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Modified Agglutination Test for *Pasteurella tularensis*

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ABSTRACT

ENGELFRIED, JOHN J. (Naval Medical Research Unit #1, University of California, Oakland), AND FRANCES SPEAR. Modified agglutination test for *Pasteurella tularensis*. Appl. Microbiol. **14**:267-270. 1966.—Several modifications in technique were incorporated into the standard agglutination test for *Pasteurella tularensis*. Reciprocal shaking of all tubes in a Kahn shaker was introduced to increase the rate of agglutination and quantity of agglutinated cell mass, making it possible to report preliminary results within 4 hr. Increased incubation time at a higher temperature was used to favor the rate of agglutination. A serum control for each serum tested was necessary to detect false positive tests. Finally, a verification procedure with 5% NaCl used as the diluent was instituted to prevent these false positive reactions.

In a study of antibody titers, many possible pitfalls were noted in the standard agglutination procedure of *Pasteurella tularensis*. This procedure, published in 1926 (4) and later confirmed by others (3, 7, 8, 9), consists of adding a suitable antigen suspension to serial dilutions of serum. The tubes are incubated from 2 hr to overnight in a water bath at temperatures ranging from 37 to 50 C. After incubation the tubes are read macroscopically and reread after overnight storage at 2 to 4 C. Positive and negative serum controls are always included.

This standard procedure was followed in this laboratory to determine antibody titers for *P. tularensis*. It was noted that many tests were difficult to read due to various amounts of precipitate which resembled *P. tularensis* agglutination. This occurred less frequently with human sera than with other animal sera. Some of these tests were questionable and others were weakly positive. Further investigation revealed that, if a 1:5 dilution of these problem sera was further diluted with an equal volume of 0.9% NaCl, a precipitate difficult to distinguish from agglutination of *P. tularensis* antigen would occur. Since numerous positive human sera were available, a study was undertaken to improve the procedure for this agglutination test.

MATERIALS AND METHODS

All agglutination tests were performed in Kahn tubes. The serum was centrifuged when necessary. The dilutions were made by doubling dilution beginning at 1:5. *P. tularensis* antigen obtained from Walter Reed Army Medical Center was diluted 1:60. The diluent for the antigen and for the serum was always the same concentration of NaCl or other reagent. An equal quantity of antigen suspension was added to the serum dilutions, resulting in a final dilution of 1:10, 1:20, etc. Positive and negative serum controls were included with each run. In addition, a serum control for each serum tested was used in later tests. The tubes were covered and incubated as illustrated in the following protocols. After incubation, the image of the contents of the tube was examined by microscope mirror for evidence of agglutinated antigen particles. The degree of agglutination was recorded as 4+, 3+, 2+, 1+, ±, and negative. A reaction of 2+ or more was considered positive. The tubes were stored overnight at 2 C unless otherwise indicated and were re-evaluated the following morning.

Effect of incubation temperature and time of agglutination was studied by preparing larger quantities of serum dilutions in 50-ml tubes and transferring 0.5 ml of each dilution to each of 16 Kahn tubes. A 0.5-ml amount of antigen suspension was added to each tube. The 16 tubes for each dilution were separated into four sets, three containing five and the fourth set containing only one tube. The last set was refrigerated immediately. Of the other three sets, one remained at room temperature, one in a water bath at 37 C, and the other at 50 C (five tubes for each serum dilution in each of three racks). One tube of each dilution in each rack was removed at 1-, 2-, 4-, and 6-hr intervals, examined, and refrigerated. All tubes were re-examined the next morning. The fifth tube was examined once at 24 hr. The set placed immediately in refrigeration was examined at the previously indicated hour intervals and returned to refrigeration.

Effect of agitation on agglutination of *P. tularensis* antigen was investigated through two different avenues of approach: one, the use of a Kahn shaker [275 to 285 oscillations per min, with a stroke of 1.5 inches (3.8 cm)]; the other, the low-speed centrifuge. Serial dilutions of positive sera were prepared in larger quantities in 50-ml tubes. Equal amounts of antigen suspension were added to each dilution and mixed, and 1-ml samples of each mixture were immediately transferred to 15 Kahn tubes. All tubes were incubated at 50 C. Five tubes of each dilution were removed from the water bath at 1-, 2-, and 4-hr intervals. At each time interval, one tube was examined without agitation; the second tube was centrifuged at approximately 1,500 rev/min for 5 min before examination. The other three tubes of each dilution were shaken (on Kahn shaker) before examination: one for 3, one for 5, and one for 10 min. After the initial reading, all tubes were refrigerated overnight, and the final reading was performed the next morning. This complete protocol was repeated with four positive sera.

