

Analysis of lipopolysaccharide antigens of *Treponema hyodysenteriae*

D. J. HAMPSON, J. R. L. MHOMA AND B. COMBS

*School of Veterinary Studies, Murdoch University, Murdoch,
Western Australia, 6150*

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SUMMARY

Lipopolysaccharide (LPS) extracts obtained from *Treponema hyodysenteriae* of serogroups A, B, D and E, and from *T. innocens* were examined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), silver-staining, and immunoblotting with hyperimmune rabbit sera. All organisms possessed multiple LPS bands, but their position and number differed.

Immunoblotting of LPS with grouping sera identified three or four major antigenic LPS components in the 10–42 kDa range in all organisms: these components were largely specific to each type-organism of a serogroup, and presumably represented group antigens. Although some minor cross-reactivity occurred between LPS from organisms in the different groups, this was insufficient to merit changes to the current LPS serogrouping system for *T. hyodysenteriae*. Besides this LPS 'complex', other higher-molecular-weight material which appeared to be a common component of the treponemes examined was present in low concentrations. Organisms with different serotypes within a serogroup apparently possessed common LPS bands, but also had unique LPS bands which may account for their serotype specificity. One 'untypable' organism lacked group-specific LPS and was thought to be a mutant of a group B organism. The loss of serogroup LPS by the isolate suggested that this material is an external component of the cell wall. The availability of an atypical organism lacking LPS components may facilitate further studies on the pathogenesis of swine dysentery.

INTRODUCTION

Treponema hyodysenteriae is a large anaerobic spirochaete which is the essential aetiological agent of swine dysentery (1, 2). The organisms possess biologically active lipopolysaccharide (LPS) as a component of their cell wall (3) the effects of which have been correlated with severity of disease in mouse models (4).

LPS antigens have also been used to serotype the organisms. Initially *T. hyodysenteriae* was divided into four serotypes by agarose gel diffusion precipitation tests (AGDP) using LPS and hyperimmune rabbit antisera (5). Three 'new' serotypes were later described by Mapother & Joens (6). Recently it has been proposed that organisms be placed into five serogroups (7): members of each group are defined by their reactivity with unabsorbed sera raised against a type

organisms for the group, with isolates possessing additional unique LPS antigens being regarded as serotypes within the serogroup.

'Serotype-specific' LPS has also been used as antigen in ELISA tests to obtain serological evidence of *T. hyodysenteriae* infection (8). The possession of titres against LPS presumably confers some protection, since vaccines consisting of bacterins confer better protection against challenge with organisms of the same serotype than they do against heterologous serotypes (9). Furthermore, in experimentally infected ligated colonic loops, protection against reinfection is serotype specific (10). Presumably this protection is directed against serotype-specific surface LPS antigens: it is also known that serotype-specific opsonization of *T. hyodysenteriae* occurs (11).

Despite the importance of *T. hyodysenteriae* LPS in pathogenicity, serotyping and immunity, little is known about this material. Chatfield and colleagues (12) have suggested that the organisms possess an LPS 'complex' in the 14000–24000 molecular-weight region. Using AGDP with cross-absorbed sera, Hampson and colleagues (7) have inferred the presence of multiple LPS antigens in *T. hyodysenteriae*. The purpose of the present work was to make a preliminary analysis of *T. hyodysenteriae* LPS using SDS-PAGE, silver-staining and immunoblotting.

MATERIALS AND METHODS

Microorganisms

The strains of *T. hyodysenteriae* used in this study are listed in Table 1, and were from the same sources as previously described (7). B78, WA1, A1 and WA6 are the type strains of *T. hyodysenteriae* for serogroups A, B, D and E respectively. B256 is the type strain of *T. innocens*. B204 is a serotype of *T. hyodysenteriae* within serogroup B. WA15 and WA16 are a pair of *T. hyodysenteriae* isolates recovered from the same pig, WA15 being of serogroup B and WA16 being untypable. B169, the type strain for group C, was not available for use at the time of this study.

Growth of bacteria, preparation of LPS, preparation of rabbit antisera to whole formalized treponemes and absorption of sera was as previously described (7).

