Identification of targets of monoclonal antibodies that inhibit adhesion and growth in *Mycoplasma mycoides* subspecies *mycoides*

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1. Introduction

Many mycoplasmas that infect livestock adhere to and colonize epithelial surfaces of various tissues in their hosts. Adhesion is thought to be tissue and host specific, and a prerequisite for colonization by pathogenic mycoplasmas (Barile and Rottem, 1993). The loss of adhesion by mutation results in loss of infectivity, and reversion to the cytoadherent phenotype is accompanied by requisition of infectivity and virulence (Razin and Jacobs, 1992; Baseman and Tully, 1997). Cytoadherence mechanisms of many *Mycoplasma* species, including those of *M. pneumoniae* (Chaudhry et al., 2007), *M. genitalium* (Ueno et al., 2008), *M. suis* (Zhang et al., 2015), *M. hominis* (Brown et al., 2014), *M. hyopneumonia* (Burnett et al., 2006), *M. agalactiae* (Fleury et al., 2002) and *M. bovis* (Song et al., 2012) have been described. In these studies, adherence was shown to be a complex multifactorial process involving one or more adhesion molecules.

In our previous study, we established a cytoadherence assay that measured the interaction of *Mmm* with various host cells and showed that *Mmm* cytoadherence is tissue and host specific (Aye et al., 2015). In this study we further analyzed the adhesion of *Mycoplasma mycoides* subspecies *mycoides* (*Mmm*), the causative agent of contagious bovine pleuropneumonia (CBPP), an important disease of cattle in sub-Saharan Africa.

The aim of this study was to identify potential *Mmm* vaccine targets by developing a panel of monoclonal antibodies against *Mmm* and use them to investigate their inhibitory effect on the adherence of *Mmm* to primary bovine lung epithelial cells (BoLEC), and to further identify an antigen to which any vaccine candidate(s) and should be subsequently tested for their ability to induce a protective immune response in vivo.
one of the inhibitory antibodies bound. Using an indirect flow cytometry assay, we identified 13 anti-Mycoplasma mycoides subspecies\
mycopoides antibodies (AMMY 1–13) that inhibited adhesion of Mmm to BoLEC. Subsequent to this initial work, Schieck et al. (2016) char-
acterized AMMY 10 as an Mmm-specific capsular polysaccharide (CPS) antibody. We further identified pdhC, part of the pyruvate dehy-
drogenase (PDH) complex, as an antigen that bound to AMMY 5 and provided evidence of its possible involvement in the cytoadhesion.
Mycoplasma molecules involved in the adhesion process, as well as molecules essential for growth or important biological functions are candidate vaccine targets.

2. Materials and methods

2.1. Mmm-specific monclonal antibody production

Five 6–8 week old BALB/c mice were immunized intraperitoneally with 100 μg Mmm strain Afadé whole cell lysate in 100 μl PBS mixed with equal volumes of TitterMax adjuvant (Sigma) on days 0 and 14 and 21. Seven days after the last immunization, antibody titers were mea-
sured by indirect enzyme-linked immunosorbent assay (ELISA—see below). Mice with highest antibody titers were given a booster dose of 100 μg Mmm lysate without adjuvant three days before aseptic spleen removal for fusion. Mice were used in accordance with the International Livestock Research Institute Institutional Care and Use Committee guidelines (IACUC Ref nr 2010.06)

Fusion was carried out as previously described in detail (Naessens et al., 1985) using X63Ag8.653 murine myeloma cells. The hybridomas were cultured in 96-well Costar tissue culture plates (Sigma) in the presence of thymocytes, hypoxanthine, aminopterin, and thymidine (Sigma) at 37 °C in 5% CO2 for 10–14 days.

Hybridomas were screened for antibody secretion by standard indirect ELISA. Briefly, Immunon 2 HB plates (Dynatech Laboratories) were coated with 5 μg/ml Mmm strain Afadé whole cell lysate (100 μl/well) and incubated overnight at 4 °C. Non-specific binding sites were blocked with PBS containing 0.1% Tween 20 (PBST) and 5% skim milk for 1 h at room temperature. The plates were incubated with 100 μl hybridoma culture supernatants and then peroxidase-conjugated anti-
mouse antibody (Sigma) for 1 h at room temperature. Plates were washed thrice with PBST after each incubation step. Reactions were vi-
sualized by adding 3,3′,5′,5′-tetramethylbenzidine (TMB) substrate and the reaction stopped with 75 μl 2 N hydrochloric acid. Plates were read at 450 nm using a microplate ELISA reader. Positive hybridomas were cloned by limiting dilution before being scaled up for further testing.

