SEROLOGICAL DIAGNOSIS

Preparation of diagnostic polyclonal and monoclonal antibodies against outer envelope proteins of *Serpulina pilosicoli*

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The purpose of this study was to prepare specific sera for use in the rapid detection and identification of the intestinal spirochaete *Serpulina pilosicoli*. In Western blot analysis, with pig antiserum which was raised against whole cells of *S. pilosicoli* and absorbed with outer envelope protein extracts from *S. hyodysenteriae* and *S. innocens*, a prominent protein with M_r of c. 72 kDa was consistently identified in outer envelope preparations of *S. pilosicoli* strains. Immunogold labelling demonstrated that this was located on the outer surface of intact *S. pilosicoli* cells. Two monoclonal antibodies (MAbs), designated C12 and M96, were raised against the protein. Although C12 reacted with a protein band of c. 72 kDa, this was also present in preparations from strains of other *Serpulina* spp. examined. MAb M96 reacted with an 80-kDa protein which was present only in preparations made from strains of *S. pilosicoli*. This was used in Western blot analysis and in an immunodot-blot assay with outer envelope extracts to specifically identify *S. pilosicoli* strains isolated from man, pigs, dogs and poultry. An indirect immunofluorescence test with MAb M96 also was used to detect and identify whole *S. pilosicoli* cells. Therefore, both the cross-absorbed antiserum and MAb M96 are potentially useful reagents for the detection and identification of *S. pilosicoli*.

Introduction

There are currently five officially named species of intestinal spirochaetes in the genus *Serpulina*: *S. hyodysenteriae*, *S. innocens*, *S. pilosicoli*, *S. intermedia* and *S. murdochii* [1–3]. *S. hyodysenteriae* is the agent of swine dysentery, a severe mucohaemorrhagic colitis of pigs, and *S. pilosicoli* was recently named as the causative agent of porcine intestinal spirochaetosis (PIS) [2]. The latter condition is an infection of the caecum and colon, is milder than swine dysentery, and has been recorded in pigs in Australia, North America, Europe and Scandinavia [4–7]. The disease is associated with poor feed efficiency and depressed growth rates [4, 8]. A striking histological feature which is often seen is the attachment of the spirochaetes by one cell end to the colonic epithelium, forming a dense ‘false brush border’ [2, 4, 8–10]. This feature is not seen in pigs with swine dysentery. End-on attachment of spirochaetes also has been observed in the large intestine of other host species, including man [11, 12], dogs [13, 14] and

poultry [15, 16]. These organisms – originally isolated from individuals with diarrhoea and intestinal disturbances, and provisionally named ‘*Anguillina coli*’ [17] – are now known as *S. pilosicoli* [18, 19].

Like other members of the genus, *S. pilosicoli* is anaerobic and takes 3–10 days to grow on selective agar. Once isolated, *S. pilosicoli* strains can be distinguished from other spirochaetes in the genus *Serpulina*, for example, on the basis of their multi-locus enzyme electrophoresis (MEE) profile [17] and their reactivity in a specific polymerase chain reaction that amplifies a 1330-bp sequence of the 16S rRNA gene [18, 20]. Characteristically, *S. pilosicoli* cells also hydrolyse hippurate and use the pentose sugar D-ribose as a growth substrate [2, 19, 21]. As it is relatively difficult and time-consuming to isolate and identify the spirochaete, a monoclonal antibody (MAb) specific to a 29-kDa outer envelope protein of *S. pilosicoli* was prepared and characterised in a previous study [22]. Although this MAb could be used in immunofluorescent and Western blot assays, it had the disadvantage that it was of the IgM class. The main purpose of the current study was to identify specific immunogenic outer envelope proteins of *S. pilosicoli*,

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prepare antisera against these and develop the sera for use in the rapid identification of *S. pilosicoli* isolates from man and animals.

