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Population Structure of Australian Isolates of *Streptococcus suis*

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The genetic diversity of 109 isolates of *Streptococcus suis*, which were recovered mainly from Australian pigs, was examined by multilocus enzyme electrophoresis. The collection was genetically diverse. Sixty-five electrophoretic types (ETs) were recognized, with a mean genetic diversity per enzyme locus of 0.512, or 0.431 when the number of isolates in each ET was considered. Serotype diversity varied, being greatest for isolates of capsular serotype 15 (0.364), and then diminishing in the order of serotypes 9, 1, 4, 1/2, 2, 7, and 3 (0.120). On average, isolates from these eight serotypes represented 4.13 separate clonal groups per serotype. This diversity indicated that serotyping of *S. suis* for subspecific differentiation is not a reliable technique for identifying specific strains and is not a good predictor of the genetic background of a given isolate. No tendency for isolates recovered from healthy pigs to be genetically distinct from those from diseased animals was found, nor were there consistent differences between isolates recovered from animals with different disease syndromes (meningitis, pneumonia, and septicemia). Danish reference strains of serotypes 1, 2, and 7 each belonged to one of the same clonal groupings of these types found in Australia, but Danish strains of serotypes 3, 4, 6, and 8 and a strain of serotype 1 from the United Kingdom were each genetically distinct from the Australian isolates. Generally, isolates in the same ET belonged to the same serotype, but one ET contained isolates of types 6 and 6/16, and three were made up of isolates of types 2 and 1/2. One isolate of serotype 2, which was recovered from a human with meningitis, belonged to the same ET as two isolates of serotype 2 that were recovered from pigs. The human infection was therefore likely to have been zoonotic.

Streptococcus suis is a common and important pathogen of swine. Although *S. suis* infection may cause meningitis, septicemia, arthritis, or bronchopneumonia (22), many healthy pigs carry the bacteria in their nasal cavities and tonsils (3, 5, 21). *S. suis* also infects a variety of ruminants and horses (7, 8, 14) and is a zoonotic cause of meningitis in humans (1).

S. suis has been divided into 29 capsular antigenic serotypes (types 1 to 28 and type 1/2) (10, 11, 20), and other antigenic types probably exist (22). In a recent survey in Quebec and western Canada, type 2 was found to be the most prevalent type (32.1% of isolates), with capsular types 3, 1/2, 8, and 4 being the next most commonly isolated types (13). In contrast, in Scandinavia, capsular type 7 is more frequently isolated than type 2 (20, 25). In Australia, capsular types 1 through 9 have been detected, with type 2 being the most commonly isolated type in South Australia (19) and type 9 being the most commonly isolated type in weaned pigs in New South Wales (9).

Genetic diversity among strains of *S. suis* has been investigated by DNA-DNA hybridization (15). Although the species appears to be genetically homogeneous, to date only 13 strains from a limited range of capsular types have been examined (15). Genomic fingerprinting has also revealed the existence of genetic diversity among isolates of some serotypes (2, 17).

The purpose of the present study was to obtain an overview of the population structure and diversity of a collection of mainly Australian isolates of *S. suis* by multilocus enzyme electrophoresis. This technique is particularly well suited for large-scale studies of bacterial populations (23).

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MATERIALS AND METHODS

Streptococcal isolates and strains. The 109 isolates of *S. suis* that were analyzed included 99 from Australian pigs, 1 each from a cow and a human being, and 1 British and 7 Danish porcine reference strains. Seventy-one of the Australian porcine isolates were from clinical samples (brain or viscera); of these, 51 were from South Australia, 10 from New South Wales, 6 from Victoria, and 4 from Tasmania. Twenty-eight isolates were recovered from the tonsils of healthy South Australian pigs at slaughter. The bovine isolate was from an abscess, and the human isolate was from a case of meningitis in a recent immigrant to Australia from Vietnam. The sources of the isolates are indicated in Table 1.

