Characterization of Dutch porcine *Serpulina* (*Treponema*) isolates by restriction endonuclease analysis and DNA hybridization

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Genomes of 55 Dutch porcine *Serpulina* (*Treponema*) *hyodysenteriae* and non-pathogenic *Serpulina* isolates were characterized by restriction endonuclease analysis (REA) and DNA hybridization. The Dutch porcine isolates were compared with American Type Culture Collection (ATCC) strains of *S. hyodysenteriae* and *S. innocens* and isolates of *S. hyodysenteriae* with known serotypes (reference strains). REA of the Dutch *S. hyodysenteriae* isolates resulted in two main patterns, while the non-pathogenic isolates had many distinct REA patterns, all different from the *S. hyodysenteriae* strains. The *S. hyodysenteriae* reference strains all had distinct REA patterns, different from the Dutch strains. Upon Southern hybridization with a *S. hyodysenteriae* DNA fragment encoding a flagellar protein, all *S. hyodysenteriae* strains could be divided in two groups. The non-pathogenic *Serpulina* strains had many distinct hybridization patterns and hybridized less intensely. Upon hybridization with a *S. hyodysenteriae* DNA fragment encoding a haemolysin, DNA of all *S. hyodysenteriae* strains reacted in the same band. DNA of non-pathogenic Dutch *Serpulina* strains and *S. innocens* did not hybridize. It was concluded that there are two main genotypes of *S. hyodysenteriae* in the Netherlands. This could be of importance for recombinant DNA vaccine development.

Introduction

Serpulina (*Treponema*) *hyodysenteriae*, an anaerobic β -haemolytic spirochaete, is the major aetiologic agent of swine dysentery, a diarrhoeal disease of swine characterized by inflammation, excess mucus production and necrosis of the large intestine. This leads to haemorrhage, dehydration, weight loss and eventually death (Harris *et al.*, 1972; Taylor & Alexander, 1971).

S. hyodysenteriae is differentiated from the non-pathogenic, weakly β -haemolytic *Serpulina* (*Treponema*) *innocens* by strong β -haemolysis on blood agar plates or by enteropathogenicity testing in pigs or mice (Kinyon & Harris, 1979; Kinyon *et al.*, 1977).

Control of swine dysentery can be achieved by drug medication (Olson, 1986) or vaccination and the latter should be feasible since natural immunity in pigs against the disease has been demonstrated in convalescent animals (Joens *et al.*, 1979). The immunity which developed in pigs was partially serotype specific (Joens *et*

al., 1983). Attempts to induce immunity to swine dysentery with whole-cell preparations had limited success and resulted only in partial protection (Fernie *et al.*, 1983; Glock *et al.*, 1978; Lysons & Burrows, 1989).

Thus it might be necessary to develop a recombinant DNA vaccine, containing proteins with epitopes common to all types. Since flagella and haemolysin(s) are considered to be important virulence factors of *S. hyodysenteriae* (Koopman *et al.*, 1992; Muir *et al.*, 1992; Boyden *et al.*, 1989; Lysons *et al.*, 1991; Huurne *et al.*, 1992), we focused on these proteins. Genes encoding some of these proteins have been cloned (Koopman *et al.*, 1992; Muir *et al.*, 1992).

For the development of a recombinant DNA vaccine for swine dysentery, we are interested in the prevalence of pathogenic clones of *S. hyodysenteriae* in the Netherlands. It was our objective to study the genetic diversity of porcine *Serpulina* isolates with special emphasis on genes encoding flagellar proteins and haemolysin.

Genotyping by restriction endonuclease analysis (REA) and DNA hybridization has been useful for a number of bacterial species since it not only differentiated between strains but also demonstrated previously

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Abbreviations: REA, restriction endonuclease analysis.

Table 1. Haemolysis, hybridization patterns with Fla and Tly probes and REA patterns of porcine *Serpulina* strainsTly, DNA fragment from a haemolytic clone of *S. hyodysenteriae*; Fla, DNA fragment of *S. hyodysenteriae* coding for a 43 kD flagellar protein.

