Serotypes of Beta-Hemolytic Treponema hyodysenteriae

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Cultures from 13 isolates of pathogenic, beta-hemolytic Treponema hyodysenteriae from 11 geographically separate outbreaks and 2 experimentally induced cases of swine dysentery were lyophilized and extracted with hot phenol-water. The resulting water phases were examined serologically with antisera produced in rabbits against whole-cell bacterins of the 13 isolates for evidence of antigenic classes within the species. Water-phase antigens gave precipitin reactions with homologous antisera. Results from cross-testing of each water phase with each antiserum showed four serologically distinct groups among the isolants examined. Based on precipitin reactions in agarose gel, four serotypes of pathogenic, beta-hemolytic T. hyodysenteriae are proposed.

Treponema hyodysenteriae has been shown to be the primary etiological agent of swine dysentery (6, 8, 9, 28, 29). Presently, two major classes of T. hyodysenteriae are recognized on the basis of hemolytic activity in blood agar: beta-hemolytic T. hyodysenteriae and weak beta-hemolytic T. hyodysenteriae (16). The beta-hemolytic isolates can induce swine dysentery when given orally to conventional and specific-pathogen-free swine (7–9, 16, 20–23). The weak beta-hemolytic isolates have been isolated from cases of canine diarrhea (2, 4, 16), normal swine (29), and postweaning diarrhea in swine (16). Weak beta-hemolytic T. hyodysenteriae has failed to produce clinical signs and lesions typical of swine dysentery when introduced orally into specific-pathogen-free swine (16).

Lipopolysaccharide is a compound from the outer envelope of gram-negative bacteria that is responsible for endotoxicity and antigenic specificity among gram-negative bacteria (12, 30) and is readily extracted with hot phenol-water (2, 30, 31). Electron microscopy of Treponema has shown that this genus has an outer envelope similar to that of other gram-negative bacteria (1, 10, 11, 14, 17, 18, 24–26). It was presumed, therefore, that phenol-water extraction of beta-hemolytic T. hyodysenteriae could yield lipopolysaccharide-like antigens that would make serological grouping of the organism possible.

The objective of this study was to investigate antigens extracted from 13 isolates of pathogenic, beta-hemolytic T. hyodysenteriae to determine whether they could be used effectively for serological classification of beta-hemolytic T. hyodysenteriae.

MATERIALS AND METHODS

Isolates. Beta-hemolytic isolates A-1, B78, B140, B169, B204, B234, Dys 7, and 9605 and weak beta-hemolytic isolates 4/71, B256, B297, and Puppy were obtained from the Veterinary Medical Research Institute, Iowa State University, Ames, Iowa, through J. M. Kinyon; isolates T3 and T4 were submitted by Joe Welter, Dallas Center, Iowa; isolates T5 and T6 were reisolated from pigs experimentally infected with B204 and B234, respectively, at the National Animal Disease Center, Ames, Iowa; isolate T7 was isolated from B140 and designated according to National Animal Disease Center nomenclature. All isolates were received frozen in broth.

Media. Blood agar plates were prepared using Trypticase soy agar (BBL Microbiology Systems, Cockeysville, Md.) supplemented with 5% citrated bovine blood (1 g of sodium citrate per 100 ml of blood). Trypticase soy broth (TSB) was rehydrated and prepared by the aerobic method of Kinyon and Harris (15). This method was also used for the preparation of 200 ml of TSB in 500-ml round-bottom flasks and 1,000 ml of TSB in 2,000-ml adapted, round-bottom flasks (L. A. Joens, Ph.D. thesis, Iowa State University, Ames, 1977). All broth contained a 10% supplement of fetal calf serum (National Animal Disease Center, Ames, Iowa; GIBCO, Grand Island, N.Y.) that was added at the time of inoculation. Broth cultures were incubated under an atmosphere of deoxygenated H2-CO2 (50:50) at 37°C on a reciprocating shaker (90 rpm).

