Immune response to and pathogenic mechanisms of contagious bovine pleuropneumonia infection. Investigation of the importance of the capsular polysaccharide and assessment of its vaccine potential.

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A mes parents

et mon frère,

Abstract

Mycoplasma mycoides subsp. *mycoides* small colony type (*Mmm*SC) is the causative agent of contagious bovine pleuropneumonia (CBPP), a major disease of cattle in Africa for which current vaccines exhibit a poor efficacy. *Mmm*SC possesses a capsular polysaccharide (CPS) believed to be an important virulence factor. Antibodies directed against CPS are bactericidal in an *in vitro* growth inhibition test (GIT). Therefore, CPS is a good vaccine candidate.

The aim of this thesis was to investigate the vaccine potential of *Mmm*SC CPS.

Before using CPS as a vaccine, the immunogenic structure of CPS needed to be studied. *Mmm*SC strains were tested with rabbit antisera (raised against different *Mmm*SC strains) in a GIT. The results showed that CPS was conserved between the strains. Purified CPS from different *Mmm*SC strains were then investigated with monoclonal antibodies (mAbs) in an enzyme linked immunosorbent assay (ELISA). It appeared that CPS from all the strains were recognised by the mAbs, except the strain PG1 that had a low signal with two of the mAbs. GIT was used with mAbs that recognised *Mmm*SC CPS to test their growth inhibiting (GI) activity; all of the mAbs used in the GIT were bactericidal.

The specificity of these mAbs was then investigated, using an ELISA, against other mycoplasmas, *Mycoplasma mycoides* subsp. *mycoides* large colony and *Mycoplasma mycoides* subsp. *capri*. The results obtained suggested that the mAbs recognised at least three different epitopes on CPS.

A competitive ELISA was performed to clarify the number of epitopes that these mAbs recognised. No conclusion could be drawn from this experiment since the results were unclear.

Since antibodies to CPS are bactericidal *in vitro*, this suggested they were protective against *Mmm*SC. Passive immunisation of mice with a bactericidal mAb directed against CPS was used to investigate the protective efficacy of anti-CPS antibodies *in vivo*. After injection with the antibody, mice were challenged with an *Mmm*SC strain. The incidence and the duration of mycoplasmaemia were used to assess the protection given by this antibody. No significant difference could be seen between mice passively immunised with anti-CPS antibody and the control group, suggesting that a bactericidal antibody was not protective *in vivo* in mice against challenge with *Mmm*SC.

The type of immune response to CPS was examined in three groups of cattle: *Mmm*SC-intubated animals, CBPP-vaccinated and unvaccinated animals in contact with *Mmm*SC-intubated cattle. The results showed that only a quarter of CBPP-vaccinated cattle had an immune response against CPS. The immune response against CPS in the three different groups was of IgM type even after a second exposure to the pathogen where it could have been expected an IgG type immune response.

The lack of immune response to CPS in cattle might be due to cross-reactions with bovine lung. Cross-reactions between bovine lung and *Mmm*SC CPS were confirmed by western-blot with anti-*Mmm*SC or anti-CPS sera from rabbits, mice

and mAbs. It was not possible to know whether antibodies recognising both CPS and bovine lung were present in anti-*Mmm*SC cow sera because of a background signal due to the secondary antibody specific for cow immunoglobulins.

To minimise the cost of CPS vaccine production, polysaccharides from other sources could be used as a vaccine. A western-blot was then performed to examine the cross-reactions between *Mmm*SC CPS and carbohydrates from cereals with *Mmm*SC-infected cow sera, *Mmm*SC-immunised rabbit sera and mAbs. The results showed that CPS shared epitopes with the polysaccharides from the cereals used in this experiment.

Declaration

I hereby declare that I composed this thesis entirely myself, that it describes my own research and that the work has not been submitted for any other degree or professional qualification.

Signature

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Abbreviations

ADRI: Animal Diseases Institute

AEBSF: 4-(2-aminoethyl)benzenesulfonyl fluoride

AGPT: agar gel precipitin test

APS: ammonium persulfate

BR: blocking reagent

CBB: cold binding buffer

CBPP: contagious bovine pleuropneumonia

CF: complement fixation

cfu: colony-forming unit

CM: casein medium

CPS: capsular polysaccharide

DNAse: desoxyribonuclease

DW: distilled water

EBL cells: embryonic bovine lung cells

EDTA: ethylene diamine tetraacetic acid

ELISA: enzyme linked immunosorbent assay

FCS: foetal calf serum

FVM: Faculty of Veterinary Medicine

GA: Gourlay's agar

GB: Gourlay's broth

GI: growth inhibiting

GIT: growth inhibition test

HAT: hypoxanthine aminopterin thymidine solution

HRP: horseradish peroxidase

Ig: immunoglobulin

LAT: latex agglutination test

LB: loading buffer

L-glu: L-glutamine

LNIV: Laboratório Nacional de Investigaçao Veterinária

LSC: liquid scintillation counting

mAb: monoclonal antibody

*M*bg7: Mycoplasma species "bovine group 7"

M. bovis: *Mycoplasma bovis*

Mccp: *Mycoplasma capricolum* subspecies *capripneumoniae*

MDC: medium-derived carbohydrate

ME: 2-mercaptoethanol

MEM: minimum essential medium Eagle

Mmc: *Mycoplasma mycoides* subspecies *capri*

*Mmm*LC: *Mycoplasma mycoides* subspecies *mycoides* large colony

*Mmm*SC: *Mycoplasma mycoides* subspecies *mycoides* small colony

MRI: Moredun Research Institute

OD: optical density

OPD: o-phenylenediamine

PBS: phosphate buffered saline

PBST: 0.05% Tween 20 PBS solution

PEG 1500: polyethylene glycol 1500

PMSF: phenylmethylsulfonyl fluoride

PS: penicillin streptomycin solution

RNAse: ribonuclease

RPMI: RPMI 1640 medium

SDS: sodium dodecyl sulphate

SDS-PAGE: sodium dodecyl sulphate polyacrylamide gel electrophoresis

TD: thymus dependent

TEMED: N-tetramethylethylenediamine

TI: thymus independent

TS: 154 mM NaCl 10mM Tris solution

TX: lower phase in TX144 fractionation

TX114: 10% Triton X114 solution

VLA: Veterinary Laboratories Agency

WB: western-blot

1. Introduction.

1.1. Contagious bovine pleuropneumonia (CBPP).

1.1.1. History.

In the 18th century, contagious bovine pleuropneumonia (CBPP), confined to the alpine region of Europe, disseminated with export of animals and was present in many countries in the world. A policy of cattle movement restriction and slaughter of diseased and suspect animals with compensation has allowed it to be eradicated from the United States and many European countries, although outbreaks were reported in France in 1980, in Spain in 1989, in Italy in 1990 and reappeared in Portugal in 1983. In Australia, cattle movement restriction and slaughter have been associated with vaccination. [1-4]. No outbreaks were reported in Europe in 1999. Despite mass vaccination campaigns, CBPP has spread alarmingly in Africa. In 1999, the disease was present in at least 27 countries in equatorial, central and southern Africa and it is now considered as the most important threat to the cattle industry in Africa [5]. The disease causes important economic losses due to mortality, loss of weight, reduced work ability, reduced fertility and growth rate, cattle trade restriction, quarantine and vaccination costs [4,6,7].

1.1.2. Clinical picture.

CBPP is an infectious disease caused by *Mycoplasma mycoides* subspecies *mycoides* small colony (*Mmm*SC) [8]. This disease only affects cattle (species *Bos*) and related animals such as buffalo, yak, bison and reindeer [2,4,9], although *Mmm*SC has been isolated from sheep's milk and from goats suffering from pneumonia [10]

and from a goat reportedly suffering from contagious caprine pleuropneumonia [11] suggesting a possibility of transmission from sheep and goats to cattle. CBPP is characterised by a severe exudative pleuropneumonia with secondary lymphatic and vascular involvement leading to the typical lung lesions due to necrosis, sequestration and encapsulation [4,7,9,12-14]. In adult cattle, the symptoms are essentially restricted to the respiratory system [15]. Young calves are rarely affected and the disease is less severe, with infections generally causing arthritis, with lesions in joints and synoviae instead of the lungs [15-17]. The incubation period is between 5 and 207 days [4]. Different forms of CBPP exist: in a herd nearly half of the animals will develop the subacute/chronic form, 30 to 40% of animals will be affected by either the acute form or the hyperacute form, the rest of the animals of the herd will be resistant to the disease. Only the acute and hyperacute forms of the disease permit a clinical diagnosis [9,13]. In the chronic form, a cough may be the only symptom but the post-mortem examination reveals necrotic lungs, walled off by fibrous tissue and encapsulated to form sequestra containing viable *Mmm*SC organisms [2,18-20]. In the first stage of the acute form, the animal shows anorexia, irregular rumination, fall in milk production with a moderate fever and persistent coughing. Excess of mucus in the nose and the mouth can be seen. In the next stage, there is no rumination and total anorexia, the animal is in distress with painful respiration, is reluctant to move and the animal dies soon after. At necropsy, a large excess of pleural fluid is found in the thoracic cavity along with lung lesions [9,15,18-21]. The hyperacute form exhibits the same clinical symptoms as the acute form, but is much accelerated and the animal dies within a week. In adult cattle, the clinical signs are due to lesions developing in the lungs.

A large number of the cases are subclinical and cannot be detected except at slaughter. The subacute form is the most dangerous form in the sense that it maintains infection in a herd or an area undetected for months before lesions are seen in abattoirs [13,15,22,23].

Lesions are usually unilateral, localised in the diaphragmatic lobe and present a characteristic marbling appearance [2,17,21]. They are thought to be caused by autoimmune and hypersensitivity reactions [7,9].

Infected animals may recover from CBPP. These animals often present sequestra of various sizes in one or both lungs. In some cases, lesions may resolve leaving scarring on the lungs [2,6,17,24]. Recovered animals appear to be resistant to reinfection with CBPP [6,20]. One experiment looked at the resistance of recovered animals to CBPP reinfection by challenging cattle intravenously and by endobronchial intubation (4 cattle for the intravenous route and 4 others for the endobronchial route) with 50 ml of Gladysdale culture containing 10^{10} mycoplasmas/ml. The recovered animals challenged both by the intravenous and the endobronchial route exhibited a secondary immune response as measured by the complement-fixation test (the titres were lower for the cattle challenged intrabronchially) but no mycoplasma could be found in the blood and tissues of any of the animals [25]. However, the significance of these results may be questioned due to the small size of the animal sample.

Since the sequestra contain viable mycoplasmas, recovered animals (with old lesions) were thought to be a potential risk for healthy animals, as it has been suggested that these lesions could break down under stress and transmit the disease [26]. However, when animals where submitted to different conditions of stress

that can be encountered in Africa (exercise, starvation, water deprivation) it was shown that it was difficult to break down these lesions. When stressed cattle with old sequestra were put into contact with normal cattle, none of the latter was infected [24].

The main routes of transmission are by direct close contact between animals excreting Flugge-type droplets by coughing and inhalation of bronchial secretions [1,7,9,27,28]. Transplacental transmission has also been suggested as *Mmm*SC can cross the placental barrier [29]. Experiments have shown the presence of *Mmm*SC in calves born to cows with extensive lung lesions. The presence of *Mmm*SC could not be due either to parturition or to suckling as *Mmm*SC was isolated from various tissues (lungs, kidney, spleen, lymph nodes, joints, blood) from a calf removed from a cow at necropsy immediately after cow slaughter and from calves that did not suck colostrum before being killed within 6 hours after birth. No CBPP lesions were found in the calves but signs of arthritis were observed in the stifle joints. However, it is not known whether these calves would develop CBPP and transmit the disease [30]. Other sources of contagion exist. Viable *Mmm*SC has been isolated from urine of cattle infected with CBPP. The small droplets coming from the splashing of urine during micturition may be inhaled in the same way as droplets of bronchial secretions [31]. The excretion of *Mmm*SC in a viable form was confirmed in another experiment, where the titres of *Mmm*SC in urine ranged from 10^1 to 10^3 organisms per ml. *Mmm*SC was detected in urine from cattle with acute cases of CBPP [32]. Viable *Mmm*SC has also been demonstrated in the nasal discharge of sick animals [2,7] and in semen and preputial washings of bulls [33]. Infection of cattle through fomites and contaminated fodder has also been observed under experimental conditions [34]. It has also been reported that *Mmm*SC can be isolated from ticks

collected from CBPP-infected cattle, but so far the attempts to infect cattle with *Mmm*SC-infected ticks have been unsuccessful [35].

Differences in susceptibility to *Mmm*SC infection have been noted. Contradictory reports have been made concerning age susceptibility. It was suggested that calves and old cattle were more often affected by CBPP, but with a difference in the symptoms, calves showing signs of arthritis and old cattle developing pleuropneumonia [6,15,20,22]. In other studies however, animals under the age of 3 were highly susceptible to CBPP when compared to those over 3 years of age since the mortality due to CBPP was higher and the lung lesions more severe in the first group [36,37]. Differences have also been reported between beef steers and dairy cows (the latter appearing to be more susceptible to infection), and between breeds [15,20]. Cattle from breed *Bos indicus* (zebu) succumbed more severely to CBPP than cattle from breed *Bos taurus* (European-type cattle), under the same conditions, when the results were based on the death alone, but when the results were based on the infection rate, no statistical difference could be seen between the 2 types of cattle [38].

Few treatments are available to cure CBPP. Some antibiotics such as Tilmicosin and Enrofloxacin have been shown to be mycoplasmacidal against *Mmm*SC [39]. In another study, Tilmicosin and Danofloxacin have been shown to have both inhibitory and mycoplasmacidal activity *in vitro* on *Mmm*SC strains [40]. However, the use of these substances is discouraged because, although they may reduce the severity of the disease and improve the conditions of the infected animals, they do not eliminate the mycoplasmas and can mask symptoms, leading to chronic carriers that would be sources of infection [4,5,41].

The disease seems to be less severe in Europe than in Africa, as seen with the lack of mortality and obvious morbidity in European outbreaks, although at necropsy the infected animals present identical pathological lesions both in Africa and Europe [5,27]. The subacute and chronic forms of the disease are the most common in Europe, the typical acute form is rare compared with Africa. This difference could be due to the administration of antibiotics, good livestock management and the immediate slaughtering of the animals. It was also suggested that it could be due to the lower virulence of the European strains in comparison with African strain [27].

1.2. Causal agent of CBPP.

1.2.1. Mycoplasmas in general.

Mycoplasmas are the smallest self-replicating organisms. They are extracellular parasites, widespread in humans, animals, plants and insects [42-44]. The majority of human and animal mycoplasmas colonise mucosal surfaces and generally cause diseases involving the respiratory and the urogenital tracts, the mammary glands, joints and eyes [42,45-47]. Some mycoplasmas have invasive properties and can disseminate and affect serosal surfaces of the thoracic, abdominal and articular cavities causing severe clinical cases [46]. Mycoplasma infections seem to be host specific but some of them can be pathogenic in one host species and colonise other hosts without any pathogenicity [46,48]. However, cases of pleuritis and diseases of the lower respiratory tract in horses caused by *Mycoplasma felis* have been reported [49,50].

In addition to their small size, mycoplasmas do not possess a cell wall and consequently lack cell-wall associated proteins. They have a small genome with a

low guanine plus cytosine content. In mycoplasmas, the UGA codon codes for tryptophan (unlike other prokaryotes in which it is a stop codon) [5,46].

In order to colonise the host, some mycoplasmas need to adhere to the host cells and the loss of adhesion means a loss of infectivity. In an experiment with *Mycoplasma pneumoniae*, hamsters were intranasally inoculated with the wild type strain B25C and its mutants which showed a significantly reduced attachment to hamster tracheal rings. Hamsters inoculated with the wild type strain developed pneumonia while hamsters infected with the mutants did not [51]. *In vivo*, mycoplasmas adhere via their membrane surface to a wide range of substrates such as mucous membranes, host-cell appendages and projections, and the host-cell surface itself [44,48,52,53].

Mycoplasmas are able to evade the host immune response and to elicit chronic inflammation by various mechanisms such as molecular mimicry, immunosuppression, release of soluble antigens, formation of immune complexes (that attract inflammatory cellular infiltrates to the area of infection by fixing complement), antiphagocytic properties (due in a part to the presence of a capsule), induction of autoantibodies (by behaving as potent mitogens for lymphocytes), defective mycoplasmacidal antibodies (antibodies that have mycoplasmacidal properties on resting mycoplasmas but not on multiplying mycoplasmas), altered lymphocyte responsiveness and antigenic variation [43,52,54,55]. The lesions observed in mycoplasmal disease are largely due to the host immunological reactivity rather than the microorganisms themselves [42].

1.2.2. *Mycoplasma mycoides* subspecies *mycoides* small colony (*Mmm*SC).

Mycoplasma mycoides subspecies *mycoides* small colony (*Mmm*SC), the causal agent of CBPP, was isolated in 1898 by Nocard and his collaborators [47,56]. This mycoplasma shares many immunological, biochemical and genetic properties with *Mycoplasma mycoides* subspecies *mycoides* large colony (*Mmm*LC), *Mycoplasma mycoides* subspecies *capri*, *Mycoplasma capricolum* subspecies *capricolum*, *Mycoplasma capricolum* subspecies *capripneumoniae* and *Mycoplasma* species "bovine group 7" (*M*bg7). These are all pathogens of cattle, sheep and goats and comprise the "*Mycoides* cluster" [23,57]. *Mycoplasma putrefaciens* was later added to the "*Mycoides* cluster" when the clustering was based only on the 16S rRNA sequences [cited in 58].

*Mmm*SC are invasive mycoplasmas causing systemic disease, they have been found in the lungs, pleural fluid and thoracic lymph glands, in liver, spleen, kidney and circulating blood [59].

As with all mycoplasmas, *Mmm*SC does not possess a cell wall. *Mmm*SC grows readily in cell-free systems in serum-enriched medium, where the culture may grow as visible threads. Virulent and moderately virulent *Mmm*SC strains have been shown to produce threads, unlike avirulent strains, however, the experiment was performed with 4 strains only and this might not be true for all *Mmm*SC strains [60]. *Mmm*SC also grows readily on agar plates, where the colonies formed have a typical "egg-fried" appearance [15,20,21]. The addition of glucose to the liquid culture medium allows the formation of thicker threads indicating that the threads may be formed by

products of glucose metabolism [61]. *Mmm*SC is surrounded by a polysaccharide capsule [2].

Some of the biochemical properties of *Mmm*SC include resistance to penicillin, the fermentation of glucose, the oxidation of maltose, trehalose and (at low concentrations) mannose and glucosamine, and the reduction of tetrazolium. *Mmm*SC is unable to hydrolyse arginine [5,15,20,21].

The major precipitating antigen has been identified as a polysaccharide [62]. Other antigens have been identified after the study of the protein patterns of 13 *Mmm*SC strains obtained by SDS-PAGE. Protein profiles demonstrated a high similarity among the strains, with 49 to 59 distinct polypeptides ranging from 14 to 150 kDa. The major components had apparent molecular weights of 109, 95, 74, 67, 62, 60 and 48 kDa, the immunodominant proteins by western-blot had molecular weights of 109, 95, 62 and 48 kDa [63,64].

Although closely related to the other mycoplasmas from the "*Mycoides* cluster" and antigenically similar, *Mmm*SC strains can be differentiated on the basis of the insertion sequence analysis of their DNA. Insertion sequences are transposable genetic elements of about 800 to 2,500 bp often present in multiple copies in bacterial genomes. Three insertion sequences have been identified in *Mmm*SC: IS*1296*, IS*1634* and IS*Mmy1* [57,65,66]. The analysis of the IS*1296* banding pattern of 64 *Mmm*SC strains from various countries and continents showed that these strains could be classified into two main clusters. One cluster is composed of the European strains that form a single homogeneous group. The other cluster contains the African and Australian strains with a more heterogeneous grouping; the type strain PG1, of unknown origin, belongs to the cluster of strains from Africa and Australia [57]. It was

later discovered that the difference in the IS*1296* profile between European and African/Australian strains was due to a lack of an 8.84 kb genetic segment in the DNA of the European isolates [67]. Unlike IS*1296* that was found in a low copy number in few strains of *Mmm*LC and *M*bg7, IS*1634* is highly specific for *Mmm*SC. No copies were found in any of the other members of the "*Mycoides* cluster", or in other closely related mycoplasma species of ruminants [65]. IS*Mmy1* was found in 15 *Mmm*SC strains; they showed the same banding pattern except for the type strain PG1, the vaccine strain T_1SR and the strain Afadé. This insertion sequence is not specific for *Mmm*SC since copies were also found in a *Mycoplasma bovis* strain [66].

Differences in virulence among *Mmm*SC strains have been observed. The European strains are thought to be less virulent than their African counterparts because the disease seems less severe in Europe. One experiment looked at the immunological reactions in cattle infected either with the African strain Afadé or the European strain L2. Cattle were infected intrabronchially with 10^9 colony-forming units (cfu) of strains Afadé (one animal) and L2 (two animals), and two cattle were put in contact with the infected cattle in each group. It was observed that Afadé induced stronger and earlier immune reactions than L2. Although only one strain from Africa and from Europe were compared and a small number of animals was used for each strain, the authors concluded that European strains were generally less virulent than strains from Africa [68]. However, the strong immune response to Afadé might clear the mycoplasma faster leaving less time to multiply in the host, and the poorer immune response to L2 might be a consequence of immunosuppressive properties of this strain, in that case, L2 could be considered as more virulent than Afadé.

In addition, it was found that European strains were unable to oxidise glycerol, unlike the African isolates. It was suggested that this inability could be a factor in the reduced morbidity and mortality of CBPP in Europe since the oxidation of glycerol by *Mmm*SC strains produces hydrogen peroxide, which may be a significant factor in the pathogenicity of CBPP, and consequently may contribute to virulence [69]. However, it should be noted that *Mmm*SC strain KH_3J, known for its avirulence in cattle, was able to oxidise glycerol with a high rate of oxygen uptake [J.B. March, personal communication], and consequently should be considered as a virulent strain according to the authors' conclusions.

1.3. Immunity to CBPP.

Humoral and cell-mediated immunity seem to play an important part in the recovery from and the protection against CBPP.

1.3.1. Humoral immunity.

Cattle recovered from CBPP are known to be resistant to subsequent infections with *Mmm*SC [6,20]. For this reason, it was thought that immunity might be conferred by inoculating healthy animals with sera from these cattle. An experiment has been carried out with 10 cows, 5 inoculated twice with 1 litre each of serum from susceptible cattle and 5 inoculated twice with 1 litre each of serum from CBPP-recovered animals. These animals were then put in contact with cattle infected with *Mmm*SC strain Gladysdale. The results showed that only one out of the five animals given serum from CBPP-recovered cattle became infected. The four others had no lesions due to CBPP, though *Mmm*SC was found in two of them. All the cattle in the

control group died from CBPP. These experiments suggested that cattle inoculated with serum from recovered animals were protected against a challenge with *Mmm*SC [70] although the significance of the results was diminished due to the small sample size of the animals.

This passive transfer of immunity has also been demonstrated in mice. Mice inoculated subcutaneously with sera from mice immunised with a significant dose of viable *Mmm*SC and then challenged intraperitoneally with live microorganisms did not develop bacteraemia [71]. This test was also used to show that serum from cattle inoculated intravenously with *Mmm*SC produced "mouse-protective" antibody (i.e. mice inoculated with *Mmm*SC immune serum from cattle did not exhibit mycoplasmaemia after challenge with *Mmm*SC) [72]. Therefore, antibodies seem important in the immunity to *Mmm*SC.

However, when the antibody titre was measured by complement-fixation test (CF test), it did not seem to have correlation between the antibody titre and protection against CBPP. In 30 cattle vaccinated with freeze-dried mycoplasmas, only 13 exhibited CF antibodies after vaccination. When these animals were challenged by in-contact method, 29 out of 30 animals had CF antibodies following challenge, and 24 out of 30 were resistant to CBPP. In the 15 unvaccinated control animals, all of them showed CF antibodies following in-contact challenge and all of them except one animal were susceptible to CBPP [73]. This demonstrated that vaccination is not good at inducing CF antibodies, unlike infection.

These results do not necessarily mean that humoral immune response is not important in protection against the disease. They rather suggest that other

mechanisms, along with humoral immune response, might be involved in protection against *Mmm*SC like cell-mediated immunity.

1.3.2. Cell-mediated immunity.

Cell-mediated immunity against CBPP was measured by three different methods: transformation of lymphocytes sensitised by a specific antigen, inhibition of leukocyte migration by a specific antigen and an intradermal allergic reaction. A study looking for these three effects has been performed with *Mmm*SC in (i) susceptible control cattle, (ii) cattle vaccinated with T_1 broth culture and (iii) cattle infected endobronchially with *Mmm*SC [74]. The intradermal allergic test consisted of the injection of membrane antigens or polysaccharide of *Mmm*SC in the neck and the measurement of any increase of skin thickness. A positive reaction was observed in all infected cattle, but not in control and vaccinated animals. Lymphocyte transformation was measured by labelling DNA with tritiated thymidine and quantifying its uptake. The results were expressed using a lymphocyte transformation index with the ratio of mean counts per minute per culture in the presence of antigen being compared to the mean counts per minute per culture in the absence of antigen. Lymphocyte proliferation was observed in five out of six infected animals and two out of six vaccinated cattle. Only a slight difference was seen in control animals [74].

Inhibition of leukocyte migration was studied using polystyrene leukocyte migration plates with the results given using a leukocyte migration index that was the mean of migration area in the presence of antigen divided by the mean of migration area in the absence of antigen. In infected animals, inhibition of the migration was observed after 30 days following infection. Vaccinated animals and control cattle did

not show any inhibition of migration. The previous results indicate that cell-mediated immunity is involved in infection with *Mmm*SC but not in vaccinated animals since the results of this group of animals is similar to those of the control cattle [74].

The cell-mediated immunity was explored in endobronchially infected animals and T_1SR/T_144-vaccinated cattle challenged by contact with the infected animals. In the group of vaccinated cattle, animals resistant to CBPP exhibited an increase of the CD4 T-cell population after challenge, with a significant release of interferon γ *in vitro* whereas vaccinated animals that died of CBPP did not. In infected cattle, animals that died of acute CBPP did not show an increase in the CD4 T-cell population and no production of interferon γ could be detected *in vitro*, while animals with inapparent disease demonstrated an increase in the CD4 T-cell population with release of interferon γ *in vitro*. Animals with subclinical CBPP showed intermediary results [75]. These results suggested the involvement of cell-mediated immunity with CD4 T cells in protection of cattle against CBPP.

1.4. Vaccines against CBPP.

The first vaccines used consisted of a subcutaneous inoculation of *Mmm*SC-infected pleural fluid. In the early 1900s, these vaccines were replaced by the subcutaneous injection of field broth cultures of *Mmm*SC but these vaccines were not reliable since batch to batch variation in the number of live organisms in the inoculated dose occurred, often resulting in post-vaccinal reactions or poor efficacy [18,76]. Egg-based vaccines were used in Africa in the 1950s and early 1960s, made by growing *Mmm*SC in embryonated eggs [77]. They were subsequently abandoned because of the severe post-vaccinal reactions at the site of injection and in

the lungs [78,79]. Inactivated vaccines have also been tested but have not progressed beyond the experimental stages as they did not induce good protection [3,71]. It has been reported that subcutaneous vaccination of cattle with killed *Mmm*SC Gladysdale strain provided a complete protection to CBPP when cattle were challenged by direct contact with CBPP-infected animals. But it should be noted that this was obtained by using a very large dose (20 ml) of killed mycoplasmas inoculated with complete Freund's adjuvant [80].

The current vaccines used in Africa are attenuated freeze-dried broth culture vaccines containing viable microorganisms from strains $T_1$44 (has still some residual virulence in some breeds), T_1SR (streptomycin resistant, causes less severe post-vaccinal reactions than $T_1$44 while maintaining the same level of immunogenicity), KH_3J and KH_3JSR (do not cause any reactions in cattle but confer a poor immunity, the latter is streptomycin resistant). $T_1$44 and T_1SR are the most commonly used [3,71].

The minimum dose of vaccine recommended by the Office International des Epizooties is 10^7 mycoplasmas per animal. This dose has been shown to be sufficient to produce immunity in cattle in an experiment with *Mmm*SC vaccine strain T_1 [81]. A study examining the storage of freeze-dried T_1 vaccine has shown that the vaccine was still potent at 4°C, 23°C and 42°C after 14 months, 4 months and 3 weeks respectively [82].

As these vaccines contain live organisms attenuated by serial passages, one concern was the risk of reversion to full virulence when they are used, but no increase in virulence of the T_1 and KH_3J strains has been shown after inoculation [83]. However, with $T_1$44 vaccine, it has recently been reported that cattle in CBPP-free

areas, vaccinated with $T_1$44, with no contact with other herds, developed CBPP. It was suspected that these outbreaks might be due to the vaccination [84].

Different routes are used to administer the vaccines. In English-speaking countries, the vaccine is injected into the tail tip, while in French-speaking countries, a subcutaneous inoculation into the chest wall or the shoulder is preferred [2]. These two routes of inoculation have been shown to give a solid immunity to cattle immunised with *Mmm*SC T_1 strain, unlike the intradermal route where a quarter of inoculated cattle developed typical lung lesions after exposure to CBPP by contact with *Mmm*SC-infected animals [85]. As the subcutaneous route was as effective as the tail-tip route in vaccination against CBPP, it was suggested that the former should be preferred in vaccination programmes as it is faster, more convenient and cleaner [85] and because the inoculation into the tail tip is difficult due to the high pressures required [2].

The length of the immunity induced after vaccination with T_1 broth vaccine has been investigated in different studies. One experiment compared the protection against CBPP in cattle vaccinated with 0.5 ml of T_1 broth vaccine into the tail tip then challenged 6 months and one year after vaccination by contact with endobronchially-infected animals. The results showed that cattle challenged 6 months after vaccination did not show *Mmm*SC in their respiratory system, while mycoplasmas were found in cattle challenged one year after vaccination [86], suggesting the immunity conferred by the vaccination lasted less than a year. However, in other studies, antibodies were demonstrated in cattle 23 months after vaccination (even though it does not mean cattle are protected against CBPP) [87]. Cattle challenged by contact with *Mmm*SC-infected animals for 9.5 months, two

years after being vaccinated with T_1 broth culture (0.5 ml dose containing $3x10^{10}$ organisms/ml), did not show serological responses to *Mmm*SC, did not present any lesions of CBPP and no *Mmm*SC were found after challenge [88]. These results suggested that vaccination with T_1 broth culture protected cattle against CBPP for at least two years. The effect of a booster dose on the immunity of cattle to CBPP vaccines was also investigated [89]. For this experiment, cattle were vaccinated with either one ml of a Formalin-inactivated oil-adjuvanted Gladysdale vaccine or with 0.5 ml of live T_1 vaccine strain. Each group contained 10 animals. Half the cattle from each vaccinated group received a booster dose of the appropriate vaccine three weeks after the primary vaccination. Three months after vaccination, vaccinated cattle were then challenged by contact with animals intubated with *Mmm*SC strain Gladysdale. All vaccinated cattle survived the challenge while the control and the intubated animals died of CBPP. One animal vaccinated with the inactivated adjuvanted vaccine that had received a booster dose showed congested lungs at necropsy and *Mmm*SC were isolated from this animal. From this study, it was concluded that a booster dose was not necessary when animals have a strong immunity after vaccination [89].

Freeze-dried vaccines are now used. They might give different protection compared to broth culture vaccines. It is commonly accepted that $T_1$44 and T_1SR vaccines induce only a short lasting immunity (less than a year) and regular vaccinations are needed [70,90,91]. Recent field trials have shown that the protection induced was not satisfactory. In the first trial, the protection rate for both freeze-dried $T_1$44 and T_1SR vaccines three months after vaccination was 37% after challenge by contact with intubated animals [92]. In the second trial, the protection rate was 59%

(for $T_1$44 vaccine) and 68.2% (for T_1SR vaccine) when the vaccinated animals were challenged three months after vaccination. When the vaccinated cattle were challenged 15 months after vaccination, the protection rate was 28% for T_1SR vaccine while the protection rate was 78.2% for $T_1$44 vaccine. A second vaccination one year after the primary vaccination gave a better protection against CBPP as seen with the protection rate of 80.4% (for T_1SR vaccine) and 95% (for $T_1$44 vaccine) in the animals challenged three months following the second vaccination [93].

Studies have also shown that the efficiency of T_1 broth culture vaccines could be affected by the use of antibiotics or by infection of the vaccinated animals by other diseases. A comparison of vaccinated cattle, treated or not treated with the antibiotic Tylosin (10 mg/kg) one week or four weeks after vaccination (with 0.5 ml of T_1 broth culture vaccine at $3.3x10^9$ organisms/ml) showed that in the group treated with Tylosin four weeks after vaccination, animals presented lesions typical of CBPP after challenge by the in-contact method with animals intubated with *Mmm*SC strain Gladysdale. The untreated vaccinated animals had no lesions. In the group treated with Tylosin one week after vaccination, some cattle had lesions that were not related to CBPP [94]. Since Tylosin might kill the mycoplasmas, this suggests that live mycoplasmas are important in inducing protection against CBPP.

Intercurrent diseases can also affect the efficacy of vaccines. A study has shown that half the cattle infected with trypanosomes prior to vaccination against *Mmm*SC (minimum dose of 10^8 cfu of T_1 broth culture vaccine) contracted CBPP after challenge by contact with CBPP-infected animals [95], suggesting trypanosome infections depress the protective immune response engendered by CBPP vaccination (*Trypanosoma congolense* and *Trypanosoma vivax* are two trypanosomes largely

spread in Nigeria where CBPP is also present). It has also been demonstrated that animals tail-tip vaccinated with T_1 broth vaccine that were already severely infected with *Dermatophilus congolensis* (causing dermatophilosis, a disease prevalent in Africa) had an immune response (measured by haemagglutination test) to CBPP vaccine markedly lower than animals mildly infected and control cattle [96], although immune response does not necessarily mean protection.

1.5. Capsular polysaccharide (CPS).

*Mmm*SC possesses a capsule made of polysaccharide [4]. This antigen, known to play a part in the virulence of strains of *Mmm*SC, is found in broth culture and in tissues from infected animals (heart, lungs, pleural fluid, and urine) [23].

1.5.1. Structure of CPS.

The capsular polysaccharide (CPS) of *Mmm*SC forms a slime layer and comprises about 10% of the dry weight of cells in young culture [61]. This polysaccharide has an apparent high molecular weight (several hundred thousand kDa) [97]. An early study of the structure was performed using hydrolysis and has allowed the main sugar component to be identified as galactose [98]. Further experiments have extracted this polysaccharide using warm aqueous phenol before digestion by partial acid hydrolysis. These digests produced a galactobiose due to the degradation of a disaccharide with the structure 6-*O*-β-D-galactofuranosyl-D-galactose [99]. Additionally, electron impact mass spectrometry has indicated the presence of fucose, mannose, galactose, glucose, *N*-acetylated galactosamine and *N*-acetylated glucosamine in the proportions 1:2:4:1:4:8 [97]. However, a more recent

study showed that CPS was composed of galactose, mannose and glucose, but since a carbohydrate component present in the culture medium contains mannose and glucose, it was suggested the CPS was composed of galactose only, the other sugars coming from the culture medium [100].

CPS is the main complement-fixing antigen. Experiments on the inhibition of complement fixation in the presence of sugars or disaccharides have suggested that this carbohydrate possessed terminal residues with the structure β-glucopyranosyl-galactofuranosyl, as the monosaccharide β-glucopyranosyl and the disaccharide 1-6-β-glucopyranosyl-galactose were the most efficient inhibitors [101].