All isolates were grown overnight on 10% sheep blood agar at 37°C, and alpha-hemolytic colonies of gram-positive cocci were identified biochemically with an API Strep 20 test kit (API Systems SA, Montalieu Vercieu, France), as recommended by the manufacturer. Isolates were serotyped by direct slide agglutination (4) with rabbit antisera against serotypes 1 through 22, supplied by J. Henrichsen of the Danish State Serum Institute, Copenhagen, Denmark. Macroscopic and microscopic agglutination were assessed immediately, and control preparations were examined for autoagglutination. Reference strains obtained from Denmark, for serotypes 1 through 8, were included as positive controls for the agglutination reactions.

Bacterial growth and enzyme preparation. The bacteria were subcultured from 10% sheep blood agar to Todd-Hewitt broth (Oxoid) supplemented with 1% yeast extract (Oxoid). After being cultured overnight at 37°C on a rocking platform, aliquots were subcultured to sheep blood agar to check for alpha-hemolysis or were Gram stained and checked for contamination and adequate growth. Cells were harvested from the broth by centrifugation at 20,000 × *g* for

TABLE 1. ETs, sources, and serotypes of *S. suis* isolates

ET	Strain or isolate designation(s)	Origin[a]	Tissue[b]	Serotype[c]
1	C5-3	SA	Tonsil	15
2	A228 (Elliot)	UK	NR	1
3	B1-24	SA	Brain	UT
4	B1-14	SA	Abscess (bovine)	20
5	SA10-11	SA	Brain	10/11
6	C4-15	SA	Lung	9
7	C4-41	SA	Lung	4
8	2	SA	Clinical	5/9/12/22
9	19	VIC	Brain	UT
10	C6-36	SA	Lung	UT
11	B1-33	SA	Blood	UT
12	B1-44	SA	Brain	9
13	WN89/2485	NSW	Nose	1
14	5	SA	Liver	3/12/15/16/21
15	C8-41	SA	Lung	15
16	D8	DEN	NR	8
17	C8-12	SA	Tonsil	1/2
18	C7-2	SA	Tonsil	1/2
19	C8-42	SA	Tonsil	15
20	C9-13	SA	Tonsil	15
21	C4-18	SA	Lung	13
22	12	SA	Clinical	4
23	C6-18	SA	Lung	6/16
	C9-28	SA	Tonsil	6
24	B1-19	SA	Brain	9
25	7	SA	Clinical	6/16
26	WN87/478	NSW	Middle ear	1
27	C9-22	SA	Tonsil	10/11/12
28	C9-40	SA	Tonsil	10/11/12
29	C6-39	SA	Lung	6/16
30	D1	DEN	NR	1
	WN87/1037	NSW	Brain	1
	WN87/047, WN87/478, WN89/105, SS43/6, GN85/2833	NSW	NR	1
31	C9-21	SA	Lung	18
32	C8-12	SA	Lung	6
33	C6-10	SA	Brain	2
	C9-10	SA	Tonsil	2
	C5-16	SA	Brain (human)	2
34	13	TAS	Joint	2
	D2	DEN	NR	2
	10	SA	Brain	1/2
35	D7-20	SA	Brain	9
36	8	SA	Clinical	15/16/21
37	C9-30	SA	Tonsil	6
38	C9-32	SA	Tonsil	9
39	D6	DEN	NR	6
40	D7-21	SA	Spleen	9
41	C5-14	SA	Tonsil	4
42	B1-36	VIC	Lung	4
	16	SA	Brain	4
43	C18-8	SA	Tonsil	4
44	17	VIC	Brain	7
45	C4-4, C9-16	SA	Lung	8
46	D7	DEN	NR	7
47	C9-31	SA	Lung	7
48	C8-2	SA	Lung	7
49	C5-27	SA	Brain	1/2
50	15	TAS	Brain	2
	WN84/428/1, WN84/428/2	NSW	Brain	2
51	D7-42	SA	Brain	2
	B1-13	SA	Spleen	2
	C7-25, C7-26, C7-27, C7-32, C8-22, C8-27	SA	Tonsil	2
	C7-1, C7-3, C7-12, C7-24	SA	Tonsil	1/2
52	D3	DEN	NR	3
53	C8-38, C8-39, C9-20, C9-29	SA	Tonsil	2
	C6-4, C8-10, D7-24	SA	Lung	2