The effect of agitation was further studied by preparing serial dilutions of 15 positive sera. A 0.5-ml amount of each dilution was transferred to three rows of Kahn tubes (three sets of serial dilutions for each serum). Antigen suspension (0.5 ml) was added to all tubes. The racks were agitated by hand and incubated at 50 C. One tube of each dilution was removed after 30 min, shaken on a Kahn shaker for 5 min, read, and refrigerated. The other two sets were removed from incubation at 4 hr. One set was examined without shaking; the other set was shaken for 5 min before examination. All tubes were re-examined after overnight refrigeration.

An additional group of 37 positive and 94 negative sera was tested with 4-hr incubation at 50 C with and without shaking.

A precipitate resembling agglutination of the antigen suspension was encountered in the 1:10 dilution of some sera. In an attempt to overcome this nonspecific precipitation, which invalidated apparent antigen-antibody reactions in low dilutions of serum, various procedures and techniques were investigated. The following concentrations of sodium chloride were utilized in later experiments: 0.9, 2, 3, 5, 8, 10, and 14%. Other additives studied were 5, 10, and 20% polyvinylpyrrolidone (PVP), 5 and 10% egg albumin, and 5 and 10% bovine albumin, all diluted with 0.9% NaCl.

Results after overnight refrigeration were photographed by use of dark-field illumination. The tubes were stoppered and taped in a horizontal position on a glass plate.

RESULTS

Four positive sera, tested at various incubation times and temperatures, gave positive results with *P. tularensis* antigen after 24 hr. The time required to obtain positive results decreased as the incubation temperature increased. At 2 C, one serum gave positive agglutination tests at 4 hr and another required 6 hr, whereas two sera did not respond until 24 hr. The agglutination response at room temperature (25 to 32 C) was 2, 4, 4, and 6 hr, whereas at 37 C positive results were obtained as early as 1, 2, 4, and 4 hr. At 50 C, two specimens were positive after 1 hr of incubation and two at 2 hr.

Preliminary studies with four known positive sera showed that agitation on a Kahn shaker increased the degree of agglutination in many of the serum dilutions. In the majority of instances, maximal agglutination was obtained with 3 min of shaking. However, slightly better results were obtained in a few instances with 5 min of shaking; therefore, this time interval was adopted for use in later studies. Centrifugation after incubation did not appear to enhance the degree of agglutination in these four positive specimens.

Fifteen other positive sera examined after 30 min of incubation plus shaking and 4 hr of incubation plus shaking gave positive tests. Very little difference in degree of agglutination was observed between the 30-min and the 4-hr incubation periods. However, only 10 of these sera gave positive results after 4 hr of incubation without shaking, but after overnight refrigeration all tests were positive.

In another group of 37 positive and 94 negative specimens, 30 were positive after 4 hr of incubation plus shaking. The other seven did not become positive until refrigerated overnight. Only 21 of these positive sera gave positive results after 4 hr of incubation without shaking. Furthermore, 17 of the 21 positives showed a lower titer than the corresponding specimen that had been shaken after 4 hr of incubation. In one test without shaking, a positive serum remained negative after overnight refrigeration. None of the 94 negative sera reacted. The effect of shaking on the degree of agglutination is shown in Fig. 1. A 1:80 dilution of positive serum was read as 1+ after 4 hr of incubation without shaking; the

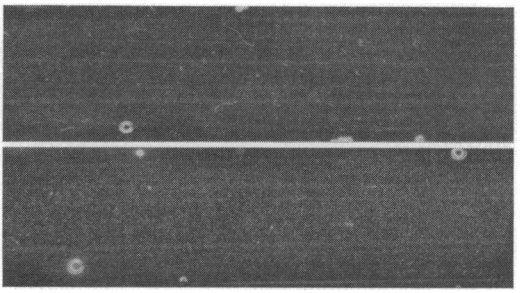

FIG. 1. *Effect of shaking on Pasteurella tularensis agglutination (human serum dilution, 1:80. Top: 4 hr of incubation, without shaking (+). Bottom: 4 hr of incubation, plus shaking (+++).*

