CURRENT MICROBIOLOGY Vol. 15 (1987), pp. 233–239

Current Microbiology

Species-specific Antigens of *Mycoplasma hyopneumoniae* and Cross-reactions with Other Porcine Mycoplasmas

Göran Bölske, Marie-Louise Strandberg, Katrin Bergström, and Karl-Erik Johansson

National Veterinary Institute, Uppsala, Sweden

Abstract. Cell proteins from the porcine mycoplasmas *Mycoplasma hyorhinis, M. hyopneumoniae,* and *M. flocculare* have been analyzed by SDS–gel electrophoresis and immunoblotting. The protein profiles of *M. hyopneumoniae* and *M. flocculare* were similar, but the protein profile of *M. hyorhinis* was quite different from the others. Antisera prepared against whole cells of the above three mycoplasmas were used in immunoblotting of electrophoretically separated antigens and in enzyme-linked immunosorbent assay. One major antigen, which had a molecular weight of 73 k, was found to be common to all three mycoplasmas. Another major antigen, with a molecular weight of 41 k, was common to *M. hyopneumoniae* and *M. flocculare* and may also be present in *M. hyorhinis*. Several antigens of comparatively high molecular weights (108 k, 102 k, 93 k, 89 k, and 87 k) seemed to be specific for *M. hyopneumoniae*. Three antisera prepared by immunization of rabbits with immunoprecipitates obtained by crossed immunoelectrophoresis of *M. hyopneumoniae* were also used in blotting experiments. One of these antisera was found to be directed against the 73 k antigen common to the three porcine mycoplasmas investigated. The other two antisera were directed against *M. hyopneumoniae*-specific antigens with molecular weights of 74 k, 58 k, 45 k, 44 k, and 38 k.

Mycoplasma hyopneumoniae is, among the mycoplasmas, the most important pathogen for swine and causes porcine enzootic pneumonia [27]. Serological cross-reactions among some of the porcine mycoplasmas (*M. hyopneumoniae, M. hyorhinis,* and *M. flocculare*) have been observed in ordinary serological methods [6, 24] as well as in enzyme-linked immunosorbent assay (ELISA) [3] and crossed immunoelectrophoresis (CIE) [23]. This imposes a serious obstacle to a reliable serodiagnosis of *M. hyopneumoniae* infection and may also interfere with identification of strains [3]. More specific antigens and antisera are thus needed for diagnostic purposes. Partially purified antigens from *M. hyopneumoniae* have, for instance, been used in ELISA [19]. Purified antigens from other mycoplasmas have been prepared by CIE [1] and line immunoelectrophoresis [9] in order to raise monospecific antisera. The former technique has also been used to analyze serologically cross-reacting antigens of *M. hyopneumoniae, M. hyorhinis,* and *M. flocculare* [6, 23]. Characteristic protein profiles can be obtained by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) of mycoplasma cell proteins [22], and this may be used for identification of porcine mycoplasmas [20, 21]. When blotting techniques were introduced for proteins (Western blotting), it became possible to perform immune assays on antigens separated by SDS–PAGE [25], which has also been utilized for mycoplasmas [13, 28].

We have identified common and species-specific antigens in the three porcine mycoplasmas by immunoblotting of electrophoretically separated antigens. Rabbits were immunized with excised immunoprecipitates for production of more specific antisera against *M. hyopneumoniae*. These antisera were characterized by different methods and used for further analysis of the antigens. The specificities of the antisera were found to be dependent on the assay method used.

Materials and Methods

Cultivation of mycoplasmas. Strains used for antigen preparation were *M. hyopneumoniae*, J, (received from the National Collection of Type Cultures, London); *M. flocculare,* Ms42 (received from N. F. Friis, Copenhagen, Denmark); and *M. hyorhinis,*

Address reprint requests to: Göran Bölske, National Veterinary Institute, Box 7073, S-75007 Uppsala, Sweden.

