Microagglutination Test for Detecting and Measuring Serum Agglutinins of *Francisella tularensis*

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A microagglutination method utilizing stained antigen for detecting and measuring serum agglutinins against *Francisella tularensis* is described. The microagglutination and standard tube agglutination techniques were demonstrated to be comparable in sensitivity and specificity. Advantages of the micro method are rapidity and ease of performance, economy of reagents and, in particular, ease of interpreting specific reactivity.

The agglutination procedure for tularemia was described initially by Francis and Evans in 1926 (4). Since then, the agglutination test has proven to be a useful tool for investigative studies and diagnosis because of its sensitivity, specificity, and relative ease of performance. However, the procedure recommended for the standard tube agglutination (STA) test for tularemia (1) has certain disadvantages. Visual examination of each tube with adequate illumination is a time-consuming, cumbersome process. In addition, scoring the degree of agglutination to determine the end point for a titration presents an even more serious difficulty. Differentiation between a 2+, or negative, and a 3+, or positive, reaction is highly subjective, and leads to variation in titers reported by different laboratory workers as well as in those recorded at different times by one individual.

This report describes a microagglutination (MA) test for tularemia that utilizes stained antigen to facilitate reading of agglutination patterns and requires micro amounts of serum, antigen, and diluents. A comparison is made of the diagnostic capabilities of the MA and STA tests for tularemia.

**MATERIALS AND METHODS**

**Sera.** Sera were obtained from 50 laboratory personnel prior to and 6 weeks after vaccination with the live vaccine strain (LVS) of *Francisella tularensis* (2). Sera were stored at -20°C until used for testing. Titers for each serum sample were determined simultaneously by the STA and MA techniques.

**Antigens.** A single lot of Formalin-inactivated, bacterial test antigen, prepared from the virulent SCHU S5 strain of *F. tularensis* (National Drug Co., Swiftwater, Pa.), was used. The optimal concentration for STA tests, a 1:80 dilution in 0.85% NaCl, hereafter referred to as saline, was established by titrating dilutions of unstained test antigen with dilutions of an antibody-positive reference serum.

**Staining of antigen.** A modification of the staining procedure described by Fiset et al. for Q fever antigen (3) was employed. Test antigen was centrifuged at 4,500 rpm for 30 min, and the sediment was washed once with saline. After a second centrifugation at 4,500 rpm for 30 min, the volume of sediment was recorded. Hematoxylin, without acetic acid (5), was added to the antigen sediment to a final concentration of 10% (vol/vol), and the mixture was incubated at 56°C for 48 h in a water bath with occasional shaking. Excess stain was removed by repeatedly washing the stained antigen with saline until a clear supernate was obtained. After resuspension in saline to restore the original volume, stained antigen was stored at 4°C.

**Standardization of stained antigen.** The optimal amount of stained antigen for MA tests was determined in the microtitr system. Dilutions of stained antigen, ranging from 1:10 to 1:60, were titrated against serial twofold dilutions of STA-positive and STA-negative control sera. The highest dilution of stained antigen that duplicated most closely the STA titer of the positive control serum was selected as the standard dilution for MA tests.

**Stability of stained antigens.** Stained antigen preparations stored at 4°C have shown no detectable change in specific reactivity when reevaluated by the above standardization procedure at periodic intervals for 3 years.

**MA test procedure.** The MA test was performed in disposable hard plastic plates with “V” bottom wells (no. IS-MVC-96, Linbro Chemical Co., New Haven, Conn.). All dilutions were prepared in saline. After 0.05 ml of diluent was added to every well, 0.05 ml of a 1:5 dilution of test serum was delivered to the first well of each row and serial twofold dilutions of serum were prepared with micro-diluters calibrated to transfer 0.05 ml. Finally, 0.05 ml of the standard dilution of stained antigen was added to all wells, making twofold dilutions of serum 1:20, 1:40, 1:80, etc.
Appropriate controls included the following: (i) a mixture of diluent and antigen, and (ii) titrations of antibody-negative and antibody-positive reference sera. Plates were sealed, gently shaken, and incubated at 37 C for at least 5 h and preferably overnight. A microtiter viewer (Cooke Engineering Co., Alexandria, Va.) was employed to evaluate the sedimentation patterns; complete or partial formation of a tight button on the bottom of the well indicated a negative reaction whereas a film covering the sloping walls of the well indicated a positive agglutination reaction. The highest serum dilution showing agglutination was recorded as the titer for that serum.