Materials and methods

Bacterial strains and culture conditions

Twenty-nine strains of anaerobic intestinal spirochaetes were used in this study (Table 1). These were obtained from a collection held at the Reference Centre for Intestinal Spirochaetes, Murdoch University, Western Australia, where their identity had been confirmed by MEE analysis [17, 23]. They included 20 strains of *S. pilosicoli* isolated from pigs ($n = 8$), man ($n = 8$), dogs ($n = 2$) and chickens ($n = 2$); two strains each of *S. hyodysenteriae*, *S. innocens*, *S. intermedia* and *S. murdochii*, as well as one strain of *Brachyspira aalborgi* [24]. The organisms were grown for 3–5 days at 37°C on a reciprocal shaker in Kunkle's prerduced anaerobic Trypticase Soy Broth (BBL) supplemented with fetal bovine serum 2% and a 1% ethanolic cholesterol solution [25], and harvested in mid-log phase ($c. 10^8$ cells/ml).

Preparation of outer envelope proteins

Outer envelope proteins were extracted from whole spirochaete cells with the detergent Triton X-114, as

described previously [22]. Spirochaetes in 300 ml of broth were centrifuged at 2800 *g* for 15 min at 4°C, resuspended and washed three more times with 0.05 M Tris buffered saline (TBS), containing 1 M NaCl, 1 M Tris (pH 8.0) and resuspended in 30 ml of extraction buffer, comprising Triton X-114 0.1% v/v, 10 mM Tris and 5 mM EDTA (pH 7.5). The preparation was mixed gently by inversion at 4°C for 18 h and then centrifuged at 20 000 *g* for 30 min at 4°C. The protein in the soluble supernatant fraction was precipitated with 10 volumes of absolute acetone at –20°C for 18 h, and collected by centrifugation at 1000 *g* for 10 min at 4°C. The pellet was resuspended with phosphate-buffered saline (PBS, pH 7.2) and its protein concentration was estimated with a commercial protein assay kit (BioRad Laboratories, CA, USA).

SDS-PAGE and Western blotting

Extracts were boiled for 5 min in sample-reducing buffer (0.125 M Tris-HCl, pH 6.8, SDS 4% w/v, 2- β -mercaptoethanol 10% v/v, glycerol 20% v/v and bromophenol blue 0.001% w/v) and subjected to SDS-PAGE through 4% stacking and 12.5% resolving gels (BioRad Protean and Mini-protein systems). Proteins in the gel were visualised with Coomassie Brilliant Blue R-250 (BioRad), with low M_r pre-stained markers as standards (BioRad). For Western blot analysis, the separated proteins were transferred

Table 1. Results of testing strains of intestinal spirochaetes with MAb M96

Isolates tested	Strain	Origin*	Western blotting†	Immunodot-blot analysis‡	IFAT§
<i>S. pilosicoli</i>	Pig isolates	1648	NSW	+	+++
		3295	WA	+	+++
		Gut A	USA	+	++
		Gut B	USA	+	+++
		MIA	UK	+	++
		883769	Can	+	+++
		891069	Can	+	++
		T4527	Tas	+	++
	Human isolates	11A	Oman	+	++
		N26	Oman	+	++
		Gap 183	Sydney	+	+++
		Gap 401	Sydney	+	++
		HRM 1A	Italy	+	+++
		HRM 2B	Italy	+	++
		Naomi	WA	+	++
		Disley	WA	+	++
	Dog isolates	1624294	USA	+	++
		MeyersK912	USA	+	+++
	Chicken isolates	42167	USA	+	+++
		Q94035406	USA	+	+
<i>S. hyodysenteriae</i>	B78	USA	–	–	–
	B204	USA	–	–	–
<i>S. innocens</i>	B256	USA	–	–	–
	4/71	UK	–	–	–
<i>S. intermedia</i>	889	NSW	–	–	–
	PWSA	UK	–	–	–
<i>S. murdochii</i>	15521	WA	–	–	–
	Growden	WA	–	–	–
<i>Brachyspira aalborgi</i>	ATCC43994	Denmark	–	–	–

*Can, Canada; Tas, Tasmania; NSW, New South Wales; WA, Western Australia.

†Western blot analysis of outer envelope proteins: –, negative reaction; +, positive reaction.

‡Immunodot-blot analysis of outer envelope proteins: –, negative reaction; +, positive reaction.