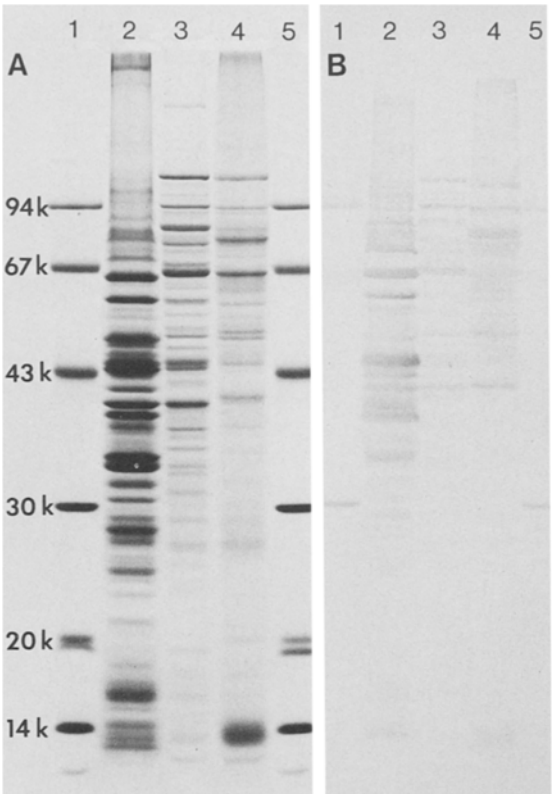

Fig. 1. Analysis of porcine mycoplasmas. The following samples were applied: (1) low molecular weight markers (LMW), (2) *Mycoplasma hyorhinis*, (3) *M. hyopneumoniae*, (4) *M. flocculare*, and (5) LMW. (A) The SDS–gel stained with Coomassie blue. (B) Staining of the NC membrane after electrotransfer and immune assay.

BTS-7 (received from FAO/WHO Collaborating Centre for Animal Mycoplasmas, Aarhus, Denmark). The two former were serially passaged in the appropriate medium 5–8 times before antigen production.

The medium used for cultivation was that of Friis [5] with the following modifications: 20% horse serum, but no swine serum, was used in broth for cultivation of antigens for serologic tests. In broth for cultivation of immunization antigen, 20% rabbit serum replaced horse and swine sera, and fresh rabbit meat infusion replaced brain–heart infusion broth and PPLO broth. Before use, the media were frozen and then incubated at 37°C for 24 h and finally filtered through 0.22-μm filters to remove media precipitates. Strains to be used as antigens were grown in 1.5 liters of medium with an inoculum of 100 ml. When the pH of the medium had dropped from 7.5 to 7.0, the culture was harvested by centrifugation, and the antigen was washed four times with phosphate-buffered saline (PBS). Purity and identity were controlled [4], and the quantity was estimated by viable count and protein determination [17].

Antisera. Antiserum against whole cells of *M. flocculare*, Ms42, produced in rabbits, was obtained from the FAO/WHO Collaborating Centre for Animal Mycoplasmas. Antisera against *M. hyopneumoniae*, J, and *M. hyorhinis*, BTS-7, cells were raised in rabbits according to the following immunization schedule: day 1, 2 ml Freund's complete adjuvant i.m.; day 6, 1 ml (0.8 mg protein) antigen + Freund's incomplete adjuvant i.p.; day 11, 1 ml antigen + Freund's incomplete adjuvant s.c.; day 15, 1 ml antigen + Freund's incomplete adjuvant i.m. and 1 ml antigen i.v.; and days 35, 39, 53, 54, 55, and 56, 1 ml antigen i.v. Blood was drawn on days 35, 42, and 64.

Antisera against specific antigens were prepared by immunization of rabbits with excised immunoprecipitates obtained after CIE of *M. hyopneumoniae* [9, 18]. Before immunization, rabbits were injected on days 1 and 5 with 0.3 ml Freund's complete adjuvant into the footpad of a hind leg in order to stimulate and enlarge the popliteal lymph node. On day 9, 0.2 ml saline suspension of immunoprecipitate mixed with 0.2 ml Freund's incomplete adjuvant was injected into the enlarged lymph node. This was repeated on days 11 and 15. On days 35, 36, and 38, 0.2 ml of saline-suspended antigen was administered i.m. Blood was drawn 1 week after the last injection.

Preparation of samples for electrophoresis. A sample cocktail containing sodium dodecyl sulfate (SDS) and dithiothreitol was added to the antigen samples [10]. If the samples were too viscous, DNase I (Sigma, St. Louis, MO, USA) was added to a final concentration of 20 μg/ml, and the samples were left on ice for 15 min or until the viscosity had been reduced. The samples were boiled for 3 min, clarified by centrifugation, and then applied to the polyacrylamide gel.