Continued on following page

TABLE 1—*Continued*

ET	Strain or isolate designation(s)	Origin[a]	Tissue[b]	Serotype[c]
	C4-5, C6-13, D7-16, D7-37	SA	Brain	2
	14	TAS	Joint	2
	20	VIC	Brain	2
	21	VIC	Heart	2
	C5-38	SA	Lung	1/2
54	C6-7	SA	Lung	5
55	C8-35	SA	Tonsil	2
56	24, B1-7, C8-4, C9-33	SA	Lung	3
57	C9-25	SA	Lung	3
58	D4	DEN	NR	4
59	C8-6	SA	Lung	15
60	C5-20	TAS	Brain	9
61	C4-19	SA	Lung	3/5/10/11/12
62	C8-25	SA	Lung	UT
63	C8-1	SA	Lung	7
	22	SA	Brain	7
64	C6-41	SA	Lung	UT
65	18	VIC	Brain	9

[a] SA, South Australia; VIC, Victoria; NSW, New South Wales; TAS, Tasmania; DEN, Denmark; UK, United Kingdom.
[b] From pigs unless indicated. Clinical, unspecified site in diseased pig; NR, not recorded; tonsil, from tonsils of healthy pigs at slaughter.
[c] Reactivity with capsular typing sera (1 to 22) in slide agglutination. UT, untypeable (weak or negative). Where there are several uneven reactivities, the strongest reaction is with the serum that is underlined.

10 min at 4°C. The bacterial pellets were resuspended in phosphate-buffered saline (pH 7.2) and centrifuged again, and the pellet was resuspended in sonication buffer (40 mM K_2HPO_4, 10 mM L-cysteine HCl, 5 μg of bovine serum albumin per ml, 3 mM dithiothreitol [pH 7.5]) to give approximately 10^{11} cells in 2 ml. The bacteria were lysed by four 1-min cycles of sonication at 4°C with a Braun Labsonic 1510 sonicator set at 100 W. The cell debris was removed by centrifugation at 20,000 × *g* for 10 min, and the supernatant was dispensed into 100-μl aliquots and stored at −70°C until required for electrophoresis.

Electrophoresis. The cell 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, as recommended by Selander et al. (23): leucyl-tyrosine peptidase, glucose-6-phosphate dehydrogenase, mannose-6-phosphate isomerase, leucyl-glycyl-glycine peptidase, nucleoside phosphorylase, α-naphthyl esterase, leucyl-proline peptidase, glutamate dehydrogenase, phosphoglucomutase, lactate dehydrogenase, 6-phosphogluconate dehydrogenase, nucleoside phosphorylase, adenylate kinase, glyceraldehyde-3-phosphate dehydrogenase, alanine dehydrogenase, and phosphoglucose isomerase. Electrode and gel buffers were all Tris-citrate (pH 8.0) (buffer system A of Selander et al. [23]).

Analysis. Each isolate showed only one band for each enzyme, and this was consistent with a single locus coding for each enzyme. Mobility variants of the enzymes were interpreted as the products of different alleles at the corresponding locus. Groups of one or more isolates with the same alleles at all loci were referred to as being an electrophoretic type (ET). 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 *i*th allele and *n* is the number of ETs or isolates in the sample (18). Total genetic diversity (*H*) was calculated as the mean of *h* over all loci. Genetic diversities among isolates of serotypes 1, 2, 1/2, 3, 4, 7, 9, and 15, which all were represented by five or more isolates, were also calculated (24, 28). 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 clustering fusion strategy was used to create a phenogram to show the relationships between isolates (26).