2.2. Inhibition assay

2.2.1. Mycoplasma mycoides subspecies mycoides culture

Mmm strains Afadé, Gladysdale and PGI were cultured in 20 ml “pleuropneumonia –like organism” (PPLO) medium (Becton Dickinson, Park, USA) supplemented with 10% horse serum (Sigma, St Louis, USA), 0.9 g yeast extract/l, 0.5% glucose and 0.03% penicillin G at 37 °C for 48 h to a density of 10^8 CFU/ml. Titration of Mycoplasma in the culture was carried out by the standard method of microtitration and color change and calculation of the titre by using the Spearman–Karber formula

(Litamoi et al., 1996) before centrifugation. The mycoplasmas were harvested by centrifugation at 6000 × g at 4 °C for 30 min, washed once in Dulbecco’s modified eagle’s medium (DMEM) without supplements and suspended in 10 ml of the same medium. Final titers were calcu-
lated using optical density at 665 nm and readings plotted on a standard curve based on correspondence between 665 and Mmm numbers as determined by TaqMan Real Time PCR.

2.2.2. Cytoadherence inhibition assay

All ELISA-positive Mmm-specific monclonal antibodies were tested for their ability to inhibit adhesion to BoLEC. Primary BoLEC were cultured using the protease digestion technique as described elsewhere (Thomas et al., 2003) and cultured in 24 well plates to a density of approximately 1.5 × 10^5 cells per well.

Mmm strain Afadé specific monoclonal antibodies (AMMY) were diluted in PBS and pre-incubated with 200 μl of Mmm (approximately 1.5 × 10^8 mycoplasmas) for 1 h at 37 °C before adding them to the BoLEC. Experiments were performed three times in duplicate.

The cytoadherence assay and flow cytometry analysis were performed as previously described (Aye et al., 2015). The proportion of cells to which mycoplasmas had bound was calculated using FlowJo (FLOWJO, 2014).

Percentage inhibition was calculated by the formula

\[
\% \text{ + ve cells of sample without antibody} - \% \text{ + ve cells of sample with the antibody} \times \frac{\%}{100}
\]

Antibodies that inhibited adhesion were isotyped using the rapid ELISA mouse isotyping kit (ThermoFisher Scientific) according to the manufacturer’s instructions.

2.3. Antigen identification

2.3.1. SDS-PAGE and Western blotting

Mmm strain Afadé was cultured as described above, a lysate pre-
pared by ultra-sonication and the protein concentration determined by micro BCA (ThermoFisher Scientific) according to the manufacturer’s instructions.

Approximately 250 μg of lysate was diluted 1:2 in SDS sample buffer and incubated for 5 min at 95 °C, loaded on to a single comb discontinuous Tris/glycine SDS-PAGE mini-gel (12.5% polyacrylamide resolving gel; 3% acrylamide stacking gel) and subjected to electro-
phoresis as described previously (Laemmli, 1970). 5 μl of a prestained molecular weight marker (PageRuler™ Prestained Protein Ladder, Thermo Scientific) was loaded in a separate lane to permit determina-
tion of the apparent molecular weight of protein(s) of interest. Elec-
trophoresis was performed using the Hoefer electrophoresis system (Serva Electrophoresis GmbH, Heidelberg, Germany) at 120 V for 2 h.

After SDS-PAGE, Mmm proteins were transferred onto nitrocellulose membrane (0.45 μm; Bio-Rad Laboratories, Inc., Hercules, CA) using the Hoefer electrophoretic system (Serva Electrophoresis GMbH, Heidelberg, Germany) at 50 V for 1 h. Non-specific binding sites on the membrane were blocked using 5% BSA/PBS for 1 h at room temperature, washed thrice in PBST, air dried for 30 min at room temperature and cut into 4 mm strips before being incubated with the different AMMY mAbs (1:1000) for 1 h at room temperature, followed by washing and incubation with polyvalent goat anti- mouse antibody conjugated to HRP (1:2500; Sigma) for 1 h at room temperature. After a further 5 washes with PBST, bands were visualized by incubation with 1% diaminobenzidine (DAB) and 1% hydrogen peroxide in Tris-buf-
fered saline until sufficient resolution was achieved. The reaction was
stopped by washing the membrane with distilled water.

