Evidence for *Serpulina hyodysenteriae* being recombinant, with an epidemic population structure

Darren J. Trott, Sophy L. Oxberry and David J. Hampson

Author for correspondence: David J. Hampson. Tel: +61 89 360 2287. Fax: +61 89 310 4144.
e-mail: hampson@numbat.murdoch.edu.au

Division of Veterinary and
Biomedical Sciences,
Murdoch University,
Murdoch, WA 6150,
Australia

The population structure of *Serpulina hyodysenteriae* was investigated using multilocus enzyme electrophoresis. A total of 231 isolates were divided into 50 electrophoretic types (ETs), with a mean genetic diversity of 0.29 for the number of ETs and 0.23 for the number of isolates. Subsets of isolates from two Australian states (71 isolates from Victoria and 68 isolates from Queensland) exhibited as much genetic variation as the entire collection. The calculated index of association (I_A) for the number of ETs (0.29 ± 0.17) was not significantly different from zero, and hence provided evidence for the occurrence of significant genetic recombination accounting for the observed variation between strains. In contrast, the I_A for the number of isolates (3.93 ± 0.03) was significantly different from zero, with seven of the 50 ETs (ETs 4, 6, 13, 14, 20, 33 and 35) containing 51% of all the isolates. Even when multiple isolates from the same farm were removed from the analysis, the I_A value for the number of isolates remained significantly greater than zero (I_A 9.87 ± 0.04), indicating that it was not biased by their inclusion. The results suggest that *S. hyodysenteriae* has an epidemic population structure.

Keywords: *Serpulina hyodysenteriae*, population structure, index of association, multilocus enzyme electrophoresis

INTRODUCTION

The intestinal spirochaete *Serpulina hyodysenteriae* is the aetiologic agent of swine dysentery (SD), a severe mucohaemorrhagic diarrhoeal disease of pigs (Stanton, 1992). *S. hyodysenteriae* also naturally infects rheas (Jensen *et al.*, 1996), and will colonize a range of experimentally infected animals, including mice (Joens & Glock, 1979), guinea pigs (Joens *et al.*, 1978) and chicks (Sueyoshi & Adachi, 1990). In Australia, SD has been considered to be the most serious production-limiting endemic disease of pigs (Cutler & Gardner, 1988). Many aspects of the causative organism remain unknown or are incompletely characterized, particularly its genetic structure, gene organization and regulation, its pathogenesis, and the immune response to its presence in the host (ter Huurne & Gaastra, 1995). Control measures for SD are expensive and have been hindered by the lack of an economical vaccine that is protective against all strains of the organism (e.g. Fernie

et al., 1983; Lysons *et al.*, 1987). Immunity against SD is serotype specific (Joens *et al.*, 1983). The serogrouping system devised for *S. hyodysenteriae* is based upon the immunoreactivity of hyperimmune rabbit sera raised against LPS antigens in agarose gel double-diffusion tests (Baum & Joens, 1979; Hampson *et al.*, 1989). In our laboratory, 11 specific sero strains representative of each serogroup (A–K) are recognized, with multiple serovars recognized in some of the serogroups (Lau & Hampson, 1992; Hampson *et al.*, 1997).

Strain diversity in *S. hyodysenteriae* has been investigated using restriction endonuclease analysis (Combs *et al.*, 1989, 1992; Harel *et al.*, 1994; ter Huurne *et al.*, 1992a) and random amplification of polymorphic DNA analysis (Dugourd *et al.*, 1996), with considerable genetic diversity being identified. Multilocus enzyme electrophoresis (MLEE) has also been used to investigate diversity amongst 99 isolates of *S. hyodysenteriae*, and identified 29 electrophoretic types (ETs) divided into four genetic divisions (Lee *et al.*, 1993a). In that study, the population structure was thought to be clonal; however, only a small number of isolates was examined

Abbreviations: ET, electrophoretic type; I_A , index of association; MLEE, multilocus enzyme electrophoresis; SD, swine dysentery.

and no attempt was made to calculate the index of association (I_A), a convenient measure of the degree of linkage disequilibrium operating in a population (Maynard Smith *et al.*, 1993). The I_A is calculated on the basis of the distribution of allelic mismatches between pairs of isolates, ETs or clonal groups among all loci, and is expressed as the ratio of variance observed in mismatches (V_o) to variance expected at linkage equilibrium (V_e), where $I_A = V_o/V_e - 1$ (Maynard Smith *et al.*, 1993). Measurements that are significantly different from zero indicate clonality (i.e. the population structure is approaching linkage disequilibrium caused by the non-random association of alleles that have arisen largely through ancestral genetic mutations), whereas those that are not significantly different from zero indicate that recombination is common in the species (i.e. random association of alleles in the population has been caused by rapid recombination, disrupting any linkage disequilibrium). The majority of bacterial species are clonal; however, some, including the important human pathogen *Neisseria gonorrhoeae*, have been shown to have a recombinant population structure (Maynard Smith *et al.*, 1993). Others such as the closely related *Neisseria meningitidis* superficially appear to be clonal (i.e. the I_A value for the number of isolates examined is significantly different from zero) but have significant underlying rates of recombination that have shaped the population structure (i.e. the I_A value for the number of ETs examined is not significantly different from zero). Such populations are said to have an epidemic population structure, with certain common clonal groups with selective advantage emerging to dominate the overall recombinant population (Maiden & Feavers, 1995).

In addition to the previous MLEE study of porcine isolates (Lee *et al.*, 1993a), we also have shown by MLEE that four *S. hyodysenteriae* strains isolated from rheas were closely related to or shared the same ET as certain porcine strains of the species (Trott *et al.*, 1996). In the present study, we have extended our previous observations by examining a further 128 porcine *S. hyodysenteriae* isolates at the same 15 enzyme loci used previously (Lee *et al.*, 1993a). These included reference strains from the USA and Canada, and field isolates from Australia, the UK and the Netherlands. Our major aims were to analyse a large collection of isolates to discover the extent of genetic diversity and distribution of haplotypes in *S. hyodysenteriae*, and to use the MLEE data to calculate the I_A value, and hence to provide insight into genetic factors underlying the population structure of the species.