SDS-PAGE

Lipopolysaccharide extracts were subjected to SDS-PAGE using the Laemmli electrophoresis system (13), incorporating 4 M urea and 1% SDS in a 14% acrylamide separating gel. A Mini-Protean II electrophoresis apparatus (Bio-Rad Laboratories, Richmond, California) was used, giving a gel length of approximately 6 cm. Six microlitres of each treponemal lipopolysaccharide, containing 2 µg of hexose, was mixed with an equal volume of a solubilizing solution consisting of 0.1 M Tris-hydrochloride buffer (pH 6.8), 2% (wt./vol.) SDS, 4% (vol./vol.) 2-mercaptoethanol, 20% (wt./vol.) sucrose and 0.01% (wt./vol.) bromophenol blue, and was boiled for 10 min immediately prior to loading onto the gel. Electrophoresis was at 6 mA per gel (constant current) continuing until the bromophenol blue marker reached the bottom of the gel (approximately 1 h). The running buffer was Tris-glycine (pH 8.3) containing 1% SDS. Pre-stained molecular weight markers (Bio-Rad) were run in the first lane of each gel: these

Table 1. *Treponemal isolates used in the study*

Strain	<i>T. hyodysenteriae</i> LPS-serogroup*	Origin
B78	A	USA
WA1	B	WA
A1	D	UK
WA6	E	WA
B204	B	USA
WA15	A	WA
WA16	NT	WA
B256	<i>T. innocens</i>	USA

* Hampson *et al.* (7). WA, = Western Australia; NT, Not typed.

were phosphorylase b, bovine serum albumin, ovalbumin, carbonic anhydrase, soyabean trypsin inhibitor and lysozyme, with apparent molecular weights ($\times 10^3$) respectively of 130, 75, 50, 39, 27 and 17. For silver staining, Coomassie blue staining and for several immunoblot preparations double the above concentrations of LPS was loaded onto the gel. Duplicate preparations of B204 and A1 LPS were also incubated with an excess of proteinase-K before being subjected to SDS-PAGE and immunoblotting.

Immunoblotting

LPS were electrophoretically transferred from the gels onto nitrocellulose membranes (Hybond-C extra, 0.45 μm pore size; Amersham, Sydney), using the method of Towbin, Staehelin & Gordon (14). The transfer was performed at 100 V (constant voltage) for 1 h using a mini trans-blot electrophoretic transfer cell (Bio-Rad). The blotting buffer consisted of 20 mM Tris-hydrochloride (pH 8.3), 150 mM glycine and 20% (vol./vol.) methanol.

After blotting the membranes were equilibrated in Tris buffered saline (TBS: 20 mM Tris, 500 mM sodium chloride, pH 7.5) for 10 min and then blocked for 1 h in a solution of 20% (wt./vol.) low-fat skim-milk powder made up in TBS containing 0.05% (vol./vol.) Tween-20 (TBS-Tween). The milk powder was then rinsed off in TBS-Tween, and the membranes were incubated for 1 h at 37 °C on a rocking platform in appropriate rabbit antiserum diluted 1:100 in TBS-Tween. The membranes were then washed twice for 10 min in TBS-Tween, and then incubated for 1 h as above in goat-anti-rabbit IgG horseradish peroxidase labelled conjugate (blotting grade: Bio Rad) diluted 1:3000 in TBS-Tween. The membranes were again washed twice for 10 min in TBS-Tween and then developed in a freshly-prepared solution containing either 60 mg of 3,3'-diaminobenzidine tetrahydrochloride dihydrate or 60 mg 4-chloro-1-naphthol (Bio-Rad) in 20 ml methanol, with 100 ml TBS containing 60 μl of 30% hydrogen peroxide added.

Silver staining

Silver staining was undertaken using the technique of Tsai & Frasch (15), using periodic acid to oxidize the LPS in the gel prior to staining.

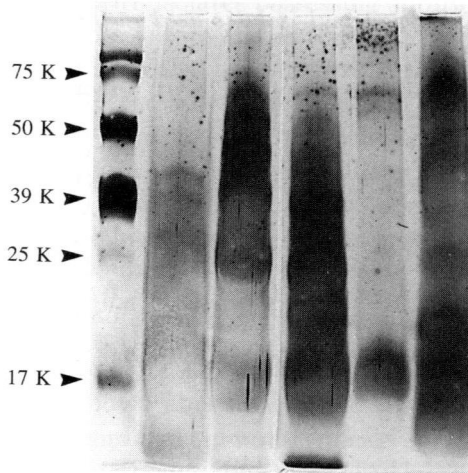


Fig. 1. Silver-stained LPS profiles of *T. hyodysenteriae* and *T. innocens*. Lanes from left to right: (1) molecular weight markers; (2) B78 (group A); (3) WA1 (group B); (4) A1 (group D); (5) WA6 (group E); (6) B256 (*T. innocens*)

Coomassie blue staining

Selected gels were stained with a 0.1% (wt./vol.) solution of Coomassie brilliant blue R-250 (Bio-Rad) in 40% (vol./vol.) methanol and 10% (vol./vol.) acetic acid, and destained in the same solution without the Coomassie brilliant blue.