Cross-reactions have been observed between CPS and barley and oat glucans and polysaccharides from coccobacillus, agar, gum guar, from bovine, human and rabbit lungs [4,23,102,103]. Agar consists of β-1-linked D-galactopyranose connected by 1-4 linkage to L-galactose. In gum guar, β-1-4-linked D-mannopyranose units are linked with D-galactopyranose units by 1-6 linkages [102]. The polysaccharide produced by bovine lung epithelial cells contains D-galactose [104]. Its structure consists of a main chain of D-galactopyranose units connected by β-1-6 linkages. Every second unit of this backbone structure has one D-galactopyranose attached in the β-1-3 position [105,106]. Bovine pneumogalactan may also contain a small proportion of glucuronic acid [107]. The linkages in agar, gum guar and pneumogalactan are 1-3 and 1-6 and the sugars of these polysaccharides are in pyranose form. Since antibodies to CPS cross-react with these polysaccharides, CPS may contain these linkages as well. CPS is composed mainly of sugars in the furanose forms, but it may also contain some galactopyranose rings. It is possible that both ring types are present in CPS [102].

The antigenic relation between CPS and pneumogalactan might play a role in the pathogenesis of CBPP. It was suggested this could lead to a partial immune tolerance of the host to *Mmm*SC, to autoimmune allergic type reaction if the immune tolerance is overcome, and anti-galactan antibodies are produced [17,102].

1.5.2. Role of the CPS.

1.5.2.1. Effects on the host.

In infected animals, galactan can be found in various part of the body (circulating blood, lung fluid, pleural fluid, pericardial fluid, urine) [2,20] in such quantities that it saturates the antibodies creating the formation of circulating immunocomplexes that damage pulmonary cells [2,108].

CPS has been shown to have toxic effects, as it can cause necrosis in cattle in the absence of mycoplasmas [4,23]. Moreover, when injected intravenously, calves suffered from transient apnoea, increased arterial and decreased systemic blood pressure. Haemorrhages associated with alveolar ducts and vessel walls, areas of pulmonary oedema and capillary thrombosis were observed at the necropsy [109]. In another similar study, some of the animals died following the injection of CPS and autopsies revealed a congestion of the trachea, bronchial tubes, lungs, jejunum, ileum and small colon, along with haemorrhaging in the right ventricle [110]. In addition to its inflammatory action, CPS may also stimulate the development of connective tissue around the sequestra [2].

1.5.2.2. Relation to virulence.

Differences in virulence have been observed between strains of *Mmm*SC. For instance, comparison of two previously used vaccine strains, V5 and KH_3J, showed that V5 could induce local reactions at the tail tip (the site of injection), which in some cases proved fatal. None of these effects have been noted with KH_3J [cited in 111]. Inoculation intraperitoneally or intravenously with these two strains in mice resulted in extended bacteraemia with the V5 strain [111,112]. These results suggest a greater virulence for V5 than for KH_3J.

Virulence of *Mmm*SC strains has been shown to be related to the amount of CPS produced by these strains. A comparison of cattle which had received either an injection of CPS at 0.66 mg/kg, or at 0.22 mg/kg, followed by a subcutaneous injection of mycoplasmas (10^9 cfu/ml of V5 strain), showed that cattle inoculated with the higher dose of CPS had a more pronounced mycoplasmaemia than the group inoculated with the lower dose or than the control group (inoculated with mycoplasmas only). In addition, two groups of cattle were injected with CPS: one with CPS from Gladysdale (a virulent strain), the other one with CPS from KH_3J (an avirulent strain). The origin of the CPS was different but all the animals received the same concentration (2 mg/kg). These animals then received an injection of mycoplasmas of the V5 strain. Both groups showed similar proportions of samples positive for the presence of mycoplasmas by blood culture [110]. The conclusion of this study was that higher levels of CPS increased the virulence of a strain because animals treated with the highest dose of CPS had a more marked bacteraemia. Also, when CPS from a virulent strain and from an avirulent strain were injected at the same concentration, they produced the same effects. This suggests that the virulent

strains produce more CPS than their avirulent counterparts, rather than increased virulence being due to differences between the CPS of Gladysdale and KH_3J. Moreover, cattle inoculated intravenously with CPS at the same time as cultures of avirulent KH_3J and of virulent V5 strains had an earlier and a prolonged mycoplasmaemia when compared with cattle inoculated with the same strains but without the addition of CPS [113].

In an experiment studying two colony types (smooth and rough), isolated in a steer previously infected with Gladysdale, it appeared that the smooth colony type, associated with a higher production of CPS than in the rough type, produced a greater loss of weight of cattle than the rough type (cattle infected endobronchially with 50 ml of broth culture containing 10^9 cfu/ml for each colony type). Cattle infected with the smooth type also developed arthritis and pleural serofibrinous exudate [114].

Other studies have also shown that *Mmm*SC mutant strains with reduced capsular synthesis were phagocytosed more effectively by polymorphonuclear lymphocytes *in vitro* [4].

Therefore, CPS is an important virulence factor since it is involved in the spread of the mycoplasmas and in the pathogenesis of CBPP.

1.5.3. Antibodies against CPS.

The growth inhibition test (GIT) consists of placing filter paper discs impregnated with antiserum in contact with mycoplasmas cultured on agar plates. Three days later the zone of inhibition between the disc and the mycoplasmas is measured. Two experiments have shown that the antibodies responsible for the

inhibition were directed against CPS. The first study [115] used monoclonal antibodies produced after immunisation with membrane fraction. Some of these antibodies were shown to inhibit the growth of mycoplasmas. Subsequently, they were tested against *Mmm*SC antigens either treated or not treated with periodate. The results showed that periodate treatment prevented the binding of antibodies, suggesting that the recognised antigens were polysaccharides. The second study [116] used rabbit antisera obtained by immunisation with *Mmm*SC. Sera that gave positive results in GIT were used. When these antisera were pre-absorbed with purified CPS before use, the inhibition activity disappeared, showing it was caused by anti-CPS antibodies. Moreover, comparison between the sensitivity of a strain to growth-inhibiting antisera and the CPS production by this same strain showed an inverse correlation, i.e. the more CPS produced by a strain, the less it is inhibited by antisera. The inhibiting antibodies are therefore those directed against CPS.

1.6. CPS in other microorganisms.

1.6.1. CPS and virulence.

Many microorganisms possess a capsule consisting of polysaccharides. This CPS is often a virulence factor. In *Erwinia stewarttii*, the most virulent strains are those that produce the most extracellular polysaccharides [117]. Similarly, in *Mycoplasma gallisepticum*, those strains that presented a dense-staining capsule after staining with ruthenium red were those that had a greater pathogenicity in chickens [118]. The presence or the amount of CPS produced has been reported to be related to virulence in *Klebsiella pneumoniae* [119], *Vibrio vulnificus* [120,121],

Cryptococcus neoformans [122], *Mycoplasma dispar* [123], *Bacteroides fragilis* [124], *Streptococcus suis* [125] and *Rhodococcus equi* [126].

The presence of CPS is associated with the dissemination of several bacteria in the host. It has been shown with *Actinobacillus pleuropneumoniae* that encapsulated strains were able to produce bacteraemia in mice, unlike strains that did not produce a capsule [127]. Another study, using mice challenged intraperitoneally with a strain of *Escherichia coli* and a capsule-defective mutant of the same strain, has shown that LD50 (the lethal dose able to kill half of the animals in a specific group) was higher for the group infected with the mutant strain than for the group inoculated with the capsulated isolate, supporting the important role of CPS in pathogenesis [128].

The role of CPS in colonisation via adhesion is not well established. In mice fed either with encapsulated or non-capsulated strains of *Klebsiella pneumoniae*, the encapsulated strain colonised the intestinal tract at a level of 10^8 cfu/g of faeces, while the non-capsulated strain had a level of 10^4 cfu/g of faeces. When mice were fed simultaneously with both the capsulated and non-capsulated strains, the acapsular isolate was rapidly outcompeted by the capsular strain. This suggested that CPS has an important role in the colonisation of the intestinal tract by *Klebsiella pneumoniae* [129]. An adhesion experiment was also performed using a capsule-defective mutant and an encapsulated wild-type strain of *Klebsiella pneumonia*. It showed that the mutant adhered more efficiently to intestinal cell lines, laryngeal cells and lung epithelial cells than the wild-type strain. However, when the experiment was repeated with mucus-producing cells, the acapsular strain adhered less efficiently to the cells than the encapsulated strains. This demonstrated that CPS

of *Klebsiella pneumoniae* partially inhibits the adhesion of the bacteria to epithelial cells unless they produce mucus [130]. Another adhesion experiment with capsulated and non-capsulated strains of *Pasteurella multocida* has shown that the non-capsulated variant adhered in significantly higher numbers to respiratory tract cells. Unlike *Klebsiella pneumoniae*, the acapsular isolate was shown to have a greater affinity for the respiratory tract mucus [131].

However, it should be mentioned that this was observed with CPS from other bacteria and there is no evidence that *Mmm*SC CPS is involved in adhesion of these mycoplasmas, nor is it clear the importance of adhesion in *Mmm*SC pathogenicity.

CPS has been shown to protect bacteria from phagocytosis. Encapsulated microorganisms are resistant to phagocytosis unless they are opsonised. In *Cryptococcus neoformans*, the phagocytosis by alveolar macrophages was also influenced by the size of the capsule present on different strains. Phagocytic cells were less able to phagocytose strains with the CPS with the highest molecular size [132]. In addition to its antiphagocytic property, CPS is also able to influence the host immune response by activation of phagocytic cells and complement components of the alternative pathways to the induction of specific antibody, T-suppressor cells, delayed type hypersensitivity response and cytokines [122]. The CPS of *Mycoplasma dispar*, for example, has been shown to possess immunosuppressive properties since it is able to suppress the production of tumour necrosis factor and interleukin 1 by bovine alveolar macrophages [123].

Components of CPS may also "hide" the bacteria from the host immune system, i.e. CPS would appear as a self-antigen to the host that therefore would not elicit an immune response to CPS (capsules containing sialic acid for instance) [133].

1.6.2. Antibodies against CPS and conjugate vaccines.

Capsular polysaccharides are important virulence factors that help microorganisms invade by protecting the bacterium from the host defences, i.e. they prevent phagocytosis in presence of complement alone [87] or in absence of specific antibodies [125,134,135]. Antibodies against polysaccharides have been shown to be protective against invasive diseases caused by encapsulated bacteria [136]. In *Actinobacillus pleuropneumoniae*, the main opsonic antibodies are directed against CPS. Additionally, an antiserum pre-absorbed with purified CPS was still able to opsonise the bacteria but phagocytosis was reduced [127].

Various studies have shown that antibodies directed against CPS from different bacteria have a protective effect against disease. Subjects immunised with Vi+ strains of *Salmonella typhi* or with sera taken from subjects immunised with Vi+ strains were protected from infection, while those immunised with Vi- strains were not [137]. Only vaccines able to elicit the production of anti-CPS antibodies gave efficient protection. Similarly, immunisation of rats with purified CPS from *Klebsiella pneumoniae* protected them against lethal experimental pneumonia [138]. Rats that received an intravenous injection of monoclonal antibodies raised against CPS (10 mg/kg), prior to an intrabronchial inoculation with a virulent strain of *Klebsiella pneumoniae*, presented lung lesions and inflammation signs at a much reduced rate compared to rats in a control group that presented a pneumonia associated with haemorrhage and necrosis. Treatment with monoclonal antibodies did not prevent the penetration of organisms in the interalveolar space and the first inflammatory reactions were comparable between the treated group and the control group. However, the administration of monoclonal antibodies increased the bacterial

clearance in the spleen and the liver [119]. When hamsters were vaccinated intramuscularly or intranasally with the polysaccharide purified from *Mycoplasma pneumoniae*, then challenged intranasally with the microorganism, the number of viable organisms in the lung was significantly reduced and the lung lesions were less severe than in the unvaccinated group [139].

Vaccines consisting of purified CPS only are not feasible because, in most cases, CPS is a poor immunogen and does not induce a cellular immunity. CPS is a thymus-independent (TI) antigen [140]. TI antigens are divided in two groups: type 1 TI (TI1) antigens and type 2 TI (TI2) antigens. TI1 antigens are bacterial products that tend to have attached lipids and function by non-specific polyclonal activation of most B cells. For example, bacterial lipopolysaccharides are TI1 antigens. They do not induce immunological memory, isotype switching of antibodies and T-helper cell involvement [136,141]. TI2 antigens are high molecular weight polymers with repeating units, slowly metabolised *in vivo*, such as CPS. They may be tolerogenic in large doses and generate few (if any) memory B cells [136,141,142]. Some TI2 antigens may activate the alternative complement pathway [142]. Unlike TI1 antigens, they do not act as B-cell mitogens and can only activate mature B cells by cross-linking surface exposed immunoglobulins, leading to the production of antigen-specific antibodies [141]. These antibodies have a low affinity IgM and IgG isotypes [140,143]. The immune response to TI antigens is short-lived since T cells are not involved in the development of the immune response [140].

Thymus-dependent (TD) antigens are soluble proteins, whole cells, viruses and parasites. The immune response to TD antigens (which require T-cell help to elicit an immune response) is characterised by a long lasting immune response with

the production of memory B and T cells. They produce high affinity antibodies of multiple isotype. The memory B cells will allow a quicker response upon a secondary exposure to the antigen [136,141].

Antibodies to polysaccharides have been shown to protect against diseases, which make them good candidates as vaccines. To be used as a vaccine, the strategy is to convert TI antigens into TD antigens. This can be achieved by conjugating CPS to immunogenic proteins. In *Salmonella typhi*, Vi antigen has been conjugated to porins. Both porins and Vi antigen were isolated from the same microorganism. The inoculation of this conjugate induced both systemic and mucosal immune responses. The protection provided against *Salmonella typhi* was also better than the one observed with Vi alone [144].

Such vaccines have already been successfully developed (pneumococcal [145] and meningococcal vaccines [146]).

1.7. Aims.

CBPP, caused by *Mmm*SC, is a disease of major importance in Africa. In Europe and the USA, CBPP has been eradicated mainly by cattle movement restriction and slaughter associated with compensation. These measures are hard to apply in Africa because of the political, social and cultural situations in many countries and of the cost involved. Therefore, the only realistic way to eradicate CBPP in Africa at present is by vaccination. Vaccines against CBPP exist but they are not efficient enough and produce a short lasting immunity. As a consequence a better vaccine needs to be found.

The most "protective" antigens against a pathogen are generally the toxic and virulence factors of the pathogen [41]. *Mmm*SC possesses CPS that has been shown to be toxic and to play an important part in the virulence of *Mmm*SC strains.

In other microorganisms, antibodies against CPS have been shown to be protective against the disease. Since CPS are thymus-independent antigens, they need to be conjugated to a protein to efficiently produce antibodies and elicit immunological memory. This has led to the development of CPS-conjugate vaccines.

Another important consideration for CPS vaccines is the duration of the immunity induced. Studies on the duration of the protection conferred by polysaccharide vaccines, such as pneumococcal multivalent vaccine and Vi capsular polysaccharide vaccine have suggested they were efficient for between 5 to 10 years [147].

For the reasons cited above, CPS of *Mmm*SC seems a good candidate vaccine and merits further investigations.

The aim of my project was to investigate the vaccine potential of *Mmm*SC CPS.

The immunogenic structure of CPS needed to be investigated. A vaccine should be efficient against most, if not all, strains of *Mmm*SC. Therefore, it was necessary to know if CPS was conserved between *Mmm*SC strains and if some epitopes were present only in some of the strains. The bactericidal activity of anti-CPS antibodies should also be examined since some epitopes may produce antibodies with mycoplasmacidal activity and some others may not: the former would then be more important in protection against CBPP, assuming the protection is

conferred by anti-CPS antibodies. *Mmm*SC CPS should also be compared with the CPS from other mycoplasmas in the "*Mycoides* cluster".

The protective efficacy of CPS-specific antibodies should also be explored *in vivo*. This cannot be done in the natural host of *Mmm*SC because of the large size of the animals and the cost involved. Mice inoculated with *Mmm*SC intraperitoneally have been shown to present bacteraemia without any signs of disease [111,148]. The protective efficacy of sera against *Mmm*SC has already been tested in mice: mice inoculated subcutaneously with immune mouse serum raised against *Mmm*SC or with serum from cattle inoculated intravenously with *Mmm*SC did not develop mycoplasmaemia after challenge with *Mmm*SC [71,72,148]. Moreover, comparisons of virulence between different *Mmm*SC strains, based on the duration of mycoplasmaemia in mice, showed a correlation between the virulence observed in mice and that observed in cattle [111,148,149]. Consequently, although the mice are not a perfect model for CBPP, passive immunisation of mice with anti-CPS bactericidal antibody followed by challenge with live *Mmm*SC could help us to evaluate the efficacy of this antibody against mycoplasma infection.

Current vaccines lack efficacy in protecting cattle against CBPP. The study of the immune response against CPS in vaccinated cattle could provide an answer. A poor immune response to CPS might explain the results obtained with vaccination. Therefore it was decided to examine the immune response to *Mmm*SC CPS following vaccination and natural infection.

A vaccine should be as safe as possible with minimal post-vaccinal reactions. Serological similarities have been reported between CPS and bovine lung. Antibodies recognising both CPS and lung tissue could lead to immunopathology in

cattle. Thus, the presence of this kind of antibodies in naturally infected cattle will be examined. The antigenic similarity between CPS and bovine lungs will also be studied with monoclonal antibodies to try to identify epitopes present on CPS that might not be found on bovine lungs.

CPS shares serological similarities with polysaccharides from various sources. In order to reduce the cost of the production of a CPS vaccine (due to the growth of *Mmm*SC in specific facilities and CPS purification), other polysaccharides, such as polysaccharides from cereals, could be used instead of CPS. To explore this issue, the antigenic similarities between CPS and carbohydrates from various cereals will be investigated.

In some other microorganisms, CPS seems to play a part in the adhesion to the host cell. Little is known about the adhesion of *Mmm*SC. *Mmm*SC CPS is a virulence factor and could be involved in adhesion, increasing the pathogenicity of the strain. The role of CPS in adhesion of *Mmm*SC will then be explored.

The results obtained will then be discussed.

2. Materials and methods.

2.1. Mycoplasma strains and growth conditions.

Mycoplasma mycoides subspecies *mycoides* SC (*Mmm*SC) strains used are shown in Table 1. *Mmm*SC strains were grown in Gourlay's broth (GB, see Appendices), casein medium (CM, see Appendices) and on Gourlay's agar (GA, see Appendices).

Table 1 *Mmm*SC strains used.

Strains	Origin	Isolation date	Host/specimen type	Source
PG1 type strain	unknown	1931	cattle	LNIV[1]
Gemu Goffa	Ethiopia	1974	cattle	LNIV[1]
Filfili	Senegal	pré-1988	cattle	LNIV[1]
$T_1$44	Tanzania	1952	cattle/ vaccine strain	BVI[2]
T_1SR	Tanzania	1952	cattle/ vaccine strain	BVI[2]
KH_3J	Sudan	1940	cattle/ vaccine strain	LNIV[1]
Tan1	Tanzania	1996	cattle	ADRI[3]
Tan8	Tanzania	1996	cattle/ pleural fluid	ADRI[3]
N6	Botswana	1996	cattle/ lung	NVL[4]
M375	Botswana	1995	cattle/ lung	NVL[4]
Afadé	Chad	1968	cattle	VLA[5]
Gladysdale	Australia	pré-1964	cattle	VLA[5]
V5	Australia	1936	cattle/ vaccine strain	VLA[5]
6479	Italy	1992	cattle/ lung	LNIV[1]
O697	Spain	1989	cattle	LNIV[1]
PO2	France	1980	cattle/ lung	LNIV[1]
B773	Portugal	1991	cattle/ semen	LNIV[1]
425	Portugal	1993	goat/ lung	LNIV[1]
B820/124	Portugal	1991	cattle/ preputial washing	LNIV[1]
O512	Portugal	1993	sheep/ milk	LNIV[1]
BF138	Italy	1992	buffalo	LNIV[1]
B103	Portugal	1986	cattle/ lung	LNIV[1]

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2.2. Antisera.

2.2.1. Anti-*Mmm*SC rabbit polyclonal sera.

Rabbit hyperimmune sera used are shown in Table 2. Sera from Moredun Research Institute (MRI) were produced according to the following protocol. The aqueous solution was made with 625 µl of whole mycoplasmas (5-10 mg/ml) added to the same volume of 0.02% glutaraldehyde solution (Sigma, G-6257) and mixed for 30 min. A volume equal to 1.25 ml of 0.01 M glycine solution (Fisher scientific, G/0800/60) was added to the mixture. Then the solution was mixed and left at room temperature for 5 min. Fifty µl of 1% thimerosal solution (Sigma, T-5125) was added. The aqueous solution was mixed with an equal volume of adjuvant Montanide ISA 50 (Seppic). The rabbits were immunised twice subcutaneously with an interval of 11 days. One month later, the rabbits received an intravenous injection of the aqueous solution without adjuvant and they were bled approximately 5 days later.

Table 2. Anti-*Mmm*SC rabbit polyclonal sera.

Strains	**Source**	**Strains**	**Source**
PG1	LNIV[1]	V5	MRI[2]
Gemu Goffa	LNIV[1]	6479	LNIV[1]
Filfili	LNIV[1]	O697	LNIV[1]
T_144	MRI[2]	PO2	LNIV[1]
T_1SR	MRI[2]	B773	LNIV[1]
KH_3J	LNIV[1]	425	LNIV[1]
Tan1	MRI[2]	B820/124	LNIV[1]
Tan8	MRI[2]	O512	LNIV[1]
N6	MRI[2]	BF138	LNIV[1]
M375	MRI[2]	B103	LNIV[1]
Afadé	MRI[2]	B345	LNIV[1]
Gladysdale	MRI[2]		

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2.2.2. Monoclonal antibodies (mAbs).

MAbs 6D11, 3F10, 3H12, 6E4, 2A3, 1D3, 1E6 and 3H7 were supplied by H. Ball (Department of Agriculture for Northern Ireland, Veterinary Sciences Division, Belfast, Northern Ireland, United Kingdom). MAbs 1C9 and 1B9 were produced at MRI (this thesis, section 2.2.5.). MAb PK2 was supplied by Sankale Shompole (Kari, Kabete, Kenya, Africa). All the mAbs were of IgM isotype.

2.2.3. Anti-*Mmm*SC bovine polyclonal sera.

Antiserum N28 was supplied by Willie Amanfu (National Veterinary Laboratory, Gaborone, Botswana) and came from an unvaccinated, naturally infected cow (Botswana, 1996). Antiserum PP34 was supplied by Benedict Lema (Animal Diseases Research Institute, Dar-es-Salaam, Tanzania) and came from an unvaccinated, naturally infected cow (Pawaga, Tanzania, 1997). Antiserum 5754 was supplied by J. Habyarimana (Veterinary Diagnostic and Epidemiology Laboratory, Entebbe, Uganda) and came from a mixed-Zebu breed animal, vaccinated subcutaneously with *Mmm*SC vaccine strain $T_1$44. Antiserum 125 was supplied by Bakary Cissé (Laboratoire de Production Animale, Bingeville, Ivory Coast) and came from a Holstein-breed animal, vaccinated in the tail tip with *Mmm*SC vaccine strain T_1SR. Antisera from bulls 180 and 184 were from a trial performed in Kenya with local breeds of Kenyan cattle vaccinated subcutaneously with *Mmm*SC vaccine strain T_1SR.

2.2.4. Anti-bovine lung mouse sera.

Six 12-week-old female Balb/c mice were immunised with 100 µl of lung homogenate (section 2.3.1.) diluted with an equal volume of Montanide ISA 206 (Seppic) injected subcutaneously to each mouse on day 0 and day 21. Mice were bled on day 28.

2.2.5. Production of monoclonal antibodies.

Five 8-12 week old female Balb/c mice were injected with 100 µl each (equivalent to 50 µg) of a mixture of a membrane fraction from mycoplasma strains T_1SR, V5 and B820/124 after fractionation with Triton X114 (Sigma, X-114) (section 2.2.6.), diluted with an equal volume of Montanide ISA 206 (Seppic). The injections were made subcutaneously, at day 0, day 21 and day 42. On day 50, mice were boosted with 100 µl (equivalent to 50 µg) of the antigen, without adjuvant, intraperitoneally. The mouse with the highest anti-capsular polysaccharide (CPS) antibody titre (measured by ELISA, section 2.4.2.) was killed 3 days later and the spleen was removed and placed in RPMI 1640 medium (RPMI) (produced at the Faculty of Veterinary Medicine (FVM), Edinburgh, United Kingdom). NSO myeloma cells (obtained from Deborah Allen, Department of Veterinary Pathology, FVM, Edinburgh, United Kingdom) were centrifuged 3 times with RPMI without foetal calf serum (FCS) at 420 *g* for 5 min. A single cell suspension from the spleen was made and spun 3 times with RPMI with 1% 200 mM L-glutamine solution (v/v) (L-glu) (Life technologies, 25030-024) and 1% 50 mM 2-mercaptoethanol solution (v/v) (ME) (Life technologies, 31350-010) at 420 *g* for 5 min. Polyethylene glycol 1500 (PEG 1500) (50% PEG 1500 (w/v) in 75 mM HEPES, pH 8.0, Boehringer

Mannheim, 783641) was heated at 37°C. The spleen and NSO cells were mixed and spun at 270 *g* for 5 min. The supernatant was removed and the tube containing the pellet was placed in a water bath at 37°C. One ml of PEG 1500 was added to the cell pellet over a period of 1 min while the cells were continually stirred with a pipette in the water bath at 37°C. The cells were stirred for a further 1 min. Fifteen ml of RPMI with L-glu and ME, pre-warmed at 37°C, was added over a period of 3 min while stirring the cells. The cell solution was incubated for 6 min at 37°C then spun at 100 *g* for 5 min. The supernatant was removed and the cells were resuspended in RPMI with 20% FCS (PAA Laboratories, A15-326), L-glu, ME, 2% 5 mM hypoxanthine-20 μM aminopterin-0.8 mM thymidine solution (v/v) (HAT) (Life technologies, 21060-017) and 1% 100 mM sodium pyruvate solution (v/v) (Life technologies, 11360-039) to give a final cell density of 10^6 NSO cells/ml. One hundred μl/well of this cell suspension was plated out in 96-well cell culture plates (Corning incorporated, 3595) that already contained 100 μl/well of mixed thymocyte medium (section 2.2.7.). The plates were incubated at 37°C with 5% CO_2. The supernatants from the wells were checked, using an enzyme linked immunosorbent assay (ELISA) (section 2.4.1.), to identify the positive wells against *Mmm*SC CPS. The positive hybridomas were cloned on agar plates, made with 400 ml of RPMI containing L-glu, ME, HAT and sodium pyruvate mixed with 50 ml of 5% agar solution (Difco, 214010). The agar solution was melted then cooled down to 45°C; RPMI with L-glu, ME, HAT and sodium pyruvate was warmed to 45°C. Positive hybridomas were diluted from 10^5 to 10^2 cells/ml. Two ml of the previous cell dilution was mixed with the same volume of the medium used to make agar plates. Two ml of this mixture was poured on the agar plates. Plates were incubated

at 37°C with 5% CO_2 for a week. Individual colonies were picked out with a Pasteur pipette and placed in 24-well cell culture plates (Corning incorporated, 3524) with RPMI with L-glu, ME, HAT and sodium pyruvate. The clones were checked for their specificity against *Mmm*SC CPS by ELISA (section 2.4.1.).

2.2.6. Triton X114 fractionation of *Mmm*SC.

Three grammes of *Mmm*SC strains were mixed, in a chilled tube, with 154 mM NaCl (Fisher scientific, S/3160/60) 10mM Tris (Sigma, T-1503) solution (TS), pH 7.4 containing 100 mM phenylmethylsulfonyl fluoride (PMSF) (Sigma, P-7626), stored at 4°C, to have a final volume equal to 9 ml. One ml of 10% Triton X114 (Sigma, X-114) solution (TX114), kept at 4°C, was added. The mixture was vortexed then shaken at 4°C for 2 h. The tube was centrifuged at 12,000 *g* for 5 min at 4°C. The supernatant was placed in a water bath at 37°C for 5 min then centrifuged at 8,000 *g* for 3 min at 25°C. The lower (TX) phase was chilled on ice then mixed with 9 ml of TS-PMSF, vortexed, placed for 3 min on ice, incubated for 5 min at 37°C then centrifuged at 8,000 *g* for 3 min at 25°C. The TX phase was saved and the previous procedure was repeated 3 times. The last TX phase obtained was stored at - 80°C. To remove the detergent, 9 volumes of methanol at 4°C were added and mixed to the solution then placed at - 80°C overnight. The sample was centrifuged at 12,000 *g* for 10 min at 4°C and the methanol completely removed. The pellet was resuspended in sterile phosphate buffered saline (PBS).

2.2.7. Mixed thymocyte medium.

Thymus from two 6-week-old female Sprague rats and two 6-week-old female Wistar rats were taken aseptically, put in tubes containing Hanks' balanced salt solution (produced at the Department of Veterinary Pathology, FVM, Edinburgh, United Kingdom). The thymuses were cut separately into pieces and placed in tubes with RPMI with 1% L-glu (Life technologies, 25030-024) and 1% 10,000 units/ml penicillin 10,000 μg/ml streptomycin solution (PS) (Life technologies, 15140-122) pre-warmed at 37°C. The cell suspensions were pooled and centrifuged at 400 *g* for 5 min. The supernatants were discarded and the pellets were resuspended in 40 ml RPMI with L-glu and PS. The tubes were spun at 400 *g* for 5 min. This operation was repeated 3 times. Then, the cells were mixed with 200 ml of RPMI with 15% FCS (PAA Laboratories, A15-326), L-glu and PS to give a final cell density of $4.5x10^6$ cells/ml and placed in tissue culture flasks (Nunc, 178905). The flasks were incubated at 37°C with 5% CO_2 for 2 days. The cell suspension was centrifuged at 850 *g* for 10 min at 4°C. The supernatant was filtered-sterilised, aliquoted under 40 ml and frozen at - 80°C.

2.2.8. Immunoglobulin (Ig) M purification.

One litre of 3F10 cell culture supernatant was concentrated to 8.7 ml by centrifugation at 2,000 *g* using a centrifugal filter device (Millipore Ultrafree-15 with Biomax-50K membrane, Sigma, Z36,465,7) then stored at 4°C. An IgM affinity column (ImmunoPure immobilized mannan binding protein, Pierce, 1855580), stored at 4°C, was placed at room temperature and prewashed with 5 ml of column preparation buffer (ImmunoPure mannan binding protein preparation buffer, Pierce,

21018) at room temperature. The column was placed at 4°C then equilibrated with 20 ml of cold binding buffer (CBB) (ImmunoPure IgM binding buffer, Pierce, 21016) kept at 4°C. Cold mAb 3F10 was diluted with the same volume of CBB. The column was filled with a part of the sample, then capped at the bottom and incubated for 30 min at 4°C. The column was then opened and the second part of the sample was allowed to enter completely the gel (while the first part passed through the column). Five hundred µl of CBB were added. The column was capped at the bottom and incubated overnight at 4°C. After incubation, still at 4°C, the column was washed with 42 ml of CBB. The column was removed from the cold and 3 ml of elution buffer (ImmunoPure IgM elution buffer, Pierce, 21017) kept at room temperature was added. The column was capped at the bottom and incubated at room temperature for 1 h. Forty-two ml of elution buffer at room temperature was added and the eluate was collected in 3 ml fractions. The elution of IgM was monitored using absorbance readings at 280 nm. Fractions with absorbances above or equal to 0.02 were pooled then dialysed against PBS. The concentration of protein was assessed by Pierce protein assay (Pierce, 23223 and 23224). The purity was checked by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and western-blot (WB) using anti-mouse IgM antibody (Serotec, STAR86P).

2.2.9. Labelling of monoclonal antibody and ascitic fluids.

Five mg of horseradish peroxidase (HRP) (Roche, 108090) was dissolved in 1.2 ml of distilled water (DW). Three hundred µl of freshly prepared 0.1 M sodium periodate solution (Sigma, S-1878) in 10 mM sodium phosphate (Sigma, S-7907), pH 7, was mixed with the HRP solution. The mixture was then incubated at room

temperature for 20 min then dialysed against 1 mM sodium acetate solution (BDH, 102363P) pH 4 at 4°C overnight with 3 changes. One ml of ascitic fluid or culture supernatant of monoclonal antibody secreting cells was mixed with the HRP solution and incubated for 2 h at room temperature. One hundred µl of sodium borohydride (Sigma, S-9125) at 4 µg/ml in DW was added and the solution was incubated for 2 h at 4°C. The solution was then dialysed against PBS overnight with 3 changes.

2.3. Antigen preparation.

2.3.1. Bovine lung homogenate.

Normal bovine lung was cut into small pieces and placed in tubes (Ribolyser Kit Red, Hybaid) containing glass beads and 10 mM Tris (Sigma, T-1503)-1 mM EDTA (Sigma, E-5134) buffer, pH 7.5. The tubes were shaken using a Hybaid ribolyser at speed 6 for 2 min. The tubes were put in ice to cool them down, then centrifuged. The supernatants were kept at - 20 °C.

2.3.2. Galactan extraction from lung homogenate.

A fraction of lung homogenate (2.4 ml) was extracted 4 times with phenol-chloroform-isoamyl alcohol (25:24:1) (Fisher scientific, BPE1752I-400) and once with chloroform (Fisher scientific, C/4960/17), then precipitated with 9.6 ml of ethanol (Fisher scientific, E/0650DF/17) and 1 ml of 3 M sodium acetate solution (BDH, 102363P).

2.3.3. CPS purification.

Two hundred ml of supernatant from *Mmm*SC strains grown in GB or CM, containing 0.05% of sodium azide (Sigma, S-2002) were adjusted to pH 5 with chlorhydric acid (BDH, 18036 5D) and autoclaved at 100°C for 30 min. The solution was then centrifuged at 15,600 *g* for 15 min. Two volumes of ethanol (Fisher scientific, E/0650DF/17) were mixed with the supernatant and the mixture was kept at 4°C for 5 days to precipitate. The precipitate was resuspended with 20 ml of DW and centrifuged at 15,600 *g* for 10 min. The pellet was resuspended with 10 ml of DW. One hundred µg of DNAse I and II (Sigma, D-5025 and D-4138) and of 100 µg of RNAse A and B (Sigma, R-5125 and R-7884) were added. The tube was incubated in a water bath at 37°C for 18 h. Sodium dodecyl sulphate (SDS) (Sigma, L-4509) was then added to a final concentration of 0.05%. One hundred µg of proteinase K (Sigma, P-2308) was added and the solution was incubated in a water bath at 45°C for 24 h. The solution was then dialysed in a 12-kDa dialysis tube (Sigma, D-9652) against running tap water for 4 days. The dialysates were mixed with 1 volume of phenol-chloroform-isoamyl alcohol (25:24:1) (Fisher Scientific, BPE1752I-400) and centrifuged at 1,600 *g* for 30 min. The upper (aqueous) phase was removed, and to this was added 1 volume of chloroform (Fisher Scientific, C/4960/17), well mixed and then centrifuged at 1,600 *g* for 30 min. The upper phase was removed, and to this were added 3 volumes of ethanol. The tube was centrifuged at 1,600 *g* for 30 min. The pellet was resuspended with 5 ml of 80% ethanol solution then centrifuged at 1,600 *g* for 30 min. The supernatant was discarded and the pellet was dissolved with 1 ml of DW then spun at 21,000 *g* for 3 min. The supernatant was

dialysed against running tap water for 5 h then against DW overnight. The dialysate was spun at 21,000 *g* for 2 min. The supernatant was then stored at – 20°C.

