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Evaluation of a Safranin-O-Stained Antigen Microagglutination Test for *Francisella tularensis* Antibodies

SUSAN L. BROWN,* FREDERICK T. McKINNEY, GEORGE C. KLEIN, AND WALLIS L. JONES

Center for Disease Control, Atlanta, Georgia 30333

A microagglutination test for *Francisella tularensis*, with 0.025-ml amounts of diluted sera and 0.025-ml amounts of safranin-O-stained antigen in U-bottom microtitration plates, was compared with a tube agglutination test by using 137 sera. There was 86.3% agreement (±1 dilution variation) between the microagglutination results and the tube agglutination results for sera with tube agglutination titers of ≥20. There was 100% agreement (±1 dilution variation) for sera with titers of <20. The advantages of the microagglutination test are: (i) it requires fewer man hours to perform; (ii) it requires only one-twentieth of the amount of antigen; and (iii) it is easier to read.

In our laboratory the routine test for assay of antibodies to *Francisella tularensis* has been the tube agglutination (TA) test (4); however, this test is time consuming and cumbersome in both performance and reading. Gaultney et al. (2) evaluated the microagglutination (MA) technique for febrile agglutination tests. Their work suggested that the MA technique, with diluent containing safranin-O could be used to replace the TA test for tularemia antibodies. They pointed out that the MA procedure is less time consuming than the TA procedure and requires less serum and antigen. Massey and Mangiafico (3) described an MA procedure for detection of *F. tularensis* antibody. Their procedure involves use of 0.05-ml amounts of hematoxylin-stained antigen and 0.05-ml amounts of diluted sera in V-bottom microtitration plates. The procedure of Gaultney et al. (2) also involves use of V-bottom microtitration plates and 0.05-ml amounts of diluted sera and antigen, along with safranin-containing diluent.

We have modified the MA test by using 0.025-ml amounts of antigen stained with safranin-O and 0.025-ml amounts of diluted sera in U-bottom microtitration plates. The present study compares this MA test with the TA test. A representative group of negative and positive sera were studied to test the reproducibility of the MA procedure.

MATERIALS AND METHODS

Stock antigen and control sera. The stock antigen used, for both MA and TA tests, in this study was obtained from the Center for Disease Control (CDC) (lot 76-0319) in sealed ampoules suspended in 0.5% formaldehyde in saline. The density of this stock antigen is such that a 1:10 dilution equals a no. 4 McFarland standard. High, low, and negative control sera were also obtained from the CDC and were run in parallel with each day's run. These controls are defined as having tube agglutination titers as follows: high, ≥320; low, 40 to 80; and negative, ≤20.

Sera. One hundred thirty-seven sera were used in this study. Of these, 93 were from patients suspected of having tularemia and were sent in to the CDC Bacterial Immunology Branch to be tested for *F. tularensis* agglutinating antibodies. Thirty-three were kindly provided by the CDC Bureau of Epidemiology and were from patients suspected of having tularemia. Eleven were positive high-titer sera obtained from the CDC Serum Bank.

MA technique. Initial 1:10 dilutions (0.1 ml of serum + 0.9 ml of diluent) of sera were made in 0.85% saline in 1-dram (ca. 1.2-g) screw-cap vials. The stock antigen suspension was diluted 1:10 in 0.85% saline containing 0.5% Formalin and 0.005% safranin-O (prepared from a 0.5% aqueous stock solution). Formalin was added to the antigen as a preservative, and it did not appear to have any effect on the titer. The MA test was performed by placing 0.05 ml of a 1:10 dilution of serum in the first well of a row in a U-bottom plate and adding 0.025 ml of 0.85% saline to the remaining 11 wells in the row. The sera were serially diluted with an Automatic Diluter (Dynatech Laboratories, Inc.), and 0.025 ml of antigen was added to each well by using a 0.025-ml pipette dropper (Dynatech Laboratories, Inc.). The dilutions of antisera tested therefore ranged between 1:20 and 1:40,960. The plates were then sealed with plastic microtiter plate sealers (Dynatech Laboratories, Inc.), mixed on a vertical vibrator (Arthur H. Thomas Co.) for 20 s, and incubated overnight (18 to 20 h) at 37°C. The plates were read with a test reading mirror (Dynatech Laboratories, Inc.) and a fluorescent lamp. A piece of thin white paper was placed on top of the plate to make reading easier. A well was rated positive for agglutination if it contained either a mat of agglutinated cells or nothing visible in the bottom of the well. Negative reactions contained a well-defined button of cells. The endpoint is the highest dilution of serum showing agglutination. The titer was the dilution factor of this endpoint.

Tube agglutination. The tube test for *F. tularensis* was performed according to the method described

by Saslaw and Carhart (4). Each serum was serially diluted in 0.85% saline in 10 tubes (13 by 100 mm); 0.5 ml of diluted antigen was added to 0.5 ml of diluted serum, and the tests were read after overnight incubation at 37°C. The beginning serum dilution was 1:20 after antigen was added. The stock antigen was diluted 1:10 in 0.85% saline containing 0.5% Formalin.

Reproducibility study. The reproducibility study consisted of three sets of 1:10 dilutions in saline from a set of 12 sera labeled with the serum number and day number. Each 1:10 dilution was tested in triplicate on three consecutive days.