Strain*	Haemolysis	Hybridization pattern with probe:		REA pattern	Strain*	Haemolysis	Hybridization pattern with probe:		REA pattern
		Fla	Tly				Fla	Tly	
ATCC 31287 (ST 1)	Strong	P	A	XVI	Z5	Strong	P	B	II
ATCC 27164 (ST 1)	Strong	P	A	XVII	Z6	Strong	P	B	II
ATCC 31212 (ST 2)	Strong	P	B	XVIII	Z13	Strong	P	B	II
B169† (ST 3)	Strong	P	B	XIX	Z14	Strong	P	B	II
A1† (ST 4)	Strong	P	A	XX	V4	Strong	P	B	II
B8044† (ST 5)	Strong	P	A	XXI	V5	Strong	P	B	II
B6933† (ST 6)	Strong	P	B	XXII	V6	Strong	P	B	II
Ack 300/8† (ST 7)	Strong	P	A	XXIII	V7	Strong	P	B	II
A1	Strong	P	A	I	V10	Strong	P	B	II
A8	Strong	P	A	I	D1	Strong	P	B	II
A9	Strong	P	A	I	D2	Strong	P	B	II
B1	Strong	P	A	I	D3	Strong	P	B	II
B2	Strong	P	A	I	D10	Strong	P	B	II
B7	Strong	P	A	I	C2	Strong	P	B	II
B8	Strong	P	A	I	C3	Strong	P	B	II
Z10	Strong	P	A	I	B4	Strong	P	A	III
Z11	Strong	P	A	I	B5	Strong	P	B	IV
V9	Strong	P	A	I	Z4	Strong	P	A	V
D4	Strong	P	A	I	V8	Strong	P	A	V
D5	Strong	P	A	I	Z7	Strong	P	A	VI
D6	Strong	P	A	I	ATCC 29796 (<i>S. inn.</i>)	Weak	O	J	XXIV
D8	Strong	P	A	I	A2	Weak	O	C	VII
C1	Strong	P	A	I	A3	Weak	O	C	VII
C4	Strong	P	A	I	A7	Weak	O	D	VIII
C5	Strong	P	A	I	Z2	Weak	O	E	IX
C6	Strong	P	A	I	Z3	Weak	O	F	X
A4	Strong	P	B	II	Z8	Weak	O	G	XI
A5	Strong	P	B	II	Z9	Weak	O	H	XII
A6	Strong	P	B	II	Z12	Weak	O	I	XIII
B3	Strong	P	B	II	D7	Weak	O	D	XIV
Z1	Strong	P	B	II	D11	Weak	O	I	XV

* Strains B1–8, Z1–9, V4–9 and D1–11, isolates from samples from Animal Health Centres (AHC) Boxtel, Zwolle, Velp and Drachten respectively; A1–8 and C1–6, isolates received from AHC Boxtel and Central Veterinary Institute Lelystad, respectively; ATCC, American Type Culture Collection; †, strains with known serotypes obtained through Dr S. Muir from L. A. Joens; ST.1–7, *S. hyodysenteriae* serotypes 1–7; *S. inn.*, *S. innocens*.

unknown genetic relationships (Collins *et al.*, 1990; Ellis *et al.*, 1991; van Eys *et al.*, 1991; LeFebvre *et al.*, 1989; Thiermann *et al.*, 1986).

We have characterized *Serpulina* isolates obtained from pigs from various regions in the Netherlands by REA and DNA hybridization. REA and DNA hybridization patterns of these *Serpulina* isolates were compared with those of *Serpulina* strains of the American Type Culture Collection (ATCC) and several isolates with known serotypes.