2.3.4. Chromatography.

2.3.4.1. Chromatography on PD10 column.

The PD10 column (Pharmacia, 17-0851-01) was equilibrated with 25 ml of DW (because the CPS used was in DW). A volume of 2.5 ml of CPS solution purified from *Mmm*SC strain V5 was added to the column. Twenty-five ml of DW were then added to the column. The eluates were collected under 500 µl fractions in Eppendorf tubes, straight after the addition of the CPS solution to the column. The fractions were checked by SDS-PAGE followed by staining with Coomassie Blue and Schiff's reagent.

2.3.4.2. Chromatography on G50 microcolumn.

CPS from Gemu Goffa and PO2 were precipitated with 4 volumes of ethanol. The pellets were resuspended in 50 µl of 1% ME (Sigma, M-3148) STE buffer (see Appendices). The G50 microcolumn (Pharmacia, 27-5335-01) was vortexed to resuspend the resin, then placed into a 1.5 ml Eppendorf tube and centrifuged for 1 min at 750 g. The samples were applied on the resin and centrifuged at 750 g for 2 min. The eluates were collected in 1.5 ml Eppendorf tubes. The column was washed 9 times with 100 µl of 1% ME-STE buffer by centrifugation at 750 g for 2 min. The eluates from each wash were set aside. The first 7 eluates from the two samples were used in an SDS-PAGE followed by Schiff's reagent staining and Silver staining.

2.3.5. Carbohydrate concentration of purified CPS.

D-glucose (Sigma, G-6152) was used as a standard and diluted in PBS from 200 µg/ml to 3.125 µg/ml. The solutions of purified CPS were diluted in PBS (1:1, 1:3 and 1:7). Twenty-five µl of each solution was put in triplicate in 96-well plates (Greiner, 655061). Twenty-five µl of a 5% phenol (Fisher Scientific, P/2315/PB17) solution was added; the plates were mixed for 30 s then placed on ice. One hundred and twenty-five µl of concentrated H_2SO_4 (Fisons, S/9240/PB17) was added to the wells. The plates were shaken for 30 s then incubated in a water bath at 80°C for 30 min. The OD was measured at 492 nm.

2.3.6. Protein concentration of purified CPS.

Bovine serum albumin (Sigma, A-4503) was used as a standard. It was diluted in PBS from 2,000 µg/ml to 1.953 µg/ml. The solutions of purified CPS were diluted in PBS (1:1, 1:3 and 1:7). Twenty-five µl of each solution was put in triplicate in 96-well plates (Greiner, 655061). Two hundred µl of protein assay reagent (Pierce, 23223 and 23224) was added to each well. The plates were incubated in a water bath at 37°C for 30 min. The OD was then measured at 540 nm.

2.3.7. Cereal extraction.

Three hundred mg of corn flour, oatmeal, rice, wheat semolina and wheat flour were mixed in 15 ml of DW and boiled for 30 min. The solutions were left for a week at 4°C. Ten ml of DW was added then the solutions were sonicated for 2 min. They were spun at 4,800 *g* for 10-30 min. Two hundred and fifty µg of proteinase K (Sigma, P-2308) was added to the supernatants. The solutions were incubated at

37°C for 5 days. The solutions were then placed at 4°C then pelleted. The supernatants were removed stored in 1.5 ml Eppendorfs at 4°C.

2.3.8. Sonicated mycoplasmas.

One ml of mycoplasma culture ($5x10^9$ cells/ml) containing sodium azide at 0.05% (Sigma, S-2002) was centrifuged at 21,000 *g* for 5 min. The pellet was resuspended in 1 ml of PBS then the mixture was spun at 21,000 *g* for 5 min. The pellet was resuspended in 1 ml of 0.05% Tween 20 (Sigma, P-2287) in PBS (PBST) then the solution was sonicated (Jencons Ultrasonic processor) by pulsation (50% duty cycle) with the intensity sets on 2-3 for 60 s.

2.4. Immunoassays.

2.4.1. ELISA.

Plates (Greiner, 655061) were coated with 100 µl/well, in triplicate, of antigen (CPS at 5 µg/ml in PBS or sonicated mycoplasma diluted 1/100 in PBS) and incubated overnight at 4°C. They were washed 3 times with PBST then blocked with 100 µl/well of blocking reagent (BR) for 1 h at 37°C. After 3 washes with PBST, plates were incubated with 100 µl/well of primary antisera diluted in BR, for 1 h at 37°C. The plates were washed 3 times. One hundred µl/well of specific HRP-conjugated secondary antibody diluted in BR were incubated in the plates for 1 h at 37°C. After 3 washes with PBST, 100 µl/well of H_2O_2-o phenylenediamine (OPD) (1 tablet of each dissolved in 20 ml of DW) (Sigma, P-9187) was added and incubated for 10 min at room temperature in the dark. The reaction was stopped by

the addition of 50 µl/well of 3 M H_2SO_4 solution (Fisher scientific, S/9240/PB17) then the OD was read at 492 nm.

Primary antibodies were:

- sera from intubated, vaccinated and in-contact cows with *Mmm*SC diluted 1/5,000;

- undiluted hybridoma supernatants;

- mAbs diluted 1/400 for isotyping;

- mAbs diluted 1/100 (1E6, 3H7), 1/400 (1C9, 1D3, 1B9), 1/1,000 (2A3) and 1/2,000 (PK2, 6D11, 3F10, 3H12, 6E4) to look at CPS epitopes on *Mmm*SC strains;

- mAbs diluted 1/25,000 (3H12), 1/100 (2A3), 1/50,000 (3F10), 1/12,500 (6E4, 6D11), and 1/800 (PK2) to look at CPS epitopes on *Mycoplasma mycoides* subspecies *mycoides* LC and *Mycoplasma mycoides* subspecies *capri* strains.

HRP-conjugated secondary antibodies were:

- anti-cow IgM antibody (Serotec, AAI19P) diluted 1/1,000, anti-cow IgA antibody (Serotec, AAI20P) diluted 1/1,000, anti-cow IgG antibody (Serotec, AAI23P) diluted 1/50,000;

- anti-mouse immunoglobulin antibody (Dako, P0447) diluted 1/2,000, anti-mouse isotype immunoglobulin antibodies (Serotec, STAR81P, STAR82P, STAR83P, STAR84P, STAR85P and STAR86P specific for IgG_1, IgG_{2a}, IgG_{2b}, IgG_3, IgA and IgM respectively), diluted 1/5,000.

2.4.2. Titration ELISA.

The plates (Greiner, 655061) were coated with 50 µl/well of 5 µg/ml of pure CPS from *Mmm*SC strain PO2, diluted in PBS, for 2 h at room temperature. They

were rinsed 3 times with PBST, and then incubated with 50 µl/well of BR for 1 h at 37°C. After 3 rinses with PBST, 50 µl/well of each antiserum diluted from 1/50 to 1/819,200 in BR was added. The plates were incubated for 1 h at 37°C. The plates were rinsed 3 times and incubated with 50 µl/well of HRP-conjugated anti-rabbit immunoglobulin antibody (Dako, P0448) diluted 1/2,000 in BR. After 3 rinses with PBST, plates were developed with H_2O_2-OPD (Sigma, P-9187) as described in section 2.4.1. but with an incubation time of 15 min instead of 10 min. A line was drawn from the linear part of the curve and the titre was the intersection point between this line and the x-axis.

2.4.3. Competitive ELISA.

Plates (Greiner, 655061) were coated with 50 µl/well of CPS from PO2 grown in CM, diluted at 5 µg/ ml in PBS and incubated overnight at 4°C. They were washed 3 times with PBST, and then blocked with 50 µl/well with BR for 1 h at 37°C. After 3 washes with PBST, 50 µl/well of unconjugated mAbs (3H10, 3H12, 6D11, 6E4 and PK2), serially diluted from 1/50 to 1/104,857,600 in BR, were added, in duplicate, to the plates and incubated for 1 h at 37°C. Plates were washed 3 times with PBST. The conjugated mAbs (3F10, 3H12, 6D11, 6E4 and PK2, respectively diluted 1/32,000, 1/500, 1/10,000, 1/1,600 and 1/50 in BR) and the anti-mouse immunoglobulin antibody-HRP (Dako, P0447), diluted 1/2,000 in BR, 50 µl/well, were incubated for 1 h at 37°C (the conjugated anti-mouse immunoglobulin antibody was used to detect the signal from the unconjugated mAbs). After 3 rinses with PBST, plates were developed with H_2O_2-OPD (Sigma, P-9187) as described in section 2.4.1. but with an incubation time of 12 min instead of 10 min.

2.5. Western-blots.

Antigens were diluted with an equal volume of loading buffer (LB) and loaded on a single large well 12.5% SDS gel (see Appendices) using a Mini Protean II module (Bio-Rad, 165-2944). The gels were run at 100 V then transferred onto nitrocellulose membranes (Amersham, RPN203E) for 45 min to 1 h at 300 mA using Trans-Blot SD semi-dry transfer cell (Bio-Rad, 170-3949). The membranes were stained overnight in Ponceau Red (Sigma, P-3504) staining solution (see Appendices). After destaining (see Ponceau Red destain in Appendices) until no staining background was visible, the membranes were rinsed in DW for 10 min. The membranes were incubated in BR for 1 h at room temperature, under agitation. For the next steps, a Mini-Protean II Multiscreen was used (Bio-Rad, 170-4017). The sheets were rinsed 3 times in PBST. Primary antibodies were diluted 1/400 in BR and incubated with the membranes at room temperature for 1 h 30 under agitation. Primary antibodies were removed and the membranes were rinsed 3 times with PBST. The secondary antibodies conjugated to HRP specific for the primary antibody were diluted 1/400 in BR and incubated at room temperature for 1 h 30 under agitation. After 3 rinses with PBST, the substrate, 50 µl of H_2O_2 (Sigma, H-1009) and few grains of diaminobenzidine (Sigma, D-5637) in 100 ml of PBST, was added and left for 10 min. The reaction was stopped by rinsing the membrane with DW.

Antigens were:

- 100 µl of each cereal extract and 100 µl of purified CPS (1.08 mg/ml) from *Mmm*SC strain V5 grown in CM;

- 225 µl of lung homogenate, boiled for 3 min and spun at 21,000 *g* for 3 min, lung extract and 1 ml (803 µg/ml) of purified CPS from *Mmm*SC strain Afadé grown in CM, precipitated with ethanol and resuspended in 200 µl of LB;

- 100 µl of purified CPS (1.08 mg/ml) from *Mmm*SC strain V5 grown in CM and 100 µl of lung homogenate.

Primary antibodies were pre-immune cow sera, anti-*Mmm*SC cow sera, anti-*Mmm*SC rabbit sera, mAbs, and anti-bovine lung mouse sera.

HRP-conjugated secondary antibodies were anti-cow immunoglobulin antibody (Dako, P0159), anti-rabbit immunoglobulin antibody (Dako, P0448) and anti-mouse immunoglobulin antibody (Dako, P0447).

2.6. Western-blot adherence assay.

Preparation of the membrane:

Strains of *Mmm*SC were pelleted by centrifugation at 21,000 g for 5 min. The pellets were resuspended in PBS and centrifuged again at 21,000 g for 5 min. The previous step was repeated another time. The pellets were then resuspended in LB and boiled for 5 min, then centrifuged for 3 min at 21,000 g. The supernatants were loaded onto a 12.5% SDS gel which was run overnight at 50 V and then transferred onto a membrane (Immobilon-P, Millipore) at 300 mA for 1 h using Trans-Blot SD semi-dry transfer cell (Bio-Rad, 170-3949). The membrane was stained overnight in Ponceau Red (Sigma, P-3504) staining solution (see Appendices). Finally, the blot membrane was destained and stored wet until use.

For the transfer, two sheets of Whatman paper were soaked in Anode buffer I (see Appendices), one sheet of Whatman paper was soaked in Anode buffer II (see

Appendices) and two sheets of Whatman paper were soaked in Cathode buffer (see Appendices). The membrane was incubated for 15 s in 100% methanol solution, then soaked in DW for 2 min. The membrane was then put for 5 min in Anode buffer II. The sheets of Whatman paper incubated in Anode buffer were placed under the membrane covered with the gel; the sheets of Whatman paper incubated in Cathode buffer were placed on top of the gel.

Preparation of the cells:

Two flasks of embryonic bovine lung (EBL) cells, in 5 ml of minimum essential medium Eagle (MEM) with 10% FCS and 50 µg/ml gentamicin (Sigma), were incubated at 37°C and 5% CO_2 for 18 h. Four µl (0.75 MBq) of ^{35}S-methionine (Amersham) were added in each flask. The cells were then incubated for 18 h at 37°C and 5% CO_2. The culture medium was removed from the flasks. Cells were quickly washed with 5 ml of PBS. Five hundred µl of 10X trypsin (Boehringer Mannheim)-EDTA in 4.5 ml of DW were added to each flask which were shaken gently for 1 min. About 90% of the liquid was removed. The flasks were agitated until the cells dettached. Twenty µl of protease inhibitor (100 mM AEBSF) were added to the flasks. The cells were then resuspended with 4 ml of Buffer A (see Appendices).

Adherence assay:

The blot membrane was soaked in methanol then in DW. The membrane was incubated in 20 ml of 2% skimmed milk powder in PBST for 1 h then rinsed twice for 5 min with Buffer A. The membrane was incubated with EBL cells at 37°C for 4 h under agitation. The membrane was rinsed three times for 5 min with 20 ml of PBST then put on Whatman paper to remove the rest of liquid on the membrane. The

membrane was placed in a cassette for autoradiography for 4 days and then developed.

2.7. Adherence inhibition assay in tissue-culture plates.

*Mmm*SC strains N6 and M375 were inoculated in 200 ml of culture medium with 60 µl of ^{3}H-palmitic acid (Amersham) and incubated at 37°C until early log phase. *Mycoplasma bovis* (*M. bovis*) strain PG45 was used as a control. EBL cells were cultured in 24-well plates ($2.5x10^{5}$ cells/ml), in MEM with 10% FCS and 50µg/ml gentamicin (Sigma) for 20 h at 37°C and 5% CO_2. The culture medium was removed and 200 µl of 1% bovine serum albumin in Buffer A (see Appendices) were added to each well. The plates were incubated under agitation at 37°C for 3 h. Mycoplasmas were centrifuged at 10,000 g for 15 min. The pellets were washed with 50 ml of PBS and centrifuged again at 10,000 g for 15 min. The pellets of mycoplasmas were resuspended with 2 ml of PBS and spun at 14,000 g for 10 min. Mycoplasmas strains N6 and M375 were resuspended with 800 µl of Buffer A, PG45 with 1,600 µl of Buffer A. Different volumes (275 µl, 125 µl and 30 µl, respectively 56.1 µg, 25.5 µg and 6.1 µg) of purified mAb 1C9 (directed against *Mmm*SC CPS) were added to the tubes with 100 µl of *Mmm*SC strain N6. Different volumes (20 µl, 14 µl and 10 µl, respectively 87 µg, 60.9 µg and 43.5 µg) of mAb 4D7 (specific for *M. bovis*) were added to the tubes with 100 µl of either *Mmm*SC strain M375 or *M. bovis* strain PG45. Each volume of both mAbs was previously diluted with Buffer A to give a final volume of 300 µl. The mixtures of mAb-mycoplasma were incubated at 4°C for 2 h. The culture medium was removed from the culture plates. The different solutions of mycoplasmas were added to the corresponding wells. The

plates were then incubated at 37°C for 30 min under agitation. The liquid was removed with a pipette and the wells were washed twice with 500 µl of cold Buffer A. Five hundred µl of 10% SDS solution in PBS were added to the wells. The plates were sealed with a plastic film and incubated overnight under agitation at room temperature. The content from each well was then transferred to liquid scintillation counting (LSC) tubes and 10 ml of LSC cocktail (Roth) were added to the tubes. The β-irradiation was then measured.

2.8. Growth inhibition test (GIT).

Mycoplasma strains were diluted in GB to give equal colony densities when cultured on GA. Ten ml of different mycoplasma solutions was put on GA in 100 mm square Petri dishes (Bibby sterilin, 109). The plates were left at room temperature for 30 min then the excess of the mycoplasma solution was removed with a pipette. The plates were left to dry for at least 5 minutes. Filter paper discs (Mast diagnostics, BD0638W) impregnated with 15 µl of serum or mAb solution were placed in contact with the mycoplasmas on agar plates. The plates were incubated at 37°C in 5% CO_2 for 3 to 4 days. The zone of clearance between the disc and the mycoplasma culture was then measured under light microscopy.

2.9. Passive immunisation.

Twenty-four 8-week-old female Balb/c mice were divided into 3 groups: two groups of 10 and one group of 4. Mice from the first group were injected with 200 µl/mouse of sterile PBS subcutaneously. Mice from the second group were injected with 200 µl/mouse of mAb 3F10 (250 µg/ml in PBS), IgM specific for

*Mmm*SC CPS. Mice from the third group did not receive any injection. Six hours later, mice were challenged with 500 µl/mouse of 10^8 N6 mycoplasmas/ml, intraperitoneally. One drop of blood was collected from each mouse in a bijoux containing 3 ml of GB, 100 µl of this solution was diluted in 900 µl of GB in Eppendorfs. Bijoux and Eppendorfs were incubated at 37°C with 5% CO_2 for a week. Mice were bled on day 0 (prior to injection), 1, 2, 3, 4 and 7. The positive results could be seen by the change of colour in bijoux and Eppendorfs, indicating the presence of mycoplasmas.

2.10. Staining protocols.

2.10.1. Coomassie Blue staining of proteins.

The gel is incubated in Coomassie Blue staining solution (see Appendices) overnight at room temperature under agitation. The gel is then destained by soaking in Coomassie Blue destain solution (see Appendices) until the gel becomes clear.

2.10.2. Silver staining of proteins

The gels were soaked three times for 10 min in 40% methanol (Fisher Scientific, M/4000/17)-10% acetic acid (Fisher Scientific, A/0400/PB17) solution then rinsed three times for 5 min with DW. They were put in silver equilibration solution for 30 min (see Appendices). The gels were rinsed for 20 s with DW and then, incubated in development solution (see Appendices) for 30 min. The gels were placed in stop solution (see Appendices) for 5 min and rinsed three times for 5 min with DW. The gels were put in reducer solution (see Appendices) for 30 s and rinsed under running tap water for 1 min and three times in DW for 5 min.

2.10.3. Schiff's reagent staining of carbohydrates.

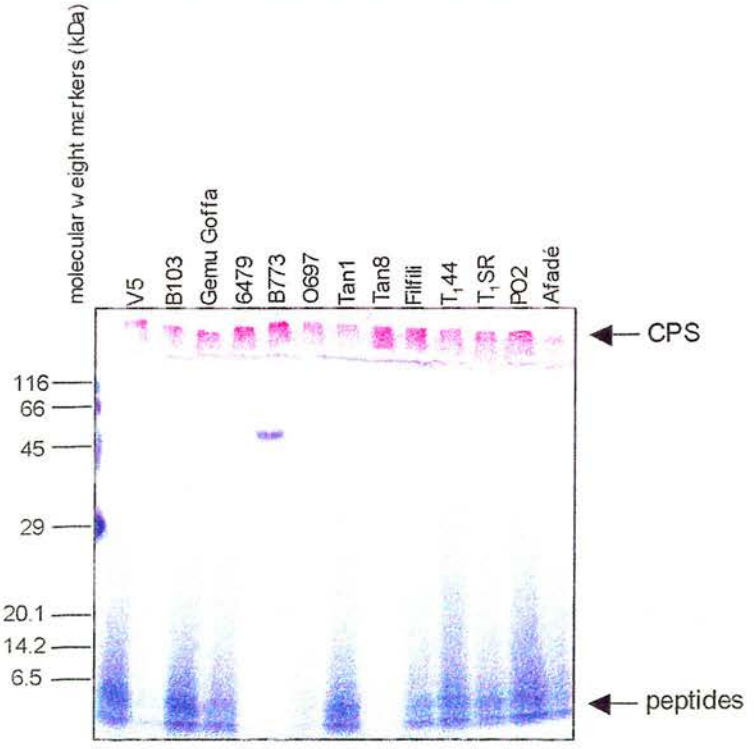
The gel is incubated for 10 min in 40% methanol (Fisher Scientific, M/4000/17)-10% acetic acid (Fisher Scientific, A/0360/PB17) solution. The gel is then transferred in 1% periodic acid (Sigma, P-7875)-3% acetic acid (Fisher Scientific, A/0360/PB17) solution for 20 min and rinsed for 5 min twice with DW. Then, it is incubated overnight in Schiff's reagent (Sigma, S-5133). After 4 rinses of 5 min with DW, the gel is soaked in 10% glycerol solution (Sigma, G-6279).

3. Results.

3.1. Purification of the capsular polysaccharide (CPS) from *Mycoplasma mycoides* subspecies *mycoides* small colony (*Mmm*SC).

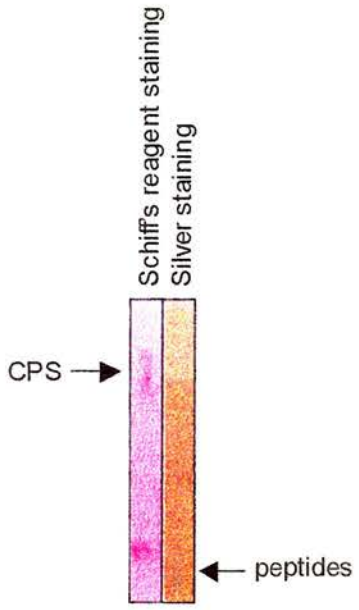
CPS was purified from culture supernatant of 22 *Mmm*SC strains grown in Gourlay's broth (GB) (see section 2.3.3.). The analysis of the purity of the different CPS solutions was assessed by a SDS-PAGE. The stacking gel was stained with Schiff's reagent (see section 2.10.3.) and the separating gel with Coomassie Blue (see section 2.10.1.). The results are given in Fig. 1. CPS appears in pink on the top of the gel. The different solutions of CPS contained also peptides as shown in blue on the bottom of the gel. A band around 50 kDa was present in the solution from the strain B773. The nature of this protein is not known.

Fig. 1. SDS-PAGE of different *Mmm*SC CPS solutions.



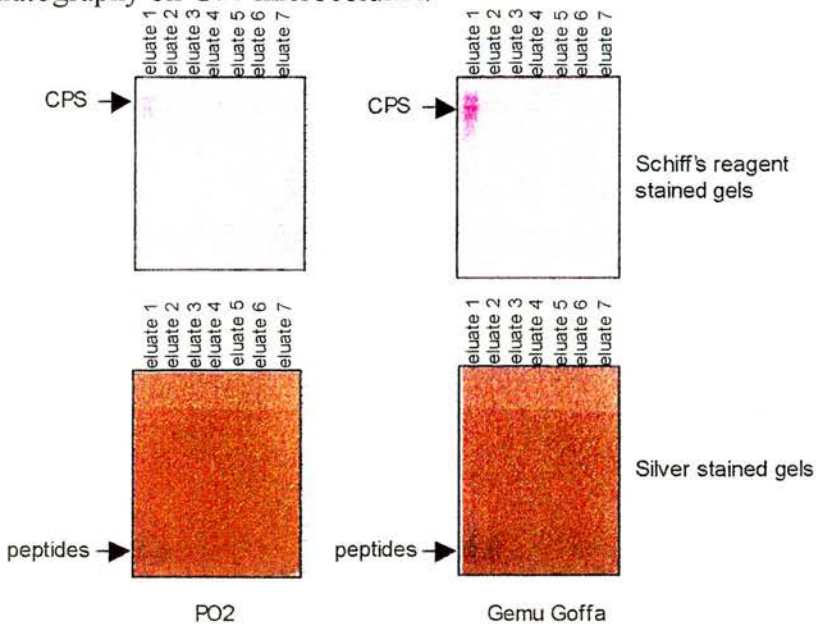
The presence of these peptides in the CPS solutions could have led to a problem in ELISA since they could have interfered with the adhesion of CPS in the ELISA plates. The removal of these peptides was attempted by gel filtration chromatography using a PD10 column (see section 2.3.4.1.) with a fractionation range for globular proteins of 1,000-5,000 Da. As shown in Fig. 2, peptides (on the right) were still present in the CPS fraction (CPS on the left).

Fig. 2. SDS-PAGE of CPS solution from *Mmm*SC strain V5 after chromatography on PD10 column.



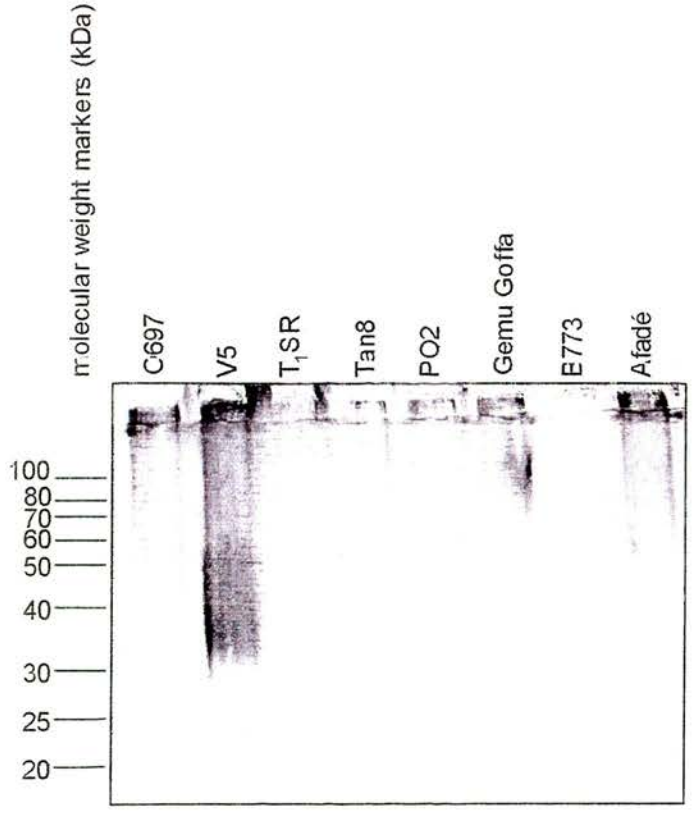
It was then decided to do another gel filtration chromatography with a G50 microcolumn with a bigger fractionation range (1,500-30,000 Da) (see section 2.3.4.2.). These attempts proved unsuccessful as the eluates containing CPS (top gels) also contained peptides (bottom gels) (Fig. 3.).

Fig. 3. SDS-PAGE of CPS solutions from *Mmm*SC strains PO2 and Gemu Goffa after chromatography on G50 microcolumn.



The different attempts to remove the peptides from purified *Mmm*SC CPS solutions did not work. A western-blot with CPS solutions from different *Mmm*SC strains was performed, using rabbit anti-*Mmm*SC strain N6 serum. The antiserum did not recognise the peptides present in the different solutions of CPS, while showing a signal against CPS, as shown in Fig. 4.

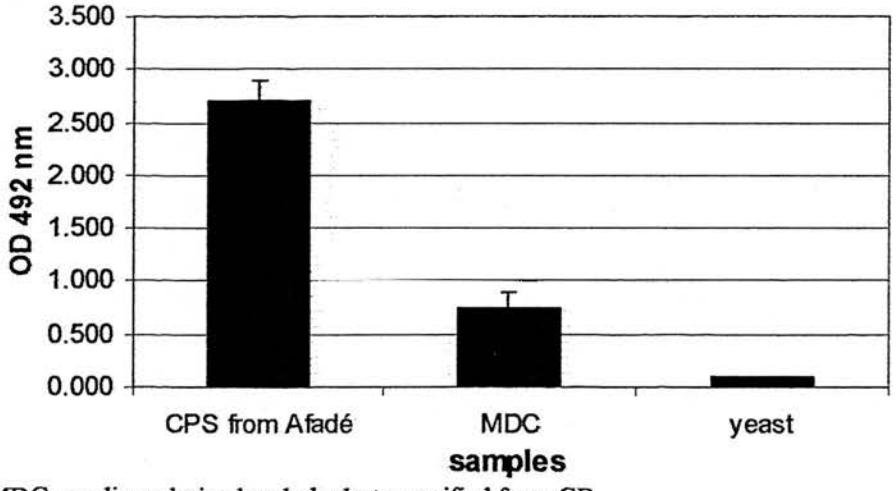
Fig. 4. Western-blot of CPS solutions from different strains of *Mmm*SC with rabbit serum raised against *Mmm*SC strain N6.



Since the peptides were not recognised by the antiserum and that the use of CPS in ELISA would not be quantitative but qualitative (i.e. it would not be the quantity of CPS that would be detected but the presence or absence of epitopes on this CPS) it was decided to use the CPS solutions with no further purification procedures.

The use of these solutions in ELISA along with solutions from purified GB (according to the same protocol as the purified *Mmm*SC CPS solutions, used as a negative control) showed that a component present in GB was recognised by antisera directed against *Mmm*SC (Fig. 5 and 6). It was then necessary to identify this contaminant to prevent false positive reactions from CPS solutions. Firstly, it was thought that the reaction was due to the yeast present in the composition of the culture medium, but an ELISA, with a solution of yeast only along a solution of CPS purified from culture supernatant of *Mmm*SC strain Afadé and carbohydrate solution purified from GB, proved that this component was not responsible for the reaction (Fig. 5) since the serum raised against *Mmm*SC strain N6 did not give any signal against the yeast.

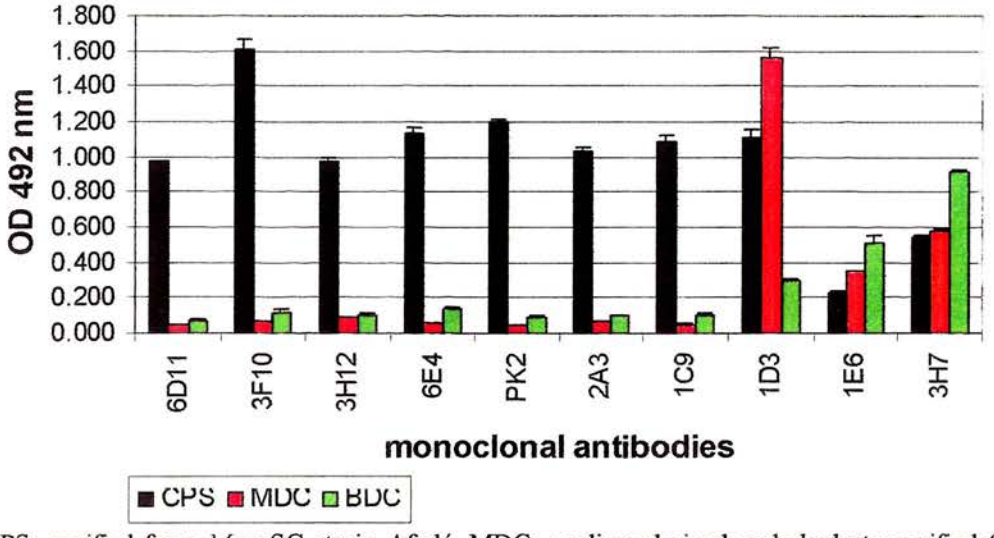
Fig. 5. Recognition of different samples by rabbit anti-*Mmm*SC strain N6 serum.



MDC: medium-derived carbohydrate, purified from GB

The same experiment was performed with Bactotryptose, another component of GB, and monoclonal antibodies (mAbs) against *Mmm*SC CPS (Fig. 6). MAbs that recognised carbohydrates from GB also exhibited a signal against carbohydrates from Bactotryptose, suggesting that this was the component responsible for the false positive reactions. Casein was subsequently used to replace Bactotryptose in the *Mmm*SC culture medium since it appeared that *Mmm*SC strains grew similarly in casein medium and GB and that this medium did not contain a carbohydrate that would be recognised by sera raised against *Mmm*SC [Emma Waite, personal communication].

Fig. 6. Recognition of different carbohydrate solutions purified from *Mmm*SC strain Afadé, Gourlay's broth and Bactotryptose by monoclonal antibodies.



CPS: purified from *Mmm*SC strain Afadé, MDC: medium-derived carbohydrate purified from GB, BDC: Bactotryptose-derived carbohydrate purified from Bactotryptose.

3.2. Production of monoclonal antibodies (mAbs) against *Mmm*SC CPS.

The investigation of the epitopes of CPS from different *Mmm*SC strains was performed using polyclonal sera (see section 3.3.) and monoclonal antibodies (see section 3.2.).

The production of mAbs was undertaken using spleen cells from mice immunised with a membrane fraction of *Mmm*SC (see section 2.2.6.) fused with NSO cells (see section 2.2.5.). After the fusion, hybridomas were cultured in 96-well culture plates. Hybridomas which supernatants gave a positive signal in ELISA against whole mycoplasmas (Table 3) or *Mmm*SC CPS (Table 4) were frozen in liquid nitrogen.

Table 3. Scheme of 96-well culture plates containing hybridomas with results of ELISA against whole mycoplasmas, *Mmm*SC strains T_1SR, V5 and B820/124.

	1	2	3	4	5	6	7	8	9	10	11	12
1A												
1B		3.894	1.204	1.287	**3.231**	1.098	1.953	**3.920**	**3.640**	**3.714**	1.185	
1C		3.911	1.340	**3.500**	1.567	1.510	1.277	**3.879**	**3.559**	1.228	**3.253**	
1D		3.437	1.605	1.013	1.378	1.583	**3.903**	1.952	1.139	**3.665**	1.707	
1E		3.840	1.591	0.956	0.779	1.002	1.297	1.442	**3.718**	1.388	**3.688**	
1F		3.672	1.630	1.270	1.666	**2.149**	**3.631**	1.150	1.582	1.153	**3.029**	
1G		3.683	0.985	1.624	1.892	1.017	1.735	0.921	1.066	1.058	1.277	
1H												

	1	2	3	4	5	6	7	8	9	10	11	12
2A												
2B		1.058	1.223	**2.188**	**3.748**	1.428	1.486	1.328	**3.803**	3.756		
2C		1.660	**3.088**	1.097	0.897	1.216	1.147	1.045	**3.604**	3.713		
2D		0.951	1.745	**2.157**	**3.617**	1.222	1.224	1.533	1.828	3.749		
2E		1.201	1.222	1.225	1.653	**2.468**	1.075	1.158	0.827	3.592		
2F		1.438	1.906	1.742	**3.686**	1.249	1.338	**3.623**	1.221	3.572		
2G		0.945	**3.722**	**3.445**	1.017	1.262	1.335	1.175				
2H												

data: results from ELISA at OD 492 nm, cells with bold borders: wells containing unfusioned spleen cells, data in bold: hybridomas to be frozen.

Attempts to clone these hybridomas on agar plates were made several times for most of them. Unfortunately, problems occurred in the process including the contamination of the cultures by fungi or bacteria. When the problem of contamination was solved, it appeared that the hybridomas were not in a healthy state after thawing as they did not multiply but died.

Table 4. Scheme of 96-well culture plates containing hybridomas with results of ELISA against CPS from *Mmm*SC strains T_1SR, V5 and B820/124.

