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

AGNES A. H. M. ter Huurne,* MARINA van HOUTEN, MARCEL B. H. KOOPMAN, BERNARD A. M. van der ZEIJST and WIM GAASTRA

Institute of Infectious Diseases and Immunology, Department of Bacteriology, Faculty of Veterinary Medicine, University of Utrecht, Yalelaan 1, PO Box 80.165, 3508 TD Utrecht, The Netherlands

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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; Boydén *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
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 (Senger 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 [B2341 ATCC 31287, B2768 ATCC 27164 (both serotype 1) and B2047 ATCC 31212 (serotype 2)] and one S. innoxens 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), B044 (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 × 10⁵ treponemes were harvested and washed in phosphate-buffered saline

### Table 1. Haemolysis, hybridization patterns with Fla and Tly probes and REA patterns of porcine Serpulina strains

<table>
<thead>
<tr>
<th>Strain*</th>
<th>Haemolysis</th>
<th>Hybridization pattern with probes:</th>
<th>REA pattern</th>
<th>Strain*</th>
<th>Haemolysis</th>
<th>Hybridization pattern with probes:</th>
<th>REA pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATCC 31287 (ST 1)</td>
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<td>P A</td>
<td>XVI</td>
<td>Z5</td>
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<td>P B</td>
<td>II</td>
</tr>
<tr>
<td>ATCC 27164 (ST 1)</td>
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<td>P A</td>
<td>XVII</td>
<td>Z6</td>
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<td>P B</td>
<td>II</td>
</tr>
<tr>
<td>ATCC 31212 (ST 2)</td>
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<td>P B</td>
<td>XVIII</td>
<td>Z13</td>
<td>Strong</td>
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<td>II</td>
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<tr>
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<td>P A</td>
<td>XX</td>
<td>V4</td>
<td>Strong</td>
<td>P B</td>
<td>II</td>
</tr>
<tr>
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<td>XXII</td>
<td>V6</td>
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<tr>
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<td>P A</td>
<td>XXIII</td>
<td>V7</td>
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<td>P A</td>
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<td>Strong</td>
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<td>II</td>
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</tr>
<tr>
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<td>D10</td>
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<td>IV</td>
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<tr>
<td>D6</td>
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<td>P A</td>
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<td>O</td>
<td>JX</td>
</tr>
<tr>
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<td>P A</td>
<td>1</td>
<td>A2</td>
<td>Weak</td>
<td>O</td>
<td>VII</td>
</tr>
<tr>
<td>C1</td>
<td>Strong</td>
<td>P A</td>
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<td>Weak</td>
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</tr>
<tr>
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<td>A7</td>
<td>Weak</td>
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<td>VIII</td>
</tr>
<tr>
<td>C5</td>
<td>Strong</td>
<td>P A</td>
<td>1</td>
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<td>Weak</td>
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<td>IX</td>
</tr>
<tr>
<td>C6</td>
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<td>P B</td>
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<td>Z5</td>
<td>Weak</td>
<td>O</td>
<td>X</td>
</tr>
<tr>
<td>A4</td>
<td>Strong</td>
<td>P B</td>
<td>II</td>
<td>Z8</td>
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<td>O</td>
<td>XI</td>
</tr>
<tr>
<td>A5</td>
<td>Strong</td>
<td>P B</td>
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<td>D7</td>
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<td>O</td>
<td>XIV</td>
</tr>
<tr>
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<td>Strong</td>
<td>P B</td>
<td>II</td>
<td>D11</td>
<td>Weak</td>
<td>O</td>
<td>XV</td>
</tr>
</tbody>
</table>

* 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. innoxens.
Characterization of porcine Serpulina isolates

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
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/cytoxin 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 genotypic 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 hardjbovis 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 above-mentioned reference strains were all distinct (Table 1). The S. hyodysenteriae contained reference strain hardjo-prajitno, while all isolates could be divided in two main 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 above-mentioned reference strains). Thiermann (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 hardjbovis genotype.

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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 Fla 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.

We thank Dr S. Muir for providing a haemolytic S. hyodysenteriae clone; Dr J. G. Kusters and Ing. K. A. Zwaagstra for critical comments; the Dutch Animal Health Centres and the Central Veterinary Institute for providing isolates and/or samples.
References