SDS–polyacrylamide gel electrophoresis. SDS–PAGE was performed as described by Laemmli [14], with some small modifications [10]. Pore gradient gels (10%–15%) with a thickness of 1.5 mm were used, and the electrophoresis experiments were performed in the GE-2/4 LS apparatus (Pharmacia, Sweden) at 100–150 V for 2500 Vh. Low molecular weight (LMW) markers (Pharmacia) were used as reference proteins for molecular weight (M_r) estimates [26]. The gels were stained with Coomassie blue.

Immunoblotting. Proteins were electrotransferred from the gels to nitrocellulose membranes (Bio-Rad, Richmond, CA, USA, 0.45 μm) in the Trans-Blot Cell (Bio-Rad) essentially as described [7]. But the double replica electroblotting technique was used to obtain two identical replicas from a single gel [11]. The protein profiles, transferred to the NC membranes, were visualized by staining with Amido black. The free binding sites of the NC membranes were quenched with 2% Tween 20 [8], which made it possible to stain them with Amido black also after the immune assay. Hyperimmune sera were diluted 100-fold in Tris–saline (pH 10.2), and the NC membranes were incubated in this solution on an oscillating table for at least 5 h [8]. Horseradish peroxidase-conjugated antirabbit immunoglobulins (Dakopatts, Denmark), diluted 2000-fold in PBS, were used as secondary antibodies. The NC membranes were incubated in this solution for 2 h, and the color was developed with 4-chloro-1-naphthol (Bio-Rad, HRP substrate) as detection reagent [7].

Immunoelectrophoresis. CIE [15] was performed with Tween-20-solubilized *M. hyopneumoniae* as described [12]. Immunoprecipitates were excised from the CIE plates with a scalpel before staining. Rocket immunoelectrophoresis (RIE) [16] was performed with the same buffer system and under the same conditions as used for the second dimension of the CIE experiments. Antigens were solubilized in Tween 20, and for *M. flocculare* also with sodium deoxycholate (DOC) [12].

Enzyme-linked immunosorbent assay. ELISA was performed according to Engvall and Perlman [2], but was carried out in microplates, Microelisa M 129 A (Dynatech, FRG). Antigens for ELISA were sonicated briefly (5 s), mainly to disrupt aggregates, diluted with PBS to 100 μg/ml, and kept frozen in aliquots at −70°C until used. For coating, the antigens were diluted in 0.05 *M* sodium carbonate buffer (pH 9.6) to optimal concentrations: 1.0 μg/ml for *M. hyopneumoniae* and *M. hyorhinis*, and 3.0 μg/ml for *M. flocculare*. Coating was performed at room temperature overnight. Antiserum was diluted in PBS (pH 7.3) with 0.05% Tween 20 in tenfold dilutions from 10^{-1} to 10^{-6} and was then incubated for 4 h at room temperature. Alkaline phosphate-conjugated antirabbit immunoglobulins (Dakopatts), diluted 1:2000, was applied overnight. The substrate *p*-nitrophenylphosphate was developed until the positive control gave a color reaction estimated to represent about 1.0 in absorbance. The absorbance was then measured at 405 nm with a spectrophotometer (Titertek Multiscan, Flow, McLean, Va, USA), and the absorbance values were normalized for deviation from 1.0 of the positive control in each plate. A homologous serum, diluted 1:1000, was used as a positive control. End-point titer was defined as the serum dilution giving an absorbance of 0.3 and was obtained from the absorbance-dilution plot of each serum.

Results

Cultivation. *Mycoplasma hyorhinis* and *M. hyopneumoniae* grew well in the above media, and the cells were harvested in logarithmic growth phase at pH 7.0 after 2–3 days of incubation. *Mycoplasma flocculare* could be serially passaged in the medium with horse serum but grew more slowly, and the final culture was harvested after 9 days of incubation. This culture also had lower yield (10^6 color-changing units/ml) than the others (10^7–10^8 color changing units/ml). In the medium with rabbit serum–rabbit meat infusion, *M. flocculare* grew poorly, and antigen for immunization was not produced from this mycoplasma.

