

A comparative study of spirochaetes from the porcine alimentary tract

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(Received 22 May 1980)

SUMMARY

Strains of *Treponema hyodysenteriae* capable of inducing swine dysentery in specific pathogen-free pigs were compared with other spirochaetes from the porcine alimentary tract by biochemical and serological tests and by electrophoresis of their proteins. Carbohydrate fermentation and esculin hydrolysis were similar in all the spirochaetes. Indole was produced by *T. hyodysenteriae* and by some of the other spirochaetes. Analysis of the fatty acids produced from glucose showed a difference between *T. hyodysenteriae* and other spirochaetes only in the amount of *n*-butyric acid produced. The indirect fluorescent antibody test showed extensive cross-reactions between all the spirochaetes unless antisera were first absorbed. A microtitre agglutination test and a growth-inhibition test were both more specific; strains of *T. hyodysenteriae* could be distinguished from the other spirochaetes using unabsorbed sera. Both tests revealed some antigenic heterogeneity among strains of *T. hyodysenteriae*. The cell proteins of a single strain of *T. hyodysenteriae* gave an electrophoretic pattern distinct from those of the other spirochaetes.

Two of the six spirochaetes not associated with swine dysentery, PWS/B and PWS/C, were indistinguishable serologically and electrophoretically. The other four strains were serologically distinct from one another and from PWS/B and PWS/C. Only two of these spirochaetes were examined electrophoretically, but each gave a different pattern from PWS/B and PWS/C. The diversity observed among spirochaetes not associated with swine dysentery indicates that their suggested inclusion in a single species, *T. innocens*, may prove to be unjustified.

INTRODUCTION

Since the large spirochaete *Treponema hyodysenteriae* was implicated in swine dysentery (SD) (Taylor & Alexander, 1971; Harris *et al.* 1972), it has become a matter of diagnostic importance to be able to distinguish it from other spirochaetes which occur in the porcine alimentary tract. The most reliable method of identification is testing for enteropathogenicity in conventionally reared pigs (Kinyon, Harris & Glock, 1977), or in isolated colonic loops (Hughes *et al.* 1972; Whipp *et al.* 1978; Wilcock & Olander, 1979). However, these methods are not

practicable for routine use. The type of haemolysis produced on blood agar is a useful guide in identifying *T. hyodysenteriae*, since this spirochaete produces areas of complete haemolysis whereas most other intestinal spirochaetes produce only partial haemolysis. Cultural conditions can affect the reaction, however, and a few non-enteropathogenic strains can produce a degree of haemolysis almost indistinguishable from that of *T. hyodysenteriae*.

Fluorescent antibody tests for the identification of *T. hyodysenteriae* in faecal smears have been introduced (Terpstra, Akkermans & Ouwerkerk, 1968; Saunders & Hunter, 1974; Hunter & Clark, 1975) but non-specific reactions occur unless conjugate or antiserum against *T. hyodysenteriae* is first absorbed with non-pathogenic intestinal spirochaetes (Hudson, Alexander and Lysons, 1976; Hunter & Saunders, 1977). Although agglutination tests have been used primarily to detect antibody to *T. hyodysenteriae*, the results obtained by Joens *et al.* (1978) suggested that agglutination in microtitre trays using an unheated antigen might be a useful method for identifying the spirochaete itself.

Recently, an amended description of *T. hyodysenteriae* was published and a new species, *T. innocens*, was proposed for non-enteropathogenic spirochaetes (Kinyon & Harris, 1979). Of the 33 characters enumerated, only enteropathogenicity, haemolysis, indole production, fructose fermentation and possibly gelatin and hippurate hydrolysis were described as being different in the two species. DNA homology studies, however, revealed significant differences between the two species, although only two strains of each were examined (Miao, Fieldsteel & Harris, 1978).

The present study of SD-associated and other spirochaetes from the porcine gut was initiated to examine some of the existing criteria for identifying *T. hyodysenteriae*, to explore other methods of identification, and to try to elucidate the relationships of intestinal spirochaetes.

