Clonal analysis and virulence of Australian isolates of *Streptococcus suis* type 2

C. G. MWANIKI, I. D. ROBERTSON, D. J. TROTT, R. F. ATYEO, B. J. LEE AND D. J. HAMPSON*

School of Veterinary Studies, Murdoch University, Murdoch, Western Australia 6150, Australia

SUMMARY

Multilocus enzyme electrophoresis was used to divide 124 Australian isolates of *Streptococcus suis* type 2 into 17 electrophoretic types (ETs). Isolates in ET 1 were the most frequent cause of disease amongst Western Australian pigs, but isolates of ET 8 were more commonly associated with disease in other Australian states. Multiple isolates from 10 of 19 farms all belonged to the same ET, whilst isolates from the other farms belonged to between 2 and 4 different ETs. Some isolates could be differentiated further by DNA restriction endonuclease analysis, whilst others with the same restriction pattern were located in different, but closely-related ETs. Fourteen isolates were tested for their virulence in mice. Most caused disease if given in high numbers, but isolates in ET 1 were virulent at lower dose rates. This virulent clone also was distinguished by the fact that 80% of isolates produced extracellular factor (EF).

INTRODUCTION

Streptococcus suis can cause a variety of clinical problems in young pigs, especially meningitis, pneumonia, septicaemia and arthritis [1–3]. The bacterial species has been divided into 29 capsular serotypes, designated 1 to 28, and type 1/2 [4–6]. Of these, serotype 2 is the most important causing disease in pigs in most parts of the world [7, 8], although serotype 7 predominates in Scandinavia [6, 9]. Despite the association of these bacteria with disease, they may also be recovered from the nasal cavities and tonsils of healthy pigs [10–12].

Outbreaks of disease may follow periods of stress, such as when weaned pigs are moved and mixed, or are overcrowded. The build-up of fumes from slurry, combined with poor ventilation, also predisposes to disease [13]. Outbreaks also may occur following the introduction of new pigs to a herd [14]. It is presumed that these are healthy carrier animals which bring new virulent strains into the herd, and that these strains then spread to susceptible pigs. Strains of *S. suis* type 2 with differing degrees of virulence to pigs have been described [14, 15]. Virulent strains produced a muramidase-released protein (MRP) and, particularly, an extracellular factor (EF) [16, 17]. Strains that produced a higher molecular weight

* Correspondence and requests for reprints to: Dr D. J. Hampson.

form of EF (EF*) were of intermediate virulence in newborn germ-free pigs, whilst strains lacking both proteins did not induce any signs of disease [17].

The species *S. suis* is genetically homogeneous as assessed by levels of DNA sequence homology between 13 strains [18]. The use of multilocus enzyme electrophoresis (MEE) to examine a much larger collection of *S. suis* isolates of various serotypes has, however, shown the species to be relatively diverse [19]. For instance 31 isolates of serotype 2 had a mean genetic diversity of 0·264, and were divided into 6 electrophoretic types (ETs), representing 3 clonal groups. Genetic differences between strains of *S. suis* type 2 also have been demonstrated using DNA restriction endonuclease analysis (REA), and ribotyping with an *Escherichia coli* rDNA probe [20, 21]. These techniques have also been used to study the distribution of strains of the bacteria amongst pigs during outbreaks of *S. suis*-associated disease.

The purpose of this study was to determine the diversity of Western Australian (WA) isolates of *S. suis* type 2, and the association of specific strains with clinical problems on piggeries. MEE was used as it is applicable to studies of bacterial populations [22], and is a useful technique for analysing isolates of *S. suis* [19]. Results were compared with those previously obtained for other Australian strains of *S. suis* type 2 [19]. In addition REA was used to help clarify the epidemiology of the infections. The virulence of isolates in different genetic groups was assayed in mice, and correlated with the presence or absence of MRP and EF in Western blot analysis of culture supernatants, using specific monoclonal antisera.