RESULTS

The silver-stained LPS profiles of the treponemes from serogroups A, B, D and E and *T. innocens* are shown in Fig. 1. No protein was detected in samples run on duplicate gels stained with Coomassie brilliant blue. Each organism possessed multiple LPS bands; judging from the protein molecular weight standards the most prominent had molecular weights of less than around 45 kDa, except in WA16 where this material was absent. All the organisms examined possessed different but consistent LPS profiles. Individual bands were broad and stained either red, grey or yellow-brown.

The results of immunoblotting with *T. hyodysenteriae* of serogroups A, B, D and E, and with *T. innocens*, using corresponding antisera are shown in Fig. 2. Antiserum prepared against *T. hyodysenteriae* in each of the four lanes reacted strongly with the homologous organism, with the bulk of the activity being against 3 or 4 broad heavy bands with apparent molecular weights of 42 kDa and less. All sera also reacted weakly with high molecular weight material in *T. innocens*. With higher LPS concentrations antisera also reacted weakly with additional high-molecular-weight bands from homologous and heterologous strains, and from *T. innocens*. In particular two sharp bands with molecular weights around 75 and 90 kDa were present in all organisms examined, and cross-reacted with most of the sera used. Antiserum against B78 (group A) identified four major bands amongst its LPS: two broad bands at around 11 and 17 kDa,

