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Immunoblot studies in the differential diagnosis of porcine brucellosis: an immunodominant 62 kDa protein is related to the mycobacterial 65 kDa heat shock protein (HSP-65)

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Abstract

Smooth *Brucella* spp. share certain lipopolysaccharide antigens with other bacteria, resulting in serological cross-reactions which can prevent the definitive diagnosis of brucellosis. To identify other antigens with serodiagnostic potential, immunoblot studies following sodium dodecyl sulphate – polyacrylamide gel electrophoresis were carried out. Sera from pigs experimentally infected with *Brucella suis* and naturally infected feral pigs, sera from pigs from a farm with a known history of *Yersinia enterocolitica* 0:9 infection, *Brucella* Complement Fixation Test (CFT) reactor pigs (aetiology unknown) and pigs from consistently *Brucella* CFT negative farms were examined. Although *B. suis* infected pigs recognized a total of nine *B. melitensis* antigens, individual pigs rarely recognized more than three antigens in the range. A 62 kDa antigen was recognized by the majority (73%) of the *Brucella* infected pigs, but only by 10 to 23% of pigs from the other groups. This antigen was shown to be the *Brucella* homologue of the ubiquitous 65 kDa heat shock protein (HSP-65) family by immunoblot studies with 14 monoclonal antibodies to the *Mycobacterium leprae* HSP-65. Only four of these monoclones (Y1.2, ML-30, D7C and IIIC8) identified the *B. melitensis* 62 kDa protein suggesting that unshared, potentially *Brucella* specific, regions exist.

Sera from *Y. enterocolitica* 0:9 infected pigs, CFT reactor pigs (aetiology unknown), CFT negative pigs and hyperimmune pig serum raised to *Y. enterocolitica* 0:9 also recognized *B. melitensis* antigens, most notably a 17 kDa protein. This antigen appears to be a common cross-reactive protein.

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1. Introduction

Since 1988 there has been a marked increase in the number of pigs failing export tests for brucellosis in Great Britain. Wrathall et al. (1993) confirmed the *Brucella* free status of British pigs by showing that serological reactions observed in these tests were due not to *Brucella* infection but to infection with *Yersinia enterocolitica* serotype 0:9. The serological cross-reaction between *Yersinia enterocolitica* 0:9 and the *Brucella* A antigen is well known (Ahvonen et al., 1969; Corbel and Cullen, 1970; Mittal and Tizard, 1979a) and Corbel et al. (1984) showed that infection with *Y. enterocolitica* 0:9 can produce serological reactions to *Brucella*. Furthermore, studies with monoclonal antibodies have shown that the O-chain of the lipopolysaccharide (LPS) of these organisms is identical (Bundle et al., 1984; Palmer and Douglas, 1989). This antigen has been characterized as a polymer of 4,6-dideoxy-4-formamido-D-mannopyranosyl residues linked by α 1–2 bonds (Bundle et al., 1989). Since *Brucella* LPS forms the basis for the complement fixation (CFT) and serum agglutination tests (SAT) currently used for the diagnosis of brucellosis, pigs infected with *Y. enterocolitica* 0:9 will tend to fail these tests. To differentiate between these infections Corbel and Cullen (1970) and Mittal and Tizard (1979b) developed methods to quantify antibodies to the flagellar antigens of *Y. enterocolitica* 0:9, whilst Schoenerer et al. (1990) developed an enzyme linked immunoassay, using *Yersinia* plasmid encoded outer membrane proteins, to detect *Yersinia* infection. The former approach involves laborious absorption procedures, but most importantly neither test specifically detects *Brucella* infection nor excludes the possibility of dual infection. In addition, cross-reactions caused by other organisms (Corbel et al., 1984) may be wrongly attributed to brucellosis.

It is important, therefore, to identify antigens that demonstrate greater specificity for the definitive diagnosis of brucellosis. Previous immunoblot and immunoprecipitation studies with sera from *B. ovis* infected rams (Chin and Pang-Turner, 1990) and dogs infected with *B. canis* (Carmichael et al., 1989) have identified antigens in cytoplasmic extracts of *Brucella* which may be of diagnostic potential. In this study, sera from pigs infected with *B. suis* or *Y. enterocolitica* 0:9 and control animals were examined by immunoblot analysis after sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) of whole *B. melitensis*, to characterize the range of antigens recognized by the humoral response in porcine brucellosis. Lysozyme digests of digestion buffer insoluble material were also examined as Sowa et al. (1991) have shown the presence of additional group 2 and 3 outer membrane proteins (OMP) in such extracts. A rough strain of *Brucella melitensis* was used as immunoblot antigen to prevent smearing profiles due to lipopolysaccharide. The use of this antigen is discussed later.