RESULTS

Enzyme activities and genetic diversity. All 16 enzyme loci were polymorphic, ranging from two (phosphoglucose isomerase) to nine (mannose-6-phosphate isomerase) alleles. The mean number of alleles per locus was 5.6. Sixty-five ETs were identified, with a mean genetic diversity per locus of 0.512, or 0.431 when the number of isolates in each ET was included in the calculations. The phenogram that was produced is shown in Fig. 1.

Serotypes and ETs. The isolates were divided into 14 recognized capsular types, including type 1/2, a group of six isolates that reacted weakly with a range of the 22 serum samples used and were classed as untypeable, and six groups of isolates other than type 1/2 that reacted with several sera (Table 1). Only 11 of the 65 ETs contained more than 1 isolate (range, 2 to 15 isolates), and 4 of these 11 contained isolates of different serological types. Three of these ETs contained mixtures of types 2 and 1/2, and one was a mixture of type 6 and type 6/16 (Table 1).

The number of isolates of each serotype and their distribution into ETs are indicated in Table 2. Isolates of the same serotype that differed at 3 or fewer of the 16 enzyme loci were considered to belong to the same clonal grouping. The number of these clonal groupings for each serotype and the genetic diversity amongst isolates of the same serotype are also shown in Table 2. Serotype diversity is expressed in relation to allelic frequencies among the ETs and to the number of isolates expressing a particular allele at a locus. Of the eight recognized capsular types that contained five or more isolates, type 15 was the most genetically diverse, followed in decreasing order of diversity by types 9, 1, 4, 1/2, 2, 7, and 3. Although type 6 was not analyzed, it was also diverse, since the four isolates also belonged to four different

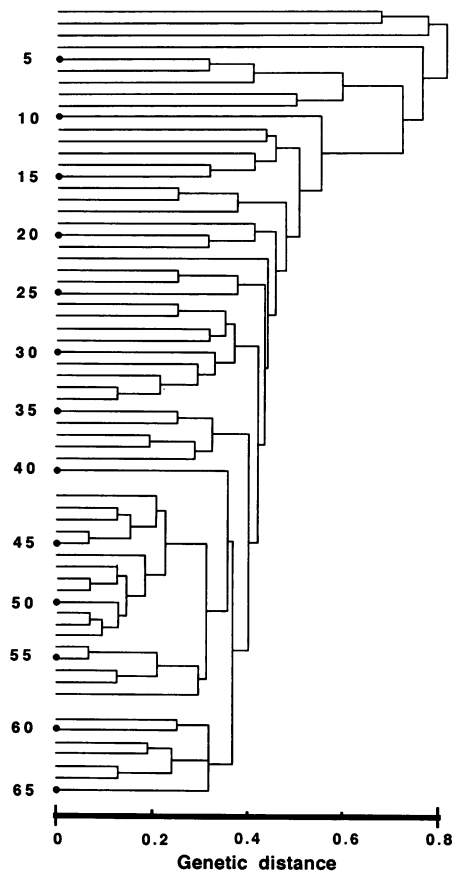

FIG. 1. Phenogram of genetic distance (expressed as percent fixed allelic differences) among 65 ETs, containing isolates of *S. suis*, clustered by the unweighted pair group method with averages strategy.

clonal groups. Each of the untypeable isolates and each of those reacting with more than one serum sample were representatives of separate clones having the same serological reactivity.

Eight non-Australian strains of seven serotypes were included in the study. In serotype 1, the British strain made up a distinct clone, whereas the Danish strain was located in the largest of the Australian clonal groups of this serotype. The Danish strain of serotype 2 belonged to one of three clonal groups of this serotype, together with an isolate from a human being. Danish serotype 7 also belonged to one of three Australian clonal groupings of serotype 7 isolates. Danish serotypes 3, 4, 6, and 8 each were the only representatives among two, four, four, and two clones of these serotypes, respectively.