MATERIALS AND METHODS

Bacterial isolates

The isolates of *S. suis* type 2 ($n = 124$) were from 4 main sources (Table 1). Source 1 comprised 67 isolates collected during a survey of pigs from 19 Western Australian (WA) piggeries (designated A to S respectively). The isolates were from the tonsils or lungs of healthy pigs following their slaughter at local abattoirs, except for 2 isolates from farm S which were obtained by swabbing the nasal cavities of 2 healthy grower pigs. Between 2 and 5 isolates were examined from slaughtered pigs from each herd, with an average of 3·4 isolates per herd. Source 2 comprised 15 isolates recovered from 6 litter-mates on farm Q. These animals were followed as a cohort from weaning to slaughter, with nasal swabs taken from each pig at 1, 2, 3 and 4 months of age, and swabs from the nose, tonsils and lungs taken following slaughter at 6 months. Source 3 comprised 11 isolates from WA pigs clinically affected with streptococcal meningitis. These were received from N. B. Buller of the Veterinary Diagnostic Microbiology Laboratory, WA Department of Agriculture, South Perth, or were isolated from dead pigs submitted for diagnosis to the Murdoch University Veterinary Hospital. These animals came from 4 farms (R to U respectively). The 4th source was 31 strains of *S. suis* type 2 studied previously [19], and included isolates from pigs in South Australia ($n = 22$), Tasmania ($n = 3$), Victoria ($n = 2$), and New South Wales ($n = 2$). Seventeen were from pigs with meningitis or septicaemia, and 12 were isolated from the tonsils of healthy animals at slaughter. One isolate was recovered in Australia from the meninges of a human, and the other was a porcine reference strain from the State Serum Institute, Copenhagen, Denmark.

Table 1. *Electrophoretic types of* Streptococcus suis *type 2 isolated from Australian pigs*

Electrophoretic type	WA piggeries*	Other states†
1	G(3); I(3); J(3); L(1); M(2); N(3); R(3); R(6)‡; S(2)‡; U(1)‡	SA(1); SA(1)‡
2	—	TAS(1)‡
3	Q(3)	—
4	C(2)	—
5	S(1)	—
6	—	TAS(1)‡; NSW(2)‡
7	Q(1); R(2)	SA(6); SA(2)‡
8	A(3); C(1); D(3); E(3); F(3); H(3); L(2); P(3); Q(14); T(1)‡	SA(4); SA(7‡); TAS(1)‡; VIC(2)‡
9	M(3)	—
10	B(3); L(1)	—
11	H(1)	SA(1)
12	K(2); R(1)‡	—
13	K(1); O(1)	—
14	O(1)	—
15	S(2)	—
16	S(3)	—
17	S(2)	—

* Twenty-one piggeries in Western Australia, designated A to U. Numbers in parentheses are the number of isolates.

† SA, South Australia. TAS, Tasmania. NSW, New South Wales, VIC, Victoria.

‡ Indicates isolates recovered from pigs with disease. All the other isolates are from healthy pigs. ET 1 also contained an isolate from a human with meningitis, and ET 2 contained a Danish porcine reference strain.

Bacterial isolation and characterization

Swabs were taken from the live and the slaughtered pigs as described previously [23]. These were used to inoculate medium incorporating 3·9% (w/v) Columbia agar base (Oxoid), 1·5% (w/v) streptococcal selective antibiotic supplement (Oxoid), and 5% (v/v) specific sheep antiserum raised against a South Australian reference strain of *S. suis* type 2 [24]. Plates were incubated aerobically for 18–24 h at 37 °C, and then for 24 h at 4 °C. Colonies of *S. suis* type 2 were identified by the presence of bright broad haloes of immunoprecipitation around them when viewed by indirect light in semi-darkness [25, 26]. Specificity of the method was confirmed by testing representatives of each batch of plates with *S. suis* isolates of serotypes 1, 3–9, 13, 15, 18, 20, and untypable isolates of *S. suis* [19], as well as with *Enterococcus faecalis*, *S. pyogenes*, *S. equi*, *S. zooepidemicus*, *S. bovis*, and a group G streptococcus.

Positive colonies were subcultured to (i) Columbia agar containing 5% (v/v) defibrinated ovine blood (SBA plates), and incubated overnight at 37 °C, and (ii) the selective agar, which in this case contained specific sheep antiserum to *S. suis* type 1. Colonies that reacted positively on both antiserum plates were recorded as being type 1/2, and were not investigated further.

Small alpha-haemolytic colonies of Gram positive cocci growing on the SBA plates were confirmed as being *S. suis* by testing for absence of growth on 6·5%
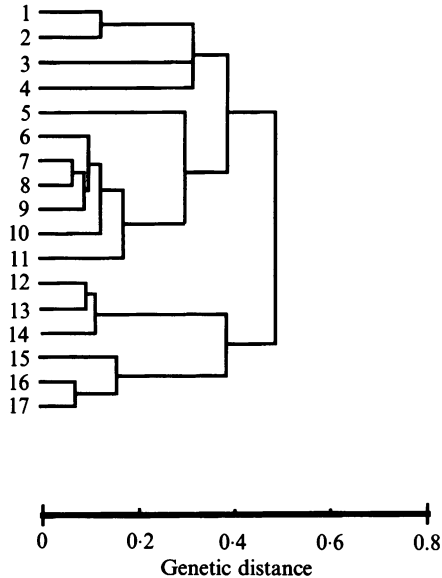
Fig. 1. Phenogram of genetic distance (expressed as percent fixed allelic differences) among 17 ETs, containing 124 isolates of *S. suis* type 2, clustered by the unweighted pair group method with averages (UPGMA) strategy.

