Treponema hyodysenteriae was incubated in 20% normal swine sera (NSS) at 37°C for 4 h, and viability was determined by a plate dilution method. NSS was bactericidal for nonpathogenic T. innocens and avirulent T. hyodysenteriae, but not for virulent T. hyodysenteriae isolates. Heat inactivation at 56°C for 30 min, treatment with EDTA or EGTA (ethylene glycol-bis[β-aminoethyl ether]-N,N'-tetraacetic acid), or removal of immunoglobulin M eliminated the bactericidal activity of NSS. However, removal of the alternate complement pathway with 10 mg of bentonite per ml did not remove bactericidal activity of NSS. Incubation of virulent isolates of T. hyodysenteriae in the presence of specific antisera plus NSS resulted in bactericidal activity. These data suggest that complement and natural antibody may be involved in protecting the host from T. innocens or avirulent T. hyodysenteriae and that T. hyodysenteriae antibody plus complement are involved in protecting convalescent pigs from re-exposure to swine dysentery.

Treponema hyodysenteriae is a gram-negative, anaerobic spirochete found in the large intestine of pigs (7–9; R. D. Glock, Ph.D. thesis, Iowa State University, Ames, 1971). The organism is virulent in pigs producing lesions of catarrhal inflammation, edema, hyperemia, and hemorrhage (6; Glock, Ph.D. thesis). Continuous passage of the organism in laboratory media renders a majority of the isolates avirulent.

T. innocens is a gram-negative, anaerobic spirochete found in fecal material from animals with no apparent disease. The spirochete is similar to T. hyodysenteriae in morphology and growth characteristics and shares many of the same antigens, but is nonpathogenic in swine (11–13, 18). The reason for the differences between the two organisms is unknown. Variations in pathogenicity have been demonstrated in other spirochetes (14, 15). Investigations have shown that avirulent Leptospira biflexa is susceptible to a heat-labile component found in normal human or rabbit serum, whereas virulent L. interrogans is resistant (15). These data also demonstrated that, if immune sera were used in the assay instead of normal sera, virulent Leptospira organisms were killed (14).

Studies on various gram-negative bacteria have demonstrated the presence of both serum-resistant and serum-sensitive strains. The smooth strains which have a complete lipopolysaccharide (LPS) structure are usually serum resistant and virulent, whereas the strains which are rough or lack a complete LPS structure are serum sensitive and usually less virulent. It appears that the lytic portion of the complement cascade (C5 to C9) is able to assemble on the membrane of the serum-resistant organism but is shed due to an unstable hyphophobic bond and therefore fails to sustain lethal membrane damage (16).

The need for the classical pathway versus that of the alternative pathway for killing has also varied between strains of gram-negative bacteria. Investigators have shown that both pathways are able to kill the organisms in the presence of immunoglobulin, either combined or as separate entities. In addition, the alternative pathway has been shown to kill gram-negative organisms in the absence of immunoglobulin (4, 20).

Serum components are released into the lumen of the pig during the acute stage of swine dysentery due to superficial necrosis of the mucosal surface (8; Glock, Ph.D. thesis). Following recovery from the disease, gross lesions are no longer seen in the intestine. However, microscopic lesions can be detected in the intestinal lamina propria of pigs throughout the convalescent period, indicating that small amounts of serum proteins may be leaking into the lumen of the pig long after recovery.

This study was conducted to examine the effect of normal swine sera (NSS) and sera from recovered pigs on the growth of T. hyodysenteriae and T. innocens in vitro and to determine if spirochetes from the intestinal tract of swine can activate either the classical or the alternative pathway of complement.

MATERIALS AND METHODS

Isolates. T. hyodysenteriae isolates B234, B204, and ACK 300/8 and T. innocens isolates B359 and B3143 were initially obtained from J. M. Kinyon, Iowa State University, Ames, and stored frozen at −70°C. T. innocens isolate D95 was obtained from the laboratory of A. Sanna, Universita’ Cattolica Del Sacro Cuore, Rome, Italy, and stored frozen at −70°C. T. hyodysenteriae isolates B234 and B204 were virulent in pigs and mice, whereas ACK 300/8 was avirulent due to continuous laboratory passage. The T. innocens isolates were nonpathogenic in pigs and mice.

Culture. Both species of spirochetes were cultured in Trypticase soy broth (TSB) (BBL Microbiology Systems) supplemented with 5% heat-inactivated fetal bovine serum (GIBCO Laboratories) as previously described (L. A. Joens, Ph.D. thesis, Iowa State University, Ames, 1977). Cultures used in the assay were in the log phase of growth (approximately 5.0 × 10⁹ viable cells per ml).

