polyacrylamide gel. The standards used were *HinfI*-digested pBR322 fragments indicated in base pairs.

From the sequence data, we developed a specific PCR method common to the P62 gene of both *M. mycoides* subsp. *mycoides* LC and *M. mycoides* subsp. *capri* by designing primers matching both ends of P62 lipoprotein gene. Genomic DNA from type- and reference strains of the different members of the *M. mycoides* cluster, as well as from field strains, was submitted as a template to this PCR assay. All *M. mycoides* subsp. *mycoides* LC and *M. mycoides* subsp. *capri* strains tested amplified a 1kb-fragment. No amplification product was detected in the other species of the *M. mycoides* cluster. The products obtained were digested with *Alu*I restriction enzyme and subsequently examined on an 8% polyacrylamide gel (Fig. 1). Profiles obtained showed a certain heterogeneity among the different strains analyzed, but did not cluster *M. mycoides* subsp. *mycoides* LC and *M. mycoides* subsp. *capri* strains.

In order to study the expression of P62 in the type strains and different field strains of *M. mycoides* subsp. *mycoides* LC and *M. mycoides* subsp. *capri*, and to study the antigenic specificity of P62 to these two species, we have produced polyclonal mouse antiserum directed against recombinant P62 expressed in an *E. coli* UGA_{Trp} suppressor strain (13). These antibodies were reacted with immunoblots containing whole cell proteins from the type- and reference strains of the *Mycoplasmas* of the *M. mycoides* cluster and from field strains of *M. mycoides* subsp. *mycoides* LC and *M. mycoides* subsp. *capri*. The immunoblots showed that anti-P62 antibodies specifically reacted with a 62 kDa protein band in all *M. mycoides* subsp. *mycoides* LC and *M. mycoides* subsp. *capri* strains tested, but not with the other *Mycoplasmas* of the *M. mycoides* cluster.

CONCLUSIONS

M. mycoides subsp. *mycoides* LC and *M. mycoides* subsp. *capri* were shown to contain a common gene encoding a lipoprotein of 62 kDa, P62. Similarity analysis revealed that P62 belongs to the family of lipoproteins which includes P72 of *M. mycoides* subsp. *mycoides* SC and P67 of *Mycoplasma* sp. bovine group 7.

Immunoblots performed with antibodies raised against recombinant P62 reacted selectively with a 62 kDa protein band in both *M. mycoides* subsp. *mycoides* LC and *M. mycoides* subsp. *capri*, but not with the other members of the *M. mycoides* cluster. Immunoblot analyses further showed the presence of this lipoprotein in all *M. mycoides* subsp. *mycoides* LC and *M. mycoides* subsp. *capri* field strains tested, thus revealing that P62 is a specific and common antigen to these two *Mycoplasmas*.

The specific PCR assay developed in this study allowed the selective amplification of P62 gene in *M. mycoides* subsp. *mycoides* LC and *M. mycoides* subsp. *capri*. We suggest the application of this PCR assay as a genetic tool in the identification of these subspecies and their differentiation from the other members of the *M. mycoides* cluster. The serological method described above could also be useful for their immunogenic identification and for the development of a specific serological test.

Our serological and genetic results show that the major lipoprotein P62, respectively its gene is common and specific to *M. mycoides* subsp. *mycoides* LC and *M. mycoides* subsp. *capri*. In addition, it had been shown previously that the genes encoding 16S rRNA (*rrs*) are practically the same in these two Mycoplasmas and do not allow their phylogenetic differentiation (9). These results indicate that *M.*

mycoides subsp. *mycoides* LC and *M. mycoides* subsp. *capri* could be regarded as the same taxonomic entity.

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DEVELOPMENT OF COLOURED *MYCOPLASMA MYCOIDES* COLONIES ON SOLID MEDIA AS AN AID TO RAPID DIAGNOSIS

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INTRODUCTION

Mycoplasma mycoides subspecies *mycoides* SC (*Mmm*SC), the cause of contagious bovine pleuropneumonia (CBPP), may be isolated from a variety of post mortem tissues of affected bovines. Infected organs may include lung, lymph nodes, liver, kidney, and organisms may be found in blood, pleural fluid and urine (1). Generally, lung tissue or sequestered lesion material is submitted to the laboratory for further investigations which involve mycoplasma culture and identification with specialised media and biochemical reactions. Bacteriological investigations are necessary for the confirmation of disease and for the declaration of outbreaks. This procedure often requires several weeks of laboratory time for satisfactory results and may necessitate ultrafiltration before passages when the culture is contaminated with bacteria (2). *Mmm*SC must be isolated and identified from samples which may contain other pathogenic mycoplasmas such as *M. bovis*, *M. dispar*, and *Ureaplasma diversum* (3). A diagnostic solid medium is presented here which may aid the primary isolation of the causative agent of CBPP because it is able to support rapid growth of coloured *M. mycoides* colonies.

MATERIALS AND METHODS

M. mycoides strains from a variety of geographical areas were tested. *Mmm*SC strains used as reference were Afadé, Gladysdale, V5, 57/13 and PG1; and *Mmm*LC *was* represented by strain Y goat and F30. Strains were maintained in ME liquid medium. Post mortem clinical material from pathologically positive bovines collected from an outbreak of CBPP in Portugal were also tested (4). Samples from lung lesions were taken from 20 animals and stored at –70°C. Pieces of defrosted tissue about 0.5 cm^2 were smeared onto the surface of the CBPP Diagnostic media plates and then removed. Smears were streaked onto the remainder of the agar with sterile disposable plastic loops. Reference strains, from liquid cultures, were plated onto the solid medium in a similar way. Plates were incubated at 37°C in an atmosphere generated by Anaero*Gen* sachets (Oxoid, Hampshire, UK) and observed after 3 and 7 days. Coloured colonies were taken from the agar and inoculated directly into Biochemical test media for the determination of glucose catabolism, arginine hydrolysis, tetrazolium reduction, and phosphatase activity. ME liquid medium, CBPP Diagnostic medium and Biochemical test media were provided by Mycoplasma Experience (Reigate, UK). Molecular tests included DNA

amplification by polymerase chain reaction (PCR) as described in the *pleuro*TRAP kit (AMRAD, Melbourne, Australia). Organisms from coloured colonies were sampled with plastic lances and placed into PCR tubes containing the reaction mixture. Amplified DNA was further analysed with restriction enzyme digestion with *Asn*1 as described previously (5).

RESULTS

The production of characteristic red colonies on the surface of the solid agar medium within 3 days of anaerobic incubation at 37°C was observed with all the reference strains of *Mmm*SC and *Mmm*LC used in this study. *Mmm*SC produced red colonies which were visible after the third day of incubation (Figure 1). The colour of these colonies intensified in 7 days to dark red which were easily visible to the naked eye and developed extensive crystalline deposits which radiated from the centres (Figure 2). Biochemical tests on coloured colonies taken from plates showed typical reactions for *Mmm*SC and *Mmm*LC (Table 1).

Lung tissue from pathologically positive bovines which were smeared on the medium produced positive colonies in 32% of the cases. Positive smears were evident from the colour of the colonies and several agar plates are shown in Figure 3. Microscopically, the colonies from tissue resembled those from pure cultures. In these samples colourless colonies were not observed. PCR tests performed directly on coloured colonies taken from the solid medium produced amplification products which were specifically identified as *Mmm*SC.

CONCLUSIONS

The coloured crystalline product resulting from the growth of *M. mycoides* colonies did not interfere with further biochemical identification nor was it inhibitory for the PCR. Thus, the medium represents a considerable time saving in the primary isolation and complete diagnosis of *Mmm*SC from clinical material, because it may directly precede standard and molecular systems for mycoplasma identification. Further studies involving other mycoplasma species occuring in bovine respiratory tract may indicate that this, or a similar medium, would allow presumptive identification of *Mmm*SC directly from clinical material.

ACKNOWLEDGEMENTS

We wish to thank Luciano Duarte of Direcção Regional de Agricultura de Entre-Douro e Minho, Braga, Portugal for his kind assistance in making this herd available to us which was sampled as part of the work for EU contract FAIR1-CT95-0711.

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Table 1. Biochemical reactions of *Mmm*SC and *Mmm*LC taken from CBPP Diagnostic medium. The production of acid was indicated by a yellow colour of the medium. Numbers specify the day on which the reaction was observed. Weak (±) and negative (–) reactions are indicated.

	*Mmm*SC	*Mmm*LC
Arginine hydrolysis	acid	acid
Glucose utilisation	acid	acid
Phosphatase activity	7 ±	3
TTC reduction	–	2

Figure 1

A culture of *Mmm*SC strain Afadé was inoculated onto CBPP Diagnostic medium plates at a density of about 600 cfu/ml. Growth after 3 days of incubation in an anearobic environent may be seen as coloured colonies 1-2 mm in diameter.

Figure 2

A culture of *Mmm*SC strain PG1, after incubation for 7 days, showing the intensification of the colour in the colonies. The presence of red crystals radiating from some colonies may be seen.

Figure 3

Plates onto which lung samples were smeared and streaked showing coloures colonies. Incubation was for 7 days at 37°C, anaerobically.

Figure 1

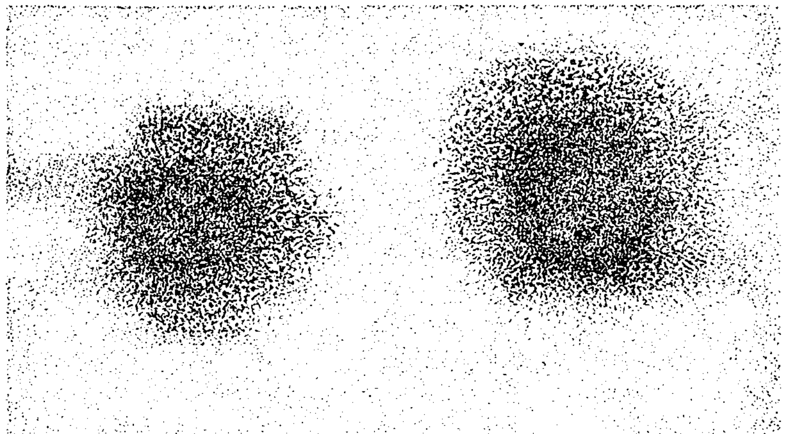

Figure 2

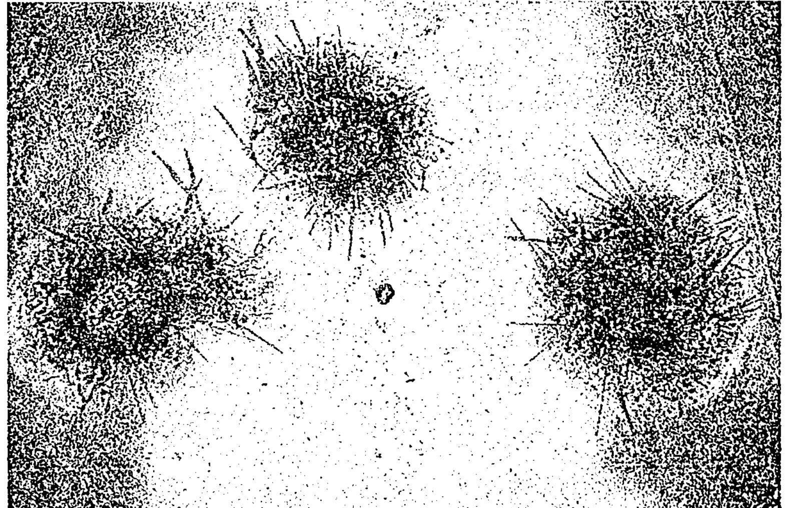

Figure 3

TEMPERATURE TOLERANCE OF MYCOPLASMAS IN THE *MYCOPLASMA MYCOIDES* CLUSTER

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INTRODUCTION

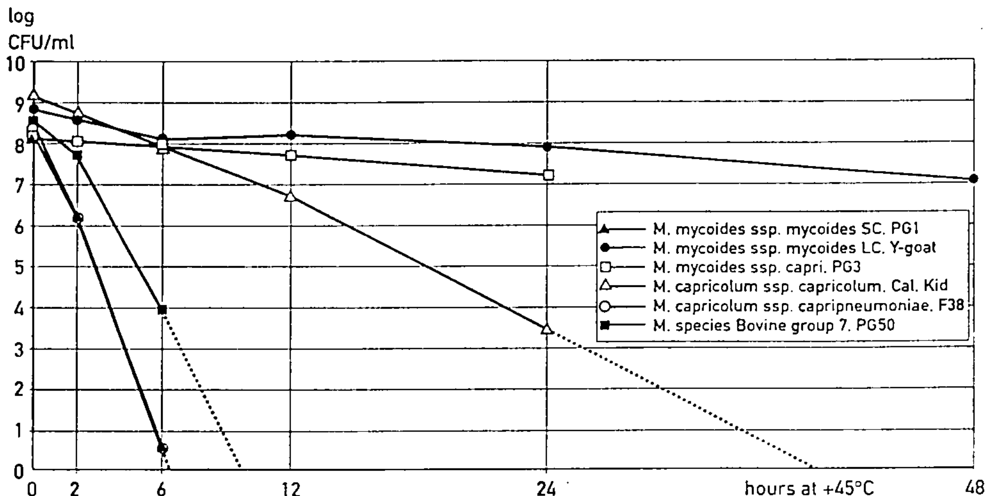
Determination of survival at +45°C is a classical test to distinguish the two types of *Mycoplasma mycoides* subsp. *mycoides*. The SC type dies within 12 h, but the LC type survives more than 24 h at +45°C (1). The other mycoplasmas in the *M. mycoides* cluster also differ in their sensitivity to +45°C (2,3). We have now investigated several strains from each group belonging to the *M. mycoides* cluster.

MATERIAL AND METHODS

Strains: *M. mycoides* subsp. *mycoides* LC (5 strains), *M. mycoides* subsp. *mycoides* SC (15 strains), *M. capricolum* subsp. *capripneumoniae* (12 strains), *M. capricolum* subsp. *capricolum* (11 strains, Table 1), *Mycoplasma* sp. bovine group 7, (8 strains, Table 2) and *M. mycoides* subsp. *capri* (3 strains).

A log phase culture of each strain was subcultured to fresh medium and placed in a water bath holding +45°C ±0.5°C. CFU was determined before the start, after 2 h, 6 h, 12 h, 24 h and 48 h incubation time.

FIG 1.



RESULTS

Viable counts for the type strains have been plotted against time of exposure to +45°C (Fig 1). These strains do well represent the temperature tolerance features of the different mycoplasmas in the *M. mycoides* cluster.

Strains of *M. mycoides* ssp. *mycoides* SC died after about 7 h exposure to +45°C and *M. capricolum* ssp. *capripneumoniae* died after about 6 h exposure. Strains of *Mycoplasma* sp. bovine group 7 survived around 9 h (Table 2) and *M. capricolum* ssp. *capricolum* strains survived around 40 h (Table 1). *M. mycoides* ssp. *mycoides* LC and *M. mycoides* ssp. *capri* survived more than 48 h with little reduction in CFU titre.

TABLE 1. Survival at +45°C for strains of *Mycoplasma capricolum* ssp. *capricolum*.

Strain	*Origin, host*	*Supplied by*	*Survival time, hours*
Cal. Kid	USA, goat	WHO/FAO	39
GM262G	USA, goat	A.J.D.	32
7714	France, goat	F.T.	40
4214	Israel, goat	S.L.	46
GM13	USA, goat	A.J.D.	39
2712/77	Australia, goat	J.G.T.	47
74/3220	Australia, goat	J.G.T.	47
B304	Portugal, cattle	J.R.	25
M153/81	Sweden, goat	SVA	48
M144	Sweden, goat	SVA	48
M4528/76	Zimbabwe, sheep	J.G.T.	24

WHO/FAO: WHO/FAO Collaborative Centre for Animal Mycoplasmas, Aarhus, Denmark.
A.J.D.: A.J. DaMassa, Davies, Calif., USA. S.L.: S. Levisohn, Bet Dagan, Israel
F.T.: F. Thiaucourt, CIRAD/EMVT, Montpellier, France
J.G.T.: J.G. Tully, Bethesda, Md., USA. J.R.: J. Regalla, Lisbon, Portugal
SVA: National Veterinary Institute, Uppsala, Sweden

TABLE 2. Survival at +45°C for strains of *Mycoplasma* sp. bovine group 7

Strain	*Origin, host*	*Supplied by*	*Survival time, hours*
PG50	- , cattle	WHO/FAO	10
QR1	Australia, cattle,	D.G. P.	8.2
Calf I	Nigeria, cattle	H. E.	12
D318b	Germany, cattle	H. E.	12
C2306	Portugal, cattle	H. E.	7.2
D424	Germany, cattle	H. E.	13
4055	- , cattle	D.G. P.	6.0
CP 291	Portugal, goat	M. M.	7.2

D.G. P.: D.G. Pitcher, London, England
H. E.: H. Ernö, Aarhus, Denmark
M. M.: M. Machado, Porto, Portugal

TABLE 3. Survival time at +45°C for the different species/subspecies of the *M. mycoides* cluster.

	No. of strains	*Mean survival time, h.*	*Range survival time, h.*
M. mycoides ssp. *mycoides* SC	15	7.3	4.0 - 13
M. mycoides ssp. *mycoides* LC	5	> 48	> 48
M. mycoides ssp. *capri*	3	> 48	> 48
Mycoplasma sp. bovine group 7	8	9.5	6.0 - 13
M. capricolum ssp. *capricolum*	11	40	24 - 48
M. capricolum ssp. *capripneumoniae*	12	≈ 6	- 7

CONCLUSIONS

The differences in temperature tolerance can be used to differentiate *M. capricolum* ssp. *capripneumoniae* from *M. capricolum* ssp. *capricolum* and most strains of *Mycoplasma* sp. bovine group 7.

M. mycoides ssp. *mycoides* LC and *M. mycoides* ssp. *capri* can not be differentiated with the test for survival at +45°C.

ACKNOWLEDGEMENTS

This work has been financially supported from the Commission of the EU (DG XII) for "Contagious Caprine Pleuropneumonia: distribution, evaluation of vaccines, and development of new tools" (contract IC18-CT95-0007).

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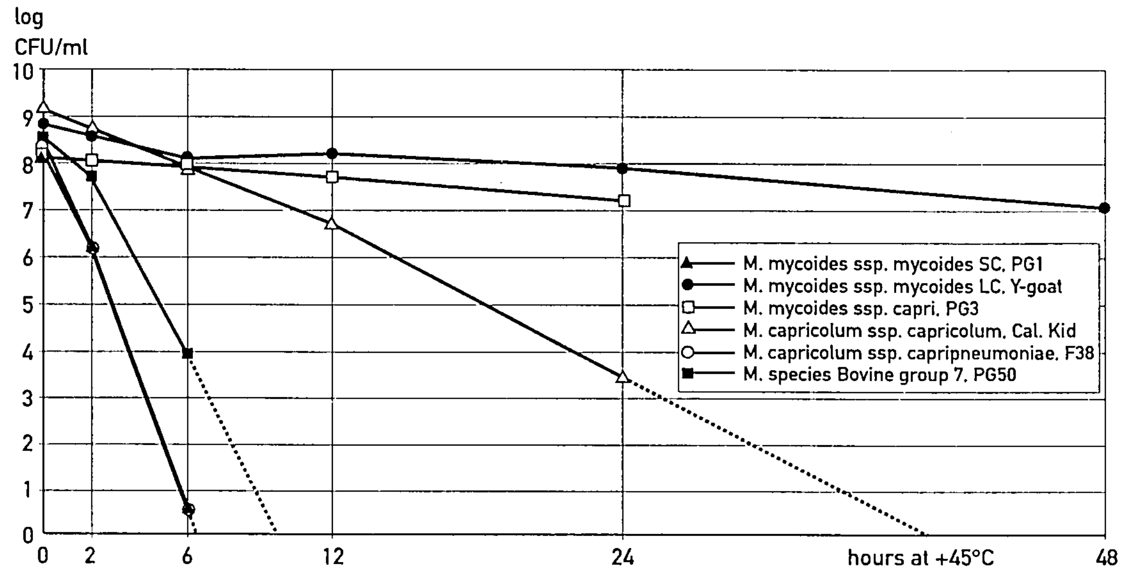
log
CFU/ml
10
9
8
7
6
5
4
3
2
1
0
0
2
6
12
24
hours at +45°C
48
M. mycoides ssp. mycoides SC, PG1
M. mycoides ssp. mycoides LC, Y-goat
M. mycoides ssp. capri, PG3
M. capricolum ssp. capricolum, Cal. Kid
M. capricolum ssp. capripneumoniae, F38
M. species Bovine group 7, PG50

HUMORAL IMMUNE RESPONSE IN EWES EXPERIMENTALLY INFECTED WITH *MYCOPLASMA MYCOIDES* SUBSP. *MYCOIDES* SC ISOLATED FROM CATTLE AND SHEEP

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INTRODUCTION

In Portugal, contagious bovine pleuropneumonia (CBPP) is restricted to the north western and central western areas. In the central eastern region (Beira Interior), where CBPP has not been detected so far, *Mycoplasma mycoides* subsp. *mycoides* SC (*Mmm* SC) was isolated from sheep and goats with mastitis and pneumonia, respectively (1). Based on this observation, experimental infections were carried out in sheep. The present study aims to get a better knowledge of the IgG immune response in this species, after endobronchial inoculation of either a *Mmm* SC CBPP strain or an ovine strain.

MATERIALS AND METHODS

Animals - The animals used were adult ewes, randomly separated into three groups and placed in separate pens as follows: Experiment 1 - Control group (n=10); Experiment 2 - Inoculated and contact group with B421 isolate (CBPP strain) (n=10); Experiment 3 - Inoculated and contact group with O526 isolate (ovine strain) (n=16). Venous blood was collected weekly, for 140 days, to monitor anti- *Mmm* SC antibody response by complement fixation (CFT) (2) and immunoblotting.

Strains and inocula - The *Mmm* SC strains used were: B421 isolate from a CBPP lung and O526 isolate from sheep milk. They were grown in Gourlay medium at 37ºC until logaritmic growth phase and inoculated. The colony forming units (CFU) were calculated (3). For second inoculation the cultures were mixed with agar or harvested by centrifugation and washed in phosphate buffered saline (PBS), pH 7.2.
Control and contact ewes were inoculated endobronchially with 3 ml of the vehicle itself. In Experiment 2, five ewes were inoculated with 1.4×10^5 CFU ml^{-1} of the bovine isolate in Gourlay broth. At the second inoculation, three ewes received 3×10^8 CFU ml^{-1} mixed with 1.5‰ of agar. In Experiment 3, eight ewes were given 5.2×10^7 CFU ml^{-1} of the ovine isolate in Gourlay broth. Three of them received $3 \times x10^9$ $CFUml^{-1}$ mixed with 1.5‰ of agar ,66 days later, and two were given 2.7×10^9 CFU ml^{-1} in PBS.

Antigens, SDS-PAGE and Westernblotting - The production of antigens, separation of samples by SDS-PAGE and Westernblotting procedures were performed according methods previously described (4). Control group sera were tested with both antigens used (O526 or B421 isolates). Serum samples were tested with their homologous antigens in Western blots. Sera were diluted to 1:5 in PBS with 0.1% skim milk and 0.1% egg albumin. Positive control was performed with a CBPP serum. The strips were incubated with anti-sheep IgG (H+L chains) conjugated with horseradish peroxidase.

RESULTS

Control group animals (Experiment 1) showed nonspecific reactions, both to immunoblotting and CFT, such as a weak response for some of the bands considered immunogenic, and serologic titres up to 1:20 (Fig 1).

In the contact group of Experiment 2, one ewe did not respond to contact throughout the experiment. Another one was in "immunologic silence" until 42 days post-contact (p.c.) for some of the bands considered specific (110, 98, 95, 60/62 and 48 kDa) (Fig. 2).The IgG response to all of these bands appeared only on day 77 p.c.. In spite of showing nonspecific reactions before contact, the remaining three ewes, presented an increased humoral response for the above polypeptides, between days 49 and 91p.c., which persisted until the end of the study. Titres up to 1:20 appeared before and after contact.

In this assay, the IgG response was not observed in two contact sheep of Experiment 3. One ewe showed nonspecific reactions before contact, to bands at 110, 98,95,62 and 60, but not to 48KDa polypeptide. The remaining two sheep, in spite of showing nonspecific reactions before contact, presented an increased humoral response for the above polypeptides and others (59, 57.5, 55, 54, 39, 38, 37kDa), between days 21 and 77 p.c., which were maintained until the end of the experiment (Fig 3). CFT titres with values up to 1:20 appeared before and/or after contact.

In the inoculated ewes of Experiment 2, the strongest reaction was observed on day 14 post inoculation (p.i.) for two animals inoculated only once (Fig 4). For three animals who received two inoculations, the strongest reaction was obtained later (77 days after the first inoculation which corresponded to 11days after the second inoculation). CFT titres with values up to 1:40 appeared after inoculation. Only one ewe had a titre of 1:160 after the challenge (Fig 5).

In Experiment 3, in animals inoculated only once (n=6), the earliest response was observed on day 11 in three sheep (Fig 6), which corresponded to those animals receiving the highest dose of inoculum. For those ewes inoculated twice (n=5), the earliest response for some of the immunogenic bands was detected for day 28. However, the strongest response for most 110, 98, 95, 62, 60, 48, 39 and 38 bands occurred at day 77 post exposure (Fig 7). CFT titres with values up to 1:40 appeared before and/or after inoculation.

DISCUSSION AND CONCLUSIONS

The immunoblotting analysis was based on previous serological work performed on cattle naturally and experimentally infected with *Mmm* SC (5, 6, 7, 8). These studies indicated that in infected bovine, *Mmm* SC polypeptides 110, 98, 95, 80, 72, 62, 48 and 39 KDa are immunodominants. In the present study, since almost every ewe, control animals included, presented nonspecific bands considered immunogenic for cattle, these results can not be explained exactly as for CBPP. However, after inoculation or contact, some sheep showed an increase in the strength of some of those polypeptides, as well as others. These polypeptides, migth play some specific role in the humoral response in sheep. Taking into consideration the inoculum dosis, animals inoculated with the bovine isolate had an earlier humoral response, when compared to those given the ovine isolate. This might be due to virulence diversity between strains isolated from different animal species. When compared with strain Afadé, the European strain L2 caused a weaker and delayed reaction on contact infected cows (8). The presence of IgG humoral response by immunoblotting analysis in sheep in contact with inoculated ewes, suggests a possibility of infection by contact. In contrast, bovine infected with strain Afadé did not cause infection in contact goats and antibodies were not detected by CFT (9). Serologic titres considered positive for cattle were found in control sheep and in ewes before inoculation. In others, positive titres

became negative after inoculation and only one inoculated ewe presented high titres after the challenge. Therefore, CFT results indicated this technique lacks diagnostic value for sheep. However, our results are in disagreement with work on sheep, which presented titres up to 1:640, after intravenous inoculation with a sheep milk *Mmm* SC strain (10).

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ACKNOWLEDGEMENTS

This work was support by the grant PBIC/AGR/2319/95 from Fundação para a Ciência e Tecnologia, Ministério da Ciência e Tecnologia, Portugal.

The authors are thankful to Dr. José Regalla for encouragement in this investigation and revision of manuscript.

