

## Demonstration of cross-reactive antigens in F38 and related mycoplasmas by enzyme-linked immunosorbent assay (ELISA) and immunoblotting

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(Received 20 December 1984; accepted 28 January 1985)

### SUMMARY

The ELISA and an immunoblotting technique were used to study F38-type mycoplasmas – an important cause of contagious caprine pleuropneumonia – and a number of related mycoplasma species, subspecies, types or serogroups.

Two-way ELISA cross-reactivity was demonstrated between five mycoplasmas, namely strain F38, *Mycoplasma mycoides* subsp. *mycoides* (LC strain), *M. equigenitalium*, *M. primum* and bovine serogroup 7. In addition one-way cross-reactivity was demonstrated between F38 and each of the following mycoplasmas: *M. mycoides* subsp. *mycoides* (two SC strains), *M. mycoides* subsp. *capri*, and bovine serogroup L. F38 and *M. capricolum* did not cross-react.

Immunoblot analysis, unlike ELISA, revealed that F38 and *M. capricolum* were closely related. At least four major protein antigens were shared between F38, *M. mycoides* subsp. *mycoides* (SC and LC strains), *M. mycoides* subsp. *capri* and bovine serogroup 7. The ELISA cross-reactions (above) shown by *M. equigenitalium* and *M. primum* with each other, with F38 and with other mycoplasmas were not apparent by immunoblotting.

### INTRODUCTION

Contagious caprine pleuropneumonia is of great economic importance in many countries (McMartin, MacOwan & Swift, 1980). In Kenya, the F38-type mycoplasma is the main causative agent (MacOwan & Minette, 1976). Recently similar organisms have been isolated from goats in the Sudan (Harbi *et al.* 1981), North Africa (Perreau, 1981), and the Yemen (Bari, 1984); and their prevalence in other parts of the world needs to be studied.

Initial studies of the Kenyan F38 strain suggested that it might represent a new species (Ernø, Leach & MacOwan, 1979). Further characterization has shown that members of the F38-like group cross-react in serological tests (gel double diffusion, complement fixation, growth precipitation, growth inhibition, immunofluorescence) with several already established mycoplasma species, namely *Mycoplasma mycoides*

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subsp. *mycoides* (SC and LC strains), *M. capricolum*, *M. primatum*, *M. equigenitalium* and mycoplasmas of 'bovine serogroup 7' (MacOwan & Minette, 1976; Ernø & Salih, 1980; Ernø, 1983; Ernø *et al.* 1983).

DNA hybridization experiments suggested that F38-like mycoplasmas were closely related to, and possibly variants of, *M. capricolum* (Christiansen & Ernø, 1982). Two-dimensional electrophoresis of cell proteins indicated that the F38-like group was related to *M. mycoides* subsp. *mycoides*, *M. capricolum* and bovine serogroup 7 (Rodwell, 1982). Two-dimensional electrophoresis of acidic proteins showed, however, a close relationship with *M. capricolum* but only a distant one with *M. mycoides* subsp. *mycoides* and bovine serogroup 7 (Andersen, Christiansen, & Christiansen, 1984). On the other hand electrophoretic analysis showed that the F38-like organisms had isoenzyme patterns distinct from those of other mycoplasmas (Salih, Ernø & Simonsen, 1983). In cross-immunization tests in mice, F38-like mycoplasmas protected mice partially but not completely against challenge with a bovine serogroup 7 strain but failed to protect against an SC strain of *M. mycoides* subsp. *mycoides* (Kanyi Kibe & Smith, 1984).

The purpose of this study was to explore further the antigenic cross-reactions between the F38-like group and related mycoplasmas by means of the enzyme-linked immunosorbent assay (ELISA) and immunoblotting techniques.

## MATERIALS AND METHODS

### *Mycoplasma strains*

The F38-like group was represented by strains F38, G183/82 and G275/82. Other organisms used were: *M. mycoides* subsp. *mycoides* strains Blenheim (SC type), O goat (SC) and Y goat (LC); *M. mycoides* subsp. *capri* strain Smith 1423; *M. capricolum* (NCTC 10154); *M. equigenitalium* (NCTC 10176); *M. primatum* (NCTC 10163); bovine serogroup 7 (NCTC 10133); and bovine serogroup L (strain B144P). Full details of these strains were given by Kanyi Kibe & Smith (1984).

