Identification of novel species-specific antigens of *Mycoplasma hyopneumoniae* by preparative SDS-PAGE ELISA profiling

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Mycoplasma hyopneumoniae, *M. hyorhinis* and *M. flocculare* are commonly isolated from the respiratory tract of pigs and are phylogenetically related. The identification and characterization of antigens specific for *M. hyopneumoniae* is crucial for the development of serological reagents and for understanding the mechanisms of pathogenicity of this pathogen. Protein and antigen profiles of six strains of *M. hyopneumoniae*, four strains of *M. hyorhinis* and a type strain of *M. flocculare* were compared using SDS-PAGE and immunoblotting. Five strains of *M. hyopneumoniae* originally isolated from diverse geographical regions produced similar protein and antigen profiles. One strain, C1735/2, produced a unique protein profile and was poorly immunoreactive, suggesting that some strains of *M. hyopneumoniae* may possess a structurally modified repertoire of antigens. Major *M. hyopneumoniae* antigens with molecular masses of approximately 36, 43, 48, 52, 76, 78, 80, 82, 94, 106, 114 and 200 kDa were identified by immunoblotting using hyperimmune pig sera raised against both high and low passage strains of *M. hyopneumoniae*. Porcine hyperimmune sera raised against the GDL type strain of *M. hyorhinis* reacted strongly with all *M. hyorhinis* strains although the profiles displayed considerable variation. Major antigens of molecular mass 42, 49, 52, 78, 80 and 82 kDa were identified in type strains GDL and BTS-7 and field strain 2; however, field strain 1 produced a unique profile. A preparative SDS-PAGE profiling (PPP) technique was developed which enabled quantification of the immunoreactivity of denatured antigens with porcine serum by ELISA. PPP facilitated the rapid identification of species-specific and cross-reactive antigens among the three mycoplasma species. PPP studies revealed several strongly immunoreactive *M. hyopneumoniae*-specific antigens of 43, 76, 94, 114 and 200 kDa as well as antigens of molecular mass between 52 and 62 kDa which were not apparent in immunoblotting studies. Rabbit monospecific anti-43 kDa serum reacted specifically with a 43 kDa antigen in whole cell lysates of geographically diverse strains of *M. hyopneumoniae* and failed to cross-react with *M. flocculare* or *M. hyorhinis* whole cell lysates. This study has identified a number of *M. hyopneumoniae*-specific antigens which warrant further investigation to determine their potential as diagnostic reagents and the role they play, if any, in pathogenicity.

Keywords: *Mycoplasma hyopneumoniae*, *Mycoplasma hyorhinis*, diagnostic antigens, preparative SDS-PAGE profiling, immunoblotting

INTRODUCTION

Mycoplasma hyopneumoniae, *M. hyorhinis* and *M. flocculare* are commonly found in the porcine respiratory tract. *M. hyopneumoniae* is the cause of porcine enzootic pneumonia (PEP), which imposes a severe economic burden on swine production. *M. hyorhinis* has been implicated as an agent of arthritis and polyserositis (Ross, 1992; Friis & Feenstra, 1994) whilst *M. flocculare* is generally regarded as non-pathogenic (Strasser *et al.*, 1992). DNA-DNA hybridization studies and 16S rRNA sequence analysis have demonstrated that these porcine mycoplasma species exhibit phylogenetic similarities and form a sub-cluster within the fermentans phylogenetic group (Stemke *et al.*, 1985, 1992). Further, more than 95% sequence homology exists between the 16S RNA sequences of *M. hyopneumoniae* and *M. flocculare*, indicating a close phylogenetic relationship between these species (Stemke *et al.*, 1992).

Serological cross-reactivity can be demonstrated between these three species using a variety of techniques including growth and metabolic inhibition assays (Friis, 1971), immunoelectrophoresis (Ro & Ross, 1983), complement fixation (Freeman *et al.*, 1984), immunoblotting (Bolske *et al.*, 1987; Young & Ross, 1987; Mori *et al.*, 1988; Thirkell *et al.*, 1991; Strasser *et al.*, 1992) and ELISA (Freeman *et al.*, 1984). The majority of these studies have focussed on antigenic differences between laboratory-adapted type strains of these three species. Earlier studies using growth and metabolic inhibition assays and the complement fixation test were unable to detect serological differences between the J type strain and several field strains of *M. hyopneumoniae* (Roberts & Little, 1970; Rose *et al.*, 1979). Ro & Ross (1983) compared eight field strains of *M. hyopneumoniae* by two-dimensional immunoelectrophoresis and noted some antigenic diversity but were unable to define the molecular masses of similar and variant antigens. Thus, little is known about the molecular identity of antigens amongst strains isolated from different geographical locations and maintained in different laboratories.

Several studies have demonstrated that increasing *in vitro* passage reduces the virulence of *M. hyopneumoniae*. Experimental challenge of pigs with low passage strains of *M. hyopneumoniae* induces lung lesions whilst inoculation with high passage J strain failed to cause disease (Tajima & Yagihashi, 1982; Zielinski & Ross, 1990). Low passage strains of *M. hyopneumoniae* specifically attach to cilia in porcine tracheal organ cultures inducing ciliostasis and loss of cilia, but attempts to reproduce these pathogenic effects using strains of *M. hyopneumoniae* which had undergone increasing *in vitro* passage either failed or were significantly diminished (DeBey & Ross, 1994). Furthermore, Ross & Young (1993) found that hyper-immune serum raised against a low passage virulent strain of *M. hyopneumoniae*, preabsorbed against a high passage avirulent strain, contained antibodies predominantly directed against several antigens with

molecular masses between 46 and 145 kDa. These antigens, which were specific to or more abundant in the low passage strain, may be important virulence antigens. Such studies suggest that strains of *M. hyopneumoniae* varying in their degree of laboratory passage may display a variable antigenic repertoire.