2. Materials and methods

Sera

Fifteen sera from pigs experimentally infected with *B. suis*, and from culture positive *B. suis* infected feral pigs were received from the Diagnostic Bacteriology Laboratory of the National Veterinary Services Laboratories (Ames, USA). Organs were cultured for *B. suis* as described by Payeur et al. (1990).

Eighteen sera were examined from pigs from a farm with a known history of *Y. enterocolitica* 0:9 infection.

Sera from reactor pigs exhibiting serological reactions to *Brucella* (aetiology unknown) were obtained from three geographically distinct farms. Four individual sera from one of these farms, with titres of <8, 17, 40 and 106 international CFT units (ICFTU) were examined. Three pools were prepared from each of the remaining reactor farms by combining equal volumes of serum from at least eight pigs with similar CFT titres (<8, 8–17 and >20 ICFTU).

The *Brucella* negative group comprised serum from five feral pigs negative by CFT and culture (received from the Diagnostic Bacteriology Laboratory, NVSL), and four British farms which were consistently CFT negative. Two or three individual sera from each of three farms, and a pool comprising equal volumes of 100 sera from the fourth farm were tested.

Hyperimmune pig sera were prepared using dense suspensions of either heat killed (60°C for 1 h) *B. melitensis* (88/131) or formalin inactivated (0.5% v/v) *Y. enterocolitica* 0:9 (M90001, our own isolate) emulsified in Freunds incomplete adjuvant (Sigma). The antigens were administered twice, intramuscularly, at two week intervals and serum taken three weeks after the second injection.

Complement fixation test

The CFT was performed as described by Morgan et al. (1978) with reference to the 3rd British National Standard serum. The results are expressed as International CFT Units (ICFTU). The pass level for international trade is <20 ICFTU per ml (Office International des Epizooties, 1986).

Antigen preparation

A rough strain of *Brucella melitensis* (88/131) from the Central Veterinary Laboratory (CVL) *Brucella* culture collection was grown on serum dextrose agar (SDA) at 37°C in air for five days. Pure growth was harvested as a dense suspension in electrophoresis digestion buffer consisting of stacking gel buffer (see below) containing glycerol (20% v/v), 2-mercaptoethanol (2-ME, 2% v/v) and sodium dodecyl sulphate (SDS, 4% w/v). Suspensions were heated at 100°C for 10 min, cooled and centrifuged at 11600 g for 10 min. Supernatants were stored at 4°C. The deposit was digested with lysozyme as described by Sowa et al. (1991). Briefly, the pellet was washed twice in Lutkenhaus buffer (10 mM Tris/HCl, 5 mM EDTA and 1 mM 2-ME, pH 7.8) and resuspended in buffer containing egg white lysozyme (grade II, Sigma) at a ratio of one part enzyme to 10 parts detergent soluble protein. After incubation at 37°C for 17 h, two volumes of electrophoresis digestion buffer were added, the mixture was heated at 100°C for 10 min and the supernatant harvested as described above.

SDS-PAGE

Electrophoresis was carried out by the method of Laemmli (1970) in a vertical slab gel system (Hoefer SE600) with 1.5 mm thick, 18 cm × 16 cm gels using a trough (blank) comb. The separation gel contained 10% or 15% acrylamide in 375 mM Tris/HCl buffer, pH 8.8, with a 5% stacking gel in 125 mM Tris/HCl buffer, pH 6.8. The tank buffer comprised 250 mM Tris, 192 mM glycine, pH 8.3 containing 0.1% SDS (w/v). Separation

was carried out at 10 mA per gel constant current for 16 h at 10°C. Rainbow molecular weight markers (Amersham) covering the range 200 to 14.2 kDa were used.

