Microagglutination Procedures for Febrile Agglutination Tests

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Febrile agglutination tests were done by using as antigens *Brucella abortus*, *Salmonella* group D, *Proteus* OX19, and *Pasteurella tularensis*. Comparison of results from 23 sera showed that the microtechnique, rapid slide, and test tube methods gave similar titers, although those from the microtechnique were generally higher. The sensitivity of the microtechnique depended upon the concentration of antigen, and, to obtain reproducible results, the optimal concentration of antigens had to be determined by preliminary titrations against specific, positive control antisera. Readability of reactions in the microtechnique was enhanced by adding the dye Safranin O to diluent for antigen and by use of V-type, rather than U-type, microtiter plates. Tests were also done to determine the effects of dye and salt concentrations, pH, and temperature of incubation upon the titer of agglutinations by the microtechnique. Our results indicated that the microtechnique could be used for agglutination tests involving febrile antigens. The procedure is less time-consuming than the tube method and requires less antigen and serum than the latter method or the rapid slide method.

Febrile agglutination tests are still performed in the clinical laboratory in almost the same way as they were when first introduced. Two tests are presently accepted: the classic test tube dilution method introduced by Widal in 1869 and the rapid slide method (8). Optimal concentrations of antigens are generally not determined for individual tests in either method, and a standard control serum is usually not included for the specific antigen. The tube method is expensive because of the number of dilutions and large amounts of antigen required. The fine agglutination of somatic antigens makes end points difficult to read, and various laboratories often report different titers with the same sera. Even with its disadvantages, the tube dilution procedure is the preferred test for agglutination reactions.

Slide agglutination is a rapid, simple test that requires little equipment. Small amounts of a large number of different antigens can be examined. Although recommended only as a screening test, to be confirmed by the tube method, slide agglutination has replaced the tube method in some laboratories because of the simplicity of the procedure and the clearly visible agglutination. However, several workers (2, 6, 11) reported difficulty in duplicating with slide agglutination the results obtained by the tube method.

Recently, various investigators have introduced the microtechnique, originally described by Takatsy (12) and modified by Sever (10), for agglutination procedures. Vedros and Hill (13) described a microagglutination test for *Neisseria meningitidis*; DeMello and DeMello (3) and Elek and Vizy (4) described methods for *Brucella abortus*; Fiset et al. (5) described procedures for *Coxiella burnetii*, *Rickettsia prowazekii*, and *R. mooseri*; and Kirkman et al. (9) described a procedure for typing of *Diplococcus pneumoniae* by agglutination. These investigators recommended the technique as a reliable procedure, economical in time and equipment. We have evaluated the microtechnique as a procedure to supplement, or to replace, the methods presently used for febrile agglutination tests in clinical laboratories. To our knowledge, such an evaluation has not been previously reported.

MATERIALS AND METHODS

Antigens and antisera. The following antigens for macroscopic slide and tube agglutination tests were obtained from Lederle Laboratories, Pearl River, N.Y.: *B. abortus* antigen, *Salmonella* group D antigen, *Proteus* OX19 antigen, and *Pasteurella tularensis* antigen. In some experiments, similar antigens prepared by the Texas State Department of Health Laboratories, Austin, were used.

Positive control antisera specifically prepared for the above antigens were used in experiments to de-

termine the optimal concentration of antigen for the microtechnique. These antisera, also obtained from Lederle Laboratories, were: *B. abortus* control serum; *Salmonella* polyvalent control serum, (somatic) groups A, B, C, D, and E; *Proteus* polyvalent control serum OX2, OXK, OX19; and *P. tularensis* control serum. The Texas State Department of Health Laboratories kindly furnished 23 sera already tested by them for use in evaluating the microtechnique. Of these sera, seven had a positive titer by test tube agglutination for *B. abortus*, seven were positive for *Salmonella* group D, and five were positive for *P. tularensis*; four sera had no titer to these three antigens; and none of the 23 showed a titer to *Proteus* OX19. The sera were heat at 56 C for 30 min before titration.