§IFAT, indirect fluorescent antibody test: –, negative reaction; +, weak positive reaction; ++, strong positive reaction; +++, very strong positive reaction.

electrophoretically to nitrocellulose membranes (Hybond-C extra, 0.45 μm pore size; Amersham, Sydney, Australia), by the method of Towbin *et al.* [26]. They were treated with appropriate antisera (pig, rabbit or mouse) diluted 1 in 50 in PBS, followed by horseradish peroxidase (HRP)-conjugated goat antiserum raised against immunoglobulins from the appropriate species (BioRad), diluted 1 in 3000 in PBS. Membranes were stained with 4-chloro-1-naphthol.

Preparation of bacterins

Bacterial cells from mid-log phase broth culture were harvested by centrifugation, washed three times with PBS and resuspended to *c.* 10^9 cells/ml in 10 ml of PBS containing formaldehyde 0.3%. Suspensions were inactivated by stirring overnight at 4°C, and stored at -20°C in small volumes.

Preparation of porcine hyperimmune sera

Antisera against *S. pilosicoli* (strain 1648), *S. hyodysenteriae* (type strain B78^T) and *S. innocens* (type strain B256^T) were prepared in three 4-week-old pigs. A blood sample was taken for serum, then each pig received 1.5 ml of bacterin suspended in 1.5 ml of Freund's complete adjuvant (Sigma) at two deep intramuscular sites in the neck. This was repeated 2 weeks later, then each pig was vaccinated weekly for 4 weeks with bacterin emulsified in Freund's incomplete adjuvant. Finally, 1.5 ml of bacterin without adjuvant was given intravenously twice with a 1-week interval. Blood was collected by jugular puncture 1 week after the last immunisation, and the sera was harvested and stored in divided volumes at -20°C.

Absorption of pig hyperimmune serum raised to S. pilosicoli

Five ml of antiserum raised against *S. pilosicoli* strain 1648 were mixed with 2 ml of pooled pellets of outer envelope proteins from both *S. hyodysenteriae* strain B78^T and *S. innocens* strain B256^T (*c.* 500 mg of bacterial protein obtained from 1500-ml lots of fresh broth), incubated at 37°C in a waterbath for 30 min and then left overnight at 4°C on a rotary mixer. The serum was centrifuged at 15 000 *g* for 10 min, and the supernate was collected. The specificity of this absorbed serum was confirmed by Western blot analysis against outer envelope preparations from a range of strains of *Serpulina* spp. Where necessary the absorption was repeated several times until the serum reacted with a specific protein band present only in *S. pilosicoli* strains.

Purification of a 72-kDa S. pilosicoli protein

For the production both of a more specific polyvalent rabbit antiserum and subsequently of MAbs, outer envelope proteins from *S. pilosicoli* strain 1648 were

prepared and separated by SDS-PAGE. A major protein band with a molecular mass of *c.* 72 kDa, previously identified as being specific for *S. pilosicoli* in Western blot analysis with absorbed pig antiserum, was excised and sliced into small pieces and the protein was extracted from it by electro-elution (BioRad electro-eluter apparatus). The protein was precipitated by the addition of 10 volumes of absolute acetone at 4°C for 18 h, centrifuged at 1000 *g* for 30 min, resuspended in PBS, and the protein content was measured. The protein was subjected to SDS-PAGE and Western blot analysis with unabsorbed pig antiserum to *S. pilosicoli* to confirm its presence. The protein nature of the purified 72-kDa band was confirmed by adding proteinase K (2 mg/ml) in proteinase K buffer (150 mM Tris, 7 mM EDTA, SDS 0.05%), heating at 50°C for 2 h, then subjecting this to SDS-PAGE and Western blot analysis with the absorbed pig antiserum.

Preparation and absorption of a rabbit antiserum

Rabbit antiserum was prepared against the purified protein in a 6-week old New Zealand White rabbit. The rabbit was inoculated with 1 ml of purified protein (50 $\mu\text{g}/\text{ml}$) emulsified in 1 ml of Freund's incomplete adjuvant and injected twice at two intramuscular sites at an interval of 14 days. One ml of the protein solution was then given intravenously at weekly intervals for a further 4 weeks. The rabbit was bled 1 week after the last immunisation and the serum was absorbed as for the porcine serum.

