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Evaluation of Microagglutination Test for Differentiation between *Serpulina (Treponema) hyodysenteriae* and *S. innocens* and Serotyping of *S. hyodysenteriae*

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Swine dysentery is a mucohemorrhagic diarrheal disease caused by the anaerobic spirochete *Serpulina hyodysenteriae*. At present, the serotyping is done by immunodiffusion testing with lipopolysaccharide (LPS) extract as antigen and rabbit hyperimmune sera produced against different serotypes of *S. hyodysenteriae*. Since the preparation of LPS is time-consuming and requires a large quantity of bacteria, it is desirable to use a serotyping method which does not require the extraction of LPS. In the present investigation, microagglutination was evaluated by using both formalinized whole- and boiled-cell suspensions as antigens and rabbit hyperimmune sera produced against formalinized whole-cell suspensions of reference strains of *S. hyodysenteriae* and *S. innocens* B256. Use of boiled cell suspension as antigen permitted the differentiation between isolates of *S. hyodysenteriae* and *S. innocens* as well as serotyping of *S. hyodysenteriae* strains accurately. A total of 18 isolates were identified as *S. hyodysenteriae*, and 3 isolates were identified as *S. innocens*. The microagglutination test was found specific, sensitive, and easy to perform; thus, it was judged suitable for routine identification and serotyping of *S. hyodysenteriae* isolates.

Serpulina (Treponema) hyodysenteriae is recognized as the causative agent of swine dysentery (4). The disease may affect swine of all ages and often produces a severe mucohemorrhagic diarrhea (6). Serodiagnosis of swine dysentery has long been plagued with problems. There are significant cross-reactions between *S. hyodysenteriae* and *S. innocens*, which is recognized as a nonpathogenic spirochete (9, 10). Serological diagnosis and vaccination programs have been used in an attempt to control the disease (8, 11). Both these programs require an extensive knowledge of the different serotypes existing in a particular region, as this organism is known to be antigenically heterogenous with different serotypes showing different geographical distributions (5, 14, 15). Several tests have been used to differentiate *S. hyodysenteriae* from *S. innocens* and to serotype *S. hyodysenteriae* isolates (1, 5, 14).

Isolates of *S. hyodysenteriae* have been grouped into nine different serotypes by an immunodiffusion (ID) technique with lipopolysaccharide (LPS) extract as antigen and hyperimmune sera prepared in rabbits (3, 15, 17). Currently the major drawback of serological typing of *S. hyodysenteriae* is the necessity to grow the isolates in large quantities and to extract LPS for use in ID testing. Since the preparation of LPS takes at least 2 to 3 days, it would be desirable to use a serotyping method which does not require the extraction of LPS. For this reason, Lemcke and Bew (14) examined the possibility of using a slide agglutination test with absorbed sera and whole-cell antigens as a rapid and simple means of serotyping isolates of *S. hyodysenteriae*, but they concluded that this was not practical because of the antigenic diversity. With a view to improve the method used by Lemcke and Bew (14), Hampson (5) proposed the method of serogrouping instead of serotyping of *S. hyodysenteriae* isolates by slide agglutination testing using a whole-bacterial-cell suspension as the antigen. The agglutination reaction was made serogroup specific by cross-absorbing the typing sera with whole-cell suspensions of all the other remaining serogroups. However, there was only 89.6% correlation between the results obtained by ID and slide agglutination tests, which limits the use of slide agglutination testing for accurate serotyping. The present study was undertaken to develop a faster differentiation assay which improves upon ID, the current method of choice for serotyping *S. hyodysenteriae*.

MATERIALS AND METHODS

Bacterial strains. *S. hyodysenteriae* reference strains representing serotypes 1 (B234), 2 (B204), 3 (B169), 4 (A-1), 5 (B8044), 6 (B6933), 7 (AcK 300/8), 8 (FM 88-90), and 9 (FMV 89-3323) and *S. innocens* reference strain B256 were used. Among 24 field isolates, 18 strains identified as *S. hyodysenteriae* and 6 strains identified as *S. innocens* by biochemical tests and hemolytic pattern were used (1, 12, 18).