METHODS

Bacterial isolates. The strain designation, origin and serotype (where known) of each of the 231 *S. hyodysenteriae* isolates used in this study may be obtained by contacting the author for correspondence. All isolates were obtained in pure culture from the collection held at the Australian Reference Laboratory for Intestinal Spirochaetes, Murdoch University, Perth, Western Australia. One hundred and three of the

isolates have been analysed previously by MLEE (Lee *et al.*, 1993a; Trott *et al.*, 1996). Isolates were from pigs, except for four isolated from rheas with necrotizing typhlitis (Jensen *et al.*, 1996). One hundred and ninety isolates were from Australian field cases of SD submitted between 1986 and 1996. These included 71 isolates from Victoria, 68 from Queensland, 26 from Western Australia, 15 from New South Wales, six from South Australia, and four from Tasmania. The remaining 41 isolates included 14 from the UK (including serostrains A1, P35/2, MC52/80, KF9 and the non-virulent strain VS1), 12 from the USA (including reference strains B204, B78^T and B234 and serostrains B6933, B8044 and Ack 300/8), 11 field isolates from the Netherlands, three serostrains from Canada (B169, FM88-90, FMV89.3323), and a single field isolate from South Korea.

The isolates in the collection had previously been confirmed as being *S. hyodysenteriae* by their strong β -haemolysis on blood agar, production of indole, and their positive reactivity in a PCR test based upon a cloned DNA sequence shown to be specific for *S. hyodysenteriae* (Atyeo *et al.*, 1996a). One hundred and sixty-five isolates (71.4%) had been serotyped by the method of Lau & Hampson (1992) and allocated to the 11 serogroups defined by Hampson *et al.* (1997).

Culture conditions. Stock cultures held at -80°C were thawed and inoculated into 7 ml Kunkle's anaerobic broth medium (Kunkle *et al.*, 1986), incubated at 37°C on a rocking platform for 72–96 h, transferred into 250 ml Kunkle's medium and again incubated at 37°C on a rocking platform for 72–96 h. After reaching mid-exponential phase (10^8 – 10^9 cells ml^{-1}), cultures were centrifuged (10000 g, 4°C , 20 min), washed in sterile PBS, pH 7.2, 0.15 M, and centrifuged again (10000 g, 4°C , 20 min). The resulting pellet was suspended in 5 vols sterile distilled water for MLEE analysis.

MLEE. The methods used for cell preparation, buffers, enzyme systems and running conditions for MLEE analysis were as previously described (Selander *et al.*, 1986; Lymbery *et al.*, 1990; Lee *et al.*, 1993a). Briefly, the suspended pellet was sonicated for two 30 s cycles on ice using a Branson B30 sonifier, centrifuged at 20000 g for 20 min and the supernatant was extracted and immediately used for electrophoresis in horizontal starch gels as described by Selander *et al.* (1986). The allelic profiles of 15 constitutive enzyme loci were examined (Lee *et al.*, 1993a): acid phosphatase, alcohol dehydrogenase, hexokinase and nucleoside phosphorylase were assayed using a Tris/maleate (pH 7.4) buffer system; alkaline phosphatase, phosphoglucose isomerase, guanine deaminase and mannose phosphate isomerase were assayed using a phosphate (pH 7) buffer system; esterase, fructose-1,6-diphosphatase, L-leucyl-glycyl-glycine peptidase, phosphoglucomutase and superoxide dismutase were assayed using a discontinuous buffer system (Tris/citrate gel buffer, LiOH electrode buffer); and arginine phosphokinase and glutamate dehydrogenase were assayed using a discontinuous buffer system (Tris/citrate gel buffer, borate electrode buffer). The variation in the mobility of an enzyme was interpreted as the products of different alleles at a specific locus encoding that enzyme. Distinctive mobility variants were numbered in order of decreasing rate of anodal migration, except for acid phosphatase allele 8, which appeared directly above allele 1. Isolates with identical enzymic profiles at all loci were grouped into an ET. ETs obtained for the new isolates were directly compared with standards obtained in the previous MLEE studies (Lee *et al.*, 1993a; Trott *et al.*, 1996) using the same numbering system for allele designation. The numbering system has been used to characterize other intestinal spiro-

Fig. 1. Phenogram depicting relationships between 231 isolates of *S. hyodysenteriae* analysed by MLEE. The phenogram is divided into four divisions, with divisions A and B comprising three subdivisions, and 50 ETs. The old ETs (1–29) of Lee *et al.* (1993a) are indicated in parentheses. The number of isolates in each ET, the origin of the isolates and their serotype where known are also indicated. Additional information such as the strain designation of each isolate, and the allele profiles of each ET is available from the corresponding author upon request. The unweighted paired group method of arithmetic means was used to construct the phenogram. CAN, Canada; KOR, Korea; NL, The Netherlands; NSW, New South Wales; Q, Queensland; SA, South Australia; T, Tasmania; UK, United Kingdom; US, United States; V, Victoria; WA, Western Australia. Serogroups A–K, serotyping system according to Hampson *et al.* (1997); NT, not tested; ?, not typable.

chaete species (Lee *et al.*, 1993b, c; Lee & Hampson, 1994) and thus is not completely sequential for *S. hyodysenteriae*. Gel runs were repeated up to six times to ensure the correct allele designation.

