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Proposed revisions to the serological typing system for *Treponema hyodysenteriae*

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SUMMARY

Antisera were prepared in rabbits against seven well-characterized strains of *Treponema hyodysenteriae* of known serotype, and reacted in agarose gel double immunodiffusion tests (AGDP) with lipopolysaccharide (LPS) extracted from 18 Western Australian isolates of the organism. Eight isolates were provisionally typed by this method, but sera raised against one 'typed' and two 'untypable' local isolates reacted in an unexpected fashion with LPS from other local and type strains. Serum raised against the 'typed' local isolate reached with LPS from other previously untyped local isolates: this indicated the presence of more than one major LPS antigen amongst certain local isolates, and was confirmed by cross-absorption of sera. Sera raised against apparently untypable local isolates reacted with LPS from certain type organisms, thus suggesting the presence of complex antigenic relationships between LPS antigens.

The serotyping system for *T. hyodysenteriae* which was proposed by Baum & Joens (1979) uses unabsorbed antisera and is made unworkable by these observations. Instead we propose placing organisms which share common LPS antigens into serogroups A to E, members of which are defined by their reactivity with unabsorbed sera raised against a type organism for the group. We suggest strains B78, WA1, B169, A1 and WA6 respectively as being the most suitable type organisms for the five serogroups identified so far. Isolates possessing additional unique LPS antigens can be regarded as serotypes within the serogroup. However the serotype of an isolate can only be established if antiserum is prepared against it, and this serum continues to react homologously after cross-absorption with bacteria from other serotypes within the serogroup.

INTRODUCTION

Treponema hyodysenteriae is a large anaerobic spirochaete which is the essential aetiological agent of swine dysentery (Taylor & Alexander, 1971; Harris *et al.* 1972). The organisms are usually differentiated from non-pathogenic intestinal spirochaetes such as *T. innocens* (Kinyon & Harris, 1979) by their greater beta haemolytic activity (Kinyon, Harris & Glock, 1977). Baum & Joens (1979) divided *T. hyodysenteriae* into serotypes 1 to 4 using antisera raised in rabbits reacted in double diffusion agarose precipitation tests (AGDP) against lipopolysaccharide extracts of the organisms. Lemcke & Bew (1984) described three 'new' serotypes

on the basis of AGDP and passive haemagglutination, and Mapother & Joens (1985) found three more serotypes which may or may not have been related to those of Lemcke & Bew. The new serotypes of Mapother & Joens were defined in AGDP only after sera which had been raised against the 'new' serotype were absorbed with cross-reacting strains. One of these new serotypes (Ack 300/8) had previously been placed in serotype 2 by Lemcke & Bew.

The initial purpose of the present study was to serotype Australian isolates of *T. hyodysenteriae* for epidemiological studies. During this project it became apparent that the current serotyping scheme had deficiencies, and suggestions are therefore made for its modification.

MATERIALS AND METHODS

Microorganisms

Seven isolates of *T. hyodysenteriae*, including reference strains for serotypes 1, 2 and 4 and two of the 'new' types of Lemcke & Bew (1984), as well as three non-pathogenic spirochaetes, including the reference strain of *T. innocens*, were received from the A.F.R.C. Institute for Research on Animal Diseases, Compton, England through Dr R. J. Lysons (Table 1). A verified isolate of B169, the organism described as being of serotype 3 by Baum & Joens (1979), was not available for this study. Eighteen isolates of *T. hyodysenteriae* (W.A.1 to W.A.18), recovered between mid-1986 and the end of 1987 from outbreaks of swine dysentery in Western Australia, were received from the Western Australian Department of Agriculture.

Media

All spirochaetes were initially grown, in anaerobic jars under an atmosphere of 94% H_2 and 6% CO_2, on Trypticase Soy Agar (BBL) supplemented with 5% defibrinated bovine blood, and passed by agar plug transfer of individual colonies. Single colonies were suspended in Trypticase Soy Broth (BBL) and injected into the prereduced anaerobically sterilized autoclaved liquid media of Kunkle, Harris & Kinyon (1986), which was supplemented with 2% foetal bovine serum and a 1·5% cholesterol solution. Broths were incubated on a rocking platform at 37 °C, and adequate growth of the organisms and absence of contamination was monitored by examining aliquots under a phase-contrast microscope. Broth containing early passages of the organism were stored at −80 °C. Large quantities of the bacteria were obtained by growing them for 3–4 days in duplicate 250 ml lots of serum bottles, and storing these at −20 °C until required.