Culture method. Frozen cells of reference and field strains were thawed and cultured on blood agar media containing 5% bovine blood (Oxoid Ltd, Hampshire, England). Plates were incubated anaerobically at 42°C for 72 h in a jar (Oxoid) using a GasPak plus generator envelope for production of an anaerobic atmosphere (BBL Microbiological Systems, Cockeysville, Md.) (1).

Antigen preparation. The organisms were harvested from plates and washed twice in phosphate-buffered saline (PBS, 0.01 M, pH 7) by centrifugation at 800 × *g* for 10 min. The pellets were resuspended in PBS and treated with 0.5% formalin for 2 h. The bacterial suspension was adjusted to an optical density of 0.5 at 540 nm and divided into two aliquots. One portion was kept at room temperature (22°C); the second was heated at 100°C for 1 h in a water bath. The two preparations, referred to as whole-cell suspension and boiled-cell suspension, respectively, were used as antigens in a microagglutination test (MAT) for serotyping.

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TABLE 1. Identification of field strains of *S. hyodysenteriae* and *S. innocens* by MAT using boiled-cell suspensions and absorbed rabbit hyperimmune sera

Isolate group (no.)	Antibody titer[a] in:	
	Pool of antiserum against *S. hyodysenteriae*[b]	Antiserum against *S. innocens*[c]
S. hyodysenteriae isolates (18)	≥100	0
S. innocens isolates (3)	0	≥800
Untypeable *S. innocens* isolates (3)	0	0

[a] Titers of <100 (negative) are shown as zero. Titers of ≥100 are considered positive.
[b] Pool of antiserum against *S. hyodysenteriae* serotypes 1 to 9 absorbed with boiled cells of *S. innocens*.
[c] Antiserum against *S. innocens* absorbed with pool of boiled cells of serotypes 1 to 9 of *S. hyodysenteriae*.

Preparation of antisera in rabbits. Antigens for immunization were prepared from 72-h-old cultures of various reference strains. The bacteria were harvested in PBS containing 0.3% formalin and kept at room temperature for 24 h. A cell suspension adjusted to an optical density of 1 at 540 nm was used for immunization of two rabbits for each strain. Briefly, equal volumes of formalin-killed bacterial cell suspension of the reference strain and Freund's incomplete adjuvant (Difco) were emulsified and injected in rabbits using an inoculation schedule as described by Mapother and Joens (17). Rabbits were bled, and sera were collected and stored at −20°C until used.

Absorption of antisera. Hyperimmune sera against all of the nine reference strains of *S. hyodysenteriae* with the same initial antibody titers were pooled in equal volumes and heat inactivated at 56°C for 30 min. The pool of antisera was absorbed twice with a 10% packed volume of boiled cells of *S. innocens* B256. Similarly the antiserum against *S. innocens* B256 was absorbed with 10% packed volume of boiled antigens of each of 9 reference strains of *S. hyodysenteriae* pooled together in equal volumes. The absorption was carried out by incubating the cell-serum mixture at 37°C for 1 h. The absorbing bacteria were removed from sera by centrifugation at 800 × *g* for 10 min. The pooled serum rendered specific for *S. hyodysenteriae* after absorption with *S. innocens* B256 was verified for the presence of antibodies against each of the reference strains of *S. hyodysenteriae* as well as for the absence of antibodies against *S. innocens* by MAT before use. Similarly, serum rendered specific for *S. innocens* B256 after absorption with *S. hyodysenteriae* antigens was also verified.

MAT. The agglutination test was performed in U-bottom microdilution plates (Flow Laboratories). PBS was delivered in 0.05-ml volumes in all the wells. Rabbit hyperimmune sera (diluted 1:100) in PBS were added to the first well in each row successively. The sera were diluted twofold. The last column was left without serum as control. The antigen was added to each well in 0.05-ml volumes. The plates were covered and incubated overnight at 37°C. Antibody titer was expressed as the reciprocal of the highest dilution of serum showing a definite positive pattern (flat sediment) as compared with the pattern of the negative control (smooth dot) in the center of the well.