Statistical analysis. Genetic diversity (h), a measure of the amount of allelic variation at each enzyme locus, was calculated for both the number of ETs and the number of isolates as $h = (1 - \sum P_i^2) / [n / (n - 1)]$, where P_i is the frequency of the indicated allele and n the number of ETs or isolates (Nei, 1977). Comparison of the genetic diversities generated using either the number of ETs or the number of isolates is a

convenient indicator of the influence that isolate distribution into ETs has on the overall genetic diversity. Haplotypic diversity (H), a measure of the frequency of haplotypes within the population (the term haplotype referring to a set of haploid organisms sharing a common set of alleles and belonging to the same ET), was calculated as $H = 1 - \sum P_i^2$, where P_i is the frequency of the i th haplotype (Fusté *et al.*, 1996). Genetic distance between ETs was calculated by pairwise comparison as the proportions of loci at which dissimilar alleles occurred. A phenogram was generated to illustrate the genetic relationships between ETs using the unweighted pair group method of arithmetic means clustering

fusion strategy (Burr, 1968, 1970). The I_A , an indicator of the degree of linkage disequilibrium operating in the population, was calculated according to the measurement devised by Maynard Smith *et al.* (1993).

RESULTS

MLEE genetic relationships between isolates

The 231 *S. hyodysenteriae* isolates were divided into 50 ETs depicted as a phenogram in Fig. 1. The phenogram was divided into four major divisions: division A (ETs 1–31), division B (ETs 32–45), division C (ETs 46–48) and division D (ETs 49–50). Divisions B and C were separated by a genetic distance of 0.30, division A was separated from B and C by a genetic distance of 0.32, and division D was separated from A, B and C by a genetic distance of 0.4. The divisions roughly approximate those of Lee *et al.* (1993a) but include more ETs. The majority of ETs contained one to eight isolates; the exceptions were ET 4 (19 isolates), ET 6 (11 isolates), ET 13 (15 isolates), ET 14 (10 isolates), ET 20 (15 isolates), ET 33 (10 isolates) and ET 35 (36 isolates). The isolates from farmed rheas were located in ETs 15 (two isolates), 17 and 20, all of which also contained porcine isolates.

Statistical analysis

The mean number of alleles per locus was 2.7: three of the loci (alcohol dehydrogenase, esterase and superoxide dismutase) were monomorphic and the remaining 12

were polymorphic. Isolates yielded reproducible banding patterns, except for two (VS1 and VIC79) which had null alleles for the enzyme L-leucyl-glycyl-glycine peptidase. The mean genetic diversity per locus was 0.29 based on the number of ETs and 0.23 based on the number of isolates (Table 1). The mean genetic distance per locus was 0.14. Haplotypic diversity was calculated as 0.94. The I_A was calculated to be 3.94 ± 0.03 using the number of isolates, and 0.29 ± 0.176 using the number of ETs. The I_A for the number of isolates was significantly different from zero ($P < 0.05$), whilst the I_A for the number of ETs was not significantly different from zero. The results are consistent with the epidemic model of population structure described by Maynard Smith *et al.* (1993). When isolates that were located in the same ET and obtained from the same piggery, or from piggeries with known movements of pigs between them, were removed from the I_A calculation to leave just one representative from each piggery, the I_A value for the number of isolates was still significantly different from zero ($I_A 9.87 \pm 0.04$).

Geographical distribution

Seven of the 15 ETs which contained non-Australian field isolates and reference strains also contained isolates from Australia. These included ETs 20 and 35, which both contained isolates from the Netherlands and the USA. Of the 21 ETs that contained more than one

Table 1. Allele frequencies and genetic diversities (h ; mean genetic diversity 0.288) calculated using n = number of ETs at 15 enzyme loci analysed for 231 *S. hyodysenteriae* isolates divided into 50 ETs

Enzyme*	Frequency of the indicated allele									h
	0	1	2	3	4	5	6	7	8	
MPI	0.000	0.000	0.220	0.080	0.100	0.420	0.060	0.120	0.000	0.756
NP	0.000	0.000	0.360	0.360	0.000	0.000	0.100	0.000	0.000	0.746
LGG	0.020	0.340	0.600	0.040	0.000	0.000	0.000	0.000	0.000	0.533
HK	0.000	0.000	0.000	0.700	0.080	0.220	0.000	0.000	0.000	0.464
PGI	0.000	0.000	0.000	0.340	0.000	0.660	0.000	0.000	0.000	0.458
ACP	0.000	0.740	0.180	0.000	0.000	0.040	0.000	0.000	0.040	0.425
GDA	0.000	0.760	0.100	0.140	0.000	0.000	0.000	0.000	0.000	0.401
GDH	0.000	0.020	0.920	0.060	0.000	0.000	0.000	0.000	0.000	0.153
FDP	0.000	0.020	0.000	0.920	0.060	0.000	0.000	0.000	0.000	0.153
ALP	0.000	0.060	0.000	0.940	0.000	0.000	0.000	0.000	0.000	0.115
AK	0.000	0.960	0.040	0.000	0.000	0.000	0.000	0.000	0.000	0.078
ADH	0.000	0.000	0.980	0.000	0.020	0.000	0.000	0.000	0.000	0.040
PGM	0.000	0.000	0.000	1.000	0.000	0.000	0.000	0.000	0.000	0.000
EST	0.000	0.000	0.000	0.000	1.000	0.000	0.000	0.000	0.000	0.000
SOD	0.000	0.000	1.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000

* MPI, mannose phosphate isomerase; NP, nucleoside phosphorylase; LGG, L-leucyl-glycyl-glycine peptidase; HK, hexokinase; PGI, phosphoglucose isomerase; ACP, acid phosphatase; GDA, guanine deaminase; GDH, glutamate dehydrogenase; FDP, fructose-1,6-diphosphatase; ALP, alkaline phosphatase; AK, arginine phosphokinase; ADH, alcohol dehydrogenase; PGM, phosphoglucomutase; EST, esterase; SOD, superoxide dismutase.