Diluents. Sodium chloride solutions were prepared over a concentration range of 0.1 to 1.0% (w/v). Solutions of 0.15 M NaCl and 0.2 M Na_2HPO_4 and of 0.5 M NaCl and 0.2 M NaH_2PO_4 were mixed in the proper quantities (7) to obtain buffered saline solutions over a pH range of 5.5 to 8.0, as checked with a pH meter. All solutions were made in distilled water.

Safranin O dye (Gram Safranin; Difco) was added to diluents to enhance visibility of sedimented antigen in the microagglutination. Several concentrations of dye from 0.1 to 1.0% (v/v) were examined by adding appropriate amounts of the Safranin O to diluents. The concentration finally selected was 0.2%, prepared by adding 1.0 ml of the Safranin O solution to 500 ml of diluent. Although some commercial febrile antigens already contain a dye, addition of Safranin O improved visibility of agglutination.

Agglutination procedures. We tested each of the 23 antisera against the four antigens by slide agglutination and by microagglutination. Appropriate positive and negative serum controls were included with all tests, as well as controls for diluent and antigen. The procedure furnished with the antigens from Lederle Laboratories was followed for slide agglutination. Dilutions of antisera ranged from 1:20 to 1:320. Slide agglutinations were read macroscopically after 3 min of mixing at room temperature. Tube agglutinations were done by the Texas State Department of Health Laboratories. Antisera were diluted over a range of 1:40 to 1:10,240 by making appropriate twofold dilutions in 0.5 ml of 0.85% saline and then adding 0.5 ml of the appropriate antigen to each series of test tubes. After incubation at 56 C for 4 hr, the tubes were placed overnight in a refrigerator at 4 C and then read visually the following morning.

Before examination of the unknown sera by the microtechnique, each of the four antigens was tested to determine its optimal concentration or that dilution of antigen which gave the highest agglutination titer with specific antiserum in the microtechnique. To determine the optimal concentration, serial twofold dilutions of antigen were tested against similar dilutions of each control serum in a "block" or "checkerboard" titration (Fig. 1), such as used in virological investigations (1). Dilutions of antisera from 1:5 to 1:640 were made directly in microtiter plates by using 0.05-ml Microdilutors and 0.05-ml pipette droppers (Cooke Engineering Co., Alexandria, Va.), according to the procedure of Sever (10). Antigen was first diluted in

test tubes by the following procedure: 2.0 ml of 0.9% saline was added to the first five test tubes, and 4- and 8-ml amounts were added to tubes 6 and 7, respectively. With a 0.05-ml pipette dropper, eight drops (0.4 ml) of each antigen were added to tube 1; four drops (0.2 ml) to tube 2; two drops (0.1 ml) to tube 3; and one drop (0.05 ml) to tube 4. Then one drop (0.025 ml) of antigen was added from a 0.025-ml pipette dropper to tubes 5, 6, and 7. This procedure resulted in dilutions of 1:6, 1:11, 1:21, 1:41, 1:81, 1:161, and 1:321, respectively, although they were designated as 1:5 to 1:320 for use in block titrations. Accuracy in precise designation of dilutions of antigens was sacrificed for convenience and speed in preparation. The various dilutions of antigens were then added in 0.05-ml amounts with a 0.05-ml pipette dropper to wells containing the appropriate dilutions of antisera.

Plates were closed with plastic covers and then were incubated for 18 to 24 hr at 37 C in a walk-in incubator or at 50 or 56 C in water baths. The latter plates were sealed with plastic tape and floated on the water. After incubation, plates were placed in a refrigerator at 4 C for 2 to 4 hr. The optimal dilutions of antigen were determined by visually examining the pattern of agglutination in the plates.

The titer of unknown sera was measured in V-type microtiter plates. With a 0.05-ml pipette dropper, 0.1 ml of diluent (0.9% saline containing 0.2% of Safranin O) was added to the first well of a row, and 0.05 ml was added to the next seven wells. Sera to be tested were added to the first well with a 0.025-ml Microdilutor to make a dilution of 1:5. Then, twofold dilutions of the antisera were made by using a 0.05-ml Microdilutor to transfer diluted antiserum from the first well serially to the second, from the second to the third, etc., and then by discarding 0.05 ml from tube 7. After the addition of 0.05 ml of the optimal dilution of antigen to each well with a 0.05-ml pipette dropper, the final dilutions of serum were 1:10 to 1:1,280. The microtiter plates were sealed with plastic covers and incubated at 37 C overnight. The plates were then placed in a refrigerator at 4 C for 2 hr before being read.