Immunogold labelling of whole spirochaete cells

Cultures of *S. pilosicoli* strain 1648 (positive control) and *S. hyodysenteriae* strain B78^T (negative control) were harvested by centrifugation of mid-log phase broth culture at 1000 *g* for 10 min, and the cells were washed three times with 0.02 M MgCl_2 -PBS. Resuspended cells were fixed in Karnovsky's fixative for 2 h at room temperature, washed in PBS and suspended in 0.1 M sodium cacodylate buffer (pH 7.2). The fixed cells were centrifuged at 1000 *g* for 10 min and suspended in 0.5 ml of undiluted absorbed rabbit serum. Cells were incubated overnight at 4°C, washed three times with 0.02 M MgCl_2 -PBS and treated with goat anti-rabbit IgG 10-nm gold conjugate (Sigma) diluted 1 in 10 in PBS. After incubation at 30°C for 2 h, the suspensions were washed three times in PBS. A drop of each suspension was placed on the surface of a Formvar-coated grid for 30 s, blotted off, a drop of sodium phosphotungstate 1% was added for 30 s, and then this was also blotted off. The grids were examined with a Phillips 301 transmission electron microscope.

Production of MAbs

MAbs were prepared as described previously [22], in this case with the purified electro-eluted 72-kDa protein

as immunogen. Hybridoma supernates were screened for specific antibody production in ELISA, with 100 μ l of purified 72-kDa protein (*c.* 5 μ g/ml) in each well as the plate-coating antigen. The absorbance of the substrate in the well was read at 450 nm. A positive to negative OD₄₅₀ ratio of two or more was considered to be a positive reaction. Hybridomas giving a positive ELISA reading were cloned by limiting dilution, again tested by ELISA, and subsequently tested in Western blot analysis against outer envelope extracts to determine their specificity. MAbs C12 and M96 were selected for further study, and their isotype was determined with an ELISA-based mouse monoclonal isotyping kit (BioRad).

Western blot and immunodot-blot assays

Outer envelope preparations from all strains were tested blindly by Western blotting, as described previously and in an immunodot-blot assay, by the method of Hawkes *et al.* [27]. Briefly, for the immunodot-blot assay, suspensions of extracts in PBS (about 50 μ g/ml) were aspirated on to a nitrocellulose filter (Hybond-C extra, 0.45 μ m pore size; Amersham) with a dot-blot apparatus (BioRad). The membrane was soaked in skimmed milk powder 5% in Tris-buffered saline (TBS, pH 7.5) for 1 h at room temperature with gentle agitation, washed with TBS twice for 5 min each, air dried and exposed to undiluted culture supernate at 4°C overnight. The membrane was incubated with a dilution of 1 in 3000 blotting grade HRP-conjugated goat anti-mouse serum in PBS, and developed with HRP-colour reagent. The reaction was stopped by transferring the membranes into distilled water, and positive reactions were indicated by the development of brown staining reactions. All assays were repeated at least twice, and the code was broken only when all results were obtained.

Indirect fluorescent antibody test (IFAT)

Spirochaete cultures were washed three times, diluted in PBS, placed on glass microscope slides at a density of *c.* 10 spirochaetes/high-powered light microscope field, air dried and fixed with cold acetone for 10 min. The fixed cells were then treated with single drops of undiluted MAb M96 culture supernate, and each slide was coded before being incubated in a moist chamber at 37°C for 30 min. After incubation, the smears were washed thoroughly three times with PBS for 5 min each, then incubated with 100 μ l of a 1 in 80 dilution of fluorescein-conjugated goat-antimouse IgG+M+A (Capple Laboratories, Organon Teknika Corp, Durham, NC, USA) in PBS at 37°C for 30 min. After washing three times with PBS for 5 min, the slides were covered with cold glycerol 90% solution in PBS and examined with an Olympus BH-2 incidence fluorescent microscope. Positive reactions were characterised by the presence of bright yellowish green fluorescence over the whole bacterial cells and were scored according to

the brightness of the reaction (+, weak reactions; ++, strong reactions; and +++, very strong reactions). All assays were repeated at least twice, and the code was broken only when all the results were available.