MATERIALS AND METHODS

Microorganisms

The spirochaetes P18A, KF9 and S75/1 were isolated from pigs with experimentally induced or naturally occurring SD. All three were capable of inducing SD when administered to conventionally reared, specific pathogen-free pigs (Lysons and Bew, personal communication; Taylor, personal communication) and can therefore be regarded as strains of *T. hyodysenteriae*. The spirochaetes PWS/A, PWS/B, PWS/C, 4/71 and M1 were isolated from pigs with no history of SD in the herd (Taylor & Alexander, 1971; Hudson *et al.* 1976; Lysons and Bew, personal communication). Strain B256 was the type strain of *T. innocens* (Kinyon & Harris, 1979). Three of the non-SD-associated strains, PWS/A, 4/71 and B256 had been tested for enteropathogenicity and found to be negative. *Leptotrichia buccalis* (NCTC 10249), *Fusobacterium necrogenes* (NCTC 10723) and *F. necrophorum* (NCTC 10575) were obtained from the National Collection of Type Cultures.

Media

For routine cultivation, spirochaetes were grown on 5% sheep blood agar incubated in 10% carbon dioxide in hydrogen, or in rabbit serum broth (RSB) under 10% carbon dioxide in nitrogen (Lemcke *et al.* 1979). Spectinomycin was omitted from RSB in which strain B256 was grown because this spirochaete is sensitive to that antibiotic. All cultures were incubated at 37 °C.

Biochemical tests

The fermentation of carbohydrates and hydrolysis of esculin by spirochaetes grown on 7% horse blood agar was determined by the method of Phillips (1976) and included the recommended controls. Carbohydrate fermentation was also tested in RSB in which glucose-containing Trypticase-soya broth was replaced by Oxoid Nutrient broth No. 2 which contains no glucose. Carbohydrates were added to a final concentration of 1% (w/v). Inocula were grown in medium without substrate. Two tubes of each medium were inoculated and pH values were determined on a Radiometer pH Meter 27 after incubation for 7 and 14 days at 37 °C. Uninoculated controls without substrate were also included. Fermentation was assessed in terms of reduction in pH compared with inoculated broths without substrate, and scored from 4+ to -. A score of 4+ represented a drop of at least 1.0 pH unit, 3+ a drop of 0.75 to 0.99 units, 2+ a drop of 0.5 to 0.74 units, 1+ a drop of 0.25 to 0.49 units and - a drop of less than 0.25 units. Tubes were also checked for growth and freedom from contamination by subculturing on blood agar immediately before determining the pH. Indole formation was tested at intervals between 3 and 14 days on cultures grown in RSB. Because of the serum in the medium, 0.2 ml of ether was added to each 2 ml culture before addition of Kovac's reagent.

Gas-liquid chromatography

Spirochaetes were grown in RSB which contained 0.5% glucose until maximum turbidity was reached (4–7 days). Supernatants for analysis were obtained by centrifugation and were stored at -20 °C until all were available for extraction. Supernatants from two sets of cultures grown at different times were examined and each supernatant was analysed twice. Analyses were carried out on a Pye Unicam GCD gas chromatograph equipped with flame ionization detectors. A 1.5 m coiled glass column of internal diameter 2 mm, and packed with 10% FFAP on 100–200 mesh acid-washed Diatomite C (Pye Unicam) was used. Volatile fatty acids were determined using ether extracts, and non-volatile fatty acids were analysed after methylation (Holdeman, Cato & Moore, 1977). One micro-litre samples were analysed under the following conditions: nitrogen carrier gas flow, 30 ml/min; injector and detector temperatures, 150 °C; column temperature, 125 °C. Hydrogen and air-flow were optimized for the detection of acetic acid. Standard solutions of fatty acids at 1 mequiv./100 ml aqueous solution, and extracts of the spirochaetes and uninoculated medium were run consecutively.