Preparation of lipopolysaccharide (LPS)

Treponemes were pelleted from 750 ml lots of frozen-and-thawed broth by centrifugation at 15000 g for 20 min at 4 °C, and were then washed twice in phosphate buffered saline (PBS, pH 7·2, 0·01 M). The organisms were then lyophilized and LPS was extracted from 200 mg batches of dried cells by the Westphal hot water-phenol method (Westphal, Lüderitz & Bister, 1952) as adapted by Baum & Joens (1979). The final acetone precipitate was made up to

Table 1. *Reference strains of* T. hyodysenteriae *and related spirochaetes used in the study*

Strain	LPS-serotype*	Origin
B78	1	USA
B234	1	USA
B204	2	USA
P18A	4	UK
A1	4	UK
MC52/80	'New'	UK
KF9	'New'	UK
B256	*T. innocens*	USA
PWS/A	Non-pathogenic	UK
4/71	spirochaetes	

* Baum & Joens, 1979; Lemcke & Bew, 1984.

1 ml in distilled water and an estimate of its hexose content made using the Dubois phenol-sulphuric acid method (Dubois *et al.* 1956), and glucose standards.

Preparation of bacterins

Treponemes were pelleted from 500 ml lots of fresh broth, washed twice in PBS and suspended in 0·3% formaldehyde in PBS. This suspension was adjusted to an absorbance of 1·0 at 420 nm, inactivated for 24 h at room temperature and stored in aliquots at −20 °C.

Preparation of antisera

Antisera against the isolates indicated in Table 2 were prepared in rabbits of 2–3 kg body weight. Each received 1 ml of bacterin suspended in 1 ml Freund's Complete Adjuvant at two intramuscular sites twice at fortnightly intervals, followed by 1 ml bacterin intravenously at weekly intervals for 5 weeks. Sera for typing was obtained 1 week after the last inoculation.

Absorption of sera

Selected absorption of sera was made with appropriate organisms. Sera against B204, WA1 and WA4 were individually absorbed with each of these strains; sera against B78 and B234 were cross-absorbed; sera raised against MC52/80, KF9 and WA6 were absorbed with each of these strains. Fresh organisms pelleted from 250 ml of broth were washed twice in PBS, and the pellet mixed with an equal volume of sera. The cell suspension was incubated at 37 °C for 30 min and then left overnight at 4 °C on a rotary shaker. The absorbing bacteria were removed from the serum by two cycles of centrifugation at 15000 g for 10 min.

Immunodiffusion

Antisera and LPS were all tested against each other at least twice. Pre-immune rabbit sera were also tested against each LPS. Immunodiffusion was in 1% agarose in barbital-EDTA buffer (0·05 M, pH 8·6). Agarose was poured into plastic petri dishes to a depth of 5 mm, and 4 mm diameter holes were punched out in a hexagonal configuration plus a central well, each well being 3 mm apart (edge to

Table 2. *Agarose gel double immunodiffusion between lipopolysaccharide extracts of* Treponema hyodysenteriae *and rabbit antisera*

Strains isolates (LPS)	LPS serotype	Antisera against									
		B78 1	B234 1	B204 2	P18A 4	A1 4	MC52/80 'New	KF9 'New'	WA1 NT	WA4 NT	WA6 NT
B78*	1	+	+								
B234*	1	+	+								
B204*	2			+					+	±	
P18A*	4				+	+					
A1*	4				+	+					
MC52/80*	'New'						+	+			+
KF9*	'New'						+	+			±
WA1	NT			+					+	+	
WA2	NT								+	+	
WA3	NT						±				+
WA4	NT								+	+	
WA5	NT						±				+
WA6	NT										+
WA7	NT		+						+	+	
WA8	NT		+						+	+	
WA9	NT								+	+	
WA10	NT								+	+	
WA11	NT								+	+	
WA12	NT		+						+	+	
WA13	NT								+	+	
WA14	NT	+									
WA15	NT	+									

* Reference strains received from Dr R. J. Lysons, England. Other isolates from Western Australia, received from the Department of Agriculture.
NT, Not typed when received; ±, Reaction present but weak.