We have previously described an ELISA, based on a 43 kDa antigen, that detected serum antibodies against *M. hyopneumoniae* with a high sensitivity and specificity in naturally and experimentally infected pigs. This antigen was shown to be highly immunoreactive against porcine hyperimmune serum raised against *M. hyopneumoniae* and did not cross-react with anti-*M. hyorhinis* and anti-*M. flocculare* sera (Djordjevic *et al.*, 1994).

The aim of this study was to identify the repertoire of antigens amongst a small group of geographically diverse strains of *M. hyopneumoniae* using anti-*M. hyopneumoniae* antisera raised against both high and low passage strains of *M. hyopneumoniae* by SDS-PAGE and immunoblotting. In addition, anti-*M. hyopneumoniae*, anti-*M. hyorhinis* and anti-*M. flocculare* sera were used to determine the molecular masses of cross-reactive antigens between these three mycoplasma species and identify *M. hyopneumoniae*-specific antigens. Furthermore, we developed and evaluated a preparative SDS-PAGE profiling (PPP) technique which combined the advantages of the electrophoretic separation of proteins with the convenience and speed of ELISA to identify species-specific and cross-reactive antigens. PPP offered the potential to quantify the humoral immune response against the entire repertoire of mycoplasmal antigens, facilitating the identification of highly immunoreactive and species-specific serological reagents. Finally, we evaluated the diagnostic utility of monospecific antiserum raised to a 43 kDa antigen against whole cell lysates of strains of these three species of porcine mycoplasma by immunoblotting.

METHODS

Growth, preparation and identification of mycoplasma strains. The mycoplasma strains used in this study (Table 1) were obtained from the Australian Mycoplasma Reference Collection (AMRC), South Australian Department of Primary Industries, Central Veterinary Laboratory, Adelaide, Australia. Methods used to culture porcine mycoplasmas have been described by Sheldrake & Romalis (1992). Briefly, both field (low passage) and laboratory adapted (high passage) strains of *M. hyopneumoniae*, *M. hyorhinis* and *M. flocculare* were grown in modified Friis medium at 37 °C and harvested during the exponential phase when the medium became honey coloured and pH was in the range 7.2–6.9. Mycoplasma cells were harvested by centrifugation at 7000 *g* for 20 min and the pellet was washed three times with Tris-buffered saline (TBS: 10 mM Tris/HCl, 0.9 M NaCl; pH 7.4). Mycoplasma field isolates had been originally typed using immunodiffusion (Taylor-Robinson *et al.*, 1963) and growth-inhibition tests (Clyde, 1964) prior to storage in the AMRC. The identity of all strains of *M. hyopneumoniae* and *M. flocculare* type strain Ms42 was confirmed using species-specific PCR primers

Table 1. Mycoplasma strains used in this study

Species	Strain	Source*
<i>M. hyopneumoniae</i>	J (NCTC 10110)	A. Pointon
<i>M. hyopneumoniae</i>	232	T. Young (Bereiter <i>et al.</i> , 1990)
<i>M. hyopneumoniae</i>	YZ	M. Kobisch
<i>M. hyopneumoniae</i>	Beaufort	AMRC
<i>M. hyopneumoniae</i>	Sue	AMRC
<i>M. hyopneumoniae</i>	C1735/2	AMRC
<i>M. hyopneumoniae</i>	OMZ407	AMRC
<i>M. flocculare</i>	Ms42	AMRC
<i>M. hyorhinis</i>	Field strain 2	AMRC
<i>M. hyorhinis</i>	BTS-7	AMRC
<i>M. hyorhinis</i>	GDL	AMRC
<i>M. hyorhinis</i>	Field strain 1	AMRC

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(Stemke *et al.*, 1994) which amplified portions of the 16S rRNA gene of these two species.

Preparation of sera. Porcine hyperimmune sera were prepared against *M. hyopneumoniae* J strain, *M. hyopneumoniae* Beaufort strain, *M. hyorhinis* GDL strain and *M. flocculare* Ms42 strain as described by Sheldrake & Romalis (1992). Briefly, these strains were freshly grown, washed and harvested as described. Each strain of mycoplasma (1.0 g cell pellet, wet weight) was emulsified in Freund's complete adjuvant (total vaccine volume 2 ml) and 1 ml was administered in the neck and rump of each of two pigs for each strain of mycoplasma. One month later, 2 ml of a booster vaccine was prepared and administered as before (except that Freund's incomplete adjuvant was used). Animals were bled before vaccination (prebleed) and weekly after the second vaccination and the serum antibody response was monitored by immunoblotting and ELISA. Pigs were killed 4–6 weeks after the second vaccination and the serum was collected and stored at -20°C . Although minor variations in immunoblotting profiles were observed using sera collected from each animal vaccinated with the same antigen, antiserum that recognized the greatest number of antigens (determined by immunoblotting) was chosen for these studies.

Rabbit (New Zealand White) polyclonal antiserum raised against *M. hyopneumoniae* strain C1735/2 was prepared as described by Harlow & Lane (1988). Briefly, two rabbits were vaccinated on two occasions (one month apart) each with 3.5 mg of C1735/2 whole cells emulsified with Freund's complete adjuvant (2 ml total volume), injecting approximately 0.75 ml intramuscularly into each of three limbs (both hindlimbs and one forelimb) on each occasion. The second vaccination was identical to the first except that Freund's incomplete adjuvant was used. The serum anti-*M. hyopneumoniae* response was monitored weekly by immunoblotting and ELISA and the animals were exsanguinated 1 month after the booster vaccination. The serum was collected and stored at -20°C .