### *Antigen preparations*

Mycoplasma antigens for the ELISA and for SDS (sodium dodecyl sulphate) polyacrylamide-gel electrophoresis were obtained from mycoplasmas grown in BVF-OS broth (Turner, Campbell & Dick, 1935) supplemented with calf serum (Calf Serum No. 1; Wellcome Reagents Ltd) 10% (or 50% for the F38-like group) and bacterial inhibitors (penicillin 100 units/ml and thallium acetate 0.05%).

Cultures (500 ml) aged 4–7 days were harvested by centrifuging at 30000 *g* in a Superspeed 50 Ultracentrifuge (Measuring and Scientific Equipment Ltd, London) for 1 h at 4 °C. The pelleted mycoplasmas were washed three times in phosphate-buffered saline (PBS) pH 7.2 and resuspended in a 20 ml volume. The cells were disrupted ultrasonically by means of an Ultrasonic Power Unit (Measuring and Scientific Equipment Ltd) for 2 min in ice. The antigen preparations were then stored at –20 °C until used.

### *Preparation of antisera*

Rabbits (New Zealand White, *ca.* 3 kg) were hyperimmunized with living whole cultures grown for 3–4 days in broth that resembled BVF-OS except that (1) the

muscle, liver and serum used in its preparation were all obtained from rabbits, (2) pepsin 10 g/l was used for the digestion, and (3) glucose 0.5 %, yeast extract 0.1 %, glycerol 0.5 %, and a 20 % supplement of serum (50 % for F38-like mycoplasmas) were added. Initially each rabbit received three subcutaneous doses of 0.5 ml of culture emulsified with an equal volume of Freund's complete adjuvant, given simultaneously in three well-separated sites. Six weeks later each animal received by the intravenous route the first of eight doses (0.1, 0.1, 0.5, 0.5, 0.8, 0.8, 1.0 and 1.0 ml) of culture given every 3–4 days. Serum, collected 24 h before the initial injection and 10 days after the final one, was stored at  $-20^{\circ}\text{C}$ .

#### *Absorption of F38 antiserum*

Aliquots of hyperimmune serum were each absorbed with one of eight mycoplasma suspensions (one homologous and seven heterologous), a procedure that removes antibody to surface antigens (Nichols & Kenny, 1984). A 10 ml volume of antiserum, diluted 1 in 100 in PBS, was first absorbed with PBS-washed (three times) pelleted mycoplasmas from *ca.* 30 ml of BVF-OS culture. This was repeated four times, except that in the third, fourth and fifth absorptions the volumes of diluted antiserum were 5 ml, 5 ml and 3 ml respectively. The ELISA reactivity of the antiserum did not change significantly with the fifth absorption.

#### *Enzyme-linked immunosorbent assay (ELISA)*

Horseshoe peroxidase was conjugated with antibody to rabbit IgG, prepared in goats. The ELISA was performed essentially as described by Voller, Bidwell & Bartlett (1979). Optimal concentrations of antigens, sera and conjugate were determined by 'checkerboard titrations'. The wells in flat-bottomed micro-ELISA plates (M129A; Dynatech Laboratories Ltd) were each coated with 100  $\mu\text{l}$  of antigen preparation diluted optimally in coating buffer ( $\text{Na}_2\text{CO}_3$  1.59 g,  $\text{NaHCO}_3$  2.93 g,  $\text{NaN}_3$  0.2 g, distilled water to 1 l; pH 9.6). The plates were covered and placed in a humid chamber at  $4^{\circ}\text{C}$  overnight. They were then washed three times in PBS containing 0.05 % Tween 20 (PBS/T). Rabbit antisera diluted 1 in 100 in PBS/T were added in 100  $\mu\text{l}$  volumes to appropriate wells. The plates were incubated in a humid chamber for 2 h at room temperature and washed as before. Conjugate diluted 1 in 8000 in PBS/T was added in a volume of 100  $\mu\text{l}$  to each well and the plates were again incubated for 2 h at room temperature and washed three times. Freshly prepared enzyme substrate (ortho-phenylene diamine 40 mg and hydrogen peroxide 40  $\mu\text{l}$ , dissolved in citrate buffer 100 ml, pH 5.0) was added in a volume of 200  $\mu\text{l}$  to each well. After incubation for 15 min at room temperature, the reaction was stopped by the addition to each well of 50  $\mu\text{l}$  of 2.5 M HCl. Absorbance of the reaction mixture was determined at 490 nm by means of a MR 600 Microplate Reader (Dynatech Laboratories Ltd).