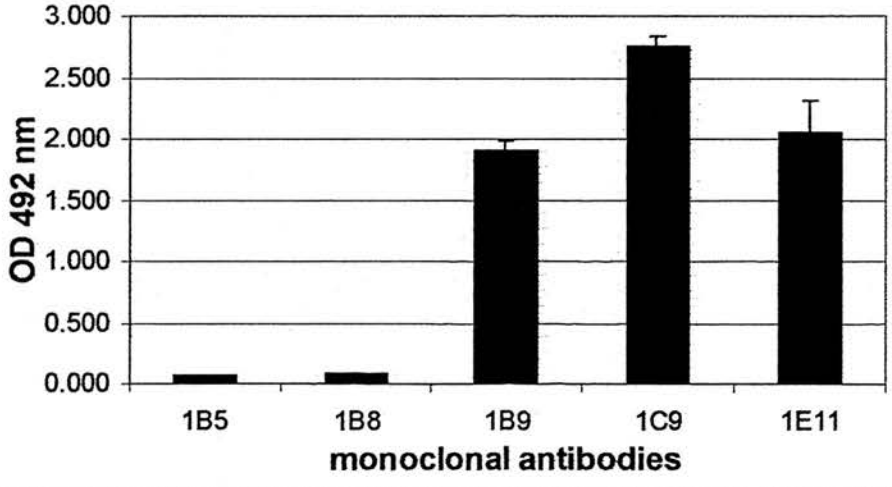
	1	2	3	4	5	6	7	8	9	10	11	12
1A												
1B		0.376	0.185	0.170	**1.560**	0.065	**0.915**	**3.473**	**3.588**	**3.608**	0.075	
1C		0.297	0.074	**2.755**	0.078	0.089	0.157	**3.461**	**3.566**	0.072	**1.395**	
1D		0.651	0.080	0.062	0.094	0.071	**3.272**	0.071	0.419	**3.329**	0.068	
1E		0.269	0.064	0.066	0.064	0.078	0.108	0.313	**3.112**	0.083	**3.326**	
1F		0.301	0.064	0.025	0.051	0.042	**3.370**	0.021	0.303	0.024	**1.980**	
1G		0.438	0.069	0.051	0.240	0.070	0.078	0.052	0.060	0.057	0.054	
1H												

	1	2	3	4	5	6	7	8	9	10	11	12
2A												
2B		0.079	0.223	0.092	**2.712**	0.064	0.059	0.061	**2.650**	0.603		
2C		0.078	**1.622**	0.069	0.065	0.107	0.073	0.070	**2.904**	0.463		
2D		0.067	0.067	0.542	**2.303**	0.063	0.054	0.064	0.053	0.659		
2E		0.060	0.062	0.087	0.079	0.191	0.055	0.074	0.049	0.559		
2F		0.037	0.448	0.171	**2.217**	0.031	0.030	**2.828**	0.289	0.450		
2G		0.056	**2.726**	**2.493**	0.062	0.057	0.058	0.056				
2H												

data: results from ELISA at OD 492 nm, cells with bold borders: wells containing unfusioned spleen cells, data in bold: hybridomas to be frozen.

Five hybridomas, 1B5, 1B8, 1B9, 1C9 and 1E11, were finally cloned. For two of them (clones from hybridomas 1B5 and 1B8), no antibodies specific for CPS appeared to be present in the culture supernatants when tested by ELISA (Fig. 7).

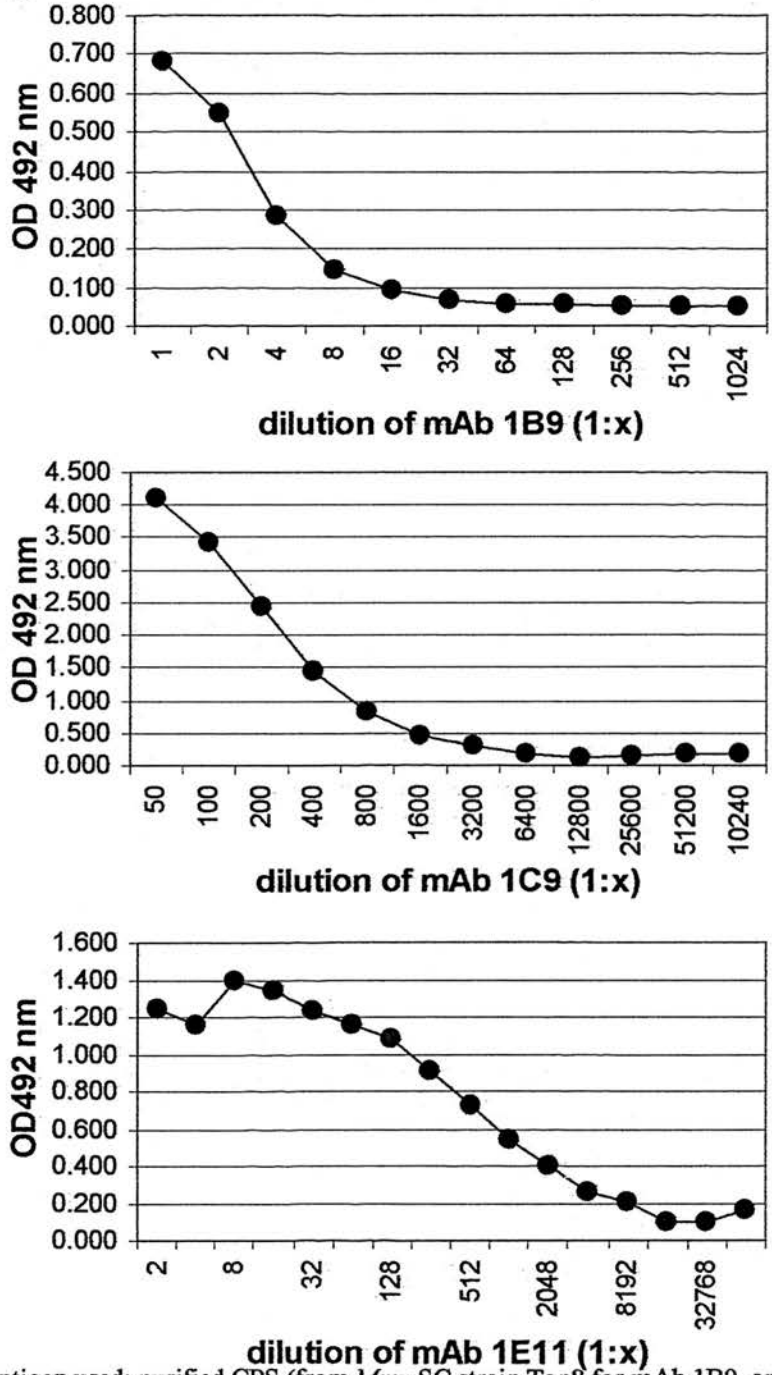
Fig. 7.Specificity of monoclonal antibodies 1B5, 1B8, 1B9, 1C9 and 1E11.



antigen used: purified CPS (from *Mmm*SC strain Tan8 for mAbs 1B5, 1B8 and 1B9, and from mixed *Mmm*SC strains T1SR, V5 and B820/124 for mAbs 1C9 and 1E11), the mAbs came from undiluted cell culture supernatants.

The antibody titre against CPS for the three mAbs that recognised CPS (1B9, 1C9 and 1E11) was very low after subculture, as measured by ELISA (Fig. 8). It should be noted that these clones grew very slowly and consequently, it was difficult to obtain a large amount of culture supernatants.

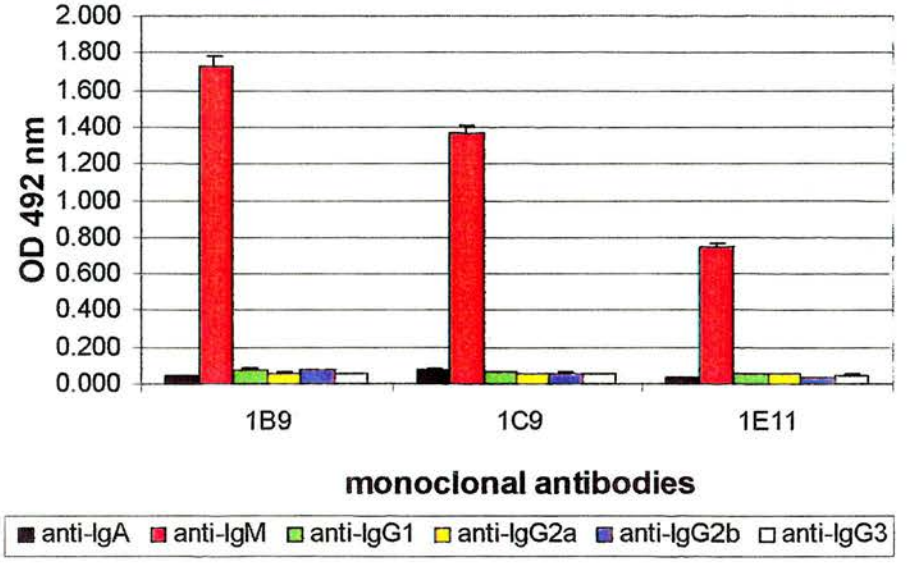
Fig. 8. Titration of monoclonal antibodies 1B9, 1C9 and 1E11.



antigen used: purified CPS (from *Mmm*SC strain Tan8 for mAb 1B9, and from mixed *Mmm*SC strains T1SR, V5 and B820/124 for mAbs 1C9 and 1E11).

All three mAbs 1B9, 1C9 and 1E11 were of IgM isotype (Fig. 9).

Fig. 9. Immunoglobulin isotype of monoclonal antibodies 1B9, 1C9 and 1E11.



antigen used: purified CPS (from *Mmm*SC strain Tan8 for mAb 1B9, and from mixed *Mmm*SC strains T1SR, V5 and B820/124 for mAbs 1C9 and 1E11), box: anti-mouse immunoglobulin antibody.

The results are summarised in Table 5.

Table 5. Summary of the production of mAbs against *Mmm*SC CPS.

Hybridomas	Anti-CPS antibodies	Attempts of cloning	Successful cloning	Remarks
1B5	Y	$4^{1,2}$	X	Abs non specific for CPS
1B7	Y	$4^{1,2}$		
1B8	Y	$5^{1,2}$	X	Abs non specific for CPS
1B9	Y	$4^{1,2}$	X	
1B10	Y	$5^{1,2}$		
1C4	Y	$4^{1,2}$		
1C8	Y	$6^{1,2}$		
1C9	Y	2^{1}	X	
1C11	Y	0		
1D7	Y	$2^{1,2}$		
1D10	Y	$3^{1,2}$		
1E9	Y	$3^{1,2}$		
1E11	Y	$3^{1,2}$	X	
1F7	Y	1^{2}		
1F11	Y	2^{2}		
2B5	Y	2^{2}		
2B9	Y	$4^{1,2}$		
2C3	Y	2^{2}		
2C9	Y	2^{2}		
2D5	Y	3^{2}		
2F5	Y	$4^{1,2}$		

2F8	Y	0		
2G3	Y	1[2]		
2G4	Y	0		
1F6	N	0		
2B4	N	0		
2D4	N	0		
2E6	N	0		

Y: hybridomas producing anti-*Mmm*SC CPS antibodies, N: hybridomas producing antibodies specific for *Mmm*SC (not against CPS), 1: contamination either by fungi or bacteria, 2: no multiplication of the hybridomas after thawing and culture.

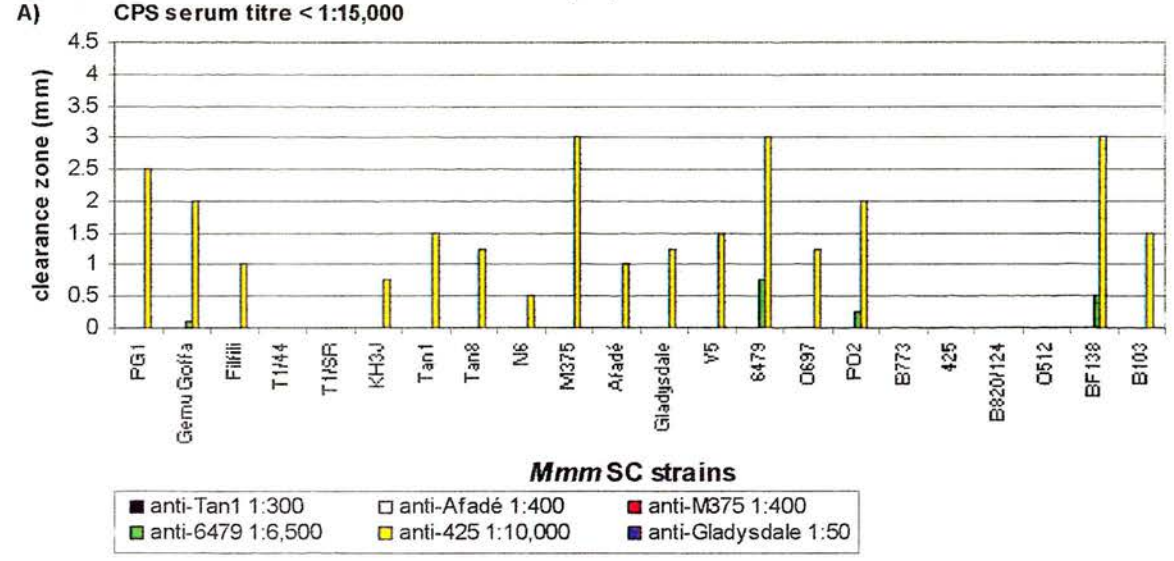
3.3. Growth inhibition test (GIT) with *Mmm*SC strains and rabbit polyclonal sera.

An efficient vaccine is one able to protect against a maximum, if not all, strains of the microorganism. In previous studies, it was shown that antibodies responsible for the inhibition in GIT were antibodies directed against CPS. When the sera were pre-absorbed with CPS they did not exhibit growth-inhibiting activity anymore [116]. The most virulent strains produce more CPS [110] and CPS has been shown to have toxic effects in cattle when injected on its own in the absence of mycoplasma [4,23]. Strains producing the most CPS are also more resistant to inhibition by antisera in GIT: CPS seems like a good potential vaccine. However, for a vaccine using *Mmm*SC CPS, it was necessary to know if CPS was conserved between all strains.

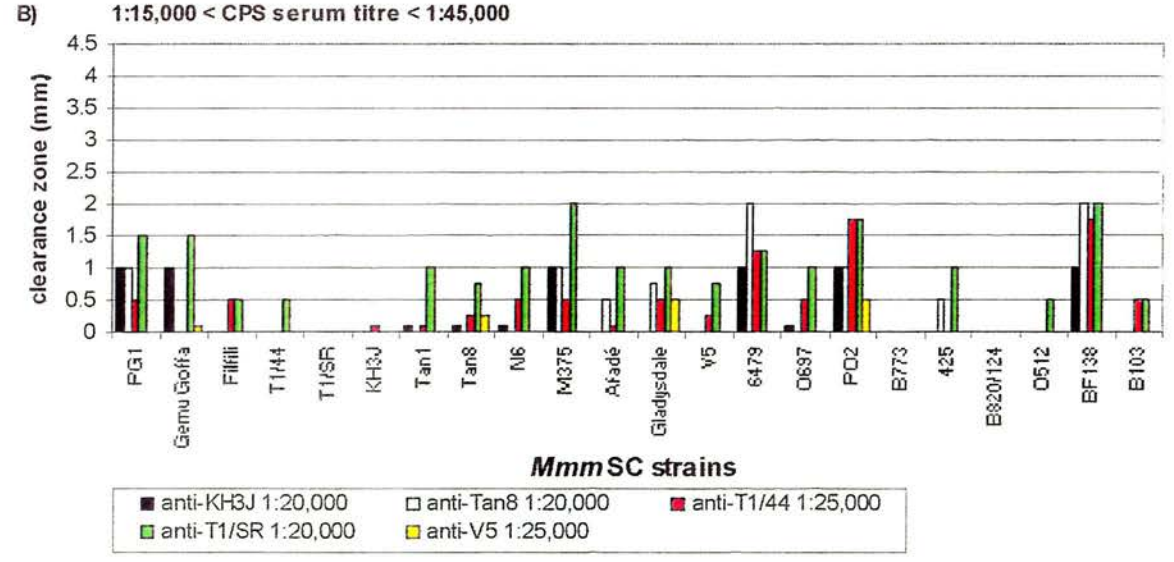
Twenty-two *Mmm*SC strains were tested with 23 antisera. Each antiserum was raised against one of the *Mmm*SC strains, as a consequence each antiserum contained antibodies specific for CPS of the strain it was raised against. If these antibodies also recognised CPS from other strains, they should inhibit the growth of these strains.

Growth inhibiting activity is shown in Fig. 10. Results were divided according to the anti-CPS antibody titre of the rabbit sera (measured as the dilution factor of the serum where the line from the linear part of the titration curve crossed the x-axis).

Fig. 10. GIT of *Mmm*SC strains with rabbit polyclonal sera.

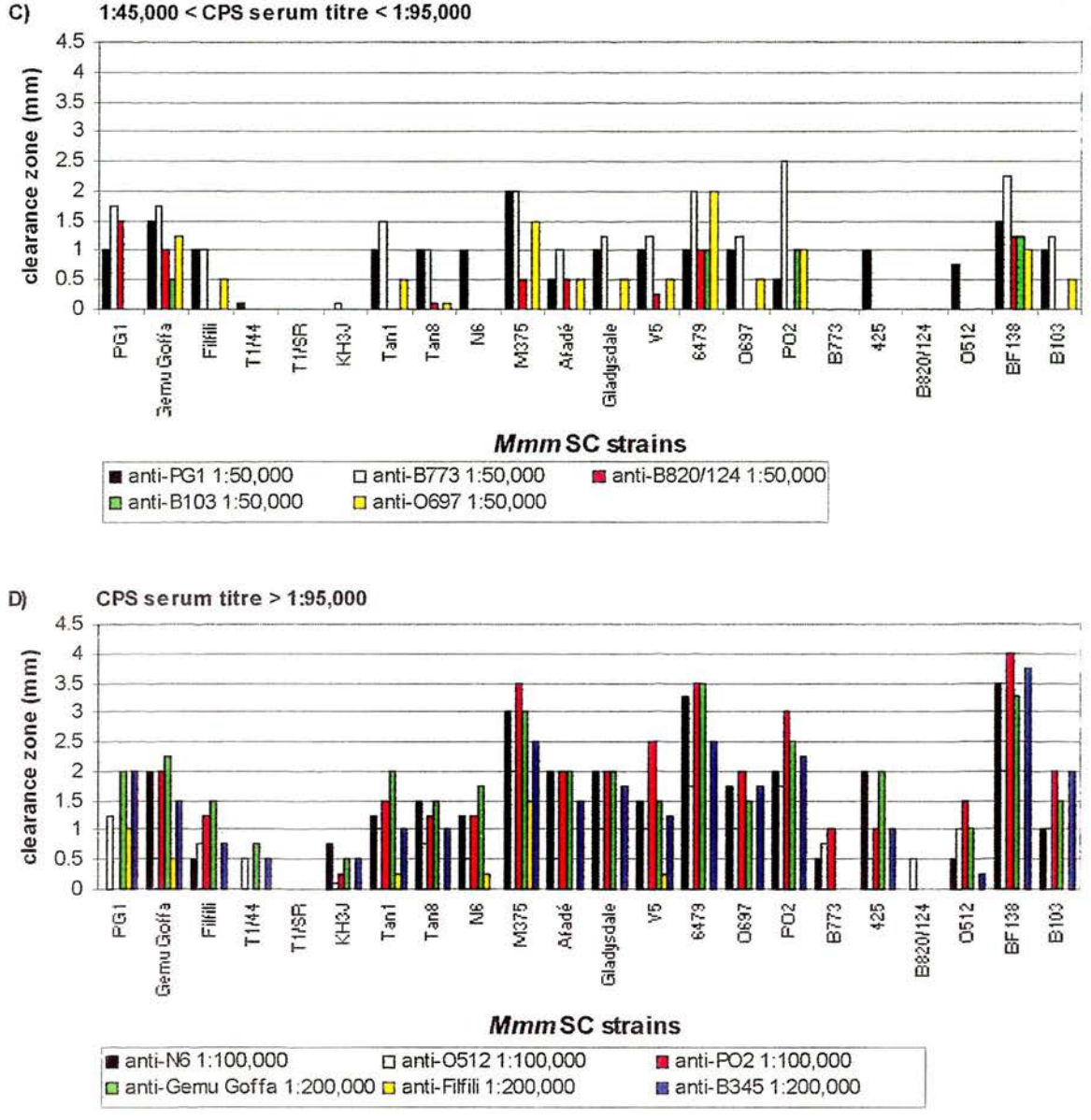


Results obtained with sera against *Mmm*SC strains Tan1, Afadé, M375 and Gladysdale do not appear on the graph because no clearance zone could be seen with these antisera against any of the *Mmm*SC strains tested.



*Mmm*SC strains on the x axis, zone of clearance on the y axis. 1:x: anti-CPS antibody titre indicated behind the serum of which it applies.

Fig. 10 (concluded). GIT of *Mmm*SC strains with rabbit polyclonal sera.



*Mmm*SC strains on the x axis, zone of clearance on the y axis. 1:x: anti-CPS antibody titre indicated behind the serum of which it applies.

For sera with a titre below 1:15,000 (Fig 10. A), none of the sera exhibited GI activity apart from anti-425 and anti-6479 (these latter sera had only a small amount of growth inhibiting activity). For sera with a titre between 1:15,000 and 1:45,000 (Fig. 10. B), some GI activity was seen, but against less than half of the strains. This activity was low as the zone of clearance was mostly below 1 mm. For sera with a titre between 1:45,000 and 1:95,000 (Fig. 10. C), the sera exhibited GI activity

against 4 to 18 of the strains. The zones of clearance measured were mostly 1 mm or above. Finally, most of the sera with a titre above 1:95,000 (Fig. 10. D) were inhibitory. In this case, the zones of clearance observed were largely above 1 mm, with many of them equal to or above 2 mm.

It is known the inhibition is due to antibodies against CPS present in the serum as pre-absorbed antisera with CPS do not exhibit GI activity [116]. Serum raised against any specific *Mmm*SC strain contains antibodies against the CPS of this specific strain. In the GIT, a specific antiserum not only inhibited the strain against which it was raised, but also inhibited other strains. The activity of antisera was related to the CPS-specific antibody titre and not to the strain they were raised against. These findings suggest that epitopes responsible for GI activity are present on all strains of *Mmm*SC (i.e. CPS is probably conserved between strains). Moreover, sera with the highest anti-CPS antibody titre exhibited the greatest GI activity. However, some exceptions exist as it can be seen, for example, with anti-425 serum (showing a low anti-CPS antibody titre but a marked GI activity) and with anti-Filfili serum (a high CPS-specific antibody titre but a low GI activity). This might be due to the nature of CPS-specific antibodies in the sera. Antibodies against CPS might not be all involved in growth inhibition. In the case of anti-425 serum, maybe the total amount of antibodies against CPS is low but most of them are inhibiting. And for the anti-Filfili serum, the sera may contain a large amount of antibodies specific for CPS but most of them may not be growth inhibiting. It cannot be excluded that, for some strains, antibodies against other components of the mycoplasma might have growth inhibiting activity as well.

3.4. Analysis of CPS from mycoplasma strains with mAbs.

Previous data (see section 3.3.) suggested CPS epitopes were shared between all strains of *Mmm*SC. However, it cannot be excluded that some epitopes might be present in only some of the strains. CPS was investigated using monoclonal antibodies in order to see if strain-specific CPS epitopes existed.

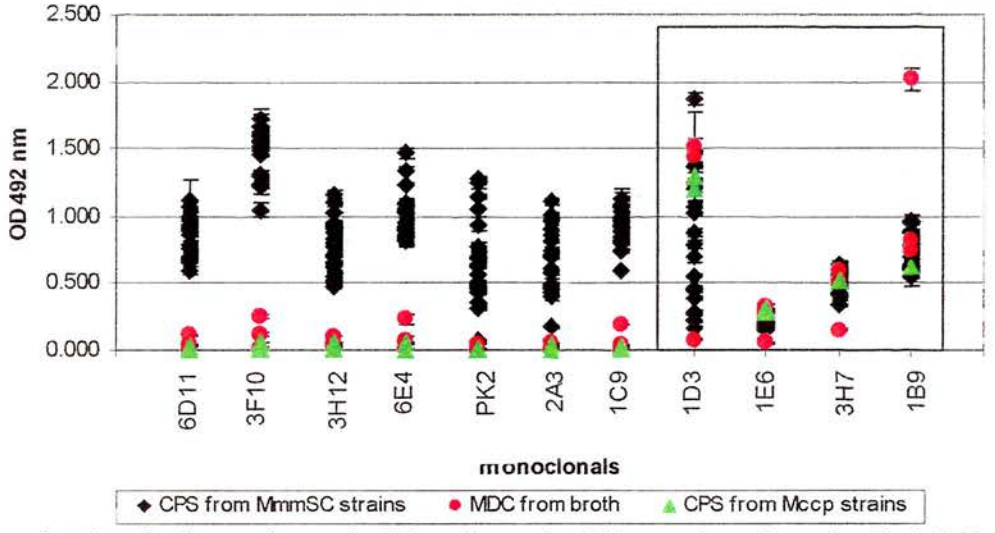
3.4.1. Analysis of *Mmm*SC CPS.

Eleven mAbs (see Materials and methods, section 2.2.2) were tested by ELISA against CPS purified from 22 individual strains of *Mmm*SC and from two strains of *Mycoplasma capricolum* subsp. *capripneumoniae (Mccp)* (these mycoplasma strains were grown in Gourlay's medium). *Mccp* is related to *Mmm*SC, causing a similar disease in goats (contagious caprine pleuropneumonia). Three batches of Gourlay's medium derived carbohydrates (MDC), purified following the same protocol as mycoplasma CPS, were also tested with the mAbs. MDC was used as a control to be sure the signal observed from CPS solutions incubated with mAbs was from CPS and not from a contaminating medium component. A high molecular weight polymer was found in the culture medium (as shown on a SDS-PAGE stained with Schiff's reagent), as a consequence it was present in solutions of purified CPS [150].

All the monoclonals recognised CPS from the different *Mmm*SC strains (Fig. 11). In addition, 4 of them (boxed data) also reacted with CPS from *Mccp* strains and with MDC, with a signal as high as the one observed with CPS from *Mmm*SC. It is unclear whether these mAbs recognised a common epitope present

both on CPS and on the culture medium carbohydrate, or if they only recognised the contaminating MDC. CPS from all the strains appeared to be recognised in the same way with each of the antibodies except with mAbs PK2 and 2A3, which exhibited a low signal with the CPS from the strain PG1. This was at a similar level as the reaction with CPS from the *Mccp* strains and MDC suggested that this strain might be antigenically different from the others concerning the epitope recognised by these two mAbs.

Fig. 11. Analysis of CPS from different *Mmm*SC strains with different monoclonal antibodies.



Monoclonal antibodies on the x axis, OD on the y axis. CPS: capsular polysaccharide. MDC: medium derived carbohydrate. MmmSC: *Mycoplasma mycoides* subsp. *mycoides* small colony. Mccp: *Mycoplasma capricolum* subsp. *capripneumoniae*. broth: uninoculated mycoplasma culture medium.

Five monoclonals were tested in GIT with *Mmm*SC strain Gemu Goffa to look at their inhibiting activity. Monoclonals that had the highest titre in the previous ELISA were chosen (see Materials and Methods section 2.4.1.). Gemu Goffa was chosen because it showed a good sensitivity to inhibition by rabbit polyclonal antisera raised against various *Mmm*SC strains. All the monoclonals were growth inhibiting (Table 6). It is not known if the bactericidal activity is related to the CPS-specific antibody titre as no titration has been performed on these mAbs.

Table 6. GI activity of monoclonal antibodies against *Mmm*SC strain Gemu Goffa.

mAbs	Clearance zone (mm)
3F10	3.5
6E4	3.5
6D11	2
3H12	2
PK2	1

3.4.2. Analysis of *Mycoplasma mycoides* subspecies *mycoides* large colony (*Mmm*LC) and *Mycoplasma mycoides* subspecies *capri* (*Mmc*) with anti-CPS monoclonal antibodies.

*Mmm*LC and *Mmc* have high similarity according to 16S rRNA sequence analysis and have the same tRNA profile [151,152]. These two species are classified in the *Mycoplasma mycoides* subgroup along with *Mmm*SC and can cause pneumonia in goats. *Mmm*LC has immunological similarities with *Mmm*SC: for instance, anti-*Mmm*LC polyclonal sera are growth inhibiting for both *Mmm*LC and *Mmm*SC [153]. Assuming the inhibiting activity in anti-*Mmm*LC sera is due to anti-CPS antibodies as it has been shown in antisera raised against *Mmm*SC, this suggests the CPS from *Mmm*SC and *Mmm*LC could share common epitopes. In consequence, it was interesting to investigate whether the epitopes on *Mmm*SC CPS recognised by the 5 previous mAbs were also present on CPS from *Mmm*LC and *Mmc*. MAbs were used in ELISA (Fig. 12) against sonicated mycoplasmas and in GIT (Table 8) against live mycoplasmas. The mycoplasma strains used are listed in Table 7. *Mmm*SC strain N6 was used as a positive control.

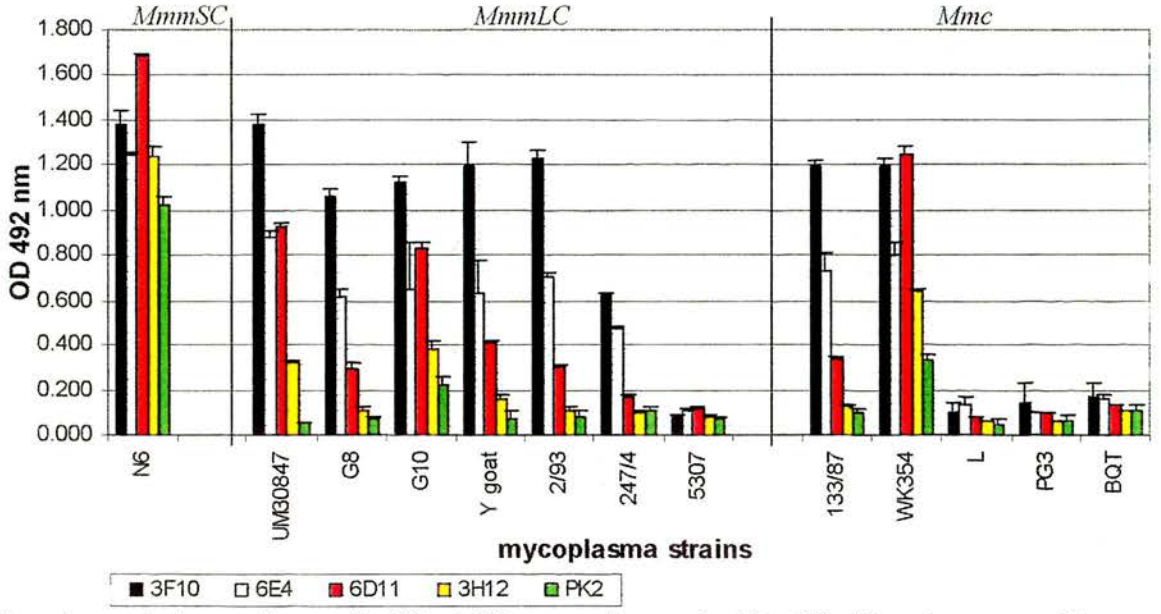
Table 7. Mycoplasma strains used in Experiment 3.4.2.

Strains	Species	Strains	Species
N6[1]	*Mmm*SC		
UM30847[2]	*Mmm*LC	133/87[2]	*Mmc*
G8[2]	*Mmm*LC	WK354[2]	*Mmc*
G10[2]	*Mmm*LC	L[2]	*Mmc*
Y goat[1]	*Mmm*LC	PG3[1]	*Mmc*
2/93[3]	*Mmm*LC	BQT[3]	*Mmc*
247/4[3]	*Mmm*LC		
5307[3]	*Mmm*LC		

[1]strains supplied by MRI, Penicuik, United Kingdom, [2]strains supplied by Göran Bölske, Uppsala, Sweden and [3]strains supplied by Robin A.J. Nicholas, Addlestone, United Kingdom.

In the ELISA, as expected, N6 showed a signal with all the mAbs. MAb 3F10 gave a signal with *Mmm*LC strains UM30847, G8, G10, Y goat and 2/93 and with *Mmc* strains 133/87 and WK354 to the same intensity as the one observed with *Mmm*SC strain N6. The signal intensity for *Mmm*LC strain 247/4 was half that observed with the other strains. This could indicate that less CPS was present in the cell extract used to coat the ELISA plate with this strain since the signal was less high with all different mAbs when compared to the signal observed with the other strains. MAb 6E4 recognised the same mycoplasma strains as 3F10, i.e. all *Mmm*LC strains apart 5037 and two *Mmc* strains 133/87 and WK354. MAb 6D11 showed a signal with *Mmm*LC strains UM30847, G10 and Y goat and with *Mmc* strain WK354. MAbs 3H12 and PK2 did not show a good signal with any of the *Mmm*LC and *Mmc* strains when compared with N6. *Mmm*LC strain 5307 and *Mmc* strains PG3, BQT and L were not recognised by any of the monoclonals. These results are summarised in Table 8, optical densities below 0.4 were considered as negative.

Fig. 12. Analysis of CPS from different mycoplasma strains with monoclonal antibodies.



Mycoplasma strains on the x axis, OD at 492 nm on the y axis. *Mmm*SC: *Mycoplasma mycoides* subsp. *mycoides* small colony, *Mmm*LC: *Mycoplasma mycoides* subsp. *mycoides* large colony, *Mmc*: *Mycoplasma mycoides* subsp. *capri*.

The previous mAbs used in the ELISA were then used in GIT (Table 8). They were all bactericidal against *Mmm*SC as previously tested against *Mmm*SC strain Gemu Goffa (see section 3.4.1., Table 6). MAbs 3F10 and 6E4 recognised epitopes on *Mmm*LC strains UM30847, G8, G10, Y goat, 2/93 and 247/4 and *Mmc* strains 133/87 and WK354 by ELISA. These mAbs were also growth inhibiting with all these strains apart *Mmm*LC strain G8 that showed no inhibition and *Mmm*LC strain 2/93 which was inhibited only by 3F10. The growth inhibition is not affected only by whether a strain exhibits an epitope on their CPS but also by the magnitude of CPS production by a strain since a strain producing a lot CPS will be more resistant to inhibition by CPS specific antibodies. The lack of inhibition of G8 and 2/93 might be because they produce more CPS than the other strains and consequently do not show inhibition of their growth. MAbs 6D11, 3H12 and PK2 did not exhibit any growth

inhibiting activity against *Mmm*LC and *Mmc* strains this could suggest that antibodies directed against CPS are not all bactericidal.

Table 8. Summary of the results of ELISA, GIT and LAT against *Mmm*LC and *Mmc*.

Strains	LAT	mAbs									
		3F10		6E4		6D11		3H12		PK2	
		ELISA	GIT	ELISA	GIT	ELISA	GIT	ELISA	GIT	ELISA	GIT
N6[1]	+	+	nd[4]	+	nd	+	nd	+	nd	+	nd
UM30847[2]	+	+	2	+	2	+	0	-	0	-	0
G8[2]	+	+	0	+	0	-	0	-	0	-	0
G10[2]	+	+	2	+	2	+	0	-	0	-	0
Y goat[2]	+	+	2	+	2	+	0	-	0	-	0
2/93[2]	+	+	2	+	0	-	0	-	0	-	0
247/4[2]	-	+	2	+	1.5	-	0	-	0	-	0
5307[2]	-	-	0	-	0	-	0	-	0	-	0
133/87[3]	+	+	2	+	2	-	0	-	0	-	0
WK354[3]	+	+	2	+	2	+	0	-	0	-	0
L[3]	-	-	0	-	0	-	0	-	0	-	0
PG3[3]	-	-	0	-	0	-	0	-	0	-	0
BQT[3]	-	-	0	-	0	-	0	-	0	-	0

[1]*Mmm*SC strain, [2]*Mmm*LC strains and [3]*Mmc* strains.[4]nd: not done. LAT: latex agglutination test, "+" means agglutination and "-" means no agglutination. ELISA: enzyme linked immunosorbent assay, "+" means recognition of the mycoplasma strain by the mAb and "-" no recognition of the mycoplasma strain by the mAb. GIT: growth inhibition test, results expressed the clearance zone in mm.