Preparation of SDS-PAGE columns. Mycoplasma cells (50–100 mg in 2 ml) were reduced in Laemmli mixture by boiling (Laemmli, 1970) and several crystals of bromophenol blue were added to dye the sample. Cell preparations were loaded onto either 5% or 9% polyacrylamide gel columns (Bioprep 491 cell, Bio-Rad), 8 cm or 12 cm in height respectively with a 1 cm stacking gel consisting of 4% polyacrylamide. Identical volumes (i.e. identical column heights) were used for the preparation of all polyacrylamide columns. Protein concentration was estimated using a BCA protein test kit (Pierce).

Columns were run at 40 mA constant current (150–300 V) for 24 h and fractions (9.5 ml) were collected immediately after elution of the bromophenol blue dye. The electrophoresis and elution buffers comprised SDS-PAGE buffer (Towbin *et al.*, 1979) and the flow rate was 0.53 ml min^{-1} . Fractions were stored at 4°C for several weeks or at -20°C for up to one year. Protein profiles detected either by SDS-PAGE or ELISA remained unchanged throughout storage. Molecular masses of peak fractions were estimated by SDS-PAGE and silver staining with known commercial molecular size markers.

SDS-PAGE and immunoblotting analysis. Column fractions (undiluted 30 μl aliquots from 9.5 ml fractions) were analysed by one-dimensional polyacrylamide gel electrophoresis under reducing conditions (Laemmli, 1970) in a Hoeffer mini-gel system. Protein samples were heated to 95°C for 5 min immediately prior to loading onto a 10% polyacrylamide resolving gel containing a 4% stacking gel. Electrophoresis was performed with constant current (20 mA) until the tracking dye (bromophenol blue) had migrated to the bottom of the resolving gel. Polyacrylamide gels were stained with either silver or Coomassie blue. Western blots were performed using a Hoeffer Trans-Blot as described previously (Djordjevic *et al.*, 1994). Molecular masses were calculated using pre-stained and unstained low range markers (Bio-Rad). Immunoblots were blocked using 5% skim milk in TBS (10 mM Tris/HCl, 0.9 M NaCl; pH 7.4), reacted with porcine hyperimmune sera (1:500 in TBS with 0.1% skim milk) and incubated with slow shaking at room temperature for 1.5 h. Immunoblots were reacted (1 h) with goat anti-swine Ig (H+L) horseradish peroxidase conjugate (Southern Biotechnics) (1:1000 in TBS with 0.1% skim milk). Blots were washed ($3 \times 5\text{ min}$) in Tris/HCl (100 mM, pH 7.6) prior to colour development with 3,3'-diaminobenzidine tetrahydrochloride (DAB) (0.05% in Tris/HCl, 100 mM, pH 7.6).

ELISA. Aliquots (10 μl) of the column fractions were loaded into 96-well ELISA plates (Nunc) and diluted 1:10 with carbonate loading buffer (30 mM sodium carbonate, 20 mM sodium hydrogen carbonate, pH 9.6) and left overnight in a humid chamber at 21°C . Plates were washed ($5 \times 400\text{ }\mu\text{l}$) with TW (Milli Q water/0.05% Tween 20) using a Titertek microplate washer. Primary antibody (hyperimmune sera against *M. hyopneumoniae* strains J, Beaufort, *M. hyorhinis* strain GDL and *M. flocculare* strain Ms42) was diluted 1:200 in 10% normal goat serum in PBST (phosphate buffered saline, pH 7.2 containing 0.05% Tween 20) and 100 μl was added to the wells. Plates were incubated for 2 h at 21°C and washed ($5 \times 400\text{ }\mu\text{l}$) as described above. Horseradish-peroxidase-conjugated goat anti-swine Ig (H+L) (Southern Biotechnics, 100 μl) diluted 1:1000 in 10% normal goat serum in PBST was added and the plates incubated at room temperature for 1 h. After a final washing step ($5 \times 400\text{ }\mu\text{l}$) colour development was achieved by the addition of 100 μl of 1 mM 2,2'-azinodiethylbenzthiazolinsulfonate (ABTS) diluted in citrate/phosphate buffer (pH 4.2) and incubated at room temperature until the desired colour developed. Absorbance was read at 405 nm (Titertek plate reader) when the colour

Fig. 1. SDS-PAGE (10%) of whole cell lysates of 11 porcine mycoplasma strains (40 µg per lane) stained with Coomassie blue. Molecular mass markers are indicated in kDa. Lane 1, *M. flocculare* strain Ms42; lanes 2–7, *M. hyopneumoniae* strains J, 232, Beaufort, Sue, OMZ407, C1735/2, respectively; lanes 8–11, *M. hyorhinis*, field strain 2, GDL, BTS-7, field strain 1, respectively.

development reached an absorbance for fractions containing the 43 kDa antigen approximately 10-fold higher than fractions containing only electrophoresis buffer (negative control). Anti-swine HRP conjugate did not cross-react with any PPP fraction in control experiments.

RESULTS

Whole cell protein analysis by SDS-PAGE

Whole cell extracts of *M. hyopneumoniae*, *M. hyorhinis* and *M. flocculare* were resolved by SDS-PAGE and the proteins stained with Coomassie blue. Electrophoretic profiles of six strains of *M. hyopneumoniae*, four strains of *M. hyorhinis* and a type strain of *M. flocculare* are shown in Fig. 1. *M. hyopneumoniae* strains J and 232 (both high passage laboratory adapted strains), Sue, Beaufort and OMZ407 (low passage strains) had very similar protein profiles (although none of the profiles were identical), with some variation evident in the region between 80 and 85 kDa. *M. hyopneumoniae* strain C1735/2 (low passage field strain) showed considerably greater variation compared with the other five strains, particularly in the 80–140 kDa region. *M. flocculare* type strain Ms42 produced a unique electrophoretic profile; however, many bands aligned at similar molecular sizes with those typical of *M. hyopneumoniae*. All four *M. hyorhinis* strains produced very similar electrophoretic profiles and were easily distinguished from the profiles representative of the other two mycoplasma species.