Polyacrylamide gel electrophoresis. Figure 1A shows the result of an SDS–PAGE experiment of whole cell extracts from three porcine mycoplasmas. It was possible to detect about 75 protein bands within the M_r range 10–100 k in the three extracts. The protein profiles of *M. hyopneumoniae* and *M. flocculare* were very similar (sample lanes 3 and 4) although not identical, and many protein bands seemed to be in common or had at least the same M_r. The protein patten of *M. hyorhinis* (sample lane 2), however, was quite different from the others and could easily be distinguished. The M_r values of the different antigens must be regarded as apparent and might well differ in other electrophoresis systems.

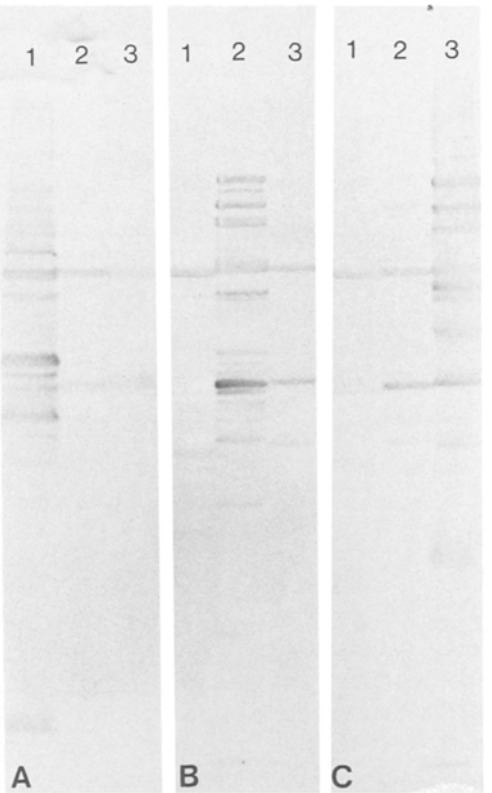

Fig. 2. Immune assay of NC membranes with the following samples applied: (1) *Mycoplasma hyorhinis*, (2) *M. hyopneumoniae*, and (3) *M. flocculare*. Antisera against the following mycoplasma were used as overlay: (A) *M. hyorhinis*, (B) *M. hyopneumoniae*, and (C) *M. flocculare*.

Figure 1B shows the protein profiles obtained after SDS–PAGE of the same samples as were analyzed in Fig. 1A and electrotransferred to an NC membrane. The high resolution of the protein profiles are well conserved, although small proteins (M_r < 20 k) were less efficiently retained on the NC membrane. Some of the LMW markers were also visible.

Immunoblotting with antisera raised against the different species. Results obtained when antigens prepared from the three porcine mycoplasmas were separated by SDS–PAGE and transferred to NC membranes are presented in Fig. 2. Antiserum against *M. hyorhinis* was used as overlay in Fig. 2A. The homologous reactions (left lane) can easily be distinguished from the heterologous ones, since more antigens (about 15) were visualized. But interestingly, two antigens present in both *M. hyo-*

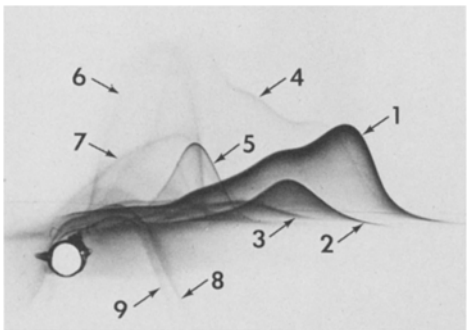

Fig. 3. Crossed immunoelectrophoresis of Tween-20-solubilized antigens of *Mycoplasma hyopneumoniae*. The immunoprecipitates obtained after staining with Coomassie blue were numbered 1–9, and the corresponding antigens are termed hp 1–9.

