SHORT ARTICLE

Lipo-oligosaccharide profiles of *Serpulina pilosicoli* strains and their serological cross-reactivities

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The purpose of this study was to determine the presence of lipopolysaccharide-like material in the intestinal spirochaete *Serpulina pilosicoli* and the extent of antigenic cross-reactivity of this material in different strains of the species. Hot water-phenol, aqueous-phase extracts from five porcine and three human strains of *S. pilosicoli*, and from seven strains of four other *Serpulina* spp., were separated by SDS-PAGE and silver-stained profiles were obtained. All *S. pilosicoli* strains had a predominant band at c. 16 kDa. Some also had a partial ladder-like profile, consistent with the presence of semi-rough lipo-oligosaccharide (LOS); this was more obvious in Western immunoblot analysis. LOS from each *S. pilosicoli* strain was serologically distinct in immunoblot analysis and there was no cross-reactivity with other *Serpulina* spp. The serological diversity found amongst the LOS of *S. pilosicoli* strains may help to explain why individual people and animals can suffer repeated infections with different strains of the organism.

Introduction

Serpulina pilosicoli was named recently as the aetiological agent of porcine intestinal spirochaetosis [1]. It colonises the large intestine and may induce a colitis and diarrhoea [1, 2]. *S. pilosicoli* is also recognised as the agent of intestinal spirochaetosis in man [3, 4], birds [5] and dogs [6]. In all species, masses of the spirochaete may be found attached by one cell end to the mucosa, forming a 'false brush border' [1, 4]. Other interesting features of the infection include the occurrence of prolonged and repeated episodes of colonisation (on average occurring once yearly and lasting for 4 months in villagers in Papua New Guinea [7]), and the presence of multiple strains of the organism both in pig herds [8] and in closed human communities [3, 9]. Such persistence and repeated occurrence of infection suggest that the spirochaete may have mechanisms for avoiding the host immune response.

Very little is known about the composition of the outer envelope of *S. pilosicoli* although, by analogy

with the related intestinal spirochaete *S. hyodysenteriae*, the agent of swine dysentery, it would be predicted to contain lipopolysaccharide (LPS)-like material [10]. In *S. hyodysenteriae*, this material has a rough or semi-rough character, consistent with it being lipo-oligosaccharide (LOS) [11, 12]; all strains possess one or more components of mol. wt 10–16 kDa, representing lipid A-core polysaccharide regions, with some strains apparently also having short O-antigen side chains [12]. This material is extremely important in *S. hyodysenteriae* because, in convalescent pigs, the protective immune response is largely directed against it [13]. LOS extracts also have been used widely as the basis for serological typing of *S. hyodysenteriae* strains [10, 14]. Eleven serogroups have been described, some of which contain multiple serovars [15].

The purpose of the present study was to investigate the occurrence of LPS-like material in *S. pilosicoli*, and to characterise and define the extent of antigenic relationships amongst extracts from different strains.

Materials and methods

Bacterial strains and culture conditions

Fifteen strains of intestinal spirochaetes from the collection held at the Reference Centre for Intestinal Spirochaetes at Murdoch University, Western Australia

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Table 1. Sources of spirochaetes used in examination of LOS profiles and antigenicity

Species	Strain no.	Origin	ET*
<i>S. pilosicoli</i>	P43/6/78 ^T	Pig, UK	30
	3295/90B	Pig, Victoria, Australia	31
	HRM2	Man, Italy	32
	HRM7	Man, Italy	33
	155-4	Pig, Western Australia	53
	WesB	Man, Western Australia	66
	7082	Pig, New South Wales	88
	181792	Pig, Queensland, Australia	89
<i>S. hyodysenteriae</i>	B78 ^T	Pig, USA	...
	WA1	Pig, Western Australia	...
<i>S. intermedia</i>	PWS/A ^T	Pig, UK	...
	2818.5	Pig, Tasmania	...
<i>S. innocens</i>	B256 ^T	Pig, USA	...
	155-5	Pig, Western Australia	...
<i>S. murdochii</i>	155-21	Pig, Western Australia	...

*Electrophoretic types of *S. pilosicoli* in multilocus enzyme electrophoresis analysis, according to Lee and Hampson [3].

^TType strain.

were used (Table 1). They comprised eight strains of *S. pilosicoli*, two strains each of *S. hyodysenteriae*, *S. intermedia* and *S. innocens*, and one strain of *S. murdochii* [16]. Three human isolates of *S. pilosicoli* were from patients with diarrhoea or intestinal disease (WesB, HRM2 and HRM7); all the other spirochaetes were from pigs with diarrhoea (Table 1). The *S. pilosicoli* strains had all been subjected to analysis by multilocus enzyme electrophoresis [3], and their electrophoretic types (ETs) are presented in Table 1. The eight strains used were selected from four different parts of the dendrogram, but included one group of four strains (in ETs 30–33) and one of two strains (in ETs 88 and 89) which were closely related. This selection was intended to give a representation of strains from across the species, and to include closely related strains to determine their potential similarities.