Table 3. *Agarose gel double immunodiffusion between lipopolysaccharide of* Treponema hyodysenteriae *of proposed serogroup B and cross-absorbed rabbit antisera*

Absorbing organism	Antisera against	Bacterial strains and isolates (LPS) B204	WA1	WA4
—	B204	+	+	—
B204	B204	—	—	—
WA1	B204	—	—	—
WA4	B204	—	—	—
—	WA1	+	+	+
WA1	WA1	—	—	—
B204	WA1	—	+	+
WA4	WA1	—	—	—
—	WA4	+	+	+
WA4	WA4	—	—	—
B204	WA4	—	+	+
WA1	WA4	—	—	—

edge). Wells were filled to the top and reactions were examined after 6 and 16 h incubation at room temperature in a humid chamber.

RESULTS

Results of initial immunodiffusion reactions are presented in Table 2. Organisms which gave no reactions with the sera are not included in the Table. No preimmune sera reacted with any extract. Antisera against B256, the type strain of *T. innocens* reacted homologously, but did not react with any of the *T. hyodysenteriae* isolates. Sera prepared against the overseas reference strains of *T. hyodysenteriae* reacted homologously, and with other known members of their own serotype: the two strains of 'new' serotypes also cross-reacted. Only 8 of 18 Australian isolates reacted with these sera: 2 with B78 antisera, 4 with B204 antisera, and 2 weakly but repeatably with MC52/80 antisera.

Antisera raised against local isolate WA1 (which was put in serotype 2 on the basis of its reaction with B204 antisera) not only reacted with B204 and the four Australian strains which reacted with the B204 antisera, but also reacted with an additional six previously untypable Australian isolates. Antisera raised against WA4 (one of the latter six 'untypable' isolates) reacted weakly with LPS from B204, but otherwise reacted with all the same isolates as did sera against WA1. Sera raised against WA6 reacted homologously, and against WA3 and WA5 (which had both reacted weakly with sera against MC52/80), as well as strongly against reference strain MC52/80 and weakly against LPS from KF9.

Mutual cross-absorptions of antisera followed by AGDP were then made between (i) B204, WA1 and WA4 (Table 3), (ii) MC52/80, KF9 and WA6 (Table 4) and (iii) B78 and B234. In the case of B204, WA1 and WA4, only where sera against WA1 and WA4 were absorbed with B204 was any activity left, and this was only directed against LPS from WA1 and WA4. Considering MC52/80, KF9 and WA6, homologous activity was retained after each absorption. MC52/80 and

Table 4. *Agarose gel double immunodiffusion between lipopolysaccharide of* Treponema hyodysenteriae *of proposed serogroup E and cross-absorbed rabbit antisera*

Absorbing organism	Antisera against	Bacterial strains and isolates (LPS) MC52/80	KF9	WA6
—	MC52/80	+	+	—
MC52/80	MC52/80	—	—	—
KF9	MC52/80	+	—	—
WA6	MC52/80	+	—	—
—	KF9	+	+	—
KF9	KF9	—	—	—
MC52/80	KF9	—	+	—
WA6	KF9	—	±	—
—	WA6	+	±	+
WA6	WA6	—	—	—
MC52/80	WA6	—	—	+
KF9	WA6	+	—	+

± Reaction present but weak.

WA6 also apparently possessed a shared antigen not present on KF9. Cross absorption of B78 and B234 on each of two occasions removed all activity from both sera.

