DETECTION OF BRUCELLAE AND THEIR ANTIBODIES BY
FLUORESCENT ANTIBODY AND AGGLUTINATION TESTS

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The laboratory diagnosis of brucellosis often is delayed because of the difficulty of isolating and
growing the organisms. Once isolated in pure cul-
ture, the brucellae can be identified by the agglu-
tination test, the standard method requiring 48 hr at 37 C. Similarly, antibodies from patients
with brucellosis can be detected with antigens of
brucellae. It was postulated that fluorescent
antibody techniques would facilitate the detec-
tion of smaller numbers of brucellae much earlier,
since relatively heavy concentrations of the or-
ganisms in pure culture would not be required.
Fluorescent antibody investigations with other bacteria have indicated that well defined condi-
tions for producing antibody which will be spe-
cific for the homologous organism must be set up
and the conditions of staining and interpretation
of the tests carefully investigated (Moody, Goldman, and Thomason, 1956; Thomason, Mood-
y, Ellis, and Updyke, 1958; Cherry and Freeman, 1959; Winter and Moody, 1959a, b; Moody and Winter,
1959). It has not been possible to predict re-
liably the specificity and suitability of a given
antiserum as a fluorescent antibody reagent merely because it is satisfactory as a reagent in
the agglutination or precipitin test.

The present report describes conditions
whereby fluorescent antibody reagents for the
laboratory diagnosis of brucellosis can be pre-
pared and used to detect either brucellae or their
antibodies in serum.

MATERIALS AND METHODS

Strains. Antisera were produced with three
species of reduced virulence obtained from the
Bureau of Animal Industry and designated
Brucella abortus (BAI), Brucella suis (BAI), and
Brucella melitensis (BAI). Additional strains were
obtained from Forrest Huddleston, Michigan
State University, W. Spink, University of
Minnesota, and Virginia Allen, Wisconsin State
Public Health Laboratories. The Jap strain of
Pasteurella tularensis was used for homologous
antiserum production.

Production of antisera for brucellae and P.
tularensis. Antigen for immunizing rabbits was
prepared as follows: Dehydrated trypticase soy
broth (BBL) was dissolved in \(\frac{1}{10}\) the required
volume (20 ml) of distilled water, placed in a
dialysis sac, and transferred to an Erlenmeyer
flask containing 180 ml of distilled water. After
autoclaving and allowing the medium to cool,
the liquid surrounding the cellophane bag of me-
dium was inoculated with organisms collected
from trypticase soy agar (BBL) slants which had
incubated overnight at 37 C. The flasks were
shaken overnight at 37 C and the cultures
checked for purity by Gram's method of staining.
Phenol (final concentration, 0.5%) was added and
the flasks held at 0 to 5 C for 48 hr or longer.
Sterility was ascertained on trypticase soy agar
and the cells collected by centrifugation and sus-
pended in a 0.85% NaCl solution containing 0.5% phenol.

Rabbits weighing 4 to 6 lb were injected in-
traperitoneally with approximately \(5 \times 10^8\)
brucellae over a period of 5 days. When the agglu-
tinin titers had reached at least 1:1,280, the
rabbits were bled by cardiac puncture and sera
of equivalent titer pooled. All sera were pre-
served with Merthiolate (1:10,000), quick-frozen
and stored in the CO\(_2\) box. Homologous agglutinin
titers determined on the sera in the undiluted
state, and in 1:5 and 1:10 dilutions made in
saline, were found to remain unchanged during
storage for at least 6 months in the CO\(_2\) box.

Antiserum for P. tularensis was prepared and
stored in the same manner, except that Snyder's
broth (Snyder et al., 1946) was used to grow the
cells.

Production of antihuman and antirabbit sera.
Antihuman serum was produced in rabbits using
antigen prepared by the method of Proom (1943).
This antigen consists of a residue of whole serum
after precipitation with alum (alum potassium
sulfate, crystal, baker’s analyzed, Al₂(SO₄)₃·K₃SO₄·2H₂O; mol wt, 948.76).

For immunization, 3 rabbits each were injected intramuscularly with 5 ml of antigen into each hind leg on the 1st and 15th days of the experiment. One milliliter of whole human serum was injected intravenously on the 40th day. By the 54th day, sera from all 3 rabbits had a precipitin titer of 1:51,200 using the antigen dilution method and a titer of 1:64 using the antiserum dilution method. Antisera produced by immunizing goats with rabbit globulin and whole serum were produced in a similar manner.

In some instances fluorescein isocyanate-labeled horse antihuman or sheep antirabbit globulin (Sylvana Chemical Company) were utilized successfully in indirect tests.

Agglutination tests. The agglutinin titers of brucellae antisera or their derivatives were determined by incubating mixtures of serum dilution and phenolized brucellae 2 hr at 37 C on a reciprocating shaker. Readings were taken immediately after shaking and again after refrigeration overnight. Slide agglutination tests were performed with similar dilutions of serum. Equal parts of serum dilution and antigen were mixed in a slide concavity and placed on a slowly rotating shaker at room temperature under a petri dish fitted with moist filter paper. Reactions were read microscopically at a magnification of 100× after incubation 15, 30, and 60 min. Formalin-killed antigens of heterologous organisms also were tested.