Preparation of antisera

Antisera were prepared in rabbits against the nine spirochaetes P18A, KF9, S75/1, PWS/A, PWS/B, PWS/C, 4/71, B256, and M1. At first, spirochaetes to be used as immunizing antigens were grown in diphasic medium with rabbit serum in the liquid phase (Lemcke & Burrows, 1979), but later in RSB. Rabbits were immunized by intravenous inoculation (i.v.) only or by subcutaneous inoculation of spirochaetes in incomplete Freund's adjuvant followed by a series of i.v. inoculations (Lemcke and Burrows, 1979). Antisera against P18A, KF9, S75/1 and M1 were prepared by both methods, but antisera against 4/71, PWS/A, PWS/B, PWS/C and B256 only by the i.v. method.

Serological tests

For the indirect fluorescent antibody (IFA) test, thin films of spirochaetes in 1/4 strength Ringer's solution were prepared on slides, air-dried and fixed for 10 min in acetone. Films were treated with dilutions of test sera for 30 min at 37 °C, washed three times in phosphate-buffered saline (PBS, pH 7.3) and, after removing excess moisture, covered with fluorescein-conjugated anti-rabbit globulin (Nordic Laboratories) at a dilution of 1:20, for 30 min at 37 °C. After washing three times in PBS and blotting off excess buffer, films were mounted in FA Mounting Fluid (Difco) and examined with a Zeiss RA microscope fitted with a IV FI Epifluorescence condenser and a mercury lamp. For absorption, antiserum against P18A diluted 1:10 with PBS was incubated for 4 h at 37 °C with an equal volume of washed organisms of PWS/A, PWS/B or 4/71 harvested from 10 blood agar plates. After removal of the absorbing spirochaetes by centrifugation, the serum was re-absorbed in the same way. For absorption with both PWS/A and 4/71, 5 plates of each organism were used for each absorption.

Suspensions of spirochaetes for use as antigens in microtitre agglutination (MA) tests were prepared from 3–5-day cultures in RSB by centrifugation, washing three times in PBS, resuspending to Opacity Tube No. 4 (Burroughs Wellcome & Co.) and heating in a boiling waterbath for 10 min. Antigens were stored at 4 °C. For the test, twofold dilutions of antiserum were prepared in 0.025 ml amounts in microtitre trays and 0.025 ml of antigen was added to each well. Trays were incubated for 2 h at 37 °C and left at 4 °C overnight before reading. In determining the relationship of one spirochaete to another, the antiserum titre with a particular spirochaete antigen was compared with the homologous titre obtained in the same test. When titres did not differ by more than fourfold, antigens were regarded as essentially identical.

The disc growth-inhibition (GI) test was carried out as previously described (Lemcke & Burrows, 1979).

Electrophoresis of cell proteins

Polyacrylamide disc electrophoresis of proteins from 5 species was carried out in urea-acetic acid gels as described by Razin (1968). Gels were scanned on a Gilford spectrophotometer 240 at 500 nm with a linear transit rate of 2 cm/min and a recording speed of 5 cm/min.

Table 1. Biochemical reactions of spirochaetes from the pig gut

Spirochaete	Acid production from								Indole formation	Esculin hydrolysis*
	Glucose	Fructose	Maltose	Sucrose	Lactose	Trehalose	Mannitol	Rhamnose		
P18A†	3+	4+	2+	2+	2+	4+	—	—	+	+
KF9†	2+	2+	+	2+	2+	4+	—	—	+	+
S75/1†	3+	3+	2+	2+	4+	3+	—	—	+	+
PWS/B	4+	4+	2+	4+	3+	4+	—	—	—	+
PWS/C	4+	4+	2+	4+	2+	3+	—	—	—	+
4/71	3+	3+	2+	4+	+	2+	—	—	—	+
PWS/A	3+	3+	2+	2+	3+	4+	—	—	+	+
M1	4+	2+	3+	4+	+	4+	4+	—	—	+
B256	3+	+	+	+	+	2+	—	—	—	+

* Determined on solid medium.

† Strains of *Treponema hyodysenteriae*.

Acid production in liquid medium after 7–14 days at 37 °C (decrease in pH): 4+ > 1.0 unit, 3+ 0.75–0.99 units, 2+ 0.5–0.74 units, + 0.25–0.49 units, — < 0.25 units. Each score represents the mean of readings from three experiments.