Sera. Pooled NSS were used as the source of complement. Blood was obtained from 10- to 12-week-old pigs (n = 5) the same day of the assay. The sera were harvested from the blood by centrifugation and filter sterilized. A portion of the NSS was heat inactivated for 30 min at 56°C, and both the
heat-inactivated and the complement-active NSS were stored on ice (4°C). Hyperimmune sera were produced in rabbits against *T. hyodysenteriae* isolates B234 and B204, using formalized cells as antigen (1). Immune sera were obtained from pigs which had recovered 30 to 44 days from infection with either isolate B234 (*n* = 2) or B204 (*n* = 4) *T. hyodysenteriae*. Hyperimmune and immune sera were harvested, heat inactivated for 30 min at 56°C, filter sterilized, and stored at -20°C. Hyperimmune rabbit sera and sera from convalescent pigs had titers of >1:128 when reacted against *T. hyodysenteriae* LPS in the enzyme-linked immunosorbent assay.

**Bactericidal assay.** The assay for antitreponemal activity of NSS was conducted in duplicate with three repetitions according to the method of Nuessen (Ph.D. thesis, Iowa State University, Ames, 1982), with the following modifications. A 1.0-ml portion of treponemal culture (−10^7 viable cells) was incubated in 20% NSS in TSB or 20% NSS in TSB containing 1.0 ml antisera to a final volume of 5.0 ml at 37°C for 4 h. Growth was measured at various times by plating 10-fold serial dilutions of the samples on blood agar plates and counting the presence of individual PFU at the highest dilution after incubation of the plates at 42°C for 4 days.

**Hemolysis assay.** A modification of the procedure by Platts-Mills and Ishizaka (23) was used to determine the hemolytic activity of NSS. Treated and untreated NSS were serially diluted in phosphate-buffered saline (pH 7.2; 0.01 M) buffer in microtiter wells. A 50-μl volume of a 1.0% suspension of either washed sensitized sheep erythrocytes or washed rabbit erythrocytes was added to the serially diluted NSS. The suspensions were mixed and incubated at 37°C for 2 h. After the incubation period, the plate was shaken and the hemolysis of the erythrocytes was read.

**Modifications of NSS.** Fresh NSS was depleted of peridin with 10.0 mg of washed bentonite per ml according to the methods of Inai et al. (10) and Steele et al. (26). Essentially, 100 mg of bentonite was washed three times with 20 ml of sterile phosphate-buffered saline (pH 7.2; 0.01 M) and resuspended into 10.0 ml of NSS. The NSS-bentonite preparation were incubated for 10 min at 37°C. The bentonite was removed from the NSS by centrifugation, and the sera were filter sterilized. To restore antibody removed from NSS by bentonite absorption, 50 μg of swine anti-*T. hyodysenteriae* immunoglobulin G (IgG) (3) or heat-inactivated NSS (1:1 ratio, vol/vol) was added to the treated sera. The bentonite-treated sera had no detectable alternative pathway activity at a 1:2 dilution as measured by hemolysis of rabbit erythrocytes. In contrast, the treated sera had hemolytic activity at this dilution when sensitized sheep erythrocytes were used to measure classical pathway-mediated hemolysis.

The classical pathway of complement was inactivated by adding 4 mM Mg^{2+} and 40 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-N,N-tetraacetic acid (pH 7.4); Sigma Chemical Co.] to 1.0 ml of NSS. This reagent was then assayed for bactericidal activity at a 20% concentration. Alternate pathway hemolytic activity was detected as assayed by rabbit erythrocyte hemolysis, but classical pathway hemolytic activity as measured by hemolysis of sensitized sheep erythrocytes was not detected.

A serum reagent which lacked both complement pathways was produced by using two different methods. In the first method, NSS were depleted of both pathways by heat inactivation at 56°C for 30 min. These sera did not support the lysis of rabbit erythrocytes or sensitized sheep erythrocytes at a 1:2 dilution. In the second method, complement was removed from NSS by chelating Mg^{2+} and Ca^{2+} ions with 40 mM of EDTA per ml. This reagent did not support the lysis of rabbit erythrocytes or sensitized sheep erythrocytes at a 1:2 dilution.

NSS were depleted of IgM by using affinity chromatography. Anti-swine IgM (Miles Laboratories, Inc.) was bound to activated Sepharose 4B beads and reacted to NSS for 45 min at 4°C. The NSS were removed from the beads by aspiration, filter sterilized, and assayed. The absorbed sera were depleted of detectable IgM as noted by Ouchterlony testing against anti-swine IgM, but retained complement activity.

Doubling dilutions were performed on NSS, using TSB as the diluent (15). The bactericidal effects of the sera were assayed against *T. hyodysenteriae* and *T. innocens* isolates.

**Statistical analysis.** All data were subject to a transformation of log_{10} units (X + 1) and analyzed by using least significant difference (*P* = 0.05) (25).