DISCUSSION

The typing sera prepared against reference strains of *T. hyodysenteriae* reacted appropriately with LPS from other reference strains, except that the two 'new' serotypes of Lemcke & Bew (1984) mutually cross-reacted (Lemcke & Bew found that they reacted only with their homologous antisera). Mapother & Joens (1985) recommended that the AGDP be read after 6 h, however even homologous reactions with reference strains were frequently not visible at this time, so all reactions were therefore read after overnight incubation (16 h).

Attempts to serotype Western Australian isolates using the sera prepared against overseas strains resulted in only 8/18 (44%) being typable. However, two antisera raised against local isolates (WA1 and WA6) reacted with 13/18 (72%) of the local isolates, seven of which had not previousy been 'typed'. Sera raised against local isolate WA1 (which was provisionally assumed to be of serotype 2 because of its reactivity with antisera against B204) reacted with six 'untypable' local isolates, as well as with four other isolates provisionally assumed to be of serotype 2. Sera raised against one of these five 'untypable' local isolates (WA4) also reacted with all the isolates which reacted with antisera against WA1. These results indicate the presence of more than one major LPS antigen in certain isolates, and suggest that certain common antigens may have different physical locations or chemical arrangements in the cell wall of certain strains. In the case of B204 and WA4, serum against WA4 reacted with LPS extracted from B204, but serum against B204 would not react with LPS from WA4 (Table 3). The organisms presumably possessed common LPS antigen(s), but in B204 these were arranged in such a way that they did not induce an antibody response measurable

in AGDP. The LPS in question may have been superficial in B204, and lost during preparation of bacterin, physically or chemically arranged so that it was poorly accessible for processing by the immune system of the immunized rabbits, or the relative quantities of the LPS may have been different in the different strains. Alternatively the common antigen(s) may have been lost during preparation of LPS from WA4, but again this would imply a different physical location from that found in B204. LPS preparations were consistent in their yield and quality, since repeated extractions from different batches of any one strain always reacted with the same set of antisera (results not shown). Cross-absorptions between B204, WA1 and WA4 were carried out in an attempt to define the antigenic relationship between these organisms, and WA1 and WA4 appeared to possess at least one major antigen not possessed by B204, as well as a common LPS antigen possessed by all three. Failure to identify antigenic differences between WA1 and WA4 was surprising considering their different reactivities with sera against B204, and may reflect differences in quantity rather than the antigenic type of their LPS.

Mutual cross-absorption of antisera between B78 and B234 removed homologous and heterologous activity, suggesting that these two strains had identical serotypes. Using agglutinin-absorption, Lemcke & Bew (1984) demonstrated antigenic differences between this pair of organisms; however this technique does not necessarily relate only to differences in LPS antigens. The present finding that sera against B78 reacted with the local isolates WA14 and WA15 but sera against B234 did not react with them, does nevertheless tend to support the suggestion that the LPS of B78 and B234 have antigenic differences. Cross-absorption of sera followed by AGDP may not be an optimal method of demonstrating antigenic similarities and diversities amongst LPS, and work is in progress to determine whether polyacrylamide gel electrophoresis and Western blotting may be more appropriate for this purpose.

Lemcke & Bew (1984) used agglutinin-absorption to demonstrate that MC52/80 and KF9 were different serologically, and our precipitin absorption tests confirmed differences in LPS antigens (Table 4). Although sera raised against MC52/80 and KF9 did not react with LPS from WA6, sera raised against WA6 reacted with both MC52/80 and KF9, as well as with the two local isolates which reacted weakly with sera against MC52/80 (WA3 and WA5). Again these findings suggest the presence of several LPS antigens, some of which are probably normally in different locations, arrangements or quantities in the different strains. Cross-absorption between MC52/80, KF9 and WA6 suggested that whilst each was distinct, WA6 possessed an additional antigen shared with MC52/80.