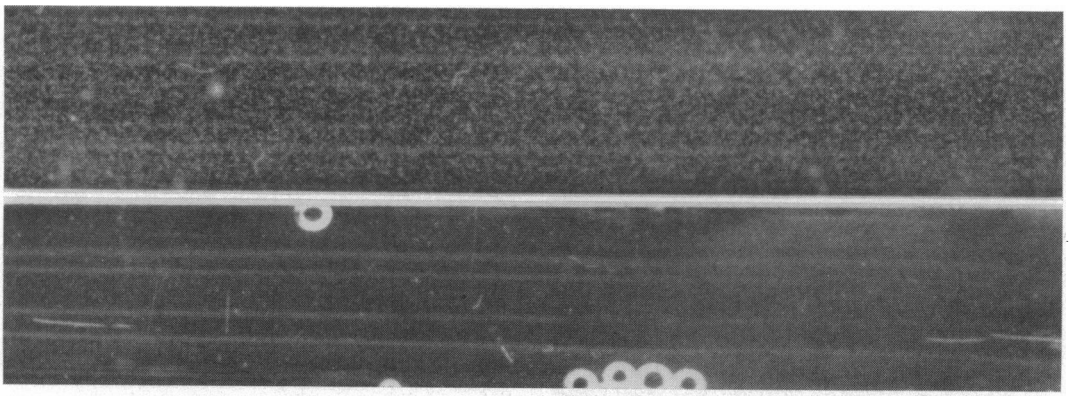

FIG. 2. *Effect of salt concentration on avian serum (specimen 658, 1:10 serum dilution, without antigen). Top: 0.9% NaCl. Bottom: 5.0% NaCl.*

TABLE 1. *Results of verification test specimen #907 (avian serum)*

Date of test	NaCl concn	Control	Serum dilutions				
			1:10	1:20	1:40	1:80	1:160
	%						
3 June 1964	0.9	++	++	+	−	−	−*
		+++	+++	++	−	−	−†
21 September 1964	0.9	++++	+++	+	−	−	−
		++++‡	++++‡	+++	+	−	−
21 September 1964	5.0	−	−	−	−	−	−
		−‡	−‡	−	−	−	−

* Reading at 4 hr.
† After overnight refrigeration.
‡ Photographs of these reactions are illustrated in Fig. 3.

corresponding dilution that had been shaken was read as 3+.

Increasing NaCl concentrations from 0.9% to 3, 5, and 8% did not interfere with agglutination and prevented precipitation that occurred in some 1:10 serum dilutions as illustrated in Fig. 2. This high NaCl concentration did not produce false positives in 199 negative human sera.

Other additives, PVP, egg albumin, and bovine albumin did not enhance agglutination or prevent nonspecific precipitation.

DISCUSSION

The degree and rapidity of agglutination of *P. tularensis* was favored by an elevated incubation temperature, suggesting that this antibody is a semiwarm agglutinin. Temperature affected the rate of agglutination more than the degree of agglutination, making a rapid presumptive test possible.

Reciprocal shaking improved the quality and increased the quantity of agglutinated cells. This was anticipated, since agitation favors small amounts of agglutinins coming in contact with opposing agglutinogens, and brings small masses of agglutinated cells in contact with one another to form larger masses. A greater agglutinated cell mass favors fewer unattached bacteria remaining in suspension, improving the clearness of the supernatant fluid. Dependable positive results occurred in the majority of positive tests after 30 min of incubation plus shaking; however, in a few instances 4 hr of incubation was necessary to obtain maximal agglutination. Therefore, 4 hr of incubation with 5 min of shaking was chosen for future studies. Low-speed centrifugation did not enhance agglutination.

The 10-fold dilution of clear serum with 0.9% saline in some instances resulted in a fine precipitate resembling agglutination of the antigen suspension. This may be due to the fact that compounds such as nucleo-proteins and globulins