The results obtained in ELISA and GIT with *Mmm*LC and *Mmc* strains are summarised in a same table (Table 8) along with the results obtained with the same strains in a latex agglutination test (LAT) [John B. March, personal communication]. The LAT was performed using latex beads coated with *Mmm*SC CPS-specific rabbit IgG thus CPS-positive strains agglutinated.

When the results are examined in respect of the mAbs, it seems that mAbs can be classified in three categories. The first category contains antibodies 3F10 and 6E4 that bind to CPS in ELISA and show a growth inhibiting activity. The second category concerns the antibody 6D11 that binds to CPS in ELISA but exhibits no growth inhibiting activity. And finally the third category regroups antibodies 3H12

and PK2 that show no signal with the CPS of *Mmm*LC and *Mmc* strains in ELISA and no growth inhibiting activity. Since these mAbs are divided in three types, this indicates that at least three different epitopes are recognised by these mAbs.

When the results are examined in respect of the mycoplasma strains, it seems they can be divided in two groups. One group represents the strains recognised by the mAbs, *Mmm*LC strains UM30847, G8, G10, Y goat, 2/93 and 247/4 and *Mmc* strains 133/87 and WK354. The other group contains the strains that are not recognised by the mAbs, *Mmm*LC strain 5307 and *Mmc* strains L, PG3 and BQT. These data fit closely with the LAT data.

It has been suggested that according to their 16S rRNA sequence analysis and tRNA profile, *Mmm*LC and *Mmc* should be classified as the same species of mycoplasma [151,152]. The results obtained with the monoclonals suggest that CPS of *Mmm*LC/*Mmc* exhibit at least two different serotypes. Further work is necessary to obtain proper re-classification of the *Mmm*LC and *Mmc* species. If these strains represent a single mycoplasma species with two "serotypes", this is a novel finding for mycoplasmas.

3.4.3. Competitive ELISA.

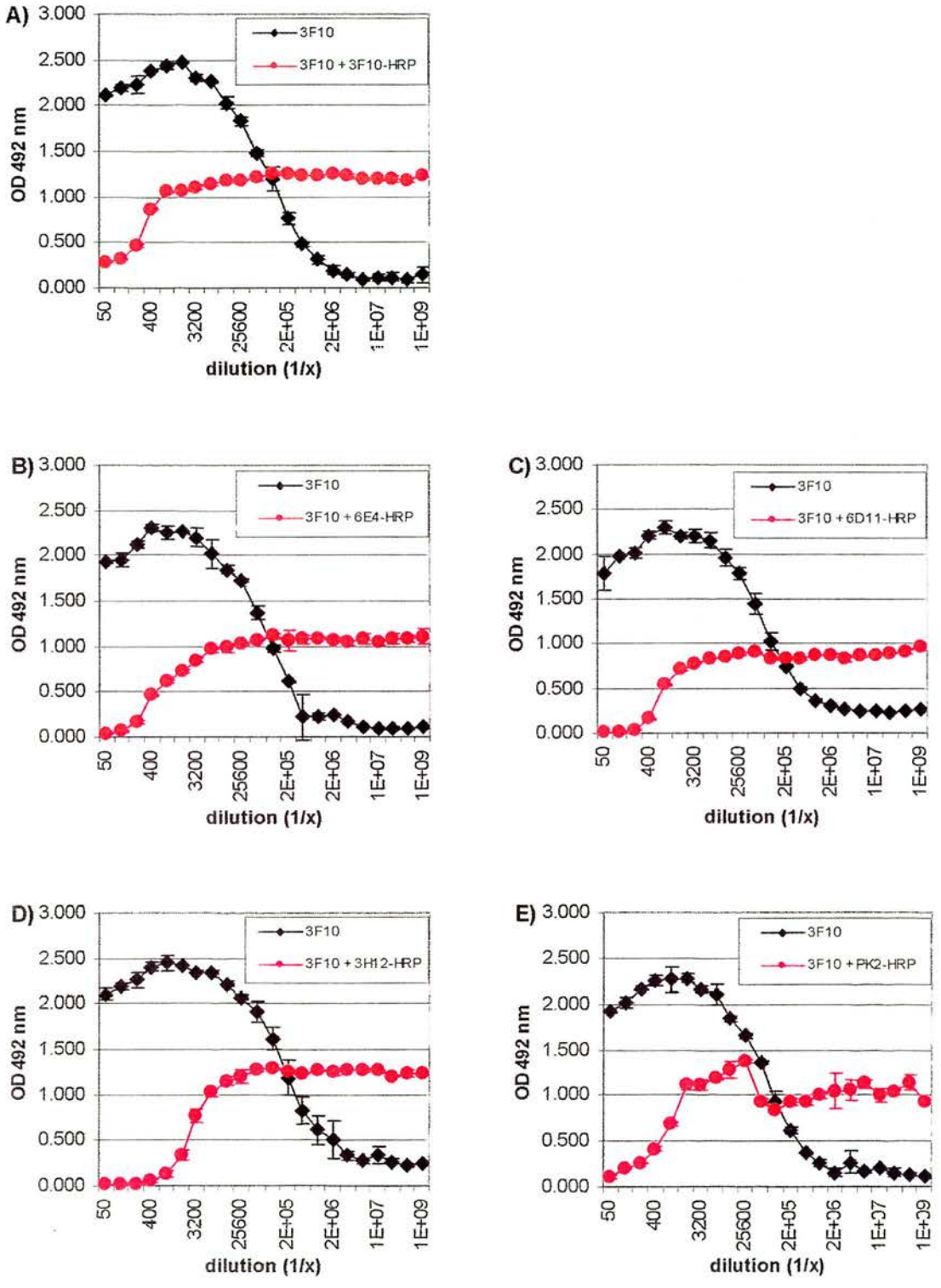
Previously (section 3.4.2.), it seemed that different CPS epitopes were recognised by these 5 mAbs 3F10, 6E4, 6D11, 3H12 and PK2. To clarify the number of epitopes recognised by these 5 mAbs, competition ELISAs were performed using mAbs conjugated to horseradish peroxidase (HRP). Binding of each mAb to CPS was competed against itself (i.e. for example, 3F10 with 3F10-HRP) as a positive control to determine if mAbs recognised the same or different epitopes.

It was decided that at least three data points should also be under the horizontal linear part of the curve to be considered as a drop in the curve.

The results obtained with unconjugated 3F10 in competition with various HRP-conjugated mAbs are shown in Fig. 13. The graph obtained with 3F10 in competition against itself is used as positive control (Fig. 13. A). Assuming equal antibody concentration, mAb recognising the same epitope as 3F10 should give the same curve as seen in Fig. 13. A. When 6E4-HRP (Fig. 13. B) was in competition against unconjugated 3F10, a drop similar to the one observed on the graph with 3F10-HRP competing against 3F10 (Fig. 13. A) could be seen. The same results were observed with unconjugated 3F10 in competition against 6D11-HRP (Fig. 13. C), 3H12-HRP (Fig. 4. 13) and PK2-HRP (Fig. 13. E). According to these graphs, all 5 mAbs seemed to compete equally against 3F10, and this would suggest that they all recognise an identical epitope on CPS, unless a phenomenon of steric hindrance occurs and prevent the binding of the mAbs.

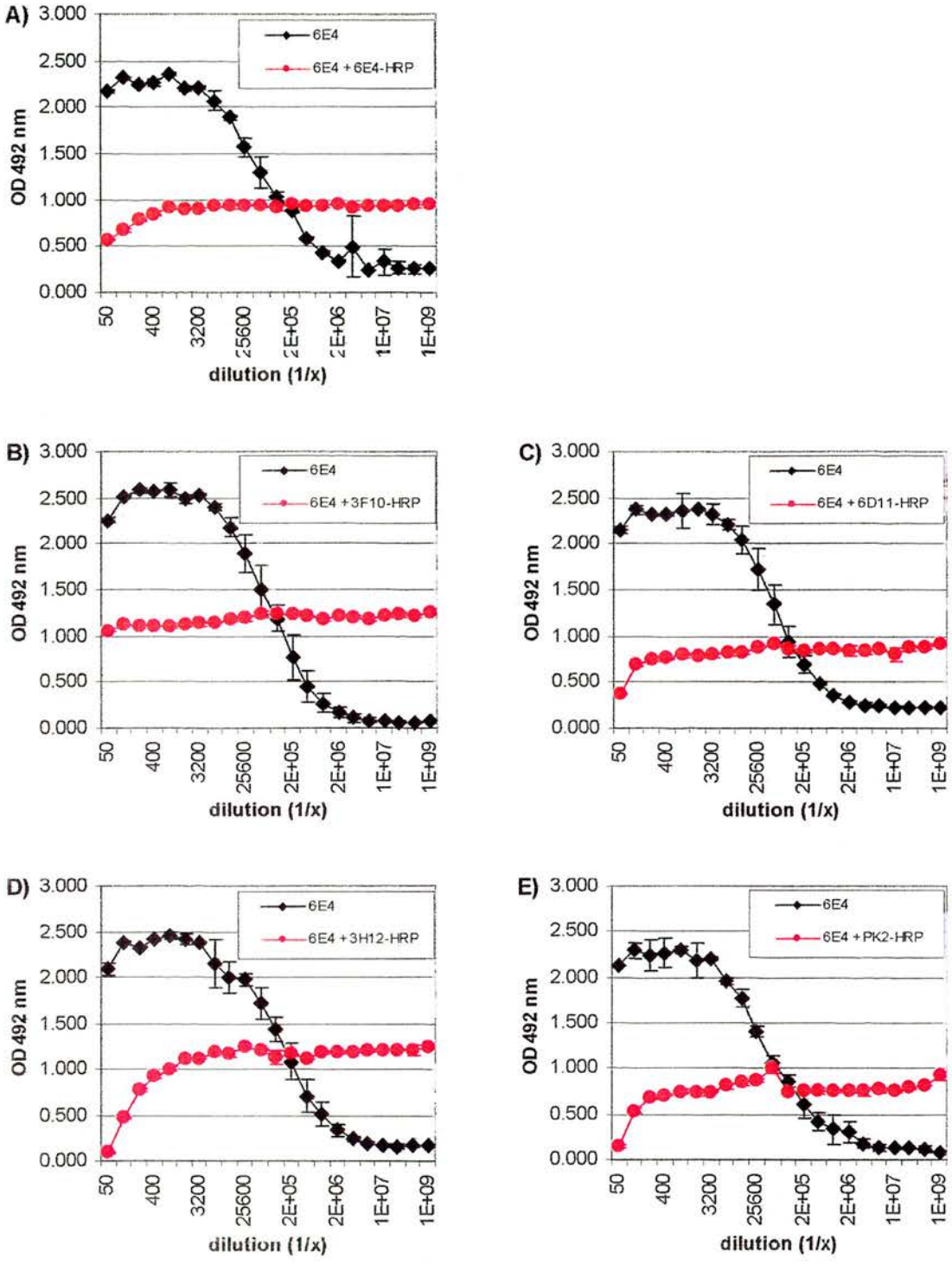
The same conjugated mAbs were competed against unconjugated 6E4 (Fig. 14). As previously described for the mAb 3F10, the graph obtained with the 6E4 competing against 6E4-HRP (Fig. 14. A) was used for comparison with the graphs obtained with the other conjugated mAbs in competition against unconjugated 6E4. No drop in the curve could be seen with 3F10-HRP (Fig. 14. B), 6D11-HRP (Fig. 14. C) and PK2-HRP (Fig. 14. E) suggesting 6E4 and 3F10, 6D11 and PK2 do not recognise the same epitope on CPS.

Fig. 13. Competitive ELISA between unconjugated 3F10 (dilution from 1/50 to $1/10^9$) and HRP-conjugated 3F10, 6E4, 6D11, 3H12 and PK2 (constant dilution).



-◆-: incubation of 3F10 followed by incubation of HRP-conjugated anti-mouse secondary antibody.
-●-: incubation of 3F10 followed by incubation of HRP-conjugated mAb.

Fig. 14. Competitive ELISA between unconjugated 6E4 (dilution from 1/50 to $1/10^9$) and HRP-conjugated 3F10, 6E4, 6D11, 3H12 and PK2 (constant dilution).



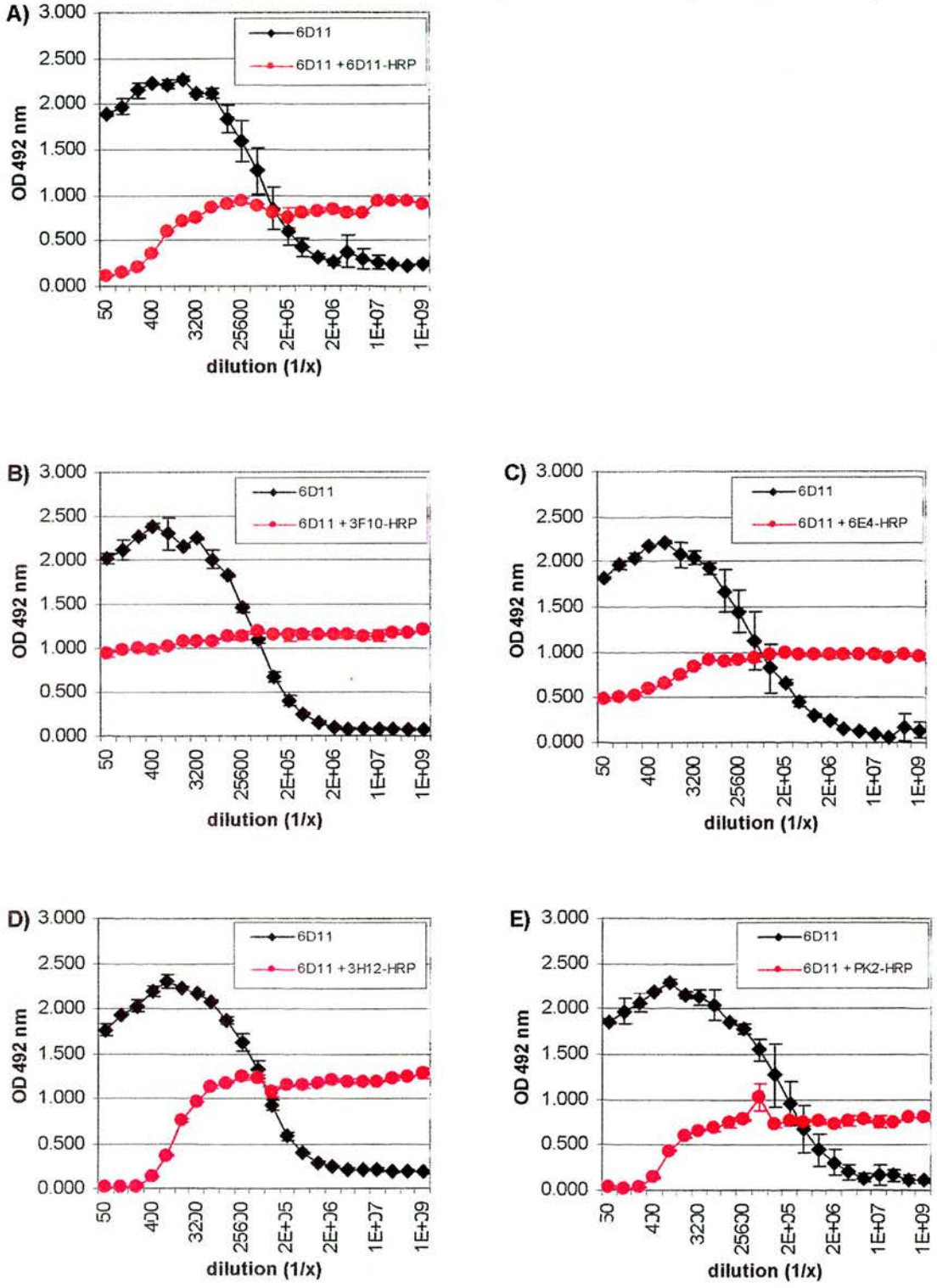
-◆-: incubation of 6E4 followed by incubation of HRP-conjugated anti-mouse secondary antibody.
-●-: incubation of 6E4 followed by incubation of HRP-conjugated mAb.

The same procedure as before was followed with unconjugated 6D11; the results with conjugated 6D11-HRP are shown on Fig. 15. A. No drop with 3F10-HRP (Fig. 15. B) and a less marked drop with 6E4-HRP (Fig. 15. C) was observed when put in competition against unconjugated 6D11, suggesting 3F10 and 6E4 recognise a different epitope on CPS than the one recognised by 6D11. The curves from the competition ELISA of 3H12-HRP (Fig. 15. D) and PK2-HRP (Fig. 15. E) against unconjugated 6D11 showed a clear drop indicating these three mAbs might recognise the same epitope on CPS.

The curve of 3H12 in competition against itself, 3H12-HRP is shown on Fig. 16. A. This curve was compared with the ones showing the competition of 3H12 against 3F10-HRP (Fig. 16. B), 6E4-HRP (Fig. 16. C), 6D11-HRP (Fig. 16. D) and PK2-HRP (Fig. 16. E). None of the 4 mAbs showed a drop, suggesting these 4 monoclonals do not bind to the same epitope as 3H12.

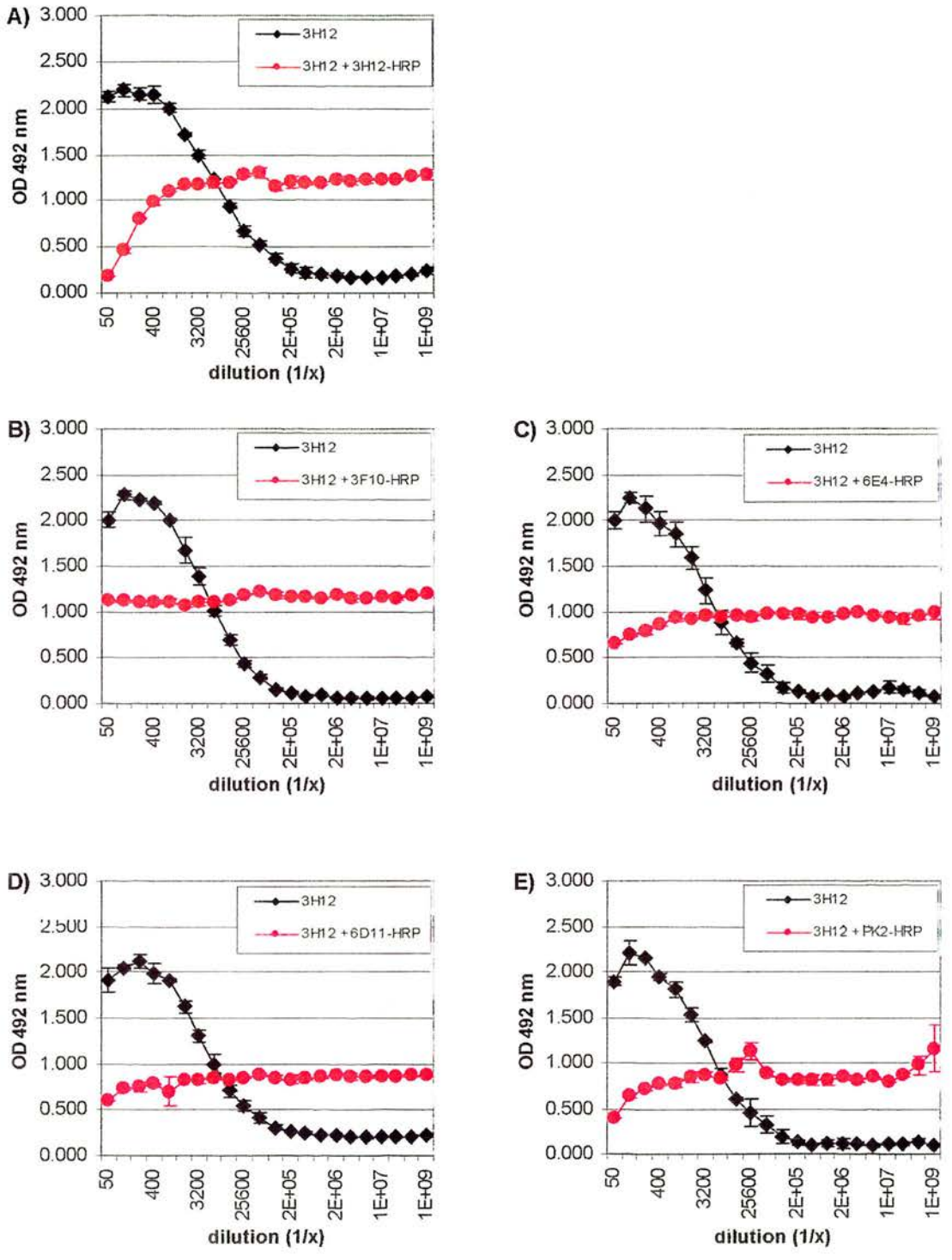
MAb PK2 was put in competition against itself, conjugated to HRP (Fig. 17. A). In contrast to the results observed with the other monoclonals in competition against themselves, the curve obtained did not show a marked drop. As a consequence, the results obtained from the competition of PK2 against the other conjugated mAbs were dubious. No drop could be seen on the graph concerning the competition of PK2 against 3F10-HRP (Fig. 17. B), 6E4-HRP (Fig. 17. C), 6D11-HRP (Fig. 17. D) and 3H12-HRP (Fig. 17. E), suggesting they bound to a different epitope on *Mmm*SC CPS.

Fig. 15. Competitive ELISA between unconjugated 6D11 (dilution from 1/50 to $1/10^9$) and HRP-conjugated 3F10, 6E4, 6D11, 3H12 and PK2 (constant dilution).



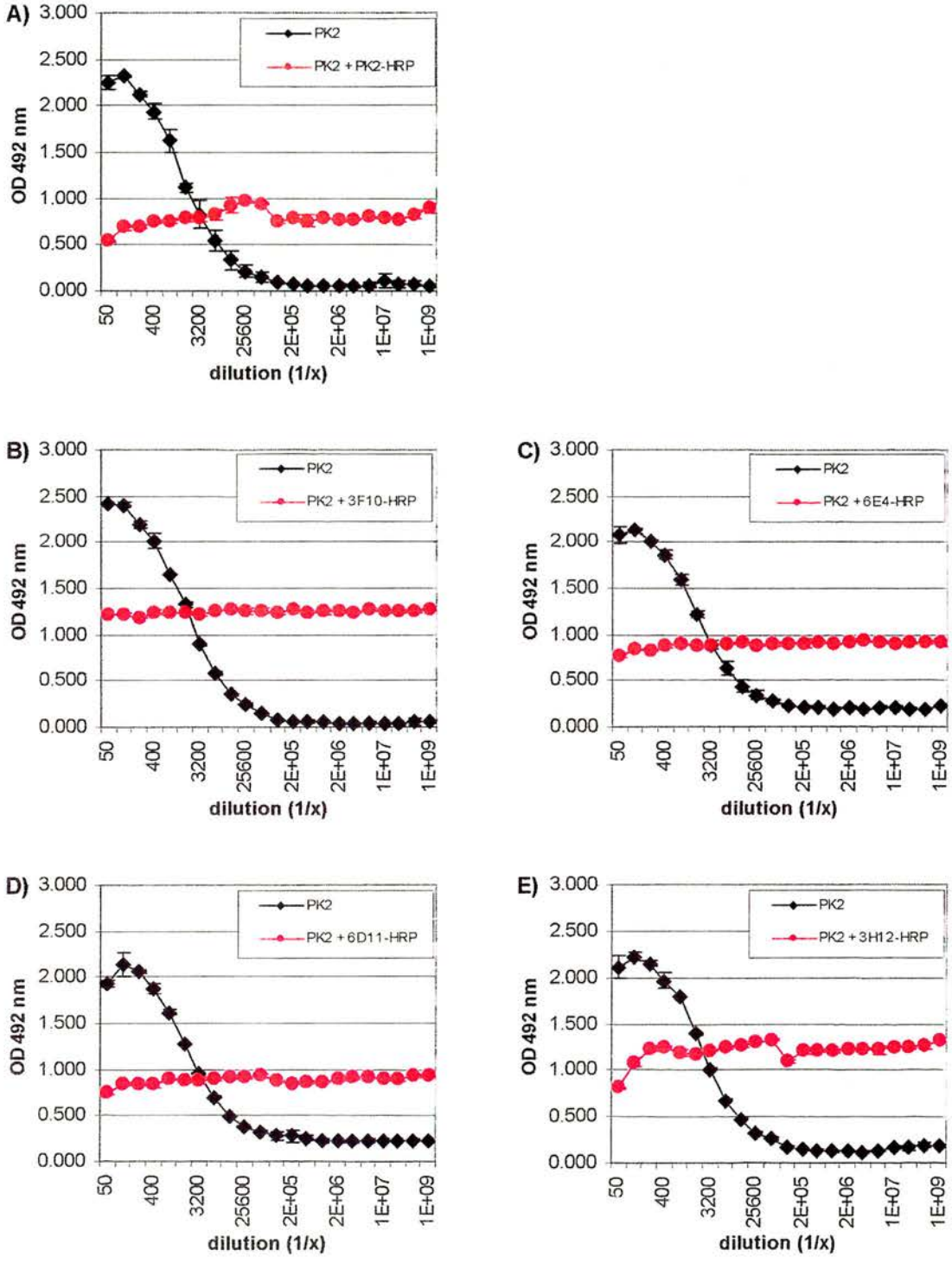
-◆-: incubation of 6D11 followed by incubation of HRP-conjugated anti-mouse secondary antibody.
-●-: incubation of 6D11 followed by incubation of HRP-conjugated mAb.

Fig. 16. Competitive ELISA between unconjugated 3H12 (dilution from 1/50 to $1/10^9$) and HRP-conjugated 3F10, 6E4, 6D11, 3H12 and PK2 (constant dilution).



-◆-: incubation of 3H12 followed by incubation of HRP-conjugated anti-mouse secondary antibody.
-●-: incubation of 3H12 followed by incubation of HRP-conjugated mAb.

Fig. 17. Competitive ELISA between unconjugated PK2 (dilution from 1/50 to $1/10^9$) and HRP-conjugated 3F10, 6E4, 6D11, 3H12 and PK2 (constant dilution).



-♦-: incubation of PK2 followed by incubation of HRP-conjugated anti-mouse secondary antibody.
-●-: incubation of PK2 followed by incubation of HRP-conjugated mAb.

The competition ELISA data are summarised in Table 9. Each mAb was tested in competition against the other monoclonals, for example, unconjugated 3F10 against 6E4-HRP, then unconjugated 6E4 against 3F10-HRP. In the reciprocal experiment, identical competition results should occur in each experiment, i.e. either these mAbs recognise the same epitope on CPS and compete against each other in both experiments or they bind to different epitopes and should not compete against each other. As it is shown in Table 9, this was not observed.

For example, unconjugated 3F10 competing against 6E4-HRP (Fig. 13. B) suggested these 2 mAbs bind to the same epitope on CPS (as indicated by a "+" in the table).However, unconjugated 6E4 did not compete ("-" in the table) against 3F10-HRP (Fig. 14. B) suggesting the 2 mAbs bind to a different epitope. Such contradictory results were not only obtained from the pair 3F10-6E4 but for most of the pairs of mAbs competing against each other (see Table 9). Similar results were obtained with the pairs 6E4-6D11, 6E4-PK2 and 3H12-PK2 where it seemed that they recognised a different epitope on *Mmm*SC CPS.

Table 9. Competitive ELISA between monoclonal antibodies.

	3F10-HRP	6E4-HRP	6D11-HRP	3H12-HRP	PK2-HRP
3F10		+	+	+	+
6E4	-		-	+	-
6D11	-	-		+	+
3H12	-	-	-		-
PK2	-	-	-	-	

"+": identical epitope recognised by the 2 mAbs. "-": different epitope recognised by the 2 mAbs.

The results obtained from competitive ELISA performed to explore the epitopes recognised by these 5 mAbs were quite confusing. The experiment with unconjugated PK2 did not seem to work. It was not possible to state with certainty which monoclonals recognise the same structure on *Mmm*SC CPS except maybe for

6E4, 6D11, 3H12 and PK2 that seemed to recognise different epitope on *Mmm*SC CPS. As shown in Table 9, the results for the other mAbs were different according to the order of incubation of the monoclonal antibodies, which could be explained by overlapping epitopes or steric hindrance. The latter seems likely since IgM are huge molecules associated together to form a pentamer. A different affinity of two mAbs for the same CPS epitope might also explain these results. If one of these two antibodies has a higher affinity for the epitope, when it is incubated in second position (i.e. conjugated to HRP), it could replace the antibody incubated in the first step and consequently, it will look like these two antibodies recognise a different epitope.

3.5. Passive immunisation.

With other microorganisms, it has been shown that CPS can give protection against diseases caused by these organisms when administered as a vaccine [137]. Our hypothesis was that if animals could elicit antibodies against mycoplasmal CPS, they would be protected against disease caused by that organism. No successful mycoplasma vaccines based upon purified CPS have yet been described. Mice were used as an animal model for CBPP. They develop bacteraemia (without any signs of disease) when inoculated intraperitoneally with *Mmm*SC and a correlation was seen between the virulence of *Mmm*SC strains observed in mice and that observed in cattle, based on the duration of mycoplasmaemia in mice, the most virulent *Mmm*SC strains producing a longest mycoplasmaemia [111]. To explore if anti-CPS antibodies are protective against the disease, 10 mice (group I) were passively immunised with purified mAb 3F10, which recognises *Mmm*SC CPS, and 10 other mice (group II)

were given PBS as negative control. Four mice (group III) did not receive any mAb or PBS as a control for the bacteraemia. All mice were then challenged with *Mmm*SC strain N6 to see if protection occurred in the group given anti-CPS mAb 3F10. This strain was chosen because it has been shown it produces a high degree of mycoplasmaemia in mice [154]. Mycoplasmaemia was monitored by blood culture in Gourlay's medium diluted at 10^0 and 10^{-1} (Table 10). The blood culture was diluted at 10^{-1} because sometimes a slight colour change in the medium can be seen following the addition of blood with no mycoplasma present. Mice were all free of mycoplasmas before the beginning of the assay as all the pre-bleeds were negative. Mice were bled 1 (D1), 2 (D2), 3 (D3), 4 (D4) and 7 days (D7) after infection.

Table 10. Mycoplasmaemia in mice after passive immunisation.

	Number of mice showing mycoplasmaemia												**GS**
	D0		**D1**		**D2**		**D3**		**D4**		**D7**		
	10^0	10^{-1}	10^0	10^{-1}	10^0	10^{-1}	10^0	10^{-1}	10^0	10^{-1}	10^0	10^{-1}	
I	0	0	10	4	8	2	4	0	0	0	0	0	10
II	0	0	10	6	10	1	4	0	1	0	0	0	10
III	0	0	4	3	4	0	0	0	0	0	0	0	4

(I) Mice immunised with 3F10, anti-CPS antibody, and challenged with *Mmm*SC strain N6. (II) Mice immunised with PBS only and challenged with *Mmm*SC strain N6. (III) Mice challenged with *Mmm*SC strain N6 only. GS: group size, number of mice in each group. D0, D1, D2, D3, D4 and D7: days of bleeding, 10^0 and 10^{-1}: mouse blood culture dilution.

No statistical difference could be seen in the duration and the extent of mycoplasmaemia between mice having received PBS and mice having received mAb 3F10. Therefore, no protection against *Mmm*SC using anti-CPS antibody was then observed using this assay using this strain. These findings suggest that a bactericidal antibody against *Mmm*SC does not protect against the disease *in vivo* in a mouse. In other studies [155], it has been observed that mice vaccinated with CPS conjugated to ovalbumin, although exhibiting a higher immune antibody response to CPS than mice vaccinated with CPS only, were not protected against infection with *Mmm*SC.

Mice immunised with sonicated mycoplasma, although showing a low anti-CPS immune response, were protected against a challenge with live mycoplasma and did not exhibit an inhibiting activity in their sera, suggesting the involvement of cell-mediated immune response against *Mmm*SC in mice.

Another explanation could be that the amount of antibody injected, was not high enough to induce protection against mycoplasma in mice.

3.6. CPS immune response in cattle.

Current vaccines against CBPP are not very efficient and induce a short lasting immunity. This lack of immunity could be due to a poor immune response against CPS in vaccinated animals. Our hypothesis was that animals showing an immune response against CPS would be protected against CBPP as it has been shown in other microorganisms where vaccination with CPS protected against the disease. First, we looked at the IgM immune response in a group of vaccinated animals to see how many of them were immunologically positive against CPS. Then, we determined the isotype (i.e. IgM, IgG and IgA) of the anti-CPS immune response in three groups of cattle: vaccinated, intubated and unvaccinated in-contact animals. IgM immune response was determined as, in a typical humoral immune response, these are the major antigen-specific immunoglobulins to be produced. IgG is the main antibody in a secondary immune response and IgA is present predominantly in mucosal secretions [156]. CPS is a thymus-independent antigen, meaning it does not produce readily an immunological memory [140]. However, the degradation of mycoplasmas in the lung could release pieces of membrane containing CPS and proteins that could act like CPS conjugate which would therefore becomes a thymus-

dependent antigen that could lead to affinity maturation of B cells and T cells, isotype switching and then production of immunoglobulins other than IgM.

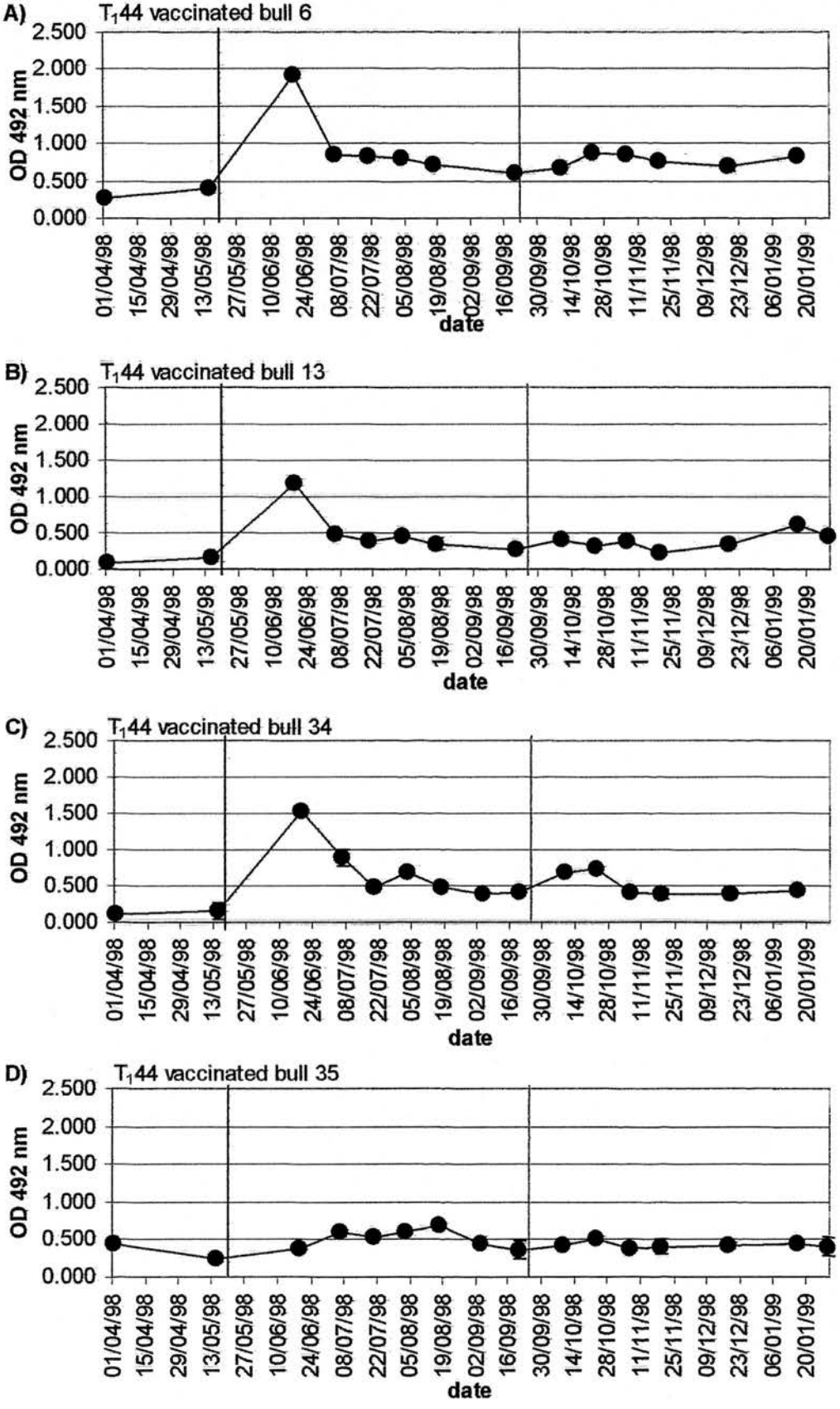
Bovine sera to be tested were from a trial performed in Kenya. The trial consisted of 120 male local breeds of Kenyan cattle aged 3 or more selected at random. All the animals were bled twice at the start of the trial. Two groups of 20 animals were vaccinated one month after the first pre-bleed (date of vaccination: 15-20/05/1998). Reconstituted freeze-dried $T_1$44 and T_1SR vaccines were injected subcutaneously. On the 21/09/1998, 40 animals were intubated with a virulent *Mmm*SC strain from Kenya. One week later (28/09/1998), the intubated animals were put in contact with 40 unvaccinated cattle and the 40 vaccinated ones. Animals were regularly bled 16 times from the beginning of the trial to the end (from 01/04/1998 to 29/01/1999).