Antigenic analysis by immunoblotting

Homologous immunoreactivity. Immunoblot analysis using anti-*M. hyopneumoniae* hyperimmune serum prepared against a high passage, laboratory adapted strain J (Fig. 2a) and a low passage (< 5) Beaufort isolate (Fig. 2b) were used to probe whole cell extracts of the 11 porcine mycoplasmas described in Table 1. *M. hyopneumoniae* strains J, 232, Sue, Beaufort and OMZ407 produced similar profiles using both hyperimmune sera. Hyperimmune sera raised against the J strain produced immunoreactive bands with approxi-

mate molecular masses at 36, 43, 48, 52, 76, 78, 80, 82, 94 and 114 kDa (Fig. 2a, lane 1). Some *M. hyopneumoniae* strains lacked some of these immunoreactive bands.

Hyperimmune serum raised against the Beaufort strain identified antigens of approximate molecular mass 30 (J strain only), 36–38, 42 (weak), 43, 48, 65 (weak), 72 (weak), 76, 78, 80–82, 94, 106, 114, 200 kDa and a band greater than 200 kDa (Fig. 2b). Immunoreactivity against antigens of molecular mass greater than 85 kDa (particularly 94, 106, 114 kDa antigens) was more intense using the anti-Beaufort hyperimmune serum. Immunoreactive *M. hyopneumoniae* antigens in the molecular mass range of 34–36, 94, 106 and 114 kDa showed minor intra-species size variation; however, immunoblots probed with monoclonal or monospecific polyclonal antibodies against these antigens would be required to confirm these observations.

Antigens of *M. hyopneumoniae* strain C1735/2 reacted poorly with both hyperimmune anti-*M. hyopneumoniae* sera; these sera identified antigens with molecular masses of 43, 46–48 (weak), 78 and (anti-Beaufort antiserum only) 80 kDa (Fig. 2a, b). This antigen profile was more similar to the profile generated by whole cell antigens of *M. flocculare* strain Ms42. In an attempt to further characterize the antigen profile of strain C1735/2, rabbit hyperimmune anti-C1735/2 antiserum was raised and used to probe an immunoblot containing whole cell antigens of the six *M. hyopneumoniae* strains. A typical *M. hyopneumoniae* antigen profile showing antigens with approximate molecular masses of 36, 43, 48–52, 76, 78, 94 and 114 kDa was observed for all *M. hyopneumoniae* strains except C1735/2, which produced a smearing pattern without clearly defined banding (data not shown).

Heterologous immunoreactivity. *M. flocculare* antigens of molecular masses 43, 55 and 78 kDa cross-reacted with hyperimmune antisera raised against both the J and Beaufort strains (Fig. 2a and b, respectively, lanes 8) of *M. hyopneumoniae*. *M. flocculare* antigens of molecular masses 76, 114 and 200 kDa reacted faintly with the anti-Beaufort antiserum. *M. hyorhinis* antigens also

Fig. 2. Immunoblot analysis of 11 porcine mycoplasma strains. Whole cell lysates (20 µg per lane) were resolved on 10% SDS-polyacrylamide gels, transferred onto PVDF membrane and reacted with antisera raised against *M. hyopneumoniae* J strain

cross-reacted with both anti-*M. hyopneumoniae* sera recognizing 78 kDa antigens, and 85 kDa and approximately 200 kDa antigens reacting only with the anti-Beaufort antiserum. Pig anti-*M. flocculare* sera cross-reacted weakly with *M. hyopneumoniae* antigens of molecular masses 25, 28, 48 kDa and more strongly with a 52 kDa antigen (data not shown). Anti-*M. flocculare* sera also detected cross-reactive *M. hyorhinis* antigens of 48, 52 (strong) and 114 (extremely faint) kDa (data not shown).

Immunoreactive antigen profiles using porcine anti-*M. hyorhinis* hyperimmune serum

Fig. 2(c) shows an immunoblot containing whole cell mycoplasma lysates reacted with hyperimmune sera raised against *M. hyorhinis* type strain GDL. Strongly immunoreactive *M. hyorhinis* antigens were identified at 42, 49, 52, 78, 80 and 82 kDa. Weakly immunoreactive antigens were observed at 36, 44, 47, 85, 94, 106 and 114 kDa. This antigen profile was identical for both type strains GDL and BTS-7 (except for the 36 kDa antigen, which was identified only in strain BTS-7). *M. hyorhinis* field strain 2 displayed strongly immunoreactive antigens of molecular masses 49, 52, 80, and 85 kDa and weakly immunoreactive antigens of 44, 82, 84 and 114 kDa showing similarity with profiles typical of *M. hyorhinis* type strains BTS-7 and GDL. *M. hyorhinis* field strain 1 displayed strongly immunoreactive antigens of molecular masses 47, 49, 80, 82, and 83 kDa and weakly immunoreactive antigens of molecular masses 52 and 85 kDa, producing a unique profile. Although the four strains of *M. hyorhinis* display very similar protein profiles (Fig. 1) and cross-reactive antigen profiles with anti-*M. hyopneumoniae* sera (Fig. 2a, b), considerable antigenic heterogeneity is evident using anti-*M. hyorhinis* serum. Similar observations have been described for many other species of mycoplasma (Andersen *et al.*, 1987; Städtlander *et al.*, 1991; Poumarat *et al.*, 1994). Faintly immunoreactive antigens ranging in molecular mass from 50 to 78 kDa and differing in molecular size by approximately 2 kDa are evident in lanes containing all four *M. hyorhinis* strains (Fig. 2c). Further studies are required to determine the degree and significance of antigenic variability amongst strains of *M. hyorhinis*.