Immunoblotting

Following electrophoresis, gels were equilibrated in transfer buffer (25 mM Tris, 110 mM glycine buffer, pH 8.6 and 20% v/v methanol) for 1 h at room temperature (RT). Separated antigens were transferred to Immobilon-P (Millipore) at 100 mA per gel constant current for 22 h, then 200 mA per gel for 1 h (LKB Transphor 2005). The membranes were stored dry between filter paper at RT. For use membranes were cut into 1 cm wide strips, wetted in neat methanol and blocked for 1 h at 37°C in 3% (w/v) skimmed milk in phosphate buffered saline-Tween (PBS-T, PBS pH 7.2 with 0.02% v/v Tween 20). After washing the strips in PBS-T (3×5 min), serum (diluted 1/100 in PBS-T with 1% skimmed milk) was added and incubated for 1 h at 37°C. The strips were then washed (3×5 min) and incubated at 37°C with horseradish peroxidase (HRP) conjugated rabbit anti-swine IgG (Nordic, 1/1000 in PBS-T with 1% w/v skimmed milk). After washing (6×5 min), 4-chloro-1-napthol substrate was added (stock solution of 30 g/l in methanol, diluted 1/100 in 50 mM Tris/HCl pH 7.6, and 100 μl 30 vol. H_2O_2 added just prior to use). After incubation at 37°C for 10 min the reaction was stopped by washing with distilled water. For monoclonal antibodies the primary antibody was incubated at RT for 90 min or overnight, and the secondary antibody was HRP conjugated goat anti-mouse IgG (Bio-Rad or Sigma, diluted 1/1000).

Protease digestion

Stock solutions (40 u/ml) of pronase E (protease XXV, Sigma) and proteinase K (protease XXVIII, Sigma) were prepared in cold (4°C) PBS pH 7.6 and stored at 4°C. Just prior to use an aliquot of pronase E stock solution was diluted 1/10 in PBS pH 7.6 at 37°C and 5 ml added to each strip of blotted antigen. A similar volume of warmed buffer was added to control strips. After incubation at 37°C for 2 h with shaking, all strips were washed (3×5 min) in buffer at 37°C. This procedure was repeated with a 1/10 dilution of proteinase K. Control strips received 5 ml of warmed buffer. After the final wash the strips were immunostained as described above.

Monoclonal antibodies

Mouse IgG monoclonal antibodies Y1.2, IIC8, IIIC8, IIIE9, C1.1, D7C, D5H, F67–2, F67–19, F67–13, F47–10, ML-30, IVD8 and IIH9, raised to the HSP-65 of *Mycobacterium leprae*, were gifts from Dr T.P. Gillis (LSU School of Veterinary Medicine, LA, USA) (IIC8, IIIC8, IIIE9, IVD8, IIH9), Dr A.H.J. Kolk (Royal Tropical Institute, Amsterdam, The Netherlands) (F67–2, F67–19, F67–13, F47–10), and Dr T.M. Shinnick (Centres for Disease Control, Atlanta, USA) (ML-30, originally cloned by Prof. J. Ivanyi, MRC Tuberculosis and Related Infections Unit, London, UK). They were received as tissue culture supernatants or ascites and were used at 1/100 or 1/2000 respectively.

3. Results

Preliminary immunoblot studies using whole cell extracts of smooth *B. suis* showed smearing which obscured the banding profile (data not shown). The use of rough *B. melitensis* overcame this smearing to give clearer profiles.

B. suis infected pigs

Sera from the 15 *B. suis* infected pigs identified a total of nine antigens in the range 85 to 11 kDa, but no one antigen was recognized by all the sera (Table 1). The 62 kDa antigen was the most frequently recognized with 11 of 15 sera (73%) showing weak (±) to strong (+++) immunostaining, strongest reactions generally accompanying increased CFT titre. Recognition of this antigen tended to agree with the CFT pass/fail threshold for international trade; ie sera with a CFT titre of >20 ICFTU generally recognized the 62 kDa antigen. However, one serum (pig 91–2) with a high CFT titre (106 ICFTU) failed to recognize this antigen whilst another (pig 117–6) with a CFT titre of 17 ICFTU (ie acceptable for international trade) showed weak recognition. The immunodominance of the 62 kDa antigen for *B. suis* infected pigs is illustrated in Fig. 1, lanes c, d, e and f. A 17 kDa antigen was the next most frequently identified. This antigen was recognized by 7 of the 15 sera (47%). Antigens of 85, 55, 40, 37, 28, 19 and 11 kDa were recognized infrequently and each of these antigens was never recognized by more than three sera.