Table 2. Ratio of isolates to ETs and genetic diversity (GD) according to isolate origin

Origin	No. of isolates	No. of ETs	Ratio of isolates:ETs	GD
Australian isolates				
Queensland	68	22	3.09	0.25
Victoria	71	23	3.09	0.27
Western Australia	26	5	5.20	NT
New South Wales	15	9	1.67	NT
South Australia	6	5	1.20	NT
Tasmania	4	3	1.33	NT
Total	190	43	4.42	0.28
Non-Australian isolates				
UK	14	8	1.75	NT
USA	12	6	2.00	NT
Netherlands	11	4	2.75	NT
Canada	3	3	1.00	NT
Korea	1	1	1.00	NT
Total	41	15	2.73	NT
Combined total	231	50	4.62	0.29

NT, Not tested.

Australian isolate, 15 contained isolates from more than one Australian state. No genetic clustering of isolates according to their place of origin was evident. The ratio of isolates to ETs for each of the geographical regions is shown in Table 2. The majority of the isolates were from Victoria and Queensland, and the isolate:ET ratio (3.09) and genetic diversity (0.25–0.27) obtained for each of these subpopulations were identical. The genetic diversities were very similar to the value obtained for the entire collection (0.29).

Serogroup distribution

Division A contained representatives of 10 of the 11 recognized serogroups. Divisions B, C and D contained isolates belonging to six serogroups. Isolates belonging to the most commonly occurring serogroups in Australia, A, B, D, G and E (Hampson *et al.*, 1994), were located in both divisions A and B. Isolates of serogroups C, H, I, J and K were restricted to division A, and serogroup F to divisions B and C. Of the 23 ETs that contained more than one isolate which had been serotyped, 16 were represented by more than one serogroup, including six of the seven ETs that contained 10 or more isolates.

Distribution of avirulent strains

Three strains that were known to be avirulent in pigs, strains B78^T (Jensen & Stanton, 1993), VS1 (Burrows & Lemcke, 1981) and SA3 (Lee *et al.*, 1993a), were genetically distinct from one another, being located in ETs 12, 39 and 50, respectively.

DISCUSSION

The spirochaetes represent a distinct branch of bacterial evolution (Woese, 1987) and the heterogeneity of the group is reflected in the wide range of the mol% G + C values for each genus (24–65%) (Baranton & Old, 1995). The *Serpulina*/*Brachyspira* cluster represents a deep branch of the spirochaete 16S rRNA phylogenetic tree, and the species that have been identified within the cluster are separated by very small 16S rRNA DNA sequence differences (Hookey *et al.*, 1994; Pettersson *et al.*, 1996; Stanton *et al.*, 1996), suggesting that speciation in the cluster is a relatively recent phenomenon. All of the known members of the genus *Serpulina* are gut-associated anaerobes that share common sugar fermentation pathways and NADH oxidase activity for survival in environments of low oxygen tension (Stanton, 1997). Of the five species that have been named and characterized, *S. hyodysenteriae* has been the most thoroughly studied. Recently, gene mapping of B78^T, the type strain of *S. hyodysenteriae*, showed that its genome is circular and approximately 3.2 bp in size; however, only relatively few genes have been identified and placed on the genetic map (Zuerner & Stanton, 1994). Electroporation/recombination experiments with *S. hyodysenteriae* haemolysin, flagella and NADH oxidase genes have recently been undertaken (ter Huurne *et al.*, 1992b; Hyatt *et al.*, 1994; Rosey *et al.*, 1995; Humphrey *et al.*, 1997). The existence of mechanisms for natural gene transfer and recombination in *S. hyodysenteriae* was substantiated by the discovery of a non-lytic transducing phage that is capable of packaging and transferring *S. hyodysenteriae* genes (Humphrey *et al.*, 1995, 1997). It is likely that the plasmids previously reported in *S. hyodysenteriae* cells by Combs *et al.*

(1992) are actually activated prophage. It is not known whether *S. hyodysenteriae* is naturally transformable, and as yet no other potential mechanisms of genetic exchange, such as the presence of transposons and insertional elements, have been identified in the species.

In a previous study using MLEE, Lee *et al.* (1993a) located 99 mainly Australian isolates into 29 specific ETs, with an overall genetic diversity of 0.26 based on the number of ETs. This diversity is comparable with most species of pathogenic bacteria (Go *et al.*, 1996). In the present study, an additional 21 ETs were identified by examining a further 132 isolates; however, the mean genetic diversity (0.29) was not substantially different from the previous study (Lee *et al.*, 1993a). The haplotypic diversity was high (0.94), indicating that the distribution of isolates was relatively even, and generally they were not over-represented in a single ET. Only one new allele (allele 8 for the enzyme acid phosphatase) was identified, and was only responsible for the generation of two additional ETs. The remainder of the new ETs were the result of different combinations of existing alleles. Two to four alleles were generated for each locus, except for mannose phosphate isomerase, in which six alleles were present. The phenogram generated in the expanded study was also similar to that of Lee *et al.* (1993a) in that four major genetic divisions were apparent. Division A contained the majority of ETs and isolates in both studies. In the study of Lee *et al.* (1993a), their ETs 28 and 29 in division D (ETs 49 and 50 located in division D in the present study) were genetically distinct from the remaining ETs and shared a number of unique alleles, including allele 5 for nucleoside phosphorylase and allele 2 for guanine deaminase. Interestingly, in the present study three isolates that were clustered together in division A (ETs 29, 30 and 31) also had allele 5 for nucleoside phosphorylase and allele 2 for guanine deaminase, but their remaining allele profiles were similar to the other ETs in division A. The separation of ETs into divisions A–D was not associated with any particularly strong clonal grouping of loci with the same alleles, as evidenced by the lack of deep branching in the bush-like phenogram. Lee *et al.* (1993a) speculated that division D might represent an avirulent biological group, since it contained the non-pathogenic strain SA3. In the present study, whilst SA3 was still in division D, avirulent strain B78^T belonged to division A and VS1 to division B. Thus, although we cannot add to the speculation concerning division D organisms, it appears that strains which lack virulence are genetically diverse. Strain B78^T may have lost its virulence for pigs after repeated subculture, whereas SA3 and VS1 have reduced chemotaxis towards porcine mucin (Milner & Sellwood, 1994). It is not known whether other isolates in the collection have reduced virulence, as relatively few *S. hyodysenteriae* isolates have been tested for pathogenicity in experimentally infected animals. It is likely that a number of different factors are required for full expression of *S. hyodysenteriae* virulence in pigs.