M. hyopneumoniae strains and *M. flocculare* type strain Ms42 failed to cross-react strongly with the anti-*M. hyorhinis* serum (Fig. 2c). An extremely weak cross-

(a), *M. hyopneumoniae* Beaufort strain (b), and *M. hyorhinis* GDL strain (c). For all blots: lanes 1–6, *M. hyopneumoniae* strains J, 232, Beaufort, Sue, C1735/2, OMZ407, respectively; lane 7, *M. hyorhinis* field strain 2; lane 8, *M. flocculare* Ms42; lanes 9–11, *M. hyorhinis* GDL, BTS-7, field strain 1, respectively. Asterisks identify the presence of faintly immunoreactive bands. An extremely faint cross-reactive *M. hyopneumoniae* antigen of 81 kDa is present in all six strains, but can only be observed in lanes 1 and 2 in (c), which contain whole cell lysates of *M. hyopneumoniae* strains J and 232.

Fig. 3. (a) Silver-stained 10% SDS-polyacrylamide gel of every third fraction eluted from a 9% SDS-polyacrylamide column. Fractions contained one to three bands. (b) Immunoblot of the same fractions eluted from a 9% SDS-polyacrylamide column resolved on a 10% SDS-polyacrylamide slab gel, transferred onto PVDF membrane and reacted with antisera raised against *M. hyopneumoniae* J strain.

reactive antigen (81 kDa) was observed in all *M. hyopneumoniae* strains.

Preparative SDS-PAGE profiling (PPP)

The BioPrep 491 SDS-PAGE column has been used previously for the electrophoretic separation and purification of preparative quantities of *M. hyopneumoniae* membrane proteins (Djordjevic *et al.*, 1994). Preliminary investigations showed that 9% polyacrylamide columns adequately separated antigens within a molecular mass range of 20–80 kDa and a 5% polyacrylamide column resolved high molecular mass antigens between 80 and 250 kDa. Fig. 3(a, b) shows that fractions (9.5 ml) eluted from a 9% SDS-PAGE column contained between one and three discernible antigens when resolved on a 10% polyacrylamide mini-gel stained with silver (Fig. 3a) or immunoblotted with hyperimmune serum against *M. hyopneumoniae* strain J (Fig. 3b). Analytical columns (28 mm internal diameter) produced well-resolved antigen profiles when loaded with approximately 50–100 mg of denatured mycoplasma whole cell antigen. Higher loading concentrations reduced the resolving capabilities of the column.

In an attempt to quantify the immunoreactivity of *M. hyopneumoniae* antigens we investigated the possibility

of adsorbing aliquots from each fraction onto 96-well ELISA plates and probing the antigens with anti-*M. hyopneumoniae* hyperimmune serum. Preliminary experiments with titrated *M. hyopneumoniae* whole cell lysate (prepared by boiling with reducing solution) bound to ELISA plates and reacted with hyperimmune serum revealed a linear relationship between antigen concentration and ELISA reactivity as measured by absorbance (data not shown). A *M. hyopneumoniae* strain J profile representative of the first 50 fractions which eluted from a 9% polyacrylamide column and reacted with hyperimmune serum raised to *M. hyopneumoniae* strain J is shown in Fig. 4(a). Immunoreactive peaks were evident in fractions 5 and 6, 9 and 10, 12–17, 21–33 and 40–47 which corresponded to molecular masses of approximately 36, 43, 48–62, 65–83 and 94 kDa. Molecular masses of antigens in peak fractions were determined using commercial molecular mass markers on SDS-polyacrylamide mini-gels stained with silver. The majority of these immunoreactive ELISA peaks were also detected on immunoblots (Fig. 2). Notable exceptions include the ELISA reactive antigens with molecular masses between 52 and 62 kDa, which were absent or appeared as extremely faint bands on immunoblots (Fig. 2a, b). Fig. 4(b) shows an SDS-PAGE ELISA profile of a 5% polyacrylamide column loaded with whole cell lysate of the J strain of *M. hyopneumoniae*. Immunoreactive peaks of molecular sizes 94, 106, 114 and 200 kDa are well resolved. These antigens were also detected by immunoblotting (Fig. 2), although the 200 kDa antigen is not usually recognized with hyperimmune sera raised against the J strain of *M. hyopneumoniae*.

Comparison of PPP of *M. hyopneumoniae*, *M. hyorhinis* and *M. flocculare* type strains against anti-*M. hyopneumoniae* J strain serum

Fig. 5 shows PPP, eluted from 9% (Fig. 5a) and 5% (Fig. 5b) polyacrylamide columns, respectively, of antigens of the three porcine mycoplasma type strains reacted with hyperimmune sera raised against the J strain of *M. hyopneumoniae*. Strongly immunoreactive *M. hyopneumoniae* antigens were identified with molecular masses of 43, 76, 94, 114 and 200 kDa which appeared specific for *M. hyopneumoniae*. Strong cross-reactivity was observed with *M. flocculare* antigens of molecular masses 36, 55 and 78 kDa. Interestingly, cross-reactivity of the 36 kDa antigen was not observed by immunoblotting (Fig. 2). Weak cross-reactivity was observed with *M. hyorhinis* antigens of molecular masses 55 and 78 kDa. PPP generated from mycoplasma antigens eluted from 5% polyacrylamide columns using the anti-strain J hyperimmune serum showed that the majority of the cross-reactivity was associated with the first three fractions (comprising antigens of molecular masses up to 80 kDa) which migrate just behind the dye front.