The specificity of AMMY mAbs for only members of a particular subspecies in the closely related Mycoplasma mycoides cluster was as-
tered. Lysates from four subspecies including Mmm (strains Afade, B237, B66 and T1/44), M. mycoides subspecies capri (strains 80/93, 83/
90 and 153/90), M. capri subspecies capripneumoniae (strains Calf kid and 7714) and M. leachii (strains FRD42 and 4146) were tested. The proteins were loaded onto a 15 well gel, and electrophoresis and Western blotting were performed as described above.

2.3.2. 2DE, immunoblotting, trypsin digest and mass spectrometry

Protein preparation and 2-dimensional electrophoresis (2DE) were performed as described by Jores et al. (2009). First dimension focusing was performed on 24 cm non-linear pH gradient (4–7 NL) strips (GE
overnight using the EttanTM DaltSix Electrophoresis System (GE Healthcare, Germany) for 21 h (3 h at 150 V, 3 h at 300 V, 6 h at a 1200 V gradient, 3 h at an 8000 V gradient, and 6 h at 8000 V).

Percent inhibition of Mycoplasma mycoides subspecies mycoides adhesion to by anti-Mycoplasma mycoides subsp. mycoides monoclonal antibodies (AMMY) at various dilutions.

| Antibody dilutions | Pre-immun serum | AMMY 1 | AMMY 2 | AMMY 3 | AMMY 4 | AMMY 5 | AMMY 6 | AMMY 7 | AMMY 8 | AMMY 9 | AMMY 10 | AMMY 11 | AMMY 12 | AMMY 13 |
|---------------------|-----------------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|
| 1:1000              | 0               | 0      | 0      | 9      | 9      | 12     |        |        |        |        |        |        |        |        |
| 1:500               | 0               | 0      | 0      | 11     | 17     | 18     |        |        |        |        |        |        |        |        |
| 1:250               | 26 (16.0)       | 32 (6.5) | 32 (6.8) | 33 (7.5) | 37 (5.3) |        |        |        |        |        |        |        |        |
| 1:100               | 30 (12.2)       | 30 (12.2) | 33 (10.4) | 40 (2.9) | 44 (2.2) |        |        |        |        |        |        |        |        |
| 1:30                | 36 (1.4)        | 36 (1.4) | 43 (10.8) | 53 (5.7) | 56 (2.5) |        |        |        |        |        |        |        |        |
| 1:10                | 38 (4.9)        | 38 (4.9) | 44 (4.6) | 52 (2.4) | 52 (10.9) |        |        |        |        |        |        |        |        |
|                     | 33 (6.1)        | 33 (6.1) | 40 (4.1) | 47 (3.3) | 49 (6.3) |        |        |        |        |        |        |        |        |
|                     | 35 (4.6)        | 35 (4.6) | 43 (10.1) | 53 (3.8) | 57 (5.0) |        |        |        |        |        |        |        |        |
|                     | 39 (9.7)        | 39 (9.7) | 52 (4.1) | 69 (2.5) | 73 (4.6) |        |        |        |        |        |        |        |        |
|                     | 31 (9.1)        | 31 (9.1) | 46 (8.2) | 50 (9.8) | 52 (7.9) |        |        |        |        |        |        |        |        |
|                     | 35 (6.4)        | 35 (6.4) | 50 (9.8) | 52 (7.9) | 52 (7.9) |        |        |        |        |        |        |        |        |
|                     | 43 (12.4)       | 43 (12.4) | 44 (4.6) | 52 (2.4) | 52 (10.9) |        |        |        |        |        |        |        |        |
|                     | 37 (3.4)        | 37 (3.4) | 44 (4.6) | 52 (2.4) | 52 (10.9) |        |        |        |        |        |        |        |        |
|                     | 37 (13.0)       | 37 (13.0) | 44 (4.6) | 52 (2.4) | 52 (10.9) |        |        |        |        |        |        |        |        |
|                     | 36 (13.0)       | 36 (13.0) | 44 (4.6) | 52 (2.4) | 52 (10.9) |        |        |        |        |        |        |        |        |
|                     | 36 (8.3)        | 36 (8.3) | 44 (4.6) | 52 (2.4) | 52 (10.9) |        |        |        |        |        |        |        |        |
|                     | 35 (9.9)        | 35 (9.9) | 44 (4.6) | 52 (2.4) | 52 (10.9) |        |        |        |        |        |        |        |        |
|                     | 38 (4.9)        | 38 (4.9) | 44 (4.6) | 52 (2.4) | 52 (10.9) |        |        |        |        |        |        |        |        |
|                     | 35 (9.7)        | 35 (9.7) | 44 (4.6) | 52 (2.4) | 52 (10.9) |        |        |        |        |        |        |        |        |
|                     | 25 (1.8)        | 25 (1.8) | 31 (9.1) | 35 (6.4) | 45 (2.9) | 55 (8.5) |        |        |        |        |        |        |        |
|                     | 30 (7.1)        | 30 (7.1) | 46 (8.2) | 50 (9.8) | 52 (7.9) | 52 (7.9) |        |        |        |        |        |        |        |
|                     | 25 (10.6)       | 25 (10.6) | 46 (8.2) | 50 (9.8) | 52 (7.9) | 52 (7.9) |        |        |        |        |        |        |        |
|                     | 27 (8.8)        | 27 (8.8) | 46 (8.2) | 50 (9.8) | 52 (7.9) | 52 (7.9) |        |        |        |        |        |        |        |
|                     | 27 (3.2)        | 27 (3.2) | 46 (8.2) | 50 (9.8) | 52 (7.9) | 52 (7.9) |        |        |        |        |        |        |        |