#### *Proteolytic treatment of ELISA antigens*

Antigen-coated plates were treated with trypsin (Difco) or pronase (BDH) in concentrations (mg/ml PBS) of 0.01, 0.1 and 1.0 for 1 h at  $37^{\circ}\text{C}$  as described by Minion, Brown & Cassell (1984). The plates were washed three times in PBS/T and an ELISA was then performed as before.

*SDS polyacrylamide gel electrophoresis (PAGE)*

Washed organisms were subjected to ultrasonic disruption as above and centrifuged at 10000 rev./min for 10 min to remove undisintegrated cells. The pellet was discarded and the supernate used as the antigen preparation for SDS PAGE. The antigen preparation was mixed with an equal volume of sample buffer (1% SDS, 1 mM EDTA, 25 mM-dithiothreitol, 50 mM Tris HCl, pH 6.8). Samples were heated at 100 °C for 2 min, cooled, and then layered on a running gel.

Slab-gel electrophoresis was performed by the method of Laemmli (1970) with a 10% running gel. An LKB Model 2117 Multiphor Slab Gel Unit (LKB Produkter AB, Bromma, Sweden) was used. Electrophoresis was carried out for 3–4 h at a constant current of 125 mA from a stabilized power supply (Model type V50–20D; Roband Electronics Ltd, England). Molecular weights were calculated from the standards cytochrome C (11700),  $\alpha$  chymotrypsinogen (25700), peroxidase (40000), albumin (68000), and IgG (160000).

*Electrophoretic blotting ('Immunoblotting')*

Proteins were transferred from SDS PAGE slab gels to nitrocellulose sheets (0.45  $\mu$ m pore; Bio-Rad Laboratories Ltd, Watford) by the method of Towbin, Staehelin & Gordon (1979). Briefly, running gels were laid upon wetted nitrocellulose sheets and then sandwiched between wetted filter paper. The gel sandwiches were placed in fine-mesh nylon cloth and suspended in a tank containing transfer buffer (25 mM Tris, 192 mM glycine, and methanol 20% v/v). Large electrodes were placed on either side of the sandwiched gel and a current of 12 V was applied for 24 h.

*Immunodetection of antigens on nitrocellulose*

The electrophoretic blots were soaked overnight at 4 °C in washing buffer (0.1 M Tris-HCl, pH 8.0, containing NaCl 0.9%, sheep serum 1% and Triton X 100 0.05%) and then incubated for 2 h with rabbit antiserum diluted 1 in 100 in modified washing buffer containing sheep serum 10% instead of the usual 1%. They were washed three times in washing buffer (10 min per wash) and incubated with conjugate (horseradish peroxidase-labelled antibody to rabbit IgG, prepared in goats) diluted 1 in 5000 in modified washing buffer.

After 2 h incubation at room temperature the blots were washed three times as before; and then, to detect the antigens, incubated with the substrate (3 mg diaminobenzidine and 3  $\mu$ l of hydrogen peroxide dissolved in 10 ml of 10 mM phosphate buffer, pH 6.8, containing 0.01 M EDTA). The blots were finally washed in distilled water to stop any further reaction.