Results

Identification of a 72-kDa outer envelope protein specific for S. pilosicoli

In SDS-PAGE gels stained with Coomassie blue, outer envelope protein preparations from the different species of intestinal spirochaetes contained a large number of polypeptide bands with a similar range of M_r of 10–112 kDa (not shown). Polyclonal pig serum raised against whole cells of *S. pilosicoli* strain 1648 reacted in Western blot analysis with most of these proteins, including a prominent protein band at *c.* 72 kDa which was the subject of subsequent investigation (Fig. 1). Similar reactions were observed when the separated proteins were treated with polyclonal pig sera raised against *S. hyodysenteriae* strain B78^T or *S. innocens* strain B256^T (not shown). When the pig antiserum raised against *S. pilosicoli* strain 1648 was repeatedly cross-absorbed, eventually it recognised only the prominent protein band with a M_r of *c.* 72 kDa that was present in preparations from all the *S. pilosicoli* strains examined. This reactivity was absent from the other intestinal spirochaetes examined. With the homologous strain 1648 a number of other protein bands of lower m_r also were observed in Western blot analysis (Fig. 2).

Before absorption, the rabbit serum raised against the electro-eluted 72-kDa protein reacted with a large number of bands in all the outer envelope preparations. As with the pig serum, it could be made specific for the 72-kDa band by cross-absorption (not shown).

Proteinase treatment of the 72-kDa band before electrophoresis resulted in its disappearance from the stained gel.

Immunogold labelling

Immunogold-labelling particles were found loosely associated with the outer surface of *S. pilosicoli* strain 1648 (Fig. 3), whereas this reaction was completely absent when *S. hyodysenteriae* strain B78^T was used (not shown).

Production, characterisation and use of MAbs

Two MAbs, designated C12 and M96, that gave positive readings in the ELISA were selected for further study. Both reacted with kappa light chain and were of isotype IgG1. Unexpectedly, in Western blot analysis C12 recognised a protein band of *c.* 72 kDa in outer envelope preparations from all

Fig. 1. Western blot analysis of Triton X-114 extracts from intestinal spirochaetes with a pig antiserum raised against *S. pilosicoli* strain 1648. Lane 1, *B. aalborgi* strain ATCC43994; 2, *S. hyodysenteriae* strain B204; 3, *S. hyodysenteriae* strain B78^T; 4, *S. murdochii* strain 56-160; 5, *S. murdochii* strain 155-21; 6, *S. intermedia* strain 889; 7, *S. intermedia* strain PWS/A; 8, *S. innocens* strain 4/71; 9, *S. innocens* strain B256^T; 10–13, *S. pilosicoli* isolates from man (10–11) and pigs (12–13): 10, WesB; 11, HRM2B; 12, 3295; 13, 1648; 14, M_r markers (kDa). The serum cross-reacted with preparations from all spirochaetes tested, but a prominent 72-kDa band which was studied further was present in all the *S. pilosicoli* strains (lanes 10–13) (arrowed).

Fig. 2. Western blot analysis of Triton X-114 extracts from intestinal spirochaetes with a pig antiserum raised against *S. pilosicoli* strain 1648, absorbed with outer envelope protein extracts of *S. hyodysenteriae* strain B78^T and *S. innocens* strain B256^T. Lane 1, M_r markers (kDa); 2–5, *S. pilosicoli* isolates from pigs (2, 3) and man (4, 5): 2, 1648; 3, 3295; 4, HRM2B; 5, WesB; 6, *S. innocens* strain B256; 7, *S. innocens* strain 4/71; 8, *S. intermedia* strain PWS/A; 9, *S. intermedia* strain 889; 10, *S. murdochii* strain 155-21; 11, *S. murdochii* strain 56-160; 12, *S. hyodysenteriae* strain B78; 13, *S. hyodysenteriae* strain B204; 14, *B. aalborgi* strain ATCC43994.

the *Serpulina* spp., although it did not react with preparations from *B. aalborgi* (not shown). MAb M96 recognised a protein band at c. 80 kDa in preparations of all *S. pilosicoli* strains tested, but did not react with any bands in preparations from the other intestinal spirochaetes (Fig. 4). In the immunodot-blot

assay, MAb M96 again reacted with outer envelope preparations only from the *S. pilosicoli* strains (Table 1). In IFAT with MAb M96, *S. pilosicoli* strains gave strong surface fluorescence, whilst no specific fluorescence was detected with other intestinal spirochaetes (Table 1).