2.4. Adhesion inhibition assay of identified antigens

Recombinant plasmids capable of expressing the genes encoding the proteins corresponding to the antigens identified by mass spectrometry were synthesized by GenScript. The ability of the corresponding AMMY to recognize the recombinant protein expressed from these constructs was tested by Western blotting as described above.

Polyclonal rabbit serum against the recombinant protein was produced by immunizing rabbits intramuscularly with 75 μg protein in 100 μl PBS mixed with equal volumes of Titermax adjuvant on days 0 and 14. Antibody titers were measured by standard indirect ELISA, and cytodeherence inhibition assay was performed as described above. Rabbits were used in accordance with the International Livestock Research Institute Institutional Care and Use Committee guidelines (IACUC Ref No. 2015-19).

2.5. Growth inhibition and latex agglutination

All AMMY mAbs were diluted 1:10, sterile filtered and 40 μl of each mAb was added into 2 wells of a 96 well round bottom microplate. Log phase culture of Mmm strain Afadé was diluted 1:625 (approximately 2.5 × 10^6 mycoplasmas/ml) in complete PPLO medium and 160 μl inoculated into each well of the microplate, and incubated at 37 °C for 72 h. PPLO without supplements, anti-Mmm strain Afadé rabbit serum and previously published anti-Mmm polysaccharides antibodies PK2 (Rurangirwa et al., 2000), 72/27.9/9, 72/16/. 2.14 and 72/18/11.7 (Kiarie et al., 1996) that inhibited growth of Mmm were used as controls. The mAbs that inhibited growth were then 10 fold serially diluted to determine the inhibition end point. To confirm whether the mAbs were mycoplasmacidal or mycoplasmastatic, a 50 μl sample was taken from the 72 h culture and inoculated onto PPLO agar, which was then incubated for 5 days at 37 °C and the concentration of the viable cells in the sample determined.

To further understand the mechanism of growth inhibition, a latex agglutination test was performed as previously described (Abdoel and Smits, 2007). Briefly 150 μl of 0.8 μm latex beads were mixed with 2.5 μg of anti-Mmm antibodies and the mixture incubated at room temperature for 2 h. The mixture was washed with PBST to remove excess antibody and the beads recovered by centrifugation at 15,000 x g for 3 min, and resuspended in 1 ml of 0.05% sodium azide. Thirty μl of Mmm whole cell lysate was placed on a glass slide and thoroughly mixed with 5 μl of the antibody and beads mixture (approximately 75 μg of beads), and then incubated for 10 min on a rocking shaker with gentle agitation.

3. Results

3.1. Inhibition of cytoadherence

Eighty hybridomas were cloned by limiting dilution from three fusions and 48/80 were positive on ELISA (results not shown).

Thirteen of the 48 antibodies were able to inhibit adhesion to BoLEC by at least 30% (Fig. S1). The 13 antibodies were renamed AMMY 1–13 and their ability to inhibit adhesion of Mmm strain Afadé to BoLEC was tested at varying dilutions (Table 1) and with 3 different strains of Mmm (Fig. 1). Mouse pre-immune serum and another mouse IgG mAb served as negative controls. At a dilution of 1:10, the inhibitory effect of the AMMY ranged from 36.73% to 73.17%.

The isotypes of the 13 AMMY were IgG2b (AMMY 1–10), IgG2a (AMMY 5, 7 and 8), IgG1 (AMMY 6 and 9) and IgM (AMMY 10). Schiek et al. (2016) characterized AMMY 10 as an Mmm-specific
capsular polysaccharide (CPS) antibody.