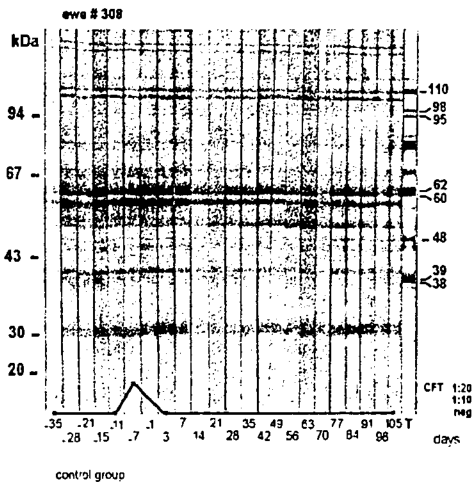

Fig 1

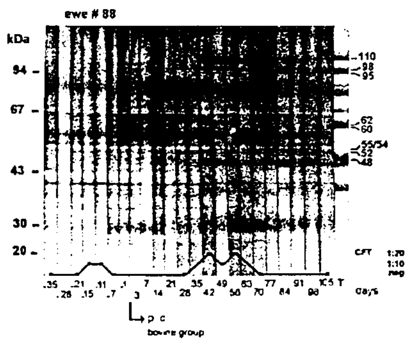

Fig 2

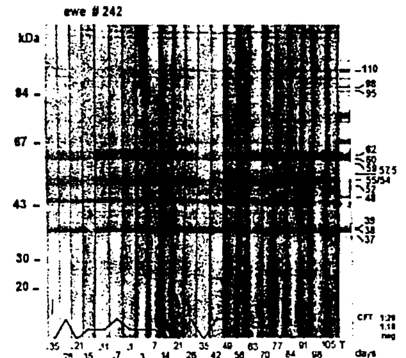

Fig 3

Legend of figures: Humoral immune response in control, contact and endobronchially inoculated ewes with *Mycoplasma mycoides* subsp. *mycoides* SC, CBPP strain (B421 isolate) and milk sheep strain (O526 isolate). The reactions of the serum samples taken sequentially every week are shown, from: Fig 1 - a control ewe (#308); Fig 2 - a contact ewe (#88) with others infected with B421 isolate; Fig 3 - a contact ewe (#242) with others infected with O526 isolate; Fig 4 - an ewe (#60) infected once with B421 isolate; Fig 5 - an ewe (#246) infected twice with B421 isolate; Fig 6 - an ewe (#136) infected once with O526 isolate; Fig 7 - an ewe (#175) infected twice with O526 isolate. Days post contact (p. c.) and post inoculation (p. i.) are given in the bottom. T- Positive control. The molecular masses of the major immunogenic antigens are given on the right. The CFT titres are given in graphics inserted in the pictures.

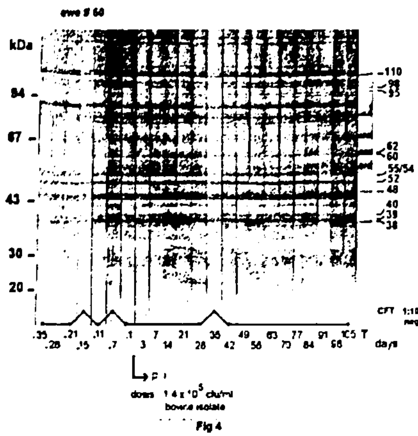

Fig 4

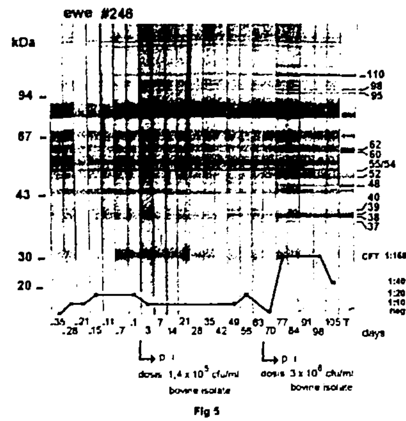

Fig 5

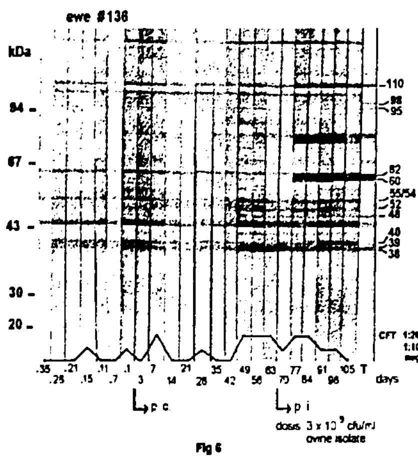

Fig 6

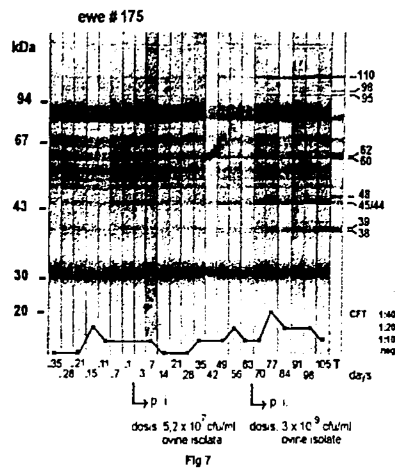

Fig 7

Legend of figures: Humoral immune response in control, contact and endobronchially inoculated ewes with *Mycoplasma mycoides* subsp. *mycoides* SC, CBPP strain (B421 isolate) and milk sheep strain (O526 isolate). The reactions of the serum samples taken sequentially every week are shown, from: Fig 1 - a control ewe; Fig 2 - a contact ewe with others infected with B421 isolate; Fig 3 - a contact ewe with others infected with O526 isolate; Fig 4 - an ewe infected once with B421 isolate; Fig 5 - an ewe infected twice with B421 isolate; Fig 6 - an ewe infected once with O526 isolate; Fig 7 - an ewe infected twice with O526 isolate. Days post contact (p. c.) and post inoculation (p. i.) are given in the bottom. The CF titres are given in graphics inserted in the pictures.

ISOLATION OF THE 60 KD-HEAT SHOCK PROTEIN OF *MYCOPLASMA ARTHRITIDIS* AND *MYCOPLASMA BOVIS* AND DETECTION OF HEAT SHOCK PROTEIN-SPECIFIC ANTIBODIES IN BOVINE SERA

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INTRODUCTION

The heat shock response is one of the most universal reactions known. Heat shock proteins (Hsps) are induced in prokaryotic and eukaryotic cells under various conditions of stress (sudden rise of temperature, changes of pH, starvation etc.). Hsps also play an important role in a number of physiological processes, e.g. folding and unfolding, assembly, repair and degradation of polypeptides. According to their apparent molecular weight Hsps are classified into different families (Hsp90-, Hsp70-, Hsp60-family, and the family of small Hsps) (1). Hsps of bacterial and mammalian species show a high degree of amino acid and nucleotide sequence homology (2). Despite their high conservation, proteins of the Hsp60- and Hsp70-family are immunodominant antigens in several bacterial and protozoal infections. They are discussed, on one hand, to be usefull as vaccines (3). Thus, in several experimental models of arthritis Hsp60 or Hsp60-specific T cells induced a protective immune response in rats (4, 5, 6). On the other hand Hsps are believed to account for autoimmune diseases (7, 8).
The immune response to Hsps of mycoplasmas and their possible effects on pathogenesis are not yet elucidated. The aim of this study is (i) to identify, isolate, and characterize Hsp60 of *Mycoplasma (M.) arthritidis* and *M. bovis*, (ii) to investigate the antibody response of cattle to this Hsp, and (iii) to investigate in vivo, whether Hsps induce protective immunity or autoimmune reactions in case of the *M. arthitidis*-polyarthritis in rats.

MATERIALS AND METHODS

Antisera directed against Hsp60

Antisera directed against Hsp60 of mycoplasmas were produced by immunization of rabbits with Hsp60 of *M. arthritidis* and *M. bovis*. In a first approach proteins of the whole cell lysate were separated by gel electrophoresis. Hsp60 was identified after Western blotting by an antiserum directed against Hsp60 of *Synechococcus* sp. Rabbits were immunnized by Hsp60 of mycoplasmas blotted onto nitrocellulose. In a second approach artificial peptides (multiple antigenic peptides) of about 13 kDa based on amino acid sequences of Hsp60 of *M. pneumoniae* and *M. genitalium* were synthesized. Furthermore two recombinant *M. arthritidis*-Hsp60 glutathion-S-transferase fusion proteins, which contain a *M. arthritidis*-Hsp60 fragment of about 28 kDa (Ma26hsp60) or a *M. arthritidis*-Hsp60 fragment of about 41 kDa (Ma410hsp60), were utilized for immunization of rabbits.

Isolation, purification and detection of Hsp60

The mycoplasma cultures (*M. arthritidis* ISR1 and *M. bovis* PG45) were grown at 32°C in a modified Hayflick medium and heat-shocked by shifting the incubation temperature up to 42°C for 30 minutes. The cells were harvested by centrifugation at

17.000 x g for 60 minutes and washed several times with phosphate buffered saline (PBS). The pellet was resuspended in 2 ml of PBS and homogenized. After lysis of the mycoplasma cells by ultrasonic treatment, the lysate was applied to a Q-Sepharose FF column (Pharmacia, Uppsala, Sweden) using a solution of 0.01 M Tris, 0.4 M NaCl, pH 9.0 as coupling buffer. Elution was carried out using 0.01 M Tris, 0.8 M NaCl, pH 9.0. Further purification was performed by immuno-affinity chromatography (Affi-Gel®Hz Hydrazide Gel; Biorad, Munich, Germany) using protein A-purified immunoglobulin directed against a multiple antigenic peptide. The chromatography fractions were separated in SDS-polyacrylamide gels (10%) and Western blotting was carried out following standard procedures. Proteins were detected by gold- and immunostaining with the rabbit antisera indicated above.
Additionally, bovine sera showing a specific antibody response to *M. bovis* in the ELISA were investigated by immunoblotting for the occurence of antibodies directed against Hsp60 using the recombinant Hsp60 fusion proteins as antigen.

RESULTS AND CONCLUSIONS

Hsp60 of *M. arthritidis* and *M. bovis* could be identified with the different antisera used, i.e. antisera raised against immunoblotted Hsp60 of *M. arthritidis* and *M. bovis*, against two different multiple antigenic peptides, and against the recombinant fusion proteins. The reaction of these sera with Hsp60 of *M. bovis* is shown in Fig. 1. Hsp60 of *M. bovis* (Fig. 2) and Hsp60 of *M. arthritidis* (not shown) could be purified by ion exchange- and immuno-affinity chromatography.
Furthermore the bovine immune response to Hsp60 was investigated. Antibodies reacting with a recombinant Hsp60 fusion protein were detected in *M. bovis* ELISA-positive bovine sera (Fig. 3). This recombinant protein is also immunogenic in rabbits. Future aspects of our studies are (i) the analysis of the amino acid sequences of Hsp60 of *M. arthritidis* and *M. bovis* and the comparison of these sequences to the Hsp60 of other organisms, and (ii) the investigation of ATPase activity, which is characteristic of these proteins in other organisms.

ACKNOWLEDGEMENT

This work was supported by a grant of the Deutsche Forschungsgemeinschaft (Ru 459/2-1). A.W. obtained a graduate student fellowship from the Tierärztliche Hochschule Hannover according to the `Graduiertenförderungsgesetz`.

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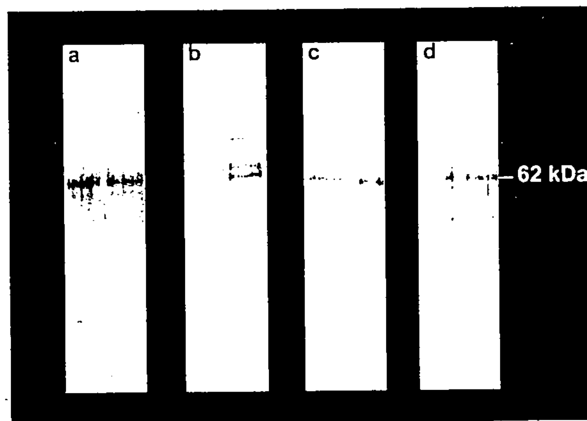

Fig. 1: Immunoblot analysis of purified Hsp60 of *M. bovis* with sera raised against a multiple antigenic peptide (a), Hsp60 of *Synechococcus* sp. (b), Hsp60 of *M. bovis* (c), and recombinant *M. arthritidis*-Hsp60 fusion protein (d).

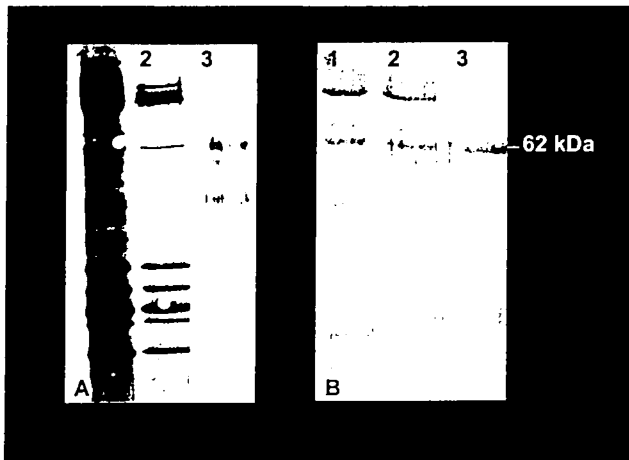

FIG. 2: Western blotting analysis of HSP60 isolated and purified from *M. bovis*.
Goldstaining (A), detection with an antiserum raised against Hsp60 (B). Lanes 1, whole cell lysates; lanes 2, Hsp60-containing fraction after ion exchange chromatography; lanes 3, fraction after immunoaffinity chromatography

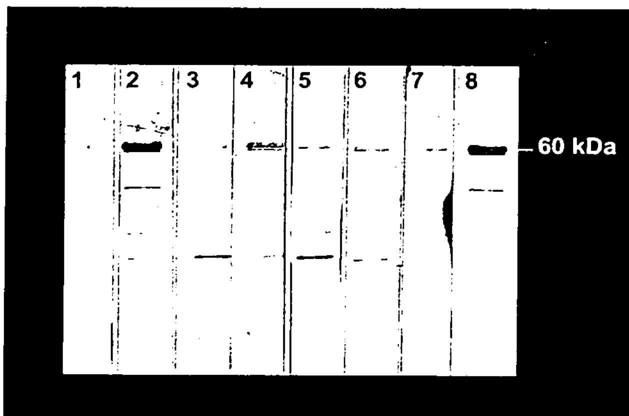

FIG. 3: Western blotting analysis of bovine sera positive in an *M. bovis* ELISA with recombinant Hsp60 fusion protein.
ELISA-negative bovine serum (lane 1), ELISA-positive bovine sera (lanes 2 - 7), rabbit serum raised against Hsp60 (lane 8).

PREVALENCE OF MYCOPLASMAS IN RESPIRATORY DISEASE IN CATTLE IN BRITAIN

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INTRODUCTION

Respiratory disease causes huge losses to the cattle industry worldwide. In Britain recent estimates indicate losses as much as £50 million per year in treatment, "set back" costs and deaths in calves (*1*). A study carried out a decade ago showed that calf pneumonia was costing between £7-15 per calf; extrapolated across Europe with a population of about 50 million calves these amount to very significant losses indeed. Pneumonia is a complex syndrome primarily caused by viruses, bacteria and, of course, mycoplasmas. It is generally believed that mycoplasmas have a secondary role in infections by exacerbating pre-existing disease. Experience suggests that the introduction of *M. bovis*, for example, on to a farm with a history of bacterial pneumonia, initiates calf mortality (*2*). In a survey by the OIE of over 48 countires, *M. bovis* was seen as a major epidiment to the cattle industry. In a US survey, *M. bovis* was isolated from one third of over 400 pneumonic lungs. In France *M. bovis* is involved in 25-30% of pulmonary disease in fattening calves. In Northern Ireland, following its introduction in 1993 the incidence of *M. bovis* in pneumonic lung samples has risen to 23%. Other mycoplasmas which have been associated with respiratory disease include *M. dispar, Ureaplasma diversum, M. bovirhinis* and of course *M. mycoides* SC. Ter laak (*3*) isolated *M. canis* from pneumonic calves but evidence on its role in disease from a survey of healthy and sick calves was not conclusive. A more potent indicator of the importance of mycoplasmas in respiratory disease is the increasing interest of international drug companies in sponsoring antibiotic trials.

In Britain, we have begun to see cases of calf pneumonia in the absence of other microbial pathogens and to see mycoplasmas not normally associated with disease. This paper presents data on mycoplasmas isolated from clinical disease in British cattle and the serological prevalence of *M. bovis* in pneumonic animals over the last two years.

MATERIALS AND METHODS

As the reference laboratory for animal mycoplasmas in England and Wales, CVL receives mycoplasma isolates from cases of clinical diseases from the 12 regional veterinary laboratories. Isolates are typed by growth inhibition, disc film inhibition (DFI), immunofluorescence tests and, increasingly, PCR specifically for *M. bovis* using the primers of Johansson *et al* (*4*). In addition serum samples are received exclusively from cattle experiencing respiratory disease. This amounts to some 2000-3000 samples a year and now also covers Scottish cattle. An indirect ELISA, using a whole

cell antigen from a British field strain, is carried out. The test shows broad agreement with the DFI tests but is more sensitive and easier scale-up. The test was set up using sera from the Channel Isles which has not imported cattle for over 150 years. The data generated from this survey is not random as different centres have different attitudes to the role of mycoplasmas in disease.

RESULTS

The most frequent mycoplasma isolated from clinically affected cattle at CVL over the last decade is *M. bovis* which accounted for nearly 60% of isolations in 1997. These isolations mainly arose from respiratory specimens but also from a small number of mastitic cases and one case of severe conjunctivitis. As expected the majority of isolations occur during the winter months from November to March extending into April when most animals are turned out but outbreaks continue in herds where animals are housed throughout the year. The results of serological diagnosis of *M. bovis* follows a similar pattern. On average the percentage of herds where *M. bovis* is about 22% and this has risen only a little over the last five years. In the vast majority of cases other pathogens such as *Pasteurella haemolytica, P. multicoda* and *Haemophilus. somnus* are also recovered but in several incidents where mortality has reached 30% no other pathogens were recovered. What these figures do not include of course are the cases where other mycoplasmas are involved.

M. bovirhinis is the next most frequently isolated but is clear from ter Laak's work and a study from Belgium last year that this mycoplasma is found equally amongst healthy and pneumonic calves. Other mollicutes also found frequently include *M. arginini* and *A. laidlawii* which are both of dubious significance. The infrequent identifications of *M. dispar* and *U. diversum*, both capable of causing experimental infection, suggest current media used for primary isolations throughout Britain is not capable of supporting these fastidious organisms.

In 1995 we reported the first isolation of *M. canis* from pneumonic cattle in Britain (*5*). Two further isolations were made in 1996 from similar conditions. Last year, however, saw 11 cases of calf pneumonia in which *M. canis* was involved; three cases this year indicate that these increased reports will continue. All cases involve animals below the age of 5 months of which half involve some mortality. Most involve other known pathogens such as *Pasteurella haemolytica, P. multicoda, H. somnus*, RSV or *M. bovis*. However in a small number, no other major pathogens were implicated.

DISCUSSION

This report confirms the surveys of many others that *M. bovis* plays an important role in respiratory disease in cattle more often with other bacterial pathogens but occasionally and with high mortality, it may be the sole cause of disease although other conditions such as weather, stress may also play apart in the disease process. Evidence over the last five years indicates no

great increase in the isolations of *M. bovis*; however it is clear from the published reports (*6*) that its role is severely underestimated.

The increasing reports of *M. canis*, although small, represents a real increase rather than greater surveillance because the mycoplasma grows readily in most media. So far three countries (Canada 1974, Netherlands 1993, Britain 1995) have reported its isolation and there may be others which have not considered the organism significant. It is possible that surveillance is not extended to this mycoplasma or that it has been misidentified as other glucose fermenters most likely *M. bovirhinis*. Biochemically the two are similar apart from the inability of *M. canis* to reduce tetrazolium salts but immunologically they are quite distinct.

Early reports suggested that close contact between dogs and calves may be responsible for the appearance of *M. canis* in cattle. The widespread distribution of *M. canis* seen in this report provides evidence that this mycoplasma may be considered to be part of the normal mycoplasma flora of cattle. Further work will examine the prevalence of *M. canis* in healthy and pneumonic cattle using culture and serological techniques. We will also examine the relationship of canine and bovine isolates for evidence of their origin

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SPECIFIC DETECTION of *Mycoplasma agalactiae* by PCR and NESTED-PCR BASED on the AMPLIFICATION of the *uvrC* GENE

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INTRODUCTION

Mycoplasma agalactiae is an important causative agent of the 'Contagious Agalactia' syndrom, which affects sheep and goats all over the world. This pathology, causing considerable economic losses, is characterized by a high contagiosity and a chronical evolution often resulting in healthy carriers longtime after primoinfection. Moreover, at a flock level, we have frequently observed in French outbreaks that isolation of *M. agalactiae* from bulk tank milk can precede symptoms coming out. For these reasons, 'Contagious Agalactia' control must be based on early and regular detection of the causative organism from different clinical specimens, and not only on serodiagnosis which can sometimes fail in precocity or in cases of asymptomatic vestibular carrying.

Isolation and identification procedures for *M. agalactiae* detection must be specific, sensitive, rapid (contagiosity) and must target conserved genetic sequences or proteic componants. Standard diagnosis tests for serological species identification of isolates generally consist in immunobinding assays. These techniques are quite specific (for *M. agalactiae*), but often laborious and time consuming ; moreover, in light of the important rate of variability of surface antigens in *M. agalactiae*, their reliability should be assessed. *In vitro* amplification of DNA fragments by PCR is a very interesting tool for 'Contagious Agalactia' diagnosis, particularly for its higher potential in sensitivity. However, the currently available PCR systems for *M. agalactiae* are based either on randomly cloned DNA fragments with unknown functions or on the conserved *rrs* (16 rRNA) gene sequence which shows high similarity with closely related species such as *M. bovis*. They are therefore less suitable for the development of highly sensitive detection systems.

We describe in this paper the use of the DNA replication repair gene *uvrC* for rapid and sensitive detection of *M. agalactiae* by PCR and nested-PCR amplifications of species specific sequences. *uvrC* is a constitutive and well conserved gene within *M. agalactiae* (see J. Frey *et al.*, this issue).

MATERIALS AND METHODS

1. Design of primers for sensitive PCR and nested-PCR

The primers for specific and sensitive detection of *M. agalactiae* are based on the *uvrC* gene encoding deoxyribodipyrimidine photolyase (EC 4.1.99.3), an essentiel gene which belongs to the DNA-repair system Uvr ABC. The DNA sequence of the *uvrC* gene is accessible on the GenBank/EMBL accession number AF003960.

The first PCR reactions performed with this pair of primers showed that the sensitivity of the amplification could be improved. For this purpose, two other pairs

of outer and two pairs of inner primers were designed (table 1) : AGANEST1O-L/R and AGANEST1I-L/R, AGANEST2O-L/R and AGANEST2I-L/R (O=outer, I=inner). These primers matched segments which differ strongly in nucleotides from *uvr*C of *M. bovis.* (alignment of *M. agalactiae* and *M. bovis* gene sequences shows 82.6% identity).

Table 1 : Oligonucleotide primers for PCR and nested - PCR reactions

Primer	Nucleotide position	Sequence	Annealing temp.	Fragment size (bp)
AGANEST1 O-L	843 - 862	5' - TTGAACTAGCCCTGTTTTTA - 3'	52 °C	816
AGANEST1 O-R	1636 - 1658	5' - TTAATGCTCTTGTTTTATGATAT - 3'		
AGANEST1 I-L	990 - 1009	5' - ACCGTTATGGCGTTTTAATA - 3'	52 °C	618
AGANEST1 I-R	1585 - 1607	5' - TATTGATGCCTAAGACTTTAAGT - 3'		
AGANEST2 O-L	363 - 384	5' - TGCAATATTTTGATGGCGCAAT - 3'	52 °C	1.199
AGANEST2 O-R	1542 - 1561	5' - TTGTAAGCCGCCATCGGTGA - 3'		
AGANEST2 I-L	633 - 655	5' - GTCCATTTCCTTCTGGTTATGGA - 3'	52 °C	762
AGANEST2 I-R	1372 - 1394	5' - AAACAATTGCTACGCCTACTGGA - 3'		

2. PCR assays

For these sets of primers, different annealing temperatures were tested : 52°C, 54°C and 56°C.

PCR reactions were carried out in 50 µl reaction mixtures containing 0.25 µM of each primer, 170 µM of each dNTP, 10 mM Tris/HCl, 1.5 mM $MgCl_2$, 50 mM KCl, and 1.5 U *Taq* DNA polymerase. They were subjected to 35 cycles of amplification with the following parameters : 30 s. at 94°C, 30 s. at the corresponding annealing temperature and 1 min. at 72°C.

The second (nested) PCR assays were performed using 1µl of amplification product from the first reaction as template.

PCR products were analysed by electrophoresis on 0.7% agarose gel at 110V for approximatively 2 hours and visualized by staining with ethidium bromide.

3. Sample preparation for PCR

Different template preparation techniques were assessed.

• Method 1 : DNA extraction and purification (modified from Razin *et al.* 1983)

This is the classical method for purification of DNA from mycoplasmal origin. Briefly, late log phase cultures were centrifuged (12000 RPM/min., 20 min.), the pellet was resuspended in 450 µl of TE buffer and incubated 2 hours at 45°C in a lysis buffer with proteinase K (20 mg/ml) and SDS (10%). Then, phenol (500 µl), chloroform (500 µl), Na acetate (50 µl) and ethanol (1ml) were successively added. Fifty µl of the final pellet in TE were used for PCR reaction.

• Method 2 : Simplified DNA extraction

Phenol-Chloroform-Isoamylalcohol (500 µl) was added to 500 µl of culture or milk; this mix was centrifuged (15000 RPM/min., 20 min.), the supernantant was mixed in a new Eppendorf tube with Chloroform-Isoamylalcohol and centrifuged again with the same conditions. The supernatant was conserved and mixed with isopropanol (500 µl) and Na acetate (50 µl). The mixture was chilled at -20°C during 20 min., then centrifuged (15000 RPM/min., 15 min., 4°C) ; ethanol was added to the pellet for washing, then removed and finally the pellet was dryed and resuspended in TE buffer.

• Method 3 : Enzymatic lysis

Two ml of culture or milk were centrifuged (13000 RPM/min., 25 min.) and the supernatant discarded. Pellets were mixed with a lysis buffer containing proteinase K (0.25 mg/ml), Tween 20 (0.05%) and Tris HCl (0.1 M pH 8.5) and incubated at 60°C during 1 hour. Inactivation of proteinase K was achieved at 97°C during 15 min.

Assessment of PCR and nested-PCR sensitivity was performed by amplifying ten fold dilutions of a PG2 culture (2.5 10^{10} CFU/ml) in NaCl or milk.

RESULTS

1. Design of primers for sensitive PCR and nested-PCR

This step was performed on decimal dilutions of a PG2 culture in NaCl. Primers AGANEST1 Outer *resp.* Inner amplified 0.816 kb *resp.* 0.618 kb fragments. After sample preparation according to method 2, they gave positive results with mixtures containing 10^7 to 10^6 CFU/ml *resp.* 10^2 to 10 CFU/ml (annealing temperature : 52°C).

Primers AGANEST2 Outer *resp.* Inner amplified 1.199 kb *resp.* 0.762 kb fragments. They gave positive results with mixtures containing 10^5 to 10^4 CFU/ml (annealing temperature : 52°C) *resp.* 10^2 to 10 CFU/ml (annealing temperature : 54°C).

Thus, sensitivity of nested PCR with AGANEST1 O/I and AGANEST2 O/I were approximatively the same, but the second one was choosen for other experiments due to better sensitivity of the first amplification.

2.Sensitivity of nested-PCR for detection of M. agalactiae *from cultures*

With AGANEST2 O/I primers, the 3 sample preparation methods were tested several times with serial ten fold dilutions of a PG2 culture in NaCl.

With method 1 (DNA extraction and purification), the sensitivity obtained after nested-PCR was 2.5 CFU/ml. With method 2 (Simplified DNA extraction), we obtained positive bands until 25 CFU/ml. Interestingly, method 3 (Enzymatic lysis), allowed routinely the detection of 25 CFU/ml, but permitted to detect 2.5 CFU/ml.

For these 3 methods, we observed 10^3 to 10^5 fold higher sensitivity of the nested-PCR as compared to single step PCR.

3. Sensitivity of PCR and nested-PCR for detection of M. agalactiae *from milk*

Preparation method 2 was tested with goat milk artificially contaminated with the same PG2 culture, giving ten fold dilutions from 10^{10} to 10^{-1} CFU/ml (use of AGANEST2 O/I primers). The first amplification (outer primers) allowed the detection of 10^5 to 10^4 CFU/ml ; after the second amplification, 10^3 to 10^2 CFU/ml were detected.

Studies comparing other milk preparation techniques are currently under investigation, but need more standardization to be enough reproducible.

CONCLUSIONS

• PCR and nested PCR systems based on the amplification of the stable *uvr*C gene of *M. agalactiae* allow species specific and very sensitive detection of this organism. With a simple enzymatic lysis performed on a culture prior to nested-PCR, the theoritical sensitivity reach 2.5 CFU/ml. This method is very simple, quick and reproducible, when more labour intensive techniques as extraction and purification are sometimes less sensitive in routine diagnosis due to loss of DNA during the multiple steps. Thus, these results would indicate that a double step-PCR break free from DNA extraction and purification.

The application of nested-PCR methods can be associated with a risk of false-positive reactions by DNA carryover. However, contamination problems can be excluded in a research laboratory by physically separating the manipulations.