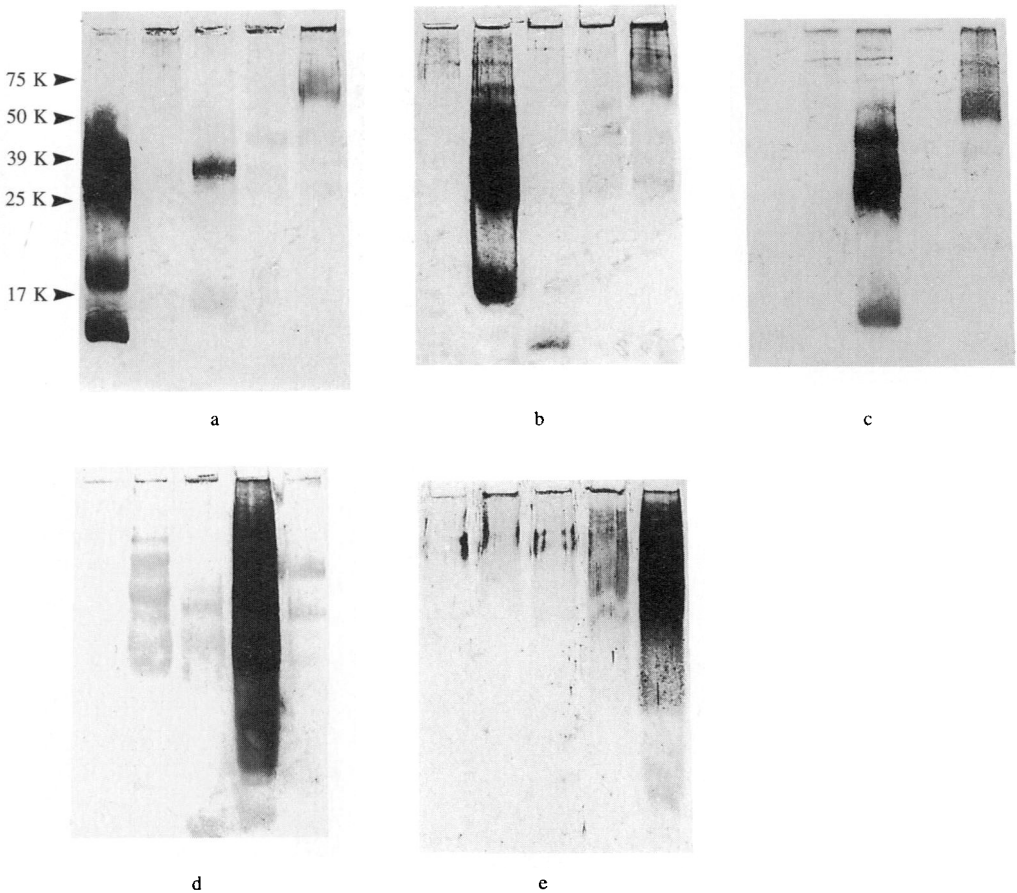


Fig. 2. Immunoblots of *T. hyodysenteriae* and *T. innocens* LPS. For each membrane, lanes from left to right: (1) B78 (group A); (2) WA1 (group B); (3) A1 (group D); (4) WA6 (group E); (5) B256 (*T. innocens*). Membranes (a), (b), (c), (d) and (e) reacted with antisera against B78, WA1, A1, WA6 and B256 respectively.

and another pair with a broad area of reactivity at around 30 and 32 kDa (not distinguishable as separate in the figure). The antiserum also reacted with a band at around 30 kDa in A1 (group D). Antiserum against WA1 (group B) reacted most strongly with a very broad and heavy band around 30 kDa, but also with material around 42 and 15 kDa. The antiserum also reacted weakly with an LPS component of A1 with molecular weight around 10 kDa, as well as with a few higher-molecular-weight (50–100 kDa) bands in all strains, including the *T. innocens*. Antiserum against A1 (group D) detected a band at around 27 kDa, with weaker bands at around 40, and 12 kDa. Antiserum against WA6 reacted with bands at around 37, 27, 22 and 13 kDa, present in a broad smear, as well as with a few distinct higher-molecular-weight bands in all strains except B78, but including *T. innocens*. Antiserum against B256 (*T. innocens*) reacted most strongly with two broad bands at around 55 and 36 kDa (not distinguishable as separate in the figure); it also reacted weakly with a few high-molecular-weight bands in WA6.

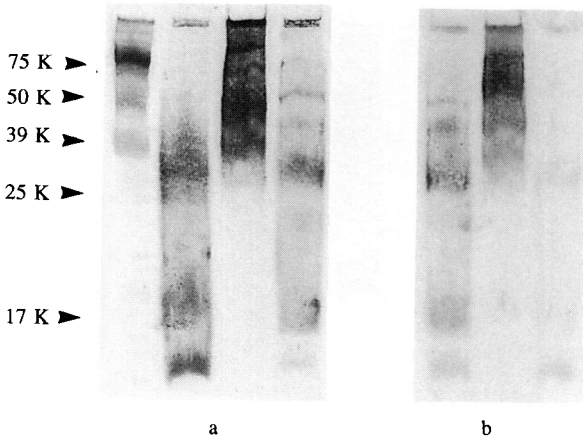


Fig. 3 Immunoblots of *T. hyodysenteriae* from serogroup A. Membrane (a) reacted with antisera against B78: lane 1, molecular weight markers; lane 2, B78; lane 3, WA16; lane 4, WA15. Membrane (b) reacted with antisera against WA16: lane 1, WA15; lane 2, WA16; lane 3, B78.

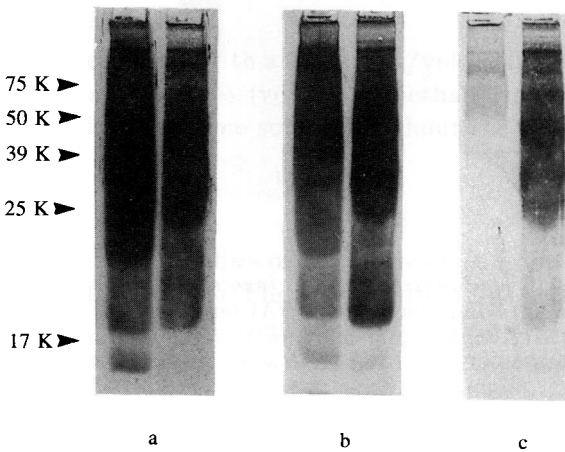


Fig. 4. Immunoblots of *T. hyodysenteriae* from serogroup B. Lane 1, B204, lane 2, WA1. Membrane (a) reacted with antisera against B204. Membrane (b) reacted with antisera against WA1. Membrane (c) reacted with antisera against WA1 which has been absorbed with B204 cells.