NaCl agar, a negative Voges–Proskauer test, and production of acid in either trehalose or salicin broths or both [27]. The serotype of selected isolates from each of the major genetic groups was subsequently confirmed using the tube precipitation and/or the capsular swelling reaction tests [27].

Multilocus enzyme electrophoresis (MEE)

The technique has been described [19]. Briefly, bacterial cells were grown overnight in 500 ml of Todd–Hewitt broth (Oxoid), harvested by centrifugation, washed in phosphate buffered saline (PBS; pH 7·2), centrifuged again, and resuspended in sonication buffer (40 mM-K_2HPO_4, 10 mM-L-cysteine HCl, 5 μg/ml bovine serum albumen, 3 mM dithiothrietol, pH 7·5). They were lysed by four cycles of sonication, clarified by centrifugation, and the supernatant collected. These lysates were subjected to electrophoresis in 11·4% horizontal starch gels, and the electrophoretic mobilities of the following 16 enzymes were determined by staining for specific enzyme activity: Leucyl-tyrosine peptidase (LT), glucose-6-phosphate dehydrogenase (G6P), mannose-6-phosphate isomerase (MPI), leucyl-glycine peptidase (LGG), nucleoside phosphorylase-1 (NP), α-naphthyl esterase (EST), leucyl-proline peptidase (LP), glutamate dehydrogenase (GDH), phospho-glucomutase (PGM), lactate dehydrogenase (LDH), 6-phosphogluconate de-hydrogenase (6PG), nucleoside phosphorylase-2 (NSP), adenylate kinase (ADK), glyceraldehyde-3-phosphate dehydrogenase (GI1), alanine dehydrogenase (ALD), and phosphoglucose isomerase (PGI). The gel and electrode buffers used were tris-citrate (pH 8·0).

Mobility variants of the enzymes were interpreted as the products of different alleles at the corresponding enzyme locus. Groups of one of more isolates with the same alleles at all loci were referred to as being an electrophoretic type (ET).

Table 2. *DNA restriction endonuclease analysis (REA) patterns of 33 Australian isolates of* S. suis *type 2 belonging to different electrophoretic types (ETs)*

Isolates*	Health status†	ET‡	REA pattern§
S9, S10, U1	d	1	1
R1-R3	h	1	2
R6-R11	d	1	3
C4-5 (SA)	d	8	4
F2	h	8	5
F1, F3	h	8	6
N3	h	8	7
13 (TAS)	d	2	8
S1	h	5	9
S2, S3	h	15	10
S4-S6	h	16	10
S7, S8	h	17	10
R4, R5	h	7	11
C7-32 (SA)	h	7	12
B1	h	10	13
R12	d	12	14
01	h	13	14
02	h	14	14

* TAS, Tasmania; SA, South Australia. All the other isolates originated from Western Australia.

† d, diseased pig; h, healthy pig.

‡ ETs in MEE analysis, shown in Fig. 1.

§ Following digestion with *Hae*III. Patterns were not obtained for 53 other isolates examined.

Genetic diversity (h) at each enzyme locus was calculated as $h = (1 - \Sigma P_i^2)(n/n-1)$, where P_i is the frequency of the ith allele and n is the number of ETs or isolates in the sample [28]. Total genetic diversity (H) was calculated as the mean of h over all loci. Genetic distances between ETs were calculated as the proportion of fixed loci at which dissimilar alleles occurred, and the unweighted pair-group method of arithmetic averages (UPGMA) clustering fusion strategy was used to create a phenogram (Fig. 1) to show relationships between isolates [29].

DNA restriction endonuclease analysis (REA)

REA was based on techniques described for *S. suis* [20, 21]. Attempts were made to obtain DNA banding patterns from 83 of the WA isolates, and 3 other Australian isolates (Table 2). The bacteria were grown overnight in 100 ml Todd–Hewitt broth, harvested by centrifugation at 10000 g, and washed twice in PBS (pH 7·2). The pellet was resuspended in 750 μl of 250 mM-Tris HCl, pH 8·0, containing 10 μg/ml freshly prepared lysozyme, and was incubated at 37 °C for 1 h, with occasional shaking. The tube was centrifuged at 15000 g for 3 min, the supernatant removed, and the pellet resuspended in 750 μl of TE buffer (50 mM-Tris, 10 mM-EDTA, pH 8·0). The tube was frozen at −20 °C, thawed, and 150 μl of pre-warmed STEP buffer (0·5% SDS, 50 mM-Tris HCl, pH 7·5, 40 mM-EDTA) added, followed by proteinase K to a concentration of 1 mg/ml. The tube was mixed by inversion and incubated at 50 °C for 1 h. 500 μl of phenol-chloroform was added and the tube repeatedly mixed by gentle inversion until an emulsion formed. Following centrifugation at 5000 g for 5 min, the upper aqueous layer was