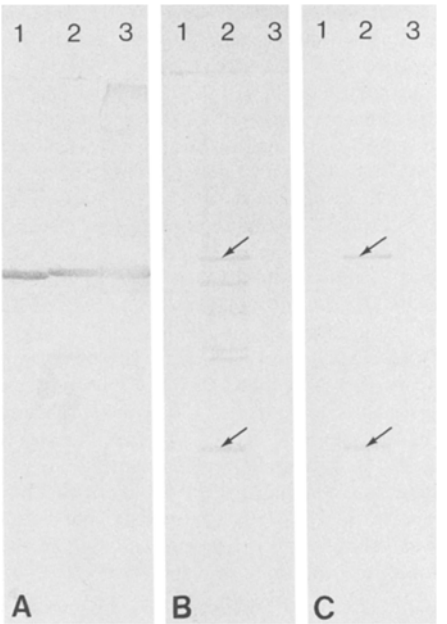

Fig. 4. Electroblotting experiments with immune assay. The following samples were applied: (1) *Mycoplasma hyorhinis*, (2) *M. hyopneumoniae*, and (3) *M. flocculare*. Antisera against the following antigens were used as overlay: (A) hp 1, (B) hp 5, and (C) hp 6.

pneumoniae and *M. flocculare*, with M_r of 73.3 k and 41.2 k, cross-reacted with antiserum against *M. hyorhinis*. These antigens, referred to as the 73 k and 41 k common antigens, are probably responsible for the serological cross-reactions occurring between some of the porcine mycoplasmas [3, 23, 24].

The result of a blotting experiment with antiserum against *M. hyopneumoniae* as overlay is shown in Fig. 2B. The homologous reactions (middle lane) are again easy to distinguish from the heterologous ones (lanes 1 and 3). Antiserum against *M. hyopneumoniae* cross-reacted strongly with the 73 k common antigen in both *M. hyorhinis* and *M. flocculare*, but only with the 41 k common antigen from *M. flocculare*. This observation agrees with the fact that *M. hyopneumoniae* and *M. flocculare* are serologically the two most closely related species. Furthermore, one or two antigens in *M. flocculare* showed a weak cross-reaction with antiserum against *M. hyopneumoniae*.

Results obtained when electroblots were overlaid with antiserum against *M. flocculare* are shown in Fig. 2C. The homologous reaction (right lane) can easily be identified. Both common antigens from *M. hyorhinis* and *M. hyopneumoniae* cross-reacted with antiserum against *M. flocculare*, although the reaction with the 41 k common antigen in *M. hyorhinis* was weak. It was evident from the SDS–PAGE experiment shown in Fig. 1A and from all of the blotting experiments shown in Fig. 2 that there are small differences in apparent M_r (about 1 k) among the 73 k antigens from the different species. The 73 k common antigen from *M. hyorhinis* was the smallest form, but for simplicity 73 k (average M_r) will be used for all three forms. Some antigens of rather high M_r (108, 102, 93, 89, and 87 k) were specific for *M. hyopneumoniae* (see Fig. 2B). Figure 2C also shows that three antigens (73, 41, and 35 k) present in *M. hyopneumoniae* cross-reacted with anti-*M. flocculare*, confirming the close relationship between these species.

Preimmune sera from the rabbits immunized with *M. hyorhinis* and *M. hyopneumoniae* were also used as antibodies in immunoblotting experiments, but the reactions were extremely weak in comparison with hyperimmune sera.

CIE of *Mycoplasma hyopneumoniae*. With CIE, nine precipitation lines were obtained when Tween-20-solubilized *M. hyopneumoniae* antigens were reacted with antiserum against whole cells of the organism (Fig. 3). Precipitation lines were numbered (hp 1–9) according to their relative speeds of migration in the first dimension of electrophoresis. Some of the immunoprecipitates were visible also before staining, and of those hp 1, 2, 5, and 6 were excised from the agarose plates and used for immunization of rabbits.

Immunoblotting with antisera raised against *Mycoplasma hyopneumoniae* immunoprecipitates. Figure 4 illustrates a blotting experiment with antigens

from the three porcine mycoplasmas (cf. Fig. 2) separated by SDS–PAGE and transferred to an NC membrane. The three antisera used as overlays were obtained by immunizing rabbits with the immunoprecipitates of *M. hyopneumoniae*. Anti-hp 1 was used as overlay in Fig. 4A, which reveals that hp 1 is identical with the 73 k common antigen, since it cross-reacted with antigens of the correct M_r, and the small difference in M_r between the different species is also confirmed. Anti-hp 5 and anti-hp 6 showed reactions only with *M. hyopneumoniae*-specific antigens, as illustrated in Fig. 4B–C. Anti-hp 6 reacted with two antigens (M_r 74.1 k and 37.6 k). Anti-hp 5 reacted with the same two antigens and in addition three other antigens (M_r = 58 k, 45 k, and 44 k). No appreciable reactions were obtained with the preimmune sera corresponding to anti-hp 1, anti-hp 5, and anti-hp 6.