Precipitin tests. Precipitin titers were determined in capillary tubes using dilutions of antigen and undiluted antiserum. When an optimal dilution of antigen was obtained, this was tested with varying dilutions of antiserum. Precipitin tests were read after incubation for 30 min at 37 C and after refrigeration overnight. The tests were controlled using normal rabbit serum and human serum antigen.

Preparation of fluorescent antibody. Globulin fractions were obtained by precipitation of serum at half-saturation with ammonium sulfate at 0 to 5 C. The protein content of the globulin was determined by means of a biuret reaction (Gornall, Bardawill, and David, 1949). Globulin was labeled with fluorescein isocyanate (Coons and Kaplan, 1950) or fluorescein isothiocyanate using minor modifications of Riggs et al. (1958) or Marshall, Eveland, and Smith (1958). Conjugates were stored either frozen or preserved with Merthiolate (final concentration, 1:10,000) and held at 0 to 5 C.

Fluorescent antibody tests. (i) Direct tests: Smears of bacteria were stained with fluorescent antibody by the direct method previously described (Moody et al., 1956). (ii) Inhibition tests: Inhibition tests were performed by a one-step procedure (Moody et al., 1956; Goldman, 1957) which consists of staining smears with mixtures of unlabeled antiserum and fluorescent antibody of the same type. A positive reaction was indicated by absence of or reduced fluorescence intensity. (iii) Indirect tests: Indirect fluorescent antibody tests were performed generally according to the method of Weller and Coons (1954) and Carter and Liese (1958).

Negative controls in fluorescent antibody tests included the use of normal serum or its respective conjugate. Positive controls were included to verify the potency of reagents at the time of use. In any case, controls consistent with the objectives of individual experiments were included.

Fluorescence microscopy and photography. Smears were examined on a monocular microscope fitted with a cardioid darkfield condenser. Fluorescence intensity of the cells was estimated visually and recorded in graded plus values, 4+ indicating a maximal reaction and 1+, a very weak reaction. Stained organisms characteristically exhibited a peripheral fluorescence, the central portion of the cell remaining nonfluorescent. The light source was an Osram HBO-200 high pressure mercury vapor bulb housed in a Reichert assembly. The filter system consisted of a 2 mm Schott BG-12 filter placed between the light source and the specimen and a 2 mm OG-1 filter in the eyepiece. Photographs were obtained with Super Anscochrome film.

Preparation of unknowns simulating clinical and bacteriological specimens. Sensitivity and specificity tests were performed with artificially prepared unknowns by adding known concentrations of various species of viable bacteria to basic substrates, such as lettuce, broth, surface water, sputum, cotton swabs, and soil. The bacteria introduced into unknowns in various combinations and concentrations were as follows: Pseudomonas pseudomallei, Actinobacillus mallei, Bacillus anthracis, Pasteurella tularensis, Vibrio comma, Pasteurella pestis, Salmonella typhosa, Brucella suis, Pseudomonas aeruginosa, Pasteu-
rela pseudotuberculosis, Bacillus cereus, Shigella flexneri, staphylococci, and Paracolobactrum intermedium.

Trained investigators studied these unknowns by conventional methods, attempting to detect and identify the pathogens. Working as rapidly as possible, cultural, bacteriophage, animal inoculation, and serological tests were performed on concentrated suspensions of bacteria prepared from the specimens. The procedures used on the original specimens were repeated, after broth enrichment and subsequently on the pure cultures that were isolated.

Duplicate sets of unknowns were examined by fluorescein antibody techniques. Reagents specific for brucellae, salmonellae, P. pseudomallei, A. mallei, V. comma, P. pestis, and P. tularensis were used. The fluorescein antibody tests were performed on smears of the sediment obtained either from original specimens after centrifuging or from saline rinsings of solid specimens.

The time required to obtain both presumptive identification and completed tests was recorded.

RESULTS

Agglutinin titers of antisera for various bacterial species. The agglutinin titer was selected as an indicator for determining (i) the specificity of brucellae antisera and (ii) the effects of fractionating and labeling procedures upon antibody. Unadsorbed antiserum produced with each brucella species reacted with antigens of all 3 brucellae species, the highest titer usually being obtained with homologous combinations. The difference between homologous and heterologous combinations was never more than 4-fold by the tube method, but was as great 8-fold by the slide method. The only cross-reaction observed was that in which P. tularensis was agglutinated by B. melitensis antiserum diluted 1:40 to 1:80.