Culture preparations. Frozen isolates were thawed at 37°C, transferred to tube TSB (11% inoculum), and subcultured first into 200 ml of TSB in 500-ml round-bottom flasks and then into 1,000 ml of TSB in 2,000-ml flasks (Joens, Ph.D. thesis, 1977). Cultures in 2,000-ml round-bottom flasks were incubated for 36 to 48 h on a reciprocating shaker at 37°C. Organisms were harvested by centrifugation at 16,000 × g at 0°C for 20 min, washed twice in 200 ml of 0.01 M phosphate-buffered saline (pH 7.2), resuspended in...
10 ml of phosphate-buffered saline, and lyophilized. Whole-cell bacteria were prepared from 36- to 48-h, 200-ml cultures of *T. hyodysenteriae*. Cells were harvested and washed as described, resuspended in 10 ml of formalized phosphate-buffered saline (0.3% formaldehyde in 0.01 M phosphate-buffered saline), inactivated at room temperature for 24 h, and stored at 4°C.

Cultures of all of the beta-hemolytic isolates except A-1, B169, B204, and B234 were purified by agar plug transfer for this study. In the context of this study, purification of cultures means the transfer of a single hemolytic plaque from blood agar to TSB. After 48-h incubation at 42°C under deoxygenated H2-CO2 (50:50), broth cultures were subcultured onto blood agar and streaked for isolation. Agar plug transfer was then repeated, and the resulting broth culture was used to seed 200 ml of TSB for subculture into 1,000 ml of broth.

**Antiserum production.** Antiserum against each isolate of *T. hyodysenteriae* was produced in white New Zealand rabbits. Bacteria (approximately 10⁶ cells per ml) were mixed with Freund complete adjuvant 1:1 (vol/vol) at the time of injection. Each rabbit received 0.5 ml subcutaneously and 1.0 ml intramuscularly on days 1 and 7. On day 14, each received an intradermal injection of 1.5 ml of bacteria in Freund incomplete adjuvant, 1:1 (vol/vol). On days 21 and 28 each received intravenous boosters of 0.5 and 1.0 ml, respectively.

Preinjection blood samples were taken from each rabbit on days 1, 14, 21, and 28. Terminal blood samples were obtained on day 35. Sera were sterilized by filtration through a membrane (0.22-μm average pore diameter) and stored at −20°C.

**Extraction of bacteria.** The hot phenol-water method (31) was adapted for the extraction of lyophilized beta-hemolytic *T. hyodysenteriae*. Hot (68°C) distilled water (10 ml) was added to 200 mg of lyophilized cells and blended with a Vortex mixer. Vortex mixing was followed by adding 10 ml of hot (68°C) 88% phenol, remixing, and heating at 68°C for 12 to 15 min. The phenol and water phases were separated by cooling the slurry to 10°C in an ice bath. Cooled extracts were centrifuged at 2,200 × g for 20 min in a swinging-bucket rotor, after which the water phase was carefully drawn from the phenol phase with a 10-ml pipette. Ten milliliters of hot distilled water was added to the phenol phase, and the extraction was repeated. The two water phases were combined and dialyzed against 50 volumes of distilled water for 24 h with six changes of water. Dialyzed water phases were concentrated under a vacuum (385 mm of Hg) to one-fifth their original volume. Carbohydrate complexes were precipitated (27) with the addition of 6 volumes of 90% ethanol plus sodium acetate and incubation at −20°C overnight. Precipitates were sedimented by centrifugation (16,000 × g), resuspended in 1.0 ml of distilled water, reprecipitated with 5 volumes of acetone, and sedimented by centrifugation. Acetone precipitation was repeated twice. Final precipitates were suspended in 1.0 ml of distilled water and diluted in distilled water to a concentration of 300 μg of hexose per ml.

**Extract analysis.** Protein was estimated by the method of Lowry et al. (19) with bovine serum albumin as the standard; carbohydrate was estimated by the method of Dubois et al. (3) with α-glucose as the hexose standard.

**Immunodiffusion.** Sera and antigens were tested (13) by double immunodiffusion in 1% agarose melted in Karrer's Veronal-buffered saline solution. Melted agarose (3 ml) was applied to a clean 25- by 75-mm frosted-edge slide. Holes were punched in the gel and filled with antisera or extract. Reactions were read after 18 h of incubation at room temperature in a humid chamber. Normal rabbit serum served as a control for each immunodiffusion test.