Analysis of antisera against *Mycoplasma hyopneumoniae* antigens by RIE. Results obtained by RIE with antigens prepared from three porcine mycoplasmas and antisera prepared by injection of rabbits with CIE-separated *M. hyopneumoniae* antigens are shown in Fig. 5. The *M. flocculare* antigens were dissolved in both Tween 20 and sodium deoxycholate (DOC), since these antigens seemed to be less soluble than the others. When anti-hp 1 was used, the homologous reaction (with *M. hyopneumoniae*) was very strong (Fig. 5A) and resulted in one heavily stained immunoprecipitate. Cross-reactions with both *M. hyorhinis* and *M. flocculare*, but not with medium components, were obtained with this antiserum. Two immunoprecipitates were obtained with anti-hp 2 in the homologous reaction, and one weak precipitate with *M. flocculare* antigens. There were no cross-reactions with *M. hyorhinis* or medium components. Two immunoprecipitates were also obtained with anti-hp 5 in the homologous reaction but not in the heterologous ones. One diffuse immunoprecipitate was obtained with *M. hyopneumoniae* against anti-hp 6.

Determination of antiserum titers by ELISA. The end-point titers for the antisera with the three porcine mycoplasmas as antigens are shown in Table 1. To facilitate the comparison among different antigens, each reaction is compared with the homologous reaction with anti-whole-cell serum. Antiserum against hp 2 is not included, since no antibodies could be detected by ELISA. The comparison is expressed as a percentage of the homologous reaction. For anti-hp 1 the homologous reaction (against

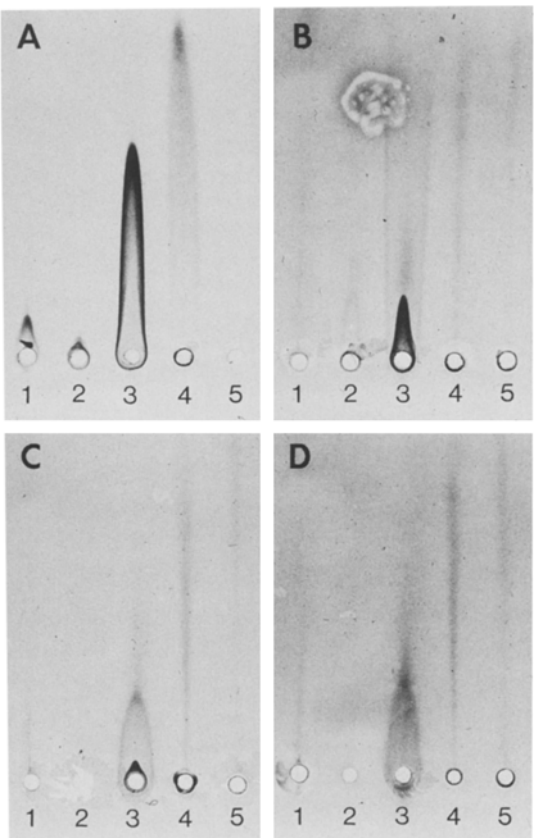

Fig. 5. Rocket immunoelectrophoresis of detergent-solubilized antigens from porcine mycoplasmas. The following samples were applied, from the left, on all plates (A–D): DOC-solubilized *M. flocculare* antigens, Tween-solubilized *M. flocculare* antigens, Tween-solubilized *M. hyopneumoniae* antigens, Tween-solubilized *M. hyorhinis* antigens, and Tween-solubilized precipitate from the growth medium. The following antisera were used: (A) anti-hp 1, (B) anti-hp 2, (C) anti-hp 5, and (D) anti-hp 6.

M. hyopneumoniae) is strong, but it is weak for anti-hp 5 and anti-hp 6. With *M. flocculare* as antigen, the titers are low for all antisera against immunoprecipitates. This implies good specificity for anti-hp 1, but for anti-hp 5 the titer reduction is questionable, and for anti-hp 6 there is no reduction. With *M. hyorhinis* as antigen, there is a titer reduction with all antisera against immunoprecipitates.