• PCR amplification is now the method of choice for identification and even for detection of *M. agalactiae.* Meanwhile, we must consider the different requirements of a research or reference laboratory and of a field one.

In the first case, as described above, nested-PCR can be performed, either from culture or from milk with method 2. This technique provides unambiguous results and *uvr*C target sequence permits systematic differenciation between *M. agalactiae* and *M. bovis.*

In field laboratories, where large number of clinical specimens (milk) must be analysed in a short time and risk of false positive or negative results avoided, we suggest to perform a single step PCR. The simplest and more reliable method is to centrifugate (20000 g, 30 min.) the milk samples and to culture the pellet in broth medium. Two days after, a single step *uvr*C PCR is performed on an aliquot of the culture.

This method, which has been used in France since one year, allows by centrifugation to concentrate mycoplasmas and to remove fat and amplification inhibitors (proteins). Isolation and identification by this method take 3 days and enhance the reliability of the result, whereas conventional cultures, subcultures and identification by immunobinding assays require at least 8 to 10 days.

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ISOLATION AND CHARACTERIZATION OF M.BOVIS FROM A CALF PNEUMONIA OUTBREAK IN GREECE.

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INTRODUCTION

Mycoplasma bovis is frequently involved in shipping fever pneumonia of young cattle and has been reported worldwide (1).

The first report of *M.bovis* infection in Greece was published in 1996 (2). This finding prompted monitoring by our laboratory of the incidence of Mycoplasmal respiratory diseases of ruminants in Greece. In this paper we report the isolation of *M.bovis* from nasal swabs and lung material, taken from Friesian calves, aged three to nineteen months, during a severe outbreak of respiratory disease in a fattening herd.

MATERIALS AND METHODS.

Description of the herd.

Fifteen Friesian calves were purchased from a Greek breeding unit at three months of age and brought into the herd. In the past (prior to the introduction of the fifteen calves) mild respiratory disease symptoms were detected in a small number of calves which subsided without complications and loss of animals. The animals were not vaccinated against any of the respiratory disease-causing viruses.

The total population at the start of the outbreak was 92 calves, aged three to nineteen months. The animals were housed in two open yards with walk-in shelter. They were fed with milk powder and calf pellets. Hay and water were also available.

Clinical symptoms.

A week after arrival of the 15 Friesian calves, acute respiratory disease developed in three of the newcomer animals. On our first visit, a week after the appearance of the outbreak, we observed that 55 of the 92 animals, (58%) were showing clinical symptoms of respiratory disease involving the upper and lower respiratory tract, apathy, anorexia, dyspnoea, coughing, high fever and rhinic secretions. Clinical examination of the lung fields revealed widespread harsh bronchial sounds. Six animals died of the disease within two weeks of the start of the outbreak.

Collection of samples.

Nasal swabs and blood samples from sixteen clinically ill animals as well as lung material from one dead calf were sent to the Microbiology and Pathology laboratories of the Veterinary Faculty for further examination.

Microbiological and Molecular assays.

The sixteen nasal swabs and the lung sample were cultured in Hayflick's medium and blood agar according to standard techniques (3,4) and yielded numerous mycoplasma and bacterial colonies.

DNA was extracted form all mycoplasma isolates and analyzed by PCR according to Sachse et al (5).

Cell lysates (total proteins) from the seventeen mycoplasma isolates were subjected to SDS-PAGE (6) and the separated proteins were either stained in Coomassie blue or electrically transferred to nitrocellulose membranes. The immobilized proteins on the nitrocellulose were probed with monospecific rabbit antisera to *M.bovis* generated in our laboratory using intact PG45 cells as immunogen.

RESULTS

Mycoplasmas with the growth characteristics of *M.bovis* were isolated from all seventeen samples. Twelves samples (70%) yielded high numbers *of Pasteurella haemolytica,* 2 samples (12%) yielded high numbers of *Actinobacillus pyogenes* and one case (6%) yielded high numbers of *Haemophilus somnus.* Only 2 samples (12%) were culture-negative in blood agar. Serological examination for Infectious Bovine Rhinotracheitis (IBR) was also performed on the blood samples, and antibodies were detected in nine of the sixteen animals.

Autopsy of the lung sample revealed extensive bronchopneumonia and emphysema. The histological examination revealed acute bronchopneumonia. The alveolar spaces were filled with alveolar macrophages and fibrinous exudate. The alveolar walls were oedematous.

All seventeen isolates were found PCR positive as indicated by the presence of the characteristic 2027 bp amplimer.

All seventeen isolates exhibited similar banding patterns on both the Coomassie stained gels and in the Western blots, indicating that they represent a single strain (Fig. 1).

CONCLUSION.

All the animals of the herd were treated with Spiramycin (Suanovil-Rh ne M rieux) at a dose of 5 ml administered once daily for two days. Anti-inflammatory non-steroid drugs (ketoprofen) were also administered. The treatment prevented further animal losses and also improved recovery.

According to the results presented here the examined herd was infected with *M.bovis.* However, since the animals were not vaccinated against any respiratory

viruses and IBR antibodies were detected in many of them, we cannot exclude the involvement of respiratory viruses such as IBR in this outbreak.

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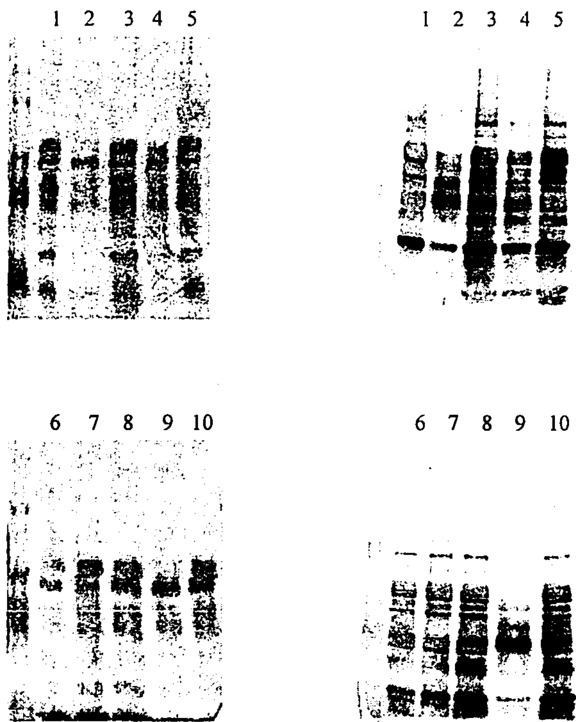

Figure 1: SDS-PAGE and Western immunoblot patterns of *Mycoplasma* isolates. Cell lysates were resolved in duplicate 8.5% SDS-polyacrylamide gels. One replicate was stained with Coomassie Brilliant Blue (left) and the other was processed for Western immunoblot analysis using the *M. bovis* antiserum (right).

APPROACHES TO THE DEVELOPMENT OF SELECTIVE MEDIA FOR *MYCOPLASMA AGALACTIAE* AND *MYCOPLASMA BOVIS*

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INTRODUCTION

Mycoplasmas are metabolically diverse. They are able to obtain energy from the fermentation of sugars, the hydrolysis of arginine, the oxidation of certain organic acids, or any combination of these reactions (1). They also vary with respect to the range of sugars and organic acids which may be utilised. This diversity presents an opportunity to develop media for specific mycoplasmas either by promoting growth of target species or inhibiting growth of other species.

Mycoplasma agalactiae and *M. bovis* are unable to ferment sugars or hydrolyse arginine. However, the type strains of both species and a range of field strains were able to oxidise organic acids (L-lactate, 2-oxobutyrate and pyruvate) and pyruvate has been shown to be an energy source for *M. agalactiae* (2). The growth of arginine hydrolysing mycoplasmas is selectively inhibited by L-citrulline, L-lysine and L-ornithine (3). L-lysine may compete with arginine for uptake by cells. Citrulline is an intermediate of the arginine hydrolysis pathway and ornithine is an end-product, the transport of which from cells may be linked to arginine uptake. The growth of sugar-fermenting mycoplasmas may be selectively inhibited by fluoride and iodoacetate (3). Iodoacetate inhibits the oxidation of glyceraldehyde-3-phosphate to 1,3-biphosphoglycerate and fluoride inhibits enolase, which metabolises 2-phosphoglycerate to phosphoenolpyruvate. In addition, we have also investigated the use of glucose analogues as selective inhibitors of glucose-fermenting mycoplasmas. The inhibitors used, α-methyl glucoside (MG) and 2-deoxyglucose (DG), are transported into cells and phosphorylated via the phosphoenolpyruvate phosphotransferse system, but are not further metabolised. Previously, we found that the growth of *M. mycoides* (glucose-fermenting) was inhibited by DG and that in medium containing the analogue only mutant cells unable to transport glucose were able to grow (4). In populations of wild-type cells, these mutants occurred at a frquency of less than 1 in 10^7 cells.

The aim of the work described here was to: (i) identify the most suitable organic acid for the growth of *M. agalactiae* and *M. bovis* and the optimum concentration; (ii) determine the maximum concentrations of the test inhibitors tolerated by *M. agalactiae* and *M. bovis*; and (iii) to formulate and test selective media for their ability to support the growth of a range of mycoplasmas. In developing selective media, inhibitors of cell-walled bacteria were also included together with the bacteriocin, nisin. Nisin is active against Gram +ve bacteria, but also kills cells of *Acholeplasma* species; *Mycoplasma* species are resistant (5), probably because of the high sterol content of their cell membrane.

MATERIALS AND METHODS

The basal broth medium (PRM) consisted of (per litre): peptone (L-72, Oxoid, Basingstoke, UK), 20 g; yeast extract (Oxoid), 5 g; glycerol, 5 g; NaCl, 5 g; HEPES, 18 g; fresh yeast extract, 100 ml; and heat-inactivated porcine serum, 100 ml. Glucose (5 gl^{-1}), organic acids (sodium salts) and inhibitors were added as appropriate.

The mycoplasmas used were type strains, except where stated. Growth was measured by optical density at 540 nm and by viable count on blood agar base number 2 (Oxoid) with 20% v/v heat-inactivated porcine serum. Substrate oxidation was determined by measurement of oxygen uptake determined from changes in dissolved oxygen tension (6).

Nisin was obtained from Sigma (Poole, UK) as a 2.5 % w/w mixture with NaCl and milk solids.

RESULTS

Type strains and field strains of *M. agalactiae* and *M. bovis* oxidised L-lactate, 2-oxobutyrate, pyruvate and ethanol, but were unable to metabolise a range of other substrates. L-lactate, 2-oxobutyrate and pyruvate, but not ethanol, also stimulated growth in PRM medium. Data for *M. agalactiae* (type strain) cultures incubated statically are shown in Table 1. Pyruvate appeared the most suitable energy source at an optimum concentration of 0.2 - 0.5 % w/v. In these experiments, yields with L-lactate were only slightly greater than in the absence of added substrate; however, cell yield with L-lactate was increased by gentle aeration.

Table 1. The effect of organic acid concentration on the growth of *M. agalactiae* in PRM medium. Cultures were incubated statically in screw-topped bottles.

Substrate	Culture optical density at 48 h				
	substrate concentration (gl^{-1})				
	0	1	2	5	10
pyruvate	0.131	0.230	0.274	0.307	0.205
2-oxobutyrate	0.131	0.191	0.208	0.304	0.189
L-lactate	0.131	0.139	0.141	0.135	0.126

The effect of inhibitors on growth of the type strains of *M. agalactiae* and *M. bovis* was assessed in PRM medium. Compared to glucose-fermenting mycoplasmas, these species were particularly resistant to glucose analogues. Data comparing the effect of MG and DG on the growth of *M. bovis* and *M. gallisepticum* (glucose fermenting) are given in Table 2.

The maximum inhibitor concentrations in PRM medium with pyruvate which did not reduce the growth yield of *M. agalactiae* and *M. bovis* at 48 h are shown in Table 3. The inhibitors were combined at these concentrations in PRM medium; however, it was necessary to further modify PRM medium, by the reduction of the NaCl concentration, as the addition of the combined inhibitors increased medium osmolarity above optimal values for their growth.

The modified media were evaluated for their ability to support the growth of a range of mycoplasmas. Results obtained using the medium designed for the growth of *M. bovis* are shown in Table 4. Growth of the *Acholeplasma* species, a representative arginine-hydrolysing (*M. arginini*) and a representative glucose-

fermenting (*M. mycoides*) species was reduced by ≥ 95 % compared to that in unmodified PRM medium. There was relatively little reduction in the growth of *M. agalactiae* and *M. bovis*. Interestingly, growth of *M. verecundum* was reduced by < 50 %. The energy source(s) for the growth of this organism are unknown (1). Inhibition of the growth of cell-walled bacteria in PRM medium was readily achieved by the inclusion of a mixture of antibiotics (ampicillin, bacitracin, penicillin, polymyxin B and sulphamethazine) and thallium acetate.

Table 2. The effect of α-methyl-D-glucoside (MG) and 2-deoxyglucose (DG) on mycoplasma growth in PRM medium with glucose and pyruvate. Cultures were incubated for 48 h.

Inhibitor (mM)	$cfu.ml^{-1}$	
	M. gallisepticum	*M. bovis*
None	8.6×10^8	8.5×10^9
MG (20)	2.5×10^5	6.6×10^9
MG (50)	1.4×10^5	not done
MG (100)	not done	4.4×10^9
DG (5)	2.2×10^7	7.4×10^9
DG (20)	4.0×10^6	9.2×10^8

Table 3. The maximum concentration of inhibitors in PRM plus pyruvate ($2\ gl^{-1}$) medium which did not reduce growth yield of *M. agalactiae* and *M. bovis*.

Inhibitor	Concentration (mM)	
	M. agalactiae	*M. bovis*
nisin	≥ 0.025	≥ 0.025
α-methyl glucoside	25	100
2-deoxyglucose	65	not determined
fluoride	0.5	not determined
iodoacetate	0.02	not determined
citrulline	30	100
lysine	10	100
ornithine	10	100
antibiotics*	not determined	0.05 - 1.0 %
thallium acetate	not determined	0.4

* ampicillin, bacitracin, penicillin, polymyxin B, sulphamethazine

Table 4. Growth of various mycoplasmas in a selective medium designed for *M. bovis*. The inhibitor concentrations were as listed for *M. bovis* in Table 3.

Species (type strains)	Culture optical density (48 h)	
	PRM medium	PRM medium plus inhibitors
A. axanthum	0.170	0.004
A. laidlawii	0.290	0.003
A. oculi	0.260	0.003
M. agalactiae	0.100	0.083
M. arginini	0.300	0.007
M. bovis	0.960	0.620
M. mycoides	0.520	0.026
M. verecundum	0.047	0.027

CONCLUSIONS

Mycoplasmas vary in the energy substrates which they use. *M. agalactiae* and *M. bovis* obtain energy for growth by the partial oxidation of organic acids (lactate, pyruvate, oxobutyrate). Media for the growth of these organisms were made selective by the addition of organic acids and of inhibitors of glucose-metabolising and arginine-hydrolysing *Mycoplasma* species. Nisin was also included in the media developed to prevent the growth of *Acholeplasma* species.

Acknowledgements. - This work was in part supported by European Commission grant FAIR1 CT-95-711.

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ISOLATION AND CHARACTERIZATION OF *M.AGALACTIAE* STRAINS ISOLATED IN GREECE

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INTRODUCTION

Contagious agalactia (C.A.) is a sheep and goats disease and the main aetiological agent is *Mycoplasma agalactiae*. In Greece there are approximately 14 million sheep and goats and C.A. is a major cause of economic loss. Mycoplasma surface antigen variability is a significant obstacle in the development of effective vaccines. In this paper we report on the characterization by PCR and Western blot analysis of 17 Mycoplasma isolates from animals afflicted with C.A. and 1 from a pneumonic calf.

MATERIALS AND METHODS

Collection of samples.

Milk samples were collected from animals with mastitis. The collection of samples was carried out over a two year period and included many flocks from Northern Greece. Samples were sent to the Microbiology laboratory for further examination. In this study 17 isolates from 17 different flocks were chosen for PCR and Western blot analysis. Also included in this study was an isolate that was identified as *M.agalactiae* by conventional methods (growth inhibition test) from a nasal swab of a pneumonic calf.

Microbiological and Molecular assays

The 18 samples were cultured in Brain-Heart Infusion Broth and Agar according to Sarris[1]. Biochemical tests were performed according to standard techniques [2].

All isolates were also tested in the growth inhibition test (GIT) using standard antisera to *M.agalactiae* (PG-2), *M.capricolum* (California kid) both obtained from the Institute of Microbiology Aarchus Denmark, *M.m.capri* generated in our laboratory and *M.bovis* obtained from K.Sachse, BgVV Institute, Jena Germany.

DNA was extracted from all isolates and analyzed by PCR using the protocols of Johansson et al [3] as well as that of Subramaniam et al [4].

Cell lysates (total proteins) from all isolates were subjected to SDS-PAGE[5] and the separated proteins were either stained in Coomassie blue or electrically transferred to nitrocellulose membranes. The immobilized proteins on the nitrocellulose were probed with monospecific rabbit antisera to *M.bovis*

and *M. agalactiae*. The *M.agalactiae* antisera were obtained from F Poumarat and the *M.bovis* antiserum was generated in our laboratory using intact PG45 cells as immunogen.

RESULTS

All isolates showed similar biochemical profiles, i.e. glucose fermentation –ve, arginine –ve, 2,3,5-TTC +ve, phosphatase +ve, digitonin +ve, film & spot formation +ve.

All 17 sheep and goat isolates as well as the calf isolate were inhibited by the *M.agalactiae* antiserum.

Sixteen out of seventeen sheep and goats isolates were found PCR positive (nos. 2 - 5 and 7 - 18), by both protocols as indicated by the presence of the characteristic 734 bp and 1,624 bp amplimers. One isolate from a sheep (no.1) and the calf isolate (no.6) tested PCR negative in both protocols. All isolates exhibited unique banding patterns on both the Coomassie stained gels and in the Western blots with the *M.agalactiae* anti-serum (fig. 1), indicating that they represent different strains. The differences observed in the banding patterns were either in the number and the molecular size of bands (e.g. compare isolates 2, 3, and 4) or in their relative intensities (e.g. compare isolates 13 and 14).

Isolates 1, 5, 6, 7, 9, 12 and 17 that exhibited weak reaction in Western immunoblotting against the *M.agalactiae* anti-serum, were also tested with the *M.bovis* antiserum. All isolates reacted relatively stronger with the *M.bovis* antiserum. Isolates 5, 6, 1, and 12 showed very similar profiles. Isolates 9 and 17 also showed similar profiles whereas isolate 7 differed from all the others (fig. 2).

CONCLUSIONS

It is clear from the results that application of Western immunoblotting with monospecific antisera can differentiate between different strains. In this study the calf isolate (no. 6) that was identified as *M.agalactiae* by the conventional techniques, tested PCR –ve for both *M.bovis* when tested by the protocol described by Sacshe et al[6] and *M.agalactiae*. This isolate showed in the Western immunoblot, a very similar banding pattern to sheep isolate 5 which tested *M.agalactiae* PCR +ve. Similarly, sheep isolate 1 that was identified as *M.agalactiae* by the conventional techniques, tested -ve for *M.agalactiae* PCR but shows a very similar banding profile to sheep isolate 12 that tested *M.agalactiae* PCR +ve.

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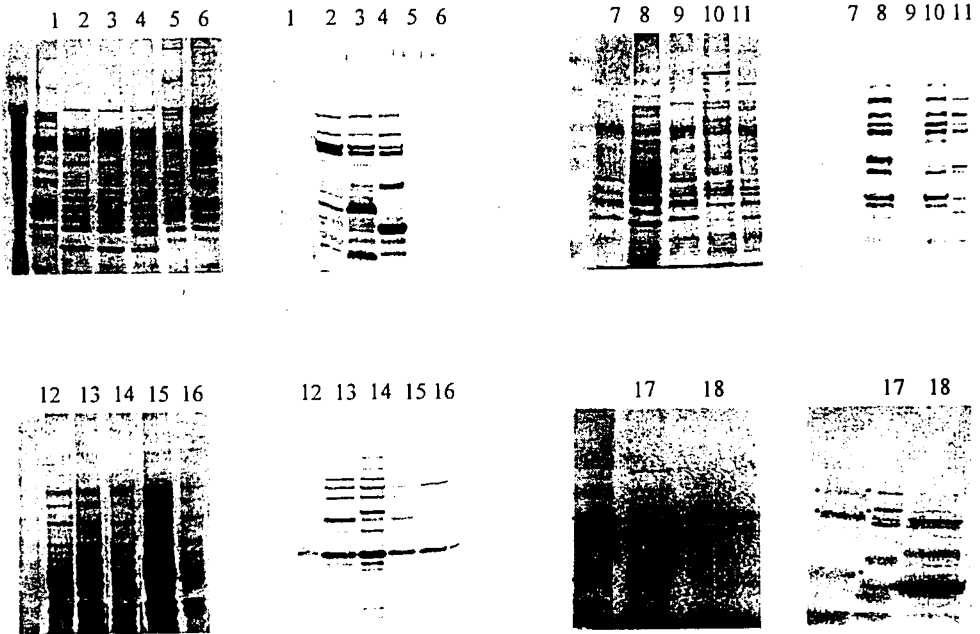

Figure 1: SDS- PAGE and Western Immunoblot patterns of *Mycoplasma* isolates. Cell lysates were resolved in duplicate 8.5% SDS-polyacrylamide gels. One replicate was stained with Coomassie Briliant Blue (left) and the other was processed for Western immunoblot analysis using the *M. agalactiae* antiserum (right).

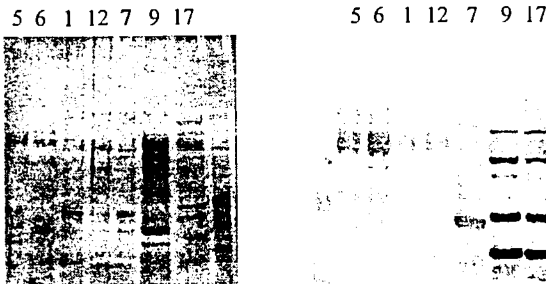

Figure 2: SDS-PAGE and Western Immunoblot patterns of Mycoplasma isolates that exhibited weak reaction to the M. agalactiae antiserum. Cell lysates were resolved in duplicate 8.5% SDS-polyacrylamide gels. One replicate was stained with Coomassie Briliant Blue (left) and the other was processed for Western immunoblot analysis using the *M. bovis* antiserum (right).

ENZOOTIC MYCOPLASMA BOVIS INFECTION IN A DAIRY HERD

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INTRODUCTION

Mycoplasma bovis infection is very much spread all over the world (1, 7). It was recognised as a pathogen in pneumo-arthritis syndrome of calves, mastitis and in the reproductive disorders of calves and bulls (3, 4, 5, 6). However very few data are *available on epidemiological and clinical appearance of the diseases caused by M.* bovis. Therefore we decided to examine appearance of the disease associated with *M. bovis* in a endemically infected cattle herd.

MATERIALS AND METHODS

Description of the farm.

The commercial dairy herd of Holstein/Friesian breed was consisting of 450-500 cows and similar number of growing heifers. The herd was closed already ten years, no animals were introduced into the farm. Six years ago a very significant (20-30%) mortality of calves younger than 1 month was observed every month due to pneumonia and arthritis. Microbiological examination of died animals at that time reviled *M. bovis* in 80 % of tested samples. Since that time various anti-mycoplasma drugs were used routinely for treatment of new born calves during first 21 days of life for prevention of the disease. Due to this measurement the mortality was reduced to about 5 %.

Design of experiment.

The prophylactic treatment of calves was stopped. The new born calves were identified with individual plastic ear tag and they received colostrum from their dams within 2 hours after birth. Calves in age of 4 days were placed into individual pens outside building. Distance between boxes were 6 meter. Calves were fed twice a day. During first 8 days animals received total milk in amount of 6 litres/animal. From day 10 animals were offered premix and hay quantum satis. Calves born during November and December 1997 and in January 1998 were included in experiment. Six consequently born calves formed a block of calves all together 6 blocks were formed. Observation started in age of 5 days and continued till 21 days.

Parameters tested for evaluation of the disease.

At the beginning and at the end of the 21 day observation period nasal swabs were collected form calves for detecting *M. bovis (2)* and *Pasteurella multocida* and *P. haemolytica*. At the beginning blood samples were also taken from calves for detecting antibodies against *M. bovis* by Western blot, as well as antibodies against

bovine virus diarrhoea, adenoviruses, parainfluensa-3, respiratory syntitial virus, infectious bovine rhinotracheitis virus. Vagina swabs form dams of calves were also collected 4 days after delivery for culturing *M. bovis* and Pasteurella

During 21 days period rectal temperature of each animals was measured every morning. Number of animals showing temperature higher than 39.5 C degree were counted in blocks and compared by Tie square test. Milk consumption was measured every day 2 times. Clinical assessment were performed every day in the morning. General clinical appearance of animals were scored (0=normal, 1=subdued slightly depressed, 2=depressed reluctant to rise, 3=depressed recumbent). Respiratory signs were also scored (0=normal, 1=hyperpnoe or slight dyspnoea, 2=moderate hyperpnoea, obvious dyspnoea, 3= respiratory distress). Nasal discharge was also scored (0=absent, 1=milde and catarrhal discharge, 2=significant and purulent). Severity of cough was scored (0=absent, 1=mild and rare, 2=severe and frequent). Animals having high rectal temperature, severe clinical scores, decreased appetite were treated individually by various anti-mycoplasma drugs. Number of treatments in various blocks of calves were counted and compared by χ^2 test. Body weights before starting the experiment, on day 10 and day 21 of experiment were measured. Average body weights, weight gains, in periods of days 0-10, 11-21 and 0-21 of experiment in each block were compared in student T-test. Animals died during experiment were necropsied and were examined for presence of pathological and histological lesions as well as for presence of *M. bovis*, *P. multocida* and *P.haemolytica* in lung, kidney, liver and spleen.

RESULTS

Presence of *M. bovis* was demonstrated in nasal cavity in 17 of 35 (48.60 %) of calves at the beginning of the observation period. 21 days later isolation rate of *M. bovis* increased to 91.4 %. All three died animals had *M.bovis* in their inner organs. 3 of 12 vagina swabs of cows was positive for *M. bovis*. Among cows 19 (27.1%) had retention of embryonic membranes. 5 cows developed mastitis. Isolation rate of *P. multocida* at the beginning as well as at the end of observation was similar, 25.7-28.6 %. The clinical signs of respiratory disease associated with *M. bovis* were observed already on second day of observation period in age of 7 days. Maximal number of affected animals (20-40 %) as well as highest scores were observed on day 3 to 8 of observation period (age 8-16 days). From day 2 to the end of experiment of period many of animals (14,2-42,8 %) showed rectal temperature higher than 39.5 C degree. From day 2 to 9 25,7-45,7 % of animals refused uptake of milk or reduced milk consumption. On days 6 and 11 of observation period 17,1 - 42,8 % of animals should be treated to reduce symptoms of respiratory disease. Average body weights (40.8-46.0 kg) of calves in different blocks did not differed significantly from each other. Body weight gains in days 0-10 varied between 1.5 and 3.2 kg, in days 11-21- 2,6-5.9 kg in period of 0-21 days between 4.5 and 7.5 kg. Severity of the disease (number clinical, respiratory and cough scores), frequency of higher rectal temperature and refusing milk uptake, body weight gains varied significantly in different blocks of calves.

DISCUSSION

The study performed showed that *M. bovis* infection in endemically infected herd is important causing significant disease of young calves. Mycoplasma infection can be detected in new born calf in about 40 % which increases significantly after 3 week period. Mycoplasma infection is probably originating from cows since *M. bovis* could be isolated from vagina swabs of mothers. Among cows 30 % had retention of embryonic membranes and some had also mastitis. Among young calves clinical symptoms or respiratory disease as depression hyperpnoea, dyspnoea, respiratory distress, nasal discharge, cough, as well as increase of rectal temperature, loss of appetite, started developed in age of 6-7days and gradually developed reaching highest incidence and highest score values in age 10-15 days. Therefore weight gains of animals in age of 5-15 days is very poor If the animals are not treated properly they may die showing severe pneumonia, pleurisy. This data showed that *M. bovis* can exist several years. Since the herd was infected with *P. multocida* (not *P. haemolytica*), this bacteria might have aggravating effect in the development of the disease. The herd was also infected with paraminfluenza-3, adenovirus and rhinotracheitis virus. which may also contribute to development of the diseases. However since the disease developed mostly in very young age, the role of viral infection should be not over estimated because colostrum antibodies were detected in sera of calves.

