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Microtitration Agglutination for Detection of *Treponema hyodysenteriae* Antibody

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A microtitration agglutination test for the detection of *Treponema hyodysenteriae* antibody in swine and rabbit sera is described. The following methods provided the best test results: antigen produced from the spirochete after a culturing period of 36 to 44 h at 38°C, washed antigen inactivated with 0.01% Merthiolate at 4°C for 24 to 36 h, sera heated at 56°C for 30 min, a diluent of phosphate-buffered saline (0.01 M, pH 7.2), and test results read macroscopically after 18 to 24 h of incubation at 38°C. The test enabled detection of antibody against pathogenic *T. hyodysenteriae* with a high level of consistency and sensitivity. Sera against nonpathogenic *T. hyodysenteriae* produced low agglutinating titers (≤1:8) when reacted against antigen from pathogenic isolates. Inactivated antigen remained stable for 7 to 10 days. Specificity of the reaction in the agglutination test was shown by absorption studies.

Serological methods used in swine dysentery (SD) research have been inadequate for the detection of antibody to *Treponema hyodysenteriae*.

The direct fluorescent-antibody test has been used to detect *T. hyodysenteriae* in fecal smears; however, because of a low degree of sensitivity, its effectiveness in this capacity has been limited (C. H. Lee and L. D. Olson, Annu. Meet. Conf. Res. Work. Anim. Dis., 54th, Chicago, Ill., Abstr. 68, 1973). The indirect fluorescent-antibody test also has been used in examining fecal specimens for *T. hyodysenteriae* antigens (1, 7, 8). Results of these tests demonstrated that spirochetes were absent from fecal samples of healthy pigs but present in the feces of pigs with clinical signs of SD (1, 8). Saunders and Hunter (7) confirmed the benefits of the indirect fluorescent-antibody test as a diagnostic test for SD and stated that the test may be used in the surveillance of pig herds for the disease.

An indirect fluorescent-antibody test also has been used in detecting *T. hyodysenteriae* antibody in swine sera (5, 6; Lee and Olson, Annu. Meet. Conf. Res. Work. Anim. Dis., 54th, Chicago, Ill., Abstr. 68, 1973; R. D. Glock, K. J. Schwartz, and D. L. Harris, Proc. Int. Pig Vet. Soc. Cong., 3rd, Abstr. L11, 1976). Although the test has a low degree of sensitivity, the results indicated a relationship between the severity and duration of diarrhea and the development of a serum titer to *T. hyodysenteriae*.

An agglutination test using World Health Organization agglutination trays has been developed for use in the diagnosis of SD (3). The test lacked the sensitivity needed for individual pig diagnosis but was recommended for detecting *T. hyodysenteriae* antibody on a herd basis.

The purpose of the following report is to describe a method for conducting agglutination tests with antigen prepared from *T. hyodysenteriae* in microtitration plates.

MATERIALS AND METHODS

Test cultures. *T. hyodysenteriae* isolates were obtained in pure culture from pigs affected with SD (pathogenic B204 and B234 isolates) or from a pig without dysentery but with signs of postweaning diarrhea (nonpathogenic B297 isolate). Cultures were frozen at −80°C for storage in 1-ml aliquots.

Culture methods. Frozen isolates B204, B234, and B297 (15 passages or less) were thawed and cultured in 7 ml of aerobically prepared Trypticase soy broth supplemented with 10% fetal calf serum (4). The organisms were subcultured (11% inoculum) in 200 ml of the above medium contained in a 500-ml round-bottom flask and grown under an atmosphere of 50:50 H_2-CO_2 for 36 to 44 h at 38°C with constant agitation by a reciprocating shaker (90 rpm).

Antigen preparation. The organisms were harvested by centrifugation (10,000 × *g* at 4°C), washed once in phosphate-buffered saline (PBS; 0.01 M, pH 7.2), and resuspended in 75 ml of PBS containing a 0.01% (vol/vol) concentration of Merthiolate. The antigen was stored at 4°C after inactivation for 24 to 36 h at 4°C.