Fig. 3. Immunogold labelling of an intact cell of *S. pilosicoli* strain 1648 with a rabbit antiserum raised against a 72-kDa outer envelope protein and absorbed with outer envelope protein extracts of *S. hyodysenteriae* strain B78^T and *S. innocens* strain B256^T. The outer cell surface location of the reactive epitope is shown.

Fig. 4. Western blot analysis of MAb M96 with Triton X-114 outer envelope protein extracts of intestinal spirochaete strains. Lane 1, M_r markers (kDa); 2–5, *S. pilosicoli* isolates from pigs (2, 3) and man (4, 5): 2, 1648; 3, 3295; 4, HRM2B; 5, WesB; 6, *S. innocens* strain B256^T; 7, *S. intermedia* strain PWS/A; 8, *S. murdochii* strain 155-21; 9, *S. hyodysenteriae* strain B78^T; 10, *B. aalborgi* strain ATCC43994.

Discussion

As in a previous study, SDS-PAGE analysis of Triton X-114-extracted protein preparations from intestinal spirochaetes showed that these contained many bands [22]. Therefore, it seems likely that these preparations included proteins from the whole outer envelope and possibly some cytoplasmic components. However, a major protein with a m_r of *c.* 72 kDa, which was unique to isolates of *S. pilosicoli*, was identified in the preparations by Western blot

analysis with both absorbed polyclonal pig serum raised against whole *S. pilosicoli* cells and absorbed rabbit serum raised against the 72-kDa protein. The absorption process apparently removed all antibody that was reactive to external proteins shared by other *Serpulina* spp. Immunogold staining in transmission electron microscopy demonstrated that the 72-kDa protein was located on the outer surface of the intact cell, suggesting that it was part of the outer membrane. Its protein nature was confirmed by its disappearance after proteinase K treatment, although

no attempt was made to determine whether it was glycosylated.

The absorbed rabbit serum produced here is a potentially useful reagent that could be used for diagnostic purposes and for further studies on the 72-kDa protein. Unfortunately, preparation of this serum is not particularly practical for widescale use. The absorption process reduces the antibody titre and is time-consuming; furthermore, the resultant sera are likely to be of variable titre and specificity. For this reason this study attempted to raise MABs against the protein, as this might overcome many of these problems. Two MABs that were both initially thought to be specific for the 72-kDa protein of *S. pilosicoli* were obtained. Unfortunately it was subsequently found that the 72-kDa protein band that MAB C12 reacted with was present in all *Serpulina* spp. examined (although not in *B. aalborgi*). Presumably, this common protein with the same M_r as the *S. pilosicoli*-specific protein was also present in the electro-eluted material used to prepare both the rabbit serum and the MABs. Although not specific for *S. pilosicoli*, MAB C12 might still be useful for differentiating intestinal spirochaetes of the genus *Serpulina* from other spirochaetes in the gut. Clearly it will be necessary to confirm the specificity of this MAB by screening it against other intestinal spirochaetes such as *Treponema succinifaciens*, as well as other enteric micro-organisms. Achacha and Mittal reported preparing *Serpulina* genus-specific MABs after using whole cells of *S. hyodysenteriae* for immunisation [28]. These recognised common antigens of *S. hyodysenteriae* and *S. innocens* with M_r in the range 26–45 kDa, but they were not tested against *S. pilosicoli* strains.

MAB M96 reacted with an 80-kDa band in extracts of all *S. pilosicoli* strains examined. This protein was distinct from the original 72-kDa protein and appeared to be a minor component in these extracts in comparison to the prominent 72-kDa band. Nevertheless, the 80-kDa band was *S. pilosicoli*-specific, and MAB M96 was effective for identification in Western blot analysis and in an immunodot-blot assay with outer envelope preparations. MAB M96 also reacted specifically in a whole-cell IFAT. This reactivity suggested that the reactive epitope was located externally, although immunogold transmission electron microscopy was not conducted to confirm this. MAB M96 correctly identified a range of *S. pilosicoli* strains from man, pigs, dogs and chickens, and hence is a potentially important reagent for diagnostic purposes, and for future studies on the outer envelope of *S. pilosicoli*.

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