3.2. Antigen identification and cytoadhesion inhibition

Ten of the residual 12 mAbs were able to recognize Mmm proteins on Western blots. AMMY 1 and AMMY 8 recognized the same proteins with molecular weights of 223, 75 and 36 kDa, whereas AMMY 5 recognized two proteins with molecular weights 75 and 50 kDa. The rest of the AMMY antibodies appeared to recognize a protein of approximately 70 kDa (Fig. 2). An anti-GlpO mAb (Mulongo et al., 2013) was used as a positive control. Since the 70 kDa protein was detected in blots probed by most of the AMMY antibodies and the positive control, we concluded that this binding was not specific and the antibodies binding to this protein were not prioritized for further analysis.

The specificity of Western blot positive antibodies for proteins of Mmm among members of the closely related Mycoplasma mycoides cluster was assessed. AMMY 5 was found to recognize proteins present in all the subspecies tested (Fig. 3A) whereas AMMY 1 and AMMY 8 recognized proteins specific for Mmm only (Fig. 3B).

Using 2-DE and Western blotting, two groups of spots recognized by AMMY 5 (Fig. 4A and B) were detected. The first group (spots 1–4) had an apparent molecular weight of 75 kDa, the second group (spots 5–10) was found at about 50 kDa- both values corresponded well with the major immunoreactive bands detected in 1D gels (Fig. 2). As non-linear strips were used for electrofocusing, the exact pIs of the spots could not be deduced from the spot positions.

Eight spots (numbers 1, 4–10) were successfully analyzed by LC–MS/MS. All eight spots analyzed were identified as containing multiple proteins and the results were sorted according to total peptide scores, sequence coverage, number of unique peptides and frequency of protein detection within the immunoreactive spots (Suppl. Table 1). The most abundant proteins found in each spot are listed in Table 2.

The antigen most probably recognized by AMMY 5 was 2-oxo acid dehydrogenase acyltransferase, encoded by the pdhC gene (MSC_0267). MSC_0267 was identified as the most abundant protein in three spots, with comparably high numbers of unique peptides. In the additional two spots, MSC_0267 was the second or third most abundant protein (Suppl. Table 1).

AMMY 5 was shown to bind recombinant MSC_0267 on Western blot (Fig. 5B), confirming our identification above. Additionally, polyclonal rabbit serum raised against recombinant MSC_0267 inhibited adherence by 41% (Fig. 5C).

3.3. Growth inhibition

One out of thirteen mAbs (AMMY 10) was able to inhibit growth (Suppl. Table 2) at a dilution of 1:10,000 (Table 3) and when added to latex beads, agglutination was observed (Fig. 6). Moreover, when the Mmm and antibody mixture was inoculation on PPLO agar, growth was inhibited on confirming the mycoplasmacidal effect of AMMY 10.

4. Discussion

Antibodies that inhibit adhesion, whether by binding to the adhesive epitope or by steric hindrance, might detect Mycoplasma antigens that are vaccine candidates, as these would induce functional antibodies with a capacity to prevent adhesion and/or growth, and potentially colonization of the lungs. Thirteen AMMYs significantly reduced adherence of Mmm to BoLEC by 30–70%. These values were with in range reported in studies on inhibition of adherence of other
Fig. 3. Species specificity of anti-\textit{Mycoplasma mycoides} subsp. \textit{mycoides} (AMMY) monoclonal antibodies. The ability of AMMY monoclonal antibodies to recognize proteins from different subspecies of the \textit{M. mycoides} cluster was assessed. (A) AMMY 5 against different subspecies of the \textit{M. mycoides} cluster. (B) AMMY 8 against different subspecies of the \textit{M. mycoides} cluster. The \textit{M. mycoides} subspecies tested include \textit{M. mycoides} subsp. \textit{mycoides} (\textit{Mmm}) strains Afadé, B237, T144 and B66; \textit{M. mycoides} subsp. \textit{capri} (\textit{Mmc}) strains 80/93, 83/93 and 153/93; \textit{M. capri} subsp. \textit{capripneumonia} (\textit{Mcc}) strains Calf Kid and 7714; \textit{M. leachii} strains FRD42 and 4146. Membrane stained with \textit{Mmm} specific monoclonal antibodies raised against \textit{Mmm} strain Afadé (1:1000) and goat anti-mouse HRP conjugated (1:2500) (Sigma) and visualized by DAB.