Collection of antisera. Pigs were injected three times at 10-day intervals either intramuscularly or subcutaneously with a 4-ml suspension of 0.01% Merthiolate-inactivated *T. hyodysenteriae* (~10^9 cells per

ml) incorporated in aluminum hydroxide adjuvant at a 1:1 ratio. Serum samples were taken from these pigs 10 days after the final injection of antigen. Convalescent sera were obtained from pigs that had been infected with pathogenic *T. hyodysenteriae* and recovered from SD. Control pigs (unexposed) were used in obtaining unexposed swine sera. Rabbits were injected twice intradermally (at 14-day intervals) with a 2-ml suspension of 0.2% Formalin-inactivated *T. hyodysenteriae* ($\sim 10^9$ cells per ml) incorporated in Freund complete adjuvant at a 1:1 ratio and injected twice intravenously (at 7-day intervals) with a 1-ml suspension of 0.2% Formalin-inactivated *T. hyodysenteriae* ($\sim 10^9$ cells per ml). Serum samples were collected from the hyperimmunized rabbits 7 days after the final injection.

Sera from hyperimmunized (isolate B204), convalescent (isolate B204), and unexposed swine were pooled within each group to form a set of reference sera for evaluation of each lot of *T. hyodysenteriae* antigen. Antigen lots that deviated more than fourfold from the mean titer were not accepted for serum evaluation.

Test methods. Either a Coleman 7 nephelometer (Coleman Instruments, Maywood, Ill.) or a Spectronic 20 spectrophotometer (Bausch and Lomb, Inc., Rochester, N.Y.) was used in the adjustment of inactivated antigen for microtitration testing. The Coleman 7 nephelometer was adjusted to zero with a PBS-Merthiolate (0.01%) blank, and the scale was adjusted to 87 with a no. 28 nephelometer turbidity standard (2). A working dilution of antigens B204 and B234 was adjusted to 105 nephelometry units, and antigen B297 was adjusted to 50 nephelometry units with PBS-Merthiolate (0.01%).

With the Spectronic 20, antigen produced from pathogenic isolates was diluted to an absorbance of 0.50, and antigen produced from nonpathogenic isolates was diluted to an absorbance of 0.35 at 420 nm.

The agglutination test was performed in microtiter plates (Linbro, no. 1S-FB-96-TC, Flow Laboratories, Rockville, Md., and Cooke, no. 1220-24A, Dynatech Laboratories, Alexandria, Va.) with U-shaped wells. A PBS diluent (0.01 M, pH 7.2) was delivered in 0.05-ml amounts to all wells. Serum was added to the first well in each row, and the sera were diluted twofold with microdiluters (Cooke Laboratory Products, Alexandria, Va.) to 1:4,096. The antigen was added to each well in 0.05-ml amounts. The plates were covered with transparent tape, shaken, and incubated for 16 to 24 h at 38°C. Antibody titers to *T. hyodysenteriae* antigens were determined macroscopically by the appearance of a shield formation (presence of antibody) or a button formation (absence of antibody) (Fig. 1).

Absorbance of antisera. Swine and rabbit *T. hyodysenteriae* antisera (2-ml amounts) were absorbed with 3.0 g (wet weight) of washed *T. hyodysenteriae* isolate B204 cells for 3 h at both 4 and 38°C. Each antiserum was absorbed twice before its agglutinating titer was examined in the microtitration agglutination test (MAT) against isolate B204 antigen.

RESULTS

Preliminary experiments. Preliminary experiments on antigen inactivation revealed that 0.05% sodium azide or 0.2% Formalin inhibited antigen settling. Antigen inactivated with 0.01% Merthiolate for 24 to 36 h at 4°C, however, did