Methods

Serpulina isolates. Fifty-five faecal and colon samples, positive in the unabsorbed, direct immunofluorescence assay (IFA) (Smit & Jongerius, 1982), were received from the Dutch Regional Animal Health Centres (AHC) at Drachten, Zwolle, Velp and Boxtel. All samples came from diseased pigs from different farms. For primary isolation all samples were streaked on Trypticase soy agar (TSA; BBL

Microbiology Systems) supplemented with 5% (v/v) sheep blood (TSAB) and 400 µg spectinomycin ml⁻¹ (Songer *et al.*, 1976) and on the improved selective medium of TSAB according to Kunkle & Kinyon (1988). For subculturing TSAB was used. Incubation took place under anaerobic conditions (Gas-Pak jar) at 37 °C for 5 d. The gaseous environment comprised CO₂ and H₂ and was produced using a gas generator envelope with palladium catalyst (BBL). A number of Dutch porcine *Serpulina* strains previously isolated at the AHC at Boxtel (nine isolates) and the Central Veterinary Institute (CVI) at Lelystad (six isolates) were also examined. Three *S. hyodysenteriae* isolates [B234^T ATCC 31287, B78^T ATCC 27164 (both serotype 1) and B204^T ATCC 31212 (serotype 2)] and one *S. innocens* isolate (ATCC 29796) were obtained from the American Type Culture Collection. DNA of isolates with known serotypes was received from Dr S. Muir: B169 (serotype 3), A1 (serotype 4), B8044 (serotype 5), B6933 (serotype 6) and Ack 300/8 (serotype 7) (Mapother & Joens, 1985). All isolates were differentiated as strongly or weakly β-haemolytic by the haemolytic pattern on TSAB (Kinyon & Harris, 1979).

Isolation of chromosomal DNA. For the isolation of chromosomal DNA, all isolates were grown on TSAB. Approximately 5 × 10⁹ treponemes were harvested and washed in phosphate-buffered saline

Fig. 1. REA and Southern hybridization of *TaqI* digests of DNA of Dutch porcine *Serpulina* isolates. Lanes 1–6 in each of (a), (b) and (c) contain DNA of *S. hyodysenteriae* strains, while lanes 7–10 contain DNA of non-pathogenic strains. Numbers and letters at the top of each lane refer to the isolates/REA or hybridization patterns listed in Table 1. (a) Gel electrophoresis of *TaqI* digests of the isolates. (b) Southern hybridization of the same gel with Fla probe. (c) Southern hybridization of the same gel with Tly probe. Molecular size markers are indicated on the right.

(PBS) pH 7.4. Chromosomal DNA was isolated by standard methods (Sambrook *et al.*, 1989).

Restriction endonuclease analysis. The restriction endonuclease *TaqI* (BRL) was selected for this study since it yielded clear, well distinguished bands. Approximately 2 µg chromosomal DNA was digested with 10 U *TaqI* and the fragments were separated in a 0.7% agarose gel in TAE buffer (Tris/acetate/EDTA) at 2 V cm⁻¹ for 16 h (Sambrook *et al.*, 1989). Subsequently, the gels were stained with ethidium bromide and photographed under short-wavelength UV light.

DNA hybridization. Fragments were transferred to a Hybond-N membrane (Amersham) by Southern blotting using standard procedures (Sambrook *et al.*, 1989). An *S. hyodysenteriae* DNA fragment of approximately 2.2 kbp coding for a 43 kDa flagellar protein (Fla probe) (Koopman *et al.*, 1992), and a 1.4 kbp DNA fragment containing the *tly* gene encoding a *S. hyodysenteriae* haemolysin (Tly probe) (Muir *et al.*, 1992) were radiolabelled and used as probes (Sambrook *et al.*, 1989). Filters were prehybridized for 8 h at 42 °C in a prehybridization mix with 50% (v/v) formamide and salmon sperm DNA. Filters were hybridized for 16 h at 42 °C in the same buffer and washed three times in 2 × SSPE (20 mM-NaH₂PO₄, 360 mM-NaCl, 2 mM-EDTA) + 0.1% SDS at 42 °C for 15 min (Sambrook *et al.*, 1989). The blots were dried and exposed to X-ray film.