3.6.1. CPS IgM immune response in vaccinated cattle.

CPS-specific IgM immune response in cattle vaccinated with $T_1$44 using 19 sera was assessed (Fig. 18). Half of the bulls exhibited a relatively high signal (OD around 0.5) against CPS before vaccination (Fig. 18. D, E, I, J, K, L, M, O and S). After vaccination, only 5 bulls showed a significant IgM immune response against CPS (Fig. 18. A, B, C, F and G). After contact with intubated animals, bulls 67 (Fig. 18. F) and 114 (Fig. 18. H) exhibited an IgM immune response against CPS. Bull 67 (Fig. 18. F) was the only animal showing an immune response following both vaccination and after being in contact with intubated animals. However, the IgM immune response was short since the level of IgM went back to normal 1 month

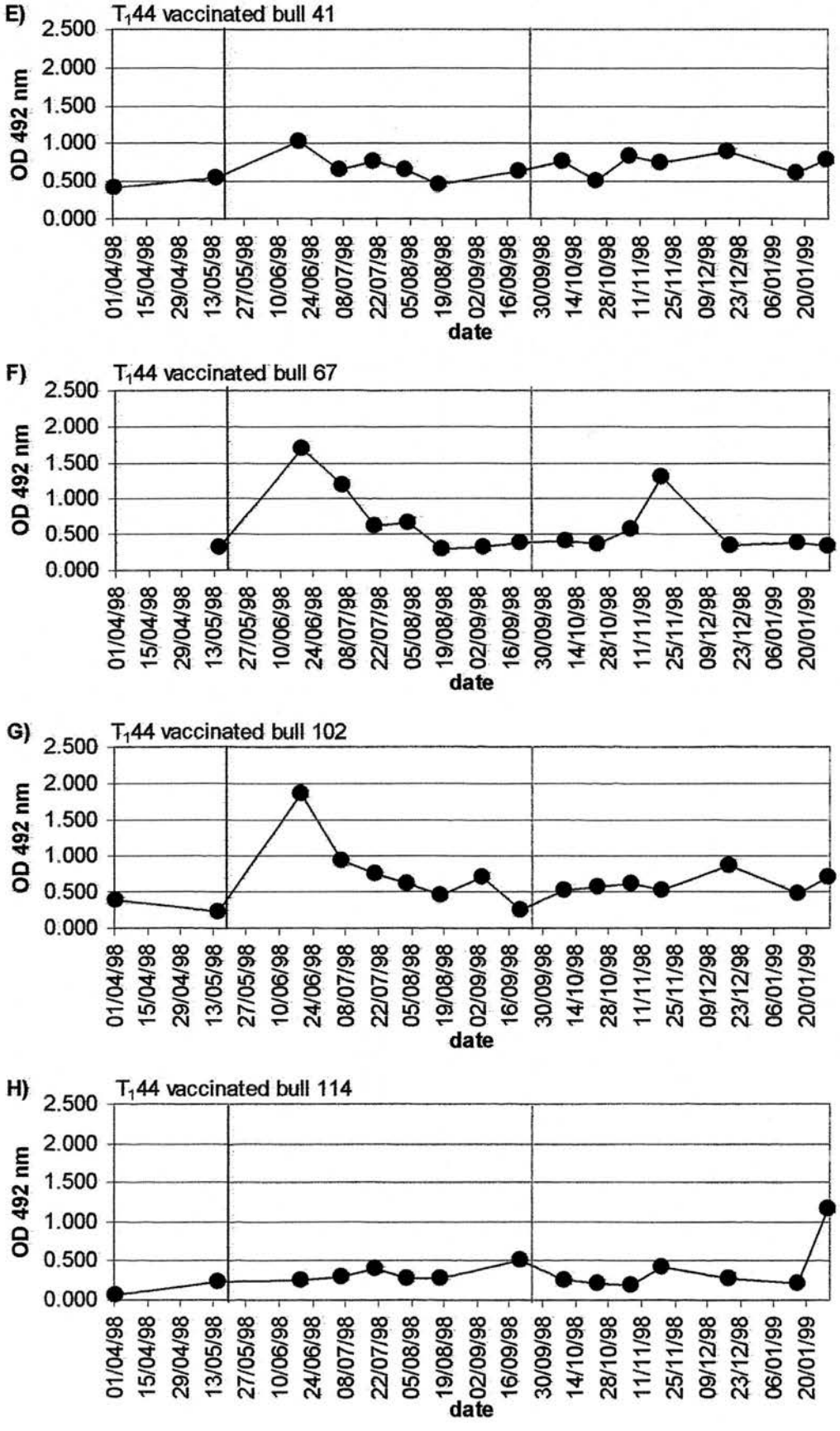
after the peak of the vaccination and two weeks after the peak following the contact with *Mmm*SC.

Fig. 18. IgM immune response against *Mmm*SC CPS in $T_1$44 vaccinated cattle.



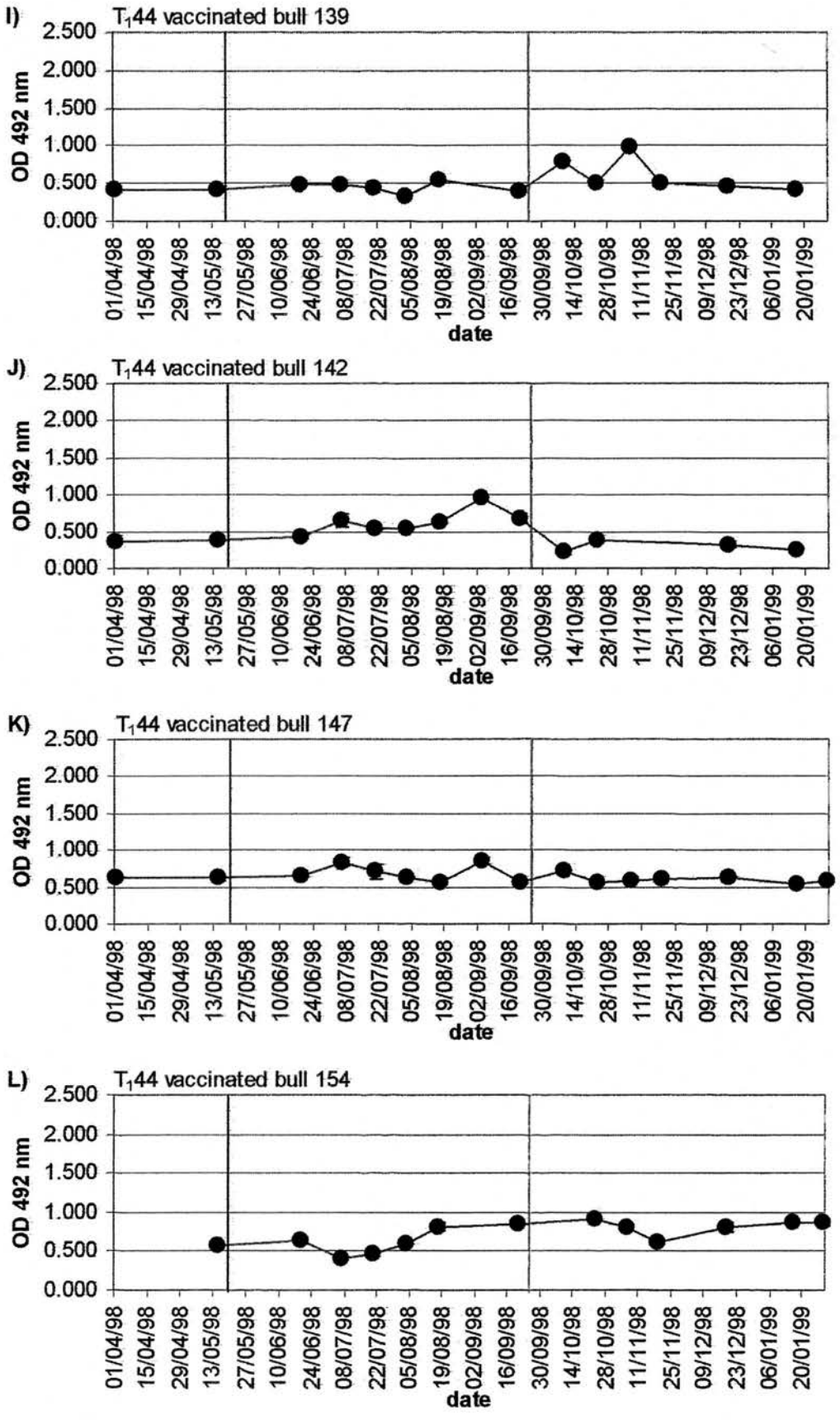
—— date of vaccination. —— date of contact with *Mmm*SC-intubated animals

Fig. 18 (continued). IgM immune response against *Mmm*SC CPS in $T_1$44 vaccinated cattle.



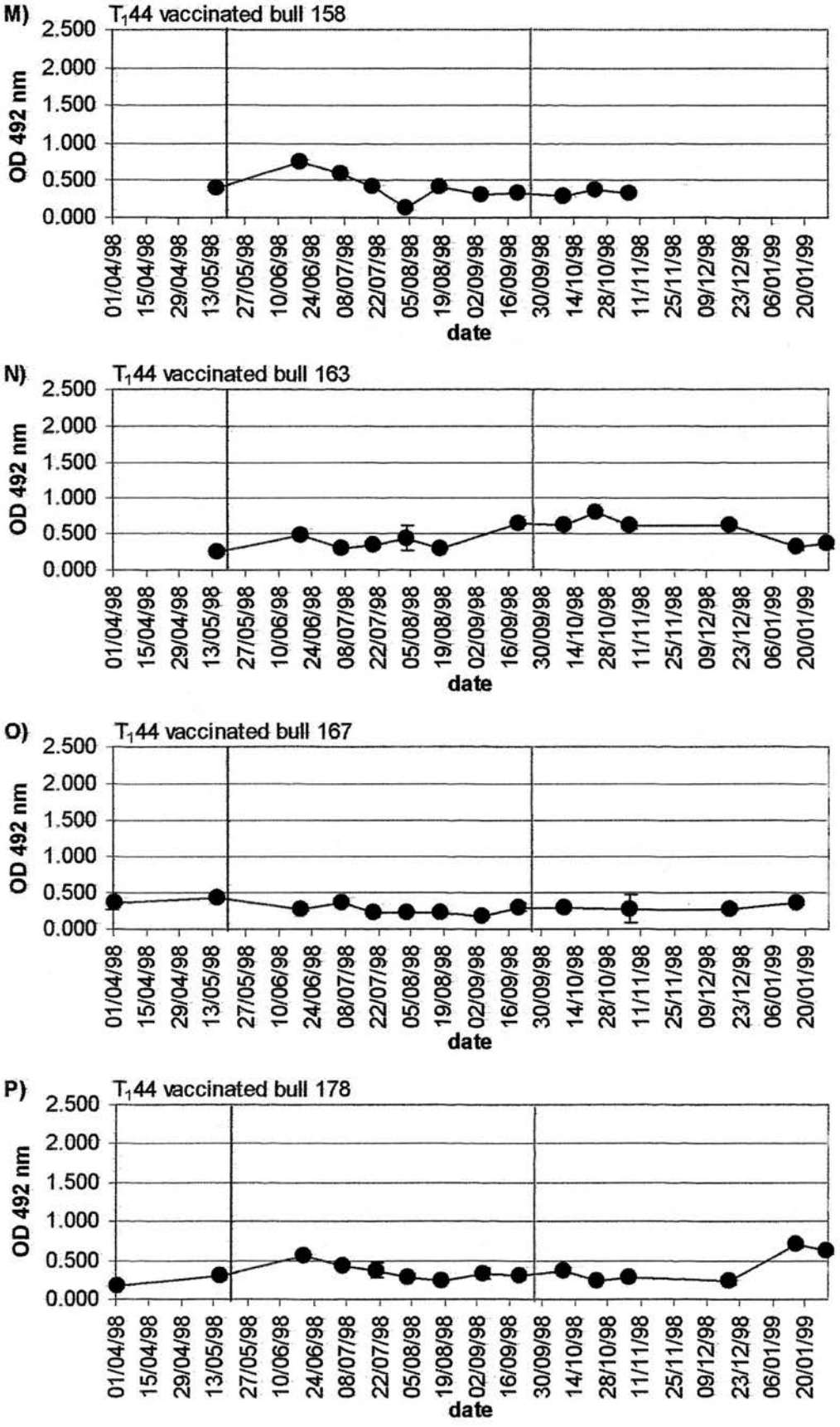
—— date of vaccination. —— date of contact with *Mmm*SC-intubated animals

Fig. 18 (continued). IgM immune response against *Mmm*SC CPS in T_144 vaccinated cattle.



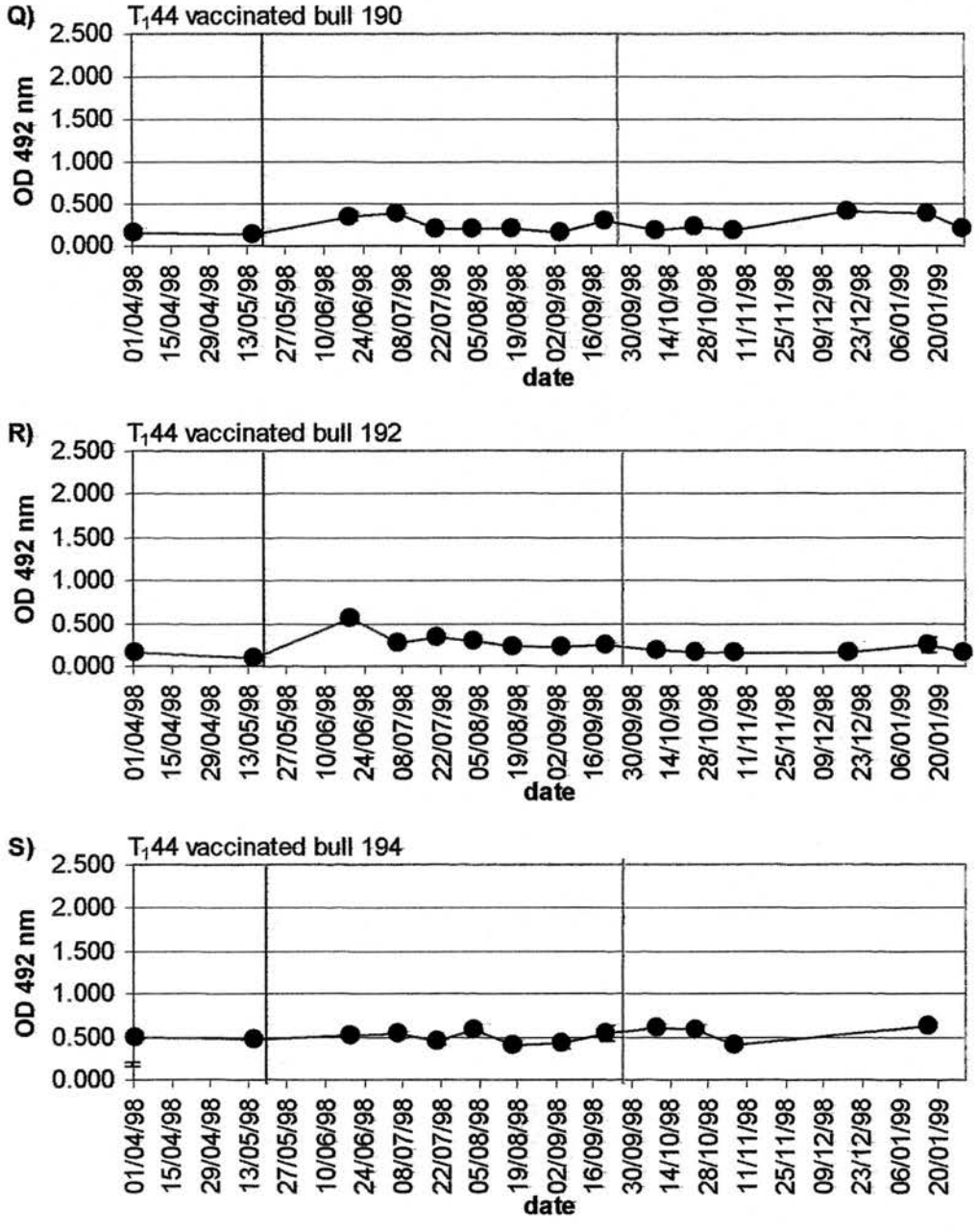
—— date of vaccination. —— date of contact with *Mmm*SC-intubated animals

Fig. 18 (continued). IgM immune response against *Mmm*SC CPS in $T_1$44 vaccinated cattle.



— date of vaccination. — date of contact with *Mmm*SC-intubated animals

Fig. 18 (concluded). IgM immune response against *Mmm*SC CPS in $T_1$44 vaccinated cattle.



—— date of vaccination. —— date of contact with *Mmm*SC-intubated animals

3.6.2. CPS IgM, IgG and IgA immune response in *Mmm*SC intubated cattle.

The immune response against CPS in 5 intubated animals was measured. The sera were from bulls chosen at random. Four out of 5 (Fig. 19. B, C, D and E) exhibited an IgM immune response but none of them showed any IgG or IgA CPS-specific sera.

Fig. 19. IgM, IgG and IgA immune response against *Mmm*SC CPS in *Mmm*SC-intubated cattle.

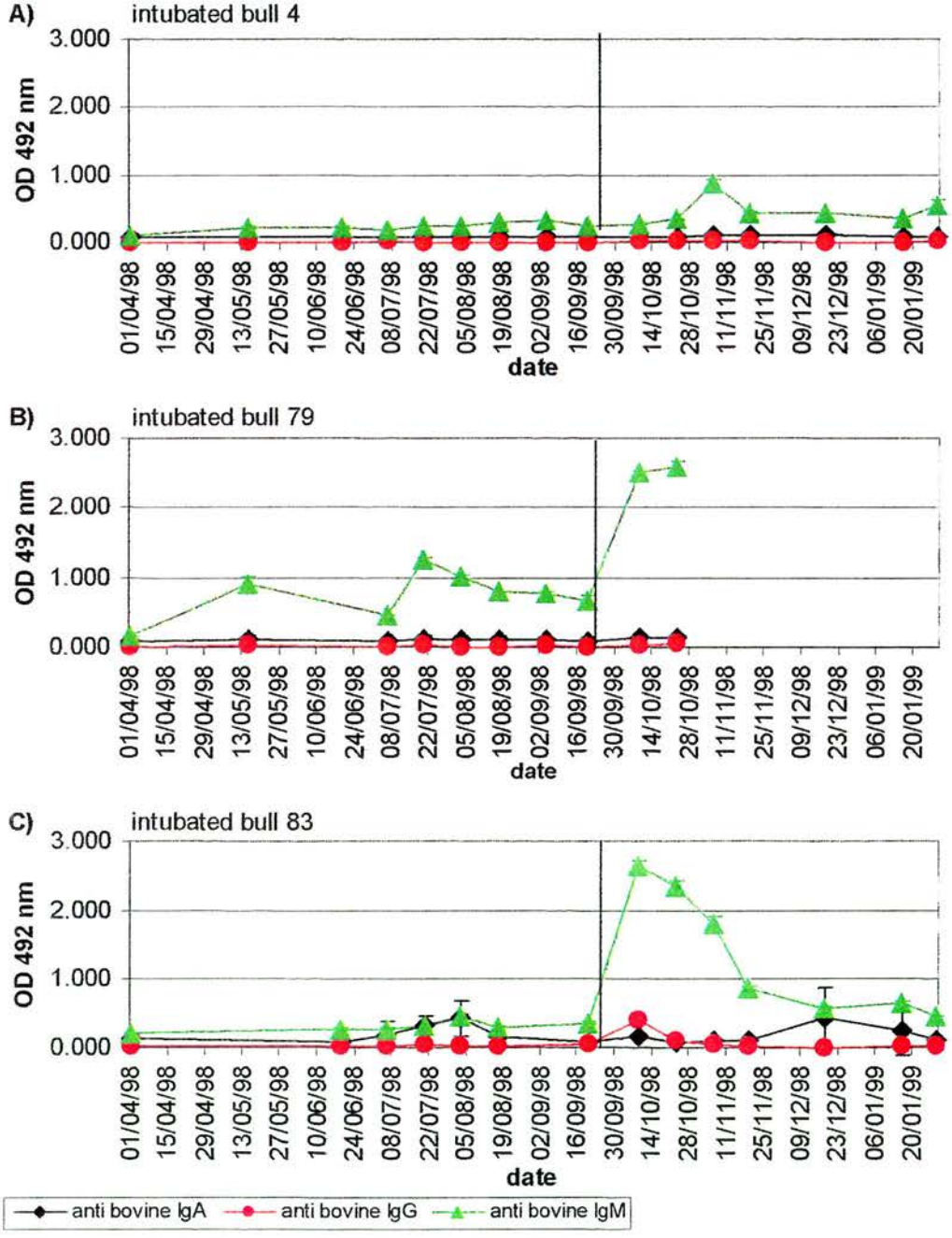
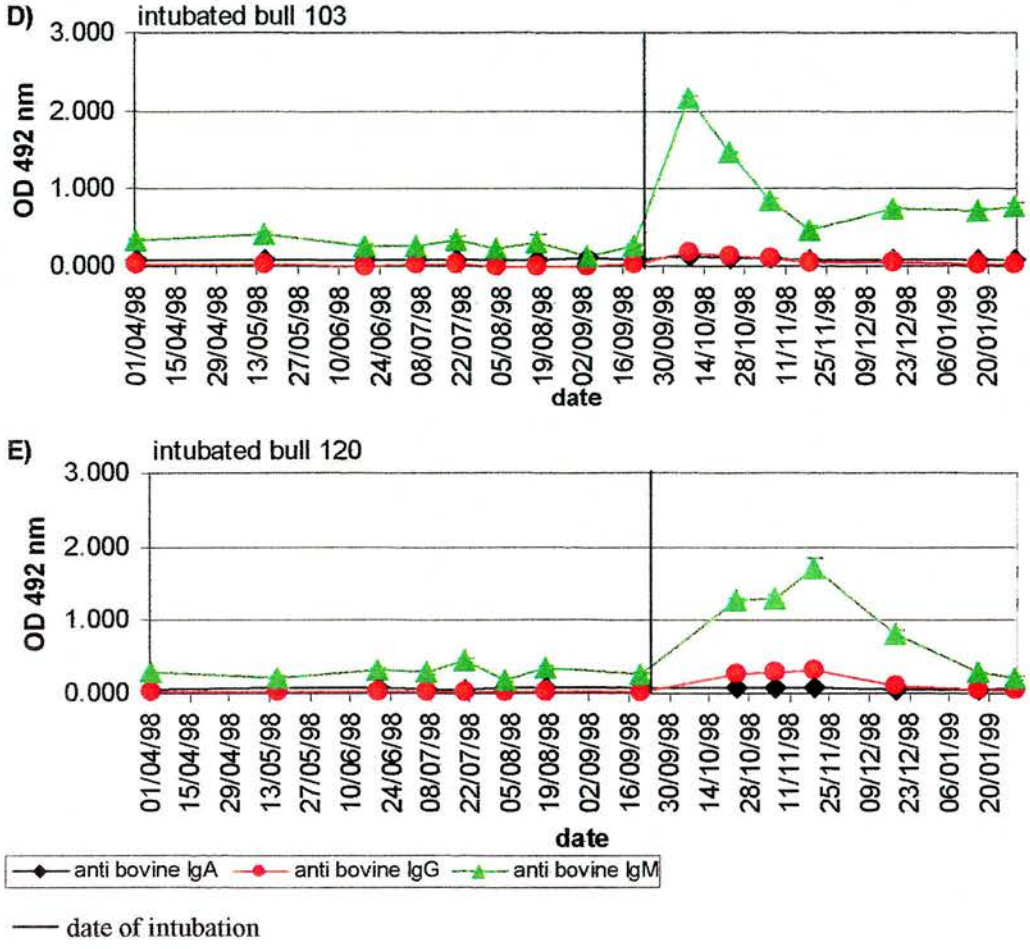


Fig. 19 (concluded). IgM, IgG and IgA immune response against *Mmm*SC CPS in *Mmm*SC-intubated cattle.



3.6.3. CPS IgM, IgG and IgA immune response in unvaccinated in-contact cattle with *Mmm*SC-intubated animals.

The sera were from bulls chosen randomly. The immunoglobulin isotype response against CPS in 5 unvaccinated bulls placed in contact with intubated animals was explored (Fig. 20). Bulls 3 (Fig. 20. A), 61 (Fig. 20. D) and 84 (Fig. 20. E) had a relatively high signal for the IgM immune response against CPS before being put in contact with intubated animals. Two bulls (Fig. 20. A and E) exhibited an IgM immune response against CPS after being in contact with intubated

cattle. None of the animals, except bull 3 (Fig. 20. A), exhibited IgG specific for CPS in their sera following challenge. No IgA specific for CPS were detected in any of the in-contact bull sera following challenge.

Fig. 20. IgM, IgG and IgA immune response against *Mmm*SC CPS in unvaccinated cattle in contact with *Mmm*SC-intubated animals.

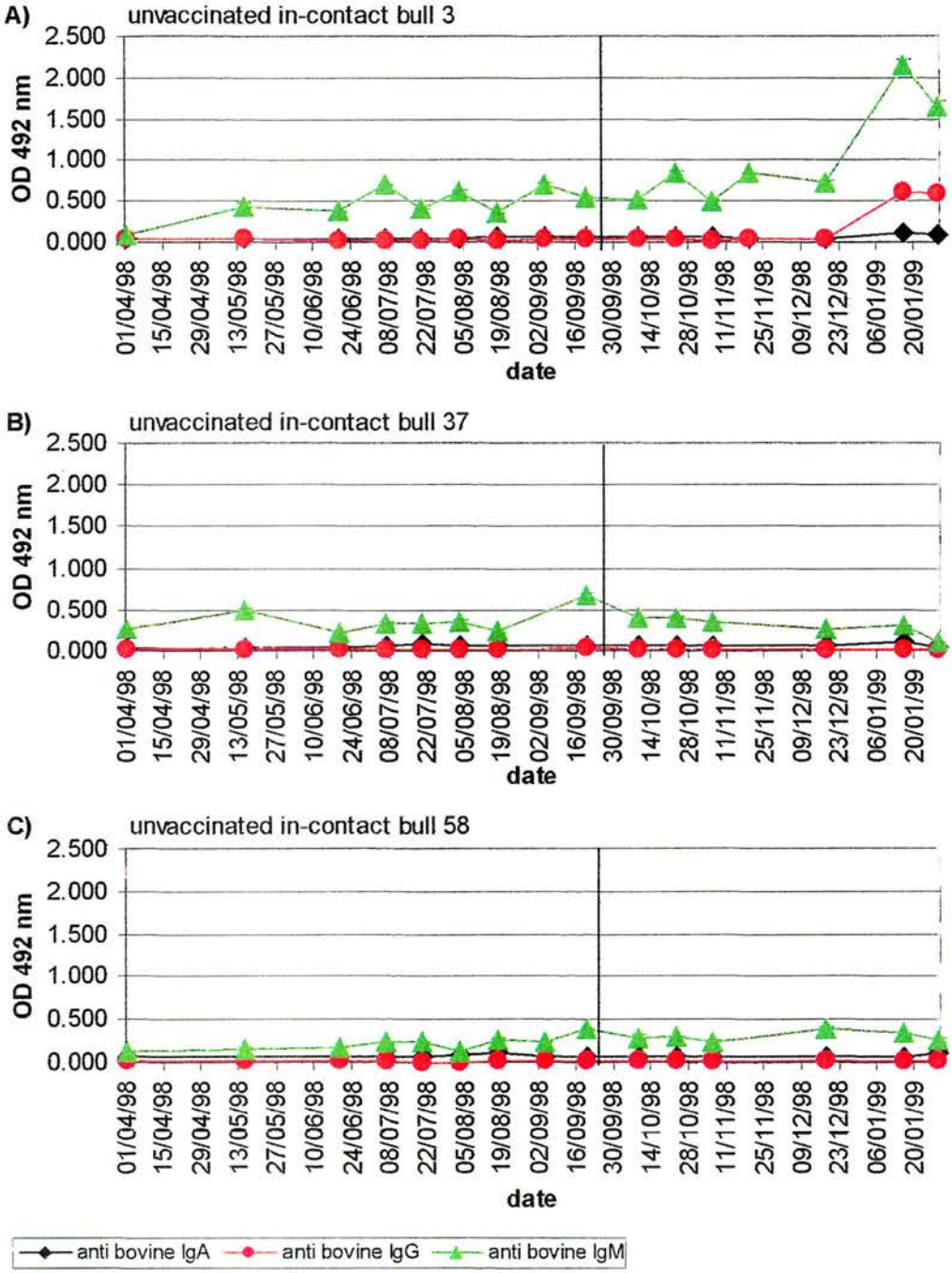
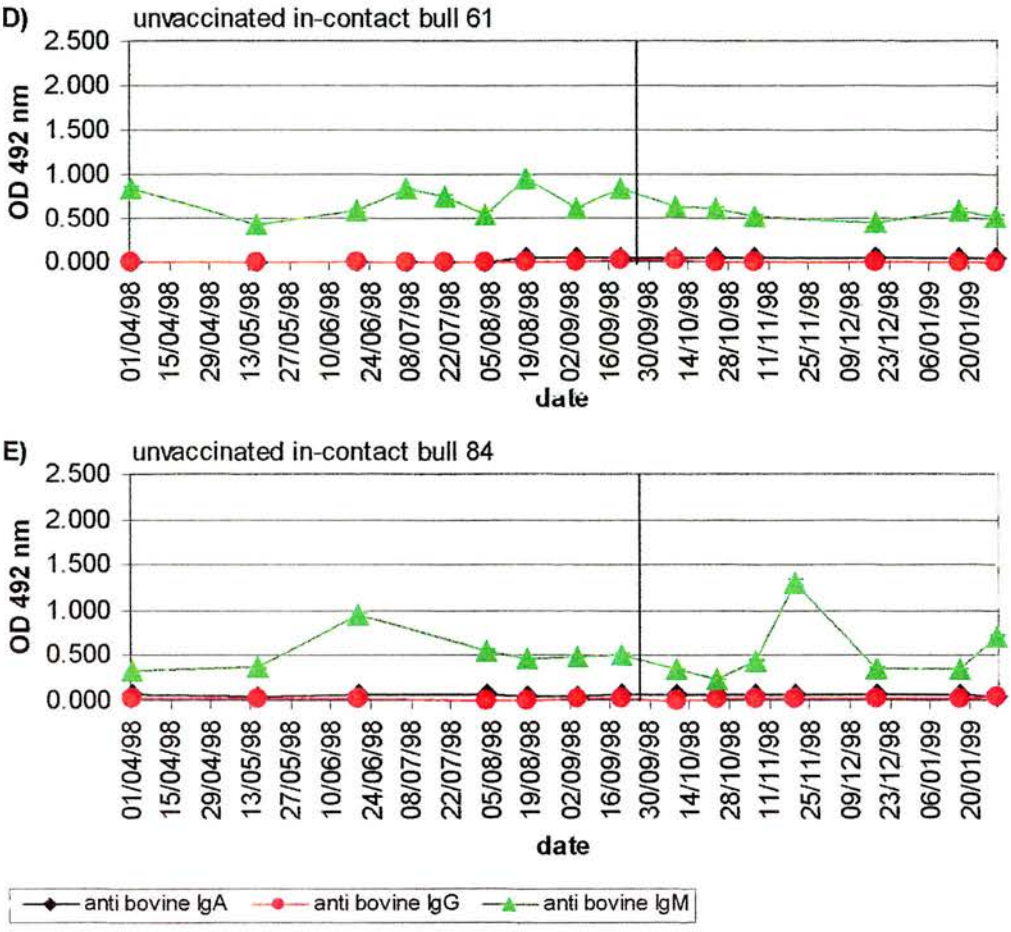


Fig. 20 (concluded). IgM, IgG and IgA immune response against *Mmm*SC CPS in unvaccinated cattle in contact with *Mmm*SC-intubated animals.



3.6.4. CPS IgM, IgA and IgG immune response in vaccinated cattle.

The immune response against CPS was investigated in 5 $T_1$44 vaccinated animals placed in contact with intubated animals (Fig. 21). The sera were chosen according to previous results (section 3.6.1.). Four of them showed an IgM-specific immune response for CPS after vaccination (Fig. 21. A, B, C and D). Among the vaccinated bulls, two exhibited an IgM immune response after being in contact with

intubated bulls (Fig. 21. C and E), one of them did not respond to vaccination (Fig. 21. E).

Fig. 21. IgM, IgG and IgA immune response against *Mmm*SC CPS in $T_1$44 vaccinated cattle in contact with *Mmm*SC-intubated animals.