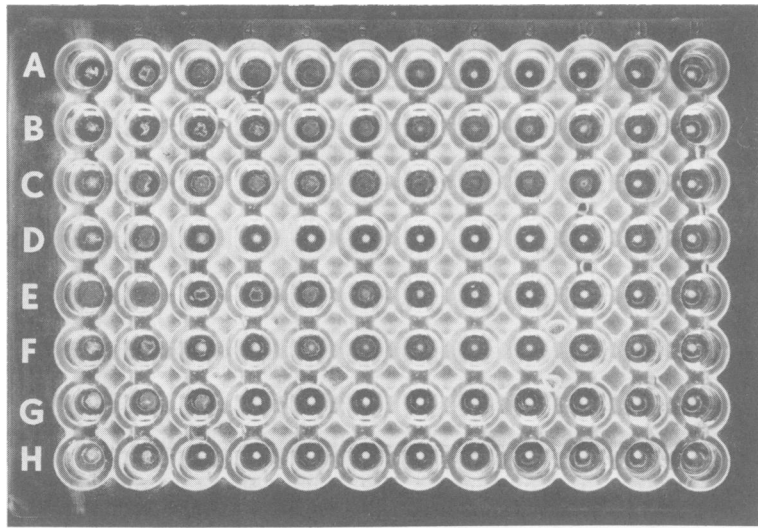

FIG. 1. *End points of the MAT were determined by the formation of shields (antibody present) or buttons (antibody absent). The sera in rows D, F, G, and H are from unexposed swine. The serum in row A is from a hyperimmunized pig (isolate B204; 1:8 dilution). The sera in rows B, C, and E are from convalescent swine. The antigen used in the test was produced from pathogenic isolate B234. Test results are as follows: row A (1:8 dilution), 1:512; row B, 1:512; row C, 1:1,024; row D, 1:8; row E, 1:64; row F, 1:32; row G, 1:8; row H, 1:4.*

not interfere with this process. Serum inactivated for 30 min at 56°C and a diluent consisting of PBS (0.01 M, pH 7.2) were optimal in the MAT for providing consistent results.

Comparison of antigen lots with reference sera. Different lots of B204 antigen were then used to evaluate the reproducibility and sensitivity of the MAT. Five lots of antigen prepared in a similar manner are compared in Table 1. A total of 24 of 29 antigen preparations were acceptable for use in the evaluation of swine and rabbit antibody to *T. hyodysenteriae* antigens. The Merthiolate-inactivated antigen remained stable for a period varying between 7 and 10 days, after which antigen settling was inconsistent.

Comparison of antigen from two pathogenic isolates. Two different lots of antigens from isolates B204 and B234 were compared in the MAT when reacted against standard swine sera (Table 2). Similar titers were demonstrated in the MAT with both B204 and B234 antigen when reacted against antisera from hyperimmunized and convalescent swine. Lower nonspecific titers were demonstrated with unexposed swine sera when reacted against B234 antigen.

Comparison of antigen from pathogenic and nonpathogenic isolates. Antigen from isolates B204 and B234 and from isolate B297 were reacted against both swine and rabbit *T. hyodysenteriae* antisera (Table 3). A minimal cross-reaction was noted when antisera to pathogenic and nonpathogenic isolates were reacted against B297 antigen and B204 and B234 antigens, respectively. Antisera to attenuated strain B78 had agglutinating activity when reacted against B204 and B234 antigen but not when reacted against B297 antigen.

Absorption of antisera. Swine and rabbit *T. hyodysenteriae* antisera were absorbed with isolate B204 cells. The agglutinating titers obtained against B204 antigen before and after absorption are shown in Table 4. Agglutinating activity of the *T. hyodysenteriae* antisera was removed after absorption with isolate B204.

Detection of agglutinating antibody in orally exposed pigs. Sera from pigs exposed to *T. hyodysenteriae* and from unexposed pigs were examined for agglutinating antibody to B234 antigen (Table 5). Clinical signs typical of SD were observed in all of the exposed pigs; however, one of the six pigs failed to develop a

TABLE 1. *Comparison of titers of reference swine sera between and within five antigen lots of isolate B204 in the MAT*[a]