removed and re-extracted with phenol-chloroform. After centrifugation, the upper aqueous layer was transferred to a new tube, and 90 μl of 3 M sodium acetate and 1 ml of isopropanol added. After mixing, the DNA was spooled out, rinsed briefly in 70% ethanol, dried, and dissolved in 250 μl of TE buffer. The concentration of the dissolved DNA was determined by measuring absorbance at 260 nm [30]. 15 μg DNA was then digested with 20 units of HaeIII (Boehringer Mannheim) in 10% medium salt buffer for 6 h at 37 °C. Digested DNA was subjected to electrophoresis for 21 h at 30 V in horizontal 0·7% agarose gels using TBE electrophoresis buffer (45 mM-Tris borate, 1 mM-EDTA, pH 8·0). Phage lambda DNA predigested with HindIII and HindIII + EcoR1 (Boehringer Mannheim) was used as a molecular mass marker. Gels were stained by soaking in ethidium bromide (0·5 mg/ml), and were photographed under u.v. light. Where distinct banding patterns could not be obtained after 2–5 attempts, isolates were recorded as being not susceptible to digestion with HaeIII. DNA banding patterns were assessed visually without knowledge of results obtained by other methods. Characteristic patterns were given a numeral, and DNAs with similar patterns were subjected to electrophoresis on the same gel to check for differences.

Virulence testing

Three-week-old outbred female Swiss ARC Albino SPF mice (Animal Resources Centre, Perth, Western Australia) were used to test the virulence of 14 selected isolates (the first 14 isolates in Table 3). This procedure was approved by the Murdoch University Animals Experimentation and Ethics Committee, and was monitored by the University's Animal Welfare Officer.

Six of the isolates were recovered from clinical cases of S. suis type 2 infections in pigs, and 8 were recovered from healthy pigs at slaughter. Eight of the isolates tested belonged to ET 1, 4 to ET 8, and 2 to ET 12 (Fig. 1). The mice were housed in 14 groups of 7, in individual mouse boxes. Six mice were inoculated initially with 2×10^8 colony forming units (c.f.u.), which had been resuspended from early log-phase culture in Todd–Hewitt broth into 0·1 ml of sterile PBS, by the intraperitoneal route; the 7th mouse acted as an in-contact control. One week later the surviving animals were challenged with 4×10^8 c.f.u. of the same strain in 0·2 ml PBS, and a week later survivors were challenged with 1×10^9 c.f.u. in a 0·5 ml volume. Surviving mice were killed 1 week later, and swabs were taken from the heart blood and cultured for S. suis type 2. Throughout the experiment the mice were observed twice daily, and any clinical signs recorded.

Analysis for MRP and EF production

The production of muramidase-release protein (MRP) and extracellular factor (EF) by the 14 isolates tested for virulence in mice, and another 17 isolates (Table 3), was assayed [16, 17]. Overnight cultures in Todd–Hewitt broth were diluted 1:10 in fresh broth, and incubated at 37 °C for 4 h. The cultures were centrifuged at 4000 g for 15 min, and the supernatants analysed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) in 8% separating gels, and by Western immunoblotting, using a mini-Protean II electrophoresis unit and transblot cell (Bio-Rad Laboratories, Richmond, Calif.). A control culture supernatant from a Dutch strain, 4005,which was positive for MRP and EF, and

Table 3. *Production of muramidase release protein (MRP) and extracellular factor (EF) by 31 isolates* S. suis *type 2*

Isolate*	Status†	ET‡	MRP§	EF§
S9	d	1	+	+
S10	d	1	+	+
U1	d	1	+	+
R6	d	1	+	+
I1	h	1	−	−
L1	h	1	+	+
N1	h	1	+	+
J1	h	1	+	−
D2	h	8	−	−
E2	h	8	−	−
Q5V4	h	8	−	−
T1	d	8	−	−
K1	h	12	+	−
R12	d	12	+	−
C5-16 (SA)‖	d	1	+	×
13 (TAS)	d	2	+	+
C5	h	4	−	−
DR31-V3	h	5	−	−
15 (TAS)	d	6	+	−
C7-25 (SA)	h	7	−	−
D7-42 (SA)	d	7	−	−
O4	h	9	−	−
B3	h	10	+	−
C8-39 (SA)	h	8	−	−
C6-13 (SA)	d	8	−	−
14 (TAS)	d	8	+	−
C4-5 (SA)	d	8	+	−
Q6	h	13	−	−
Q1	h	14	+	−
DR41-4	h	15	−	−
DR27-T	h	16	−	−

* The first 14 isolates (S9–R12)) were tested for virulence in mice, and S9, U1, I1, L1 and N1 caused disease at 2×10^8 c.f.u. when administered intraperitoneally. SA, South Australia; TAS, Tasmania. All other isolates from Western Australia.