ID test. The test was carried out essentially as described by Baum and Joens (3). The gel diffusion medium consisted of 1% agar (special Noble agar; Difco) buffered with PBS in petri dishes (60 by 15 mm). The gel (6 ml) was dispensed in the plate to a depth of 2.8 mm. Six wells, 5 mm in diameter and 2 mm deep, were punched in the agar at a distance of 6 mm from each other and from a central well. Westphal hot phenol-water method, adapted by Baum and Joens (3), was used to extract LPS. The wells were filled with antiserum and LPS antigens. The plates were incubated at room temperature in a water-saturated atmosphere and examined daily for 3 days for any precipitation reaction.

RESULTS

Absorption test and differentiation between *S. hyodysenteriae* and *S. innocens*. Use of hyperimmune sera rendered specific after absorption enabled us to identify strains of *S. hyodysenteriae* and *S. innocens* correctly. However, some field strains of *S. innocens* remained unidentified (Table 1).

Serological activity of reference strains of *S. hyodysenteriae* and *S. innocens*. Marked cross-reactions were observed between different serotypes of *S. hyodysenteriae* as well as be-

TABLE 2. Serological activity of reference strains of *S. hyodysenteriae* and *S. innocens* by MAT using whole-cell suspensions and rabbit hyperimmune sera

Serpulina spp. (reference strains)	Antibody titer[a] against:									
	Serotypes of *S. hyodysenteriae*									*S. innocens*
	1	2	3	4	5	6	7	8	9	
S. hyodysenteriae serotype:										
1	1,600	800	400	0	400	800	400	0	200	0
2	1,600	1,600	0	0	1,600	200	800	400	1,600	200
3	1,600	1,600	1,600	400	800	0	0	1,600	0	200
4	AA[b]	AA	AA	AA	AA	AA	AA	AA	AA	AA
5	800	400	800	200	400	800	400	1,600	400	200
6	1,600	0	0	0	0	200	0	0	0	0
7	1,600	1,600	1,600	200	0	400	1,600	200	0	1,600
8	0	800	1,600	1,600	800	400	1,600	1,600	400	0
9	1,600	1,600	1,600	200	1,600	1,600	1,600	1,600	1,600	1,600
S. innocens B256	400	200	200	0	0	400	0	800	0	1,600

[a] Titers in rabbit hyperimmune sera (unabsorbed rabbit antisera). Titers of <100 are shown as zero.
[b] AA, autoagglutination.

TABLE 3. Serological activity of reference strains of *S. hyodysenteriae* and *S. innocens* by MAT using boiled cell suspensions and rabbit hyperimmune sera

Serpulina spp. (reference strains)	Antibody titer[a] against:									
	Serotypes of *S. hyodysenteriae*									*S. innocens*
	1	2	3	4	5	6	7	8	9	
S. hyodysenteriae serotype[b]:										
1	800	0	0	0	0	0	0	0	0	0
2	0	800	0	0	0	0	0	0	0	0
3	0	0	800	0	0	0	0	0	0	0
4	0	0	0	1,600	0	0	0	0	0	0
5	0	0	0	0	800	0	0	0	0	0
6	0	0	0	0	0	800	0	0	0	0
7	0	0	0	0	0	0	1,600	0	0	0
8	0	0	0	0	0	0	0	1,600	0	0
9	0	0	0	0	0	0	0	0	800	0
S. innocens B256	0	0	0	0	0	0	0	0	0	1,600

[a] See Table 2, footnote *a*.
[b] Results of serotyping were confirmed by ID.

tween *S. hyodysenteriae* and *S. innocens* in MAT when formalinized whole-cell suspensions were used as antigens (Table 2). The cross-reactivities among different serotypes of reference strains of *S. hyodysenteriae* as well as between reference strains of *S. hyodysenteriae* and *S. innocens* were completely abolished when boiled-cell suspensions were used as antigens (Table 3). However, cross-reactions were encountered among 50% of the field strains tested (Table 4). The reference strain of serotype 4 showed an autoagglutinating reaction which disappeared completely upon boiling (Tables 2 and 3). None of the field strains tested autoagglutinated in PBS after boiling. Both formalinized whole-cell and boiled-cell suspensions gave the same results when used after storage at 4°C for 1 month (results not shown).