Serum	Source	Titer[b] in antigen lot:				
		A	B	C	D	E
SD771	Hyperimmunized swine, isolate B140	256	128	128	512	128
		256	128	512	256	128
		256		512	256	256
SD365	Hyperimmunized swine, isolate B204	4,096	4,096	4,096	4,096	2,048
		2,048	2,048	4,096	1,024	2,048
		4,096		2,048	2,048	2,048
SD404	Hyperimmunized swine, isolate B204	2,048	2,048	2,048	2,048	1,024
		2,048	512	2,048	1,024	1,024
		2,048		512	2,048	2,048
SD408	Convalescent swine,[c] isolate B204	256	256	256	512	512
		256	256	512	256	256
		256		512	256	128
SD635	Convalescent swine, isolate B204	512	512	1,024	1,024	512
		512	1,024	1,024	512	512
		512		1,024	512	512
SD460	Unexposed swine	32	8	16	16	4
		32	16	16	4	8
		32		32	16	16
SD472	Unexposed swine	32	32	16	32	16
		32	8	16	32	16
		32		32	32	16
SD517	Unexposed swine	8	4	4	4	4
		8	4	8	4	4
		8		4	4	4

[a] Antigen used was inactivated with 0.01% Merthiolate at 4°C and reacted against inactivated swine sera.
[b] Titers expressed as the reciprocal of the serum dilutions.
[c] Pigs 20 to 45 days convalescent from SD.

detectable agglutinating serum titer. Serum agglutinating titers of the unexposed pigs remained at a low level throughout the study.

DISCUSSION

After preliminary experiments were completed, we attempted to critically evaluate the MAT for its usefulness as a routine serological procedure. A high level of consistency and sensitivity was exemplified by the results obtained with this test. The greatest discrepancy between

TABLE 2. *Comparison of antigen preparations from two pathogenic isolates in the MAT when reacted against swine sera*[a]

Serum	Source	Titer[b]	
		Antigen B204	Antigen B234
SD365	Hyperimmunized swine, isolate B204	2,048 2,048	1,024 2,048
SD404	Hyperimmunized swine, isolate B204	4,096 4,096	2,048 1,024
SD635	Convalescent swine,[c] isolate B204	2,048 1,024	512 512
S185	Convalescent swine, isolate B204	1,024 1,024	1,024 1,024
S186	Unexposed swine	64 256	4 8
S18	Unexposed swine	8 16	8 8
SD460	Unexposed swine	8 8	8 8
SD472	Unexposed swine	8 8	4 4

[a] Antigen used was inactivated with 0.01% Merthiolate at 4°C.

[b] Titers expressed as the reciprocal of the serum dilutions. Paired numbers represent results of two lots of antigen.

[c] Pigs 20 to 45 days convalescent from SD.

TABLE 4. *Comparison of serum agglutination titers: B204 antigen used before and after absorption (2×) of swine and rabbit sera with T. hyodysenteriae isolate B204*[a]

Antiserum	Source	Titer[b]	
		Unabsorbed antiserum	Absorbed antiserum
SD404	Hyperimmunized swine, isolate B204	2,048	8
R42	Hyperimmunized rabbit, isolate B204	4,096	32
SD408	Convalescent swine[c]	128	4
SD635	Convalescent swine	256	4
R32	Hyperimmunized rabbit, isolate B78	256	16
SD771	Hyperimmunized swine, isolate B140	128	16
Tp405	Hyperimmunized rabbit, isolate B140	128,000	<200
R41	Hyperimmunized rabbit, isolate B234	256	4

[a] Antigen used was inactivated with 0.01% Merthiolate at 4°C.

[b] Titers expressed as the reciprocal of the serum dilutions.

[c] Pigs 20 to 45 days convalescent from SD.

TABLE 3. *Comparison of antigens prepared from pathogenic and nonpathogenic isolates in the MAT*

Antiserum	Pathogenicity	Source of serum	Titer[a]		
			Pathogenic isolate		Nonpathogenic isolate B297
			B204	B234	
B140	Pathogenic	Pig	128		16
B140	Pathogenic	Rabbit	32,768	4,096	8
B204	Pathogenic	Pig	2,048	1,024	8
B204	Pathogenic	Rabbit	16,384	2,048	4
B234	Pathogenic	Rabbit	2,048	32,768	16
B78	Attenuated[b]	Rabbit	256	512	8
B256	Nonpathogenic	Rabbit	4	8	>4,096
B297	Nonpathogenic	Rabbit	8	8	65,536
4/71	Nonpathogenic	Rabbit	8	8	16
Puppy	Nonpathogenic	Rabbit	8	8	32

[a] Titers expressed as the reciprocal of the serum dilutions.

[b] Pathogenic isolate attenuated by in vitro passage.