RESULTS

Biochemical tests

Carbohydrate fermentation tests carried out on *T. hyodysenteriae* strain P18A and four other spirochaetes grown on solid medium showed that all produced acid from glucose, fructose, maltose, sucrose and lactose but failed to ferment mannitol or rhamnose. Trehalose was strongly fermented by P18A and PWS/A but only weakly by 4/71, PWS/B and PWS/C. No acid production was observed on inoculated plates without substrate or in uninoculated plates containing the various substrates. The fermentation patterns of the anaerobes *L. buccalis*, *F. necrogenes* and *F. necrophorum* which were used as controls agreed closely with those of Holdeman, Cato and Moore (1977).

The results of tests on three strains of *T. hyodysenteriae* and six other spirochaetes grown in liquid medium containing the same substrates showed an essentially similar pattern of carbohydrate fermentation (Table 1), although the amounts of acid produced did not always correspond with those observed in tests on solid medium. In particular, most strains produced a decrease of less than 0.75 pH units in liquid medium containing maltose, whereas the reactions were all strongly positive on solid medium containing maltose. No distinction could be made between the three strains of *T. hyodysenteriae* and the other spirochaetes on the basis of carbohydrate fermentation; fructose, notably, was fermented by both groups. The intestinal spirochaete M1 was distinct from all the others in fermenting mannitol. In regard to indole formation, *T. hyodysenteriae* strains P18A, KF9 and S75/1 were positive, but so was PWS/A, a spirochaete not associated with swine dysentery. All the spirochaetes hydrolysed esculin.

Table 2. Results of indirect fluorescent antibody (IFA) tests

Titres* with antiserum against:

Spirochaete antigen	Titres* with antiserum against:								
	P18A*	PWS/B	PWS/C	4/71	PWS/A	P18A† (absorbed PWS/A)	P18A† (absorbed PWS/B)	P18A† (absorbed 4/71)	P18A† (absorbed PWS/A & 4/71)
P18A†	2560	2560	640	320	1280	640	640	1280	640
PWS/B	320	2560	640	80	160	80	< 40	< 40	< 40
PWS/C	640	2560	320	80	320	80	< 40	40	< 40
4/71	1280	2560	160	1280	80	80	80	< 40	< 40
PWS/A	1280	1280	320	80	2560	< 40	40	80	< 40

* Means of two titrations. Titres of preimmunization sera < 40 (lowest dilution tested).

† *Treponema hyodysenteriae*.*Gas-liquid chromatography*

All nine spirochaetes produced appreciable amounts of acetic acid from glucose. *N*-butyric acid was also formed by most of the spirochaetes, although only in trace amounts by PWS/B and 4/71. However, the three strains of *T. hyodysenteriae* produced more *n*-butyric acid, relative to the amount of acetic acid produced, than the other intestinal spirochaetes. Propionic, *iso*-butyric and *iso*-valeric acids were produced in trace amounts by most of the spirochaetes, but traces of lactic acid were produced by only four strains, KF9, PWS/B, PWS/C and 4/71. Valeric, caproic and succinic acids were not detected in any of the cultures.

Indirect fluorescent antibody tests

T. hyodysenteriae, strain P18A, was compared initially with four other spirochaetes (Table 2) but as extensive cross-reactions were observed to high titre between all five, even with antisera produced by i.v. inoculation, the comparison was not extended to other strains. Absorption of P18A antiserum with PWS/A, PWS/B or 4/71 reduced the titres of the serum with the absorbing strain to < 40, the lowest dilution tested, whereas the homologous titre was reduced only two- to fourfold. Heterologous strains other than the absorbing strain still gave titres up to 80, however. Absorption with both PWS/A and 4/71 reduced all titres with heterologous spirochaetes to < 40 (Table 2).