Table 1
Immunoblot profiles of sera from *B. suis* infected pigs. *B. melitensis* antigens were separated by SDS-PAGE and screened with sera from pigs experimentally infected with *B. suis* and *B. suis* infected feral pigs

Serum No	CFT Titre (ICFTU)	Brucella Isolated	Molecular weight of band (kDa) (Density of immunostaining ± to +++)													
			85	62	55	52	40	37	32	28	21	19	17	12.5	12	11
117-7	<8	+											+++			
117-10	12	+			++											
117-6	17	−		+									±			
117-9	23	−											±			
117-14	23	−		+												
117-1	27	+	±	++									±			
117-2	27	+		+									±			
117-13	27	+		±												
117-16	40	−	±	±												
117-5	80	−		+++	++			±								
117-3	106	+		+				+					++			
117-8	106	+		++	±											
** 91-2	160	+										++				
117-4	186	+		++			+					+	+			
** 89-6	213	+	+	++			±	++		+		++				+
Percentage of sera recognizing the antigen			20	73	20	0	13	20	0	6	0	20	47	0	0	6

**Feral pigs.

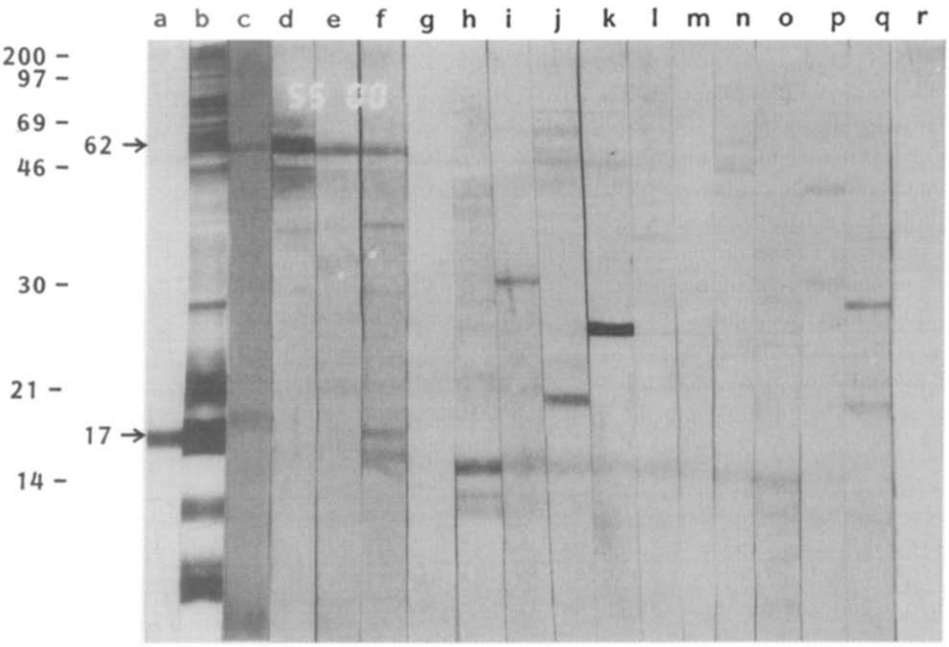

Fig. 1. Profiles of *Brucella* antigen immunoblotted with sera from *Brucella suis* infected, *Yersinia enterocolitica* 0:9 infected, CFT reactor (aetiology unknown) and CFT negative pigs. Lanes were blotted with: hyperimmune pig anti-*Y. enterocolitica* 0:9 (lane a); hyperimmune pig anti-*Brucella* sera (lane b); *Brucella* infected pig sera 117–2 (lane c); 117–5 (lane d); 117–8 (lane e) and 117–4 (lane f). CFT negative feral pig sera 89–5 (lane g) and 89–4 (lane h). *Yersinia* infected pig sera 40 (lane i); 6 (lane j); 16 (lane k) and 77 (lane l). CFT reactor sera (lanes m, n and o) and CFT negative sera (lanes p, q and r).