Fig. 4. Two-dimensional electrophoresis and immunoblot analysis of \textit{Mycoplasma mycoides} subsp. \textit{mycoides}. Total cell lysate proteins of \textit{Mmm} strain Afadé (1 mg) were separated on pH 4–7 non-linear Immobiline\textsuperscript{TM} Dry Strips and 12.5% SDS–PAGE. (A) Stained with colloidal Coomassie G250. (B) Further analyzed by immunoblot. Membrane stained with \textit{Mmm} specific monoclonal antibodies raised against strain Afadé (AMMY 5, 1:1000) and goat anti-mouse alkaline phosphatase conjugated (1:2500) and visualized by BNIP/NCIT, and proteins in the molecular mass standard are indicated on the right of the gels.

Table 2

| Spot | Accession\textsuperscript{a} | Protein name | locus tag\textsuperscript{b} | gene name | MW [kDa] | calc. pI |
|------|-----------------------------|--------------|-----------------------------|-----------|----------|---------|
| 1    | D9QWT6                      | Dihydrolipoyl dehydrogenase | MSC\_0268 | lpdA       | 64.2     | 5.72    |
| 4    | D9QWTS\textsuperscript{c}  | 2-oxo acid dehydrogenase acyltransferase | MSC\_0267 | pdhC       | 45.8     | 6.67    |
| 5    | D9QV29                      | DNA gyrase subunit B          | MSC\_0006 | gyrB       | 71.5     | 5.94    |
| 6    | D9QX02                      | Aspartate–tRNA ligase          | MSC\_0333 | aspS       | 66.6     | 6.00    |
| 7    | D9QYF8                      | Glycerate mutase              | MSC\_0825 | gpmL       | 60.1     | 6.33    |
| 8    | D9QWS9                      | Pyruvate kinase               | MSC\_0261 | pyk        | 53.8     | 6.40    |
| 9    | D9QWTS\textsuperscript{c}  | 2-oxo acid dehydrogenase acyltransferase | MSC\_0267 | pdhC       | 45.8     | 6.67    |
| 10   | D9QWTS\textsuperscript{c}  | 2-oxo acid dehydrogenase acyltransferase | MSC\_0267 | pdhC       | 45.8     | 6.67    |

\textsuperscript{a} Uniprot accession number.

\textsuperscript{b} Genome sequence \textit{M. mycoides} strain PG1 (BX293980).

\textsuperscript{c} Most probable protein to AMMY 5.
(Schieck et al., 2016), had the greatest capacity to inhibit adhesion, inhibited growth and caused agglutination of Mmm lysate when coated with latex beads suggesting that this antibody does not block the adhesin-receptor interaction but rather agglutinates and kills the mycoplasmas hence rendering them unable to adhere. The role of anti-Mmm carbohydrate in inhibition of growth has been reported previously (Rurangirwa et al., 2000). AMMY 10 is an IgM antibody and thus is likely to be particularly effective in complement activation, and might contribute greatly to opsonization (Ding et al., 2013). Immunization of cattle with a capsular polysaccharide (CPS) formulated vaccine elicited antibody responses with titers similar to those elicited by the live vaccine and reduced pathology by 57% (Mwirigi et al., 2016). CPS plays a role in the capacity of Mmm to persist and disseminate in the infected host (March et al., 2000) further underscoring the potential of Mmm carbohydrate as a vaccine candidate.

Ten monoclonal antibodies bound to multiple protein bands on western blots. This could be because they bound to proteins having two or three isoforms with mass and/or charge differences indicating post-translational modification of the proteins (Jores et al., 2009), or because of repetitive sequences in the Mmm genome, resulting in proteins/protein coding genes of differing sizes that contain the same epitope (Westberg et al., 2004; Bischof et al., 2006).