## RESULTS

*Enzyme-linked immunosorbent assay*

A two-way cross-reaction was observed between F38, *M. mycoides* subsp. *mycoides* (Y goat), *M. equigenitalium*, *M. primatum* and bovine serogroup 7 (Table 1). One-way cross-reactions were also noted. Thus F38 antigen cross-reacted with serogroup L antiserum; and F38 antiserum with all the heterologous

Table 1. Cross-reactions between F38 and related mycoplasmas in the enzyme-linked immunosorbent assay

No.	Antigen Preparation		Reaction produced by antiserum* no.									
	From		1	2	3	4	5	6	7	8	9	10
1	F38 mycoplasma		++	-	-	+	-	-	+++	+++	++	++
2	<i>M. mycoides</i> subsp. <i>mycoides</i> (Blenheim; SC type)		+	++	++	-	-	-	++	++	+	++
3	<i>M. mycoides</i> subsp. <i>mycoides</i> (O goat; SC type)		+	++	++	-	-	-	+++	+	+	++
4	<i>M. mycoides</i> subsp. <i>mycoides</i> (Y goat; LC type)		+	++	+	++	+	+	+++	-	+	+
5	<i>M. mycoides</i> subsp. <i>capri</i>		+	++	+	++	++	++	+++	+	++	++
6	<i>M. capricolum</i>		-	-	-	-	-	++	++	+	+	++
7	<i>M. equigenitium</i>		+	-	-	-	-	-	++	+	-	+
8	<i>M. primatum</i>		+	-	-	-	-	-	+++	++	+	++
9	Serogroup 7 mycoplasma		+++	-	-	-	-	-	+++	+++	++	+++
10	Serogroup L mycoplasma		-	-	-	-	-	-	+++	+	-	++

\* All antisera were diluted 1 in 100 in PBS/T. ELISA absorbance values were corrected by converting the OD at 490 nm with the homologous antiserum to give an absorbance reading of 1.0 (Voller, Bidwell & Bartlett, 1979).

+ = OD 0.4-0.8; ++ = OD 0.8-1.2; +++ = OD 1.2-1.6; ++++ = OD > 1.6; - = OD < 0.4.

Table 2. *Effect of trypsin and pronase on the ELISA antigens of mycoplasma strain F38*

Antiserum*	Corrected ELISA readings* after treatment with indicated concentration (mg/ml) of trypsin or pronase†			
	0	0.01	0.01	1
Strain F38	1.0 (1.0)	0.81 (0.56)	0.75 (0.55)	0.27 (0.46)
<i>M. mycoides</i> subsp. <i>mycoides</i> (Blenheim)	0.32 (0.41)	0.07 (0.05)	0.02 (0.04)	0.02 (0.04)
<i>M. mycoides</i> subsp. <i>mycoides</i> (Y goat)	0.64 (0.50)	0.36 (0.26)	0.28 (0.27)	0.18 (0.16)
<i>M. equigenitalium</i>	> 2 (1.91)	1.50 (1.18)	1.19 (0.88)	0.30 (0.82)
<i>M. primatum</i>	1.48 (1.59)	1.14 (0.73)	0.84 (0.71)	0.57 (0.60)
Serogroup 7 mycoplasma	0.97 (1.05)	0.45 (0.30)	0.35 (0.28)	0.11 (0.24)
Serogroup L mycoplasma	> 2 (1.91)	0.59 (0.30)	0.36 (0.23)	0.09 (0.19)
Normal serum	0.02 (0.03)	0.01 (0.01)	0.01 (0.01)	0.01 (0.02)

\* Footnote as in in Table 1. † Pronase results are in parentheses.

antigen preparations except *M. capricolum* and serogroup L antigen. A relation of some kind was shown, therefore, between F38 and each of the other nine mycoplasmas except *M. capricolum*. In some instances reactions were stronger with heterologous than homologous antisera.