Similarly individual *B. suis* sera rarely recognized more than three antigens and showed little consistency in the profile exhibited.

Yersinia infected pigs

Fourteen of the 18 sera from pigs infected with *Yersinia enterocolitica* 0:9 also recognized *B. melitensis* antigens. Most notably a 17 kDa antigen was identified by 13 of the 18 sera (72%) (Table 2), three showing strong (+ +) staining. Each of the 62, 28 and 19 kDa antigens were recognized strongly (+ +, + + +) by two or more sera, whilst antigens of 85, 52, 40, 37, 21, 12.5, 12 and 11 kDa were stained less intensely and were rarely recognized by more than two sera. *Yersinia enterocolitica* 0:9 infected pigs showed no correlation between CFT titre and recognition of the 62 kDa antigen, since a CFT negative serum (pig 80) and an international trade fail (27 ICFTU) serum (pig 3) both recognized this antigen strongly (+ + +). A range of cross-reactions shown by sera from pigs infected with *Y. enterocolitica* 0:9 is shown in Fig. 1, lanes i, j, k and l.

CFT reactor (aetiology unknown) and negative pigs

A summary of the results of the CFT reactor (aetiology unknown) and CFT negative groups are given in Table 3 together with the results from *B. suis* and *Y. enterocolitica* 0:9 infected pigs for comparison. Examples of these immunoblot profiles are shown in Fig. 1

Table 2
Immunoblot profiles of pig sera from a farm with a known history of *Y. enterocolitica* 0:9 infection. *B. melitensis* antigens were separated by SDS-PAGE

Serum No	CFT Titre (ICFTU)	Molecular weight of band (kDa) (Density of immunostaining ± to + + +)													
		85	62	55	52	40	37	32	28	21	19	17	12.5	12	11
80	<8		+ + +		±	±	±		+			+ +			
34	<8														
2	<8														
40	8							+				+			
8	10											+	+		
6	12	±								+		±			
29	13											+			
72	13										+ +	±			
21	17											±			
7	20		±						+ +			+ +			
65	23														
3	27	+ + +	+ + +						+ +			+		+ + +	
86	27		+		+			+	±			±			
53	33						±		+		+ +				
5	40	±							+	+ +	+ + +	+ +			
16	46	±						+ + +			±				
19	53														
77	53						±		±			±			
Percentage of sera recognizing the antigen		22	22	0	11	5.5	17	11	44	11	17	72	5.5	5.5	0

Table 3
Summary of the percentage of sera from *B. suis* infected, *Y. enterocolitica* 0:9 infected, *Brucella* CFT reactor and consistently CFT negative pigs recognizing *B. melitensis* antigens. The antigens were separated by SDS-PAGE and immunoblotted

Group	% of Sera with >20 ICFTU[1]	Molecular weight of band (kDa)												
		85	62	55	52	40	37	32	28	21	19	17	12.5	12	11
Brucella infected	80	20*	73	20	0	13	20	0	6	0	20	47	0	0	6
Yersinia infected	50	22	22	0	11	5.5	17	11	44	11	17	72	5.5	5.5	0
CFT reactor (aetiology unknown)	40	0	10	0	20	0	10	0	40	0	20	90	0	0	10
CFT negative	0	0	21	7	35	0	0	7	21	14	0	64	0	0	14

[1]ICFTU International complement fixation test units
* % of sera recognizing band

lanes m, n, and o (reactor) and g, h, p, q and r (negative). Complement fixation test reactor and negative groups identified fewer antigens than the *B. suis* or *Y. enterocolitica* 0:9 sera. The 17 kDa antigen was most consistently recognized by sera from the reactor (9 of 10, 90%) (Fig. 1 lanes m, n and o) and negative (6 of 14, 64%) groups (Fig. 1, lane h). The 52 and 28 kDa antigens were the next most frequently recognized, whilst the 62, 55, 37, 32, 21, 19 and 11 kDa antigens were recognized less frequently. In contrast to the results with *B. suis* sera, only one serum (105 ICFTU) from the reactor group (± immunostaining) and three sera from the negative group (± to + + + immunostaining) detected the 62 kDa antigen (data not shown).