The protein identified upon 2D-gel electrophoresis, 2-oxo acid dehydrogenase acyltransferase (Catalytic domain) (MSC_0267), is part of the pyruvate dehydrogenase (PDH) complex, which also contains pyruvate dehydrogenase (lipoamide) alpha chain (MSC_0265), pyruvate dehydrogenase (lipoamide) beta chain (MSC_0266) and dihydrolipoamide dehydrogenase (MSC_0268). In Mmm, the pyruvate dehydrogenase complex is an immunogenic protein located in the membrane (Krasteva et al., 2014) that is recognized by sera from experimentally infected cattle (Jores et al., 2009). The catalytic domain of the complex catalyzes the overall conversion of alpha-keto acids to acetyl-CoA and carbon dioxide, an irreversible step in the utilization of carbohydrate (Perham, 1991), suggesting that MSC_0267 plays a crucial role in the survival of Mmm in the host. The pyruvate dehydrogenase complex has been shown to mediate adhesion of M. pneumoniae to fibronectin (Dalło et al., 2002) and plasminogen (Thomas et al., 2013; Grundel et al., 2015), a very common component of eukaryotic cell surfaces and basement membranes. Antibodies to PDH were able to inhibit adherence of M. pneumoniae to fibronectin, but had little or no effect on adherence to human epithelial cells, leading the investigators in this study to speculate that the interaction of these proteins with host components is important in later stages of colonization. These include protection against the host’s immune response (Madureira et al., 2007; Feng et al., 2009), adherence to deeper layers of the epithelium with higher concentrations of extracellular matrix (ECM) molecules (Yavlovich et al., 2004; Grundel et al., 2016), and access of the bacteria to more favorable nutrient conditions. On the other hand, interaction with plasminogen may also increase bacterial adhesiveness. Plasminogen binds to bacterial cell surface receptors as well as to integrin molecules on eukaryotic cells and can thus also enhance bacterial adherence to host epithilia through a bridging mechanisms (Pluskota et al., 2004; Siemens et al., 2011; Sanderson-Smith et al., 2012). The process enhances surface-bound proteolytic activity that can enhance bacterial survival or spread within the host (Lähteenmäki et al., 2005). PDH is an example of a ‘moonlighting protein’. The proteins have more than one function, playing a role in essential cellular processes (canonical functions), and also an auxiliary role, such as binding to host cells and the ECM (moonlighting functions) (Kainulainen and Korhonen, 2014). In our study, polyclonal rabbit antiserum raised against MSC_0267 inhibited adhesion of Mmm to BoLEC by 41%. Further investigations may help to provide a more comprehensive picture of the mechanisms involved in the overall success of Mmm in colonizing the host respiratory tract. The studies described here suggest that MSC_0267, a component of the pyruvate dehydrogenase complex, may be a structural component of the adherence
and 3, and (++) very strong for sample 5.

system.

5. Conclusion

A panel of Mmm-specific monoclonal antibodies was developed and their ability to inhibit Mmm cyto-adherence to BoLEC and inhibit growth was assessed. The data suggest that the mechanism of Mmm adherence to BoLEC is complex, involving a variety of mycoplasmal antigens, as evidenced by the inhibitory effect of various antibodies. There were similarities to the mechanisms involved in the adherence of M. pneumoniae and M. bovis. Our approach allowed us to identify an anti-Mmm pdhC specific mAb (AMMY 5), which bound to an antigen that appears to play a vital role in cellular processes. We further described the adhesion and growth inhibitory capacity of AMMY 10, a previously characterized anti- Mmm CPS mAb. Antigens recognized by these antibodies could be vaccine candidate(s) that might induce antibodies that inhibit adhesion, growth and other important biological functions, and prevalent colonization of the lung by Mmm.

Competing interests

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary material related to this article can be found in the online version, at doi:https://doi.org/10.1016/j.vetimm.2018.09.002.

References

Abdel, T.H., Smits, I.L., 2007. Rapid latex agglutination test for the serodiagnosis of human brucellosis. Diag. Microbiol. Infect. Dis. 57, 123–128. Aye, R., Mwirigi, M., Frey, J., Jones, J., Pilo, P., Naessens, J., 2015. Cyto-adherence of Mycoplasma mycoides subsp. mycoides to primary bovine lung epithelial cells is a critical step in contagious bovine pleuropneumonia. BMC Vet. Res. 11, 27. Barile, M.F., Rottem, S., 1993. Mycoplasmas in cell cultures. In: Kabone, I., Adoni, A. (Eds.), Rapid Diagnosis of Mycoplasmas. Plenum, New York, pp. 155–193.

Fig. 6. Mycoplasma mycoides subsp. mycoides (Mmm) latex agglutination test. (1) Uncoated latex beads with Mmm total cell lysate. (2) Latex beads coated with Anti-Mmm rabbit serum plus Mmm total cell lysate. (3) Latex beads coated with unpurified Anti-Mmm (AMMY 10) plus Mmm total cell lysate. (4) Latex beads coated with control mouse IgM plus total cell lysate. (5) Latex beads coated with purified AMMY 10 plus Mmm total cell lysate. Agglutination was rated as (−) absent for samples 1 and 4, (+) strong for samples 2 and 3, and (++) very strong for sample 5.

and 3, and (++) very strong for sample 5.

system.