DISCUSSION

This study examined a large collection of mainly Australian isolates of *S. suis*. The selection of these isolates undoubtedly influenced the overall results obtained. The isolates used were identified as being typical *S. suis* isolates on the basis of their alpha-hemolysis, colony size, morphology, staining reactions, and biochemical reactivity, but less typical isolates, as described by Devriese et al. (6), were not included in the study. Moreover, the isolates were only representatives of the types found in Australia, and many capsular types were not represented. Nevertheless the genetic diversity of the collection, at 0.512, or 0.431 when the number of isolates was included in the calculations, was larger than anticipated on the basis of a previous DNA-DNA hybridization study (15). This is probably a reflection of the small number and limited range of isolates examined in the previous study. The diversity found in this study was comparable to that for a large collection of *Escherichia coli*

TABLE 2. Distribution of capsular types of *S. suis* in ETs, number of clones of each serotype detected, and serotype diversity

Serotype(s)[a]	No. of isolates	No. of ETs	ET(s)[b]	Estimated no. of clones[c]	Serotype diversity[d]
1	10	4	2; 13; 26; 30 (7)	4	0.340 (0.241)
2	31	6	33 (3), 34 (2); 50 (3), 51 (8), 53 (14); 55	3	0.264 (0.164)
1/2	9	6	17; 18; 34; 49, 51 (4), 53	4	0.290 (0.267)
3	6	3	52; 56 (4), 57	2	0.120 (0.096)
4	7	6	7; 22; 41, 42 (2), 43; 58	4	0.320 (0.292)
5	1	1	54	1	NT
6	4	4	23; 32; 37; 39	4	NT
7	6	5	44; 46; 47, 48; 63	4	0.120 (0.125)
8	3	2	16; 42 (2)	2	NT
9	8	8	6; 12; 24; 35; 38; 40; 60; 65	8	0.344 (0.344)
13	1	1	21	1	NT
15	5	5	1; 15; 19; 20; 59	5	0.364 (0.364)
18	1	1	31	1	NT
20	1	1	4	1	NT
UT	6	6	3; 9; 10; 11; 62; 64	6	NT
6/16	3	3	23; 25; 29	3	NT
10/11	1	1	5	1	NT
10/11/12	3	3	27; 28; 61	3	NT
15/16/21	1	1	36	1	NT
5/9/12/22	1	1	8	1	NT
3/12/15/16/21	1	1	14	1	NT

[a] Capsular antigenic type in slide agglutination. UT, untypeable (weak or negative).
[b] The ETs are given in Fig. 1. Numbers in parentheses are numbers of isolates when more than one is present. Underlining indicates ETs of non-Australian reference strains. Semicolons separate clonal groups; commas separate ETs within clonal groups.
[c] Bacteria of the same serotype, clustered together, with different alleles at three or fewer enzyme loci.
[d] Calculated on the basis of allelic frequency per ET and (in parentheses) per isolate. NT, not tested.

isolates (0.47) (28) but was considerably greater than diversities previously obtained by us for the porcine pathogenic species *Serpulina hyodysenteriae* (0.260) (16) and *Actinobacillus pleuropneumoniae* (0.312) (12). The isolates that were least genetically typical, in ETs 1 through 9, included five that reacted clearly with only one typing serum specimen, and one of these isolates was a reference strain from the United Kingdom for serotype 1 (A228). This indicated that the isolates used in the study were correctly identified as *S. suis* and confirmed that the species as currently defined is diverse.