Discussion

The serological analysis methods used in this work differ in some important respects. First, in ELISA, a whole-cell antigen is used under non-denaturing conditions. All epitopes are probably not accessible in such an antigen, since it has not been dissolved.

Table 1. Comparison of antisera with ELISA

Antiserum against	Antigen					
	M. hyopneumoniae		*M. flocculare*		*M. hyorhinis*	
	Reciprocal titer[a]	%[b]	Reciprocal titer	%	Reciprocal titer	%
M. hyponeumoniae	40,000	100	890	8.9	2,800	25
M. flocculare	5,000	12.5	10,000	100	700	6.4
M. hyorhinis	800	2.0	<10	<0.10	11,000	100
hp 1	8,900	22	40	0.40	450	4.1
hp 5	180	0.45	30	0.30	<10	<0.09
hp 6	200	0.50	90	0.90	<10	<0.09

[a] Reciprocal of dilution giving an absorbance of 0.3.
[b] Percent of homologous reciprocal titer.

It seems likely that only those epitopes which are exposed on the surface of the cells are important in ELISA as used here. Second, RIE is also performed under non-denaturing conditions, but the antigens have been dissolved with a neutral detergent, and insoluble antigens were removed by centrifugation. Therefore, RIE will detect only antigens that are soluble under the conditions used and, furthermore, the antigens must have at least three epitopes accessible for antibody binding. Otherwise, an immunoprecipitate will not be formed. Finally, in immunoblotting the antigens are first solubilized and separated by SDS–PAGE under denaturing conditions. During the blotting procedure, the antigens are at least partly renatured, and a prerequisite for detection with antibody probes is that at least one epitope must have attained the native conformation on the nitrocellulose membrane.

It is not surprising that the antisera against various *M. hyopneumoniae* antigens show different degrees of specificity depending on the analysis method used. The highest specificity for *M. hyopneumoniae* was obtained with anti-hp 5 and anti-hp 6 in immunoblotting. Anti-hp 1 cross-reacted with the three porcine mycoplasmas in RIE and immunoblotting. Contrary to this, anti-hp 1 showed less cross-reactions in ELISA. Anti-hp 5 and anti-hp 6 did not cross-react with *M. hyorhinis*, but probably with *M. flocculare* in ELISA. It should also be remembered that the immunoprecipitates hp 5 and hp 6 are very close in the immunoprecipitation pattern, and there is a great risk that they will become cross-contaminated upon excision from a CIE plate.

For none of the antisera against immunoprecipitates was there complete specificity in all tests, and the practical application is, therefore, connected with the assay method used. Anti-hp 1 may be used to detect specific antibodies in a competitive ELISA system with antigen preparation of whole cells as in this study. Anti-hp 5 and anti-hp 6 may be used for identification in immunoblotting or RIE or serve as markers for specific bands in electrophoresis and immunoblotting as an aid to determine specificity of a serologic reaction.

ACKNOWLEDGMENTS

We thank Prof. Bengt Hurvell and Dr. Olof Söderlind for many valuable discussions and Dr. David Eaker for the linguistic revision. Financial support has been obtained from the board of the National Veterinary Institute and from the Magnus Bergvall Foundation.

Literature Cited

1. Alexander AG, Kenny GE (1980) Characterization of the strain-specific and common surface antigens of *Mycoplasma arginini*. Infect Immun 29:442–451
2. Engvall E, Perlman P (1972) Enzyme-linked immunosorbent assay, ELISA. III. Quantitation of specific antibodies by enzyme-labelled anti-immunoglobulin in antigen coated tubes. J Immunol 109:129–135
3. Freeman MJ, Armstrong CH, Sands-Freeman LL, Lopez-Osuna M (1984) Serological cross-reactivity of porcine reference antisera to *M. hyopneumoniae*, *M. flocculare*, *M. hyorhinis* and *M. hyosynoviae* indicated by the enzyme-linked immunosorbent assay, complement fixation and indirect hemagglutination tests. Can J Comp Med 48:202–207
4. Freundt EA, Ernø H, Lemcke, RM (1979) Identification of mycoplasmas. In: Bergan T, Norris JR (eds) Methods in microbiology, vol 13. London: Academic Press, pp 391–406
5. Friis NF (1975) Some recommendations concerning primary isolation of *Mycoplasma suipneumoniae* and *Mycoplasma flocculare*. Nord Veterinaermed 27:337–339
6. Friis NF, Jensen PT (1984) Serological comparison of type