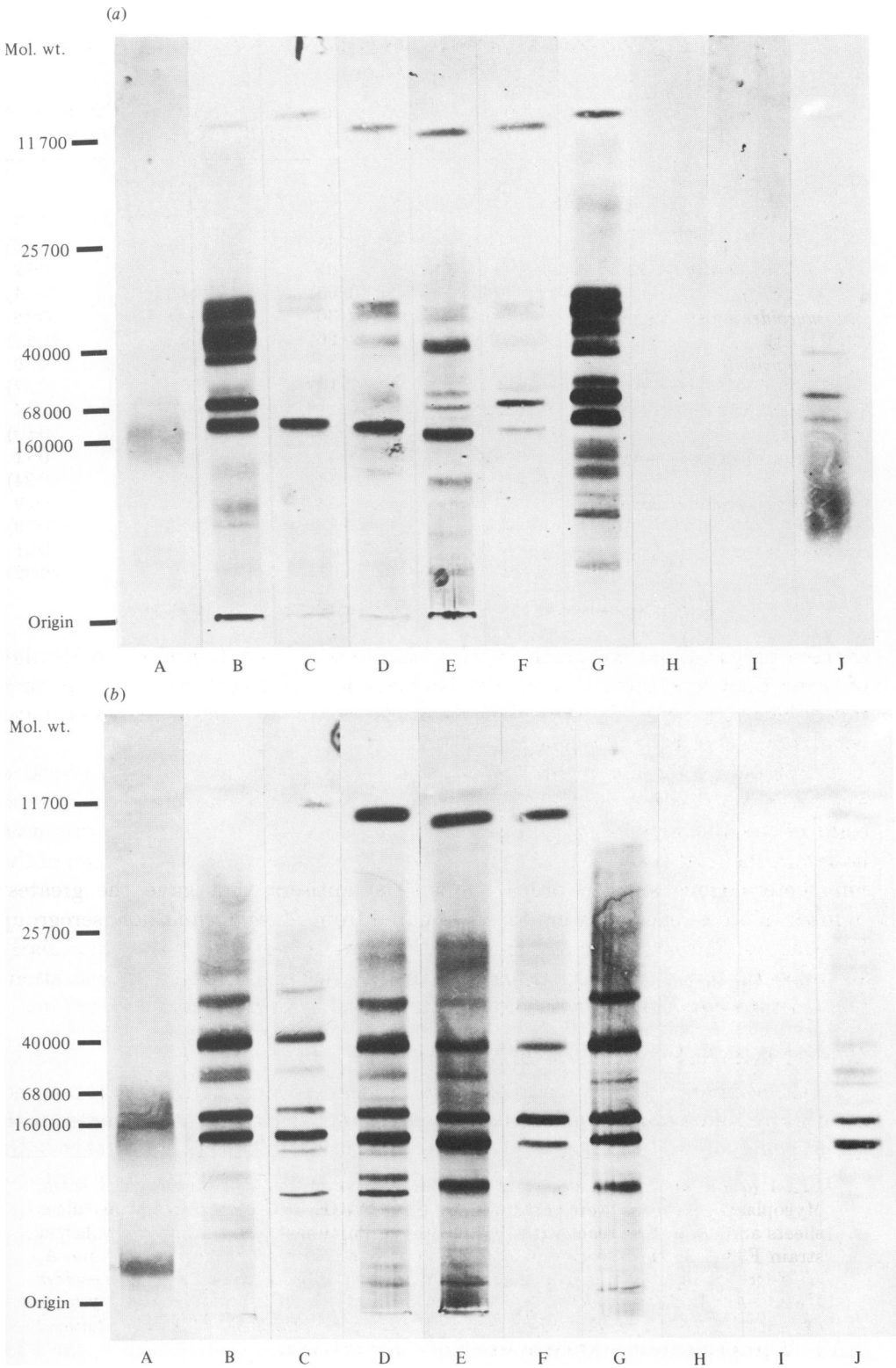
*M. mycoides* subsp. *capri* and *M. mycoides* subsp. *mycoides* (Y goat) provided the two most reactive antigen preparations, giving cross-reactions with at least eight of the nine heterologous antisera. *M. mycoides* subsp. *capri* antiserum was, however, the least reactive of the ten antisera, cross-reacting with only two of the nine heterologous antigen preparations. The antisera that gave the greatest number of cross-reactions were those prepared from *M. equigenitalium*, serogroup L, serogroup 7 and *M. primatum*; the two first-named often gave particularly strong reactions but, perhaps surprisingly, their corresponding antigen preparations reacted with only three or four of the nine heterologous antisera.