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IDENTIFICATION AND ANTIBIOTIC SUSCEPTIBILITY OF MYCOPLASMAS ASSOCIATED WITH CONTAGIOUS AGALACTIA IN SICILY

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INTRODUCTION

Contagious agalactia has been known for nearly 2 centuries in Italy where it was first described as "mal di sito" because of its persistence in the environment. In Sicily, although vaccines have been produced for nearly 70 years ago, CA remains the second biggest problem after brucellosis accounting for over 43% of mastitis cases reported over the last 5 years. Severe falls in milk production account for the major economic losses but the rates of mortality and morbidity in adults, young and foetuses as a result of septicaemia, arthritis and pneumonia are also significant.

The main aims of this project were: to identify the isolates collected by IZS, Palermo over an 8 year period from milk, lung and swabs from small ruminants affected by CA; and to determine the antibiotic susceptibility of a representative group of isolates.

MATERIALS AND METHODS

Identification of isolates

Freeze-dried isolates were reconstituted of sterile distilled water and 300 μl were added to 3 mls of Eaton's Medium. After 3-4 days incubation, cells were pelleted and DNA extracted by conventional procedures. A PCR for *M. agalactiae* was carried out (1); if results were negative the PCR for the *Mycoides* cluster was performed (2) which, if positive, was followed by the PCR for *M.mycoides* subsp. (2). If all PCRs were negative, then the isolate was identified by conventional means (3).

Minimum inhibitory concentrations

Fifteen isolates of *M. agalactiae* from the milk and lungs of small ruminants in Sicily were tested for sensitivity to a range of antibiotics. In total twenty three antibiotics were tested, twenty on Eatons plates and five on sensititre microplates. The isolates were initially cultured in Eatons broth and then sown either onto three agar plates per isolate for a qualitative assessment and onto sensititre plates for quantitative assessment. Dried antibiotic discs were placed on the Eatons agar medium containing penicillin + 10% egg yolk and incubated until there was visible mycoplasma growth. Agar plates were read either by observing growth or measuring a zone of inhibition around the antibiotic disc. The use of plates with penicillin could not be avoided so some synergistic effect could not be discounted. The isolates were also subcultured onto sensititre plates (10μl inoculum to 90μl of Eatons broth per well), containing different concentrations of freeze-dried antibiotics. Sensititre

plates were spun down after 48 hours incubation at 37° C and read by observing whether growth could be seen. The sensitivity was taken as the minimum concentration where there was no growth.

RESULTS

Over 180 mycoplasma isolates from cases of natural disease resembling clinical contagious agalactia in small ruminants in Sicily were collected and freeze-dried over a seven year period beginning 1991. The majority of isolates were from milk but other samples in order of frequency were lung, synovial fluid, vaginal swabs, nasal swabs and eye swabs. The isolates were grown in Eaton's media and subjected to PCR for *Mycoplasma agalactiae* (1) as soon as a colour change was evident. Where results were negative for *M. agalactiae*, the PCR for the *M. mycoides* cluster was carried out (2). Conventional identification techniques were carried out on isolates were were negative for both PCRs. Over 80% of isolates were identified as *M. agalactiae* (including some recovered from lungs), 6% were *M. m. mycoides* LC from goats and others identified were *M. arginini.*

In a qualitative assessment of antibiotic effectiveness on mycoplasmas, many had little or no effect including neomycin, penicillin, and nalidixic acid. The minimum inhibitory concentrations of some antibiotics were determined on a representative sample of *M. agalactiae* strains. The most effective antibiotics were danafloxacin and oxytetracycline while spectinomycin was the least effective (Table 1).

Table 1: Quantitative assessment of the sensitivity of *M. agalactiae* strains

Sample	Source	Oxytetracyclin	Spectinomycin	Florfenico	Tilmicosi	Danofloxn
420/91	Sheep Milk	1	32	16	4	1
862/91	Sheep Milk	2	4	8	8	0.25
899/91	Sheep Milk	4	1	4	4	0.5
1341/91	Sheep Milk	8	4	8	16	1
119/92	Sheep Milk	1	4	4	4	0.5
1398/92	Sheep Milk	4	4	8	8	1
1701/92	Sheep Milk	0.5	4	8	16	8
1740/92	Sheep Milk	0.25	0.5	1	0.5	0.5
1835/92	Sheep Milk	4	4	16	8	16
2645/92	Sheep Milk	0.5	2	2	2	2
2684/92	Sheep Milk	16	16	4	1	4
2685/92	Sheep Milk	8	4	8	8	2
723/93	Sheep Lung	0.5	64	128	64	4
23/94	Sheep Milk	4	4	8	8	1
453/94	Sheep Lung	32	4	16	64	0.25
	Mean	**5.72**	**10.10**	**15.93**	**14.37**	**2.80**

CONCLUSIONS

This survey confirmed the biochemical evidence seen previously in culture medium that *M. agalactiae* is the major cause of contagious agalactia in Sicily. *M. m. mycoides* LC was seen less commonly and only in goats. As expected antibiotics which target cell wall synthesis were ineffective against mycoplasmas while those which affect 30S ribosomal RNA and therefore interfere with protein synthesis prevent mycoplasmal growth. Evidence of resistance of strains, particularly lung isolates, to some antibiotics was shown and would need to be confirmed by further work including mycoplasmacidal effects.

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THE CONTROL AND ERADICATION SCHEME OF CONTAGIOUS AGALACTIA IN FRANCE: THE ROLE OF CNEVA SOPHIA ANTIPOLIS

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Contagious agalactia, due to *Mycoplasma agalactiae*, is a serious cause of mastitis in milking ewes and goats and has been recognized in various parts of France. An eradication scheme has been implemented in some regions and is coordinated by a committee called « Coordination Nationale » which gather representatives of the sheep and goat industries, scientists, government veterinary services,... This programme is mainly based on the qualification of flocks following serology, and more recently on bacteriology on bulk milk. The CNEVA Sophia Antipolis has developed and produced ELISA kits necessary to qualify the flocks. The qualification is obtained following the testing of 20 sera from each flock; titers of these 20 sera are used to define a serological profile and an index [I] leading to four classes of flocks: contagious agalactia [I>124], latent agalactia [124<I>64], presumed free from contagious agalactia [64<I>4], free from contagious agalactia [I<4]. In the recent years, the ELISA kits have been continually improved in particular by eliminating the numerous false-positive reactions[1]. Monoclonal antibodies against field strains of *M. agalactiae* have been produced and are currently tested for the development of a new generation ELISA kit. The isolation of *M. agalactiae* strains in flocks without any clinical signs has led to evaluate the virulence of these particular strains in experimentally-infected sheep according to a previously described model[2]. The results have shown that the « asymptomatic » strains still keep their pathogenicity even if their *in vivo* behaviour was slightly different to a fully virulent strain. This experiment demonstrates the necessity to follow with caution these « asymptomatic » flocks as potential reservoirs for a new outbreak.

With the combination of all these measures including the bacteriological tests, the percentage of infected flocks has fallen from 10% in 1990 to less than 1.9% in 1997.

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Outbreaks of Contagious Agalactia in Israel: Clinical and Epizootiological aspects

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INTRODUCTION

The small ruminant population of Israel comprises over 400.000 head, of which about 90.000 are goats. While about 2/3 of that population is concentrated in the southern desert ("Negev"), possessed by its Bedouin inhabitants who still breed their livestock using traditional grazing and management methods, the sheep and goat population of the North is more commercially-oriented. Many flocks and herds (some of them are of mixed population of both sheep and goats) in the North are, therefore, bred indoors, partially or totally, with genetically improved animals, intensive feeding methods, routine synchronisation of reproduction and sophisticated milking gear.

Contagious agalactia (CA) caused by *Mycoplasma agalactiae* (Ma) has been reported in Israel since the mid '50's and is always confined to certain areas of the North. In the mid '70's, when intensive Saanen goat breeding became popular in the upper north ("Galilee") of the country, many outbreaks of an "agalactia-like" disease were observed, from which *M. mycoides subsp. mycoides (LC)* (*Mmm LC*) was isolated (3). The occurrence of the disease caused by that mycoplasma has decreased markedly in the previous decade. At present, Ma is the predominant CA-causative organism, while *M. capricolum, Mmm LC* and other members of the *"mycoides cluster"* are isolated from time to time.

Up to the present, mycoplasma infections of sheep and goats constitute a permanent threat to the health of the flocks and herds all over the country, even to those in the South. Little is known about the reservoirs of infection and means of transmission of the disease. Recently we have carried out preliminary studies employing modern methods of molecular epidemiology in order to address these problems.

Except few unsuccessful vaccination trials in the late '70's, no use is made of vaccine against small ruminant mycoplasmas. In view of the lack of efficacy of the antibacterial drugs, management improvements and controlled animal movements are the only ways in which the Israeli veterinary establishment is struggling against this disease.

MATERIALS AND METHODS

The data used for the following analysis were extracted from the epizootiological computerised database of the Israeli Veterinary Services, that includes both laboratory test results and the reports of the regular epizootiological enquiries,

carried out by the field staff of the Veterinary Services whenever an outbreak of a notifiable disease, one of which is CA, occurs in the field.

Standard methods were used for isolation of mycoplasmas from clinical samples and identification by direct and indirect immunofluorescence with specific antisera. Molecular biological methods have been recently described (1,2).

RESULTS

From 1990 through 1997, 68 outbreaks of CA were diagnosed (equally in sheep and goats, in spite of the disparity in numbers of both species). Of the mycoplasmas isolated from milk or synovial-fluid samples taken from diseased animals, 50/68 were *M. agalactiae*. From the other outbreaks, 6 were of *M. capricolum* and 12 were of the "mycoides cluster", including *Mmm LC.* Despite the situation in other Middle-Eastern countries, there is, so far, no evidence, either clinical or bacteriological, for the existence in Israel of *M. capripneumoniae* (formerly: "F-38"), the agent of Contagious Caprine Pleuropneumonia.

Although most of the outbreaks occur in poorly managed small ruminant populations, some of them, which occurred in large-scale, commercially oriented, well managed sheep and goat flocks/herds are worth describing, as in those flocks an economic assessment of the damage can be made.

1) An outbreak occurred in 1988 in a large Saanen dairy herd, into which no animals had been introduced in the previous 5 years, so the origin of the infection remains unknown. During this outbreak, which started abruptly at the middle of the lactation period, many of the dairy animals had to be culled due to total drying off. Clinical mastitis cases continued to occur, but less intensively, in the next 2 milking seasons. Only in 1991 was the herd declared clinically free of CA (no guarantee can be given as to its bacteriological status). The damage to the flock at the end of the first year of the disease is as follows: out of the 320 milking does at the beginning of the lactation period, 120 were lost (20 died, 80 were culled, 20 yearling were dried-off and re-mated a year later); the expected milk yield of the herd was reduced in about 64.000 litres during that lactation period; the farm had to restock its herd with new goats. Together with the other unexpected expenses due to the outbreak, the direct financial damage assessment was of about US$ 64.000. As the separation of the offspring from their dams immediately after delivery and their adaptation to milk replacer feeding was a part of the herd's management system, almost no case of arthritis could be detected, a fact that contributed to the success in cutting the vicious cycle of re-infection.

2) A very severe outbreak of CA caused by *Ma*, which led to the liquidation of the whole flock, occurred in 1993 in a well managed dairy sheep flock, very probably after lending a ram to a neighbouring farm. It started with a sudden outbreak of mastitis in many sheep, followed by numerous cases of arthritis. As the breeder could not stop the infectious cycle within his flock, and in view of the heavy immediate economic loss, he decided to liquidate all his livestock in order to buy new stock a year later. The direct financial damage to that flock, due to the total loss of both milk and meat production and to the very low price of the adult dams and bucks sold for slaughter, is estimated at about US$ 220.000.

3) A very severe outbreak of mastitis, from which both *MM. L.C.* and *M. capricolum* were isolated, occurred in a well managed Saanen dairy herd in 1994, as a sequelae of an devastating outbreak of foot-and-mouth disease. The clinical mastitis started about a week after the onset of the vesicles, typical of foot and mouth disease, on the goats' hooves, mouths and udders, from which strain "O_{22}" of the virus could be isolated. Five years prior to the outbreak, this herd was in contact with a mycoplasma-affected herd, but had never shown any clinical signs of CA.

4) An outbreak was diagnosed in 1996, in a well managed local dairy goat when the owner bought-in a whole Saanen herd from a nearby village. Although the purchase was approved by the Veterinary Services (no previous reports of any contagious disease, including mycoplasmosis, many years backwards), many cases of mastitis, from which *Ma* has been constantly isolated, started to occur in the goats upon arrival to their new premises. The spread of the mycoplasma to the main herd on the farm was avoided thanks to the veterinary practitioner who ordered, even before sending the first milk samples to the laboratory, the immediate separation of the newly introduced animals from the existing herd. The economic assessment of the damage is not yet fully established. The source of infection in the purchased goats is unknown.

Recently the presence of high frequency genetic variation among Ma field strains was described by our group , using a gene probe derived from the *vsp*-gene of *M. bovis* (1). The initial observation was that each of the tested *Ma* clinical isolates, which originated from randomly selected field outbreaks, showed a unique pattern for the *vsp*-associated genomic fragments (2). However, when multiple Ma isolates within the same outbreak from different diseased animals are examined, many show the same or similar genetic pattern. We refer to this as an "outbreak representative" pattern. When comparing isolates from different outbreaks, the representative pattern may be similar or markedly different, suggesting a possible use of the molecular technique for epidemiological studies.

DISCUSSION

Since the almost complete disappearance of *Mmm LC,* the dominant endemic sheep and goat mycoplasma species in Israel is by far *Ma*, while the occurrence of the disease caused by the other species, although clinically similar, has become merely sporadic.

The high density of the sheep and goat population in the North, together with old livestock trade traditions, according to which uncontrolled animal transfer among the flocks/herds is practised, seem to be the main reason for the occurrence of CA in the North only. In this region poor management systems (mainly poor milking hygiene) and poor nutritional status of many flocks/herds, as well as concurrent diseases within the flock/herd, coupled with the lack of awareness of many breeders to any health risk to their livestock, contribute to the severity of the well known clinical triad of mastitis, arthritis and keratoconjunctivitis, typical to CA, in this region.

Both clinical and epizootiological observations indicate that the perpetuation of CA in the North is due, to a very large extent, to the existence of asymptomatic carrier dairy animals, who keep shedding the organisms in their milk, constantly or intermittently. This may be the case in ewes and does which were previously exposed to mycoplasma, although not necessarily with the development of a clinical

disease. In the absence of adequate epizootiological tools, it is very difficult to assess the extent of this phenomenon, either within a given flock/herd or within a whole regional population of sheep and goat.

CONCLUSIONS

1) Goats only are hosts (at least under field conditions) of the organisms of the "mydoides cluster" and of *M. capricolum.* Sheep CA seems to be due exclusively to *Ma.*

2) The first penetration of a mycoplasma into a naive sheep and goat population seems always to be via the introduction of non-clinical shedders. It is difficult with current methodology to detect such animals under field conditions.

3) Poor management practices help to perpetuate the presence of small ruminant mycoplasmas in a given population and predict the severity of the clinical and epizootiological aspects of CA outbreaks.

4) Systematic population monitoring for the degree of its exposure to mycoplasmas is mandatory for any preventive campaigns against CA. The present, molecular biology-based techniques may supply the so far lacking efficient tool for such activity.

5) For the understanding of the epizootiological pattern of *Ma*, new molecular biology-based methods that enable us to characterise strain variations are being currently developed by our team.

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This research was partially supported Research Grant Award No. IS-2540-95R from BARD, The United States-Israel Binational Agricultural Research and Development Fund.

MYCOPLASMAS IN A PROBLEM FLOCK OF SHEEP WITH CONTAGIOUS AGALACTIA

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Contagious agalactiae (CA) is a disease of small ruminants and is one of the biggest dangers to the rural economy of some Mediterranean countries. In some areas it is difficult to define the impact of the disease because the animals are moved to richer pastures during summer. Sheep and goats are the main economic resource in areas where the climate and the terrain make it impossible to keep other livestock. CA is caused by *Mycoplasma agalactiae* (*Ma*), but the symptoms of mastitis, arthritis and keratoconjunctivitis may be caused by other mycoplasmas including *Mycoplasma mycoides* subsp. *mycoides* LC (*Mmm*LC), *Mycoplasma mycoides* subsp. *capri* (*Mm*capri), *Mycoplasma capricolum* and *Mycoplasma putrefaciens*. It is present in many countries around the Mediterranean sea and has been reported from France, Italy, Spain, Portugal and Greece.

The present study considers a flock of about 400 animals affected by mastitis, and in a few cases, also by keratoconjunctivitis. After the first diagnosis of CA obtained by the isolation of *Ma* from a milk sample, the animals were treated with antibiotics (tetracycline) and vaccinated with a phenol-inactivated vaccine made from a pooled sample of milk from the same flock. The vaccination was repeated 20 days after the first treatment and a third time 20 days later. Symptoms of mastitis persisted after vaccination. Milk samples were collected from all lactating sheep (132 animals), for the bacteriological investigations, and blood was also sampled for serological tests.

MATERIALS AND METHODS

Serology. Complement fixation test (CFT) was carried out in microtiter plates using 6% sheep red blood cells, 12 haemolysin units, 2.5 guinea pig complement units. All sera were diluted 1:10 and incubated for 3 hours at 37°C with, the haemolytic system was then added and incubated 30 minutes in waterbath. The sera were considered positive at a titre of 1:10 complete fixation. The ELISA test was carried out with a kit (Chekit agalactiae, Bommelli, Switzerland) following the instructions provided by the manufacturer.

Bacteriology. A 100 µl aliquot of each milk sample was plated directly on agar plates for *Ma* (Mycoplasma Experience, UK). The plates were incubated for 4 days at 37°C in a gas jar. Standard biochemical tests and growth inhibition (GI) were then used for identification. The GI test was performed with rabbit hyperimmune serum to *Ma* strain PG2.

PCR test. A 200 µl aliquot of each milk sample was centrifuged at 14000 rpm for 10 min and the pellet was washed twice with 800 µl of TNE (10mM Tris-HCl, pH 8.0, 10mM NaCl, 10mM EDTA). DNA was extracted with phenol from the resulting pellet as previously described (1) and 1µl of it was used in PCR. The PCR test was carried out for CA using *M. agalactiae* specific primers previously established (3) at the following conditions: 2.0 mM $MgCl_2$, Amplitaq Gold (Perkin Elmer, USA) for 30 cylces (94°C/10min, 30 x 94°C/30sec-60°C/30sec-72°C/30sec, 72°C/5min). The same DNA suspension was also used for *M. mycoides* specific PCR and the amplicons were digested with restriction enzymes as already described (1).

RESULTS

The serological test with CFT showed that antibodies to *M. agalactiae* were present in 59% of the 132 animals tested, while the ELISA test revealed 87% positive samples. The CFT titre ranged between 1:10 to 1:160. Milk samples plated directly on agar showed typical colonies in 23% of the samples (Figure 1). Other mycoplasmas without the typical colonial morphology and smaller in size were also present on the agar. The identification of the *M. agalactiae* colonies was done by biochemically with glucose utilisation (–), arginine hydrolysis (–), phosphatase activity (+), tetrazolium reduction aerobically and anaerobically (+/+), film and spot production (+), and by growth inhibition test (+). The colonies showing the typical *M. agalactiae* morphology on agar were cloned and confirmed by PCR. By PCR, carried out on all milk samples, 42 positive samples (32%) were identified to have *M. agalactiae*. To identify the mycoplasmas present other than *M. agalactiae*, the same samples were processed by PCR for *M. mycoides* which resulted in 82 positive samples (62%). The identification of the *M. mycoides* PCR positive colonies was done by restriction enzymes analysis with *Asn*1. A pattern of two bands of 380bp and 180bp was obtained for all the samples, indicating the presence of PCR product from *Mmm*SC. The isolation of the organism is currently underway.

CONCLUSIONS

At the beginning of the disease there was a significant (80%) loss in milk production which remained low for the following months. After treatment and vaccination some of the animals recovered clinically from the diseases, but the milk production never reached previous levels, and progressively almost all the animals became affected by mastitis. Milk from some of these animals appeared normal but production was reduced. The cause of the observed symptoms could be infection with *Ma*, but the persistence of these signs after vaccination indicates the presence of complicating factors one of which could be the colonisation of the mammary glands by another mycoplasma. Prolongation of the syndrome may be attributable to the presence of different mycoplasmas in the same flock which in this case was a mycoplasma genetically identifiable as *Mmm*SC which requires further confirmation. The isolation of *Mmm*SC from sheep milk has been reported previously (2) and offers support for the recognition of these organisms as infectious agents of small ruminants. The epidemiological role of sheep in the maintenance of *Mmm*SC and its pathogenicity need to be investigated.

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Figure 1

Mycoplasma colonies on solid agar which was inoculated with sheep milk. Two types of colonies may be seen of which those which are larger and have dark fibrils in the centre are *M. agalactiae*.

A NEW APPROACH FOR *MYCOPLASMA BOVIS* SERODIAGNOSIS.

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Mycoplasma bovis is considered one of the most pathogenic bovine mycoplasmas, causing significant economic losses in areas with intensive dairy and meat production. In absence of an effective antibiotictherapie or vaccination, the only strategy currently available to prevent *M. bovis* outbreaks in herds is the early removal of infected animals. A rapid detection of animals that have been in contact with the pathogen is therefore a crucial step requiring sensitive and specific diagnosis approaches. Preliminary data have shown that serum antibodies from animals naturally infected with *M. bovis* reacted with major epitopes carried by components belonging to a family of abundant surface lipoproteins, the Vsps. So far, three such lipoproteins, namely VspA, VspB and VspC have been characterized and shown to oscillate in expression among and within *M. bovis* strains. To evaluate the potential of Vsp epitopes as target candidates in serological tests, sera from experimentally or naturally infected herds were tested in Western blot for their ability to recognize *M. bovis* antigens and more specifically those corresponding to the Vsps.

For this purpose, Western blots were performed using total proteins of three clonal variants derived from the PG45 type strain, each expressing a well-defined but distinct Vsp phenotype. Hyperimmune sera were collected from four different geographical locations during natural outbreaks in herds presenting distinct clinical manifestations or from animals experimentally infected either by inoculation or by contact. Results showed that despite their variability, the Vsps and in particular VspA and VspC, are the major components that trigger the bovine humoral response. In experimentally infected calves, serum antibodies directed towards the Vsps appeared within an average of 10 days following the inoculation, but also as early as 6 days. This study was extended to more than 100 sera and showed that the presence of anti-Vsp antibodies in bovine sera was independent of (i) the clinical manifestations observed during the infection, (ii) the field isolate or strain responsible for infection, (iii) the mode of infection, (iv) the animal history, and (v) the geographic location of natural outbreaks. These data demonstrated that Vsps may provide highly sensitive target antigens in serodiagnosis assays.

PATHOLOGY OF INDUCED *MYCOPLASMA BOVIS* CALF PNEUMONIA IN EXPERIMENTALLY VACCINATED ANIMALS

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INTRODUCTION

Vaccination, using a whole cell preparation of *Mycoplasma bovis*, has been shown to confer some level of protection from respiratory tract colonisation by the organism under experimental conditions (1), and to contribute to a reduction in severity of field outbreaks of respiratory disease when used in an experimental multi-valent vaccine also containing antigens of viral respiratory pathogens (2). The present study explored an hypothesis that improved vaccine protection for *M. bovis* could be stimulated by using more defined mycoplasma-specific antigens for vaccination. This is on the basis that the non-specific media contaminants in whole cell vaccine preparations, and in particular the serum proteins, might interfere with the host response to mycoplasma-specific antigen. This could occur in two ways. Firstly, by being highly antigenic themselves, they might cause a dilution of the overall immune response to mycoplasma antigens. Secondly by being attached to mycoplasma cell surface membranes, they could physically interfere with host immune effector mechanisms

For the present study, two approaches were used for the production of mycoplasma specific antigen from cultures of *M. bovis*. The antigenic extracts were used to vaccinate calves prior to challenge inoculation with the viable organism. The experimental objectives were to determine whether immunisation using the various antigenic preparations could protect calves against *M. bovis*-induced respiratory disease, and to study clinical and pathological responses in control and vaccinated calves.

METHODS

A total of eleven colostrum fed calves was used. Prior to the first vaccination, calves had very low or zero antibody titres to *M. bovis*.

Two antigenic preparations were used for vaccination. The first derived from a sonicated concentrated culture of *M. bovis* which was extensively washed with a Tris/EDTA buffer, pH 7.2, before being extracted with Triton X-114 (3). The hydrophobic membrane protein Triton X-114 fraction was used to vaccinate. The second preparation was a mixture of four antigens purified by means of monoclonal antibody (MAb) affinity columns. The antigens purified consisted of three non-variable antigens of approximate molecular weights of 5, 30 and 37 kD and one

variable surface antigen, Vsp A. The non-variable antigens were all shared with *M. agalactiae*.

Four calves (Group H) were vaccinated with the first preparation and three (Group Af) with the second. The vaccination regime consisted of three subcutaneous inoculations at fortnightly intervals; Quil A (250µg per inoculum: Superfos, Vedback, Denmark) was used as the adjuvant on each occasion. The first immunisation was given when the calves were three weeks old. Three calves comprising a control group (C) were not vaccinated. Three weeks following the final immunisation, all calves were challenged by combined intranasal and intratracheal inoculation of a recent field isolate of viable *M. bovis*. Inoculations were carried out on two consecutive days and the total challenge dose was approximately 1.4×10^9 cfu intratracheally and 0.7×10^9 cfu intranasally, per day.

Clinical examinations were carried on four occasions prior to challenge, and on a daily basis post challenge. At each examination, a clinical score was awarded to each calf on the following basis: dullness = 1, tachypnoea = 1, laboured abdominal type breathing = 2, fever = 1.

One calf in the Af group developed severe respiratory distress on post-infection day (PID) 2 and was euthanised at this time on humanitarian grounds. The remaining 10 calves were necropsied between PID 13 and 14. At necropsy, respiratory tract tissues were taken for pathological, microbiological and immunocytochemical examinations.

RESULTS

Clinical Findings

No signs of respiratory disease were noted prior to challenge. Immediately post challenge several calves (both immunised and controls) exhibited transient tachypnoea, but respiratory rates returned to normal within a few hours. On PID 2, one calf in Group Af developed an acute respiratory distress syndrome and was euthanised. The respiratory signs noted in the remaining calves over the two weeks post-challenge period ranged from very mild to moderate in severity. Respiratory signs were more severe in both groups of immunised calves than they were in the control animals. The mean clinical score of the immunised calves over the period from PID 3 to PID 13, was 10 compared with 2.75 for the control calves.

Gross Pathological Findings

Negligible consolidation was present in the lungs of the three control calves.

In Group H, mild to moderately severe consolidation was present in three of the four calves. Nodular lesions were seen within the consolidated areas and the lesions had a cranioventral distribution in the lungs (Fig 1).

The calf from Group Af sacrificed on PID 2 had dark red zones of lobular consolidation scattered throughout both cranial and caudal lung regions. There was also pulmonary oedema and severe interstitial emphysema, with bulla formation in the caudal lobes. Moderately severe pulmonary consolidation was present in the remaining Group Af calves killed on PID 13-14. Most consolidation was present in the caudal lobes (Fig 2).

Histopathological Findings

Only very mild histopathological changes were present in the lungs of the three control calves and consisted primarily of lymphoid hyperplasia around airways.

Histopathological findings in the Group H calves were dominated by the presence of numerous granulomata. These contained a central zone of coagulative necrosis and/or degenerate leucocytes, surrounded by a zone of "epithelioid" macrophages and occasional giant cells (Fig 3a). Large quantities of *M. bovis* antigen were detectable by immunoperoxidase staining in the central necrotic zones, several of which were calcified (Fig 4). Granulomata often appeared to be "centred" on airways. Other pathological findings in the Group H calves included severe parenchymal and interlobular fibrosis, bronchitis with considerable mural damage and broncho-interstitial pneumonia.

Histopathological findings in the Group Af calf sacrificed on PID 2, consisted of scattered areas of acute broncho-interstitial pneumonia with a widespread severe alveolitis, the latter especially marked in the caudal lung lobes (Fig 5). In the two remaining Group Af calves killed PID 13-14, the histopathology was dominated by a severe interstitial alveolitis with mononuclear cell infiltration of alveolar walls and pneumocyte hyperplasia (Fig 6). This was most severe in the caudal lobes. Areas of broncho-interstitial pneumonia with lymphoid hyperplasia were also present. *M. bovis* antigen was detectable by immunoperoxidase staining in only one of the calves in this group, and was noted only in a small number of airways.