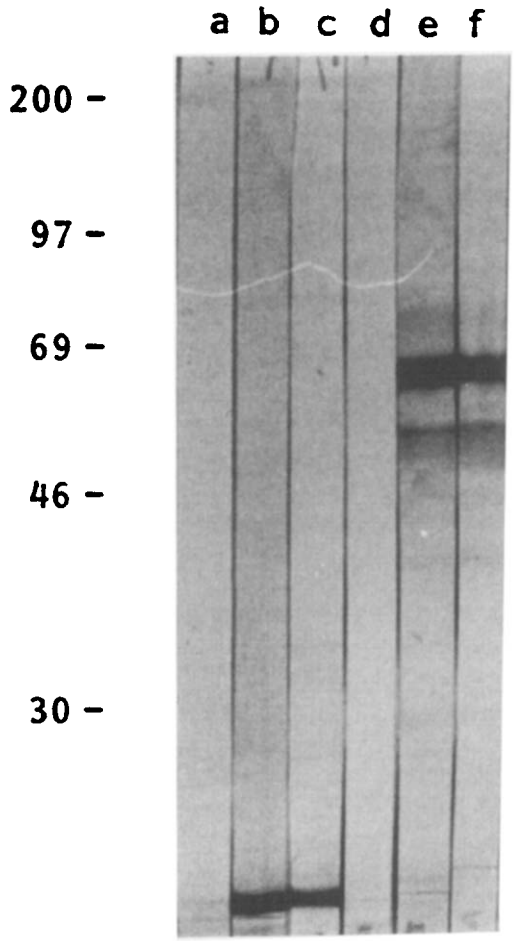

Fig. 2. Profiles of protease treated *Brucella* antigen immunoblotted with sera from pigs either hyperimmunized with *Y. enterocolitica* 0:9 or infected with *Brucella suis*. Lanes were treated with pronase E/proteinase K (lanes a and d); enzyme buffer only (lanes b and e) or untreated (lanes c and f). Sera: hyperimmune anti-*Y. enterocolitica* 0:9 (lanes a to c); *Brucella* infected pig 117–5 (lanes d to f).

Hyperimmune sera

Pig hyperimmune serum raised to *B. melitensis* recognized a broad range of *B. melitensis* antigens well, most notably those of 85, 62, 55, 28, 21, 19, 17, 12.5 and 11 kDa (Fig. 1 lane b). Serum raised to *Y. enterocolitica* 0:9 recognized the 17 kDa *Brucella* antigen strongly and demonstrated weak recognition of a 58 kDa antigen (Fig. 1 lane a).

Protease digestion

The nature of the immunodominant 62 kDa and cross-reactive 17 kDa *B. melitensis* antigens was evaluated by digestion with pronase E and proteinase K. The ability of pig sera to recognize these antigens was abrogated by protease digestion (Fig. 2), demonstrating that both antigens are proteins.

Monoclonal antibodies

The size, proteinaceous nature, immunodominance and cross-reactivity of the 62 kDa antigen suggested that this was the *Brucella* homologue of the *Mycobacterium leprae* HSP-65. The presence of epitopes common to the immunodominant 62 kDa *B. melitensis* protein and the HSP-65 of *M. leprae* was demonstrated by reactivity with the anti-*M. leprae* monoclonals Y1.2, IIIC8, D7C and ML-30 (Fig. 3, lanes a, c, f and l respectively). The remaining 10 monoclones failed to recognize the *B. melitensis* protein, demonstrating the presence of both common and unshared regions on the *Brucella* and Mycobacterial proteins.

Lysozyme digestion

Sowa et al. (1991) identified additional group 2 and 3 *Brucella* OMP's by digesting the SDS-insoluble fraction of *B. abortus* with lysozyme. In the present study the two additional group 2 proteins of 35 and 37 kDa, and one further group 3 protein of 27 kDa recovered by lysozyme treatment were not identified by immunoblotting with sera from *B. suis* infected pigs (data not shown). These proteins therefore do not appear to be immunologically significant and were not studied further.