† Disease status of pig from which the isolate was recovered. d, diseased; h, healthy.

‡ ET, electrophoretic type in Fig. 1.

§ +, protein present in the supernatant; −, protein absent; ×, higher molecular mass band present (EF*).

‖ Isolate from a case of meningitis in a human.

which was supplied by Dr Uri Vecht, DLO-Central Veterinary Institute, Lelystad, The Netherlands, was also included on the gels. Following electrophoresis, gels were stained using a silver staining kit (Bio-Rad Laboratories). Proteins that transferred to nitrocellulose membranes in Western blots were blocked for 1 h in 5% (w/v) non-fat milk powder, and then incubated with a 1:1 mixture of mouse anti-MRP monoclonal antibody (Mab) (11·3 mg/ml) and anti-EF Mab (8·4 mg/ml), each in a 1:200 dilution [16, 17], provided by Dr Vecht. After washing, the membranes were incubated with a 1:2000 dilution of a 1:1 mixture of goat anti-mouse IgG and goat anti-mouse IgM, both conjugated with horseradish peroxidase (Bio-Rad laboratories). After washing, antibodies were localized by adding the

substrate   3-3′-diaminobenzidine   tetrahydrochloride   dihydrate   (Bio-Rad Laboratories).

<div align="center">RESULTS</div>

MEE

The enzymes LDH, ADK, GP1 and ALD were monomorphic whilst the other 12 enzymes were polymorphic, with 2–4 alleles. The average number of alleles per locus was 2·69. The 124 isolates were divided into 17 ETs, with a mean genetic diversity per locus of 0·397, or 0·283 when the number of isolates in each ET was considered. Two broad genetic diversions were observed (Fig. 1). ETs 1–11 contained 111 (89·5%) of the 124 isolates, separated from ETs 12–17 at a genetic distance of 0·467.

The WA isolates were distributed in 15 of the ETs, but not in ETs 2 or 6 (Table 1). The Danish isolate and the other Australian isolates of serotype 2, from our previous study [19], were located in ETs 1, 2, 6, 7, 8 and 11. These ETs corresponded respectively to ETs 33, 34, 50, 51, 53 and 54 in our previous study [19]. Overall, there were 50 isolates in ET 8. ET 1 contained 30 isolates, ET 7 11 isolates, and the other ETs between 1 and 4 isolates.

Isolates in ET 8 were common in WA (39% of isolates), where they made up 35 of the 82 (43%) isolates from healthy pigs, but only 1 of the 11 (9%) isolates from diseased animals. Isolates in this ET also were common in the other states (14 of 29 porcine isolates; 47%), but here they were more frequently recovered from diseased pigs (10 of 17; 59%) than from healthy animals (4 of 12; 33%). In contrast, isolates in ET 1 were common in WA (29% of 93 isolates), but were much less so in the other states (2 of 29; 7%). In WA they made up 82% (9 of 11) of the clinical isolates, but only 22% (18 of 82) of those from healthy pigs.

Isolates were collected from healthy pigs on 19 farms, and on 11 of these all belonged to the same ET. Five farms had isolates belonging to 2 ETs, 2–3 ETs, whilst the 8 isolates from farm S belonged to 4 ETs. In the cohort study on farm Q, 9 isolates from 5 of 6 pigs belonged to ET 8 (as did 3 isolates collected earlier in the abattoir survey). In the sixth pig, however, 2 isolates were of ET 8, 3 ET 3, and 1 ET 7. Of the 11 isolates recovered from diseased pigs in WA (Table 1), 9 belonged to ET 1, and 1 each to ETs 8 and 12. Isolates belonging to these ETs were also recovered from healthy WA pigs. The 17 isolates from diseased pigs in other Australian states belonged to ETs 1(1), 2(1), 6(3), 7(2) and 8(10); those from healthy pigs were of ETs 1(1), 7(6), 8(4) and 11(1) (Table 1).

REA

Fourteen different REA patterns (Fig. 2) were identified for 33 isolates, using the enzyme HaeIII (Table 2). Patterns could not be obtained for 53 other isolates as these either failed to lyse adequately, yielded poor quality DNA, or the DNA was refractory to HaeIII.