Microbiological findings

M. bovis was isolated from the upper respiratory tract of three of the four control calves. In only one of the three upper respiratory colonised calves was the organism recovered from the lungs.

In Group H, *M. bovis* was isolated from upper respiratory tract and lungs of the three calves in which pulmonary lesions were present, and from neither region of the remaining calf in which lung lesions were not present.

M. bovis was isolated from the lungs of the Group Af calf killed on PID 2. Recovery was also made from the upper respiratory tract of both Group Af calves killed PID 13-14 and also from the lungs of one of these calves.

No bacterial respiratory pathogens were recovered from the lungs of any calf. Examination of the lungs for bovine respiratory syncytial virus, using an immunoperoxidase technique proved negative (4).

Calves in the vaccinated groups developed a serological serum and lung secretary immune response to the respective vaccine constituents, detectable by direct ELISA.

CONCLUSIONS

Under the conditions of the present study, vaccination of calves with the hydrophobic membrane protein fraction or with the mixture of affinity purified antigens, did not prevent induced disease. Indeed, vaccination of the calves appeared to enhance lung pathology and clinical signs although caution must be

exercised in view of the small group sizes. Also, variable histopathological patterns of pneumonia were observed with the different antigenic preparations.

The nature of the granulomatous lesions observed in the Group H calves is suggestive of involvement of a Type IV hypersensitivity reaction. Such granulomatous lesions are also observed in a proportion of naturally occurring cases of calf pneumonia associated with *M. bovis* in Northern Ireland. There was also a surprisingly severe degree of pulmonary fibrosis in the lungs of the calves in Group H, considering that the time interval from first challenge infection to necropsy was only 13-14 days.

The lung lesions seen in Group Af, particularly the severe interstitial alveolitis present in the animals killed on PID 13-14, have some similarities to extrinsic allergic alveolitis conditions of man and animals. A well known example of extrinsic allergic alveolitis is "farmers lung" resulting from exposure to fungal antigens in mouldy hay, in which both Type III and Type IV hypersensitivity mechanisms are believed to contribute to lung damage (5). Interstitial alveolitis of varying severity is a frequent lesion in natural outbreaks of calf pneumonia and can be associated with responses to a range of infectious agents.

The challenge regime used in this study was a particularly severe one in terms of the number of organisms administered, the combined respiratory route used and the repeat inoculations given on consecutive days. However in planning the experiment it was considered important to seek to induce significant lung pathology at least in the control calves, so that any protective effect in the "vaccinates" could be appreciated. In the event, several of the experimental findings were unexpected, not least the absence of significant lesions in the lungs of any of the four control calves.

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Fig. 1 Nodular consolidation in the lungs of Group H calf.
Fig. 2 Consolidated zones within caudal lobe of Group Af calf.
Fig. 3 Granuloma type lesion with central necrosis in lungs of Group H calf.
Fig. 4 *M. bovis* antigen within central necrotic areas of granuloma type lesions (immunoperoxidase).
Fig. 5 Acute alveolar reaction in the Group Af calf killed on PID 2.
Fig. 6 Interstitial alveolitis in Group Af calf killed on PID 13.

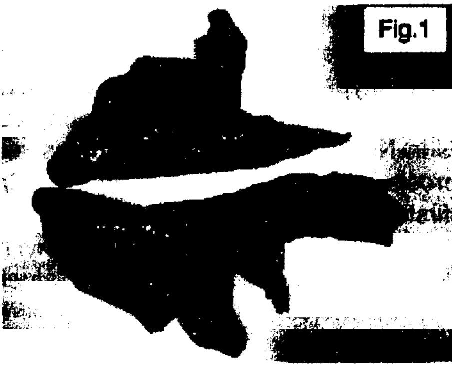
Fig.1

Fig.2

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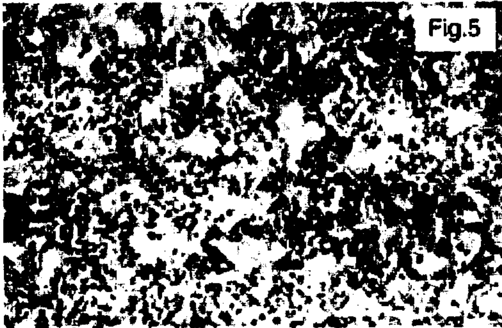
Fig.5

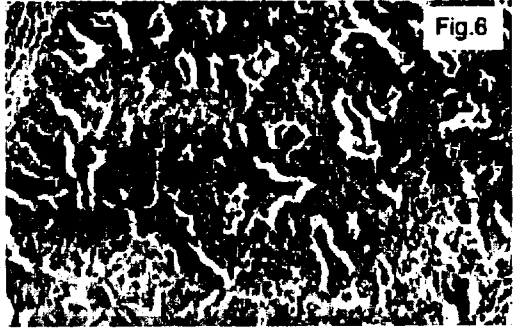
Fig.6

ISOLATION OF *MYCOPLASMA BOVIS* FROM THE ABOMASAL CONTENTS OF AN ABORTED BOVINE FOETUS

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INTRODUCTION

Mycoplasma bovis has been isolated from clinical cases of respiratory disease (1), mastitis (2), and arthritis (3, 2) occurring in calves or adult cattle in the Republic of Ireland, as also reported previously in many other countries worldwide (4). To the best of the authors' knowledge, this is the first report of an isolation of *M. bovis* from the abomasal contents of an aborted bovine foetus in either the Republic of Ireland or the U.K..

This case occurred in a small dairy herd comprising of 12 milking cows, and a bull. A two year old replacement heifer was purchased, approximately seven months earlier, from a local farmer, and was served by the bull. It was housed in the same group as five other heifers. It aborted after six months gestation without any other clinical signs of disease. The foetus was examined post-mortem and a sample of abomasal contents was collected aseptically and submitted for laboratory examination. Subsequently a serum and a milk sample were collected from this cow.

MATERIALS AND METHODS

Blood agar (Lab M) with bovine blood, Albimi agar (Difco), and Desoxycholate Citrate agar (Oxoid CM35) plates were inoculated with the abomasal contents and were incubated in 8% CO_2, at 37° C for five days. The serum sample was tested for antibodies to; *Brucella abortus* by serum agglutination, I.B.R. by serum neutralisation, *Neospora caninum* by immunoflourescent antibody (VMRD, Inc.), *Salmonella dublin* and *S. typhimurium* by tube agglutination, and *Leptospira interrogans* serovar *hardjo* by microscopic agglutination test.

Mycoplasma agar plates, using an Oxoid PPLO agar base, were used for selective mycoplasma culture and were incubated in 5% CO_2 at 37° C. An enrichment and capture procedure, using a monoclonal antibody based sandwich enzyme linked immunosorbant assay (ELISA) technique (5), was employed for selective *M. bovis* culture and identification.

An indirect ELISA was used to detect antibodies to *M. bovis* in the serum sample from the cow.

RESULTS

There were no significant antibody titres to *Brucella abortus, Neospora caninum, Salmonella dublin, S. typhimurium, Leptospira hardjo,* nor I.B.R. No bacteria were isolated from the abomasal contents. A fine transparent growth was noticed on the blood agar associated with an area of partial haemolysis. This growth was sub-cultured onto mycoplasma agar plates and mycoplasma colonies were observed after three days. This mycoplasma isolate was identified as *M. bovis* by

the enrichment and capture ELISA test. Direct mycoplasma culture of the abomasal contents also yielded *M. bovis*. *M. bovis* was not detected in the milk sample collected from the cow. Antibodies to *M. bovis* were detected in the serum sample. No other disease problems have been reported in the dairy herd since the abortion occurred.

CONCLUSIONS

M. bovis was the only infectious agent identified from this case of bovine abortion. This organism is a recognised bovine pathogen, which is capable of causing mastitis (6) and arthritis (7, 8, 9) ; it has also has been associated with specific pathological changes in the bovine respiratory tract (10, 11). *M. bovis* can also invade the bovine bloodstream (10). In addition it has also been isolated from the bovine reproductive tract (12), and from the tympanic bullae of calves with exudative otitis media (13).

M. bovis has been detected in cattle herds in many other countries for over twenty years (4). However, isolation of *M. bovis* from foetal tissue or foetal abomasal contents is seldom reported. An incidence of *M. bovis* in aborted foeti of 2.4% (6 out of 256 aborted foeti examined) was reported in Canada (12). *M. bovis* was isolated from aborted foetal and stillborn calf tissue, embryonic membranes, and from uterine and vaginal discharges during an investigation of reproductive disorders including abortion, stillbirths, retained placenta, and endometritis in a newly formed dairy herd in Hungary (14).

Salpingitis and endometritis have been reported in the reproductive tract of experimentally infected non-pregnant heifers (15). Experimental infection by intrauterine inoculation via laparotomy, of pregnant cows has resulted in abortion (16).

In this case the foetus and placenta were not submitted to the regional veterinary laboratory. Therefore, it was not possible to examine these for pathological changes. However, normal foetal abomasal contents are usually free of *M. bovis* (12), and are usually sterile. Therefore, the isolation of *M. bovis* in pure culture, from an aseptically collected sample of abomasal contents of an aborted foetus may be considered as a significant isolation. The absence of any other known abortafacient in tests carried out and the previously demonstrated ability of *M. bovis* to cause abortion following experimental infection, together with its known pathogenicity in the bovine mammary gland, synovial joints, and respiratory tract, implies that *M. bovis* should be considered as the most likely cause of this abortion.

In many veterinary diagnostic laboratories routine abortion investigation may not always include selective culture for mycoplasma species. Therefore, the relative significance of *M. bovis* as a possible cause of bovine abortion remains unknown. However, it is worth noting that *M. bovis* is detectable on blood agar plates incubated in 8% CO_2, as an area of haemolysis in the absence of any macroscopically visible bacterial colonies. Further culture of abomasal contents of bovine aborted foeti is ongoing to determine the incidence of *M. bovis* in aborted bovine foeti in Ireland.

Acknowledgements

The authors would like to thank Neall Brice of the Veterinary Sciences Division for typing this isolate and for mycoplasma serology, Jim Bradley, David

Kelly and the veterinary practitioner for facilitating this investigation, and the staff of the virology division in Abbotstown for virus serology.

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ENHANCEMENT OF *MYCOPLASMA BOVIS* DETECTION IN MILK SAMPLES BY ANTIGEN CAPTURE PRIOR TO PCR

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Mycoplasma (M.) bovis, the causative agent of mycoplasma mastitis in cattle, is difficult to identify in shedders or animals at the subclinical stage, i.e. in circumstances where it occurs at low titres. While PCR is known to facilitate high sensitivity of detection in various biological fluids and tissues, milk samples present particular problems due to high protein concentration and micellar structures. Therefore, time-consuming and rather expensive sample pretreatment is usually necessary to remove or neutralize DNA polymerase inhibitors from the matrix.

In the present work, a method was worked out which significantly reduced the extent of pre-PCR operations and at the same time permitted high sensitivity of detection. Milk samples were transferred into microtiter plates, the wells of which had been coated with a monoclonal antibody (MAb) to *M. bovis*. Overnight incubation of the plates facilitated two effects: 1) specific antigen capture due to the MAb, and 2) cultural enrichment of germs. After washing, bound mycoplasma cells were solubilized and phenol-extracted.

Detection was accomplished by PCR using *M. bovis*-specific primers. The whole procedure takes 16 hours. Because of the combination of cultural enrichment with specific antigen capturing the sensitivity of detection was significantly increased, so that we were able to detect 1 cfu mycoplasmas per ml milk. Since the procedure is carried out in microtiter format there is a possibility of automation.

COMPARISON OF FOUR SAMPLING PROCEDURES FOR IDENTIFICATION OF RESPIRATORY MYCOPLASMAS IN CATTLE

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INTRODUCTION

Most mycoplasmas species are harmless but some can be responsible for mild to very severe respiratory diseases. It leads oftenly to a dilemna when recovering such a species from clinical sample.

The purpose of this study is to compare recovery rates of various mycoplasmas species in in the nose and in the lower respiratory tract of healthy and diseased calves. Four different sampling procedures were used : nasal tampon (NT), nasal swab (NS), transtracheal aspiration (TTA) and bronchoalveolar lavage (BAL).

MATERIALS AND METHODS

Animals

First group.

Twenty-four Friesan healthy male calves were involved in this study. They were 119 ± 12 days old and weighed 113 ± 23 kg. They were colostrum-deprived and have undergone six bronchoalveolar lavages at all.

Second group.

Nine Belgian White and Blue double-muscled have presented mild respiratory signs (chronic cough and moderate nasal discharge). Four were treated with Baytril® and Mycotil® up to ten days before collecting samples.

Sampling procedures

Nasal samples.

Two different techniques were investigated.
In the first group, a tampon was put into the nose during twenty minutes and the nasal fluid was collected by centrifugation (1000g during 5 minutes).
In the second group, a sterile swab was introduced into the nose and mixed in broth as soon as possible.

Transtracheal aspirations.

Only the animals from the first group have undergone transtracheal aspirations. After shaving and disinfection of the skin, a sterile catheter (Vygon, Ecquen, France) was introduced into the trachea through a trocard. Fifty millilitres of PBS were injected and re-aspirated.

Bronchoalveolar lavages.

In the first group, they were performed with a sterile X.R.O. gastroduodenal dual flow tube® (Vygon, Ecquen, France). Fifty millilitres of PBS were injected and re-aspirated at the same time.

In the second group, lavages were performed with a sterile one flow catheter. Fifty millilitres of Ringer® were injected and immediately re-aspirated.

Identification procedure

A method of species identification of ruminant mycoplasmas by dot immunobinding on membrane filtration (MF dot) has been described by Poumarat et al. (1).Briefly, mycoplasmas from broth cultures (5% CO_2 - 37°C - 3 to 7 days) were directly trapped, by vacuum filtration, onto the surface of low-protein-binding affinity membranes of 0,22 μm pore size. Hyperimmune sera against reference strains (*M. bovis*, *M. bovigenitalium*, *M. arginini*, *M. bovirhinis*, *M. alkalescens*, *A. laidlawi*) were used.

RESULTS

First group.

1. *M. bovirhinis* is the only species found inhealthy calves. Its prevalence in the nose or in the lower respiratory tract is very high (96%) and confirms the results of ter Laak (88%) (2).
M. bovirhinis is more frequent in the BAL (92%) and in the TTA (79%) than in the nasal samples.

2. Our technique used for collecting nasal samples have found *M. bovirhinis* in only 38% of the cases.
In 17% of the cases, neither nasal tampons nor transtracheal aspirations have found *M. bovirhinis* even if bronchoalveolar lavage succeeded in it.

3. Even if 4% of the BAL are mycoplasmas-free when the corresponding TTA are positive, 17% of the animals are BAL positive when their TTA is negative. Such results, showing a possible nasal contamination, are sustained by classical bacteriology : 21% of the TTA are sterile against 4% for the BAL.

Second group.

1. The bronchoalveolar lavages and the nasal swabs have shown the same prevalence of *M. bovirhinis* in diseased calves (67%).

2. Two other species have been isolated : *M. arginini* from BAL (11%) and *M. bovis* from nasal swab (11%).

CONCLUSIONS

1. Healthy and diseased calves are frequently infected by *M. bovirhinis.*

2. *M. bovirhinis* is more frequent in lower than upper respiratory airways.

3. The ideal method to investigate the lower repiratory tract is the transtracheal aspiration avoiding nasal contamination. In practice, however, TTA is more invasive than BAL. The BAL may thus represent a good compromise between laboratory and field requirements.

ACKNOWLEDGEMENTS

This research was possible thanks to the grant from "Ministère des Classes Moyennes et de l'Agriculture - Administration Recherche et Développement - Convention 5792A section 1".

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Table 1. Isolation of *M. bovirhinis* from healthy calves (n=24).

	Nasal tampons	**Transtracheal aspirations**	**Bronchoalveolar lavages**
n = 1	-	-	-
n = 1	+++	+++	-
n = 4	-	-	+++
n = 8	+++	+++	+++
n = 10	-	+++	+++
n = 24	9+++	19+++	22+++

+++ strong reaction
- no reaction

Table 2. Isolation of mycoplasmas species from diseased calves (n=9).

	Nasal swabs	**Bronchoalveolar lavages**
M. bovirhinis n = 6	+++	+++
M. bovis n = 1	+	-
M. arginini n = 1	-	++
n = 1	-	-

+++ strong reaction
++ average reaction
+ weak reaction
- no reaction

EXAMINATION OF ANTIBODY RESPONSE OF COWS AND THEIR CALVES TO M. BOVIS BY WESTERN BLOT

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INTRODUCTION

Mycoplasma bovis infection is one of the causes of considerable economic losses in dairy herds. This organism can be responsible for the calf pneumonia and arthritis and in many cases it can be isolated from severe mastitis and genital disorders (5).

There are surface antigens of M. bovis which are high frequency phase variable and can be altered by the antibodies of the host animal during the immune procedures. (3, 4, 6)

In this short study we intended to examine what kind of mycoplasma polypeptides induce immune response in cows and whether the same immune profile appears in the sera of their calves.

MATERIALS AND METHODS

Blood sera were collected from three to five-day-old calves and their mother in a dairy herd endemically infected with M. bovis. Antigens were made from the M. bovis "MB1" strain and from a field isolate cultured from the same herd. The cultures were centrifuged and washed with PBS three times at 12,000 RPM. Mycoplasma cells were denatured in a solution of 125 mM Tris, 10% v/v 2-beta-mercaptoethanol, 20% v/v glycerol pH 6.8 and a 4% w/v solution of sodium dodecyl sulfate. The samples were boiled in separate Eppendorf tubes for 4 minutes. A 12.5 % SDS–polyacrilamide gel was prepared. The samples were stained with 0.1 % bromophenol blue. The amount of mycoplasma protein loaded into the long track was approximately equivalent to the content of 10^8 cells. The gel was electrophoresed at 180 V for about 4 hours. We used the SIGMAMARKER Wide Range molecular weight standard to compare the size of the polypeptides (1)

The proteins were transferred onto 0.45 μm nitro-cellulose membrane (Sigma Chemicals) using a wet blotting chamber. The power voltage was 30 V for 12 hours. The membrane was cut into 3-mm thick stripes, and the free surfaces were blocked by 1 ml 5% solution of non-fat milk powder (1 hour, 37 °C).

250 μl from the tested sera were incubated with each of the prepared stripes, leaving the milk solution inside the trays (4 hours, 37 ° C). Then the stripes were washed 2× with PBS, 2× with 0.05 % tween-20 (Serva) PBS, and then 2× with PBS again. After washing 40 μl, 1:100 diluted horseradish peroxidase labelled anti-bovine

IgG conjugate (Sigma Chemicals) in 1ml of milk solution was added to the stripes, and incubated at 4 ° C, overnight. After repeating the aforesaid washing procedure we stained the stripes with Zymogram staining. (2). We used the serum of a previously infected cow as a control.

RESULTS

There were several polypeptides against which antibody production was detected. The following ones occurred at a relative high frequency: p73, p63, p46, p42, p38-40 and p32-34. Comparing the Western blot profiles of cow sera with the sera of their calves the following was observed:

1. There were cases where a great similarity was observed between the two profiles. In these cases antibodies against almost all major polypeptides of M. bovis were present in both of the profiles.

2. In other cases there was not any antibody reaction against the polypeptides of M. bovis in both profiles.

3. Besides the above-mentioned examples we have found sometimes relatively strong reaction with the serum of the cow meanwhile the serum of her own calf showed poor reaction or the response was absent.

4. There were pairs where the cow and the calf showed totally different immune profiles.

DISCUSSION

The results indicate supposed colostral immunity. The cases where the profiles were similar suggest a usual intake of colostrum of the adequate cow. Therefore the maternal globulins could be normally absorbed.

In those where both of the animals showed no reaction the most probable answer is that the cow remained intact from mycoplasma infection, therefore there was not any specific immune globulin that could be transmitted in the colostrum.

The calves, which showed no reaction meanwhile the sera of their mother was positive with full profile, possibly had not get colostrum at all or the first suckling time was so late when the absorption of the maternal immunoglobulins was impossible.

The explanation of the phenomenon -where the immune-profile of the calves was totally different than the one of their own mother- can be that they had got colostrum from another cow, or the colostrum was mixed.

These variations of immune response can give explanation of various clinical and epidemiological appearances of the disease due to M. bovis infection in endemically infected herds.

These results indicate some facts affecting the prevention and the control of calf pneumonia. As well as in case of other bacterial or viral diseases it is essential to give the colostrum within the time the globulins can be absorbed.

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EPIDEMIOLOGICAL STUDY OF MYCOPLASMA INFECTIONS IN CALVES

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INTRODUCTION

This study is continuation of our previous studies dealing with monitoring of mycoplasma infections in cattle in the Czech Republic (1,2). Respiratory disorders are one of the most frequent health problems in calves. This syndrom has many etiological factors and is widespread in our republic. Aetiological factors responsible for these disorders are various species of viruses and bacterias, including mycoplasmas (3, 4). But the other factors that contribute to this diseases and that shouldn´t be underestimated are housing and climate factors (i.e. origin of animals, number of animals in a herd, the density of population, temperature, ventilation, etc.).

The great economic losses in calves due to severe respiratory disorders in one farm in South Moravia were the cause of our taking part in the project attempting to solve this problem. The complex epidemiological, clinical and laboratory examinations were carried out to determine the causative agent.

MATERIALS AND METHODS

A/ Description of the farm

The farm is central calf – house having the capacity of 241 animals and serves for separating of young stock from the mother.The calves are kept here in separated sections divided by wooden barriers during their pre – fattening period. After finishing of this period the calves are transferred to another place – the fattening farm - in the neigbouring village. This fattening farm is destinated as the checking station for efficiency testing of bulls´ progeny (male calves) - the Czech red - spotted breed. The efficiency testing is the cause of choosing and transportation of the calves from large area, comprising approximately 80 farms. But high morbidity (55%), mortality (10%) and lost of body weight became limiting factors of the farm production in the last time, including the animals in the checking station. The young calves suffer by respiratory disorders of different severity and diarrhoea is also sometimes observed during the period of their milk nutrition.

B/ Description of experimental animals

One section of newly arriving calves, comprising 32 animals, was used as an experimental group. This group was divided into 2 subgroups that differ in the type of antibiotic medication of the feed used in critical periods of their pre- fattening time. Both subgroups of calves were clinically examined, including the sampling immediately after their arrival. Further clinical observations and laboratory examinations were repeatedly carried out during following 5 months.

C/ Types of investigations

Following investigations were performed in the experimental group of calves : clinical, biochemical, haematological, parazitological, pathological and histological, zoohygienic and microbiological.

Our laboratory was responsible for providing of mycoplasmological examinations in this project.

This work was devoted to the onset and spreading of mycoplasma infections and was divided into three chronological parts:

1) detection of mycoplasmas and the frequency of their onset in the calves in the dependence upon the used antibiotic therapy (this part is the subject of the presented poster)

2) identification of isolated species of mycoplasmas with special regard to the M. bovis (it will be carried out by epi-imunofluorescence test and PCR)

3) search of the sources of the origin of mycoplasma infections in the calves in the parental herds of cows

D/ Sampling and isolation of mycoplasmas

Nasal swabs were taken from nasal cavities from all experimental calves and were placed into tubes with transport medium. Samples were store frozen (-80° C) until their culture The stored samples were thawed before culture and inoculated into modified liquid (with arginin, glucose and/or urea) and solid medium according to Hayflick (5)

RESULTS

Results of our culture examinations of the 1st sampling revealed that 30% of the calves were mycoplasma free.This sampling was performed immediately after the arrival of the calves to the calf - house and before the beginning of antibiotic medication of the feed. But the culture examination of the 2nd sampling, carried out 7 days after the arrival of the calves showed that all animals were already mycoplasma positive. Immediately after the 2nd sampling antibiotic medication of the feed was started. The calves No. 1 - 16 were treated by the preparate „A“ (commonly used in this farm) and calves No. 17 - 32 were treated by Geotrim P plv. ad us. vet. As it is evident from our results either type of medication or typ of individual antibiotic therapy given later has no permanent mycoplasmacide effect. Only the assessment of the culture examination of sampling No.5 indicated weak inhibition effect in calves No. 16, 26 and 27. But the culture examination of the last sampling (No. 6) was positive in all calves.

The antibiotic therapy has no long - term effect on health status of these calves. The range of morbidity remained without change and two cases of the death (calves No. 2 and No. 22) were recorded (due to severe bronchopneumonia).

Results of culture examinations in the whole group of experimental calves

Calf No.	Sampling No. 1	Sampling No. 2	Sampling No. 3	Sampling No. 4	Sampling No. 5	Sampling No. 6
1	+	+	+	+	+	+
2	+	D				
3	-	+	+	+	+	+
4	-	+	+	+	+	+
5	-	+	+	+	+	+
6	+	+	+	+	+	+
7	+	+	+	+	+	+
8	+	+	+	+	+	+
9	+	+	+	+	+	+
10	-	+	+	+	+	+
11	+	+	+	+	+	+
12	+	+	+	+	+	+
13	+	+	+	+	+	+
14	+	+	+	+	+	+
15	+	+	+	+	+	+
16	+	+	+	+	-	+
17	+	+	+	+	+	+
18	-	+	+	+	+	+
19	+	+	+	+	+	+
20	-	+	+	+	+	+
21	-	+	+	+	+	+
22	-	+	D			
23	+	+	+	+	+	+
24	+	+	+	+	+	+
25	+	+	+	+	+	+
26	+	+	+	+	-	+
27	-	+	+	+	-	+
28	+	+	+	+	+	+
29	+	+	+	+	+	+
30	+	+	+	+	+	+
31	-	+	+	+	+	+
32	-	+	+	+	+	+

+ = positive mycoplasma isolation
- = negative mycoplasma isolation
D = death

CONCLUSIONS

According to the negative bacteriological and virological examinations of involved laboratories in this complex study, except occasional isolation of P. multocida in affected and P. haemolytica in dead animals, we can presume that presence of mycoplasmas in the calves with respiratory disorders might be of some aetiological and epidemiological significance in this farm.

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USE OF ELISA "CIVTEST *MYCOPLASMA AGALACTIAE*" FOR THE DETECTION OF SPECIFIC IGG ANTIBODIES IN SHEEP VACCINATED WITH AG1 STRAIN.

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INTRODUCTION

Serological tests, as unique or main method for the diagnosis of Contagious Agalactiae in live animals, are under discussion by the scientific community (2;5); the evaluation of the antibody response of small ruminant against Mycoplasma spp and particularly against *Mycoplasma Agalactiae* is crucial for laboratories working on vaccine production.

Problems regarding sensitivity and specificity of different test – CFT and ELISA - used as a tools for control/eradication programs have put in evidence the limit of their use in the field if not supported by other profilaxis measures (3). The possibility of utilisation of CFT or ELISA on vaccine control have been studied at I.K.V.; a method to standardise the production have been implemented to ensure the Albanian Veterinary Services of the quality of the vaccine used in the national control program.

MATERIALS AND METHODS

An ELISA test, Civtest (1), produced by HIPRA, has been used in 200 sheep to control the IgG antibodies response to vaccine prepared at I.K.V. using AG1 strain.(2).
At I.K.V. 20 sheep, as experimental group, were controlled at Civtest before vaccination and 21 and 90 days after vaccination with AG1 strain; 2 flocks of Tirana District, respectively of 80 sheep (flock A) and 100 sheep (flock B), in field conditions, were clinically examined and found negative for signs referred to Contagious Agalactiae, flocks A+B, vaccinated with AG1, were serologically controlled at Civtest after 21 and 90 days.

AG1 vaccine is a live broth culture vaccine; the potency of the batch used in this experimental study was 5-7x 10^7/ml, mouse were vaccinated subcutaneously with double sheep doses for safety control.
Civtest is based on the principle of the enzyme immunoassay (EIA) using an inactivate specific antigen. The test was carried out following the method described by HIPRA company (1). Corrected Negative Control O.D was 0,155. Corrected Positive Control O.D was 1,349. The ratio between the Positive Control mean P and Negative Control mean N was 17,5.

Civtest ELISA detects IgG class of immunoglobulins which is an advantage for the evaluation of the immuno response of vaccinated animals: in fact IgM appears first in serum but soon IgG dominate (4).