Absorption of antisera against B78 and WA1 with A1 cells removed some but not all activity against the homologous organism, although it did remove the cross-reacting activity against A1 LPS (not shown).

The results of immunoblotting with organisms of different serotypes within serogroups A and B are shown in Figs. 3 and 4 respectively. Antiserum against B78, besides reacting with its own major LPS components, reacted with main bands in WA15 at around 39, 30, 14 and 10 kDa, but only with high-molecular-weight material in WA16 (around 75, 56, 45 and 35 kDa). Antiserum against WA16 reacted with its own high molecular weight LPS components, all the major

components of WA15, but only very faintly with similar material in B78 (Fig. 3). Considering serogroup B organisms, antisera against both WA1 and B204 reacted with a number of broad, heavy LPS bands running into each other in both organisms. B204 possessed a low-molecular-weight band at around 10 kDa not possessed by WA1, although sera against both organisms reacted with it. After absorption with B204 organisms, serum against WA1 lost most of its activity against B204 LPS, but still reacted with several high molecular weight bands of WA1 LPS. Serum against B204 which was absorbed with WA1 organisms no longer reacted with LPS from either organism (not shown).

Digestion of samples with proteinase K prior to SDS-PAGE and immunoblotting failed to remove major antigenic material from the profiles present on the nitrocellulose membranes.

DISCUSSION

Silver staining of LPS in polyacrylamide gels revealed the presence of multiple bands in most preparations, but the position and number of bands differed between organisms. The main bands observed were almost certainly LPS because of the method of preparation of samples, colour of the bands, absence of material staining with Coomassie brilliant blue (although this technique is much less sensitive than silver staining), and failure of proteinase K to disrupt major banding patterns. Red, grey or brown staining of enterobacterial LPS components in polyacrylamide gels with silver may be associated with the presence of specific sugars or fatty acid constituents of lipid A in the material (16): the presence of similar colour patterns in treponemal LPS suggests that these organisms contain similar components.

Proteins and LPS have different migration rates in acrylamide gels (17) so that the protein markers used in this work were not adequate to allow accurate determination of the molecular weight of individual LPS components. All molecular weights quoted are therefore only apparent, compared with protein markers. In any case LPS bands were also broad and heavy, making precise location of the bands difficult. The bulk of the LPS components in the silver-stained gels, and the major specific immunogenic components in immunoblots had apparent molecular weights between 10 and 42 kDa. Other higher molecular weight bands were also present, and several of these including a pair at 75 and 90 kDa reacted with sera from organisms from different serogroups. These results conflict somewhat with those of Chatfield and colleagues (12) who stated that *T. hyodysenteriae* has an 'LPS complex' in the 14 000–24 000 molecular-weight region, although they did not describe how they arrived at these conclusions.

Antisera raised against the type organisms of 4 of the 5 serogroups of *T. hyodysenteriae* reacted in immunoblots with 3 or 4 LPS components from the homologous organism. Organisms of serogroup C were not available in this study, but probably would have a similar arrangement. These major antigenic components in *T. hyodysenteriae* presumably correspond to the multiple LPS antigens which were deduced to exist by Hampson and colleagues (7) using AGDP. However unlike the current technique, solubilization of samples is not employed for AGDP. Nevertheless the basic method of preparation of LPS in this study (hot

water-phenol extraction) is the same as has been employed for most other studies using what has been called *T. hyodysenteriae* LPS.

All four of the type organisms for the serogroups possessed LPS components at around 27–30 kDa, but these were generally immunologically distinct for the group. All also possessed two or three other major LPS bands. Serum against B78 did react with the 27 kDa band of A1, and sera against WA1 reacted with a component in A1 at around 10 kDa. Interestingly antiserum against A1 did not react with this component of its own LPS. The cross-reaction of antisera against B78 and against WA1 and A1 components was removed by absorption with A1 organisms, and the two sera still reacted with their own LPS, although less strongly. This minor one-way cross reactivity was insufficient to justify changes to the serogrouping system, which was generally supported by the immunoblotting results.

All four antisera against serogroup organisms detected to a greater or lesser extent minor high molecular weight components; these were presumably components common to the groups, although not all necessarily of identical size or configuration. The sera also reacted weakly with components of similar weight which were present in *T. innocens* (B256), and were again presumably some common basic structural components present in relatively small quantities. Antisera to B256 reacted strongly with two of its own LPS components, one of which was of higher molecular weight than the major immunogenic components in *T. hyodysenteriae*. There may therefore be differences in the arrangement and size of LPS in the cell walls of *T. hyodysenteriae* and *T. innocens*.