The 12 belonging to ET 1 in MEE analysis were divided into three REA patterns. Notably, 3 nasal isolates from healthy pigs on farm R had a different pattern from 6 clinical isolates from diseased pigs on the same property. The latter had an additional band at approximately 22 kb. Five isolates belonging to ET 8 were divided into 4 REA patterns. In contrast 7 isolates, from farm S, all had
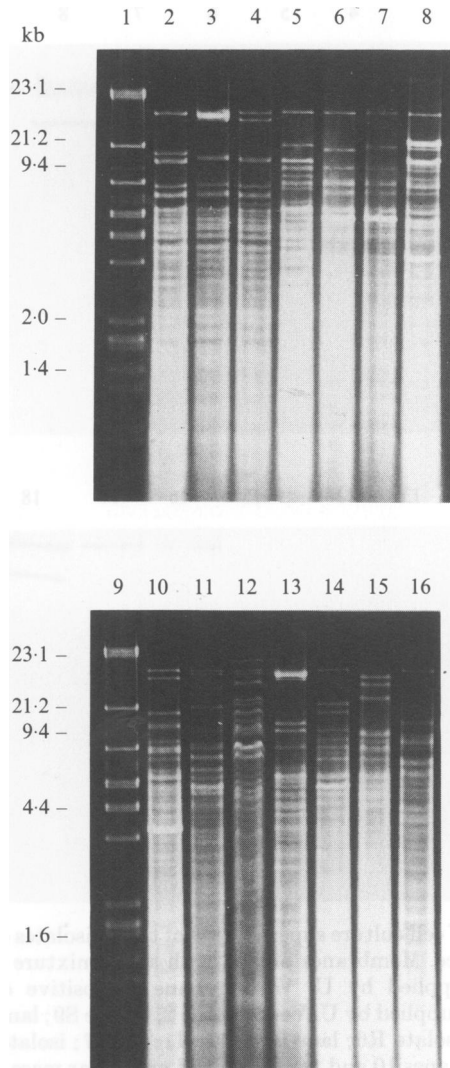
Fig. 2. Restriction fragment patterns of DNA from isolates of *S. suis* type 2. Samples digested with *Hae*III and separated by electrophoresis in 0·7% agarose. Fourteen patterns were recognized, and representative isolates from each of these are presented in ascending order in lanes 2–8, and 10–16. Lanes: 1 and 9, λ DNA size markers; 2, isolate S9; 3, isolate R1; 4, isolate R6; 5, isolate C4-5 (SA); 6, isolate F2; 7, isolate F3; 8, isolate L3; 10, isolate 13 (TAS); 11, isolate S1; 12, isolate S5; 13, isolate R4; 14, isolate C7-32 (SA); 15, isolate B1; 16, isolate R12.

REA pattern No. 10, but were separated by MEE into the closely related ETs 15, 16 and 17. Similarly, another 3 isolates with REA pattern No. 14 belonged to the closely related ETs 12, 13 and 14.