Results

Serpulina isolates

From the 55 samples received, 38 *Serpulina* strains were isolated. A total of 43 Dutch *S. hyodysenteriae* and 10 Dutch non-pathogenic porcine spirochaetal (referred to below as non-pathogenic *Serpulina*) strains were examined. They were compared with eight *S. hyodysenteriae* reference strains and with *S. innocens* ATCC 29796.

Restriction endonuclease analysis

Although the 43 *S. hyodysenteriae* Dutch porcine strains had six different REA patterns, most strains fell within pattern I or II: I (18 strains), II (20 strains), III (1 strain), IV (1 strain), V (2 strains) and VI (1 strain). The 10 non-pathogenic Dutch *Serpulina* strains had a greater variation and all differed from the *S. hyodysenteriae* strains: patterns VII to XV (only 2 strains had identical REA patterns). The *S. hyodysenteriae* reference strains

Fig. 2. REA and Southern hybridization of *TaqI* digests of DNA of *S. hyodysenteriae* and *S. innocens* reference strains. Serotypes (ST) and *S. innocens* (*S. inn.*) are indicated at the top of each lane. The strains representing the serotypes are listed in Table 1. For serotype 1, DNA of strain ATCC 31287 was used. Letters at the top of each lane refer to the REA or hybridization patterns, also listed in Table 1. (a) Gel electrophoresis of *TaqI* digests of the isolates. (b) Southern hybridization of the same gel with Fla probe. (c) Southern hybridization of the same gel with Tly probe. Molecular size markers are indicated on the right.

all had different REA patterns: XVI to XXIII. *S. innocens* had REA pattern XXIV.

REA patterns of all strains are listed in Table 1. Representative REA patterns are shown in Figs 1(a) and 2(a).

DNA hybridization

Hybridization with the Fla probe was carried out on DNA from all strains. The 18 strains with REA pattern I and 9 strains with other REA patterns hybridized strongly with fragments of approximately 4 and 14 kbp: pattern A. The 20 strains with REA pattern II and 4 strains with other REA patterns hybridized strongly with fragments of approximately 4 and 4.2 kbp: pattern B.

Hybridization of the DNA from the non-pathogenic Dutch *Serpulina* strains with the Fla probe was less intense and at different fragments: 2 strains with 3.1 kbp

(pattern C); 2 strains with 8.3 kbp (pattern D); 1 strain with 3.7 kbp (pattern E); 1 strain with 15 kbp (pattern F); 1 strain with 6.5 kbp (pattern G); 1 strain with 1.8 kbp (pattern H); and 2 strains with 8.7 kbp (pattern I). *S. innocens* hybridized, also less intensely, with a fragment of 7.2 kbp (pattern J).

Hybridization patterns with the Fla probe are listed in Table 1. Examples of these patterns are shown in Figs 1(b) and 2(b).

Upon hybridization with the Tly probe, all *S. hyodysenteriae* Dutch and reference strains hybridized with a strong band at approximately 6.5 kbp: pattern P. The non-pathogenic Dutch *Serpulina* strains and *S. innocens* did not hybridize with Tly: pattern O.

Hybridization patterns with the Tly probe are listed in Table 1. Examples of these patterns are shown in Figs 1(c) and 2(c).

Discussion

Protection from swine dysentery after vaccination with whole cell preparations (based mainly on LPS) is relatively poor (Fernie *et al.*, 1983; Glock *et al.*, 1978; Lysons & Burrows, 1989) and partially serotype specific (Joens *et al.*, 1983). In order to circumvent these drawbacks, a subunit vaccine might be needed, based on possible virulence factors other than LPS, which induces protection against challenge with all homologous and heterologous types. In the case of another bacterium, *Actinobacillus pleuropneumoniae*, the haemolysin/cytotoxin determinant was chosen for this purpose (Bosch *et al.*, 1990; Frey & Nicolet, 1990; Smit *et al.*, 1990). Likewise, we have also focused on haemolysin(s) as well as on the flagella as possible virulence factors of *S. hyodysenteriae*. To confirm that these recombinant DNA vaccines will provide universal protection, the genotypes of prevalent strains rather than serotypes are important (Ellis *et al.*, 1991; Thiermann & Ellis, 1985).