Other observations suggested that *T. hyodysenteriae* may under certain circumstances lose LPS antigens. Isolates WA15 and WA16 were recovered at the same time from the same colonic site in a pig, but colonies of WA16 spread on Trypticase Soy Agar plates whilst WA15 was non-spreading. This colony characteristic was retained after more than five passages. Restriction endonuclease analysis of DNA extracted from the two isolates failed to reveal differences, suggesting that the organisms were derived from a single clone (Combs, B., Hampson, D. J., Mhoma, J. R. L. & Buddle, J. R., submitted for publication). However, LPS extracts from WA15 reacted with sera raised against B78, whilst extracts from four separate preparations of WA16 all failed to react with this

antiserum. In turn antiserum raised against WA16 reacted with LPS from WA16 and WA15, but not with LPS from B78. Presumably WA16 had lost antigenic surface structures (LPS) which were present in WA15 and B78, and which may also have been associated with colony growth and spread. Hovind-Hougen & Hogh (1984) also recovered from single pigs *T. hyodysenteriae* isolates having different colony morphologies and antigenic properties.

Investigations of antigenic relationships between LPS from various isolates of *T. hyodysenteriae* revealed the presence in some organisms of multiple major LPS antigens, some of which may not normally induce an antibody response detectable in AGDP. These findings make the typing system of Baum & Jones (1979) unworkable, at least to the serotype level. The obvious modification is the use of absorbed sera, as was done by Mapother & Joens (1985) when describing 'new' serotypes; however this requires preparation of sera against each new isolate, as well as cross-absorption with any apparently related organisms. This procedure is time-consuming (at least 6–8 weeks to produce sera), and costly. An alternative which we are investigating is to use specific extracted LPS antigens to immunize rabbits, and then use slide agglutination tests with whole bacteria and the sera. Again this procedure will require better characterization of the LPS antigens involved.

At present, since certain organisms appear to share a common LPS antigen as detected in AGDP, it would appear useful to call such a cluster of organisms a serogroup. Serotypes with additional specific unique LPS antigens could then be placed within each serogroup. In the case of the organisms B204, WA1 and WA4, since only serum against WA1 will react with LPS from all three bacteria, WA1 should be considered as the type organism for the serogroup. WA1 and WA4 are also antigenically distinct from B204, and so should be considered as a different serotype within the serogroup.

In the case of the organisms MC52/80, KF9 and WA6, each has distinct antigenic properties and could be considered as a separate serotype. However since serum against WA6 reacts with LPS extracts from all three organisms, they should also be considered as a serogroup with WA6 being the type organism.

T. hyodysenteriae strains B78, B234, WA14 and WA15 again appear to form a serogroup, with B78 as the type organism. It seems likely that further testing will reveal antigenic differences between B78 and B234 which will place them in separate serotypes within the serogroup.

Our proposals for a revised serotyping scheme for *T. hyodysenteriae* are summarized in Table 5. Group C, represented by B169, can at present be only surmised based on the work of Baum & Joens (1979). Further elucidation of the scheme will require additional information about the content, configuration, antigenic structure and stability of the LPS of *T. hyodysenteriae*, together with the preparation of specific sera against individual major LPS antigens. In the future these sera could be used in panels to rapidly and accurately serotype organisms. At present, for epidemiological studies, sera prepared against type organisms for a serogroup can be used unabsorbed to identify isolates to the serogroup level. The type organisms from each serogroup occurring in a geographic locality would presumably also be the most suitable candidates as a source of antigenic material for serological tests, particularly the LPS-based ELISA tests of Joens *et al.* (1982) and Egan, Harris & Joens (1983).

Table 5. *Proposed serological groupings of strains of* T. hyodysenteriae

Serogroup	Type organism	Serotype 1	2	3
A	B78	B78	—	—
B	WA1	WA1	B204	—
C	B169	B169	—	—
D	A1	A1	—	—
E	WA6	WA6	MC52/80	KF9

Of the local WA isolates examined, those reacting with sera against B78 we consider as being of serogroup A, those reacting with sera against WA1 are of serogroup B, and those reacting with sera against WA6 are of serogroup E. Isolate WA16 will probably eventually be placed in a revised serogroup A, whilst the other 'untypable' isolates may be part of group C or may form the beginnings of a new serogroup or serogroups. The serotypes of these organisms cannot at present be determined without raising antisera against them and absorbing these with cross-reacting organisms.

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