PPP studies using anti-*M. hyorhinis* serum identified cross-reactive *M. hyopneumoniae* J strain antigens of molecular masses 50–55, 65 and 74–78 kDa (data not

Fig. 4. PPP of *M. hyopneumoniae* J strain whole cell lysate showing ELISA reactivity (A_{405}) for each consecutive fraction that eluted from the column. Aliquots (10 μ l) of each fraction were coated onto a microtitre plate and reacted against hyperimmune serum raised to the J strain of *M. hyopneumoniae*. Panels (a) and (b) show PPP of the first 50 fractions eluted from 9% and 5% polyacrylamide columns respectively. The numbers on the profile indicate molecular masses (kDa).

Fig. 5. Superimposed PPP of 9% (a) and 5% (b) columns of whole cell lysates derived from type strains of *M. hyopneumoniae* (J, solid line), *M. hyorhinis* (GDL, dashed line), and *M. flocculare* (Ms42, dotted line) probed with hyperimmune serum raised against the J strain of *M. hyopneumoniae*. ELISA reactivity (A_{405}) was measured for each consecutive fraction that eluted from the column. Panels (a) and (b) show PPP of the first 50 fractions eluted from 9% and 5% polyacrylamide columns respectively. The numbers above the profile indicate molecular masses (kDa).

shown), whilst anti-*M. flocculare* serum identified weakly cross-reactive *M. hyopneumoniae* antigens with molecular masses of 50–55 and 65 kDa (data not shown). The basis for the identification of species-specific antigens by the absence of cross-reacting antigens in other mycoplasma species is based on an assumption that cross-reactive antigens of different molecular masses are unrelated. Further studies are required to confirm the species specificity of these antigens.

Purification and specificity of a 43 kDa *M. hyopneumoniae*-specific antigen

Although we have determined from immunoblotting and PPP studies that antigens of 36, 43, 76, 94, 114 and 200 kDa are specific for *M. hyopneumoniae*, we chose one of these antigens to further evaluate its diagnostic potential. The 43 kDa antigen was selected for two reasons: first, whilst PPP studies identified the 43 kDa

Fig. 6. Immunoblot analysis of 12 porcine mycoplasma strains using monospecific anti-43 kDa sera. Whole cell lysates (20 µg per lane) were resolved on 10% SDS-polyacrylamide gels, transferred onto PVDF membrane and reacted with rabbit anti-43 kDa serum raised against a putative *M. hyopneumoniae*-specific 43 kDa antigen. Lanes 1–7, *M. hyopneumoniae* isolates J, 232, YZ, Beaufort, Sue, OMZ407, C1735/2, respectively; lane 8, MS42 (*M. flocculare*); lanes 9–12, *M. hyorhinis* field strain 2, GDL, BTS-7, field strain 1, respectively.

antigen as an *M. hyopneumoniae*-specific antigen (Fig. 5), immunoblotting studies detected a cross-reactive 43 kDa antigen in a whole cell lysate of *M. flocculare* using anti-*M. hyopneumoniae* serum (Fig. 2); and second, we had previously used the 43 kDa antigen as an ELISA antigen to detect porcine serum antibodies against *M. hyopneumoniae* but had not determined the presence of this antigen amongst geographically diverse field and laboratory-adapted strains of this pathogen. Fraction 9 (from a single column run) containing the 43 kDa antigen was concentrated by ultrafiltration (Amicon, YM30) and emulsified with Freund's adjuvant for the generation of monospecific polyclonal antisera. Fig. 6 shows an immunoblot of whole cell lysates of 12 porcine mycoplasma strains reacted against rabbit anti-43 kDa serum. *M. hyopneumoniae* strain YZ (originating in France) became available in the latter stages of this study and was included in this experiment to test the specificity of the anti-43 kDa antiserum against as many *M. hyopneumoniae* strains from different geographic origins as possible. A strongly reactive band of molecular mass 43 kDa is present in the whole cell lysates of all *M. hyopneumoniae* strains but absent from lanes containing whole cell lysates of *M. hyorhinis* and *M. flocculare*. A faintly reactive band of approximate molecular mass 45 kDa is evident in lanes containing *M. hyopneumoniae* strains J (lane 1) and Sue (Lane 5) and may be due to antigenic size variation in these two strains.

DISCUSSION

Protein and antigenic variation among mycoplasmas has traditionally been studied using SDS-PAGE and immunoblotting techniques. However, these techniques do not readily facilitate quantitative comparisons between strains unless densitometric analyses are performed. ELISA is one of the most sensitive, versatile and rapid diagnostic assay formats and has been used to detect and quantify the immune response against mycoplasmal antigens (Ross & Young, 1993). SDS-PAGE, immunoblotting and various chromatographic matrices