The strains were cultured in trypticase soy broth supplemented with fetal bovine serum 2% and an ethanolic cholesterol 1% solution under anaerobic conditions until they reached mid-log phase (c. 10⁸ cells/ml) [17].

Extraction of LPS-like material

LPS-like material from all the strains was extracted by the hot water-phenol method, as modified for *S. hyodysenteriae* [10, 14]. In addition to these aqueous-phase extracts, LPS-like material was also extracted from the phenol phase of five *S. pilosicoli* strains (Table 2) by the method described for *Leptospira* spp. [18]. A hexose assay was used to estimate the concentrations of the LPS-like material extracted from both phases [19]. All samples were stored at –20°C until required.

Preparation of sera for immunoblot analysis

Spirochaete cells were obtained by centrifuging 500 ml of fresh mid-log phase cultures at 10000 *g* for 20 min

Table 2. Comparison of hexose concentration of LOS from aqueous-phase and phenol-phase extracts of five strains of *S. pilosicoli*

Strain no.	LOS extract (µg/ml)*	
	aqueous phase	phenol phase
P43/6/78	400	150
3295/90B	350	80
HRM2	1000	80
155-4	500	230
WesB	400	150

*Hexose concentration of LOS.

at 4°C, washing twice in phosphate-buffered saline (PBS) and resuspending in formaldehyde 0.3% in PBS. This suspension was adjusted to an absorbance of 1.0 at 420 nm, and inactivated for 24 h with constant stirring at room temperature. The bacterin was stored at –20°C in small volumes.

Hyperimmune sera used for immunoblot analysis were obtained from rabbits of 2–3 kg body weight, as described previously for *S. hyodysenteriae* [14]. Each animal received 1 ml of bacterin, emulsified in 1 ml of Freund's incomplete adjuvant, at two intramuscular sites twice at fortnightly intervals. Three weeks later, each rabbit was inoculated intravenously with 1 ml of bacterin without adjuvant at weekly intervals for 5 weeks. Blood was collected 1 week after the last injection and the serum was removed and stored at –20°C until required.

SDS-PAGE and Western blot analysis

Extracts from all the intestinal spirochaete strains were subjected to SDS-PAGE and silver staining, as described previously for *S. hyodysenteriae* [12]. Approximately 2 µg of hexose equivalent was added to each well. Separated extracts were also analysed by Western immunoblots with rabbit antiserum raised against each isolate, again with the reagents and

conditions described previously for analysis of *S. hyodysenteriae* LOS [12].

Results

Characterisation of LOS by SDS-PAGE

Silver-stained SDS-PAGE profiles of the aqueous-phase extracts of the *S. pilosicoli* strains are shown in Fig. 1. Pre-stained protein mol. wt markers were used to obtain an estimate of the size of the LOS bands. Multiple bands were found in preparations from all the strains, and the position and number of these varied between strains. All had one or more broad predominating band at *c.* 16 kDa. Generally, the major components were of low mol. wt (<30 kDa), but less intense bands were also observed in the high mol. wt region (30–60 kDa). Individual bands were broad and stained either grey or brown. In addition, a ladder-like pattern of repeating units was clearly observed for *S. pilosicoli* strain 155-4 (Fig. 1, lane 6) and to a lesser extent for the other *S. pilosicoli* strains.

Immunoblot analysis of *S. pilosicoli*

Antiserum raised against each *S. pilosicoli* strain reacted strongly only with LOS extracts from the homologous strain. These sera did not react with the major *c.* 16-kDa components, but did react with and highlighted the ladder-like components in each strain at *c.* 20–30 kDa, even in those strains where these bands were not obvious in the silver-stained profiles (Fig. 2). None of these sera reacted with LOS preparations from the other *Serpulina* spp. tested, nor did the antisera

raised against these species react with the *S. pilosicoli* extracts (data not shown).

Comparison of LPS-like material from *S. pilosicoli* strains extracted from the phenol phase and water phase

S. pilosicoli LOS was also detected in the phenol-phase extracts, although at lower concentrations than in the aqueous-phase material (Table 2). This phenol-phase material had a similar electrophoretic profile to the material extracted from the aqueous phase (data not shown).

Discussion

The LPS of gram-negative bacteria is classically 'smooth', with repeated O-antigen oligosaccharide units forming a regular ladder-like pattern from high to low mol. wt in silver-stained polyacrylamide gels [20]. The number of repeating oligosaccharide units vary from as few as two (semi-rough) to as many as 40 (smooth-type LPS). In the case of *S. hyodysenteriae*, aqueous-phase rough material from strains of the spirochaete possess one or two predominant molecules in the 16-kDa range, with a few additional but irregular bands usually observed in the lower (<30 kDa) mol. wt region [11, 12, 21]. The predominant bands are thought to represent lipid A-core material, whilst the other low mol. wt material has been presumed to represent LOS rather than LPS, and the spirochaete has been considered to have a semi-rough LOS profile [11, 12].