DISCUSSION

The results of the present investigation (Table 1) show that isolates of *S. hyodysenteriae* and *S. innocens* can easily be distinguished by using two serum specimens, each rendered specific by absorption with boiled-cell antigens of heterologous species. Some strains identified as *S. innocens* by hemolytic pattern and biochemical tests showed negative reactions to both the antisera, indicating that *S. innocens* strains are antigenically heterogenous, as reported by Agnes et al. (2). It is suggested that rabbit antisera prepared against field strains of *S. hyodysenteriae* and *S. innocens* which are antigenically different from reference strains should be included for detection of new serotypes of both species. Results shown in Tables 2, 3, and 4 clearly indicate that antigens involved in serotype specificity are heat stable. The ID test is the standard reference method currently used for serotyping *S. hyodysenteriae* isolates; however, the major drawback of this technique is the need for LPS extraction, which requires a large quantity of bacterial growth. Major cross-reactions between *S. hyodysenteriae* and *S. innocens* as well as among different serotypes of *S. hyodysenteriae* may be caused mainly by heat-labile antigens which may be associated with outer membrane proteins; however, heat-stable antigens associated with LPS may also share common epitopes among different serotypes of *S. hyodysenteriae* (15, 17). Mapother and Joens (17) reported that cross-reactions occurred in ID tests between serotypes 5 and 2, between serotypes 1 and 6, and between serotype 7 and both serotypes 1 and 2. In addition, antisera against serotypes 3, 4, and 7 showed weak cross-reactions with LPS extracted from all the reference strains of *S. hyodysenteriae* and *S. innocens* (15).

TABLE 4. Serological activity of field strains of *S. hyodysenteriae* and *S. innocens* by MAT using boiled cell suspensions and rabbit hyperimmune sera

Field strain (no.)	Antibody titers[a] against:									
	Serotypes of *S. hyodysenteriae*									*S. innocens*
	1	2	3	4	5	6	7	8	9	
S. hyodysenteriae serotype[b]:										
9 (8)	0	0	0	0	0	0	0	400–1,600	0	0
3 (8)	0	0	200–400	0	0	0	0	1,600	0	0
1 (8)	0	0	800	0	0	400	0	1,600	0	0
1 (8)	0	0	0	0	0	400	0	800	400	0
1 (9)	0	0	0	0	0	0	0	0	800	0
2 (9)	0	0	0	0	0	0	0	400	1,600	0
1 (3)	0	0	1,600	0	0	0	0	400	0	0
S. innocens (3)	0	0	0	0	0	0	0	0	0	400–1,600

[a] See Table 2, footnote *a*.
[b] See Table 3, footnote *b*.

Lyson and Lemcke (16) used a fluorescence antibody test with absorbed antiserum for identification of *S. hyodysenteriae*. Other workers also used cross-absorbed sera in a slide agglutination test (5, 14). Each procedure has merits, but none has been free from problems, mainly due to cross-reactivity. Hampson et al. (6, 7) demonstrated that serogroup antigens apparently consist of three or four major antigenic components, and an isolate possessing additional unique LPS can be regarded as a representative of a serotype or serovar within the serogroup by using cross-absorbed sera in a Western blot (immunoblot). Lau et al. (13) also proposed similarly the serotyping scheme for *S. hyodysenteriae* by Western blot. In view of the serogroup and serotype scheme proposed by Hampson et al. (6, 7) and Lau et al. (13), results shown in Table 4 suggest that strains among serotypes 3, 6, 8, or 9 sharing cross-reacting epitopes may possibly belong to the same serogroup(s). Since cross-reacting antigens are not completely destroyed by boiling the bacterial cells for some field isolates, it is necessary to cross-absorb the typing sera as suggested by earlier workers (5, 13, 14, 16).

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