### RESULTS

NSS were bactericidal for *T. hyodysenteriae* isolate ACK 300/8 and *T. innocens* isolates D95, B3143, and B359 with a decrease in numbers of 3 to 7 logs (significant at *P* = 0.05) (Table 1). NSS had no significant effect on the growth of virulent *T. hyodysenteriae* isolates B234 and B204. Heat-inactivated NSS and fetal bovine serum had no bactericidal effect on avirulent *T. hyodysenteriae* and nonpathogenic *T. innocens*.

NSS were serially diluted (1:5 to 1:80) to determine which concentrations would kill the treponemes. Isolate ACK 300/8 *T. hyodysenteriae* was killed at a 1:10 dilution and B3143 *T. innocens* was killed at a 1:5 dilution (significant at *P* = 0.05). At higher dilutions, the serum was ineffective in killing these isolates.

To determine if antibody was fixing complement, IgM was removed from NSS by affinity chromatography. This treatment eliminated the bactericidal activity of NSS when reacted against either avirulent *T. hyodysenteriae* or *T. innocens* (significant at *P* = 0.05) (Table 2). Adding heat-inactivated sera to the affinity chromatography-treated sera restored some of the bactericidal activity of the serum (*P* = 0.05). This suggests that swine IgM is fixing complement and that both IgM and complement are needed to kill (avirulent) *T. hyodysenteriae* and *T. innocens*. However, further experimentation with purified IgM is needed to confirm the role of this immunoglobulin in the bactericidal process.

#### TABLE 1. Antitreponemal activity of NSS

<table>
<thead>
<tr>
<th>Test organism</th>
<th>Fetal bovine serum</th>
<th>ΔNSS</th>
<th>NSS</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>T. hyodysenteriae</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B234</td>
<td>1.4 × 10^7</td>
<td>9.0 × 10^6</td>
<td>6.0 × 10^6</td>
</tr>
<tr>
<td>B204</td>
<td>1.1 × 10^8</td>
<td>1.3 × 10^8</td>
<td>1.4 × 10^8</td>
</tr>
<tr>
<td>ACK 300/8</td>
<td>2.5 × 10^7</td>
<td>1.1 × 10^7</td>
<td>0^6</td>
</tr>
<tr>
<td><em>T. innocens</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B359</td>
<td>3.4 × 10^7</td>
<td>5.5 × 10^7</td>
<td>3.0 × 10^8</td>
</tr>
<tr>
<td>B3143</td>
<td>6.0 × 10^7</td>
<td>5.6 × 10^7</td>
<td>5.0 × 10^8</td>
</tr>
<tr>
<td>D95</td>
<td>5.0 × 10^7</td>
<td>2.0 × 10^7</td>
<td>0^6</td>
</tr>
</tbody>
</table>

* Growth of *T. hyodysenteriae* and *T. innocens* isolates after exposure to normal pig serum for 4 h at 37°C.

* Significant at *P* = 0.05 when compared with ΔNSS.
NSS were exposed to various treatments to test whether the classical or alternate pathway was involved in the assay. Heat inactivation or treatment of normal sera with EDTA eliminates both the classical and the alternate pathways of complement. When NSS were heated to 56°C for 30 min or treated with 40 mM EDTA, the bactericidal activity of the sera on avirulent *T. hyodysenteriae* or *T. innocens* was abolished (significant at \( P = 0.05 \)) (Fig. 1). Treatment of NSS with 40 mM Mg

\[ \text{ Growth of } T. \text{ hyodysenteriae and } T. \text{ innocens isolates after exposure to treated serum for 4 h at 37°C. } \]

\( \Delta \text{NSS} \) NSS Absorbed* \( \Delta \text{NSS} \)

<table>
<thead>
<tr>
<th>Test organism</th>
<th>Growth</th>
<th>Absorbed*</th>
<th>Absorbed*</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>T. hyodysenteriae</em> isolate ACK 300/8</td>
<td>4.0 × 10^7</td>
<td>3.0 × 10^7</td>
<td>6.6 × 10^7</td>
</tr>
<tr>
<td><em>T. innocens</em> isolate B3143</td>
<td>2.0 × 10^7</td>
<td>2.0 × 10^4</td>
<td>1.3 × 10^7</td>
</tr>
</tbody>
</table>

* Growth of *T. hyodysenteriae* and *T. innocens* isolates after exposure to treated serum for 4 h at 37°C.

* Significant at \( P = 0.05 \) when compared with NSS.