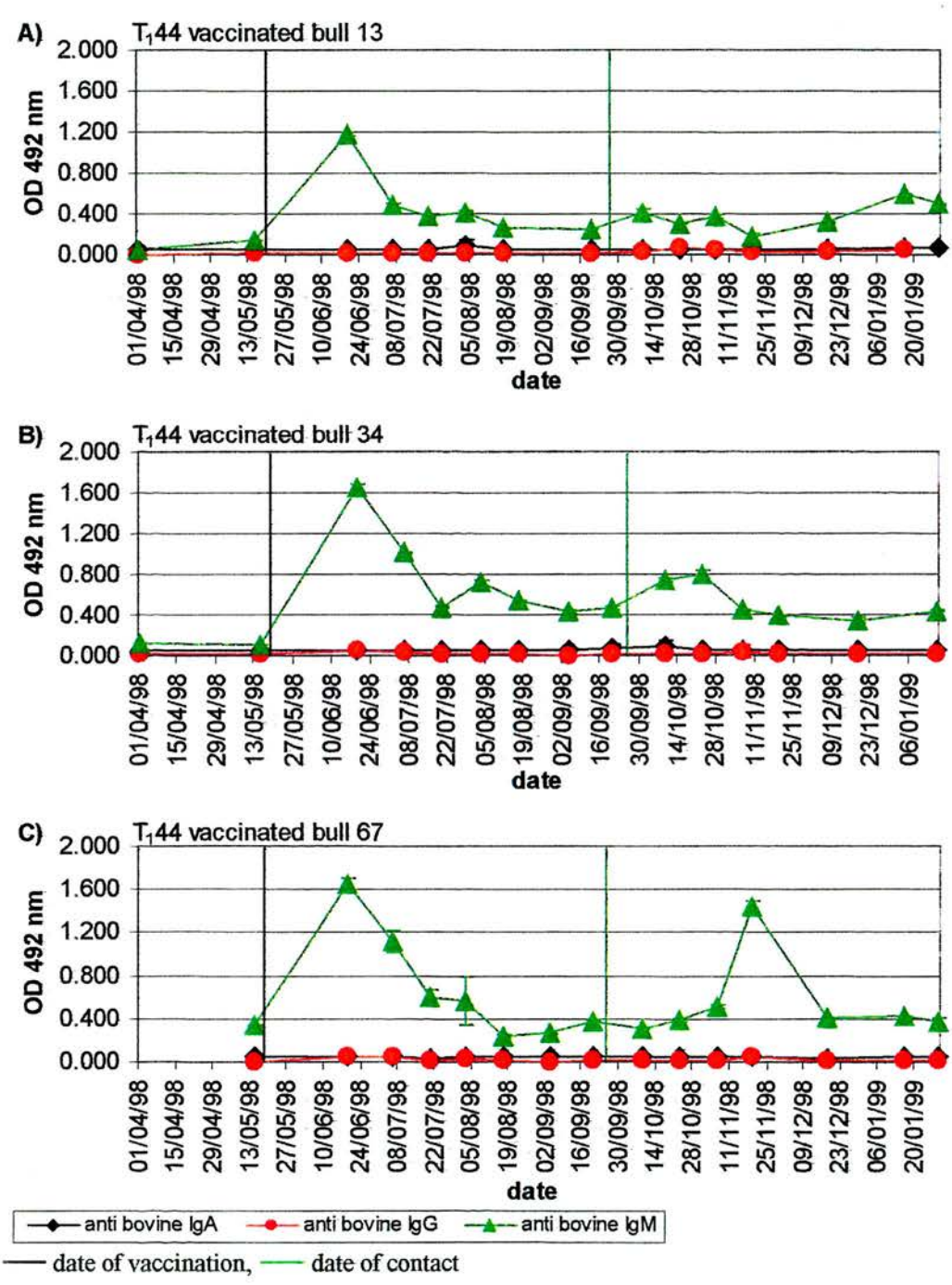
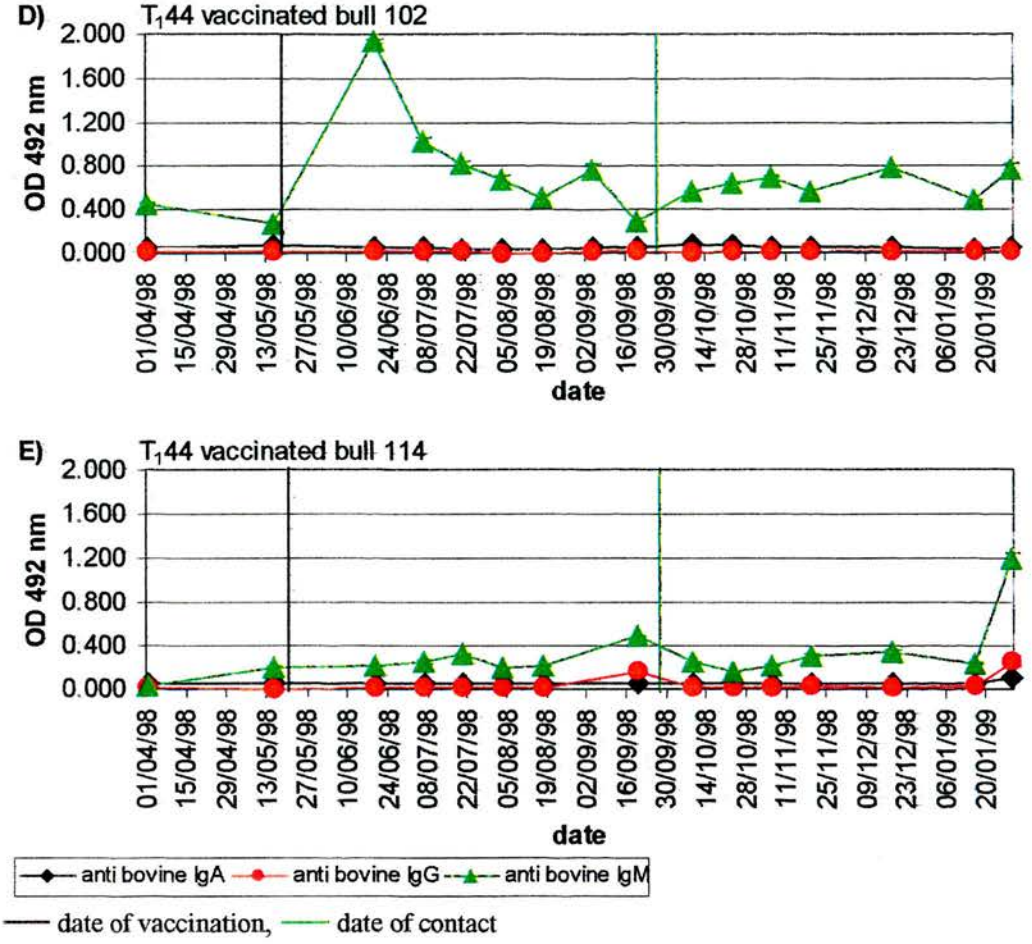


Fig. 21 (concluded). IgM, IgG and IgA immune response against *Mmm*SC CPS in T_144 vaccinated cattle in contact with *Mmm*SC-intubated animals.



3.6.5. Conclusions.

The class of immunoglobulin responses against CPS were investigated in three different groups of cattle, *Mmm*SC intubated bulls, unvaccinated bulls and T_144 vaccinated bulls both placed in contact with *Mmm*SC intubated animals, were investigated. Firstly, in the group of vaccinated cattle, only 5 out of 19 (26%) had an IgM immune response, in agreement with the fact that the current vaccine is not very efficient in the protection against CBPP, if it is assumed the protection is conferred by anti-CPS antibodies.

The immune response from the bulls in the three groups was only IgM type. No IgG could be detected against CPS except for bull 3 in the group of unvaccinated cattle in contact with intubated animals. This could be explained because of the nature of CPS, since the immune response against polysaccharides is predominantly of IgM type. As the vaccinated bulls were in contact a second time with *Mmm*SC, it was expected some of them would exhibit CPS specific IgG in their sera. Even though CPS is a polysaccharide, it could have induced the production of IgG since hyperimmunised rabbit with *Mmm*SC produced IgG specific for CPS. According to the serological response against CPS, it appears that three different categories exist: no response to CPS, response to CPS only after vaccination and response to CPS both after vaccination and after contact with *Mmm*SC intubated animals, though very rare. It would have been interesting to compare the clinical and post-mortem data from these bulls with the serological data: (i) to see if the animals showing immune response to CPS were those exhibiting signs of disease, (ii) to compare the severity of the lung lesions with the immune response to CPS, (iii) to examine if animals vaccinated against *Mmm*SC and showing CPS immune response to CPS were protected against the disease.

3.7. Cross-reactions between CPS and normal bovine lung.

Cross-reactions have been reported between bovine lung and *Mmm*SC CPS [102,105]. Previously, it was observed that in cattle vaccinated with *Mmm*SC, only a small proportion showed an immune response to CPS (see section 3.6.). Unresponsiveness to CPS might be a consequence of this cross-reaction, as the

lymphocytes recognising both the lung and CPS would be in a state of anergy or deleted and could not be stimulated and consequently produce antibodies.

It was interesting to confirm the cross-reactions between bovine lung and CPS using mAbs by western-blot (WB) as mAbs specific for CPS are more specific than the polyclonal sera that were raised against the whole microorganism. Also WBs are more accurate than agar-gel precipitin test (AGPT). Polyclonal sera and AGPT were used in the earlier experiments showing cross-reaction between CPS and bovine lung. It was also of the interest to examine if the different CPS epitopes are also present on bovine lung. If one of them is different, it could be studied for its protective activity and to be used as a vaccine. Of interest was whether antibodies recognising both CPS and bovine lung would be present in *Mmm*SC-infected cow sera; if these sera recognise both CPS and lung, this could lead to immunopathology in the lung. If only antibodies against CPS are present, these cattle might be protected against the disease.

3.7.1. Western-blot with CPS and normal bovine lung using anti-*Mmm*SC cow and rabbit sera and anti-CPS mAbs.

Different sera were tested to see if they recognised both bovine lung homogenate (consisting of disrupted normal bovine lung tissue in buffer, boiled in loading buffer before being loaded in the gel) and bovine lung galactan and *Mmm*SC CPS. Sera were from *Mmm*SC naturally-infected cows, *Mmm*SC-vaccinated cows, *Mmm*SC-hyper-immunised rabbits and CPS-vaccinated mice (CPS was conjugated to ovalbumin). Five mAbs that recognise CPS were also used: 3F10, 6E4, 6D11, 3H12 and 2A3 (Fig. 22) (see section 3.4.). WB with CPS was used as a positive control

(Fig. 22. A) and the signal was compared to that seen against bovine lung homogenate (Fig. 22. B) and bovine lung galactan (Fig. 22. C). None of the HRP-conjugated secondary antibodies reacted with CPS. Pre-immune cow serum gave a positive signal with CPS (although this was low) whereas pre-immune rabbit and mouse sera did not. The sera from *Mmm*SC-infected and -vaccinated cows, *Mmm*SC-immunised rabbits and CPS conjugate vaccinated mice showed a signal with CPS. As expected, mAbs strongly recognised CPS. When the sera from cows, rabbits and mice, and monoclonal antibodies were tested with bovine lung homogenate (Fig. 22. B) and bovine lung galactan (Fig. 22. C), the similar results were observed as those with CPS, the only difference came from HRP-conjugated anti-cow immunoglobulin that cross-reacted with bovine lung homogenate and bovine lung galactan. As a consequence, it was not possible to determine if the bovine sera gave a signal or not (different secondary antibodies against bovine immunoglobulins were tested unsuccessfully even after pre-absorption with bovine lung homogenate).

3.7.2. Western-blot with CPS and normal bovine lung using mouse sera raised against bovine lung homogenate.

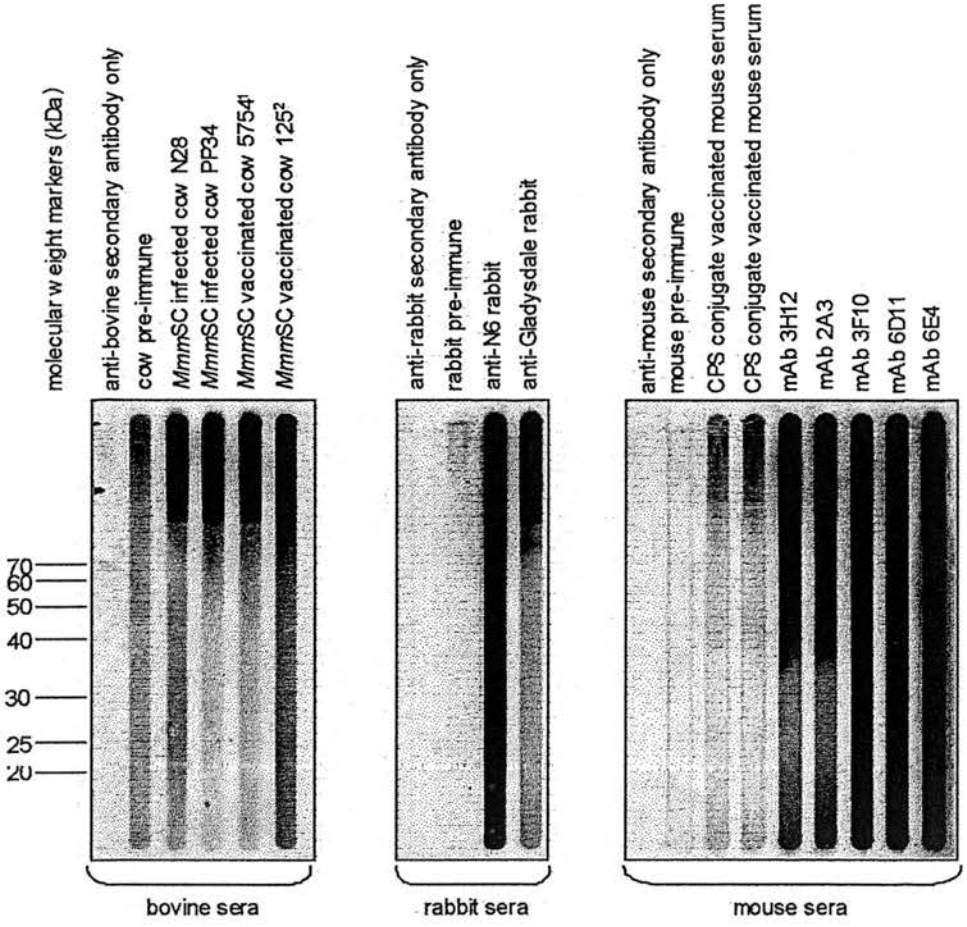
For further confirmation of the cross-reaction between bovine lung and CPS, mice were immunised with bovine lung extract. The sera obtained were tested against bovine lung (as a control) (Fig. 23. A) and against CPS (Fig. 23. B). HRP-conjugated anti-mouse immunoglobulin antibody did not cross-react with bovine lung homogenate. Pre-immune sera showed only a slight reaction with bovine lung (pre-immune serum from mouse 10 was not available). As expected, the immune sera from mice reacted strongly with bovine lung homogenate. The immune sera also

showed a positive signal with CPS, in agreement with earlier data suggesting that the two antigens shared epitopes. When tested with CPS, no reaction could be seen with the conjugated secondary antibody and with the pre-immune sera.

Sera from mice 30 and 40 were tested in GIT against *Mmm*SC strains Gemu Goffa and M375. None of them inhibited the growth of these mycoplasmas. It does not prove that it did not contain inhibiting antibodies since the sera from mice immunised with CPS conjugated to ovalbumin exhibited antibodies to CPS but did not have growth inhibiting activity [155].

Fig. 22. Immunoblots showing cross-reactions between *Mmm*SC CPS as antigen and normal bovine lung homogenate and galactan as antigen using anti-*Mmm*SC sera and CPS monoclonal antibodies.

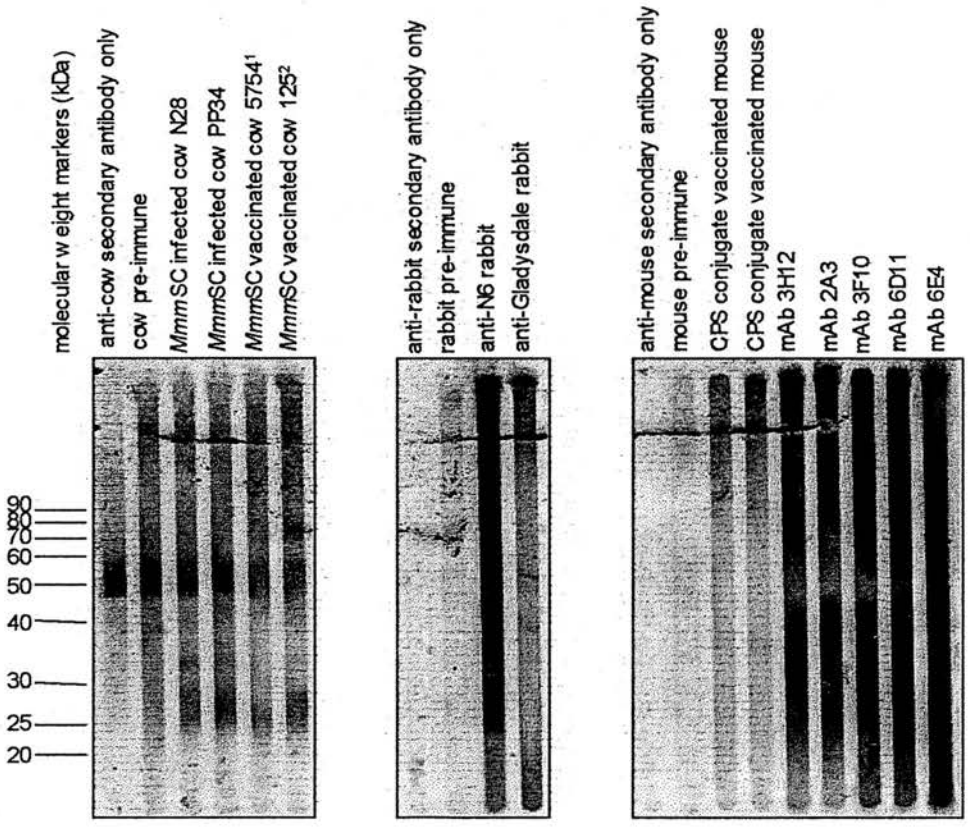
A) *Mmm*SC CPS immunoblot



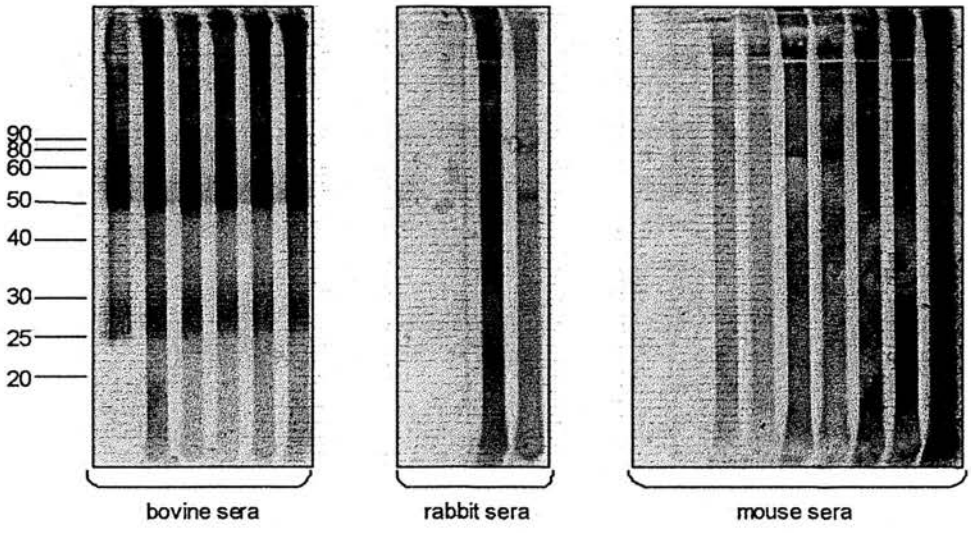
[1]10 weeks post-vaccination bleed, [2]2 weeks post-vaccination bleed.

Fig. 22 (concluded). Immunoblots showing cross-reactions between *Mmm*SC CPS as antigen and normal bovine lung homogenate and galactan as antigen using anti-*Mmm*SC sera and CPS monoclonal antibodies.

B) Bovine lung homogenate immunoblot



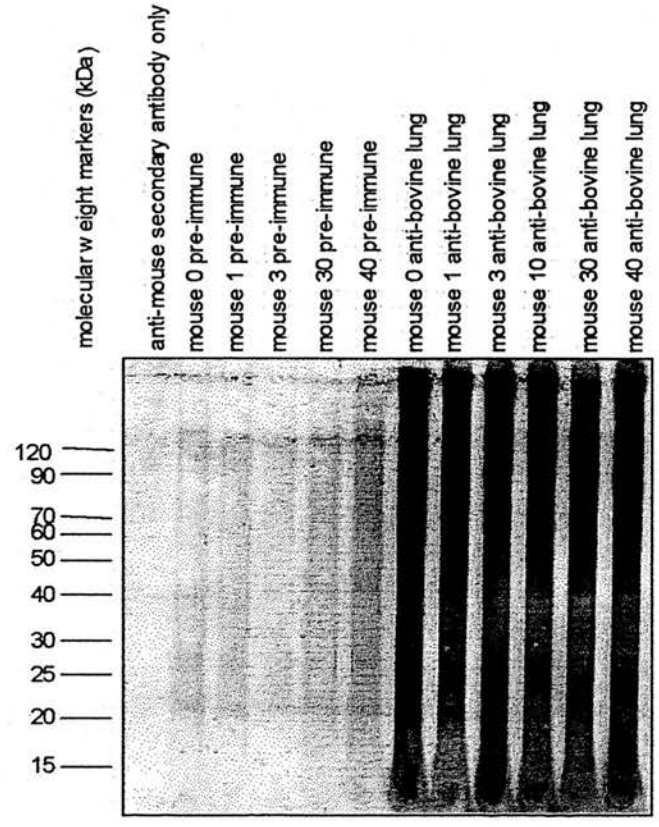
C) Bovine lung galactan immunoblot



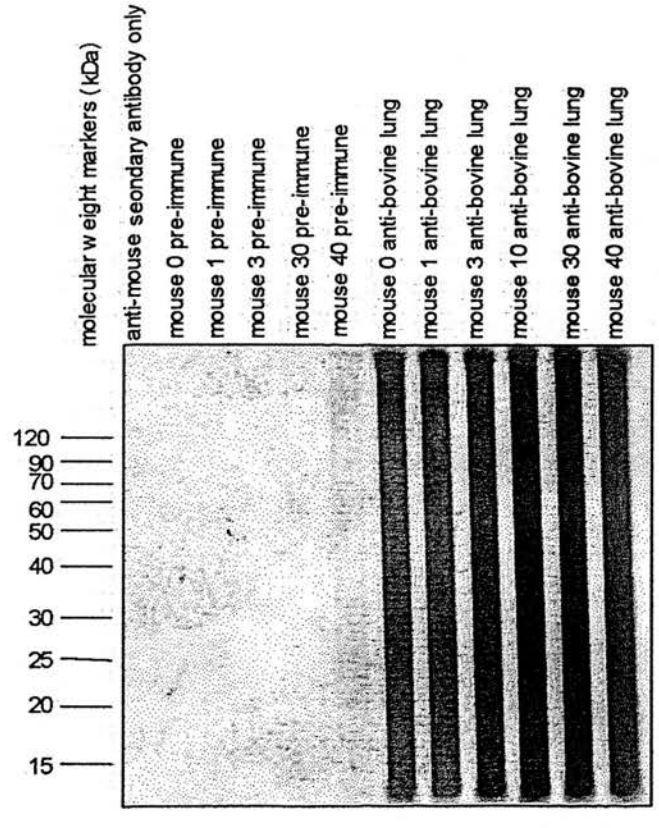
[1]10 weeks post-vaccination bleed, [2]2 weeks post-vaccination bleed.

Fig. 23. Immunoblots showing cross-reactions between normal bovine lung homogenate as antigen and *Mmm*SC CPS as antigen using mouse anti-bovine lung sera.

A) Bovine lung homogenate immunoblot



B) *Mmm*SC CPS immunoblot



3.7.3. Conclusions.

The results obtained with these WBs suggested that epitopes from CPS were also present on bovine lung. However it was not possible to determine if antibodies recognising both CPS and bovine lung were present in cow sera as the HRP-conjugated anti-cow immunoglobulin antibody reacted with bovine lung. It should be noted that the pre-immune cow serum showed a signal against CPS though lower, indicating that in a normal state, antibodies that recognise carbohydrate epitopes that can be found on CPS are present in a cow with no past of contact with *Mmm*SC. Although only one serum was tested in this assay, another experiment, using 15 normal cow sera (from cows bred in the United Kingdom) tested by ELISA against CPS, showed that 11 of them exhibited a signal with an optical density higher than 0.5 against CPS although these cows never encountered *Mmm*SC (data not shown). It is not known why antibodies that can bind to CPS are present in these sera; it can be speculated that these cows react to factors present in their environment (microorganisms, food...). This possible cross-reaction was explored. The results are exposed in the following section.

3.8. Cross-reactions between CPS and carbohydrates from cereals.

The use of CPS as a vaccine would require to grow *Mmm*SC in large quantities then to purify the supernatant, a procedure that needs time and a lot of handling and adequate laboratory facilities to culture the mycoplasmas. It has already been shown using anti-*Mmm*SC polyclonal bovine serum [cited in 102] that *Mmm*SC CPS shared epitopes with a variety of compounds such as polysaccharides from other

bacteria, barley and oat glucans. Thus, these polysaccharides might replace CPS to make a vaccine. Cereals are a common source of carbohydrates, these carbohydrates may share epitopes with CPS but the use of mAbs will permit the identification more specifically of the common epitopes between CPS and cereal carbohydrates. Crude carbohydrates were purified from different cereal preparation: corn flour, oatmeal, rice, wheat flour and wheat semolina and investigated in western blot (WB) (Fig. 24. B, C, D and E respectively). CPS purified from *Mmm*SC strain V5 grown in casein medium was used as a positive control (Fig. 24. A). Cow and rabbit immune sera against *Mmm*SC and mouse anti-CPS antibodies (mAbs and polyclonal sera) were used in these assays.

None of the secondary antibodies cross-reacted with the carbohydrate extracts. Cow pre-immune and vaccinated-immune sera gave a signal with all the different carbohydrate solutions and CPS. The serum from the rabbit immunised with *Mmm*SC strain N6 recognised the different preparations of carbohydrates and CPS. Pre-immune serum from the same rabbit did not showed any signal except with the corn flour extract, a slight signal was also shown with CPS. Four out of the 5 mAbs (3H12, 2A3, 3F10 and 6E4) presented a signal with all carbohydrate extracts. MAb 6D11 recognised wheat flour extract only (Fig. 24. D) as the same results were obtained on repeating assays. As expected, all mAbs reacted with CPS (Fig. 24. A).

These results suggested that epitopes on CPS might be relatively common because they can be found on other carbohydrates, found readily in nature. MAb 6D11 recognised wheat flour extract but not wheat semolina extract. This could be explained by a different strain of wheat, or by a difference in wheat treatment to

make these products, since one of these treatments could "present" a new epitope on the molecule or destroy it.

Fig. 24. Immunoblots showing cross-reaction between *Mmm*SC CPS as antigen and carbohydrates from cereals as antigens.

A) CPS immunoblot

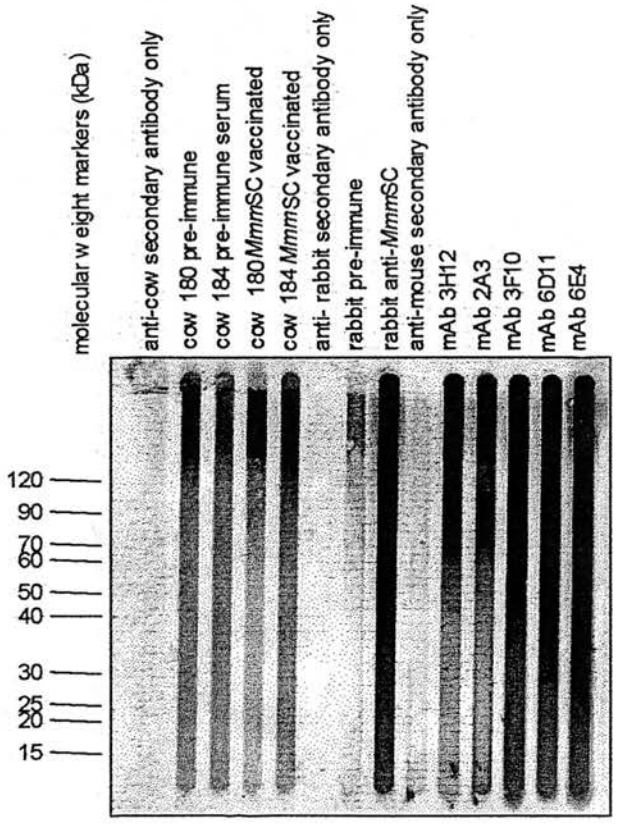
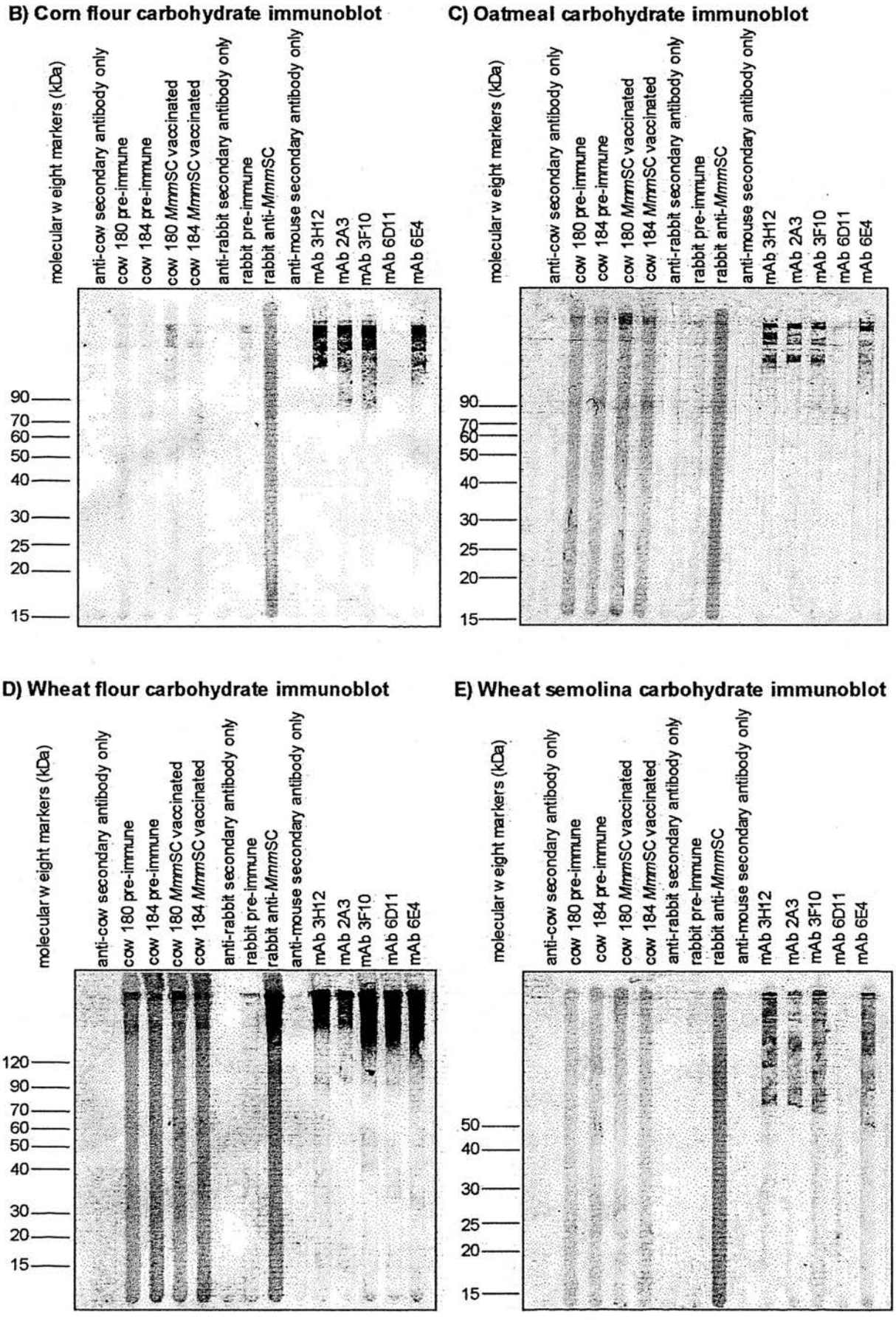


Fig. 24 (concluded). Immunoblots showing cross-reaction between *Mmm*SC CPS as antigen and carbohydrates from cereals as antigens.



3.9. Role of CPS in adhesion.

In other microorganisms, differences in adhesion have been related to the presence or absence of CPS [129,130]. The role of *Mmm*SC CPS in adhesion has never been explored so far. It was then decided to study the role of CPS in this process by an indirect way in an adherence inhibition assay in tissue-culture plates.

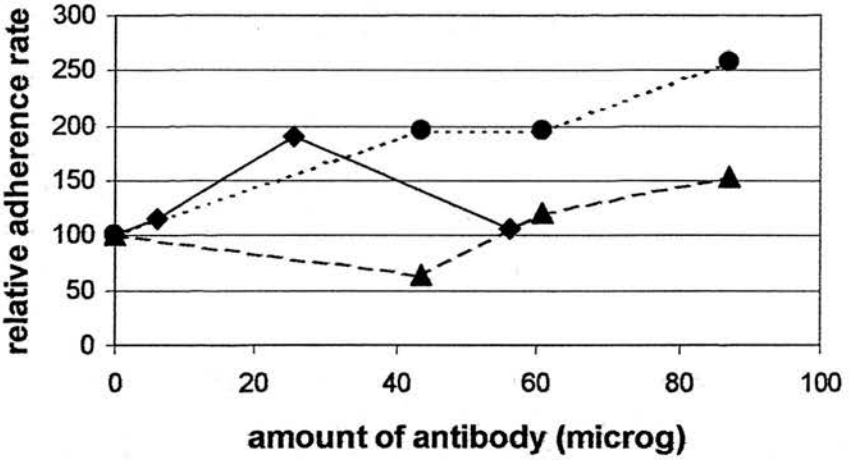
*Mmm*SC strains N6 and M375 and *M. bovis* strain PG45 were pre-incubated with a different mAb. Anti-*Mmm*SC CPS mAb 1C9 was used with the strain N6, anti-*M. bovis* mAb 4D7 was used with the strains M375 and PG45. The hypothesis was that if CPS plays a part in adhesion of *Mmm*SC, the addition of anti-CPS antibody should inhibit the adhesion to embryonic bovine lung (EBL) cells. *M. bovis* strain PG45 with mAb 4D7 was used as a positive control, i.e. the addition of mAb 4D7 has been shown to inhibit the adhesion of strain PG45 to EBL cells. *Mmm*SC strain M375 could not be pre-incubated with mAb 1C9 due to the small amount of this antibody; it was therefore decided to incubate this strain with mAb 4D7. In this case, no inhibition in the adhesion of M375 was expected.

The results obtained from mycoplasmas pre-incubated with mAbs were compared with those obtained from mycoplasmas without mAbs to determine the relative adherence rate. The results are shown in Fig. 25.

The results obtained from the adherence assay were not conclusive. The results obtained from the *Mmm*SC strain N6 could not show whether *Mmm*SC CPS was involved in adhesion of mycoplasmas because, even though no inhibition of the adhesion could be seen with 6.1 µg and 56.1 µg of mAb 1C9, the adhesion of N6 to EBL cells was nearly doubled in presence of 25.5 µg of antibody. The positive control, *M. bovis* strain PG45, showed an inhibition of the adhesion with 43.5 µg of

mAb 4D7 but not with 60.9 µg and 87 µg of mAb 4D7 where the inhibition of the adhesion was expected to be higher than with 43.5 µg of antibody. No inhibition of the adhesion of *Mmm*SC strain M375 was expected since this strain was pre-incubated with a non-specific mAb. It appeared as if the addition of mAb 4D7 increased the adhesion of M375 to EBL cells since the relative adherence rate was twice more important in presence of antibody when compared with the adhesion in absence of antibody, i.e. the relative adherence rate was 100 for M375 without mAb and was more than 250 for M375 with 87 µg of mAb (the experiment was performed in duplicate and the results were consistent between the duplicates). This seemed unlikely since mAb 4D7 was not supposed to bind to the *Mmm*SC strain and the use of this mAb induced the inhibition of adhesion of *M. bovis* in previous experiments [Konrad Sachse, personal communication].