Virulence testing

Following the first challenge, some mice (mean 1·7 of 6) inoculated with isolates U1, I1 and L1 died, and *S. suis* type 2 was isolated from these. Other mice inoculated with these cultures, as well as S9 and N1, also developed clinical signs
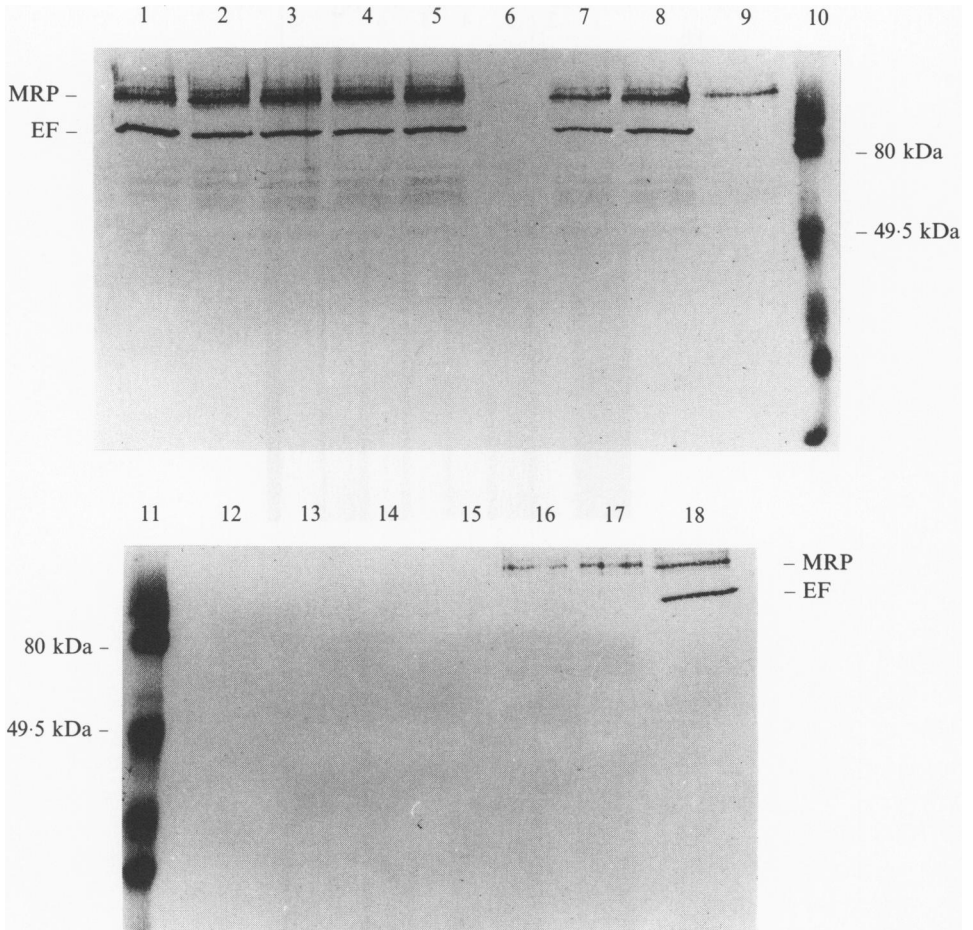
Fig. 3. Western blots of cell culture supernatants of the 14 isolates of *S. suis* type 2 that were used to infect mice. Membranes probed with a 1:1 mixture of mouse anti-MRP and anti-EF Mab (supplied by U. Vecht). Lane 1, positive control supernatant (MRP+ve; EF+ve), supplied by U. Vecht; Lane 2, isolate S9; lane 3, isolate S10; lane 4, isolate U1; lane 5, isolate R6; lane 6, isolate I1; lane 7, isolate L1; lane 8, isolate N1; lane 9, isolate J1; lanes 10 and 11, prestained molecular mass markers (low range, Bio-Rad Laboratories); lane 12, isolate D; lane 13, isolate E2; lane 14, isolate Q5V4; lane 15, isolate T1; lane 16, isolate K; lane 17, isolate R 12; lane 18, positive control supernatant.

(10 of 25 mice), which included lack of grooming, lethargy and incoordination. All five isolates belonged to ET 1, although only S9 and U1 originated from diseased pigs. Following the second challenge, with a higher inoculum, only those mice exposed to isolate L1 developed disease. Two of these 4 animals died, and the other 2 showed clinical signs. After the 3rd inoculation, deaths occurred in 12 of the 14 groups, although not in mice challenged with isolates S9 or K1. Forty-one of 77 mice died (53%), and 23 showed clinical signs (30%).

MRP and EF production

Production of MRP and EF by the 31 isolates is recorded in Table 3. Specific bands in Western blots using the Mabs are shown in Fig. 3 for the 14 isolates tested for virulence in mice. Three phenotypes were recorded: MRP and EF positive;

MRP positive, EF negative; MRP and EF negative. Isolates from diseased animals were more likely to produce MRP and EF than were those from healthy animals. Isolate C5-16 (SA), from a human with meningitis, had an EF protein with a molecular mass of approximately 150 kDa (EF*; membrane not shown).

DISCUSSION

MEE indicated that *S. suis* type 2 from healthy and diseased Australian pigs originated from diverse genetic backgrounds, confirming the conclusions of others that serotyping cannot be relied upon for strain identification of *S. suis* [20, 31]. Two broad genetic groups were identified. The smaller group comprised 13 isolates (10·5%) in 6 ETs, and included only 1 of the 28 isolates from diseased pigs. This group is, therefore, probably of minor significance clinically.

Despite the overall diversity amongst isolates, 40, 24 and 9% belonged to ETs 8, 1 and 7 respectively. The geographical distribution of ETs 1 and 8 in Australia differed, as did their association with disease. ET 1 was the most common cause of disease in WA, and ET 8 in South Australia, Tasmania and Victoria. Isolates from both ETs were also recovered from healthy pigs on farms without a history of recurrent *S. suis* type 2-associated disease. Some isolates in ETs 2, 6, 7 and 12 were also associated with disease, indicating that virulence is not restricted to the two most common ETS (1 and 8).

The distribution of strains of *S. suis* type 2 varied on different farms, and more than one ET was found in 9 of 19 (47%) WA piggeries. On the other 10 properties all isolates were of the same ET, although some of these (ETs, 1, 8, 10, 13) were found on other properties, indicating that these strains may be widespread.