NSS Antisera NSS antisera

Antisera from recovered pigs and hyperimmune rabbits were tested for bactericidal activity to virulent *T. hyodysenteriae*. Heat-inactivated antisera plus NSS (complement source) were bactericidal for both isolates (significant at \( P = 0.05 \)) (Table 3). Antisera without NSS had no effect on the growth of the isolates. Serotype differences between isolates had no effect on the bactericidal activity of the antibody present in the antisera.

### DISCUSSION

These studies have demonstrated that NSS contain factors which can kill avirulent *T. hyodysenteriae* and nonpathogenic *T. innocens*. In contrast, virulent isolates of *T. hyodysenteriae* were resistant to the bactericidal effects of NSS. These findings were consistent with those of Johnson and Muschel (15), who found that avirulent leptospires were sensitive to normal sera, whereas the virulent serotypes were resistant.

### TABLE 3. Antitreponemal activity of *T. hyodysenteriae* antibody in the presence of complement

<table>
<thead>
<tr>
<th>Test organism</th>
<th>Recovered swine</th>
<th>Rabbit</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NSS Antisera</td>
<td>NSS antisera</td>
</tr>
<tr>
<td><em>T. hyodysenteriae</em> isolate B234</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.1 × 10^7</td>
<td>6.0 × 10^7</td>
</tr>
<tr>
<td><em>T. hyodysenteriae</em> isolate B204</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.1 × 10^7</td>
<td>1.4 × 10^6</td>
</tr>
</tbody>
</table>

* Growth of *T. hyodysenteriae* isolates after exposure to treated serum for 4 h at 37°C.

* The convalescent and hyperimmunized sera were heat inactivated for 30 min at 56°C prior to performance of the assay; complement was provided in the NSS.

* Significant at \( P = 0.05 \) when compared with NSS.

Previous work has demonstrated that the heat-labile component in serum responsible for killing gram-negative bacteria is complement (21, 24, 28, 29). The complement system is composed of the classical and alternate pathways and both of these enzyme systems can be abolished by heating to 56°C for 30 min. The killing of avirulent *T. hyodysenteriae* and *T. innocens* was dependent on a heat-labile factor(s) since these treponemes were capable of growing in the presence of heat-inactivated NSS.

Investigators have shown that the alternate pathway can be activated by the presence of LPS in the absence of immunoglobulin. However, when immunoglobulin is involved in fixing complement on gram-negative bacterial membranes, it is usually of the IgM class, especially when normal sera are used. This is probably due to the antigenic structure of the O antigen in LPS; carbohydrates will generally stimulate a T-independent response in the affected host, resulting in IgM antibody. To determine if immunoglobulin was involved in the killing of the treponemes, detectable amounts of IgM were selectively removed from NSS by using affinity chromatography. Treatment of NSS with this method eliminated the bactericidal activity of the sera. By adding heat-inactivated NSS, which contained IgM, to the affinity-treated sera, bactericidal activity was restored. This suggested that antibody and not LPS from the spirochete was fixing complement and that this reaction was responsible for the lytic activity of the sera. However, the role of IgM in fixing complement on the treponemal surface is still unclear. Adding back purified porcine IgM to the absorbed
NSS may clarify the role of this immunoglobulin in future experiments.

Wright and Levine (29) showed that complement damages the outer and inner membranes of Escherichia coli simultaneously and that this damage is lethal. Further examination of this serum component showed that both pathways of complement are activated in the presence of various strains of Serratia marcescens and E. coli, except that either the alternate or the classical pathway proved more crucial for actual killing of the representative strains (28). In our studies, Ca\(^{2+}\) and Mg\(^{2+}\) ion depletion of the serum by EDTA, which abolishes both pathways of complement, rendered the serum ineffective against both avirulent T. hyodysenteriae and T. innocens isolates. In addition, depletion of only Ca\(^{2+}\) ions with 40 mM EGTA and addition of 4 mM Mg\(^{2+}\) to NSS, which abolishes only the classical pathway, eliminated the killing activity of NSS against avirulent T. hyodysenteriae and T. innocens. However, depletion of properdin from NSS with bentonite, and subsequently the alternate pathway, did not affect the bactericidal activity of NSS. This suggests that the classical pathway of complement is the major pathway involved in the killing of these nonpathogenic isolates.

All isolates of pathogenic T. hyodysenteriae T. hyodysenteriae tested were capable of growing in the presence of NSS. However, when specific antibody was added to the system, pathogenic T. hyodysenteriae were killed. The differences in susceptibility between pathogenic and avirulent T. hyodysenteriae to NSS may be due to a difference in the outer envelope of the bacteria. The LPS moiety of pathogenic T. hyodysenteriae may prevent the lytic effects of complement on the organism, as has been indicated with other gram-negative bacteria (21). If antibody and complement components are present in sufficient quantities in the convalescent pig colon, these serum proteins may be protecting the pig against swine dysentery.

ACKNOWLEDGMENTS

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