have been used to identify and recover diagnostic mycoplasmal antigens for ELISA (Sheldrake & Romalis, 1992; Bolske *et al.*, 1987; Young & Ross, 1987; Mori *et al.*, 1988; Kazama *et al.*, 1989). Comparatively lengthy time periods are often required to run and monitor fractions eluted from chromatographic columns. Other limitations include difficulties with producing preparative quantities of desired antigen(s), and atypical chromatographic behaviour of some antigens in column matrices (Sheldrake & Romalis, 1992). In this communication we report the development of a preparative SDS-PAGE profiling technique which can rapidly identify and quantify the immunoreactive responses against the spectrum of whole cell mycoplasmal proteins, facilitating the identification of potentially useful diagnostic antigens. Using hyperimmune sera to profile whole cell lysates of *M. hyopneumoniae*, *M. hyorhinis* and *M. flocculare*, antigens of molecular masses 36, 43, 76, 94, 114 and 200 kDa were shown to be *M. hyopneumoniae*-specific, highly immunoreactive antigens. One of these antigens (43 kDa) was used to produce polyclonal antisera to confirm its specificity for *M. hyopneumoniae* in an immunoblot assay. Although the 43 kDa antigen has been shown in a previous study to (i) fail to cross-react with porcine hyperimmune anti-*M. hyorhinis* and anti-*M. flocculare* antisera and (ii) form the basis of a diagnostic ELISA for the detection of serum antibodies against *M. hyopneumoniae* (Djordjevic *et al.*, 1994), the utility of anti-43 kDa sera as a diagnostic antiserum for immunoblotting studies has not been addressed. Polyclonal antibodies raised against this antigen were able to detect a 43 kDa band on immunoblots containing whole cell lysates of the seven strains of *M. hyopneumoniae* described in this study and failed to cross-react with whole cell lysates of *M. flocculare* and *M. hyorhinis*.

The presence of species-specific and cross-reactive antigens among type strains of *M. hyopneumoniae*, *M. hyorhinis* and *M. flocculare* has been described previously in immunoblot studies (Bolske *et al.*, 1987; Young & Ross, 1987; Mori *et al.*, 1988; Thirkell *et al.*, 1991). Thirkell *et al.* (1991) described cross-reactive antigens between *M. hyopneumoniae* and *M. flocculare* of molecular mass 52, 58, 64, 74, 107 and 184 kDa whilst Young & Ross (1987) identified cross-reactive antigens of 41, 50 and 110 kDa. Mori *et al.* (1988) identified cross-reactive antigens in *M. flocculare* probed with anti-*M. hyopneumoniae* serum with molecular masses of 46 and 74 kDa. Similarly, Bolske *et al.* (1987) described a 73 kDa antigen common to all three mycoplasma species and 41 and 35 kDa antigens common to *M. hyopneumoniae* and *M. flocculare*. In this study we identified cross-reactive antigens in *M. flocculare* with molecular masses of 36, 55 and 78 kDa in PPP studies (Fig. 5) and of 43, 55 and 78 kDa in immunoblotting studies using anti-*M. hyopneumoniae* sera (Fig. 2). Although it is feasible that the 36, 43 and 78 kDa antigens described here are similar to those described in other studies, further experiments are required to confirm this. The molecular masses of these antigens

vary considerably between studies and this variation could be due to any one of a number of different experimental parameters including different sources (pig versus rabbit) and type of antisera (convalescent versus hyperimmune) and the different methods used to extract antigens. Strains of mycoplasma derived from various geographical locations with different degrees of *in vitro* passage could be expected to display different antigen profiles. In addition, minor differences in relative mobilities of antigens in different studies may be a function of the different molecular mass markers (stained versus unstained) used to estimate molecular mass. Finally, antigenic size variation has recently been described for an antigen from *M. hyopneumoniae* with a molecular mass of 97 kDa (Zhang *et al.*, 1995) and this phenomenon is likely to alter the relative molecular masses of some antigens amongst different strains of this species.

Considerable genomic heterogeneity has been reported among strains of *M. hyopneumoniae* recovered from different geographical locations in both Europe and the United States (Frey *et al.*, 1992; Artiushin & Minion, 1996) and considerably greater genomic variation was observed when comparing *M. hyopneumoniae* with *M. flocculare* (Frey *et al.*, 1992; Chan & Ross, 1984). Genomic heterogeneity is common among mycoplasma species and it is thought that mycoplasmas evolve rapidly (Razin *et al.*, 1983; Christiansen & Andersen, 1988; Ionas *et al.*, 1991; Frey *et al.*, 1992; Poumarat *et al.*, 1994; Artiushin & Minion, 1996). The fastidious nature of *M. hyopneumoniae*, its slow growth and the complex medium requirements needed to culture field strains are reflected by the paucity of studies comparing protein and antigen profiles of this species. We examined six strains of *M. hyopneumoniae* isolated from diverse geographical regions together with strains of *M. hyorhinis* and *M. flocculare* using SDS-PAGE and immunoblotting techniques. The electrophoretic profiles produced by *M. hyopneumoniae* strains J, Beaufort, Sue, 232 and OMZ407 are very similar. Strains J and 232 are high passage laboratory adapted strains originally isolated in Great Britain (Goodwin *et al.*, 1965) and the United States (Bereiter *et al.*, 1990) respectively, whilst OMZ407, Sue and Beaufort are field strains with a low passage history recovered from pneumonic lungs in pigs from Victoria, Australia. Only strain C1735/2 displayed a different protein profile compared with the other *M. hyopneumoniae* strains. Although the geographical origin of strain C1735/2 is unconfirmed the circumstances of its appearance in a PEP-free herd in Queensland, Australia, suggests that it may have originated from Canada (see below). One dimensional SDS-PAGE studies carried out on denatured whole cell lysates of other mycoplasmal species have not identified major intraspecies antigenic variation (Andersen *et al.*, 1987; Städtlander & Watson, 1992; Poumarat *et al.*, 1994).