Reading the pattern of agglutination in microtiter plates. Nonagglutinated antigen sedimented as a sharply defined, round button in the microtechnique. Agglutinated antigen was either completely dispersed and did not form a sediment or, if it settled, formed a poorly defined sediment with a crenated edge that was easily differentiated from the button of nonagglutinated antigen. The titer of the agglutination reaction in microtiter plates was determined by observing the highest dilution of antiserum that permitted sedimentation of a round button of nonagglutinated antigen. In block titrations to determine the optimal concentration of antigen, the end point was determined by observing the highest dilution of antigen that allowed sedimentation of a round button of nonagglutinated antigen with the highest dilution of antiserum. The end points were thus designated by a negative reaction rather than by a positive one, as is usually the case. We purposely chose this procedure because the end point of nonagglutinated antigen was easier to define

as a round button than was the dispersed or poorly sedimented agglutinated antigen.

Although microtiter plates are available with either V- or U-shaped wells (Cooke Engineering Co., Alexandria, Va.), we prefer the V-type, because the difference between agglutinated and nonagglutinated antigen is easier to read. Addition of Safranin O to diluent enhances visibility of nonagglutinated antigen by making the button bright red. The buttons are easily observed at lower dilutions, but as the end point of titration is approached the button becomes small, and experience is required to determine the precise titer.

RESULTS AND DISCUSSION

Standardization of antigen. Preliminary experiments demonstrated that the concentration of antigen used in microagglutination procedures was a critical factor for obtaining reproducible results. If high concentrations of antigen were used, the titer of antibody was low; conversely, low concentrations of antigen resulted in high titers of antibody. Standardization of antigen concentration was accomplished by testing each antigen against its specific control antiserum in a block titration (Fig. 1). As determined by the procedure given above, the optimal concentration of antigen for *B. abortus* was 1:20, because beyond this point all combinations of antigen and antiserum dilutions showed complete dispersion of antigen. Optimal concentrations of antigen for *Salmonella* group D, *Proteus* OX19, and *P. tularensis* were 1:80, 1:80, and 1:160, respectively.

Effect of dye and salt concentrations, pH, and temperature. The influence of each of these factors on the optimal concentration of antigen was investigated by repeating the block titrations. Addition of Safranin O over the range of concentrations used showed little effect on the agglutination reactions, and a concentration of 0.2% was chosen for our experiments. A variation in *p*H of
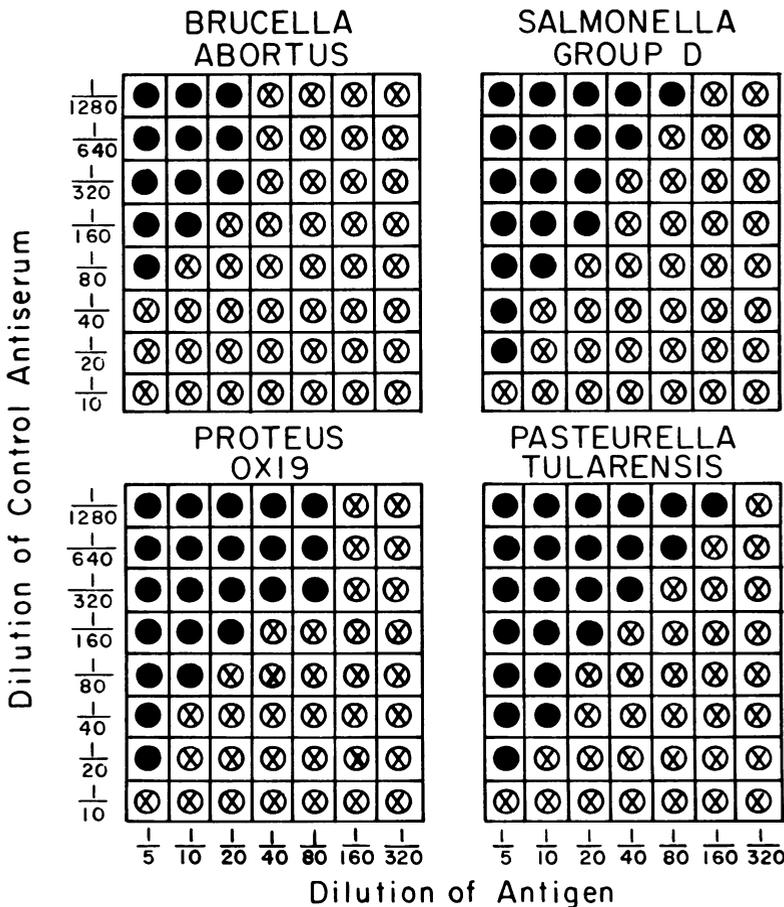