5. Conclusion

A panel of Mmm-specific monoclonal antibodies was developed and their ability to inhibit Mmm cyto-adherence to BoLEC and inhibit growth was assessed. The data suggest that the mechanism of Mmm adherence to BoLEC is complex, involving a variety of mycoplasmal antigens, as evidenced by the inhibitory effect of various antibodies. There were similarities to the mechanisms involved in the adherence of M. pneumoniae and M. bovis. Our approach allowed us to identify an anti-Mmm pdhC specific mAb (AMMY 5), which bound to an antigen that appears to play a vital role in cellular processes. We further described the adhesion and growth inhibitory capacity of AMMY 10, a previously characterized anti- Mmm CPS mAb. Antigens recognized by these antibodies could be vaccine candidate(s) that might induce antibodies that inhibit adhesion, growth and other important biological functions, and prevalent colonization of the lung by Mmm.

Competing interests

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary material related to this article can be found in the online version, at doi:https://doi.org/10.1016/j.vetimm.2018.09.002.

References

Abdel, T.H., Smits, I.L., 2007. Rapid latex agglutination test for the serodiagnosis of human brucellosis. Diag. Microbiol. Infect. Dis. 57, 123–128. Aye, R., Mwirigi, M., Frey, J., Jones, J., Pilo, P., Naessens, J., 2015. Cyto-adherence of Mycoplasma mycoides subsp. mycoides to primary bovine lung epithelial cells is a critical step in contagious bovine pleuropneumonia. BMC Vet. Res. 11, 27. Barile, M.F., Rottem, S., 1993. Mycoplasmas in cell cultures. In: Kabone, I., Adoni, A. (Eds.), Rapid Diagnosis of Mycoplasmas. Plenum, New York, pp. 155–193.

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Shevchenko, A., Tomas, H., Havli, J., Olsen, J.V., Mann, M., 2007. In-gel digestion for mass spectrometric characterization of proteins and proteomes. Nat. Protoc. 1, 2856–2860.

Shibata, K., Sasaki, T., Watanabe, T., 1995. AIDS-associated mycoplasmas possess phospholipases C in the membrane. Infect. Immun. 63, 4174–4177.

Siemens, N., Patenge, N., Otto, J., Friedler, T., Kreklumzeyer, B., 2011. Streptococcus pyogenes M49 plasminogen/plasmin binding facilitates keratinocyte invasion via integrin-integrin-linked kinase (ILK) pathways and protects from macrophage killing. J. Biol. Chem. 286, 21612–21622.

Song, Z., Li, Y., Liu, Y., Xin, J., Zou, X., Sun, W., 2012. α-Enolase, an adhesion-related factor of Mycoplasma bovis. PLoS One 7 (6), e38856.

Thomas, A., Sachse, K., Farnir, F., Dizier, I., Mainil, J., Linden, A., 2003. Adherence of Mycoplasma bovis to bovine bronchial epithelial cells. Microb. Pathog. 34 (3), 141–148.

Thomas, C., Jacobs, E., Dumke, R., 2013. Characterization of pyruvate dehydrogenase subunit B and enolase as plasminogen-binding proteins in Mycoplasma pneumoniae. Microbiology 159, 352–365.

Ueno, P.M., Timersotsky, J., Cantoraz, V.E., Wever, J.J., Cagle, M., Stein, M.A., et al., 2008. Interaction of Mycoplasma genitalium with host cells: evidence for nuclear localization. Microbiology 154 (10), 3033–3041.

Weldaregay, Y.B., Pich, A., Schiek, E., Liljander, A., Gicheru, N., Wesonga, H., Thiaucourt, F., Kiirika, L.M., Valentin-Weigand, P., Jores, J., Meers, J., 2016. Proteomic characterization of pleural effusion, a specific host niche of Mycoplasma mycoides subsp. mycoides from cattle with contagious bovine pleuropneumonia (CBPP). J. Proteomics 131, 93–103.

Westberg, J., Persson, A., Holmberg, A., Goessmann, A., Lundberg, J., Johansson, K.E., et al., 2004. The genome sequence of Mycoplasma mycoides subsp. mycoides SC type strain PG1T, the causative agent of contagious bovine pleuropneumonia (CBPP). Genome Res. 14, 221–227.

Yavinovich, A., Katzenell, A., Tzahis, M., Higazi, A.A., Rottem, S., 2004. Mycoplasma fermentans binds to and invades HeLa cells: involvement of plasminogen and urinease. Infect. Immun. 72, 5004–5011.

Zhang, Y., Zou, Y., Ma, P., Muhammad, H.M., Li, Y., Jiang, P., 2015. Identification of Mycoplasma suis MG1 interaction proteins on porcine erythrocytes. Arch. Microbiol. 197 (2), 277–283.