Combs *et al.* (1989) were able to distinguish REA patterns of strains within serotypes of *S. hyodysenteriae*. They therefore suggested that serotyping may not give an accurate reflection of the number of different pathogenic clones within an area. In the present study two ATCC strains belonging to the same serotype were found to have different REA patterns also. Thiermann & Ellis (1985) could, in this way, distinguish genotypes with different pathogenicity within *Leptospira interrogans* serogroup Sejroe serovar *hardjo*: *hardjo-bovis* and *hardjo-prajitno*. This had consequences for vaccine components, as the vaccine so far used in North America contained reference strain *hardjo-prajitno*, while all isolates examined in that study, belonged to the *hardjo-bovis* genotype.

In the present study the Dutch *S. hyodysenteriae* isolates could be divided in two main REA patterns (Table 1). The *S. hyodysenteriae* reference strains all had different REA patterns (Fig. 2a). This is in accordance with the results of Combs *et al.* (1989), who found that REA patterns of all local (West Australian) strains were different from those of overseas strains (including five reference strains used in the present study: B78^T, B234^T, B204^T, B169 and A1). Moreover, the REA patterns of the five above-mentioned reference strains were all distinct in both our study and that of Combs *et al.* (1989), although different restriction enzymes were used. The non-pathogenic Dutch *Serpulina* strains and *S. innocens* showed an extensive variety of REA patterns, all different from the REA patterns of the *S. hyodysenteriae* strains (Table 1).

DNA hybridization with defined DNA probes has been used to demonstrate genetic relationships and differences within leptospires, *Borrelia burgdorferi* and

Mycobacterium paratuberculosis (Collins *et al.*, 1990; van Eys *et al.*, 1991; LeFebvre *et al.*, 1989). Van Eys *et al.* (1991) used defined DNA probes to demonstrate four subgroups with extensive genomic homology within serogroup Sejroe of *L. interrogans*. Strains within REA patterns could be distinguished by hybridization, while a number of serovars outside serogroup Sejroe also showed homology with these subgroups.

In the present study two defined DNA probes of *S. hyodysenteriae* (Fla and Tly) were used for Southern analysis. Upon hybridization with the Fla probe all *S. hyodysenteriae* isolates fell into hybridization patterns A and B. Hybridization with the Fla probe did not distinguish strains within REA patterns, but showed homologies between strains of different REA patterns. Upon hybridization with the Tly probe, all non-pathogenic strains hybridized weakly with one band of varying size, confirming the large heterogeneity of these strains.

Upon hybridization with the *S. hyodysenteriae* Tly probe, all *S. hyodysenteriae* isolates fell into a single pattern (Table 1, Figs 1c and 2c). This is in accordance with the results of Muir *et al.* (1992), who did Southern hybridization of the identical reference strains of *S. hyodysenteriae* with the same Tly probe to examine the copy number of a haemolysin gene. None of the non-pathogenic Dutch *Serpulina* strains nor *S. innocens* hybridized with this probe. This indicated extensive genomic homology within all tested *S. hyodysenteriae* strains. Under these hybridization conditions no genomic relationship was found between the tested *S. hyodysenteriae* and non-pathogenic *Serpulina* strains.

We have called the weakly haemolytic Dutch porcine spirochaetal strains non-pathogenic *Serpulina*. Although some strains could belong to the non-pathogenic *S. innocens* group, it seems more likely that these strains belong to more than one species. The variation observed in REA pattern and hybridization pattern to the Fla probe of these non-pathogenic spirochaetes was in accordance with the serological variation of non-pathogenic spirochaetes (Adachi *et al.*, 1981; Lemcke & Burrows, 1979). Lymbery *et al.* (1990) also identified three genetically different groups among 10 isolates of non-pathogenic spirochaetes.

In view of the observed homologies, we suggest that challenge studies with swine dysentery vaccines based on recombinant DNA technology can be limited to a small number of strains. Depending on the component(s), either haemolysin and/or flagellar proteins, challenge studies need to be done with one or two genotypes only.

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