#### *Effect of proteolytic enzymes on ELISA antigens*

To throw light on the nature of the F38 antigens reacting with homologous and heterologous antisera in the ELISA, F38 antigen-coated plates were treated with

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Fig. 1 (*a* and *b*). Immunodetection of cross-reactive antigens of nine mycoplasmas. Mycoplasma antigens were separated by SDS-PAGE, transferred to nitrocellulose sheets and allowed to react with (Fig. 1*a*) hyperimmune serum against mycoplasma strain F38 and (1*b*) hyperimmune serum against serogroup-7 mycoplasma. Lane A, BVF-OS medium; B, mycoplasma strain F38; C, *M. mycoides* subsp. *mycoides* (Blenheim); D, *M. mycoides* subsp. *mycoides* (O goat); E, *M. mycoides* subsp. *mycoides* (Y goat); F, *M. mycoides* subsp. *capri*; G, *M. capricolum*, H, *M. equigenitalium*; I, *M. primatum*; and J, a bovine serogroup-7 mycoplasma.



**Fig. 1 (a and b).**

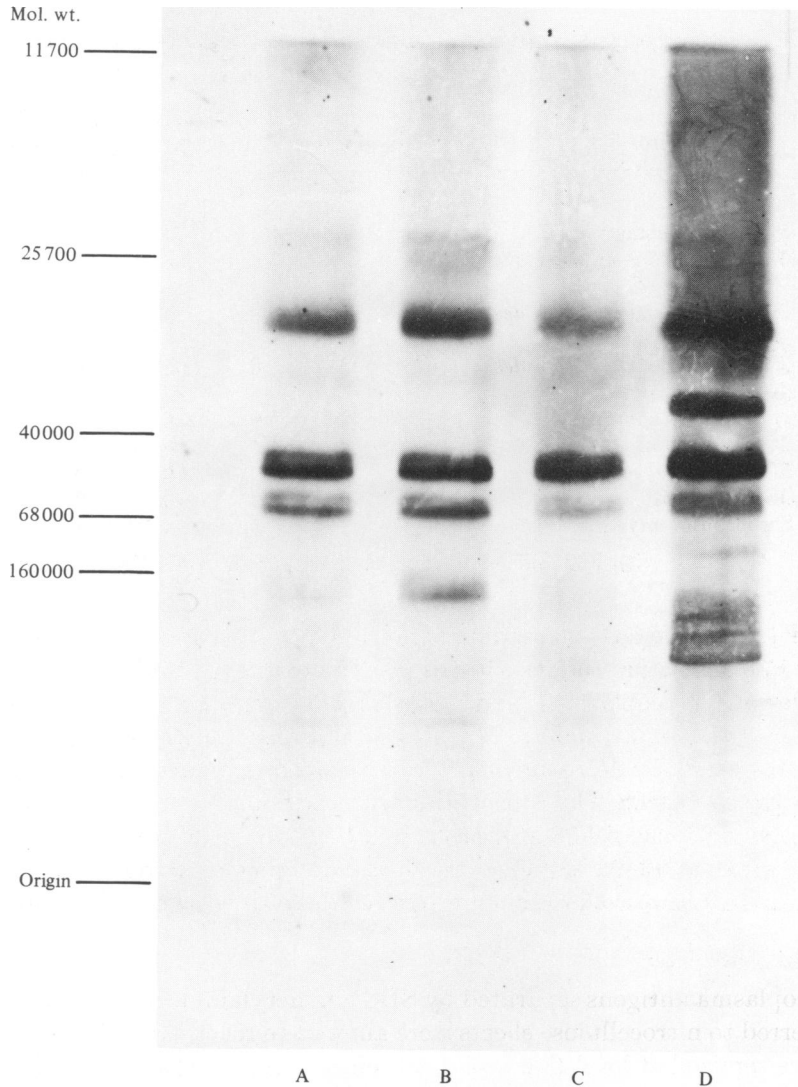


Fig. 2. Immunodetection of cross-reactive antigens in three F38-type mycoplasmas and *M. capricolum*. Mycoplasma antigens were separated by SDS-PAGE, transferred to nitrocellulose sheets and allowed to react with *M. capricolum* hyperimmune serum. Lane A, strain G275/82; B, G183/82; C, F38; D, *M. capricolum*.