Microtitre agglutination tests

The geometric mean titres obtained in tests comparing three strains of *T. hyodysenteriae* and six other spirochaetes are shown in Table 3. The results obtained with adjuvant-produced antisera against P18A, KF9, S75/1 and M1 are quoted in the Table, since these sera had higher homologous titres than those produced by i.v. inoculation.

The three strains of *T. hyodysenteriae*, P18A, KF9 and S75/1, all reacted to similar titre with antiserum against P18A, indicating that they were essentially identical (Table 3). There were, however, greater than fourfold differences between titres when the three strains were titrated against KF9 or S75/1 antisera.

Table 3. Results of microtitre agglutination tests

Spirochaete antigen	G.M. titres* with antisera against:								
	P18A†	KF9†	S75/1†	PWS/B	PWS/C	4/71	PWS/A	M1	B256
P18A†	580	57	28	50	10	28	67	28	14
KF9†	453	678	28	40	20	40	20	40	14
S75/1†	453	113	226	80	40	40	63	80	20
PWS/B	22	20	14	422	160	20	5	28	5
PWS/C	67	20	28	1280	368	20	14	40	10
4/71	67	40	20	50	20	253	50	20	20
PWS/A	20	14	20	10	5	28	538	40	10
M1	40	40	28	40	20	20	10	226	5
B256	28	20	5	40	5	20	14	14	160

* Geometric mean of at least three titrations. Titres of preimmunization sera \leq 10.

† Strains of *Treponema hyodysenteriae*.

Table 4. Results of growth-inhibition tests

Spirochaete antigen	Zone of inhibition (mm)* with antisera against:								
	P18A†	KF9†	S75/1†	PWS/B	PWS/C	4/71	PWS/A	M1	B256
P18A†	10	3	1	0	0	0	0	0	0
KF9†	2	4	1	0	0	0	0	0	0
S75/1†	1	1.5	3	0	0	0	0	0	0
PWS/B	0	0	0	6	3	0	0	0	0
PWS/C	0	0	0	6	6	0	0	0	0
4/71	0	0	0	0	0	8	0	0	0
PWS/A	0	0	0	0	0	0	3	0	0
M1	0	0	0	0	0	0	0	3	0
B256	0	0	0	0	0	0	0	0	2

* Diameter of zone measured from edge of disc.

† Strains of *T. hyodysenteriae*.

No inhibition with preimmunization sera.

Among the six spirochaetes not associated with SD, only PWS/B and PWS/C cross-reacted to a degree which indicated a close antigenic relationship (Table 3). These six spirochaetes thus fell into five serological groups. Cross-reactions between *T. hyodysenteriae* and the other six spirochaetes were at a low level not indicative of a close antigenic relationship.

Growth-inhibition tests

The results are shown in Table 4. Cross-reactions were observed only between the three strains of *T. hyodysenteriae* and between PWS/B and PWS/C. The results of both MA and GI tests thus indicated the same serological groupings among the nine spirochaetes examined. Comparison of the three *T. hyodysenteriae* isolates showed that heterologous strains were usually less strongly inhibited than the homologous strain. In particular, S75/1 was minimally inhibited by P18A and KF9 antisera, and P18A and KF9 were minimally inhibited by S75/1 antiserum, suggesting that there were some antigenic differences between the strains.

Electrophoresis of cell proteins

Visual inspection and photometric scanning of gels from the five strains tested revealed that the electrophoretic pattern of *T. hyodysenteriae* P18A was distinct from that of the other four intestinal spirochaetes. Among these four spirochaetes, PWS/B and PWS/C gave closely similar patterns, distinct from the other two. Thus, three different electrophoretic patterns were discernible among the four spirochaetes not associated with SD.