The addition of further isolates in this study also

confirmed the observations of Lee *et al.* (1993a) that serogroups are not distributed along distinct genetic lines. Divisions A and B each contained representatives of the most common serogroups in Australia, and a substantial number of ETs contained isolates of more than one serogroup. One explanation for this is that recombination may have modified the genes involved in LPS synthesis. The possibility that recombination may have influenced the overall population structure of *S. hyodysenteriae* is supported by the finding that the I_A value for the numbers of ETs (0.29 ± 0.17) was not significantly different from zero. This implies that genetic recombination has disrupted allelic clusters that are characteristic of a fully clonal population (Maynard Smith *et al.*, 1993). We assume that horizontal gene flow between strains accounts for this recombination, although the possibility of genetic modification through widespread internal sequence rearrangements (via insertion sequence or transposon-like mechanisms) cannot be excluded as an explanation for the disruption of allelic clusters at this stage.

Measurements of linkage disequilibrium are best performed on large populations where the chances of geographical or sampling bias are eliminated. Ideally isolates should be obtained from a large population of freely mixing individuals where there is opportunity for genetic exchange. Reference collections provide a ready source of isolates but are usually obtained from diseased individuals who are often unrelated, and a recombinant population could still appear clonal if the sample size was not large enough to include all possible combinations. This appeared to occur in the study of Lee *et al.* (1993a) in which 99 isolates of *S. hyodysenteriae* were examined. We calculated the I_A value for the 29 ETs identified in that study to be 0.61 ± 0.30 , a figure significantly different from zero, and therefore consistent with clonality in the species. As previously mentioned, ETs 28 and 29 were genetically distinct from the remaining ETs and possessed unique alleles. When these two ETs were removed from the calculation, the I_A was reduced to 0.45 ± 0.26 , a value that was no longer significantly different from zero, and hence suggesting a recombinant population. In the present study, the identification of additional ETs meant that the inclusion of ETs 49 and 50 (ETs 28 and 29 in the previous study) in the I_A calculation did not result in the appearance of linkage disequilibrium, and the population structure was clearly recombinant.

The I_A value for the number of isolates was 3.93 ± 0.03 . Since some of the isolates originated from the same piggery, or from piggeries with known movement of stock, and because it is unusual for more than one strain to be present in a piggery (Combs *et al.*, 1992), multiple isolates from the same piggery were then excluded from the I_A calculation. This resulted in the I_A value actually increasing to 9.87 ± 0.04 . These results strongly suggest that as a species *S. hyodysenteriae* fits the epidemic population structure model of Maynard Smith *et al.* (1993). In this type of structure, which was first described for the naturally transformable bacterium *N. meningi-*

tidis, recombination has shaped the population; however, particular selected clones that have survival or virulence advantages achieve an explosive and often widespread dissemination, giving the overall appearance of clonality. In the case of *N. meningitidis*, strains of serotype A are strongly clonal and are involved in epidemic disease, whereas certain serotype C strains are highly represented in small 'hyper-endemic' outbreaks amongst a background of genetically diverse recombinant strains involved in sporadic cases of meningitis (Maiden & Feavers, 1995). Similarly, 357 isolates of *Staphylococcus aureus* obtained from dairy cows with mastitis were divided into 39 ETs and had a recombinant structure; however, 90% of the isolates were restricted to eight ETs, resulting in a calculated I_A value for the number of isolates that suggested clonality (Kapur *et al.*, 1995). The results for *S. hyodysenteriae* showed that despite the operation of recombination, certain 'epidemic clones' have been widely disseminated around the globe; for instance ET 20 (15 isolates) and ET 35 (36 isolates) both contained isolates from several distinct geographical regions in the world. The overall results, however, must be interpreted in the light of possible geographical or temporal bias (i.e. the majority of isolates were obtained from Australia over a 10 year period). These do not seem to be significant factors, however, as the genetic diversities of isolates obtained from two distinct geographical regions in Australia were virtually identical, and were similar to the value obtained for the entire collection. It is possible that the isolates in the highly represented ETs may have survival advantages, such as differences in their virulence compared to the other less common strains. Alternatively they may have been disseminated artificially by relatively recent movement of pigs from infected nucleus or multiplier herds, both national and international, which have supplied pigs to production herds throughout Australia.

Other epidemiological and ecological factors associated with the disease must also be considered. Intensification of the pig industry in the last 30 years now means that pigs are kept in relatively discrete herds, with reduced opportunity for local transmission of pathogens between herds. The organism may enter a susceptible herd through the introduction of carrier pigs, and is then rapidly spread amongst non-immune stock. SD has largely been controlled by medication, destocking, thorough disinfection and limiting movements of pigs between herds (Hampson *et al.*, 1997). We presume that these relatively recent constraints on *S. hyodysenteriae* may have reduced the opportunity for strains of different genetic make-up to come into close contact, and then potentially transfer genetic material. It is likely, therefore, that the background population structure was created prior to intensification of the pig industry. This may in some way explain the epidemic population structure, with some ETs represented by large numbers of isolates because of limited opportunity for mixing. It may also be possible that less virulent strains of *S. hyodysenteriae* are likely to survive for longer in infected

piggeries, since they would escape detection. Similarly, in the absence of opportunities for recombination, and in the face of widespread antibiotic usage in the pig industry, strains that are more resistant to antimicrobials may become over-represented in the population.