In contrast, considerable antigen profile variability has been reported amongst mycoplasmas belonging to the same species (Andersen *et al.*, 1987; Städtlander *et al.*,

1991; Avakian *et al.*, 1991; Städtlander & Watson, 1992). Poumarat *et al.* (1994) observed marked differences in the antigenic profiles amongst 20 *Mycoplasma bovis* field strains belonging to both the same and different genomic groups. However, all *M. hyopneumoniae* strains reported in this communication (except C1735/2) produced very similar antigen profiles. The conservation of antigens amongst *M. hyopneumoniae* strains and a relatively high degree of genomic heterogeneity suggest that these antigens are evolutionarily conserved and may function in crucial biological roles important for the survival of the species.

Unlike the similarity of antigen profiles among strains of *M. hyopneumoniae*, the profiles generated by anti-*M. hyorhinis* antisera of the two field strains of *M. hyorhinis* were readily distinguishable from one another and from the profiles generated by type strains GDL and BTS-7. This result was unexpected as the electrophoretic profiles of whole cell lysates stained with Coomassie blue were very similar for all four *M. hyorhinis* strains and it suggests that considerable antigenic heterogeneity may exist amongst strains of this species. Although field strain 1 produced a unique antigenic profile against anti-*M. hyorhinis* strain GDL serum it was indistinguishable from the other three *M. hyorhinis* strains (with the exception of a high molecular mass antigen at approximately 200 kDa) when reacted against anti-*M. hyopneumoniae* sera. Consequently, immunoblotting may be a useful tool for the identification of field strains of *M. hyorhinis* and warrants further investigation, particularly in light of the studies performed by Gois *et al.* (1974), who described antigenic variation amongst seven strains of *M. hyorhinis* using a variety of immunological techniques. All four *M. hyorhinis* strains displayed marked antigenic size heterogeneity, producing a distinctive immunoreactive 'ladder' pattern on immunoblots. This antigen pattern has been reported for several species of mycoplasma including *M. hyorhinis* and is most likely indicative of size variation of immunoreactive membrane associated lipoproteins described by Rosengarten & Wise (1990).

M. hyopneumoniae strain C1735/2 is clearly unusual, displaying a unique protein profile, and is poorly immunoreactive against hyperimmune sera raised to either high or low passage strains of *M. hyopneumoniae*. C1735/2 was recovered from lung samples submitted by a piggery (previously free of PEP) in south-east Queensland, Australia, when a severe outbreak of PEP occurred soon after the importation of boar from Canada. Two mycoplasma isolates (C1735/5, C1735/2) were recovered from infected lung and the gel-diffusion test identified C1735/5 as *M. hyorhinis* whilst C1735/2 failed to give any reaction with anti-*M. hyorhinis* or anti-*M. hyopneumoniae* antisera. Strain C1735/2 was later identified as *M. hyopneumoniae* by the growth inhibition test and its species identity confirmed by PCR using *M. hyopneumoniae* specific primers (this study). Avakian *et al.* (1991) described strains of *Mycoplasma gallisepticum* that were poorly immunoreactive to anti-serum raised against another *M. gallisepticum* strain.

These observations suggest that some mycoplasma strains produce a structurally modified repertoire of antigens not recognized by some diagnostic antisera. Although hyperimmune sera failed to react strongly with C1735/2 antigens, rabbit anti-43 kDa antisera identified the 43 kDa antigen in a whole cell lysate of C1735/2, confirming the identity of this strain as *M. hyopneumoniae*. The poor immunoreactivity of strain C1735/2 highlights the limitations of gross serologically based typing methodologies and the requirement for more than one diagnostic antiserum to confirm species identity.

Whilst anti-*M. hyopneumoniae* J strain sera detected strongly immunoreactive *M. hyopneumoniae* antigens of molecular mass between 52 and 62 kDa in PPP experiments, these antigens are poorly reactive on immunoblots probed with the same antiserum. For both PPP and immunoblotting studies, antigens are denatured in reducing mixture (Laemmli, 1970) and separated by SDS-PAGE using the same buffer composition. Whilst proteins may partially renature during Western transfer (Bolske *et al.*, 1987) the conformational state of mycoplasma antigens dissolved in SDS-PAGE buffer, diluted in carbonate buffer and coated onto 96-well microtitre plates may be different compared with the same antigens used in immunoblotting studies. These differences may alter the repertoire of linear and conformational epitopes amongst different proteins, affecting the ability of some antibodies to bind to some antigens. Bolske *et al.* (1987) described different specificities of anti-*M. hyopneumoniae* serum for antigens which had undergone different preparation procedures likely to affect their degree of renaturation. Finally, antigens identified as cross-reactive species in immunoblotting studies are often of a different molecular mass to those described in PPP studies, which may also be a reflection of this phenomenon.

Recently, we described *M. hyopneumoniae* antigens of molecular masses of 76, 78, 80–82, 94, 106 and 114 kDa which afforded a significant reduction in lung pathology when administered as parenteral vaccines to pigs (Djordjevic *et al.*, 1996). Ultimately, the control of mycoplasma pneumonia will depend upon the development of reliable diagnostic reagents and a thorough understanding of the mechanism(s) of pathogenesis. Recently, we have cloned and characterized a gene encoding the 94 kDa *M. hyopneumoniae*-specific antigen described in this study. Hybridization studies have shown that the DNA encoding this antigen is present in all seven strains of *M. hyopneumoniae* described here yet fails to hybridize to genomic DNA from strains of *M. flocculare* and *M. hyorhinis* (data not shown). More importantly, the deduced amino acid sequence reveals the presence of tandemly arranged, proline-rich repeat motifs typical of bacterial and eukaryote binding proteins (Williamson, 1994). Clearly, further studies are required to characterize the *M. hyopneumoniae*-specific antigens of 36, 43, 76, 94, and 114 kDa described here to evaluate their diagnostic and prophylactic potential as a means of controlling mycoplasma pneumonia.

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