RESULTS

According to the Civtest procedure, the positive result is when sample O.D is over 0,240 . All the sheep, after vaccination, presented a positive antibody response. Experimental flock: the O.D average of 20 sheep before vaccination was 0,077 . In Figure 1 are presented test results 21 and 90 days after vaccination: the O.D average of 20 sheep vaccinated was 0,732 up to 1.031 after 21 days and 0,457 up to 0,657 after 90 days .

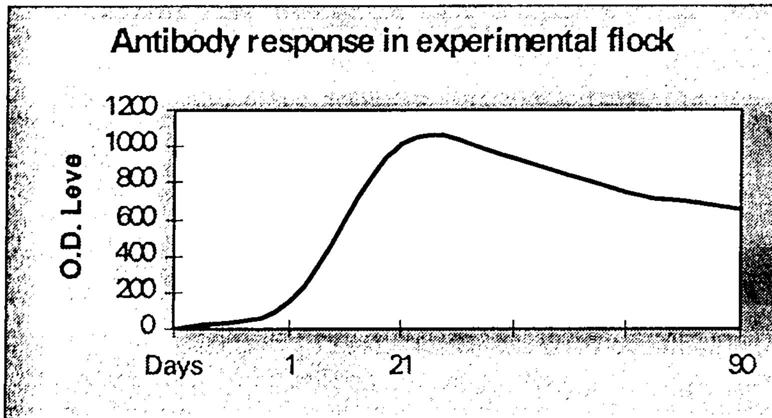

Figure 1
The O.D of both the 2 flocks of Tirana district, respectively of 80 head (flock A) and 100 head (flock B), in field conditions, was 0,457 up to 0,657 .

Flock A: the O.D average of 80 sheep before vaccination was 0,107
In Figure 2 are presented test results 21 and 90 days after vaccination: the O.D average of 80 sheep was 0,998 up to 0,746 after 21 days and 0,495 up to 0,457 after 90 days .

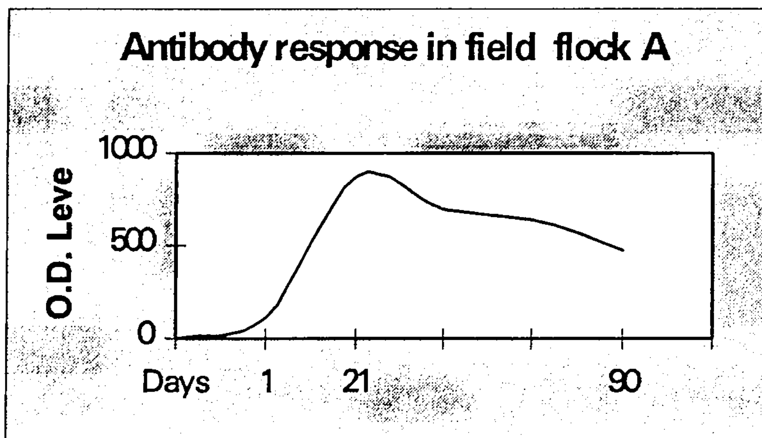

Figure 2
Flock B: the O.D average of 100 sheep before vaccination was 0,099.

In Figure 3 are presented test results 21 and 90 days after vaccination: the O.D average of 100 sheep was 0,897 up to 0,780 after 21 days and 0,552 up to 0,502 after 90 days .

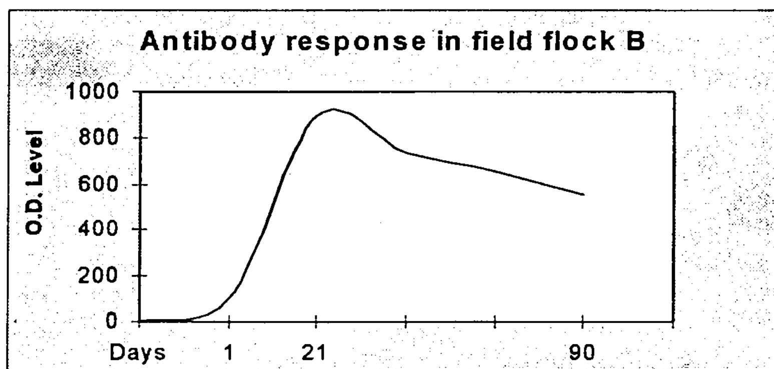

Figure 3

Comparison of the result of all the flocks at ELISA Civtest shows that the antibody response against AG1 vaccine was present in all vaccinated animals - O.D. average > 800 at 21 days - and that after 90 days O.D. average was around 0,500, see tab 4.

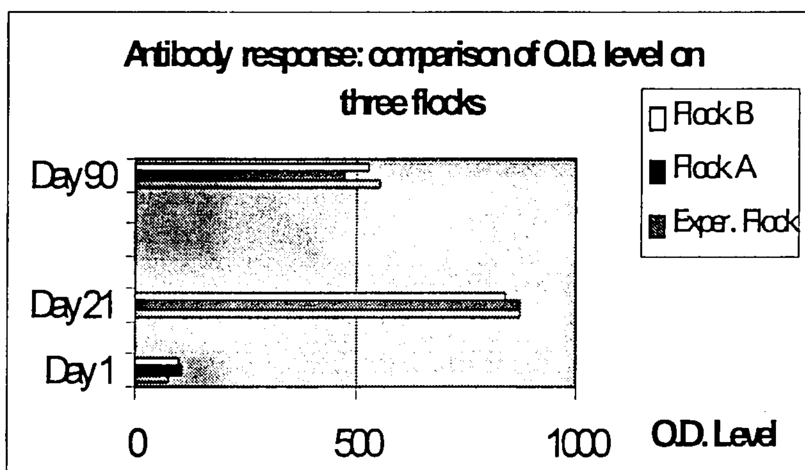

Figure 4

Discussion

Considering the actual problems linked to the difficulties to manage animal movements and serological surveillance in the country, the Albanian Veterinary Services are using AG1 vaccine to control Contagious Agalactiae in the country.

This activity started on the 1960s and the epidemiological data confirm the effectiveness of this strategy. According to the isolations made at I.K.V., up to day *Mycoplasma aalactiae* is considered the unique strain responsible of the disease in Albania. The protocol of intervention is based on two vaccination per year but for several reasons not all the farmers have followed the above instructions: some vaccination ruptures have been recorded. As for other Mycoplasma diseases, broth live vaccine against C. Agalactiae is used more as a *(short)* protection than to determine a long immunity response. The time life of the IgG immunoglobulins response is linked to the quality production of vaccine, particularly to the potency.

This control – every batch should have not minus than 10^7 cells /ml – may be accompanied by a routine serological control on sheep. The ELISA Civtest has demonstrated its utility in this vaccine quality control.

In consideration of the relationship between vaccine potency and antibody response, epidemiological situation, serological surveillance, at 21 days after vaccination O.D. range $\geq$ 0,500 should be considered as a limit to deliver a new vaccine production.

The ELISA Civtest may contribute to evaluate the time-life of IgG in order to indicate to the Veterinary Services the right number of vaccinations to be made per year in infected area: in fact the curve of the antibody response at 90 days is at the limit of the above mentioned O.D. range.

Next control, on June 98, of IgG at 6 months will better suggest the strategy to be adopted in future.

As last conclusion it is necessary to remind the needs of a new vaccine production strategy in order to have a long protection or immunity; in the meantime, considering the risks of the transmission of other diseases, such as scrapie, it is opportune to suggest to stop any C. Agalactiae vaccine production starting from brain or biological products not controlled against prions.

ACKNOWLEDGEMENTS

We wish to thank the "Programma Zootecnico Integrato" funded by the Italian Co-operation and the Istituto Agronomico per l'Oltremare di Firenze for the support in Tirana to the present research.

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VALIDATION OF A COMPETITIVE ELISA FOR CONTAGIOUS BOVINE PLEUROPNEUMONIA

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The detection of CBPP infected cattle is necessary for the implementation of control policies. Countries that are CBPP-free need to perform mass screenings in order to confirm their status and also to analyse sera from imported animals in order to prevent the introduction of CBPP. Countries that are infected should also perform a mass screening in order to determine the exact prevalence of the disease and adapt their control strategies. Complement fixation (Campbell and Turner, 1953), the currently recommended serological test (OIE Manual, 1996), is not entirely satisfactory. It lacks specificity and therefore may create problems in non affected countries. It may not detect antibodies for a long time after an outbreak and therefore mass screening in affected countries may detect only herds that were recently infected.

The newly described competitive ELISA (Le Goff and Thiaucourt, 1998) may solve some of these problems. In order to maximise the specificity, the cut-off of the test was chosen according to results obtained from Namibian cattle in CBPP-free regions. Controls on French bovine sera confirmed the specificity of the test. It also proved sensitive enough to detect antibodies in cattle infected by contact during an experimental infection with a pathogenic strain of African origin. These results seemed promising as antibodies were detected at high levels in the five animals four months after the onset of the clinical signs. It was therefore decided to perform a thorough validation of this test within the framework of the COST action 826, a Euro-African network and the joint FAO-IAEA division.

The main characteristics of a serological test are its specificity, sensitivity and repeatability.

In the case of CBPP serological testing, the measurement of the specificity can easily been done on cattle herds from CBPP-free countries. Four hundred sera from Austrian cattle were analysed at the IAEA laboratory. None of them had values exceeding the cut-off, 50% (fig 1). The distribution of the results was strictly following a gaussian curve similar with what had been found when analysing the negative Namibian cattle population. In that sense the performance of the test does not seem to be affected by breed differences.

The sensitivity of this cELISA was assessed during two different experimental infections. The first one was held in Cameroon with the Afade strain. All artificially infected animals developed a long lasting serological positivity and a sero conversion was also noted in the control animals. Similar seroconversion were observed in an experiment performed in Switzerland with animals infected with a strain of Italian origin (Pilloud 1996). The infected animal showed a high

seroconversion by cELISA throughout the study as the titer in CFT showed a decline after 60 days (fig 2). The contact animal had a concomitant seroconversion in CFT and cELISA but titers declined in parallel and returned negative after 210 days (fig 3). It is to be noted that this contact animal showed a transient mycoplasma excretion and had no visible lesions when necropsied. The sensitivity was further investigated on sera from naturally infected animals during the CBPP outbreaks in Botswana. The distribution of the cELISA results is no more gaussian and there seem to be two subpopulations, a negative one having titers below 50 and a positive one above 50 (fig 4). The data harvested at necropsy of 46 animals indicate that there is a very strong correlation between the presence of macroscopic lesions and the cELISA titers.

The correlation between cELISA and CFT was investigated in two European countries. Hundred and one sera from an Italian herd that had to be slaughtered due to CBPP were analysed by cELISA and CFT. The distribution of the cELISA results show that there was a subpopulation of positive animals in that herd. In that example the CFT seemed to detect less positives (9) than the cELISA (30). Comparable analyses were made in Portugal, they showed that there was a good correlation between the two tests, the CFT being in that case a little more sensitive (fig 5).

The serodiagnosis of CBPP in Africa could be hampered by the mass vaccinations that are performed with the attenuated strain T1. It is well known that this strain can elicit antibodies detected by the CFT. The percentage of positives after vaccination is very variable from one herd to another, hence not enabling a sero-monitoring by CFT, and in any case the seroconversion is transient, no positives being found after three to five months. The ability to detect antibodies after vaccination with the T1/44 strain, at the recommended dose of $10^{7,6}$ per animal, was investigated in Ethiopia. There is a slight seroconversion detectable by cELISA three weeks post vaccination, titers being slightly above 50%, but no positives are to be found afterwards. These results suggest that cELISA could be used in Africa to monitor the occurrence of CBPP outbreaks in spite of widespread vaccinations.

Finally the repeated use of the test in various laboratories enabled us to have an evaluation of the test in terms of repeatability and user-friendliness. Technicians with minimal training are able to obtain satisfactory results rapidly, as an example, the correlation of titers obtained at the OVI and CIRAD on a common set of sera were remarkably correlated (fig 6). It is also to be noted that when the test is handled properly, the difference between the two duplicates of a single serum is regularly inferior to 5 (mean = 0, STD= 1,3). The most common troubles encountered are the relatively high values obtained in many African laboratories with the negative control sera. This could be due to slight modifications of the protocol.

The results obtained up to now with this competitive ELISA are very promising and should enable a precise investigation of CBPP prevalence in infected regions or in suspect herds. Results obtained in Europe show that the difference of strains that seem to exist in that continent (Cheng et al. 1995) does not modify the characteristics of the test. It should be noted however that this test has to be used at the herd level. Collaborative work with the IAEA and ten African countries is underway in order to measure the exact persistence of antibodies detected by this

test. The use of the EDI software developed by the IAEA should permit a quality assurance of the results. Reliable data concerning the true prevalence of CBPP is an absolute necessity for national veterinary services and international organisations in order to be in a position to implement sound control strategies.

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A FIELD TEST FOR DETECTING ANTIBODIES TO *MYCOPLASMA MYCOIDES* SUBSP. *MYCOIDES* SMALL COLONY TYPE USING THE LATEX SLIDE AGGLUTINATION TEST.

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INTRODUCTION

Mycoplasma mycoides subsp. *mycoides* small colony (SC) type is the cause of contagious bovine pleuropneumonia (CBPP). Initial laboratory diagnosis of the disease is by serological test, usually the complement fixation test (CFT) (1). The CFT however is time consuming and false reactions occur requiring confirmation using methods such as Western Blotting (2). Diagnosis is confirmed by culture of the organism. A rapid field test for screening cattle, especially in African countries where the disease is endemic and laboratory facilities remote, would be beneficial.

Latex agglutination tests (LAT) have been used since 1956 when a rheumatoid factor test was developed (3). In these tests either antigen or antibody are used to 'sensitise' polystyrene microspheres which amplify any antigen/antibody reaction, by clumping in a positive test. Rurangirwa *et al.* (4) developed a LAT for field diagnosis of contagious caprine pneumonia (CCPP) which is caused by a closely related *'M. mycoides* cluster' member, *M. capricolum* subsp. *capripneumoniae.* A similar approach may be used to develop a successful test for CBPP, although there are conflicting reports on the antigenic activity of polysaccharides from *M. mycoides* (5, 6, 7, 8, 9, 10 & 11). Slide agglutination tests for CBPP have previously been developed (12, 13, & 14), however they are only useful on a herd basis as they give false positives at low serum dilutions and are reliable only early in infection (15). Thus the possibility of using a carbohydrate extract as an antigen in a LAT for CBPP was investigated.

MATERIALS AND METHODS

The initial investigation used *M. mycoides* subsp. *mycoides* SC Portuguese strain B103 which was grown in one litre of Eatons' medium (16) for 72 hours at 37°C in 5% CO_2. Polysaccharide extraction was carried out essentially following the method of Rurangirwa *et al.* (4). Only the supernatant of cultures was used as these authors stated it to contain ten times more carbohydrate than cells. Dialysis steps were replaced with separation through a PD10 column (Pharmacia, Uppsala, Sweden) with HPLC grade distilled water. Final filtration was through a BioSep Sec 300 column to separate the carbohydrates by size. Fractions were collected into 31 tubes before being pooled into five groups and concentrated by ethanol precipitation.

An indirect ELISA was performed on the pooled extracts and whole cells, with CBPP positive serum, negative CBPP serum and a potentially cross reacting *M. bovis* positive/CBPP negative serum. Briefly, the coated plate was washed four times in a PBS solution containing ten per cent milk powder and 0.5 per cent tween 80. The sera were diluted one in 320 with the same buffer, and 100µl placed in the

appropriate wells and incubated for one hour at 37°C. The plate was washed as before, and 100μl of a 1 in 4,000 dilution of IgG anti-cow horse radish peroxidase specific conjugate added. The plate was incubated for another hour at 37°C before washing and addition of 100μl of 3,3',5,5'-tetramethylbenzidine (TMB) substrate. The reaction was stopped when sufficient colour had developed by adding 50μl of 0.1 molar citric acid. The specific OD_{450} for each well was measured using a Labsystem plate reader.

A sample from each pool was run on a 12 per cent SDS PAGE using the standard technique of Laemmli, (17). The gels were stained using a silver staining method (Sigma Chemicals, Poole, UK). This is more sensitive than coomassie blue staining and will stain both carbohydrates and proteins.

Protein estimations on the pooled cell extracts were carried out using a BCA protein estimation kit (Pierce, UK). A general phenol-sulphuric acid assay (18) was used for carbohydrate estimations. Further analytical methods are still to be carried out.

Latex beads (1.08μm, Sigma Chemicals, Poole, UK) in a ten per cent v/v suspension were sensitised as per the method of Rurangirwa *et al.* (4). The equivalent of one ml of the latex beads were mixed with 1mg of polysaccharide extract in 1ml phosphate buffered saline, pH 7.4, containing 0.2 per cent sodium ethylene diamine tetra-acetate (Sigma Chemicals, Poole, UK) and 0.01 per cent sodium azide (Sigma Chemicals, Poole, UK). The mixture was shaken and incubated for one hour at 37°C and eight ml of the same buffer added before the concentrations were optimised using the same sera as used for the ELISA. Equal 10μl volumes of sensitised latex beads and serum were mixed on a slide and agitated for up to two minutes before recording the result, visible agglutination being recorded as positive.

The LAT was evaluated using positive field sera from Portugal, Italy and Uganda. Known CBPP negative field sera were obtained from samples submitted routinely for *M. bovis* ELISA testing at CVL; Great Britain has been free from CBPP for 100 years.

RESULTS

The OD_{280} of fractions from the column was determined. There was a small peak at fraction 9 which was included in the first pool, and a large peak at fraction 26 which was included in pool 5. The five pools run on the SDS PAGE gel gave five different profiles, but all had small background bands. Pools 1 and 2 gave a similar major band at approximately 65 KDa, and Pool 3 a double band at approximately 25 KDa, pool 5 gave no bands at all. Western Blotting using CBPP positive serum gave no detectable bands.

The ELISA results showed that all pools gave some reaction with CBPP positive serum, although none were as good as whole cells. All the pools were negative with the CBPP negative serum, but all reacted to some extent with *M. bovis* positive serum. The biggest difference being observed in Pool 1, which was used for the LAT.

Optimisation of the LAT was carried out using CBPP positive and *M. bovis* positive and negative sera. Initially cross reactions were seen but dilution of the sensitised latex from 10% to 0.2% eliminated cross reactions so that CBPP positive and negative sera were differentiated. From the 195 UK sera that has been tested

only two (false) positive results were obtained. Stored sera taken from CBPP affected areas during the Italian outbreak (17 samples) and from Uganda (32 samples) gave comparable results with CFT, ELISA and Western Blotting. Characterised sera from Portugal (169 samples) gave more varied results. All sera that were negative by all other tests were also negative by LAT. From the six Portuguese farms examined five were found CBPP positive by all tests including the LAT. However, the most sensitive methods were ELISA and Western Blotting, which on farm six gave positive results for only three of the fifteen samples, the LAT being most comparable with the CFT results.

CONCLUSIONS

It appeared from the ELISA and SDS PAGE results that Pool 1 was the most effective in the LAT. The LAT results were generally in agreement with CFT, ELISA and Western Blotting results. The LAT appears to be specific but lacks sensitivity. The lack of sensitivity became apparent in the sera tested from the Portuguese where CBPP disease is chronic rather than acute. The results obtained from Portugal samples, farm six, highlighted the lack of sensitivity of the LAT which like the CFT was negative. However, the overall results are encouraging and further development should improve the test which will have enormous benefits for use in diagnosing CBPP in field conditions.

Analysis of the extract seen in Pool 1 and its immunogenic properties is continuing with the aim of further purifying the antigen and increasing the sensitivity and specificity of this LAT. In addition, monoclonal antibody (Mab) production using the antigenic components of the extract may enable development of novel whole cell (antigen) detection systems. Such a Mab could be bound to latex beads to produce a detection system for antigen in nasal exudates or lungs at post-mortem, or as a rapid confirmatory identification test for *M. mycoides* subsp. *mycoides* SC cultures in the laboratory.

ACKNOWLEDGEMENTS

This work was jointly funded by the EC, FAIR Project CT95 0711, and by The Ministry of Agriculture, Fisheries and Food, Great Britain.

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STUDIES ON THE IMMUNOLOGICAL DIVERSITY OF TYPE, VACCINE AND WILD STRAINS OF *MYCOPLASMA MYCOIDES* SUBSP *MYCOIDES* SMALL COLONY (SC) VARIANT.

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INTRODUCTION

Contagious bovine pleuropneumonia (CBPP) has re-emerged in Africa in the 1990s causing immense losses following many years of quiescence. Although reasonably successful in the past, vaccination has been unsuccessful in containing this outbreak. The reasons behind this are unclear. One suggestion is that the vaccines used, in particular the T_1SR vaccine which has been the predominant form used in the 1990s, have lost immunogenicity during repeated passage, were mishandled in the field or were of an insufficient titre to confer protection (Tulasne *et al.* 1996). An alternative possibility is that wild strains involved in the panzootic have either mutated sufficiently to enable them to escape the protective effects of the vaccines or have acquired new virulence characteristics such as plasmid-encoded endotoxins. The purpose of this study was to compare wild strains isolated from different regions involved in the current outbreaks with vaccinal and type strains using serological and other biochemical methods.

MATERIALS AND METHODS

Mycoplasma strains. Type strains Afade, V5 and Gladysdale (obtained from R. Nicholas). Vaccine strains $T_1$44 and T_1SR (current vaccine stocks from the Botswana Vaccine Industry). Recent field isolates; N6 (Nokaneng, Botswana, October 1996), M375 (Mohembo, Botswana, November 1995) (both W. Amanfu), Tan1 (Pawaga, Tanzania, 1996) and Tan8 (Kagera, Tanzania, 1996) (both B. Lema). All mycoplasma strains were grown in Gourlay's medium (broth or agar, Gourlay 1964). Current field strains were clonally isolated and their identity as *Mmm*SC isolates confirmed by PCR (Johansson *et al.* 1997).

Antisera. (1) Bovine. Convalescent sera (Botswana) NK6 was obtained from W. Amanfu. Pre- and post- vaccinated sera (Tanzania) were obtained from B. Lema. (2) Rabbit. Hyperimmune sera were produced by two injections of inactivated mycoplasma in adjuvant, followed by one intravenous injection of an aqueous suspension. Two rabbits were used for each strain, and sera were tested individually and pooled (results from each serum sample were highly similar for each strain tested). Pre-absorption with capsular polysaccharide (CPS): 10ug of CPS was added to 0.1ml serum, clarified by centrifugation, then repeated until the CPS antibody titre dropped to background levels (measured using a CPS ELISA). Growth inhibition tests were performed by spotting 20ul of undiluted serum onto 5mm diameter filter paper discs, placing the discs onto suitable dilutions of mycoplasma cultures and incubating at 37°C for 2-4 days prior to measuring the zone of inhibition under a light microscope.

CPS was purified from cleared growth medium (acid precipitation/boiling) by phenol extraction followed by Proteinase K digestion, differential dialysis (50kD cut

off membrane) and finally size exclusion chromatography (2x) using Sephacryl S-300 media. No protein was detectable in the purified product (March *et al.,* this volume).

Molecular techniques. Standard procedures were used for DNA extractions, agarose gel electrophoresis, protein gels, Western blotting and ELISA.

RESULTS

Episomal DNA. No evidence for episomal DNA elements was observed for any of the strains tested following standard DNA extraction procedures, although this does not exclude the possibility of chromosomally-integrated prophage being present in some or all of the strains.

Morphological data. The field isolates fell into 2 distinct types. N6, Tan1 and Tan8 all gave large homogenous 'mucoid' colonies, while M375 produced a mixture of small, non-mucoid colonies and a few large 'mucoid' colonies (approximately 5% of the total). Sub-culturing of individual small or large colonies of M375 resulted in progeny displaying the same heterogeneity of colony size and appearance.

TABLE 1. Growth inhibition of rabbit hyperimmune antisera against *Mmm*SC strains.

Antiserum raised against	Growth inhibition (mm) against				
	Afade	**N6**	**M375**	**Tan 1**	**Tan 8**
Afade	0	0	0.8	0	0
Gladysdale	0	0.5	0.5	0.8	0.6
V5	1.3	1.8	2.5	2.0	1.9
$T_1$44	2.0	2.5	2.0	2.8	2.7
T_1SR	2.0	2.9	4.5	3.5	3.0
Botswana N6	2.6	3.4	5.0	3.8	3.5
Botswana M375	0	0	1.0	0	0
Tan 1	1.0	1.0	1.5	1.5	1.0
Tan 8	0.7	2.3	3.5	2.5	2.5

Legend. GI activity (mm) of rabbit hyperimmune serum. Leftmost column indicates strain used to raise antiserum. Right hand columns indicate zone of clearance of antiserum against each strain. Read downwards to asses the susceptibility of each strain to inhibition by different antisera. Read across the table to assess the potency of each antiserum against individual strains. GI activity of antisera raised against the vaccine strains T_1SR and $T_1$44 is shown boxed.

Growth inhibition (GI) tests. Rabbit hyperimmune sera. All antisera were tested using ELISA and Western blotting. These tests indicated a significant titre against whole *Mmm*SC antigens with no significant differences in the level of response to individual strains (data not shown). Results for pooled pairs of antisera in GI tests are shown in Table 1. It should be emphasised however, that these tests do not take into account any cell-mediated immunity engendered by these vaccines. Of the 9 strains used to vaccinate rabbits the two vaccine strains ($T_1$44 and T_1SR) and 1 field strain (N6) were most effective at raising GI antisera. In contrast, antisera raised against strains Afade, Gladysdale and M375 had very poor GI properties. From Table 1 it is also apparent that antisera raised against the two vaccine strains were as effective against newer 'field' isolates (N6, M375, Tan1 and Tan8) as they were against older 'type' strains such as Afade . Two conclusions can be drawn from this. In terms of GI activity (measured using rabbit hyperimmune sera), antisera

raised against current vaccine stocks is as effective as (and usually more so than) that raised against the other strains. *On the basis of this finding alone,* strains T_144 and T_1SR would appear to be appropriate choices for vaccine material. Secondly, antisera raised against these vaccine strains appear to be effective against all strains tested, including older types (Gladysdale, V5, T_144 and T_1SR (data not shown)) and contemporary field isolates. Thus antigenic drift of protective epitopes in recent field isolates does not seem to be a likely explanation for possible non-protection by current vaccines, nor does a loss of antigenicity by vaccine stocks appear to have occurred. Poor vaccine storage or administration may be a more likely explanation.

Field isolates appear to fall into two categories from this data: Tan1, Tan8 and N6 were effective at raising growth-inhibiting antisera, while M375 was very poor. M375 was also the most sensitive strain to GI, with all antisera tested displaying some degree of GI against this strain. Interestingly, strains were not more sensitive to homologous antisera. One possible explanation for this is that the GI activity of these rabbit hyperimmune sera is directed at the capsular polysaccharide (CPS) of the mycoplasma, and differences in sensitivities of each strain to GI reflect differences in the amount of CPS produced by that strain (the less CPS produced by that strain, the more sensitive it is to GI. Similarly, the more CPS antibodies in an antiserum preparation, the greater the level of GI activity of that particular serum).

Bovine Convalescent Serum: GI activity was tested against the nine strains using a field convalescent serum sample from Botswana (Table 2). Only strain M375 of the field strains was sensitive to growth inhibition with this serum, in agreement with data obtained with rabbit hyperimmune sera suggesting this strain belongs to a different group. Pre-absorption with CPS completely removed this activity. Interestingly, the 'type' strains Afade and V5 were also sensitive to NK6 antiserum, and pre-absorption to remove CPS antibodies only partially removed this activity, suggesting that the sensitivities of these two strains involved non-CPS (presumably protein) epitopes, and that these epitopes are specific to these strains. Identification of these protective epitopes may help development of a subunit protein vaccine against CBPP.

TABLE 2- Growth inhibition tests using bovine convalescent serum

ANTISERUM	Growth Inhibition (mm) against								
	Afade	Gladys	V5	T144	T1SR	N6	M375	Tan 1	Tan 8
NK6	3	0	2	0	0	0	1.5	0	0
NK6 pre-abs.	1	0	1	0	0	0	0	0	0

Protein Banding Patterns. The 9 strains were probed on a Western blot with NK6 bovine convalescent serum. No differences in banding pattern were noted between strains Afade, V5 and the 7 other strains which were consistent with the differences observed during GI tests. NK6 serum identified a 110kD band present in T_144,T_1SR, Tan 8 and M375 (but not the other strains), while a 60kD band was present in all strains with the exception of N6, Gladysdale and M375. Interestingly, strain M375 displayed two unique polymorphisms; the absence of a common 40kD band and the sole presence of a 70kD band. Whether these observed differences reflect functional serological changes is not known.