We have recently provided evidence that *Serpulina pilosicoli*, a closely related spirochaete that is the agent of intestinal spirochaetosis (IS) in pigs, humans and other species, also has a recombinant population structure (D. J. Trott, A. S. J. Mikosza, B. G. Combs, S. L. Oxberry & D. J. Hampson, unpublished). IS is a chronic endemic disease that causes a mild loss of production in pigs, and is difficult to eradicate (Taylor & Trott, 1997). This is reflected in the population structure of the organism which approaches complete panmixis (i.e. the I_A values for both the number of ETs and the number of isolates are not significantly different from zero), a situation that is analogous with *N. gonorrhoeae* (Maynard Smith *et al.*, 1993). We have often found up to seven genetically distinct strains of *S. pilosicoli* in a single pig herd (Atyeo *et al.*, 1996b; D. J. Trott & D. J. Hampson, unpublished data), giving ample opportunity for the organisms to freely recombine. The differences between the *S. hyodysenteriae* and *S. pilosicoli* population structures may have been influenced by these epidemiological factors. *S. hyodysenteriae* and *S. pilosicoli* are amongst the first primarily enteric pathogens that have been shown to have a recombinant population structure. Other enteric pathogens such as *Escherichia coli* and *Salmonella* spp. have been shown to be strongly clonal (Maynard Smith *et al.*, 1993), despite the operation of genetic recombination and being under the same ecological pressure from the gastrointestinal immune system. The two species of *Serpulina* may have much in common with *Helicobacter pylori*, which also has a recombinant population structure (Go *et al.*, 1996).

MLEE is not as sensitive for strain typing as are whole genomic techniques such as PFGE or restriction endonuclease analysis (REA), although in the study of Lee *et al.* (1993a) MLEE and REA results corresponded closely. Previously, we showed that rhea and porcine strains of *S. hyodysenteriae* that were located in the same ET could be differentiated by PFGE (Trott *et al.*, 1996). The close genetic relationships of the rhea and porcine strains suggest recent crossing of host species boundaries (i.e. the rhea strains originated in pigs, or pigs were the possible source of the outbreaks of necrotizing typhlitis in farmed rheas); however, two rhea strains failed to colonize or induce disease when orally inoculated into experimental pigs, indicating that natural cross-species transmission is unlikely (Stanton *et al.*, 1997). A more likely scenario is that the porcine and rhea strains in the same ET independently share a common set of recombinations. Given that the frequencies for alleles 2 and 3 of the enzyme nucleoside phosphorylase are identical, three of the four rhea strains had profiles that were composed of the most prevalent allele at each of the 15 enzyme loci.

This study has demonstrated that *S. hyodysenteriae* is genetically diverse, and that a relatively high level of recombination has shaped the overall population structure of the species. It is possible that similar mechanisms operating in the genus *Serpulina* may be responsible for the high rate of recombination in both *S. hyodysenteriae* and *S. pilosicoli*. A non-lytic bacteriophage capable of transducing *S. hyodysenteriae* DNA from one strain to another is a likely candidate (Humphrey *et al.*, 1997), but further research should also investigate other possible means of genetic transfer and recombination. The epidemic population structure of *S. hyodysenteriae* suggests that certain clones may be more virulent or have better survival mechanisms than others, or that the epidemiology of the disease and the measures that have been adopted for the control of SD have limited the opportunity for genetic transfer. The practical implications of a recombinant population structure in relation to the epidemiology, host immunity and control of *S. hyodysenteriae* require further investigation.

ACKNOWLEDGEMENTS

This work was supported by a grant from the Australian Pig Research and Development Corporation (PRDC). D. J. T. was in receipt of a PRDC Postgraduate Scholarship. We sincerely thank Drs Neil Jensen, Agnes ter Huurne, Richard Sellwood, Thad Stanton and Jaël Lee for the provision of non-Australian isolates used in this study. We also thank Camilla Maltas and Julie Ardley for technical assistance.