Fig. 25. Adhesion of different mycoplasma strains, in presence of mAbs, to EBL cells.



◆: *Mmm*SC strain N6 pre-incubated with anti-*Mmm*SC CPS mAb 1C9, ●: *Mmm*SC strain M375 pre-incubated with anti-*Mbovis* mAb 4D7, ▲: *Mbovis* strain PG45 pre-incubated with anti-*Mbovis* mAb 4D7.

It was not possible to show whether CPS was involved in adhesion in the adherence inhibition assay in tissue-culture plates. A western-blot adherence assay

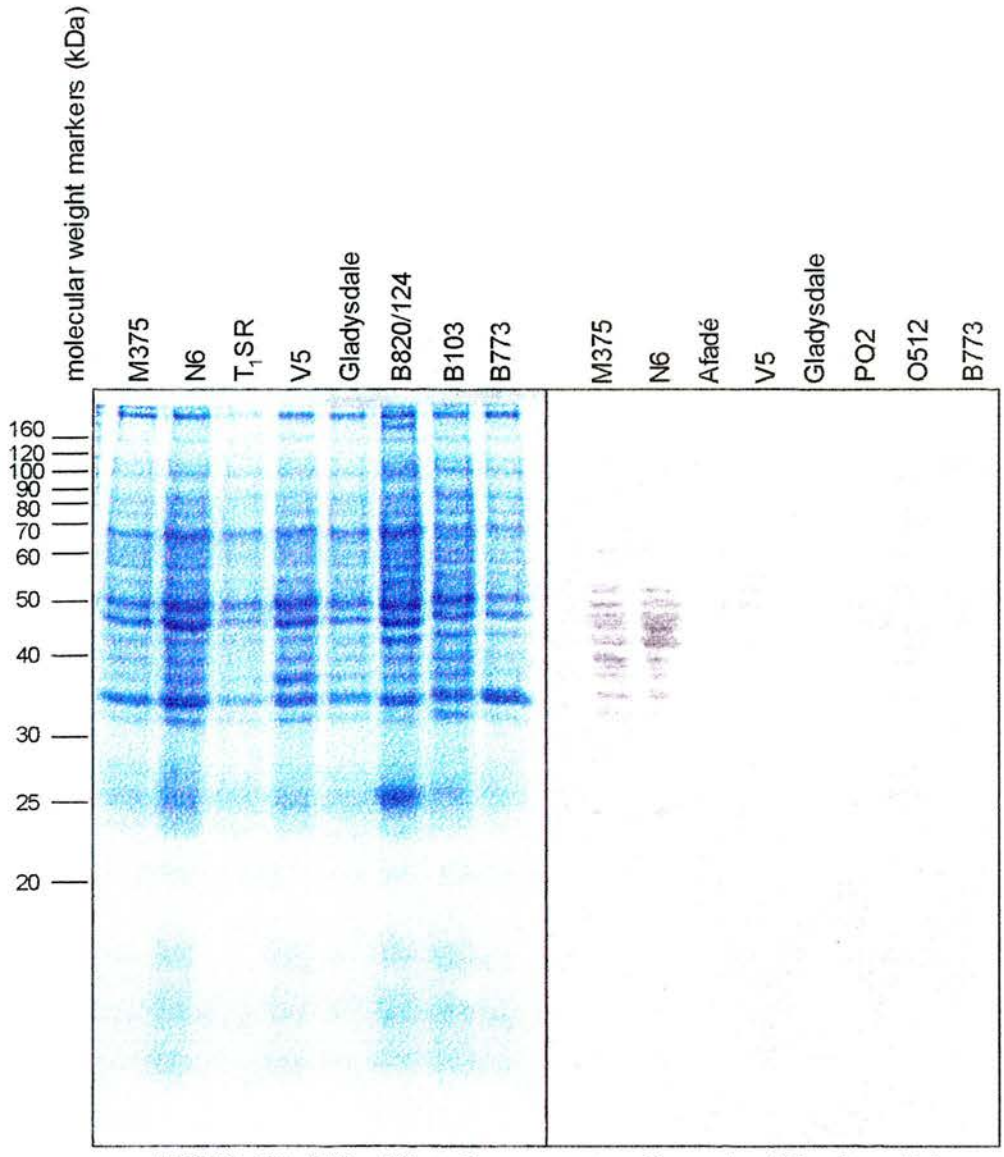
was then performed to study the direct adhesion of EBL cells to mycoplasma components.

An example of a SDS-PAGE of mycoplasmas is given in Fig. 26. The gel stained with Coomassie Blue showed numerous proteins. CPS, although not stained on this gel, is localised on the top of the gel. The adhesion of EBL cells to mycoplasma was visualised by autoradiography of the nitrocellulose membrane (Fig. 26.). The results from the autoradiography were not very good since the signal was rather weak for most of the *Mmm*SC strains used in the experiment.

No signal could be seen on the top of the autoradiography for any of the *Mmm*SC strains, suggesting that CPS is not involved in the adhesion of *Mmm*SC to EBL cells. However, it cannot be excluded that the lack of signal could be due to the poor exposure of the autoradiography.

Many proteins of *Mmm*SC strains M375 and N6 interacted with EBL cells. This would mean that these proteins are all adhesins which seems unlikely. The different peptides might be degradation fragments from a single high-molecular-weight protein that binds to EBL cells or represent a family of variable proteins expressed on the surface of the mycoplasma. These results might also be due to a non-specific binding to the EBL cells.

Fig. 26. Western-blot adherence assay of *Mmm*SC strains with radiolabelled EBL cells.



It was not possible to determine the role of *Mmm*SC CPS in adhesion from the results of the adherence inhibition assay and the western-blot adherence assay. The identification of *Mmm*SC adhesins was not possible either since the results of the autoradiography for *Mmm*SC strains M375 and N6 showed too many bands, suggesting that these bands were due to a non-specific binding to the EBL cells or that they were degradation products from a single protein or variable proteins.

4. Discussion.

Vaccination seems the only way to eradicate contagious bovine pleuropneumonia (CBPP) in Africa since the political and economical situations in the affected countries cannot allow the funding of a policy of slaughter and compensation as a policy of eradication. The current vaccines are not efficient enough against CBPP [28,90,91]. Therefore a new, better vaccine is needed. A capsular polysaccharide (CPS), produced by *Mycoplasma mycoides* subsp. *mycoides* (*Mmm*SC), appears to offer good potential as a vaccine since it is a virulence factor [110]. In addition, in other microorganisms, CPS has been shown to protect bacteria against phagocytosis and antibodies against CPS and pneumococcal and meningococcal CPS-conjugate vaccines have been shown to protect against the disease [145,146].

4.1. CPS immunogenic structure.

An efficient vaccine is one able to protect against the different strains of the targeted microorganism. The analysis of CPS by growth inhibition test (GIT) with polyclonal antisera raised against different strains of *Mmm*SC has shown that the epitopes responsible for the growth inhibiting (GI) activity were present in all *Mmm*SC strains. In general, the sera with the highest anti-CPS titres had the greatest GI activity. This suggested that antibodies against CPS were protective against *Mmm*SC. However, some exceptions occurred. The serum raised against *Mmm*SC strain 425 had a low CPS-specific antibody titre when compared to most of the sera but exhibited a good GI activity against different *Mmm*SC strains. Conversely, the serum raised against *Mmm*SC strain Filfili had a very high anti-CPS antibody titre

but its GI activity was very low. This could be explained by the property of the anti-CPS antibodies: not all antibodies against CPS might have GI activity. In the case of the anti-425 serum, the total amount of CPS-specific antibodies may be low but most of them might be growth inhibiting, whereas in the anti-Filfili serum, the total amount of anti-CPS antibodies may be high but with only a small portion exhibiting a GI activity. In addition, other components of the mycoplasmas might elicit GI antibodies, i.e. CPS-non-specific antibodies might be bactericidal.

Our results in the GIT suggested that *Mmm*SC CPS was probably conserved between strains. This was further confirmed by an enzyme-linked immunosorbent assay (ELISA) performed with different anti-CPS monoclonal antibodies (mAbs) where the CPS purified from different *Mmm*SC strains were all recognised by the mAbs 6D11, 3F10, 3H12, 6E4, PK2, 2A3 and 1C9, except for the type strain PG1. For this strain, mAbs PK2 and 2A3 gave a low signal by ELISA, suggesting the epitopes recognised by these two mAbs were not present on PG1 CPS. The mAbs 6D11, 3F10, 3H12, 6E4 and PK2 tested in GIT showed all GI activity against *Mmm*SC strain Gemu Goffa.

The ELISA with different *Mmm*SC CPS incubated with different mAbs was also performed using CPS purified from *Mycoplasma capricolum* subsp. *capripneumoniae* (*Mccp*) strains. *Mmm*SC and *Mccp* are both classified in the "*Mycoplasma mycoides* cluster". *Mccp* causes contagious caprine pleuropneumonia in goats. No cross-reaction could be seen between *Mmm*SC CPS and *Mccp* CPS although their sugar composition was reported as being very similar (fucose, mannose, galactose, glucose, galactosamine and glucosamine in an approximate ratio of 1:2:4:1:4:8 and fucose, mannose, galactose, glucose, galactosamine and

glucosamine in approximately equal quantities, respectively) [97,157]. However, the *Mmm*SC and *Mccp* strains used for the determination of CPS composition in these two publications were both cultured in Gourlay's broth. A high molecular weight carbohydrate, extracted from this culture medium, was subsequently shown to contaminate solutions of purified CPS [150]. Therefore, it is possible that this similarity between the CPS from these two species is due to contamination with carbohydrate culture medium. Another explanation could be that the two polysaccharides contain the same sugars but are structurally dissimilar and present different epitopes.

Two other mycoplasma species, *Mycoplasma mycoides* subsp. *mycoides* large colony (*Mmm*LC) and *Mycoplasma mycoides* subsp. *capri* (*Mmc*), are also classified in the *Mycoplasma mycoides* subgroup along with *Mmm*SC. These two mycoplasma species are difficult to identify from each other, and many *Mmc* isolates have been reclassified as *Mmm*LC [cited in 158]. It has been suggested that *Mmm*LC and *Mmc* should be re-classified as a single species according to their 16S rRNA sequence analysis and their tRNA profile [151,152]. In addition, *Mmm*LC and *Mmm*SC have been shown to share immunological similarities, since anti-*Mmm*LC polyclonal sera inhibited the growth of *Mmm*SC strains [cited in 153]. Five mAbs recognising CPS of *Mmm*SC (3F10, 6E4, 6D11, 3H12 and PK2) were used in an ELISA against 7 *Mmm*LC strains (UM30847, G8, G10, Y goat, 2/93, 247/4 and 5307) and 5 *Mmc* strains (133/87, WK354, L, PG3 and BQT). Six *Mmm*LC strains (UM30847, G8, G10, Y goat, 2/93 and 247/4) and two *Mmc* strains (133/87 and WK354) were recognised by both 3F10 and 6E4. Three *Mmm*LC strains (UM30847, G10 and Y goat) and the *Mmc* strain WK354 were recognised by 6D11. MAbs 3H12 and PK2

did not exhibit a signal against any of the *Mmm*LC and *Mmc* strains. This suggested that the CPS of the "*Mmm*LC/*Mmc*" species and CPS of *Mmm*SC exhibit antigenical differences.

The same mAbs were used against these *Mmm*LC and *Mmc* strains in GIT. MAbs 3F10 and 6E4 exhibited growth inhibiting activity against all *Mmm*LC and *Mmc* strains recognised in the previous ELISA except for *Mmm*LC strain G8. *Mmm*LC strain 2/93 was inhibited by 3F10 but not by 6E4 (both mAbs recognised this strain by ELISA); this might be due to a greater CPS production by G8 and 2/93 when compared to the production of CPS by the other *Mmm*LC strains. None of the other mAbs (6D11, 3H12 and PK2) were inhibitory against any *Mmm*LC and *Mmc* strains tested. The results obtained with PK2 in GIT were in agreement with those obtained in an experiment which showed that PK2 inhibited 9 *Mmm*SC strains but did not inhibited any of the 3 *Mmm*LC strains used in GIT [153].

The results obtained with the ELISA and the GIT suggest that the mAbs can be divided into three categories: (i) mAbs binding to *Mmm*LC/*Mmc* CPS and exhibiting GI activity against some *Mmm*LC and *Mmc* strains (3F10 and 6E4), (ii) mAbs binding to *Mmm*LC/*Mmc* strains with no GI activity against *Mmm*LC and *Mmc* strains tested (6D11) and (iii) mAbs that do not bind to *Mmm*LC/*Mmc* CPS and showing no GI activity against *Mmm*LC and *Mmc* strains tested (3H12 and PK2). Data suggest that CPS contains at least three different epitopes.

*Mmm*LC strains and *Mmc* strains were not all recognised by the mAbs, i.e. some strains were recognised by the mAbs and some others were not, suggesting two "serotypes", based on CPS. To our knowledge, this would be the first time that it has

been described in mycoplasmas. It would then be suggested to re-classify *Mmm*LC and *Mmc* as a single species with two serotypes.

As described in the previous paragraphs, results suggested that at least three different epitopes were recognised by the mAbs, meaning that at least three different epitopes exist on *Mmm*SC CPS. To explore further the number of epitopes recognised by the different mAbs, a competition ELISA was performed. Unfortunately, it was not possible to state with certainty which mAbs recognise the same structure on *Mmm*SC CPS except maybe for 6E4, 6D11, 3H12 and PK2 that seemed to recognise different epitope on *Mmm*SC CPS. The results for the other mAbs were different according to the order of incubation of the mAbs, which could be explained by overlapping epitopes, steric hindrance or affinity difference for the same CPS epitope.

We have shown that *Mmm*SC CPS was conserved between *Mmm*SC strains and that the molecule presented at least three different epitopes. As a consequence, the CPS from only one *Mmm*SC strain could be used in vaccination against CBPP.

4.2. CPS as a vaccine.

In the precedent section, antibodies against CPS were shown to be bactericidal *in vitro* suggesting these antibodies were protective against *Mmm*SC. It is not known whether anti-CPS antibodies are protective *in vivo*. Ideally, the best model to explore the protective efficacy of CPS-specific antibodies *in vivo* would be cattle. Unfortunately, it is not possible due to the size of the animal and the cost this would involve to maintain cattle in containment. Therefore, it was decided to use mice as a preliminary assessment of anti-CPS antibody efficacy against *Mmm*SC.

To explore the protective effect of anti-CPS antibodies against CBPP, mice were passively immunised with purified bactericidal mAb 3F10, then challenged with *Mmm*SC strain N6. As this mAb is bactericidal, it seemed likely it might protect against the disease. When compared with the group of mice immunised with PBS then challenged with N6 strain, mice that had received the injection with mAb 3F10 did not show a significant difference in the incidence and duration of mycoplasmaemia. These results suggested that *in vivo*, the bactericidal mAb was not protective against *Mmm*SC strain N6 in mice. These results were in agreement with a study showing that mice immunised with a *Mmm*SC CPS conjugate vaccine, although showing an immune response to CPS, were not protected against challenge with *Mmm*SC. Mice immunised with whole inactivated *Mmm*SC, although having a low CPS-specific antibody titre in their sera, were protected against infection with *Mmm*SC but these sera did not exhibit a growth inhibiting activity [155]. These results suggested that in mice, the protection against *Mmm*SC was cell mediated rather than humoral.

Current vaccines against CBPP are not very efficient. They induce a short-lasting immunity and do not protect all vaccinated cattle [28,90,91]. This lack of protection might be due to a poor immune response to CPS, assuming that the protection against *Mmm*SC in cattle is due to anti-CPS antibodies. Therefore, it was decided to analyse the CPS immune response in various cattle. The immune response against CPS in three groups of cattle was examined. The groups were (i) $T_1$44-vaccinated cattle subsequently placed in contact with *Mmm*SC-intubated cattle, (ii) *Mmm*SC-intubated cattle and (iii) unvaccinated cattle placed in contact with *Mmm*SC-intubated cattle (i.e. group II). Only 26% of vaccinated cattle showed an

immune response to CPS after vaccination as measured by ELISA, in agreement with the lack of efficacy of the vaccine (assuming the protection against CBPP is from antibodies against CPS).

A typical primary immune response to an antigen consists mainly of IgM class antibodies. When the host is confronted a second time with the same antigen, a typical secondary immune response is composed mainly of IgG class antibodies, which exhibit a higher affinity for the antigen. The immune system also responds faster. Examining the immune response in more detail in the experiment described in the previous paragraph, only IgM class antibodies were detected in the three groups of cattle. No IgG or IgA could be detected against CPS except for a single unvaccinated "in-contact" bull, which showed a low level IgG immune response. IgG responses were especially expected in sera from vaccinated cattle following "in-contact" challenge since they were exposed twice to *Mmm*SC. CPS on its own is a poor immunogen. As a T-independent antigen, it does not induce memory B cells and antibody class switching. But in a vaccine or following infection, CPS is presented with *Mmm*SC proteins, which could behave as protein carriers. CPS could therefore become a T-dependent antigen, enabling antibody class switching from IgM to IgG to occur. This seems to be possible since rabbits immunised with *Mmm*SC exhibit a good IgG antibody response against CPS. The lack of IgA immune response was less surprising as IgA is the predominant immunoglobulin in seromucous secretions such as tracheo-bronchial secretions and we only looked at the IgA immune response in sera. Since CBPP is a respiratory disease, it would have been interesting to investigate the immune response against CPS in lung lavages of the same group of cattle. It would also have been interesting to compare the

serological data with the clinical and post-mortem data to see if the CPS immune response was correlated with the severity of lung lesions and with protection against CBPP.

The observed lack of immune response against CPS in vaccinated cattle might be the consequence of the reported cross-reaction between bovine lung and CPS, with autoreactive B cells being anergised or deleted to prevent damage to the host (i.e. the similarity between the host lung and *Mmm*SC CPS could cause immunotolerance or immunopathology). This cross-reaction was further investigated by western-blots (WBs) using sera raised against bovine lung homogenate and CPS from different animals. The sera from *Mmm*SC immunised rabbits, CPS-conjugate vaccinated mice and CPS mAbs gave a signal against both CPS and bovine lung. The sera from *Mmm*SC naturally-infected cows and from *Mmm*SC vaccinated cows showed a positive signal against CPS but it was not possible from these experiments to determine if bovine antisera cross-reacted with bovine lung homogenate and bovine lung galactan due to a non-specific signal from the secondary antibody (different secondary antibodies against bovine immunoglobulins still exhibited this non-specific signal even after pre-absorption with bovine lung homogenate). Cow pre-immune serum was also positive for CPS but the signal was a lot weaker than the signal observed with sera from infected and vaccinated cows that showed a response, whereas rabbit and mouse pre-immune sera did not show a signal against CPS. The results obtained with the cow pre-immune serum could be considered as a background reaction, since sera from cattle with no history of CBPP were shown in this thesis to react positively with CPS in an ELISA. This background reaction could

be due to exposure to other polysaccharide antigens found in foodstuffs or from other microorganisms, i.e. these polysaccharides might give cross-reactions with CPS.

These results confirmed the cross-reaction between bovine lung and CPS. Furthermore, the different epitopes recognised by the different mAbs, which were shown to be bactericidal against *Mmm*SC strain Gemu Goffa, were also all present on bovine lung, i.e. all CPS mAbs bound to the bovine lung. The cow immune response against bovine lung homogenate and bovine lung galactan would have been of a particular interest. Although rabbit and mouse sera showed cross-reactions between the bovine lung and *Mmm*SC CPS, the sera from cows might have shown a different result, i.e. cow sera could have shown a signal against CPS and not the bovine lung since CPS might have epitopes that cannot be found on bovine lung. If antibodies against bovine lung had been detected in cow sera, this would have given evidence of immunopathology.

The cross-reaction between bovine lung and CPS was also confirmed by the reciprocal experiment i.e. the use of anti-bovine lung mouse sera which gave a positive signal against CPS as well as against bovine lung homogenate as expected.

In the WB with *Mmm*SC CPS as antigen, pre-immune cow sera gave a positive, although weak, signal against CPS. This reaction is not uncommon since in 15 sera from cows free of CBPP (bred in the United Kingdom), 11 of them showed a similar background reaction with CPS in an ELISA (optical density (OD) above 0.5) [J.B. March, unpublished results]. This background signal reaction was also visible in the ELISA with vaccinated and unvaccinated cattle in which pre-immune bleeds showed an OD close to 0.5, but following vaccination or being placed in contact with *Mmm*SC-intubated cattle, an increase in the immune response to CPS occurs in some

cattle. This background might be caused by antibodies raised against polysaccharides from foodstuffs or against CPS from other microorganisms. It has been reported that *Mmm*SC CPS shared epitopes with polysaccharides from different sources (bacteria, barley, oat glucans...) [cited in 59]. Therefore dietary exposures might have led to this background signal in many cattle.

To minimise the cost of CPS vaccine production, polysaccharides from other sources, such as cereals, could be used. Carbohydrates purified from corn flour, oatmeal, wheat flour and wheat semolina reacted with pre-immune cow sera, *Mmm*SC-vaccinated cow sera, *Mmm*SC-immunised rabbit serum and mAbs 3H12, 2A3, 3F10 and 6E4. An additional mAb, 6D11 also reacted with wheat flour carbohydrate. These results suggested that different epitopes of CPS might be very common in the environment. As a consequence, they might not be very immunogenic, hence difficult to mount an effective immune response.

Since the mAbs, which recognised epitopes on CPS and cereal carbohydrates, have bactericidal activities, this means that these polysaccharides could elicit protective antibodies if used in a vaccine. But due to the nature of these molecules, they would need to be given in an effective manner such as conjugated to proteins.

4.3. Conclusions.

*Mmm*SC CPS possesses at least three different epitopes. These epitopes are found readily in nature since *Mmm*SC CPS shares epitopes with carbohydrates from cereals such as wheat flour, rice, oatmeal and wheat semolina. In addition, these epitopes are also present in bovine lung.

We have shown that cattle vaccinated with T_144 exhibited poor immune responses to CPS. According to the hypothesis that CPS is the protective antigen against CBPP since anti-CPS antibodies are bactericidal *in vitro*, the lack of CPS-specific antibodies could explain the poor efficacy of current vaccines. Passive immunisation with anti-CPS antibodies proved unsuccessful in mice. However, this does not mean that the same results would occur in cattle. The success with other CPS vaccines for other microorganisms should lead to further study on the potential of CPS as a vaccine against CBPP.

An adhesion assay could be carried out to explore the role of CPS in the adhesion of *Mmm*SC to the bovine respiratory tract (i) by comparing the adhesion between *Mmm*SC strains producing a lot of CPS and strains producing less CPS to see if there is any difference that could be due to the production of CPS and (ii) by comparing the adhesion between a *Mmm*SC strain and the same one but pre-incubated with CPS-specific antibodies. Efforts should also be made to determine the exact structure of CPS by chemical analysis, mass spectrometry and nuclear magnetic resonance of a pure solution of CPS without any polysaccharide contaminant from the culture medium. Oligosaccharides of known sugar composition and structure could be used for epitope mapping in an ELISA with anti-CPS monoclonal antibodies to identify the bactericidal epitopes. The anti-CPS antibodies could also be used to identify molecular mimics or to screen peptides library or to produce anti-idiotype antibodies to be used as an alternative to CPS in vaccination.

In mice, the protection against *Mmm*SC seems to be through cellular immune response. Consequently, it would be interesting to examine the cellular immune response to CPS in these animals by looking at the type of cytokines produced after

vaccination and infection with whole mycoplasmas and CPS-conjugates. This could be done in different species of mice, i.e. mice deficient in B cells, deficient in the production of interferon γ for instance. The cellular immune response to CPS could also be examined in different groups of cattle ($T_1$44 or T_1SR vaccinated cattle, *Mmm*SC-infected cattle by contact with *Mmm*SC-intubated cattle, *Mmm*SC-intubated cattle themselves).

Cattle could be vaccinated with *Mmm*SC CPS-conjugates to determine the protection efficacy of this vaccine in cattle against challenge with *Mmm*SC and to look at a possible pathology in the lungs brought on by vaccination. The immune response to CPS in these vaccinated cattle should also be analysed i.e. whether it is a humoral or cell-mediated immune response. The effect of adjuvant and the route of administration of the CPS conjugate vaccine on protection should also be investigated. Intranasal vaccination might be more effective in eliciting protection since this is the route of natural infection.

But more importantly, since cross-reaction between CPS and bovine lung is suggested, it would be helpful to identify protective epitopes present on CPS but not on bovine lung by screening bovine lung galactan with many anti-CPS antibodies of which the recognised epitopes are known.

4.4. Role of CPS in *Mmm*SC adhesion.

In *Klebsiella pneumoniae*, it was shown that encapsulated strains colonised the intestinal tract and adhered to mucus-producing epithelial cells more efficiently than the non-capsulated strains [129,130]. In *Bacteroides fragilis*, it was suggested that carbohydrates present on the surface of the microorganism were involved in the

adhesion to human intestinal cells [159]. The role of *Mmm*SC CPS in adhesion has never been studied. Adherence inhibition assay in tissue-culture plates (with anti-*Mmm*SC CPS mAb used as inhibitor) and western-blot adherence assay, using embryonic bovine lung (EBL) cells, were performed to study the involvement of CPS in adhesion of *Mmm*SC. Since *Mmm*SC are bovine mycoplasmas infecting the lungs, EBL cells were preferred to perform the experiments.

Unfortunately, the results of both experiments were inconclusive concerning the part of CPS in adhesion.

Adherence inhibition assay did not seem to work since "illogical" results were obtained. The addition of anti-*Mmm*SC CPS mAb 1C9 to *Mmm*SC strain N6 did not produce any inhibition of the adhesion at 6.1 µg and 56.1 µg, but when added at 25.5 µg, an increase in the adhesion could be observed. Anti-*M. bovis* mAb 4D7 was inhibiting only when added to *M. bovis* strain PG45 at 43.5 µg. When mAb 4D7 was added at 60.9 µg and 87 µg, an increase in the adhesion of PG45 to EBL cells could be seen. Finally, the addition of mAb 4D7 to *Mmm*SC strain M375 increased the adhesion of the mycoplasma strain to EBL cells while it should have had no effect on the adhesion of M375. Clearly, these experiments need repeating. A monoclonal antibody other than 1C9 should be used since it is not known if this mAb is bactericidal and this property might affect the result, i.e. bactericidal antibody might inhibit the adhesion of mycoplasmas to EBL cells. It would also be better to use higher concentrations of antibody to be sure that a maximum of mycoplasmas are coated of antibodies.

Western-blot adherence assay did not give better answers about CPS and adhesion. Since CPS is a high molecular weight polysaccharide, it is found on the top

of the gel in a SDS-PAGE. As a consequence, any binding of EBL cells to CPS would appear at the top of the autoradiography film. No signal was observed on the top of the autoradiography, suggesting *Mmm*SC CPS was not involved in the adhesion to EBL cells. However, due to the poor exposure of the autoradiography, it cannot be excluded that the signal was not strong enough. Many proteins (around 15) for the *Mmm*SC strains M375 and N6 seemed to bind to EBL cells. It is unlikely that *Mmm*SC possesses so many adhesins. Many of them might interact with EBL cells by a non-specific binding. Some of these proteins could have been denatured by the SDS-PAGE and this would then allow the adhesion to the EBL cells that would not occur *in vivo*. They also might come from the degradation of a single high molecular weight protein or represent a family of variable proteins.

Little is known about *Mmm*SC adhesion. Other mycoplasma species have been shown to adhere to various receptors such as sialoglycoconjugates receptors, sulfatide receptors, to proteins and glycoproteins [160] and major adhesins have been identified like P1 [161] and P30 [162] for *Mycoplasma pneumoniae*, P140 for *Mycoplasma genitalium* [163] and pMGA for *Mycoplasma gallisepticum* [164]. Adhesion of mycoplasmas to host cells could play a role in pathogenicity. Then, the identification of host cell receptors for *Mmm*SC and molecules involved in the adhesion would be interesting because of its possible implication in the pathogenicity of CBPP.

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Appendices

Anode buffer I:

Tris	0.3 M	(Sigma, T-1503)
methanol	100 ml	(Fisher Scientific, M/4000/17)
distilled water	volume up to 1 l	
	pH 10.4	

Anode buffer II:

Tris	25 mM	(Sigma, T-1503)
methanol	100 ml	(Fisher Scientific, M/4000/17)
distilled water	volume up to 1 l	
	pH 10.4	

Blocking reagent:

dried skimmed milk	50 g	(Marvel)
PBST	volume up to 1 l	

Buffer A:

Tris	0.05 M, pH 7.2	(Sigma, T-1503)
NaCl	0.1 M	(Fisher scientific, S/3160/60)
$CaCl_2$	1 mM	
distilled water	volume up to 1 l	

Casein medium: for 1 l

casein	20 g	(Sigma, C-0626)
D-glucose	5 g	(Sigma, G-6152)
NaCl	5 g	(Fisher scientific, S/3160/60)
Na_2HPO_4 anhydrous	2.5 g	(Sigma, S-0876)
glycerol	5 ml	(Fisher scientific, G/P450/08)
yeast extract	1 g	(Oxoid, LP0021)
distilled water	750 ml	
autoclave at 121°C for 15 min		
horse serum	200 ml	(Life technologies, 16050-098)
10% thallous acetate solution	2.5 ml	(Sigma, T-8266)
100 mg/ml ampicillin solution	2.5 ml	(Sigma, A-9518)
phenol red	15 ml	(Sigma, P-0290)

Cathode buffer:

Tris	25 mM	(Sigma, T-1503)
glycine	40 mM	(Fisher scientific, G/0800/60)
methanol	100 ml	(Fisher Scientific, M/4000/17)
distilled water	volume up to 1 l	
	pH 9.4	

Coomassie Blue destain solution:

distilled water	500 ml	
methanol	400 ml	(Fisher Scientific, M/4000/17)
acetic acid	100 ml	(Fisher Scientific, A/0360/PB17)

Coomassie Blue staining solution:

Coomassie Blue	1 g	(Sigma, B-0149)
distilled water	500 ml	
methanol	400 ml	(Fisher Scientific, M/4000/17)
acetic acid	100 ml	(Fisher Scientific, A/0360/PB17)

Development solution:

Developer I concentrate	3 ml	(Sigma, D-4282)
Developer II concentrate	17 µl	(Sigma, D-4407)
distilled water	volume up to 27 ml	

Gourlay's agar: for 1 l

Bactotryptose	20 g	(Difco, 0124-17)
D-glucose	5 g	(Sigma, G-6152)
NaCl	5 g	(Fisher scientific, S/3160/60)
Na_2HPO_4 anhydrous	2.5 g	(Sigma, S-0876)
glycerol	5 ml	(Fisher scientific, G/P450/08)
yeast extract	1 g	(Oxoid, LP0021)
agar	9 g	(Oxoid, LP0028)
distilled water	750 ml	
autoclave at 121°C for 15 min then cool down to 47°C		
horse serum (pré-warmed at 47°C)	200 ml	(Life technologies, 16050-098)
10% thallous acetate solution (pré-warmed at 47°C)	2.5 ml	(Sigma, T-8266)
100 mg/ml ampicillin solution (pré-warmed at 47°C)	2.5 ml	(Sigma, A-9518)
phenol red (pré-warmed at 47°C)	15 ml	(Sigma, P-0290)

Gourlay's broth: for 1 l

Bactotryptose	20 g	(Difco, 0124-17)
D-glucose	5 g	(Sigma, G-6152)
NaCl	5 g	(Fisher scientific, S/3160/60)
Na_2HPO_4 anhydrous	2.5 g	(Sigma, S-0876)
glycerol	5 ml	(Fisher scientific, G/P450/08)
yeast extract	1 g	(Oxoid, LP0021)
distilled water	750 ml	
autoclave at 121°C for 15 min		
horse serum	200 ml	(Life technologies, 16050-098)
10% thallous acetate solution	2.5 ml	(Sigma, T-8266)
100 mg/ml ampicillin solution	2.5 ml	(Sigma, A-9518)
phenol red	15 ml	(Sigma, P-0290)

Loading buffer:

distilled water	1 ml	
1M Tris solution, pH 6.8	1 ml	(Sigma, T-1503)
glycerol	4 ml	(Fisher scientific, G/P450/08)
10% SDS solution	4 ml	(Sigma, L-4509)
2-mercaptoethanol	200 µl	(Sigma, M-3148)
bromophenol blue	10 mg	(Promega, H501a)

10X PBS:

NaCl	800 g	(Fisher scientific, S/3160/60)
KCl	20 g	(Fisons, P/4280/53)
Na_2HPO_4, 12 H_2O	115 g	(Fisons, S/4400)
KH_2PO_4	20 g	(Fisons, P/4800/50)
distilled water	volume up to 1 l	
	pH 7.2	

Ponceau destain:

acetic acid	10 ml	(Fisher scientific, A/0360/PB17)
distilled water	990 ml	

Ponceau stain:

Ponceau Red	1 g	(Sigma, P-3504)
acetic acid	10 ml	(Fisher scientific, A/0360/PB17)
distilled water	1 l	

Reducer solution:

Reducer A concentrate	200 µl	(Sigma, R-2006)
Reducer B concentrate	400 µl	(Sigma, R-2131)
Reducer C concentrate	70 µl	(Sigma, R-2256)
distilled water	volume up to 30 ml	

SDS gel:

separating gel (set for 1 h)		
30% acrylamide solution	4 ml	(Severn biotech, 20-2100-05)
distilled water	3.3 ml	
1.5 M Tris solution, pH 8.8	2.5 ml	(Sigma, T-1503)
10% SDS solution	100 µl	(Sigma, L-4509)
10% ammonium persulfate solution (APS)	100 µl	(Sigma, A-3678)
N-tetramethylethylenediamine (TEMED)	14 µl	(Sigma, T-8133)
stacking gel (set for 2 h)		
30% acrylamide solution	670 µl	(Severn biotech, 20-2100-05)
distilled water	2.7 ml	
1 M Tris solution, pH 6.8	500 µl	(Sigma, T-1503)
10% SDS solution	40 µl	(Sigma, L-4509)
10% APS solution	40 µl	(Sigma, A-3678)
TEMED	8 µl	(Sigma, T-8133)

Semi-dry blot buffer for WB:

Tris	5.82 g	(Sigma, T-1503)
glycine	2.93 g	(Fisher scientific, G/0800/60)
10% SDS solution	3.75 ml	(Sigma, L-4509)
distilled water	volume up to 1 l	

STE buffer:

NaCl	0,438 g	(Fisher scientific, S/3160/60)
TE buffer	40 ml	
(10 mM Tris 1 mM EDTA buffer, pH 8)		
adjust to pH 8		
adjust to 50 ml with TE buffer		

Silver equilibration solution:

Silver concentrate	75 µl	(Sigma, S-3140)
distilled water	volume up to 30 ml	

Stop solution:

glacial acetic acid	300 µl	(Fisher Scientific, A/400/PB17)
distilled water	volume up to 30 ml	

Tris-glycine running buffer for SDS-PAGE:

10 X Tris-glycine buffer	100 ml	
distilled water	890 ml	
10% SDS solution	10 ml	(Sigma, L-4509)

10X Tris-glycine buffer:

Tris	30 g	(Sigma, T-1503)
glycine	144 g	(Fisher scientific, G/0800/60)
distilled water	volume up to 1 l	

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