DISCUSSION

It was impossible to distinguish three strains of *T. hyodysenteriae* from six other intestinal spirochaetes, which were not associated with SD, by differences in carbohydrate fermentation, indole production or esculin hydrolysis. According to the descriptions of the two species given by Kinyon and Harris (1979), *T. innocens*, but not *T. hyodysenteriae*, ferments fructose with the production of acid. This is contrary to our finding that all the spirochaetes fermented fructose whether tested on solid or in liquid medium. It is possible that more luxuriant growth was obtained in the experiments described here. Phillips (1976) stressed the importance of using an adequately nutritious basal medium such as blood agar in determining the fermentation reactions of obligate anaerobes. An inherent difficulty in comparing the fermentation or the fermentation products of different spirochaetes is that some strains grow more luxuriantly than others under a given set of conditions. It was apparent from our results that the extent of the decrease in pH and the amounts of fatty acids produced were directly related to the amount of growth. In regard to indole production by *T. hyodysenteriae*, our results agreed with those of Kinyon & Harris (1979), but one indole-positive strain was also found among the six spirochaetes not associated with SD. This strain is not unique; so far we have found eight other spirochaetes from sources not associated with SD that give a positive indole reaction (Burrows & Lemcke, unpublished observations). It must be concluded, therefore, that biochemical reactions do not provide satisfactory criteria for identifying *T. hyodysenteriae*.

The pattern of fatty acid production from glucose also proved unsatisfactory as a criterion for distinguishing *T. hyodysenteriae* from spirochaetes not associated with swine dysentery. The only point of distinction was that the three strains of *T. hyodysenteriae* which we examined tended to produce more *n*-butyric acid than the other spirochaetes. Clearly, more spirochaetes of both types need to be analysed to determine whether this is a valid distinction.

The IFA test could not be used to distinguish *T. hyodysenteriae* from other intestinal spirochaetes if unabsorbed antisera were used. This confirms the observations of Joens *et al.* (1978). Absorption with a single non-SD-associated strain, although effective in removing antibody against the absorbing strain, failed to eliminate cross-reactions with other intestinal spirochaetes. Absorption with two such strains eliminated cross-reactions with the four non-SD-associated spirochaetes tested. However, if, as seems likely, these spirochaetes belong to a number

of different serological types, it would be difficult to absorb *T. hyodysenteriae* antiserum in such a way as to eliminate cross-reactions with every other intestinal spirochaete.

Both MA and GI tests were more specific than the IFA test, since *T. hyodysenteriae* could be distinguished from the other intestinal spirochaetes using unabsorbed antisera. The GI test in particular was highly specific; no cross-reactions occurred between *T. hyodysenteriae* and the other spirochaetes. Since both agglutination and growth-inhibition are generally considered to be associated with surface antigens, it seems likely that the specific antigens are located in or on the outer envelope of the spirochaetes. The cross-reactivity observed between the three strains of *T. hyodysenteriae* in both tests indicated that they constituted a recognizable serological group. In this connexion, a species-specific protein antigen has recently been identified in *T. hyodysenteriae* (Baum & Joens, 1979b). Nevertheless, the results of MA and GI tests also suggested that the strains of *T. hyodysenteriae* were not completely identical. The basis for this antigenic heterogeneity is still conjectural, although Baum & Joens (1979a) recently identified four serologically distinct lipopolysaccharides among thirteen strains of *T. hyodysenteriae*, and proposed that four new 'serotypes' should be recognized in this species. Since antigens were heat-treated in our MA tests, the differences noted between, for example, P18A and S75/1 could be ascribed to the presence of a heat-stable, type-specific antigen such as LPS. A comparison of a larger number of *T. hyodysenteriae* strains by agglutinin-absorption might help to elucidate the antigenic differences which exist within the species.

Since the results reported here showed that *T. hyodysenteriae* can be distinguished from other intestinal spirochaetes by the MA test, a slide agglutination test has been developed (Burrows & Lemcke, 1981) and is being evaluated, together with the GI test, for identification of this spirochaete among field isolates.

Among the six non-SD spirochaetes examined, five serological types were distinguished by MA and GI tests. Similarly, three different electrophoretic patterns were found among four such isolates, and there were also differences with respect to indole production. In view of this diversity, it seems doubtful whether the recent proposal to include non-enteropathogenic spirochaetes in a single species, *T. innocens*, will eventually be justified.

We are grateful to Dr D. L. Harris and to Dr D. J. Taylor for kindly supplying us with strains B256, S75/1 and 4/71,

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