FIG. 1. *Block titrations of febrile antigens against the specific, positive control antiserum for each antigen to determine the optimal concentration for use in the microtechnique. Symbols:* ●, *no agglutination or a red button of sedimented antigen;* ⊗, *agglutinated, or dispersed, antigen.*

5.5 to 8.0 showed no effect on the pattern of ag-
glutination. Salt concentrations of less than 0.5%
inhibited agglutination, but concentrations be-
tween 0.5 and 1.0% had no effect. Essentially
the same end points were observed when micro-
titer plates were incubated at 37, 50, or 56 C.
However, refrigeration at 4 C for 2 hr after incu-
bation at all temperatures showed better differ-
entiation between agglutination and no agglutina-
tion. As a result of these experiments, the optimal
concentrations of antigens were diluted in 0.9%
unbuffered saline, pH about 7.2, containing 0.2%
Safranin O. The microtiter plates were incubated
overnight at 37 C and refrigerated at 4 C for 2 hr
the following morning before being read.

**Comparison of microtiter with conventional ag-
glutination procedures.** Twenty-three sera pre-

viously tested in the Texas State Department of
Health Laboratories by the tube agglutination
method were compared. Every serum was titrated
against all four antigens by each method, but only
the positive reactions are shown in Table 1. Two
different preparations of antigen were used in the
microtechnique. Microtiter, rapid slide, and tube
agglutination procedures showed excellent agree-
ment, although the titer of agglutination varied
(Table 1). No serum showed agglutination by any
of these methods with *Proteus* OX19 antigen. No
false-positive reactions occurred with the micro-
titer procedure when compared to tube agglutina-
tion. However, in preliminary experiments not re-
ported, we discovered that false-positive titers of
1:40 or below sometimes occurred with micro-
agglutination. If sera were heated at 56 C for 30

TABLE 1. *Comparison of agglutination titers by microtiter, rapid slide, and test tube methods[a]*

| Serum | Agglutination titer | | | |
| | Microtiter antigens | | Rapid slide[b] | Test tube[d] |
	I[b]	II[c]		
Positive for *Brucella abortus*				
1	1:320	1:320	>1:320	1:320
2	1:5,120	1:640	>1:320	1:5,120
3	1:640	1:640	>1:320	1:1,280
4	1:160	1:80	1:160	1:640
5	1:80	1:40	1:160	1:160
6	1:40	1:20	1:40	1:40
7	1:640	1:320	>1:320	1:160
Positive for *Pasteurella tularensis*				
8	1:1,280	1:320	1:320	1:160
9	—[e]	—	—	1:160
10	1:2,560	1:640	>1:320	1:320
11	1:1,280	1:640	>1:320	1:320
12	1:1,280	1:320	1:160	1:640
Positive for *Salmonella* group D				
13	1:640	1:640	>1:320	1:160
14	1:160	1:320	—	1:80
15	1:5,120	1:1,280	—	1:640
16	1:640	1:80	1:160	1:80
17	1:640	1:640	1:80	1:320
18	1:40	—	—	1:80
19	1:640	1:320	1:160	1:640

[a] Although each serum was tested by the same methods against all four febrile antigens, only the
positive titers are shown; the other tests showed no titer. None of the sera was positive with *Proteus*
OX19 antigen.