**RESULTS**

Reactions in a typical MA test plate are shown in Fig. 1. Dilutions of negative reference serum (row 7) uniformly demonstrated absence of agglutination; the titer of the positive reference serum (row 8) was 1:640. Titers for test sera ranged from 1:20 in row 5 to 1:640 in row 3. Prozones rarely were observed when serum titrations started with a 1:20 dilution.

Titrations with human sera obtained from 50 individuals prior to and 6 weeks after vaccination with LVS indicated that the STA and MA techniques yielded essentially the same agglutination titers (Table 1). Identical titers were noted for 43 of 50 (86%) prevaccination sera and for 35 of 50 (70%) postvaccination samples. Titers in the MA test exceeded STA titers by one dilution in 4 prevaccination and 13 postvaccination sera; a one-dilution decrease in MA titer was observed less frequently, occurring with one postvaccination and two prevaccination specimens. One serum in each immunization category that was negative in the STA test had a 1:20 MA titer, an antibody level considered to be neither specific nor diagnostic for tularemia. Overall, the MA titer for 98 of 100 serum samples was identical with, or within one dilution of, the corresponding STA titer.

**DISCUSSION**

An MA test employing stained F. tularensis antigen has been described. The MA test has several advantages over the STA test, the most important being the ease and rapidity of performance, economy of reagents, and relatively increased capability for discriminating between positive and negative reactions. The readily visible sedimentation patterns of stained antigen make possible reduction in technician training time and in variability of interpretation of different technicians.

Titers obtained with the MA and STA tests were very similar (± one dilution) for 98% of the serum samples tested. These data demonstrate that the degree of sensitivity, specificity, and reliability of the MA test equals that of the STA test for detecting and measuring serum agglutinins of F. tularensis. Despite the number of the MA tests that had slightly higher titers than corresponding STA tests, it is postulated that the MA test is not more sensitive but rather provides an improved and reproducible capability for reading the end point of reactions.

The MA test has been in use in this Institute for the past 3 years. During this time, it has proven to be a sensitive, reliable procedure suitable for detection of antibody response to

![Typical patterns produced by stained antigen in microagglutination test. Wells 1 to 12 contain twofold serial dilutions of serum, 1:20 in well 1 to 1:40960 in well 12. Rows 1 to 6 contain test sera, row 7 contains negative reference serum, and row 8 contains positive reference serum.](image)

Table 1. Comparison of STA and MA titers for F. tularensis in human sera obtained before and after LVS vaccination

| Sample group | Reciprocal STA titer | Number of samples | MA titer relative to STA titer |
|--------------|----------------------|-------------------|--------------------------------|
|              | Same titer           | Same titer        | 1 Dilution higher | 1 Dilution lower |
| Pre vaccination | <20 35 34 | 1* 0 |
| 20 8 4 4 | 0 |
| 40 5 4 0 1 |
| 80 2 1 0 1 |
| Post vaccination | <20 1 0 1* 0 |
| 20 2 1 1 0 |
| 40 12 7 5 0 |
| 80 10 8 2 0 |
| 160 11 7 4 0 |
| 320 12 11 1 0 |
| 640 2 1 0 1 |

* Reaction in 1:20 dilution of serum was negative in STA test, but positive in MA test.
F. TULARENSIS SERUM AGGLUTININS

Tularemia vaccination or infection in man, monkey, rabbit, and rat.

Adaptation of this technique for diagnostic agglutination studies with other bacterial antigens would appear to be warranted.

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