REFERENCES

- Atyeo, R. F., Trott, D. J. & Hampson, D. J. (1996a). Use of polymerase chain reaction for the detection of swine dysentery and intestinal spirochaetosis from faecal samples. In *Proceedings of the 14th International Pig Veterinary Society Congress*, p. 291. Bologna: University of Bologna.
- Atyeo, R. F., Trott, D. J., Robertson, I. D., Buddle, J. R. & Hampson, D. J. (1996b). Epidemiological analysis of *Serpulina pilosicoli* within a high-health status pig herd. In *Proceedings of the 14th International Pig Veterinary Society Congress*, p. 287. Bologna: University of Bologna.
- Baranton, G. & Old, I. G. (1995). The spirochetes: a different way of life. *Bull Inst Pasteur* 93, 63–95.
- Baum, D. H. & Joens, L. A. (1979). Serotypes of beta-hemolytic *Treponema hyodysenteriae*. *Infect Immun* 25, 792–796.
- Burr, E. J. (1968). Division sorting with mixed character types. I. Standardisation of character values. *Aust Comput J* 1, 97–99.
- Burr, E. J. (1970). Division sorting with mixed character types. II. Fusion strategies. *Aust Comput J* 2, 98–103.
- Burrows, M. R. & Lemcke, R. M. (1981). Identification of *Treponema hyodysenteriae* by a rapid slide agglutination test. *Vet Rec* 108, 187–189.
- Combs, B., Hampson, D. J., Mhoma, J. R. L. & Buddle, J. R. (1989). Typing of *Treponema hyodysenteriae* by restriction endonuclease analysis. *Vet Microbiol* 19, 351–359.
- Combs, B. G., Hampson, D. J. & Harders, S. J. (1992). Typing of Australian isolates of *Treponema hyodysenteriae* by serology and by DNA restriction endonuclease analysis. *Vet Microbiol* 31, 273–285.
- Cutler, R. & Gardner, I. (1988). *A Blueprint for Pig Health Research*, pp. 48–49. Canberra: Australian Pig Research Council.
- Dugourd, D., Jacques, M., Bigras-Poulin, M. & Harel, J. (1996). Characterization of *Serpulina hyodysenteriae* isolates of serotypes 8 and 9 by random amplification of polymorphic DNA analysis. *Vet Microbiol* 48, 305–314.
- Fernie, D. S., Ripley, P. H. & Walker, P. D. (1983). Swine dysentery: protection against experimental challenge following single dose parenteral immunization with inactivated *Treponema hyodysenteriae*. *Res Vet Sci* 35, 217–221.
- Fusté, M. C., Pineda, M. A., Palomar, J., Vinas, M. & Lorén, J. G. (1996). Clonality of multidrug-resistant nontypable strains of *Haemophilus influenzae*. *J Clin Microbiol* 34, 2760–2765.
- Go, M. F., Kapur, V., Graham, D. Y. & Musser, J. M. (1996). Population genetic analysis of *Helicobacter pylori* by multilocus enzyme electrophoresis: extensive allelic diversity and recombinational population structure. *J Bacteriol* 178, 3934–3938.
- Hampson, D. J., Mhoma, J. R. L., Combs, B. & Buddle, J. R. (1989). Proposed revisions to the serological typing system for *Treponema hyodysenteriae*. *Epidemiol Infect* 102, 75–84.
- Hampson, D. J., Maltas, C. D., Stephens, C. P., McKechnie, K. & Buller, N. B. (1994). Serogroups of Australian isolates of *Serpulina hyodysenteriae*. *Aust Vet J* 71, 347.
- Hampson, D. J., Atyeo, R. F. & Combs, B. G. (1997). Swine dysentery. In *Intestinal Spirochaetes in Domestic Animals and Humans*, pp. 175–209. Edited by D. J. Hampson & T. B. Stanton. Wallingford: CAB International.
- Harel, J., Belanger, M., Forget, C. & Jacques, M. (1994). Characterisation of *Serpulina hyodysenteriae* isolates of serotypes 8 and 9 from Quebec by restriction endonuclease fingerprinting and ribotyping. *Can J Vet Res* 58, 302–305.
- Hookey, J. V., Barrett, S. P., Reed, C. S. & Barber, P. (1994). Phylogeny of human intestinal spirochaetes inferred from 16S rDNA sequence comparisons. *FEMS Microbiol Lett* 117, 345–350.
- Humphrey, S. B., Stanton, T. B. & Jensen, N. S. (1995). Mitomycin C induction of bacteriophages from *Serpulina hyodysenteriae* and *Serpulina innocens*. *FEMS Microbiol Lett* 134, 97–101.
- Humphrey, S. B., Stanton, T. B., Jensen, N. S. & Zuerner, R. L. (1997). Purification and characterization of VSH-1, a generalized transducing bacteriophage of *Serpulina hyodysenteriae*. *J Bacteriol* 179, 323–329.
- ter Huurne, A. A. H. M. & Gaastra, W. (1995). Swine dysentery: more unknown than known. *Vet Microbiol* 46, 347–360.
- ter Huurne, A. A. H. M., van Houten, M., Koopman, M. B. H., van der Zeijst, B. A. M. & Gaastra, W. (1992a). Characterization of Dutch porcine *Serpulina* (*Treponema*) isolates by restriction endonuclease analysis and DNA hybridization. *J Gen Microbiol* 138, 1929–1934.
- ter Huurne, A. A. H. M., van Houten, M., Muir, S., Kusters, J. G., van der Zeijst, B. A. M. & Gaastra, W. (1992b). Inactivation of a *Serpulina* (*Treponema*) *hyodysenteriae* haemolysin gene by homologous recombination: importance of this haemolysin in the pathogenesis of *S. hyodysenteriae* in mice. *FEMS Microbiol Lett* 92, 109–114.
- Hyatt, D. R., ter Huurne, A. A. H. M., van der Zeijst, B. A. M. & Joens, L. A. (1994). Reduced virulence of *Serpulina hyodysenteriae* haemolysin-negative mutants in pigs and their potential to protect pigs against challenge with a virulent strain. *Infect Immun* 62, 2244–2248.
- Jensen, N. S. & Stanton, T. B. (1993). Comparison of *Serpulina hyodysenteriae* B78, the type strain of the species, with other *S. hyodysenteriae* strains using enteropathogenicity studies and

restriction fragment length polymorphism analysis. *Vet Microbiol* 36, 221–231.

Jensen, N. S., Stanton, T. B. & Swayne, D. E. (1996). Identification of the swine pathogen *Serpulina hyodysenteriae* in rheas (*Rhea americana*). *Vet Microbiol* 52, 259–269.

Joens, L. A. & Glock, R. D. (1979). Experimental infection in mice with *Treponema hyodysenteriae*. *Infect Immun* 25, 757–760.

Joens, L. A., Songer, J. G., Harris, D. L. & Glock, R. D. (1978). Experimental infection with *Treponema hyodysenteriae* in guinea pigs. *Infect Immun* 22, 132–135.

Joens, L. A., Whipp, S. C., Glock, R. D. & Nuessen, M. E. (1983). Serotype-specific protection against *Treponema hyodysenteriae* infection in ligated colonic loops of pigs recovered from swine dysentery. *Infect Immun* 39, 460–462.

Kapur, V., Sischo, W. M., Greer, R. S., Whittam, T. S. & Musser, J. M. (1995). Molecular population genetic analysis of *Staphylococcus aureus* recovered from cows. *J Clin Microbiol* 33, 376–380.