Examination of organisms with different serotypes within serogroups A and B, and particularly the use of cross-absorbed sera on group B organisms, supported the concept that organisms within a group can have additional serotypic LPS antigens. Considering WA1 and B204 (serogroup B), serum against each reacted with both organisms. Each had bands of similar as well as different molecular weight, and in particular B204 had an extra low-molecular-weight band not possessed by WA1. Interestingly however serum against WA1 reacted with this band in B204. When serum against WA1 was absorbed with B204 cells it still reacted with several of its own higher-molecular-weight LPS bands, but only faintly with some high-molecular-weight components of B204 LPS, whilst serum against B204 after absorption with WA1 cells would not react with either organism. These results are similar to those reported by Hampson and colleagues (7) using AGDP; they confirm that WA1 has additional LPS antigens not possessed by B204, and they support the use of WA1 as the type strain for group B. Although B204 had at least one additional low molecular weight LPS band not possessed by WA1, it was detectable by both antisera, presumably shared antigenic structures with other components of WA1, and activity against it was absorbed out from B204 serum by WA1 cells. The possession of additional LPS bands is not therefore sufficient to indicate that an organism is of a different serotype from another organism within a serogroup, unless these bands are serologically distinct.

Comparison of immunoblots of LPS from B78 and WA15 (serogroup A) using antisera against B78 demonstrated that these two organisms had at least two bands of similar low molecular weight, although apparently in different quantity:

this material was presumably group specific. They also possessed unique bands in different locations, but these could not be tested for their serotype specificity because serum against WA15 was not available at the time of the study. Comparison of WA15 and WA16 LPS supported the suggestion that it is the low-molecular-weight LPS of the organisms which confers serogroup specificity. WA15 and WA16 were both recovered from the colon of a pig which had died from swine dysentery. WA16 had an unusual spreading growth and raised mucoid colony type, whilst WA15 was more typical, spreading slowly and having a flat colony type. Both were strongly beta-haemolytic and had identical DNA patterns when subjected to restriction endonuclease analysis (18); however LPS from WA16 unlike that from WA15 would not react in AGDP with antiserum against B78, although serum against WA16 reacted with its own LPS and with that of WA15, but not with B78 LPS (7). In the present work low-molecular-weight LPS components were not observed in WA16 in either the silver-stained PAGE preparations or in immunoblot preparations using sera against B78 or WA16. The classification of WA16 remains unclear, but essentially it appears to be a mutant of WA15 which has lost serogroup LPS antigens, but which retains high-molecular-weight immunogenic material which shares epitopes with the WA15 LPS. Loss of LPS presumably in some way also influences bacterial growth and colony morphology. Bacteria lacking LPS components do not usually have a mucoid colonial character, although rough strains of *Brucella canis* may do so (19).

These results are not sufficient to deduce the structural arrangement of treponemal LPS. By analogy with other Gram-negative organisms, low-molecular-weight material would be rough or core LPS, with high-molecular-weight material being outer immunogenic somatic or 'O' antigen (16). In this case however the lower-molecular-weight material bearing serogroup antigens would seem more likely to be external. The hot water-phenol extraction method used here to prepare LPS tends to select for outer rather than core LPS (20). The lower-molecular-weight material missing from the mutant WA16 was presumably lost from the external surface, with high-molecular-weight internal components retained. This assumption concerning the external arrangements of serogroup LPS antigens is supported by the observations that 'serotype' - specific antiserum specifically opsonizes *T. hyodysenteriae* (11), and by the present results where absorption of serum with homologous whole organisms removed its activity against the LPS serogroup antigens.

Further work is required to analyse the structure and composition of treponemal LPS. Specific LPS components can be excised from gels for further serological and chemical analysis, and should be useful for the preparation of serogroup/serotype specific reagents for typing and diagnosis. Immunoblot analysis may also have a role as a specific serological test using LPS from type organisms blotted onto nitrocellulose strips, and test sera from infected pigs. Finally, the availability of a *T. hyodysenteriae* mutant lacking serogroup specific LPS may be of use in establishing the role of this material in immunity to and pathogenicity of swine dysentery.

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