Antibody titre of vaccinated animals. Sera from 15 vaccinated animals (Kilombero, Tanzania) were compared with 15 unvaccinated controls (Mikumi,

Tanzania). Animals were vaccinated with T_1 freeze dried vaccine (Debre Zait) given as a single shot at four times the normal dosage. Animals were bled 35 days post vaccination. None of the sera exhibited GI activity against any of the strains tested. An ELISA against CPS, membrane proteins and whole antigen of *Mmm*SC revealed no significant antibody titre compared with pre-immune animals.

CONCLUSIONS

From this study of field, type and vaccinal strains of *Mmm*SC the following conclusions have been drawn. No evidence was found for the acquisition of episomally located virulence factors in recent field isolates, nor was there any evidence of antigenic drift in field strains compared to type or vaccine stocks. Current vaccine strains do not appear to have lost immunogenicity, and induce as much GI activity in rabbits as any of the field or type strains. Sera from field-vaccinated cattle exhibited neither GI activity nor a measurable antibody titre, in agreement with the premise that vaccine stocks may have been mishandled in the field or were of insufficient titre to confer protection. Whilst no differences were apparent between the two Tanzanian field isolates of *Mmm*SC, the two Botswana isolates displayed significant differences from each other (although one of them (N6) was indistinguishable from the Tanzanian isolates) in morphology, growth rate, pathogenicity and biochemical characteristics (protein banding pattern and CPS production). The reduced CPS production of Botswana strain M375 is interesting, since the affinity coefficient of mannose and glucosamine utilisation of this strain is different when compared with other African isolates (Roger Miles, pers. comm.). Both these sugars are constituents of the *Mmm*SC capsular polysaccharide (March *et al.*, this volume), and this biochemical difference may manifest itself in the altered CPS production of this strain. Thus we conclude that in Botswana, at least two different strains of *Mycoplasma mycoides* subsp. *mycoides* SC were implicated in the recent outbreak of contagious bovine pleuropneumonia. The question of how two strains have arisen (whether due to recent introduction, antigenic drift, vaccinal origin or a reaction to antibiotic treatments), and the possible implications for this finding on the spread and control of the current outbreak remains to be resolved.

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RENAL LESIONS IN CATTLE WITH CONTAGIOUS BOVINE PLEUROPNEUMONIA: AN IMMUNOHISTOCHEMICAL STUDY

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INTRODUCTION

Contagious Bovine Pleuropneumonia (CBPP) is an infectious disease primarly affecting cattle, caused by the small colony type of *Mycoplasma mycoides* subspecies *mycoides* (*M. m. mycoides* SC). Several outbreaks of CBPP have been reported in Northern Italy from 1990 to 1993 and more than 13,500 cattle from infected herds have been slaughtered during this period.

At post-mortem examination several cattle with typical and severe pulmonary lesions of CBPP showed renal lesions consisting of multiple ischemic infarcts or large foci of interstitial nephritis (1). Similar renal lesions have been described by Bygrave et al. (2) and Masiga et al. (3). Moreover, Scudamore (4) reported the isolation of *M. m. mycoides* SC from the kidneys and the urine of cattle with CBPP.

The present study was designed to investigate the histological lesions, to detect *M. m. mycoides* SC and to localize *M. m. mycoides* SC antigen in kidneys of cattle affected by CBPP.

MATERIALS AND METHODS

77 cattle whose lungs were bacteriologically positive for *M. m. mycoides* SC and showing typical CBPP lesions at different stages (25 acute, 14 subacute, 38 chronic) were selected for this study. Ten cattle without pulmonary lesions, bacteriologically negative for *M. m. mycoides* SC and belonging to CBPP-free herds were taken as negative controls.

The kidneys of all the animals were collected and submitted to bacteriological analysis and histological and immunohistochemical examination. As for the last two procedures, kidney samples were fixed in 10% buffered formalin and routinely embedded in paraffin. 5 µm thick section were stained with haematoxylin and eosin and with an immunoperoxidase method (5) (avidin-biotin peroxidase complex (ABC) procedure) using a policlonal rabbit antiserum directed against *M. m.mycoides* SC as primary antibody.

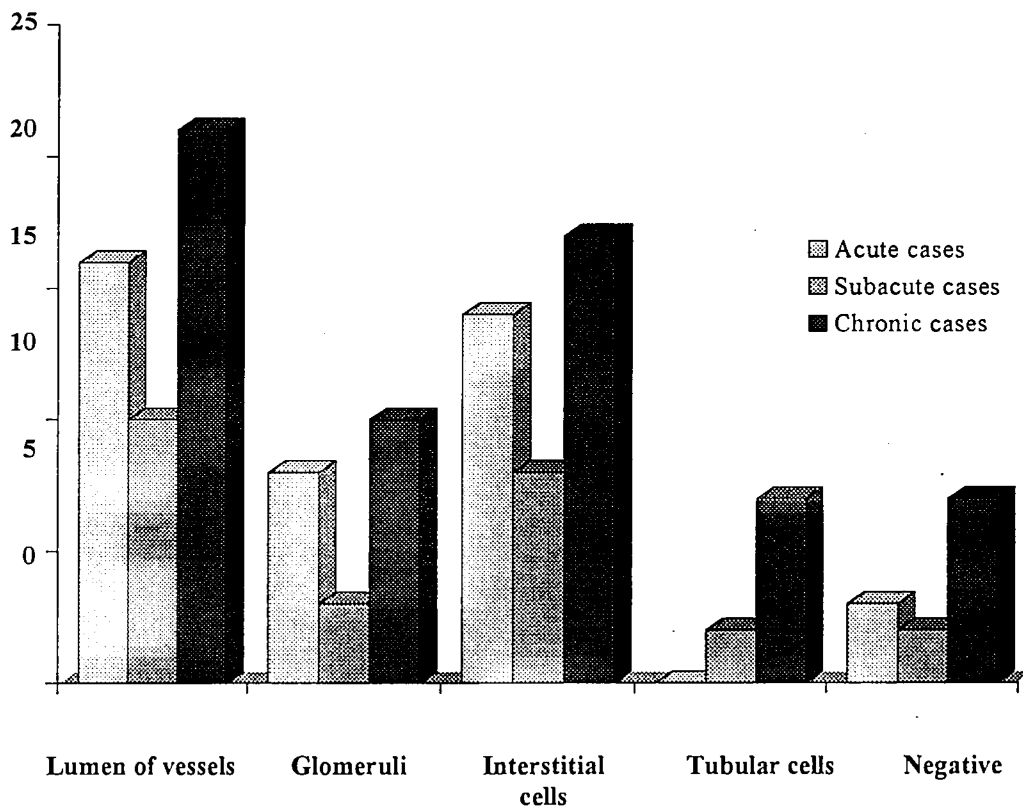
RESULTS

Multiple renal ischemic infarcts were observed in cases with acute pulmonary lesions. Cases with chronic pulmonary lesions showed renal multiple whitish areas presumably derived from infarcs and histologically caracterized by fibrosis, lymphocytic infiltration, tubular atrophy, tubular regeneration and focal

calcification. A common finding in both infected and uninfected animals was the presence of multifocal lymphoplasmacitic infiltration.

M. m. mycoides SC was isolated from the kidneys of 12 (15,6 %) animals. *M. m. mycoides* SC antigen was detected immunohistochemically in the kidneys of 62 (80,5 %) animals; positivity was localized in the lumen of renal vessels, glomeruli, interstitial cells and tubular epithelia. The results of immunohistochemical staining are schematically reported in figs 1. Kidneys from the 10 control animals tested immunohistochemically and bacteriologically negative for *M. m. mycoides* SC.

Fig. 1 - Results of immunohistochemical staining: localization of *M. m. mycoides* SC antigen in the kidneys of 77 cattle.



CONCLUSIONS

Our results confirm the association of renal lesions with severe cases of CBPP. In the acute phase of the disease these lesions are represented by multiple infarcts that probably are induced by embolism of material derived from pulmonary lesions. In the chronic phase, infarcts evolve in large foci of interstitial nephritis characterized by tubular atrophy/regeneration, lymphocitic infiltration and scattered

calcifications. Calcifications are distinctive and easily differentiate CBPP induced lesions from those that occur in the common forms of bovine interstitial nephritis.

M. m. mycoides SC can be isolated fom the kidneys of animals with acute or chronic CBPP and *M. m. mycoides* SC antigen can be detected in several renal structures. *M. m. mycoides* SC and *M. m. mycoides* SC antigen reach the kidneys through the bloodstream. The presence of circulating *M. m. mycoides* SC antigen is, in fact, common in animals with CBPP (6). *M. m. mycoides* SC antigen was often detected immunohistochemically in cases in which mycoplasma organisms could not be isolated. This may be explained by the fact the antigen remains recognizable even after organisms are no longer viable. Our results agree with previous reports that proved the presence of *M. m. mycoides* SC in the urine of cattle with CBPP (4). These findings suggest a possible role of urine in the spreading of CBPP.

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EVALUATION OF COLORIMETRIC PCR FOR THE DIAGNOSIS OF CBPP

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INTRODUCTION

The aim of the present study was to evaluate the commercially available kit *pleuro*TRAP from AMRAD, Australia, conceived for the colorimetric detection of PCR products specific of the *Mycoplasma mycoides* group (*Mycoplasma mycoides* subsp *mycoides* SC, *Mycoplasma mycoides* subsp *mycoides* LC and *Mycoplasma mycoides* subsp *capri*).

The traditional and one of the most common method for detection of PCR products has been agarose gel electrophoresis stained with ethidium bromide. Another method is autoradiography, still the most sensitive DNA detection system. For non radioactive detection other methods have been developed. The non radioactive detection can be direct (the reporter group is directly attached to the primer), or indirect (primary label is detected through interaction with a secondary system that contains a detectable reporter group). The major difference between these two approaches is found in the number of manipulations required to visualise the product. In the development of indirect detection system three aspects are involved: indirect recognition label (biotin-labelled primers); immobilisation of the amplicons (specific DNA binding site); direct reporter label (streptavidin-HRP conjugate).

In the *pleuro*TRAP kit the primers used to amplify the target sequence have been modified. One of them contains a specific DNA binding site: the GCN4 recognition sequence, so that the amplified DNA could be captured by binding to GCT-GCN4 (glutathione S-transferase-GCN4 fused polypeptide) that coats the wells of a microtiter dish (capture of the amplicons and its immobilisation); the other primer contains biotin (the indirect recognition label). Streptavidin-horseradish peroxidase conjugate (direct reporter label) is bound to the amplified DNA molecules taking advantage of the high affinity of streptavidin to biotin, and a colour reaction is developed after reaction with the chromogenic substrate TMB (tetramethyl-benzidine) as in a standard ELISA reaction.

MATERIALS AND METHODS

Mycoplasma mycoides subsp. *mycoides* SC type strain PG1 and 174 post-mortem clinical samples, which included lung tissue and lymph nodes from bovines with CBPP, were analysed.

Extraction of DNA from clinical samples and from PG1 strain was as previously described (1).

DNA from type strain PG1 was 10 fold diluted from 680 ng up to 68 fg, and 1 µl of the dilutions submitted to PCR; 1 µl of DNA samples from lung tissues was also subjected to PCR.

PCR was performed as already described (1), but using the modified primers MM450B and MM451+ included in the kit *pleuro*TRAP. The cycling conditions were: denaturation at 94°C for 5 min, followed by 35 cycles at 94°C for 30 sec, 50°C for 30 sec, 72°C for 30 sec, and a final extension step at 72°C for 5 min.

Colorimetric detection of PCR products was performed with the reagents included in the kit *pleuro*TRAP from AMRAD (cat. No. 90001240), Australia, following the instructions provided. DNA from strain PG1 or synthetic dsDNA from *M. mycoides* (included in the kit) were used as templates in the batch-positive control (positive control of the PCR reaction); template DNA was replaced by water in the batch-negative control (negative control of the PCR reaction). Plate positive and negative controls were provided in the kit. The optical density was read on a microtiter plate reader using a 450 nm filter (OD_{450}) with 620 nm as the reference.

For sensitivity assessment of the detection system, 5 µl of the PCR products of PG1strain DNA ten fold diluted were analysed both by gel agarose electrophoresis and by colorimetric detection. The PCR product of the 680 ng DNA sample was also 10 fold diluted up to 1/1000 and analysed.

To evaluate the system in the field, 5 µl of the 174 PCR reactions from clinical samples were also subjected to gel agarose electrophoresis and to colorimetric detection.

RESULTS

The sensitivity of the detection system was between 680 pg and 6.8 ng of template DNA, what corresponds to that achieved analysing the PCR product on ethidium bromide stained agarose gel. The OD_{450} values for the plate positive control had a mean value of 2.027 (0.630-2.694). OD_{450} values for the plate negative control had a mean value of 0.027 (0.015-0.044). Batch positive and batch negative controls had a OD_{450} mean value, respectively, of 2.461 and 0.030.

The analyse of lung tissues revealed that 63.2% of the samples were negative both in the detection by agarose electrophoresis and by colorimetric system, the OD_{450} mean value of these samples was 0.058 (Table 1). Positive results both in electrophoresis and in colorimetric method were presented by 14.4% of the samples with a OD_{450} mean value of 1.577. Twenty two percent of the samples showed a negative result in the detection by agarose electrophoresis, while in the colorimetric method the OD_{450} had a mean value of 1.512 (Table 1). A comparison of OD_{450} values of the lowest positive (0.438) and the highest negative (0.104) samples gave a ratio of about 4:1.

TABLE 1
ANALYSIS OF CLINICAL SAMPLES

N° of lung tissue samples	PCR *pleuro*TRAP	
	Electrophoresis	Colorimetric OD_{450} (range)
110	Negative	0.012 - 0.104 (0.058)
25	Positive	0.756 - 2.398 (1.577)
39	Negative	0.438 - 2.586 (1.512)

DISCUSSION AND CONCLUSIONS

In a general comparison between gel agarose electrophoresis detection and colorimetric detection with *pleuro*TRAP we observed that the main problem with indirect detection systems is that they require the addition of secondary detection reagents, that then must be removed by extended washing and binding steps, which are both cumbersome and time consuming. In addition, enzymatic detection complexes (e.g. horseradish peroxidase coupled to streptavidin) may bind non-specifically both to DNA and to the support matrix, producing a background that can be eliminated only by extensive matrix blocking and washing. That is why the colorimetric procedure is suitable only for mass screening samples (over 28 samples) in a routine base. In this case the use of a standard equipment for ELISA serology, such as plate washers and readers, is essential to reduce time and subjectivity of the assay.

As the sensitivity of the system was the same as the sensitivity of ethidium bromide stained agarose gel, there is no benefit in using it when less than 28 samples are concerned. When large numbers of field samples are involved the method could be advantageous, though restriction enzyme digestion of the PCR product must be performed, to fully elucidate if the mycoplasma present is the causal agent of CBPP *Mycoplasma mycoides* subsp. *mycoides* SC.

Some discrepancy was observed with 39 lung tissue samples that gave negative results in the agarose gel and positive in the colorimetric detection. This fact could theoretically be explained by a higher sensitivity of the colorimetric method, but in fact the sensitivity was the same. One aspect that might account for these results was the variance observed in the OD_{450} values of the plate positive and plate negative controls from different batches of the kit. Plate positives vary from 0.592 to 2.694, while plate negatives vary from 0.013 to 0.063 depending upon the kit batch.

The positive:negative ratio 4:1 observed is not in accordance with other results (2) in which the ratio was 8:1, nor with the value stated in the manufacturers instructions (between 10:1 and 50:1), what might hampered the interpretation of some weak positive signals.

Assuming that *Mycoplasma mycoides* subsp *capri* is specific of small ruminants and *Mycoplasma mycoides* subsp *mycoides* LC is seldom isolated from bovines, this colorimetric assay could be adequate for detection of *Mycoplasma mycoides* subsp *mycoides* SC, the causal agent of CBPP.

ACKNOWLEDGEMENTS

Financial support for this work was provided in part by the EU project FAIR1-CT95-0711.

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MODIFICATION OF THE COMPLEMENT FIXATION TEST (CFT) FOR CBPP

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INTRODUCTION

The Campbell & Turner CFT is the procedure recommended by the European Union (EU) and the OIE for the identification of antibody to *Mycoplasma mycoides* subsp *mycoides* SC (*Mmm*SC). The CFT has been harmonised in the EU (1) and is the official method carried out in Italy for the eradication of the Contagious Bovine Pleuropneumonia (CBPP). The method requires 12 U of haemolysin (hyperimmune rabbit serum to sheep red blood cells), 2,5 U of guinea pig complement (C'), and sheep red blood cells (RBC) at a concentration of 6%. In general, CFT is difficult to perform because it requires trained personnel and is time consuming. The high concentrations of the reagents required for the Campbell and Turner test increase the difficulties. This study has been carried out in an attempt to identify a simpler CFT procedure for CBPP. Various concentrations of RBC, haemolysin and C' were tested in cross titrations both in presence and absence of antigen. From the preliminary results the 2% RBC concentration was chosen. The CFT, according to Campbell and Turner, was used as control. The method was standardised with three positive reference sera as recommended by the OIE. To evaluate the performance of the test in field situation, CBPP positive sera, from naturally infected animals, and negative sera were used.

MATERIALS AND METHODS

Titration of haemolysin and complement. In the modified Campbell and Turner method 12 U of haemolysin are used, the titration is carried out in tubes; while C' is used at a concentration of 2.5 U and the titration is carried out in the presence of antigen in a 96 well format microtiter plate. RBC are used at a concentration of 6%. The method we describe used 2% RBC, 2 U of haemolysin and 2 U of C' cross titrated in presence of antigen directly in microplates. The dilution of haemolysin and complement were prepared using Veronal buffer (VB) pH 7.2. The haemolysin was diluted from 1: 500 to 1: 8000, the C' was diluted from 1: 40 to 1: 320. The haemolysin was supplied by BioMérieux (Marcy l'Etoile, France). Two different type of C' were tested; the first was obtained from fresh guinea pig serum and freeze dried, the second was purchased from BioMérieux. Both sources of C' gave identical results. The RBC used for the titration was

obtained by aseptic puncture of the sheep jugular vein, and preserved before use in Alsever's solution. The RBC were washed three times in VB and used in a 2% suspension. The haemolytic system (HS) was prepared in tubes by adding an equal volume of the 2% RBC suspension to each haemolysin dilution. The HS was sensitised in a water bath at 37°C for 15 minutes. The titration was carried out in a microtiter plate. A 25 µl amount of each dilution of the C' was distributed in all the wells of the corresponding column, another 25 µl of each HS was added to the wells of the corresponding row. A 25 µl amount of antigen at the right concentration and 25 µl of VB were added to all the wells. The microtiter plate was shaken, sealed and incubated at 37°C in a water bath for 30 min. After centrifugation, the highest dilution of haemolysin and the highest dilution of C' showing 100% haemolysis corresponding to 1 U of C' and 1 U of haemolysin were chosen. In the test 2 U of C' and 2 U of haemolysin were used.

Test procedure. In the test, three OIE positive reference sera 806, 845, 840 (5), a field CBPP positive serum (S+ TE) and a negative serum (S- TE) were tested. Moreover, 445 field sera collected during the Italian eradication programme (1992) were tested. All the sera were processed in parallel with the Campbell and Turner CFT, as described in the OIE Manual. The sera samples were diluted 1:10 in VB in tubes and inactivated at 56°C for 30 minutes. A 25 µl of each sera were dispensed in the microplate wells. The sera were diluted with a multichannel pipette from column n1 to column n6 (from1:10 to 1:320). Two CFT U of antigen and 2 U of C' each in 25 µl were subsequently added to the wells. The microplate was shaked, sealed and incubated at 37°C for 30 min in a water bath. After incubation, a 25 µl of the HS were added, the plate agitated and incubated for further 30 min at 37°C in a water bath. After centrifugation of the microplate, the assessment was carried out on the basis of percentage of C' fixed (25% fixation = +; 50% fixation = ++; 75%fixation = +++; 100% fixation = ++++).

RESULTS AND CONCLUSIONS

The two tests were compared using the Cohen measure of agreement K. The reference sera had the same titres in both methods. The total concordance was equal to 0.97, whereas the agreement beyond the chance was equal to 0.86 ($p<0.01$). In 12 cases there was no agreement between the two methods. Therefore it must be noted that in all the cases the titres ranged between 1+ 1:10 and 2+ 1:10. The titres were also converted in quantitative values in order to compare the two methods. The evaluation was done by Spearman Correlation Coefficient which gave a value of 0.86 ($p<0.01$). The results allows us to state that the two tests are interchangeable for the identification of positive and negative sera.

Table 1.

		CFT CAMPBELL & TURNER		
		POSITIVES	NEGATIVES	TOTAL
CFT ALTERNATIVE METHOD	POSITIVES	**392**	**8**	**400**
	NEGATIVES	**4**	**41**	**45**
	TOTAL	**396**	**49**	**445**

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APPLICATION OF THE POLYMERASE CHAIN REACTION FOR THE IDENTIFICATION OF RUMINANT MYCOPLASMAS ISOLATED IN BELGIUM

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INTRODUCTION

In the Belgian Veterinary and Agrochemical Research Centre, a unit involved in the diagnosis of Mycoplasmosis was set up recently. Mycoplasmas are isolated by conventional culturing and are consequently identified using a combination of biochemical and serological tests. However, these traditional methods are time-consuming, difficult and sometimes lack specificity. Last years, several reports on new identification methods, such as specific PCR tests, have been published. PCR allows a rapid and correct identification of *Mycoplasma* strains and avoid the potential problems related with conventional methods. These advantages prompted us to start some preliminary tests on the application of these techniques in our laboratory and the possibilities of using them in routine diagnosis.

MATERIALS AND METHODS

Strains and growth conditions: In this study, we used field isolates of *Mycoplasma agalactiae* (83/61), *Mycoplasma arginini* (84/338), *Mycoplasma bovis* (82/557, 97/29, 98/1, 98/21), *Mycoplasma capricolum* (85/202), *Mycoplasma mycoides* subspecies *mycoides* LC (86/203) and *Acholeplasma laidlawii* (88/215) The strains were grown in *Mycoplasma* medium, as described by Bölske (1). All strains were isolated in Belgium and were typed by biochemical and serological methods.
DNA extraction: Two methods were used to prepare the samples. 1) Cells were washed, resuspended in PBS and heated for 5 min at 95 °C. 2) DNA was isolated from the cells by phenol/chloroform extraction, followed by ethanol precipitation.
PCR: Amplifications were performed as described by the authors. Fragments were visualised by electrophoresis on 1.5 % agarose gel and staining with ethidium bromide.

RESULTS

We selected two recently published PCR methods which allow specific identification of *Mycoplasma bovis* (2, 3) and two tests specific for *Mycoplasma agalactia* (4, 5). *Mycoplasma* isolates were analysed with these PCR systems.

Mycoplasma bovis

Application of the PCR system described by Chàvez Gonzàlez (2), resulted in selective amplification of all *M. bovis* strains that were tested. For *M. agalactiae*,

which is closely related to *M. bovis,* we also obtained a positive result, the other tested *Mycoplasma* strains were not amplified.
The PCR of Ghadersohi *et al.* (3) also allows selective amplification of *M. bovis.* Other bovine strains were not amplified, but we could not discriminate *M. bovis* from *M. agalactiae.* In their paper, the authors describe the detection of a single band of 215 bp, but in our experiments, two additional bands of 500 and 900 bp were clearly visible.

Mycoplasma agalactiae

Tola *et al.* (5) described a PCR method, selective for *M agalactiae.* We did several trials with this test, altered slightly the reaction conditions, but did not obtain any positive result with our strains.
All strains were also analysed with the PCR of Johanssen *et al.* (4). As mentioned by the authors, an amplification product of 730 bp was formed and when working under high stringency conditions, only *M. agalactiae* and not *M. bovis* was amplified. When primer annealing was done at lower temperature, we equally obtained an amplification product for *M. bovis.*

CONCLUSIONS

We obtained selective amplification of *M. bovis* and *M. agalactiae* strains, isolated in Belgium, by application of the PCR methods described by Chàvez Gonzàlez *et al.*(2) and Johansson *et al.* (4). Until now the PCR tests of Ghadersohi (3) and Tola et al. (5) did not give the results we expected. Additional trials need to be done.
In the future, we will also try to use these PCR systems for analysis of clinical samples (milk samples, lung tissue, nasal swabs).

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INVESTIGATION ON THE CURRENT STATUS OF CAPRINE MYCOPLASMOSIS IN THE EASTERN HILLS OF NEPAL

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INTRODUCTION

Goats are one of the predominant ruminants in the Eastern Hills of Nepal. In numerous developing countries, above all in Asia and Africa, the keeping of goats is of particular importance. In Nepal, about 5.4 million goats are kept (1), ca. 17% thereof in the mountainous areas of eastern Nepal. Consumption of goat meat is about ten time of that of mutton, five times that of poultry meat and three times that of pork. Over the last 20 years, the price of goat meat has risen by ca. 300%. The particular necessity of a most intensive research in the field of goat as suppliers of meat, they also constitute a highly flexible financial reserve for the rural population. Beyond this, these animals play a high social and cultural role. Goats are kept by all social classes and categories of peasants of all castes in the eastern mountainous regions of Nepal. They are of special importance for the very small rural households and the poorest part of the population. Ninety-two percent of households rear goats, with an average of five goats per household. A number of infectious diseases constitute the most important causes of a reduced goat production and high mortality. Anywhere in the world, respiratory diseases play a special role in this connection. Numerous bacteria, viruses and helminths are capable of causing infections. Their presence essentially depends on the geographical, climatic, breeding and keeping conditions. For the Nepalese region, data on the causes of the high number of cases of respiratory diseases are insufficient. For comparison, the situation in India is outlined. In the mountainous regions of north-eastern India, the average mortality from respiratory diseases was 56.63%, with a peak of 59.63% during the rainy season (2).

MATERIAL AND METHODS

Animals

The results of the survey study carried out at Pakhribas Agricultural Centre, Dhankuta, Nepal, on diseases indicate that respiratory diseases are a main constraint of goat keeping. Ewes were kept under conditions combining indoor (slatted grates) and pasture keeping. The momentary health status was evident from a massive presence of mucopurulent discharge from the nose, combined with cough and in part, discharge from the eyes. Part of the animals exhibited elevated body temperature (104 - 106 °F = 40.0 - 41.1°C). An age-dependency of clinical manifestations upon age was evident. Thus, 60% of the young animals below 6 months of age generally exhibited mucopurulent discharge from the nose, which in 70% was associated with spontaneous or easily triggered cough. Ca. 30% of the

animals had an elevated body temperature and ca. 20% showed discharge from the eyes. In animals older than 6 months, the incidence of respiratory manifestations was ca. 40%. One animal presented with polyarthritis, ca. 5%, with chronic mastitis. 50% of the animals younger than 6 months exhibited clinically pronounced disease, i.e ecthyma contagiosum (Poxviridae). Systematic postmortal studies in goats has been performed at Pakhribas Agricultural Centre during the 1985 - 1991 period. In the course of these examinations it was found that pneumonia has been the cause of death in 31.6% of cases, with a peak mortality of 75% during the rainy season. Next in frequency were enteric disease (14.7%) and coccidiosis (10.5%). These conditions were found to apply to all age groups. There were, however, particularly high losses among young animals (0 - 3 months) from pneumonia-associated deaths (63.1%). In 53.3% of lung specimens with pneumonic changes examined, bacterial growth, in 2.8%, an invasion by lungworms was detected. Bacterial species isolated from pneumonic lung samples and nasal swabs were not considered as major causes of pneumonia (3). This indicated an involvement of mycoplasmas causing respiratory diseases in goats. The aim of this pilot study was the collection of preliminary data about the occurrence of mycoplasmas in diseased goats. Isolation and identification of caprine mycoplasmas were performed by cultivation, immunofluorescence and PCR. For serological screening the Campbell & Turner complement fixation test was used as standardised method. For rapid and specific detection of members belonging to the *Mycoplasma mycoides* cluster, a PCR scheme utilising a set of primer pairs derived from the CAP-21 genomic region was used for differentiation. PCR involving cluster-specific amplification at the first stage and species-specific amplification at the second stage was performed for identification of all species forming the cluster (4). Identified species of the cluster are presented in Table 1.