The method used for serotyping in this study resulted in certain isolates reacting with more than one serum sample. Only 22 typing serum samples were available for use, and they would not be expected to detect serotypes 23 through 28 (10). Furthermore, it has been recommended that *S. suis* be serotyped by a combination of capsular reaction, capillary precipitation, and coagglutination tests (11). As judged by the current results, the slide agglutination test, which has been used in the United Kingdom and Australia for typing *S. suis* (4, 19), may be more prone to cross-reactivity than some of the other typing techniques. Cross-reactivities, as found between serotypes 6 and 16, 2 and 22, and 1 and 2, have, however, also been recorded by other typing techniques and may in some cases be eliminated by absorption of the antisera (11). It was interesting that in the only cases in which ETs were found to contain different isolates that reacted with different sera, the isolates were either of types 6 and 6/16 or of types 2 and 1/2. It is therefore likely that these cross-reactivities result from genuine differences in antigen expression by closely related organisms. In view of the rapid and clear agglutination of the reference strains by the typing sera used, it can be assumed that these and the other Australian isolates, other than those that reacted with a range of sera, were probably correctly typed by slide agglutination.

Five of the eight non-Australian reference strains were genetically distinct from the Australian isolates of the same serotypes. The exceptions were in serotype 1, where the Danish strain, but not the strain from the United Kingdom, belonged to the same ET as six Australian isolates; in serotype 2, where the Danish strain belonged to one of the three clonal groupings of serotype 2 that are present in Australia; and in serotype 7, where the Danish strain also belonged to one of three clonal groupings of Australian isolates of this serotype. However, strong conclusions about the genetic uniqueness of other Australian isolates cannot be drawn, since those isolates were compared with very few non-Australian strains. A much more extensive study, with large numbers of isolates from many countries, is necessary to resolve these questions.

Generally, there was considerable genetic diversity among isolates of the same serotype. The eight serotypes that had sufficient numbers of isolates for analysis were made up of an average of 4.13 clonal groupings. Diversity varied between serotypes, with types 15, 9, and 1 being the most diverse and types 3 and 7 being the least diverse. Types 2 and 1/2 were of intermediate diversity, but of all the serotypes, only type 2 was represented by a large number of isolates. All of the serotype diversities were, however, considerably smaller than the overall diversity of the collection. Isolates of a given serotype are therefore more likely to be closely genetically related than isolates of different serotypes.

The genetic variability among isolates of a given serotype makes the use of serotyping of *S. suis* unreliable as a means of strain identification for epidemiological studies. For the same reason, the usefulness of describing further capsular antigenic types of *S. suis* can be questioned. The existence of genetic diversity among strains of serotype 2 has previously been described (2, 17). In the present study, 31 isolates of serotype 2 were located in six ETs. These were grouped into a minor clonal group containing a single isolate in one ET and two larger clonal groups containing 5 and 25 isolates. Both of these clonal groupings contained isolates from different geographical regions, and one ET (ET 33) contained an isolate from a human as well as two from pigs. The human isolate and one of the porcine isolates were from cases of meningitis. Previous studies using DNA restriction endonuclease analysis and hybridization with ribosomal DNA probes have also demonstrated genetic similarities between strains of *S. suis* from pigs and humans (2, 17), and this is consistent with what is known about the zoonotic potential of *S. suis* (1).

Strains of *S. suis* type 2 have been shown to vary in virulence, with virulent strains possessing a muraminidase-released protein and an extracellular factor (27). In the current study, 12 of the 31 isolates of serotype 2 were from the tonsils of healthy pigs, and the rest were isolated from lesions in diseased animals. The virulence of these isolates was not tested, but it can be assumed that most, if not all, of the isolates recovered from diseased pigs were virulent. There was no evidence to suggest, however, that isolates from diseased animals were more likely to come from one or the other of the two larger clonal groupings of type 2 isolates. Therefore, virulent isolates may apparently originate from different genetic backgrounds. Moreover, considering all antigenic types of *S. suis*, there was no part of the phenogram that contained isolates which were recovered from only either healthy or diseased pigs, nor were those from a particular disease syndrome, e.g., meningitis, specifically clustered together. Virulence, as well as tissue tropism within the species, is not confirmed to narrow genetic groupings of the bacteria.

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