Fig. 1. Silver-stained LOS profiles of *S. pilosicoli* strains (lanes 2–6, pig isolates and lanes 7–9, human isolates). Lane 1, mol. wt marker (kDa); 2, strain P43/6/78; 3, 3295/90B; 4, 7082; 5, 181792; 6, 155-4; 7, HRM2; 8, HRM7; 9, WesB. Note the regular ladder-like pattern which is most obvious in lane 6 (strain 155-4).

Fig. 2. Immunoblot analysis of LOS from *S. pilosicoli* strains. Antiserum raised against *S. pilosicoli* strain 3295/90B was used for detection. Lane 1, mol. wt marker (kDa); 2, strain P43/6/78; 3, 3295/90B; 4, 7082; 5, 181792; 6, 155-4; 7, HRM2; 8, HRM7; 9, WesB (lanes 2–6, pig isolates and lanes 7–9, human isolates). Note the strong homologous reaction with LOS from strain 3295/90B (lane 3) and the appearance of a regular ladder-like pattern in the region *c.* 20–30 kDa.

As with *S. hyodysenteriae* [12], the profiles of the aqueous-phase extracts of the *S. pilosicoli* strains stained grey or brown with silver, consistent with the presence of specific sugars or fatty acid constituents of lipid A [22]. The *S. pilosicoli* strains also resembled *S. hyodysenteriae* in possessing one or more predominant molecules in the 16-kDa region. Again, by analogy, these probably represent the lipid A-core material. Unlike *S. hyodysenteriae*, the *S. pilosicoli* strains all showed a regular series of low mol. wt bands to a greater or lesser extent in their silver-stained profiles. This was most pronounced in strain 155-4, with fairly regular multiple bands extending down from the *c.* 70-kDa region, but was more sparse and in more irregular patterns for the other strains (Fig. 1). The low mol. wt of this material is more consistent with it being LOS than LPS.

Repeat ladder-like patterns were more demonstrable by immunoblotting than by silver stain. For example, with homologous rabbit antisera, *S. pilosicoli* strains 3295/90B and HRM2 were seen to have ladder-like bands at 20–30 kDa, yet these were not obvious by silver staining (Fig. 2). Five bands were present in strain 3295 and seven in strain HRM2. The lipid A-core material did not stain with the antisera. These findings suggest that *S. pilosicoli* strains differ from *S. hyodysenteriae* in having a smoother type of LOS, with some strains such as 155-4 having more repeat oligosaccharide subunits than others. Clearly, these findings should be confirmed by detailed biochemical analysis of the LOS components.

Subtle differences between silver-stained and immunostained LOS profiles have been observed for *S. hyodysenteriae* strains [11]. The more obvious differences in profiles found here with the two techniques may reflect the differences between the LOS of the

two species. As *S. pilosicoli* strains apparently have relatively smooth LOS profiles, the immune response against the spirochaete is likely to be directed more at these external oligosaccharides than at the internal core material. Consequently, the core material would not react with the antiserum.

The LOS of the *S. pilosicoli* strains partitioned into both the aqueous and phenol phases of the hot water-phenol extraction. The solubility of LPS is dependent on the relative proportion of hydrophilic polysaccharides and hydrophobic lipid A [23]. Interestingly, there are examples of spirochaetes (e.g., *L. interrogans* serovar *hardjo*) which have LPS-like materials that are more soluble in the phenol phase than in the aqueous phase [24]. Again, further work is required to investigate the physicochemical basis for this partitioning in *S. pilosicoli*.

The LOS extracts from the eight strains of *S. pilosicoli* were all antigenically distinct from each other, as well as from the LOS of the other *Serpulina* spp. tested. *S. hyodysenteriae* strains can be divided into 11 LOS serogroups, although the majority of isolates examined have belonged to only two or three of these [15]. The main component involved in the serological reactivity described for *S. hyodysenteriae* is the lipid A-core material, but in the case of *S. pilosicoli*, hyperimmune serum does not appear to recognise this factor. The overall interpretation of the failure to find *S. pilosicoli* strains with antigenically similar LOS must be tempered by the fact that only eight strains were examined and that these were from two different species (pigs and man) and from different geographical localities. However, by multi-locus enzyme electrophoresis, strains P43/6/78^T, 3295/90B, HRM2 and HRM7 apparently belonged to one clonal group, and strains 7082 and 181792

were also very closely related, separated by a single allele [3]. As shown with *S. hyodysenteriae* [12], genetically related strains of *S. pilosicoli* do not necessarily have the same serological specificity.

S. pilosicoli strains are genetically diverse [8, 9], and the results of this study suggest that they are also antigenically diverse. This finding may help explain the tendency for infections to persist or recur in piggeries and human communities where there is extensive exposure to multiple strains [8, 9]: under these conditions, antigenically diverse strains will not necessarily stimulate a cross-protective immune response. Currently it is not clear to what extent the LOS antigens of *S. pilosicoli* strains are important in the development of protective immunity and further work is required to examine this.

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