**RESULTS**

**Reactivity of water phases.** Thirteen isolates of *T. hyodysenteriae* were extracted with hot phenol-water. The concentrated water phase from the first extraction of isolate T5 was tested with antisera to whole cells of beta-hemolytic *T. hyodysenteriae* (Fig. 1). The observed bands of precipitate were later shown to be identical (Fig. 2). Repeated extractions of cells from various lyophilized cultures of T5 yielded an antigen which produced the same types of precipitin reactions in gel.

After demonstration that the method of extraction could be repeated, water phases from extracts of the rest of the beta-hemolytic isolates were examined. Each water phase produced precipitin reactions in gel against homologous antiserum. Each water phase was cross-tested against each antiserum in agarose gel to determine what, if any, antigenic relationship the isolants had with each other. Final results of these tests are demonstrated in Fig. 3 and summarized in Table 1. Four distinct groups were noted among the isolates: B178, B234, Dys 7, T6, and T7 comprising one group; B140, B204, T3, T5, and 9605 comprising another.
group; B169 alone; A-1 alone. Furthermore, none of the water phases reacted with the antisera produced in this study against weak beta-hemolytic isolates.

**FIG. 2.** Precipitin test in agarose gel demonstrating the identity of the reactions seen in Fig. 1. Reactants: 1 = T5, water phase; 2 = T5, antiserum; and 3 = B140 antiserum.

**Analysis of extracts.** The water-phase precipitates contained 85 to 90% carbohydrate (based on dry weight) as estimated by the method of Dubois et al. (3) with D-glucose as the standard. Protein accounted for 5 to 10% of the precipitate (based on dry weight) estimated by the Lowry method (19).

**DISCUSSION**

The results presented in this report indicate that an antigen in the water phase from the phenol-water extraction of each beta-hemolytic T. hyodysenteriae isolate reacts with homologous antisera in gel diffusion precipitin tests. Results from immunodiffusion tests showed that certain isolants have the same variants of this extractable antigen in common. Weak but identical precipitin bands were observed among the B78-B234-Dys 7-T6-T7 group and were probably a result of less than optimal antigen concentration. However, the identity of these reactions provides the basis for inclusion of the isolates in the same group. With that, we propose that the reactions summarized in Table 1 be used as the

**FIG. 3.** Precipitin reactions among all available reactants. Peripheral wells: 1 = anti-B78; 2 = anti-B234; 3 = anti-Dys 7; 4 = anti-T6; 5 = anti-T7; 6 = anti-B140; 7 = anti-B204; 8 = anti-T4; 9 = anti-T5; 10 = anti-T5; 11 = normal rabbit serum; 12 = anti-9605; 13 = anti-B169; 14 = anti-A-1; 15 = normal rabbit serum. Center wells filled with standardized antigen: A = B78; B = B234; C = Dys 7; D = T6; E = T7; F = B140; G = B204; H = T5; I = T5; J = T5; K = 9605; L = B169; M = A-1.
Table 1. Results of double-diffusion precipitin reactions among water-phase antigens and antisera of T. hyodysenteriae isolates

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<th>B234</th>
<th>Dys 7</th>
<th>T3</th>
<th>T5</th>
<th>T7</th>
<th>B140</th>
<th>B204</th>
<th>T3</th>
<th>T5</th>
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Reactions were run at 25°C and at antigen concentration of 300 μg of hexose per ml. Blank areas = no band of precipitin; + = band of precipitin reacting identically with homologous systems.

Table 2. Proposed serotype designations of beta-hemolytic T. hyodysenteriae and included isolates

<table>
<thead>
<tr>
<th>Serotype</th>
<th>Isolates</th>
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<tr>
<td>1</td>
<td>B78, B234, Dys 7, T3, T7</td>
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<tr>
<td>2</td>
<td>B140, B204, T3, T4, T5, 9605</td>
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<tr>
<td>3</td>
<td>B169</td>
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<tr>
<td>4</td>
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LITERATURE CITED