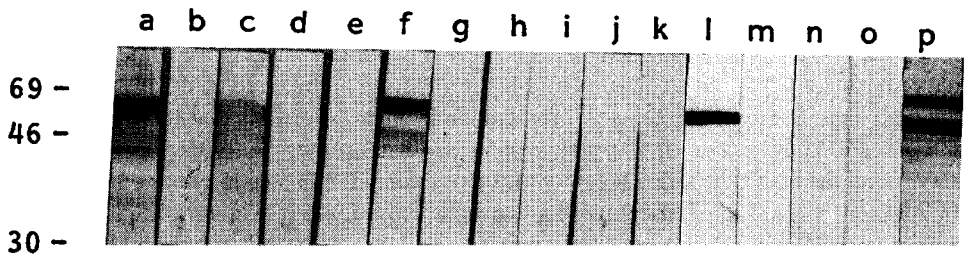

Fig. 3. Profiles of *Brucella* antigen immunoblotted with monoclonal antibodies raised to *M. leprae* HSP-65. Lanes were blotted with: Y1.2 (lane a); IIC8 (lane b); IIIC8 (lane c); IIIE9 (lane d); C1.1 (lane e); D7C (lane f); D5H (lane g); F67–2 (lane h); F67–19 (lane i); F67–13 (lane j); F47–10 (lane k); ML-30 (lane l); IVD8 (lane m) and IIH9 (lane n). Controls: mouse monoclonal raised to bovine corona virus (lane o); *Brucella suis* infected pig 117–5 (lane p).

4. Discussion

The smearing profiles in immunoblots with smooth *B. suis* antigen were believed to be due to antibodies binding both specifically and non-specifically to smooth lipopolysaccharide (S-LPS) present in these extracts (Gamazo et al., 1989; Belzer et al., 1991; Cloeckaert et al., 1992).

This problem was resolved by using a rough strain of *B. melitensis*, a strategy previously used by others (Cloeckaert et al., 1992). This was adopted in view of the high DNA relatedness of all *Brucella* species (Verger et al., 1985); the antigenic similarity of proteins of the different species of *Brucella* (Diaz et al., 1967; Berman et al., 1975; Verstreate et al., 1982; Santos et al., 1984; Holman et al., 1985; and Gamazo et al., 1989); and improved sensitivity of *B. melitensis* outer membrane proteins in the detection of human infection by all species of *Brucella* (Hunter et al., 1986).

Pigs infected with *B. suis* generally recognized *B. melitensis* antigens poorly. In similar studies *B. ovis* infected sheep produced strong antibody responses to the 63, 55, 41–42, 38–40.5, 36–37, 32–33.5, 25–27, 19–20.5 and 15–18 kDa antigens (Chin and Pang-Turner, 1990). Although a similar range of antigens was identified by *B. suis* infected pigs, individual sera rarely identified more than three antigens and, in contrast to the results obtained with infected sheep, there was little consistency in the profiles obtained. This relatively weak serological response in *B. suis* infected pigs parallels the results obtained in traditional serological tests where the detection of 80–90% of infected pigs is considered optimal (Deyoe, 1987). For diagnostic use in individual animals a single protein antigen should detect brucellosis, specifically, in a high proportion of pigs. However, to differentiate brucellosis from infection with known cross-reacting organisms (Corbel, 1985) in countries with no *B. suis* infection a test of lower sensitivity but high specificity, ie a herd test, may be acceptable.

The antigen recognized most frequently by *B. suis* infected pigs (the 62 kDa protein) was identified by 73% of these animals (Table 1). This antigen is distinct from the outer membrane proteins: group 1 (88–94 kDa), group 2 (38–40 kDa), group 3 (25–30 kDa) (Verstreate and Winter, 1984), and the 15 kDa or 8 kDa proteins identified by Dubray and Bezard (1980) and Gomez-Miguel and Moriyon (1986). Previous studies have shown that serum from *B. ovis* infected sheep (Chin and Pang-Turner, 1990) and *B. canis* infected dogs (Carmichael et al., 1989) also recognize a significantly immunogenic antigen in the range 63–68 kDa. The experiment reported here with monoclonals to the *M. leprae* HSP-65 demonstrates that the 62 kDa protein is the *Brucella* homologue of the mycobacterial antigen. This family of proteins is immunodominant in infections due to Mycobacteria (Thole and Van der Zee, 1990) and *Legionella* (Sampson et al., 1986; Plikaytis et al., 1987) and the results reported here confirm its immunodominance in porcine brucellosis. In addition, this group of proteins is known to be cross-reactive, demonstrating sequence homology and serological cross-reactions with a wide range of prokaryotic and eukaryotic organisms (Hoiby, 1975; Shinnick et al., 1988; Thole et al., 1988; Waldinger et al., 1988; Sampson et al., 1990). It is therefore not surprising that the immunodominant 62 kDa protein was also recognized by some sera from pigs infected with *Y. enterocolitica* 0:9, CFT reactor and CFT negative groups of pigs. Indeed, two *Yersinia* sera and one serum from the CFT negative group identified the protein strongly. This implies that antibodies are raised