one of three different concentrations of the proteolytic enzymes trypsin or pronase before being allowed to react with the various antisera. Table 2 shows that both enzymes reduced the antigenic reactivity of F38, strikingly for two antisera (*M. mycoides* subsp. *mycoides* strain Blenheim, and bovine serogroup L), moderately for one (bovine serogroup 7) and slightly for four (one homologous and three heterologous).

#### *ELISA reactivity of F38 antiserum absorbed with various mycoplasmas*

After complete absorption with each of eight mycoplasma suspensions, the F38 antiserum was further diluted in PBS/T to 1 in 100 and tested with F38 antigen



Table 3. Major protein antigens recognized by seven antisera in homologous and heterologous mycoplasmas

No.	Mycoplasma Designation	Number of major antigens demonstrated by antiserum no.						
		2	3	4	5	6	7	8
1	F38 mycoplasma*	4	3	4	4	4	0	0
2	<i>M. mycoides</i> subsp. <i>mycoides</i> (Blenheim: SC type)	9	3	4	5	5	0	0
3	<i>M. mycoides</i> subsp. <i>mycoides</i> (O goat; SC type)	9	6	6	5	6	0	0
4	<i>M. mycoides</i> subsp. <i>mycoides</i> (Y goat; LC type)	6	2	8	5	6	0	0
5	<i>M. mycoides</i> subsp. <i>capri</i>	5	2	6	6	5	0	0
6	<i>M. capricolum</i>	6	4	6	5	7	0	0
7	<i>M. equigenitalium</i>	0	0	0	0	0	5	0
8	<i>M. primatum</i>	0	0	0	0	0	0	3
9	Serogroup 7 mycoplasma*	4	1	3	2	4	0	0

\* Results with F38 and serogroup 7 antisera are shown in Fig. 1(a and b).

in the ELISA. The OD 490 reading of normal rabbit serum (unabsorbed) was 0.04, and of F38 antiserum (unabsorbed) 1.00. The readings obtained after absorption with various mycoplasmas were: F38 mycoplasma, 0.09; *M. mycoides* subsp. *mycoides* (Blenheim), 0.89; *M. mycoides* subsp. *mycoides* (Y goat), 0.92; *M. capricolum*, 0.94; *M. equigenitalium*, 0.27; *M. primatum* 0.22; serogroup 7, 0.11; and serogroup L, 0.08. Thus absorption with the homologous mycoplasma reduced the ELISA activity of F38 antiserum by 91%, and with four of the heterologous mycoplasmas by 73–92%. However, three heterologous mycoplasmas (Blenheim, Y goat and *M. capricolum*) failed to reduce the ELISA activity significantly.

#### Electrophoretic blots

Mycoplasma antigens separated by SDS polyacrylamide gel electrophoresis and transferred to nitrocellulose sheets were allowed to react with F38 antiserum. The latter recognized at least five major protein antigens, shown as bands in Fig. 1a, shared by the homologous mycoplasma and *M. capricolum*; and at least four shared by *M. mycoides* subsp. *mycoides* (one LC and two SC strains), *M. mycoides* subsp. *capri* and bovine serogroup 7. A major antigen (MW ca. 80000) and a fast-moving antigen (MW ca. 11500) were common to all the mycoplasmas except *M. equigenitalium* and *M. primatum*; and an antigen (MW ca. 37000), sometimes faintly staining was similarly distributed except that it was absent from the serogroup 7 mycoplasma. F38 antiserum recognized no antigens in *M. equigenitalium* and *M. primatum*, suggesting that cross-reactions previously revealed by other techniques were probably due to non-protein antigens.