[b] Antigens obtained from Lederle Laboratories.

[c] Antigens obtained from Texas State Department of Health Laboratories.

[d] Titration done by Texas State Department of Health Laboratories.

[e] Indicates titer less than 1:10, 1:20, or 1:40 for microtiter, rapid slide, or tube method, respectively.

min before titration, the number of false-positive reactions was reduced. Serum 9 did not show agglutination in the microtiter or rapid slide procedure, whereas the titer by the tube method was 1:160. Only serum 18 showed a discrepancy in the reaction obtained with the two different antigens used in the microtechnique. Four sera (9, 14, 15, and 18) were negative by the rapid slide procedure, although the tube agglutination reaction was positive.

Microtiter and tube agglutination procedures showed perfect agreement when sera were tested that had no titer for the four antigens (Table 2). However, by rapid slide agglutination, serum 23 showed a titer of 1:160 for *Salmonella* group D antigen. Since no agglutination appeared with the other two procedures, this reaction probably represented a false-positive test.

The titer of agglutination in the microtechnique varied depending upon the concentration of antigen (Table 3). *Salmonella* group D antigen was diluted 1:80, the optimal concentration as determined by block titration (Fig. 1), and also was diluted 1:40. The titer obtained with the optimal concentration was consistently greater, being one to four dilutions higher than the titers obtained with a more concentrated amount of antigen. The optimal concentration also showed generally higher titers than obtained by tube agglutination. Similar results were obtained when more concentrated amounts of *B. abortus* or *P. tularensis* antigens were tested with the corresponding positive sera. When even greater concentrations of antigens were used, the agglutination titers were even lower.

Our data demonstrate that the microtechnique

TABLE 2. *Comparison of agglutination titers of four negative sera by microtiter, rapid slide, and test tube methods*[a]

Serum	Agglutination titer[b]			
	Microtiter antigens		Rapid slide	Test tube
	I	II		
20	—	—	—	—
21	—	—	—	—
22	—	—	—	—
23	—	—	1:160[c]	—

[a] Sera were tested by the same methods against all four febrile antigens, *Brucella abortus*, *Salmonella* group D, *Proteus* OX19, and *Pasteurella tularensis*.

[b] See footnotes *b, c, d,* and *e* of Table 1.

[c] Titer for *Salmonella* group D only; no agglutination was observed with the other three antigens.

TABLE 3. *Comparison of microtiter agglutination using two concentrations of antigen with test tube agglutination of sera positive for Salmonella group D*

Serum	Agglutination titer		
	Dilution of microtiter antigen[a]		Test tube[b]
	1/40	1/80	
13	1:160	1:640	1:160
14	1:20	1:160	1:80
15	1:320	1:5,120	1:640
16	1:80	1:640	1:80
17	1:320	1:640	1:320
18	1:10	1:40	1:80
19	1:40	1:640	1:640

[a] Antigen obtained from Texas State Department of Health Laboratories.

[b] Titration done by Texas State Department of Health Laboratories.

can be used for febrile agglutination tests. The sensitivity of the procedure depends upon the concentration of antigen, and, for best results, the optimal concentration of antigen must be determined by titration against specific, control antiserum. Infrequent false-positive reactions apparently can be reduced by heating sera to 56 C for 30 min before testing. Incorporation of the dye Safranin O into the diluent permits easier differentiation between agglutination and no agglutination, and refrigeration of plates after incubation sharpens these differences. Our results suggest that the microtiter agglutination is more accurate than the rapid slide procedure, although the former method takes longer. The microtiter procedure is much less time-consuming than tube agglutination. Less antigen and serum are required by the microtiter procedure than by either of the other two.

Microtiter and tube agglutination procedures give comparable results. The advantage of requiring less time and material recommends the former method for routine use. However, laboratories must remember that, irrespective of the procedure, the significant factor for diagnostic febrile agglutination tests is to demonstrate a rising titer between paired acute and convalescent sera.

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