to conserved regions of this antigen during infection with other unidentified organisms, and confirms the cross-reactive nature of the HSP-62 in *Brucella*.

Other cross-reactions were also seen, most notably to antigens of 52, 37, 28, 19 and 17 kDa. The most extensive of these were to the 17 kDa *Brucella* protein. This antigen was frequently recognized by sera from all groups tested and was identified by hyperimmune pig sera raised to *Y. enterocolitica* 0:9 (Fig. 1). The range of cross-reactions observed indicates that the use of undefined antigen preparations in diagnostic assays will not provide adequate specificity for the diagnosis of brucellosis.

In view of the known cross-reactive and immunodominant nature of the HSP-65 family of antigens it is surprising that antibodies to the 62 kDa antigen were not detected in hyperimmune pig serum raised to *Y. enterocolitica* 0:9. Yamaguchi et al. (1990) identified an HSP-65 homologue in *Yersinia* and demonstrated cross-reactivity with a wide range of organisms, although these workers did not examine *Brucella*. The absence of antibody to the 62 kDa *Brucella* protein could be due to growth of the *Yersinia* inoculum under controlled non-stress conditions. This explanation seems unlikely as HSP's are produced constitutively and account for approximately 1% of the total protein in *E. coli* grown at 37°C (Thole and Van der Zee, 1990). Alternatively, the epitopes recognized by the humoral response to the HSP-65 family during infection may be more specific than the results with cross-reactive monoclonals imply. This may be due to suppression of the response to broadly cross-reacting epitopes (Cooper et al., 1984). Furthermore, the observation that a greater proportion of *Brucella* sera recognized the 62 kDa antigen provides some evidence for greater specificity.

The reaction of four of the 14 *M. leprae* HSP-65 monoclones with the *Brucella* homologue confirms the existence of cross-reactive epitopes on the 62 kDa protein. However, the failure of 10 monoclones to react demonstrates that many epitopes are not shared and suggests that *Brucella* specific epitopes are present. Recent studies would appear to support this since the deduced amino acid sequence of the HSP-65 homologue of *B. abortus* (Roop et al., 1992) shows regions of variability when compared to other published HSP-65 homologue sequences. Indeed, studies with the mycobacterial HSP-65 have shown an *M. leprae* specific epitope in the most variable region (amino acids 420–480) (Anderson et al., 1988), and work with the HSP-60 of *Y. enterocolitica* has demonstrated an epitope specific for this organism (Yamaguchi et al., 1990). In addition studies with *Legionella* (Steinmetz et al., 1991) have demonstrated a genus specific epitope.

The observed cross-reactions and the presence of an HSP-65 homologue in *Y. enterocolitica* (Yamaguchi et al., 1990) precludes the use of the entire *Brucella* HSP-62 in a differential diagnostic role. To develop a specific test for brucellosis based on this immunodominant antigen the epitopes which confer specificity must be defined. Specificity may be introduced by the use of specific monoclonals in a competition assay. Indeed, Levis et al. (1986) developed a highly specific enzyme-linked immunoassay for lepromatous and borderline lepromatous leprosy patients based on competition with the monoclonal IVD8 for HSP-65. Unfortunately this test detected only 32% of these patients. The use of monoclonals to more immunodominant specific regions, or a panel of specific monoclonals could improve this sensitivity. Therefore, although the *Brucella* HSP-65 homologue carries cross-reacting epitopes, its immunodominance and the potential existence of both genus and species specific epitopes show potential as a candidate for a differential serological test for

brucellosis in pigs and other animals. It is essential that fragments of recombinant *Brucella* HSP-62 or synthetic peptides are evaluated to identify the B-cell epitopes recognized specifically by *Brucella* infected pigs.

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