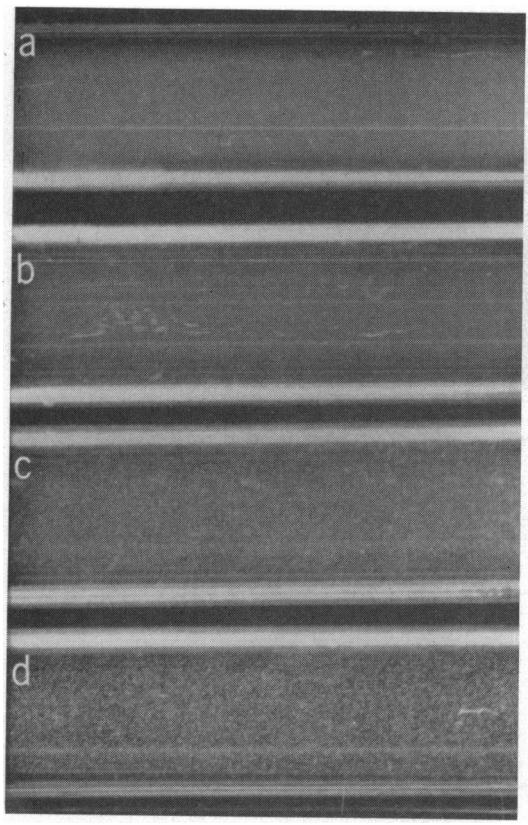

FIG. 3. *Results of verification test, avian serum specimen 907 (a) Serum and 5.0% NaCl, plus antigen; (b) serum and 5.0% NaCl, without antigen. (c) Serum and 0.9% NaCl, plus antigen; (d) serum and 0.9% NaCl, without antigen. (final serum dilution, 1:10 in all tubes.)*

are insoluble in low sodium chloride concentrations or may be conditioned by some other physiochemical situation (1, 2, 5, 6). This phenomenon occurred more frequently with avian than with mammalian sera and necessitated the routine use of serum control with every serum tested. In some instances, the serum control has been read as 3 or 4+ agglutination.

Further studies revealed that higher concentrations of sodium chloride would prevent this precipitation, as shown in Table 1 and further emphasized by photography in Fig. 3. This led to the use of 5% NaCl as the diluent in the verification procedure which is employed whenever a serum shows any degree of reaction in the control tube.

RECOMMENDED PROCEDURE

Presumptive test with 0.9% sodium chloride. Prepare 0.5 ml of graduated dilutions of serum under examination and 0.5 ml of antigen in a series of unetched agglutination tubes. Shake tubes in a reciprocal shaker for 5 min. Transfer tubes to a water bath (50 C) for 4 hr, repeat shaking for 5 min, and make the preliminary reading. (Examine the image in the concave side of a microscope mirror in a darkened room with one source of light.) Refrigerate tubes overnight and make a final reading the next morning, after the tubes are allowed to warm to room temperature. Positive and negative sera should be included with every run. A serum control (1:10 dilution of serum without antigen) must be included for each serum tested.

Verification test with 5.0% sodium chloride. A serum showing any reaction, even evidence of a slight precipitate in the serum control tube, is restudied by performing concurrently the presumptive procedure and the verification procedure. The verification test is performed in the same manner as the presumptive test, with the exception that 5% NaCl is used as diluent for serum and antigen.

Interpretation of results. The greatest dilution of serum giving a 2+ reaction indicates the end point for titer, providing the serum control has no precipitate. A 1+ reaction in the 1:10 dilution is considered doubtful (providing the serum control has no precipitate); if the serum control tube in a positive test contains interfering precipitate (when 5% NaCl is used as the diluent), the test is considered to be inconclusive.

LITERATURE CITED

1. CARPENTER, P. L. 1956. Immunology and serology, p. 96–118. W. B. Saunders Co., Philadelphia.
2. CUSHING, J. E., AND D. H. CAMPBELL. 1957. Principles of immunology, p. 241–315. Hill Books Co., New York.
3. DUBOS, R. J. 1958. Bacterial and mycotic infections of man, 3rd ed., p.123–124 and 767–768. J. B. Lippincott Co., Philadelphia.
4. FRANCIS, E., AND A. C. EVANS. 1926. Agglutination, cross agglutination and agglutinin absorption in tularemia. Public Health Rept. U.S. **41**:1273–1295.
5. KABAT, E. A., AND N. M. MAYER. 1961. Experimental immunochemistry, p.113–128. Charles C Thomas, Publisher, Springfield, Ill.
6. MUNOZ, J., AND E. L. BECKER. 1952. The use of chicken antiserum in immunochemical studies of edestine. J. Immunol. **68**:405–412.
7. RANSMEIER, J. C., AND C. L. EWING. 1941. The agglutination reaction in tularemia. J. Infect. Diseases **69**:193–205.
8. SMITH, D. T., AND N. F. CONANT. 1957. Zinsser bacteriology, 11th ed., p.151–153 and 905–906. Appleton-Century-Crofts, Inc., New York.
9. SMITH, D. T., D. S. MARTIN, N. F. CONANT, J. W. BEARD, G. TAYLOR, H. I. KOHN, AND M. A. POSTON. 1948. Zinsser's textbook of bacteriology, 9th ed., p. 937–938. Appleton-Century-Crofts, Inc., New York.