strains of porcine, bovine, and ovine mycoplasmas with atypical colony morphology. Acta Vet Scand 25:29–35
7. Haid A, Suissa M (1983) Immunochemical identification of membrane proteins after sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Methods Enzymol 96:192–205
8. Heegaard NHH, Bjerrum OJ (1987) Immunoblotting: general principles and procedures. In: Bjerrum OJ, Heegaard NHH (eds) Handbook of immunoblotting. Boca Raton FL: CRC Press (in press)
9. Johansson K-E (1983) Characterization of the *Acholeplasma laidlawii* membrane by electroimmunochemical analysis methods. In: Bjerrum OJ (ed) Electroimmunochemical analysis of membrane proteins. Amsterdam: Elsevier, pp 321–346
10. Johansson K-E (1987) Separation of antigens by analytical gel electrophoresis. In: Bjerrum OJ, Heegaard NHH (eds) Handbook of immunoblotting. Boca Raton FL: CRC Press (in press)
11. Johansson K-E (1986) Double replica electroblotting: a method to produce two replicas from one gel. J Biochem Biophys Methods 13:197–203
12. Johansson K-E, Wróblewski H (1983) Characterization of membrane proteins by crossed immunoelectrophoresis. In: Razin S, Tully JG (eds) Methods in mycoplasmalogy. New York: Academic Press, pp 257–267
13. Kenny GE, Cartwright FD (1984) Immunoblotting for determination of the antigenic specificities of antibodies to the *mycoplasmatales*. Isr J Med Sci 20:908–911
14. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685
15. Laurell C-B (1965) Antigen–antibody crossed immunoelectrophoresis. Anal Biochem 10:358–361
16. Laurell C-B (1972) Electroimmuno assay. Scand J Clin Lab Invest [Suppl 124] 29:21–37
17. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193:265–275
18. Martinsson K (1974) Preparation of antisera against different animal serum proteins, using cross-reacting specific anti-human sera. Zentralbl Vet Med [B] 21:302–306
19. Nicolet J, Paroz P, Bruggman S (1980) Tween 20 soluble proteins of *Mycoplasma hyopneumoniae* as antigens for an enzyme linked immunosorbent assay. Res Vet Sci 29:305–309
20. Nicolet J, Paroz PH, Kristensen B (1980) Growth medium constituents contaminating mycoplasma preparations and their role in the study of membrane glycoproteins in porcine mycoplasmas. J Gen Microbiol 119:17–26
21. Paroz PH, Krawinkler M, Nicolet J (1977) Verwendung der Polyacrylamid-Gel-Elektrophorese in der Diagnostik von Nutztier-Mykoplasmen. Zentralbl Vet Med [B] 24:668–677
22. Razin S, Rottem S (1967) Identification of *Mycoplasma* and other microorganisms by polyacrylamide-gel electrophoresis of cell proteins. J Bacteriol 94:1807–1810
23. Ro LH, Ross RF (1983) Comparison of *Mycoplasma hyopneumoniae* strains by serological methods. Am J Vet Res 44:2087–2094
24. Rose DL, Tully JG, Wittler RG (1979) Taxonomy of some swine mycoplasmas: *Mycoplasma suipneumoniae* Goodwin et al. 1965, a later objective synonym of *Mycoplasma hyopneumoniae* Mare and Switzer 1965, and the status of *Mycoplasma flocculare* Meyling and Friis 1972. Int J Syst Bacteriol 29:83–91
25. Towbin H, Staehlin T, Gordon J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci USA 76:4350–4354
26. Weber K, Osborn M (1969) The reliability of molecular weight determinations by dodecyl sulfate–polyacrylamide gel electrophoresis. J Biol Chem 244:4406–4412
27. Whittlestone P (1979) Porcine myoplasmas. In: Barile MF, Razin S (eds) The mycoplasmas, vol 2. New York: Academic Press, pp 133–176
28. Wise KS, Watson RK (1983) Monoclonal antibodies to *Mycoplasma hyorhinis* surface antigens: tools for analyzing mycoplasma–lymphoid cell interactions. Yale J Biol Med 56:623–629