To determine whether isolates within an ET were likely to be identical, attempts were made to differentiate them using REA. This proved possible for only a proportion of isolates and it is possible that differences in the thickness or composition of the capsule could have accounted for variations in cell lysis, and recovery of DNA. Nevertheless, using REA, it was possible to detect differences between certain isolates in ETs 1, 8 and 7, including those from the same farms, e.g. ETs 1 and 8 on farms R and F respectively. Those in ET 1, from farm R, were particularly interesting because isolates from healthy pigs (pattern 2) differed from isolates from diseased animals (pattern 3; Fig. 2). The latter had an additional DNA band (approximately 22 kb) that might have been associated with virulence. Unfortunately these isolates were lost following a freezer failure, and could not be investigated further. Other isolates from different but closely related ETs gave the same REA pattern (patterns 10 and 14). All isolates with pattern 10 were from the same farm, but comprised three related ETs (ETs 15, 16 and 17). The significance of these minor differences is uncertain.

In order to assess the relationship between genetic group and virulence, 14 isolates, from ETs 1, 8 and 12, were tested in mice. Mice were chosen because they are a convenient model of *S. suis* type 2 infection in pigs [32, 33], even though their use has never been fully compared with standardized pig models of the disease. Intraperitoneal injection also has been found to be an easy and effective route for establishing infection in mice [32, 34]. Most isolates belonging to ET 1 were more virulent in mice than are those in ETs 8 or 12, although most isolates were capable of inducing disease at higher dose rates. It was unclear why isolate S9 caused

disease at 2×10^8 c.f.u., but not following challenge with 1×10^9 c.f.u. A protective immunity may have developed after the mice received the initial doses, but a similar effect was not seen with other isolates. Three of the 5 most virulent isolates were recovered from the tonsils of healthy pigs at slaughter, showing that healthy pigs may harbour virulent strains.

Vecht and colleagues [16, 17] demonstrated that isolates of *S. suis* type 2, virulent to newborn germ-free pigs, released MRP and EF into the culture medium. Isolates that produced both MRP and EF*, a higher molecular weight form of EF, were of intermediate virulence, whilst isolates not producing either form did not induce disease. In this study, 8 of the isolates produced MRP, but not EF or EF*; this phenotype has not previously been described. Six isolates from ET 1 tested in mice produced both MRP and EF, and, in concordance with results obtained in pigs [16, 17], 4 were virulent at relatively low doses. Nevertheless isolate I1, also in ET1, did not produce MRP or EF, but was virulent at low dose rates. Other isolates, lacking either or both proteins, were virulent at higher dose rates. MRP and EF, therefore, are not essential as virulence determinants in mice inoculated by the intraperitoneal route. They may be important in the early establishment of infection in natural cases of disease in pigs, however, since 6 of 13 isolates from diseased pigs produced MRP and EF, and 4 produced MRP, whilst only 2 of 18 isolates from healthy pigs produced MRP and EF, and 4 produced MRP. Although most isolates from healthy pigs were MRP and EF negative, some that were potentially more virulent (MRP and EF positive) were carried by healthy animals in certain of the WA herds that infrequently suffer from *S. suis*-associated disease. The absence of disease in these herds may be associated with superior management practices, such as provision of good ventilation and low stocking densities.

Isolate C5-16 (SA) recovered from the meninges of a human produced an EF-like molecule (EF*) with a higher molecular mass than that produced by our porcine isolates (approximately 150 kDa, compared to 110 kDa). Similar material (EF*) has been described by Vecht and colleagues [17] in strains isolated from humans, although in relatively few porcine isolates. These results suggest that EF* may be involved in specific aspects of the pathogenesis of the infection in humans.

All the isolates producing EF belonged to the closely related ETs 1 and 2 (8 of 10 isolates analysed from these ETs were EF positive), and 9 of the 10 isolates produced MRP. In contrast only 7 of the 21 (33%) isolates from other ETs that were examined produced MRP, including only 2 of the 8 isolates from ET 8 (4 of which were from diseased pigs). ETs 1 and 2, which could be distinguished by the presence of allele 1 for esterase and allele 4 for glutamate dehydrogenase, appeared to be more virulent than other ETs. Production of EF and MRP by these isolates therefore may only be a marker for this virulent clone, rather than the proteins themselves necessarily being determinants of virulence. This possibility was suggested by Vecht and colleagues [17]. They pointed out that, in order to determine the function of the proteins, it was necessary to obtain isogenic strains that varied in their production of the proteins, and to test these strains for virulence in pigs. Currently, irrespective of the function of EF, screening for its production is a means of detecting isolates belonging to the virulent clone

represented by ETs 1 and 2. Isolates from other clones that do not produce EF however may also be capable of causing disease.

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