In a second experiment the same electroblotted antigens were allowed to react with antiserum to the bovine serogroup-7 mycoplasma. This antiserum demonstrated two major protein antigens and at least five minor ones in the homologous mycoplasma, and at least 4–7 – often shared – in the heterologous mycoplasmas, excluding *M. equigenitalium* and *M. primatum*, both of which completely failed to react (Fig. 1b).

Seven further experiments of a similar kind (Table 3) were carried out with antisera to *M. mycoides* subsp. *mycoides* (strain Blenheim, O goat, Y goat), *M. mycoides* subsp. *capri*, *M. capricolum*, *M. equigenitalium* and *M. primatum*. Antisera to all except the two last-named demonstrated 6-9 major protein antigens in the homologous strain and 1-9 in all the heterologous mycoplasmas except *M. equigenitalium* and *M. primatum*. Antisera to *M. equigenitalium* and *M. primatum* demonstrated five and three major antigens respectively in the homologous mycoplasma, but neither antiserum reacted with any heterologous strain.

As described above, F38 and *M. capricolum* produced closely similar immunoblot patterns with F38 antiserum. In a final experiment *M. capricolum* antiserum was allowed to react with antigens of *M. capricolum*, F38 and two F38-like strains (G183/82 and G275/82). The antiserum demonstrated at least five major protein antigens in the homologous mycoplasma, three of which were shared by each of the F38-type mycoplasmas (Fig. 2).

#### DISCUSSION

The ELISA revealed a one- or two-way relationship between strain F38 and *M. mycoides* subsp. *mycoides* (SC and LC types), *M. mycoides* subsp. *capri*, *M. equigenitalium*, *M. primatum* and bovine serogroups 7 and L, but not *M. capricolum*. The lack of ELISA cross-reactivity between F38 and *M. capricolum* is interesting for several reasons. (1) The two organisms produced closely similar immunoblot patterns with F38 antiserum. (2) Whole cells of *M. capricolum*, like those of *M. mycoides* subsp. *mycoides* (SC and LC types), failed completely to absorb F38 antibody from serum, thus suggesting that the shared antigens revealed by immunoblotting were internal. (3) Vaccination of mice with F38 but not *M. capricolum* gave partial protection against challenge with bovine serogroup 7 (Kanyi Kibe & Smith, 1984). (4) DNA hybridization showed a close relationship between F38 and *M. capricolum* (Christiansen & Ernø, 1982).

F38, *M. equigenitalium* and *M. primatum* showed two-way ELISA cross-reactions with each other but, surprisingly, no cross-reaction by the immunoblotting technique. The ELISA cross-reactions of these three mycoplasmas were shown by absorption tests to be due to surface antigens. These antigens were comparatively resistant to proteolytic enzymes (Table 2), suggesting that they were either of a completely non-protein nature or consisted of non-protein antigenic determinants requiring secondary protein structures such as glycoproteins or glycolipid haptens. Such antigens are not unknown in mycoplasmas (Plackett, Buttery & Cottew, 1963; Lemcke, Marmion & Plackett, 1967; Sobeslavsky *et al.* 1967; Minion, Brown & Cassell, 1984).

Immunoblot analysis is based on the detection of protein antigens (Gershoni & Palade, 1983; Towbin & Gordon, 1984), but it must be borne in mind that the chemical structure of some protein antigens may be so radically changed by SDS treatment that recognition by antibody is abolished. However, washing probably removes most of the SDS, allowing the proteins to recover structurally to some degree (Tsang, Peralta & Simons, 1983). A further technical limitation is that some proteins, especially those of low molecular weight, may bind only loosely to

nitrocellulose and as a result be lost during transfer or subsequent processing. Conceivably such complications might explain the failure of any heterologous antiserum to demonstrate immunoblotted antigens from *M. equigenitalium* or *M. primatum* (Table 3) despite the considerable ELISA cross-reactivity shown by these two organisms (Table 1). A more likely explanation is that the cross-reacting antigens of *M. equigenitalium* and *M. primatum* are not proteins, but further study is needed.

With the few striking exceptions mentioned above, the results obtained by immunoblot analysis were consistent with those obtained by the ELISA.

This study was supported by grants from the British Council and the Overseas Development Administration.

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