Kunkle, R. A., Harris, D. L. & Kinyon, J. M. (1986). Autoclaved liquid medium for propagation of *Treponema hyodysenteriae*. *J Clin Microbiol* 24, 669–671.

Lau, T. T. A. & Hampson, D. J. (1992). The serological grouping system for *Serpulina* (*Treponema*) *hyodysenteriae*. *Epidemiol Infect* 109, 255–263.

Lee, J. I. & Hampson, D. J. (1994). Genetic characterisation of intestinal spirochaetes and their association with disease. *J Med Microbiol* 40, 365–371.

Lee, J. I., Hampson, D. J., Combs, B. G. & Lymbery, A. J. (1993a). Genetic relationships between isolates of *Serpulina* (*Treponema*) *hyodysenteriae*, and comparison of methods for their subspecific differentiation. *Vet Microbiol* 34, 35–46.

Lee, J. I., Hampson, D. J., Lymbery, A. J. & Harders, S. J. (1993b). The porcine intestinal spirochaetes: identification of new genetic groups. *Vet Microbiol* 34, 273–285.

Lee, J. I., McLaren, A. J., Lymbery, A. J. & Hampson, D. J. (1993c). Human intestinal spirochetes are distinct from *Serpulina hyodysenteriae*. *J Clin Microbiol* 31, 16–21.

Lymbery, A. J., Hampson, D. J., Hopkins, R. M., Combs, B. & Mhoma, J. R. L. (1990). Multilocus enzyme electrophoresis for identification and typing of *Treponema hyodysenteriae* and related spirochaetes. *Vet Microbiol* 22, 89–99.

Lysons, R. J., Burrows, M. R., Jones, P. W. & Collins, P. (1987). Swine dysentery, a new and effective vaccine. *Pig Vet Soc Proc* 18, 87–91.

Maiden, M. C. J. & Feavers, I. M. (1995). Population genetics and global epidemiology of the human pathogen *Neisseria meningitidis*. In *Population Genetics of Bacteria*, pp. 269–293. Edited by S. Baumberg, J. P. W. Young, E. M. H. Wellington & J. R. Saunders. Cambridge: Cambridge University Press.

Maynard Smith, J., Smith, N. H., O'Rourke, M. & Spratt, B. G. (1993). How clonal are bacteria? *Proc Natl Acad Sci USA* 90, 4384–4388.

Milner, J. A. & Sellwood, R. (1994). Chemotactic response to

mucin by *Serpulina hyodysenteriae* and other porcine spirochetes: potential role in intestinal colonization. *Infect Immun* 62, 4095–4099.

Nei, M. (1977). F-statistics and analysis of gene diversity in subdivided populations. *Ann Hum Genet* 41, 225–233.

Pettersson, B., Fellström, C., Andersson, A., Uhlén, M., Gunnarsson, A. & Johansson, K. (1996). The phylogeny of intestinal porcine spirochetes (*Serpulina* species) based on sequence analysis of the 16S rRNA gene. *J Bacteriol* 178, 4189–4199.

Rosey, E. L., Kennedy, M. J., Petrella, D. K., Ulrich, R. G. & Yancey, R. J., Jr (1995). Inactivation of *Serpulina hyodysenteriae* *flaA1* and *flaB1* periplasmic flagellar genes by electroporation-mediated allelic exchange. *J Bacteriol* 177, 5959–5970.

Selander, R. K., Caugant, D. A., Ochman, H., Musser, J. M., Gilmour, M. N. & Whittam, T. S. (1986). Methods of multilocus enzyme electrophoresis for bacterial population genetics and systematics. *Appl Environ Microbiol* 51, 873–884.

Stanton, T. B. (1992). Proposal to change the genus designation *Serpula* to *Serpulina* gen. nov. containing the species *Serpulina hyodysenteriae* comb. nov. and *Serpulina innocens* comb. nov. *Int J Syst Bacteriol* 42, 189–192.

Stanton, T. B. (1997). Physiology of ruminal and intestinal spirochaetes. In *Intestinal Spirochaetes in Domestic Animals and Humans*, pp. 7–45. Edited by D. J. Hampson & T. B. Stanton. Wallingford: CAB International.

Stanton, T. B., Lee, J. I., McLaren, A. J., Trott, D. J., Hampson, D. J. & Paster, B. J. (1996). Differentiation of intestinal spirochaetes by multilocus enzyme electrophoresis and 16S rRNA sequence comparisons. *FEMS Microbiol Lett* 136, 181–186.

Stanton, T. B., Jensen, N. S., Bosworth, B. T. & Kunkle, R. A. (1997). Evaluation of the virulence of rhea *S. hyodysenteriae* strains for swine. In *Proceedings of the 1st National Animal Disease Centre Virtual Conference*, p. 100006. Ames, IA.

Sueyoshi, M. & Adachi, Y. (1990). Diarrhoea induced by *Treponema hyodysenteriae*: a young chick caecal model for swine dysentery. *Infect Immun* 58, 3348–3362.

Taylor, D. J. & Trott, D. J. (1997). Porcine intestinal spirochaetosis and spirochaetal colitis. In *Intestinal Spirochaetes in Domestic Animals and Humans*, pp. 211–241. Edited by D. J. Hampson & T. B. Stanton. Wallingford: CAB International.

Trott, D. J., Atyeo, R. F., Lee, J. I., Swayne, D. A., Stoutenburg, J. W. & Hampson, D. J. (1996). Genetic relatedness amongst intestinal spirochaetes isolated from rats and birds. *Lett Appl Microbiol* 23, 431–436.

Woese, C. R. (1987). Bacterial evolution. *Microbiol Rev* 51, 221–271.

Zuerner, R. L. & Stanton, T. B. (1994). Physical and genetic map of the *Serpulina hyodysenteriae* B78 chromosome. *J Bacteriol* 176, 1087–1092.

Received 15 May 1997; revised 4 July 1997; accepted 9 July 1997.