TABLE 5. *Agglutinating serum titers of pigs unexposed and exposed to T. hyodysenteriae*[a]

Pig no.	Treatment	Antibody titer[b]							
		Pre-bleed	2 weeks post-challenge	3 weeks post-challenge	4 weeks post-challenge	5 weeks post-challenge	7 weeks post-challenge	8 weeks post-challenge	9 weeks post-challenge
66	Exposed	6 (4–8)	1,448 (1,024–2,048)	1,024 (1,024)	2,048 (1,024–4,096)	2,896 (2,048–4,096)	2,896 (1,024–8,192)	1,024 (1,024)	
50	Exposed	4 (4)	8 (8)	2,896 (2,048–4,096)	1,448 (1,024–2,048)	4,096 (4,096)	1,448 (1,024–2,048)	256[c] (256)	512[c] (512)
79	Exposed	6 (4–8)	4 (2–8)	1,024 (512–2,048)	2,896 (2,048–4,096)	2,896 (2,048–4,096)	2,896 (1,024–8,192)	1,448 (1,024–2,048)	16 (16)
49	Exposed	4 (4)	11 (8–16)	1,024[c] (1,024)	256 (128–512)	362 (256–512)	11 (8–16)	32 (16–64)	90 (32–256)
77	Exposed	3 (2–4)	11 (8–16)	1,024[c] (1,024)	2,048 (1,024–4,096)	4 (4)	11 (8–16)	8 (8)	16 (16)
54	Exposed	6 (4–8)	6 (4–8)	3 (2–4)	6 (4–8)	6 (4–8)		8 (8)	8 (8)
80	Unexposed	6 (4–8)	11 (8–16)	6 (4–8)	6 (4–8)	6 (4–8)	4 (4)	6 (4–8)	8 (8)
61	Unexposed	6 (4–8)	8[c] (8)	4[c] (4)	4[c] (4)	4[c] (4)	8[c] (8)	8[c] (8)	16[c] (16)
67	Unexposed	4 (4)	8[c] (8)	4[c] (4)	8[c] (8)		8[c] (8)	8[c] (8)	32[c] (32)

[a] *T. hyodysenteriae* isolate B234 was used as antigen.
[b] Geometric mean titers of two determinations expressed as the reciprocal of the serum dilutions. Number(s) in parentheses designates range of titers.
[c] Titer of one determination expressed as the reciprocal of the serum dilution.

antigen lots was an eightfold difference in titer noted in only one serum sample (Table 1).

A high degree of cross-reactivity existed between B204 and B234 antigen when reacted against various swine and rabbit antisera (Table 2). This cross-reactivity between pathogenic isolates points out the possibility that these isolates have similar envelope antigens. A lower nonspecific reaction was noted in the MAT when isolate B234 was used as antigen. Because of this response, isolate B234 appears to be an excellent candidate for use in the MAT.

An interesting feature was noted with the agglutination results of antigen from both pathogenic and nonpathogenic isolates when reacted against swine and rabbit antisera. Antigen from pathogenic isolates reacted specifically to antisera from pathogenic *T. hyodysenteriae*, and antigen from nonpathogenic isolates reacted specifically to two of the four antisera from nonpathogenic *T. hyodysenteriae* (Table 3). Preliminary work in our laboratory on swine sera, using the indirect fluorescent-antibody test, has shown a cross-reactivity between pathogenic and nonpathogenic isolates. This difference in test results is probably due to the reacting of different antigens in the two serological tests.

Absorption studies of swine and rabbit antisera with isolate B204 demonstrated a reduction in agglutinating titer with *T. hyodysenteriae* isolate B204. This finding confirms the specificity of the antigen used in the MAT.

High serum agglutinating titers were detected in five of the six exposed pigs at 3 weeks postchallenge. The lack of a detectable serum titer in one of the six exposed pigs was probably due to the degree of involvement of the pig with the disease. The decline in the serum titer of the exposed pigs after 4 weeks postchallenge was expected and was probably due to a lack of host-antigen interaction. Due to these results, a titer of $\geq$256 seems to indicate a possible exposure of the pig to pathogenic *T. hyodysenteriae*.

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