Table 1: Number of PCR positive samples (lungs, nasal swabs) of 266 goats

Mycoplasma species	107
Mycoplasma mycoides cluster	13
Mycoplasma mycoides subsp. *mycoides* (to be differentiated further)	2
Mycoplasma mycoides subsp. *mycoides* LC	3
Mycoplasma mycoides subsp. *capri*	1
Mycoplasma capricolum subsp. *capricolum*	6
Mycoplasma capricolum subsp. (to be differentiated further)	3

Preliminary genetic identification using the CAP-21 genomic region indicate that two *Mycoplasma* species isolated might turn out to be *Mycoplasma mycoides* subsp. *mycoides* SC and two further isolates might be attributed to *Mycoplasma capricolum* subsp. *capripneumoniae*. The presence of *Mycoplasma mycoides* subsp. *mycoides* SC in caprine has been reported previously (5) and might be of high epidemiological importance (6), since goats and sheep are generally not considered as hosts for this *Mycoplasma*. It has, however, to be stated that the identification of

Mycoplasma mycoides subsp. *mycoides* SC and *Mycoplasma capricolum* subsp. *capripneumoniae* in this work is preliminary and is solely based on a relatively short DNA fragment named CAP-21 with unknown function (7) and hence needs to be confirmed.

Another technique used for identification of mycoplasma isolates was the immuno- fluorescence method performed with colonies transferred to nitrocellulose membrane. After incubation with rabbit hyperimmune sera, the bound antibodies were detected by a FITC-labelled secondary antibody. Using this method we were able to confirm the presence *Mycoplasma mycoides* subsp. *mycoides* LC, *Mycoplasma arginini, Mycoplasma auris* and *Acholeplasma laidlawii.* Further investigations will include epidemiological relevant factors like macro- and micro-climate, husbandry, feeding and socio-economic aspects.

This study was supported by the Pakhribas Agricultural Centre (PAC) in partial co-operation with the German Society of Technical Cooperation (GTZ).

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FURTHER EPIZOOTIOLOGICAL AND MICROBIOLOGICAL OBSERVATIONS ON AN OUTBREAK OF OVINE INFECTIOUS KERATOCONJUNCTIVITIS IN CROATIA

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INTRODUCTION

Ovine infectious keratoconjunctivitis (OIKC), also described as "pink eye" (5), is an important ocular disease in sheep, often characterized by severe inflammation of one or both eyes. The outbreaks of the disease have been reported in all continents, including many European countries (2). Concerning frequent relapses regardless of the treatment and need of special nursing during the period of reduced vision or total blindness, the disease is of equally nuisance to veterinarians and farmers (1, 2). Although being clinically well defined and easily recognizable, the etiology of OIKC still remains uncertain (6, 7). Among a great variety of microorganisms which have been involved as causative agents of the disease during the time a special attention was focused on *Branhamella (B.) ovis*, *Chlamydia. psittaci* and *Mycoplasma (M.) conjunctivae*. Recent investigations indicate that *M. conjunctivae* could be the primary etiologic agent of OIKC (2). Perpetuating study of the first outbreak of OIKC in Croatia during 1995 (8,9), this paper reports further microbiological and epizootiological data and some additional clinical observations on the epizootic.

MATERIAL AND METHODS

Animals. Related to the donation of about 6.000 sheep from Australia and New Zealand during 1995 all over the Croatia a great number of native sheep which were in contact with imported animals acquired a severe ocular disease closely resembling ovine infectious keratoconjunctivitis (OIKC). In diseased flocks 20-80% of native animals had ocular changes involving one or both eyes. Affected animals regularly showed the signs of reduced vision or total blindness. In each flock with 20 to 40 animals at least one to three animals should be eliminated from further breeding because of panophthalmia. At the same time the majority of imported animals were apparently healthy or showed only the signs of mild conjunctivitis. *M. conjunctivae* was isolated from 44% and *B. ovis* from 56% of microbiologically examined conjunctival swabs. In no case mycoplasmas were detected in the flocks free of the OIKC. In the beginning of the epizootic only topical or systemic treatment of affected sheep was predominately applied using oxytetracycline eye ointment and oxytetracycline dihydrate (Geomycin® retard, PLIVA Zagreb), respectively. The topical treatment was predominately effective only for mildly affected animals and if oxytetracycline ointment was used two or three times daily for one week. Five or six days after the initial amelioration clinical signs usually relapsed requiring further

treatment. The similar effects were achieved with single or triple parenteral administration of only long acting oxytetracycline. The most effective was a combination of daily topical application of oxytetracycline eye ointment and triple (on days 0, 3 and 6) intramuscular injection of oxytetracycline dihydrate. In this manner treated animals usually recovered for three to six days and relapsed rarely. For many reasons in the course of 1996 most of the farmers eliminated imported sheep from their flocks and only sporadic outbreaks of OIKC occurred among native sheep during this year. By the end of 1997 all 11 inspected flocks located in different places of Croatia were clinically free from OIKC.

Material. During last three months of 1997 six flocks affected with OIKC in the course of 1995 and five flocks with no history of the ocular disease underwent clinical and microbiological examinations. The flocks with 20 to 116 animals in each, are located in the regions under the care of Veterinary Stations Samobor, Jastrebarsko, Popovaca, Porec, Bjelovar and Rasinje. The sheep in the flocks are of different native breeds (Pramenka, Cigaja-Suffolk, Solcavka, Istrian Sheep, Wuertemberg cross-breed). Imported animals were eliminated from all but one previously affected flock, in which eleven Australian animals were kept together with 105 of native sheep. In each of examined previously affected flocks farmers could identify at least some animals which showed typical ocular changes during 1995 or 1996. The animals from the flocks with no history of OIKC had never been in contact with animals imported from Australia and New Zealand. At the time of monitoring all inspected sheep were clinically free of OIKC but about 10% in each flock showed the signs of mild conjunctivitis. The highest number of diseased (30%) animals was found in the flock with native and Australian sheep kept together. At this time conjunctival swabs were taken for microbiological examination from 63 animals originated from 6 previously infected flocks, and from 32 animals derived from the 5 flocks with no history of OIKC. The age of animal varied from several months to five years.

Methods. Conjunctival swab from one eye was inoculated directly into PPLO broth (3) enriched with 20% horse serum, fresh yeast extract (2.5%), dextrose (0.5%), DNA and common antibacterial agents. In the next several hours two further dilutions from inoculated broth were prepared in the laboratory. Conjunctival swab from the other eye was inoculated in the laboratory onto sheep blood agar. Mycoplasmas and bacteria were cultured and identified by the methods described by Razin and Tully (10) and Holt et al. (4). In the identification of bacteria a special attention was paid to branhamella, staphylococci, streptococci and pseudomonas. Other opportunistic and saprophytic microphlora was only provisionally identified if more than 5 colonies were grown up. The smears prepared from ocular swabs from 20 sheep were stained by Giemsa and modified Ziehl-Neelsen methods for microscopic detection of chlamydia (11).

RESULTS

Mycoplasma suspected colonies grew up wholly from conjunctival swabs of four native sheep originated from a flock affected with OIKC during 1995. Two isolates were slow growing, dextrose- and arginin-negative, and two fast growing dextrose-positive and arginine-negative strains. The isolates did not require serum

for growth and grew at room temperature. Slow growing strains were isolated from the eyes with the signs of mild conjunctivitis in pure culture or with *B. ovis*. One fast-growing strain was isolated in pure culture from the animal with mild conjunctivitis, and the other one with coliforms from apparently healthy eyes. The overall results of bacteriological examinations are presented in table 1.

Table 1. Bacteria and mycoplasma in ovine eyes

Microorganism	Flocks with history of OIKC (63 examined animals)	Flocks without history OIKC (32 examined animals)	Total (95)
Mycoplasma sp.	0	0	0
Acholeplasma sp.	4 (6.3%)	0	4 (4.2)
Branhamella ovis	27 (42.9%)	1 (3.1%)	28 (29.5%)
Escherichia coli and Coliforms	3 (4.8%)	5 (15.6%)	8 (8.4%)
Bacillus sp.	1 (1.6%)	24 (75%)	25 (26.3%)
Staphylococcus sp.	17 (27.0%)	21 (65.6%)	38 (40%)
S. aureus	5 (7.9%)	1 (3.1%)	6 (6.3%)
Streptococcus sp.	11 (17.5%)	5 (15.6%)	16 (16.8%)
Negative	22 (35.0%)	8 (25.0%)	30 (31.5%)

In the flocks affected with OIKC during 1995 *B. ovis* was customary found in pure culture or as predominant bacterial species, frequently accompanied with mild conjunctivits. Branhamellas were isolated more often from the animals less than one year old (44.4%), than from those aging 1-3 years (33.3%) and over 3 years (22.2%) respectively. The conjunctivitis was characterized with serous lachrymation and mild hyperemia of palpebral and bulbar vessels. Using microscopical evidence chlamydia were not detected in any examined specimen.

CONCLUSIONS

Our microbiological and epizootiological investigations and clinical observations during the first epizootic of OIKC in Croatia are supporting the thesis that OIKC is primary mycoplasmal disease caused by *M. conjunctivae*.

Mycoplasmal infections were commonly accompanied with bacteria *B. ovis* isolated almost exclusively from the sheep affected with OIKC or originated from affected flocks. Its implication in pathogenesis of OIKC as secondary pathogens requires further investigation.

Clinical and microbiological observations indicates that *B. ovis* might as only microbial agent sporadically cause mild conjunctivitis.

The first observations raise expectations that prompt elimination of imported animals and suspected higher resistance of native sheep resulted with eradication of OIKC in Croatia.

Related to costs of medication and need of special nursing during the period of reduced vision or total blindness, anxiety of the farmers caused by severity of

clinical signs, and other inconveniences, there is a strong suggestion that prevention and eradication of OIKC should be controled by veterinary regulations.

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EVALUATION OF BOVINE WHOLE BLOOD NEUTROPHIL VIABILITY UPON INTERACTION WITH *MYCOPLASMA MYCOIDES* SUBSP. *MYCOIDES* SC

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INTRODUCTION

The interaction between invading organisms and mammalian cells is of importance in the pathogenesis of infection.

Neutrophils, which play a central role in the nonspecific defense mechanisms of the host, have the ability to phagocytose several bacteria and mount various metabolic responses usually ending in the destruction of these organisms.

In some lung lesions from animals with Contagious Bovine Pleuropneumonia, a strong co-accumulation of neutrophils and *Mycoplasma mycoides* subsp. *mycoides* SC (*Mmm* SC) was demonstrated (1). In these lesions, especially in the areas of transition between the interlobular septa and the pulmonary parenchyma, numerous degenerating neutrophils were detected by immunohistochemistry.

The mechanisms involved in the interaction between *Mmm* SC and bovine neutrophils remains to be established, although many different papers report the relationship between invading bacteria and neutrophils.

The aim of this study was to determine whether the viability of bovine whole blood neutrophils is affected or not by *Mmm* SC when this bacteria is used under conditions similar to those of other ongoing assays. For this purpose, flow cytometry (FCM) and spectrophotometry (SPM) were carried out.

MATERIALS AND METHODS

Mycoplasma Strains

Mycoplasma mycoides subsp. *mycoides SC*, strains B345/93 LNIV, Afadê and L2, at the end of the exponential growth phase, were harvested by centrifugation at 10.000xg for 20 min, washed in HBSS and resuspended to a 5% concentration (C) of the original culture. From this concentration, two other tenfold suspensions (B and A) were done.

Exposure of whole blood to Mmm SC strains

Heparinized whole blood from a healthy female Holstein Friesian was used within 4 hours after collection.

Fifty microliters from A, B, and C mycoplasma suspensions and 100µl of blood, in a final volume of 250 µl, were incubated at 39°C for 20 min, in a shaking water bath.

Positive and negative controls consisted of 100 µl of blood plus HBSS or plus 1mM ethylmaleimide, respectively.
Erythrocytes were lysed by 0,83% NH_4Cl. The remaining leukocytes were washed in HBSS 250xg at 4°C for 5 min, and processed for FCM and SPM.

FCM

Neutrophil viability was determined by incubating the leukocytes with 20 ng/ml of fluorescein diacetate (FDA) for 10 min at RT.

Data were acquired on a FACScan flow cytometer (Becton Dickinson) and collected on basis of 20.000 cells per sample. Neutrophils were selected in a live gate according to their forward and side scatter characteristics. Fluorescence intensity was displayed in a 4 log. decade histogram and the results were analysed using a Cell Quest software (Becton Dickinson).

SPM

Neutrophil ability to respond to phorbol 12-myristate 13-acetate (PMA) was determined by the tetrazolium salt MTT reduction, based on the method originally described by Mosmann (2).

Leukocytes were incubated with 6 µg/ml PMA and 0.5 mg/ml MTT in HBSS at 39°C for 1h, and 0.04 N HCl in isopropanol was added to dissolve the dark blue crystals. Supernatants were recovered by centrifugation at 250xg (4°C for 5 min) and read at 550nm on a MR 700 Microplate spectrophotometer (Dynatech). The MTT reduction was expressed in OD units.

Statistical analysis were carried out by ANOVA. P values <0.05 were considered significant.

RESULTS

FCM

The neutrophils displaying fluorescence, as a result of FDA hydrolysis, were analysed on basis of percentage and of fluorescence intensity (mean channel, MC). Histograms of cells versus log fluorescence intensity were generated. Viability was defined as an increase in the mean green fluorescence compared with a negative control. No differences were found between the results obtained in neutrophils exposed to *Mmm* SC (88-98%; 903-1650 MC) and those of mean±SEM for positive controls (91% ± 2.9; 1274 MC ± 271), as shown in table I and expressed in Fig. 1.

SPM

The results of MTT reduction were expressed in mean OD±SEM after subtraction of the negative controls mean OD (Table II). The values obtained in the supernatants from leukocytes previously exposed to *Mmm* SC strains and submitted to PMA, were not significantly differents from those of the positive controls but significantly differents when compared to those of the resting cells.

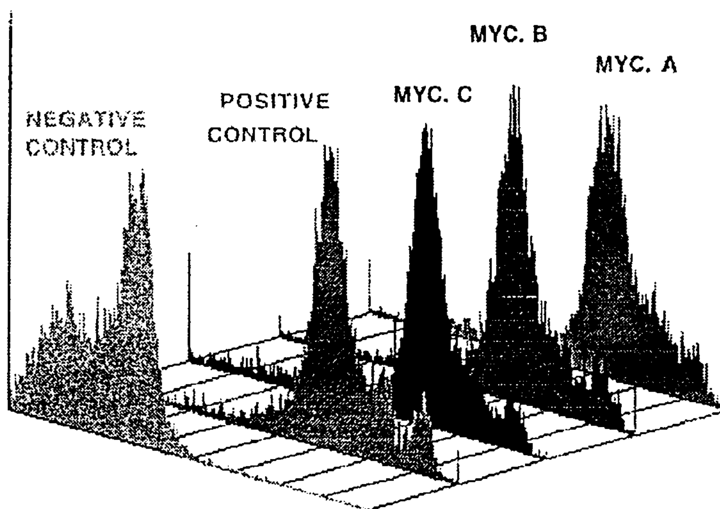

Fig. 1 - 3D Overlay hystogram of DAF fluorescence from three mycoplasma concentrations as compared to controls.

Table I - Neutrophil viability estimated by FDA hydrolysis.

Controls	Mycoplasma		%	M C
	Strains	Concentration		
Negative [a]			2 ± 0.8 [c]	57 ± 17
Positive [b]			91 ± 2.9	1274 ± 271
	B345/93 LNIV	A	95 ± 1.0 [d]	1500 ± 100
		B	94 ± 1.0	1400 ± 200
		C	92 ± 1.0	1650 ± 150
	Afadê	A	97 ± 0.9	1300 ± 133
		B	95 ± 1.8	1279 ± 228
		C	94 ± 0.9	1400 ± 150
	L2	A	98 ± 0.4	1113 ± 18
		B	97 ± 0.4	1087 ± 117
		C	88 ± 1.8	903 ± 21

[a] blood treated with 0,1mM ethylmaleimide; [b] untreated blood; [c] mean ± SEM for five experiments
[d] mean ± SEM for three experiments

Table II - Neutrophil ability to respond to PMA, after exposure to *Mmm* SC.

Controls	Mycoplasma		MTT reduction		
	Strains	Concentration	OD_{550nm}	P [c]	P [d]
Resting cells [1]			0.08 ± 0.004 [a)]		
Positive [2]			0.13 ± 0.002		
	B 345/93 LNIV	A	0.16 ± 0.003		
		B	0.18 ± 0.001		
		C	0.14 ± 0.001		
Resting cells [1]			0.07 ± 0.01 [b)]		
Positive [2]			0.15 ± 0.01		
	Afadé	A	0.15 ± 0.03	NS	< 0.05
		B	0.17 ± 0.01	NS	< 0.001
		C	0.16 ± 0.01	NS	< 0.01
Resting cells [1]			0.06 ± 0.01 [b)]		
Positive [2]			0.15 ± 0.00		
	L2	A	0.15 ± 0.02	NS	< 0.01
		B	0.16 ± 0.02	NS	< 0.01
		C	0.16 ± 0.02	NS	< 0.01

[1] Cells without PMA; [2] Cells with PMA; [a)] mean ± SEM, for a single experiment, for three wells replicates; b) mean ± SEM for three experiments; [c)] P value obtained by comparison with positive control; [d)] P value obtained by comparison with resting cells; NS not significant

CONCLUSIONS

The results of flow cytometry permitted to conclude that *Mmm* SC did not impair the ability of bovine neutrophils non-specific esterases to hydrolyse FDA into its fluorescent form.

Concerning MTT, the results of the colorimetric assays correlated well with those of flow cytometry, permitting to conclude that *Mycoplasma mycoides* subsp. *mycoides* SC, used under conditions similar to those of other ongoing assays, did not affect neutrophil integrity and not even impaired their ability to be stimulated.

ACKNOWLEDGMENTS

This work was partially supported by the "CBPP/EU Research Project " (Pan African Rinderpest Campaign nº 7-ACP-RPR-24). The authors are very grateful to Maria Joaquina Lapão for excellent technical assistance.

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EXPERIMENTAL TRANSMISSION IN SHEEP AND GOATS OF MYCOPLASMA MYCOIDES SUBSP. MYCOIDES,LC ,FROM BOVINE ORIGIN

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INTRODUCTION

Mycoplasma mycoides subsp. *mycoides*, Large Colony (*Mmm* LC), has been associated, in most cases, to situations of natural disease in goat, although experimentally sheep and goats are usually infected (1,3,4,5). The results of experimental inoculation of *Mmm* LC, from goat origine to bovine are rather controversial, suggesting that the LC type of *M. mycoides* is not a primary etiological agent in cattle. (3,4,5)

Recent reports, however, on the identification of *M. mycoides* strains in the lungs and lymph nodes from bovines, with and without CBPP lesions, either singly or associated with other mycoplasmas (*Mmm* SC, *M. bovis*) (2) prompted us to evaluated experimentally the possible pathogenic role of these strains in sheep and goats, namely if *Mmm* LC of bovine origin produce a syndrome akin to contagious agalactiae. In view of widespread prevalence of the agent, this experiment was carried out in spite of our awareness of the difficulties associated with the experimental reproduction of disease with mycoplasmal agents.

MATERIALS AND METHODS

For experimental inoculation, strain BP787 of *M. mycoides* subsp. *mycoides* LC, was used. This strain was isolated from a bovine lung with pneumonia lesions. *M. bovis* was also isolated from the same specimen.

The strain was cultured in Hayflick medium and freeze dried. The titre of the freeze dried was about 107/CFU/ml.

The assay was carried out in a farm with reasonable conditions of hygiene, desinfection and housing.

Nineteen sheep and nineteen goats, all pregnant and good health were included in the experiment.

After parturition and before inoculation, all the animals in assay were serologically screened for *Brucella* sp., *Mmm* SC and *M. capricolum* subsp. *capricolum* antibodies. Milk samples were also analysed to detect mycoplasmal agents and other pathogenic bacteria. All the above tests were negative.

EXPERIMENTAL INOCULATION

Seven sheep and seven goats, were i. v. inoculated with 5ml of the suspension in PBS of the freeze dried culture of strain BP 787 ($\pm 10^7$ CFU/ml).The same amount was used to inoculate seven sheep and seven goats by intranasal spraying.

The remaining animals (five sheep and five goats)were not inoculated - control group.

The offspring were also considered as "controls "being susceptible to a carry-over infection through their mother` s milk.

The animals were tested weekly for the anti-*M.mm* SC antibodies by CFT according to the routine procedure for CBPP. At the same time, the milk was cultured for the detection of *Mycoplasma* and other bacteria (Table I, II, III).

Table I - Antibody titre after infection with Mmm LC strain BP787

Sheep	Antibody titre by C.F.T.* (weeks p. i.)								
	1°	2°	3°	4°	5°	6°	7°	8°	9°
1 IV	(+++) 1:40	(+) 1:2 0	(+) 1:8 0	(++) 1:8 0	(++) 1:1 0	(+) 1:20	-	(++) 1:20	-
2 IV	-	(+++) 1:1 0	(+++) 1:4 0	(++) 1:4 0	(++) 1:80	(+++) 1:2 0	(+++) 1:2 0	(++) 1:4 0	(+++) 1:2 0
3 IV	(++) 1:2 0	(++) 1:4 0	(++) 1:10	(+++) 1:20	(++) 1:40	-	-	-	-
4 IV	-	(+++) 1:1 0	(++) 1:1 0	(+++) 1:1 0	(+) 1:8 0	(++) 1:1 0	-	-	(++) 1:10
5 IV	(++) 1:10	(+++) 1:2 0	(+++) 1:4 0	(+++) 1:40	(++) 1:40	(+++) 1:20	(++) 1:10	-	(++) 1:10
6 IV	(++) 1:8 0	(+) 1:2 0	(+1:32 0	(+++) 1:80	(++) 1:10	(++) 1:4 0	-	-	-
7 IV	(+++) 1:80	(+++) 1:80	(+) 1:80	(+) 1:8 0	(+++) 1:1 0	(+++) 1:40	(++) 1:40	(++) 1:4 0	(+++) 1:10
8 IN	(++) 1:10	(+++) 1:2 0	(+++) 1:40	(++) 1:20	(++) 1:1 0	(+++) 1:10	-	-	-
9 IN	-	(++) 1:10	(++) 1:10	(+++) 1:20	(++) 1:10	(+++) 1:10	-	-	-
10 IN	-	(+++) 1:10	(++) 1:40	(+++) 1:20	-	(+++) 1:10	-	(++) 1:2 0	(++) 1:10
11 IN	(++) 1:10	(++) 1:10	(+++) 1:10	(+++) 1:20	(++) 1:40	(++) 1:10	-	-	-
12 IN	(++) 1:10	(++) 1:20	(++) 1:10	(++) 1:20	(++) 1:4 0	-	-	-	-
13 IN	-	(++) 1:10	(++) 1:40	(++) 1:20	(+++) 1:10	-	-	-	-
14 IN ¤	-	(+++) 1:10	(+++) 1:10	(+++) 1:10	(++) 1:20	-	-	-	-

* C.F.T. used for PPCB IV Intravenous IN Intranasal

¤ Isolation of *Mmm* LC from milk and supramammary lymph nodes at necropsy

Table II - Antibody titre after inoculation with *Mmm* LC strain B 787

Goat	Antibody titre by C.F.T.* (weeks p. i.)								
	1°	2°	3°	4°	5°	6°	7°	8°	9°
1 IV	(+++) 1;10	(+++) 1:1 0	(+++) 1:20	(+++) 1:20	(++) 1:2 0	(+++) 1:20	(+++) 1:20	-	(++) 1:2 0
N°2 IV	(++) 1:10	(+++) 1: 20	(++) 1:4 0	⊛ (++) 1:4 0	(+++) 1: 20	(++) 1:4 0	-	-	(+++) 1:1 0
N°3 IV	(+++) 1:4 0	(+) 1:40	(+++) 1:8 0	(+++) 1:4 0	(+++) 1:4 0	(+++) 1:1 0	(+++) 1:4 0	(+++) 1:2 0	(++) 1:10
4 IV ¤	(+++) 1:20	(++) 1:1 0	⊛ (+) 1:80	⊛ (+) 1:320	⊛ (+) 1:320	(+) 1:320	⊛ (+++) 1:160	(+++) 1:160	(+++) 1:16 0
5 IV	(++) 1:10	(+++) 1:2 0	(++) 1:2 0	(+) 1:20	(+) 1:20	(++) 1:10	-	-	-
6 IV ¤	(++) 1:160	(+++) 1:20	(++) 1:40	-	⊛ -	-	-	-	-
7 IV	(++) 1:40	(+++) 1:20	(++) 1:8 0	(++) 1:4 0	(++) 1:1 0	(++) 1:2 0	-	-	(++) 1:20
8 IN	-	(++) 1:10	(++) 1:10	-	-	-	-	-	-
9 IN ¤	-	(++) 1:80	(+++) 1:80	⊛ (+++) 1:40	(+++) 1:40	(+++) 1:40	(+++) 1:20	(++) 1:40	(++) 1:10
10 IN	(++) 1:20	(++) 1:20	(++) 1:20	(++) 1:40	(++) 1:20	(++) 1:20	(+++) 1:10	-	(++) 1:10
11 IN	-	-	-	⊛ -	⊛ -	-	-		-
12 IN	(++) 1:20	(++) 1:20	(++) 1:20	(++) 1:4 0	(++) 1:2 0	(++) 1:40	-	-	-
13 IN	-	-	-	-	-	-	-	-	-
14 IN	(++) 1:10	(+++) 1:10	(+) 1:40	(++) 1:20	"(++) 1:2 0	(++) 1:20	(+++) 1:10	(++) 1:20	(+++) 1:10

* C.F.T. used for PPCB IV Intravenous IN Intranasal
⊛ Isolation of *Mmm* LC from milk during assay.
¤ Isolation of *Mmm* LC from milk and supramammary lymph nodes at necropsy

Table III - Antibody titre of Control Group

	Antibody titre by C.F.T.*								
	1°	2°	3°	4°	5°	6°	7°	8°	9°
Sheep 18	(+++) 1:10	(+++) 1:20	(+++) 1:40	-	-	(+++) 1:20	-	-	(++) 1:10
Sheep 19	-	(+++) 1:20	(+++) 1:40	(+++) 1:40	(++) 1:20	(+++) 1:20	-	-	-
Sheep 20	-	(+++) 1:20	(++) 1:40	-	(+++) 1:20	(++) 1:10	-	-	-
Sheep 21	-	(++) 1:20	(++) 1:20	(++) 1:20	(++) 1:20	(++) 1:10	-	-	-
Sheep 22	-	(+++) 1:10	(++) 1:10	(++) 1:10	-	-		-	-
Goat 18	(++) 1:10	-	(++) 1:20	(+++) 1:10	-	(++) 1:10	-	-	(++) 1:10
Goat 19	-	(++) 1:20	(+++) 1:10	(++) 1:20	(+++) 1:10	(++) 1:10	(++) 1:10	(++) 1:20	(+) 1:20
Goat 20	-	(++) 1:10	(+++) 1:10	-	-	(++) 1:2 0	-	-	(++) 1:1 0
Goat 21	-	(+) 1:20	(+++) 1:20	(+++) 1:40	(+++) 1:40	(+++) 1:40	(+++) 1:10	(+++) 1:10	(+++) 1:10
Goat 22	-	(++) 1:10	-	-	-	(++) 1:10	-	-	-

* C.F.T. used for PPCB

RESULTS AND CONCLUSIONS

There were no clinical symptoms in inoculated animals and in the control group.
The experimental exposure by intravenous and intranasal spray caused intermittent milk excretion of *Mmm* LC from the 3rd week onwards in goats but not in sheep, suggesting that sheep are less susceptible than goats to infection.
At necropsy, neither goats nor ewes, showed pneumonia lesions or signs of acute mastitis, although *Mmm* LC was isolated from supramammary lymph nodes of two goats and from an ewe inoculated via intranasal route.
The overall low titres of CF antibodies both in goats and sheep (as compared with the control group) as well as the absence of pathological changes in inoculated animals point to the attenuation (lowered virulence) of BP787 strain due to serial "in vitro" passages before the inoculation experiment.
In spite of Mycoplasma excretion through the teats of inoculated goats, their milk showed no visible alteration (flocculation), situation frequently found in the field and currently responsible for disease transmission to the kids.
Finally, the lymph nodes persistence as well as the milk excretion of *Mmm* LC, in inoculated goats, may add evidence to the carrier state of these animals in the epidemiology of *Mmm* LC infection.

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Acknowledgments: We are grateful to Prof. A. Penha Gonçalves for his critical review of the manuscript.

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European Commission

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Luxembourg: Office for Official Publications of the European Communities

1999 — viii, 191 pp. — 17.6 x 25 cm

ISBN 92-828-5222-9

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ISBN 92-828-5222-9