RESULTS

Preliminary studies. Preliminary studies were undertaken to determine which serum diluent, antigen diluent, and antigen concentrations to use. Various diluents were tested, including phosphate-buffered saline (pH 7.2); 0.85% saline, approximately pH 6.0; and phosphate-buffered saline (pH 7.2) with 0.05%, 1.0%, or 1.5% Tween 20 or 0.05% or 0.1% bovine serum albumin added. Phosphate-buffered saline or 0.85% saline as diluent yielded plates that were easy to read with titers similar to TA titers. No consistent advantage was seen when any of the concentrations of Tween or bovine serum albumin was added to the diluent, and in fact plates were often more difficult to read with these additives. Using the above information and the fact that 0.85% saline is used in the TA test, we chose to use 0.85% saline as our diluent. Antigen dilutions (1:10, 1:15, 1:20, and 1:40), serum dilution volumes (0.025 ml and 0.05 ml), and type of microtitration plates (U-bottom and V-bottom) were compared with high, low, and negative control sera.

The preliminary studies indicated that 0.025-ml amounts of antigen diluted 1:10 added to 0.025-ml amounts of diluted serum in U-bottom plates gave results that were most consistent with tube test results as well as easiest to read; therefore these conditions were used in the remainder of the study.

Comparison of MA with TA. One hundred thirty-seven serum specimens were tested; 73 had TA titers ≥20, and 64 had TA titers <20. The tests gave identical results for 63 of the 64 (<20) sera; one serum had an MA titer of 20. Results for sera with TA titers ≥20 are shown in Table 1. For sera with tube test titers of 20 or higher, agreement within ±1 dilution was 86.3%.

We were able to obtain clinical or epidemiological confirmation of tularemia for 25 patients from whom 35 serum specimens were tested. All of these patients had sera with titers of ≥320 by both tests. Acute- and convalescent-phase serum specimens were obtained on nine patients, and a rise in titer of at least 2 doubling dilutions was detected by both the TA and MA tests. The MA test tends to give somewhat higher titers than the TA test. A comparison of the highest TA titer to the corresponding MA titer for each of the 25 patients with a clinical diagnosis of tularemia is as follows: 10 sera were 1 dilution higher by MA; 3 were 2 dilutions higher by MA; 9 sera gave the same titers by both tests; and only 3 were 1 dilution lower by the MA than by the TA test.

TABLE 1. *Comparison of MA and TA titers for sera with TA titers ≥ 20*

Titer	No. of serum specimens					% Agreement (within ±1 dil.)
	TA test	MA test				
		1 dil.[a] lower	Same dil.	1 dil. higher	2 dil. higher	
20	2		2			100.0
40	4	2	2			100.0
80	2		2			100.0
160	6		5	1		100.0
320	18	1	6	8	3	83.3
640	23	3	10	7	3	87.0
1,280	17	1	2	10	4	76.5
2,560	0					
5,120	1		1			100.0
Total	73					86.3

[a] dil., Dilution.

Reproducibility study. The results of the reproducibility study are shown in Table 2. Allowing for a ±1-dilution variation, the MA test was 100% reproducible both day to day and within day. Only one serum (A) yielded titers that varied more than 1 dilution.

DISCUSSION

Eigelsbach stated that by week 3 of infection, titers to *F. tularensis* would usually be 320 or higher (1). Generally a tube test titer of 80 or greater is considered to be indicative of infection with *F. tularensis* (5, 6). However, a fourfold rise in titer (e.g., 20 to 80) between acute- and convalescent-phase sera is preferable to a single specimen titer. In our laboratory a single specimen titer of 80 is considered to be equivocal and a titer of 160 is considered to be positive. There was 100% agreement between the TA and MA tests for the determination of positive (≥160) specimens (Table 1). Higher titers were obtained with the MA test for 36 sera (26 were 1 dilution higher, 10 were 2 dilutions higher), and lower titers were found for 7 sera (all were 1 dilution lower). The reason for this finding is unknown; however, it could be due to the greater difficulty of reading TA results as compared to MA results or to the MA tests being slightly more sensitive. Since the MA test tends to yield slightly higher titers than the TA test in sera from confirmed cases, we feel that the higher titers may be true

TABLE 2. *Reproducibility study: MA titers of 12 sera tested in triplicate over 3 consecutive days*

Sera	Day 1			Day 2			Day 3		
	Titer 1	Titer 2	Titer 3	Titer 1	Titer 2	Titer 3	Titer 1	Titer 2	Titer 3
A	2,560	2,560	2,560	1,280	5,120	2,560	2,560	2,560	2,560
B	20	20	20	20	20	20	20	20	20
C	1,280	1,280	1,280	2,560	2,560	2,560	1,280	2,560	2,560
D	<20	<20	<20	<20	<20	<20	<20	<20	<20
E	20	20	20	20	20	20	20	20	20
F	1,280	1,280	2,560	1,280	1,280	1,280	1,280	1,280	1,280
G	640	320	640	640	320	640	640	640	640
H	640	640	1,280	1,280	1,280	640	640	640	1,280
I	640	1,280	1,280	640	640	1,280	640	1,280	1,280
J	160	160	160	160	160	320	320	160	160
K	2,560	1,280	2,560	1,280	2,560	1,280	2,560	2,560	2,560
L	640	640	640	640	640	640	640	640	640

titers. Massey and Mangiafico (3) also reported slightly higher titers with some of their tularemia MA tests as compared to their TA tests. The reproducibility study showed the MA test for *F. tularensis* antibodies to be very reproducible, both within day and from day to day.

The titers obtained with the MA test are reproducible and compare favorably with the titers from the tube agglutination tests. The advantages of the *F. tularensis* MA test over the TA test are: (i) it requires fewer man hours to perform; (ii) it requires only one-twentieth of the amount of antigen; and (iii) it is easier to read.

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