Identification of brucellae by direct fluorescein antibody tests. Representative strains of the 3 brucellae species were stained with fluorescein-labeled antiglobulin for B. suis. Saline suspensions of viable organisms or of nonviable organisms killed in various ways, e.g., 0.5% phenol or formalin, at 100 C for 1 hr, or autoclaving for 15 min at 121 C, were stained readily. Growing organisms under increased carbon dioxide tension did not influence the intensity of fluorescence. Controls consisting of smears stained with labeled normal rabbit globulin were negative.

Staining reactions were not demonstrated after removal of antibody by adsorption of the conjugate with heavy suspensions of formalin-killed B. suis. The fact that reactions were inhibited by antiserum for brucellae provided additional evidence for the specificity of the test.

In other experiments, fluorescein antibody for B. suis did not stain smears of 3 species of salmonellae, 2 species of shigellae, 5 strains of P. tularensis, and 1 strain each of A. mallei, P. pseudomallei, Pasteurella novicida, P. pestis, P. pseudotuberculosis, B. anthracis, B. cereus, P. aeruginosa, Staphylococcus aureus, and Serratia marcescens.

Effects of fractionation and labeling on antibody titer. Samples of 2 lots of antiglobulin for B. suis and 1 lot of B. melitensis were labeled with 5 different preparations of fluorescein isoioxyanate. Agglutinin titers dropped approximately 2-fold after fractionation of the antiserum for B. suis; no change was noted for the serum of B. melitensis. A 2- to 4-fold decrease in agglutinin titer occurred as a result of labeling with fluorescein isothioxyanate. Titers were not significantly different for conjugates labeled with the 5 different dye lots.

Brilliant staining reactions were demonstrated with homologous organisms using dilutions of 1:10 of conjugate regardless of the dye lot used for labeling the globulins. On the other hand, homologous agglutinin titers were 1:2,560 for conjugates of B. suis and 1:160 for the conjugate of B. melitensis.

Comparison of the sensitivity of agglutination and direct fluorescent antibody tests. Positive slide and tube agglutination tests performed with varying concentrations of pure cultures of B. suis, B. abortus, and B. melitensis and their homologous antisera were demonstrated by the tube method, provided the suspensions contained at least 70 to 90 million brucellae per ml, and by the slide method, 60 to 70 million. The sensitivity remained unchanged when standard suspensions of S. marcescens, V. comma, P. pseudomallei, or P. tularensis rather than saline were used for diluting the brucellae.

It was apparent that in clinical specimens considerable time would be required for propagation of brucellae to concentrations suitable for performing agglutination tests. To determine this, 1-ml amounts of heart infusion broth and heart infusion agar (Difco) were inoculated with vary-
ing numbers of *B. suis* and incubated at 37°C. Slide agglutination tests on the incubated broth were set up immediately and at various times after inoculation. Plates were examined at specified times ranging from 6 to 54 hr after inoculation and slide agglutination tests performed on colonial isolates emulsified in a small drop of saline. Organisms growing in broth were detectable within 24 to 30 hr provided the initial inoculum contained at least 2 million organisms, or within 48 hr if 200 cells were inoculated. Slightly longer incubation periods were required when agar was used.

The sensitivity of fluorescent antibody tests for detecting *B. suis* was demonstrated with smears of sediment from 1-ml volumes containing varying concentrations of brucellae or mixtures of brucellae and Serratia organisms. The average number of fluorescent bacteria observed in 15 microscopic fields was determined. Brucellae were detectable in smears of pure cultures containing as few as $2.5 \times 10^6$ cells per ml. The presence of Serratia organisms in a ratio of $6.8 \times 10^4$ to 1 brucella cell did not affect the sensitivity.

These experiments showed generally that at least $6.0 \times 10^7$ brucellae per ml of suspension must be present to obtain a positive agglutination reaction, whereas suspensions containing $2.5 \times 10^6$ cells per ml are satisfactory for detection by fluorescent antibody. In either case the level of specificity was equivalent.

*Detection of B. suis in artificially prepared unknowns by both conventional and fluorescent antibody tests.* Among the 37 unknowns tested, 10 possibilities existed for detection of brucellae. Three specimens were reported positive by conventional tests and 6 by fluorescent antibody tests. Brucellae could not be demonstrated by conventional tests earlier than 96 hr, the time required for propagating sufficient organisms to obtain positive agglutination reactions with pure or contaminated cultures. The 6 positive fluorescent antibody reactions were obtained within 1 hr, the time required for centrifuging the specimen, staining smears of the sediment, and examining on the fluorescence microscope. No false positive results were reported by either method. Brucellae were detected in fluorescent antibody tests only when at least $6.5 \times 10^4$ cells were present.

The experiment demonstrated that fluorescent antibody tests were superior to conventional tests with respect to sensitivity and rapidity. Reliable results were obtained directly and immediately by fluorescent antibody tests in 6 out of 10 cases. It is likely that more exhaustive concentration methods would have resulted in more positive fluorescent antibody tests, since results with pure cultures showed that brucellae could be detected in suspensions containing as few as $2.5 \times 10^6$ cells per ml of broth.

Detection of brucella antibody in serum by inhibition and indirect fluorescent antibody tests by agglutination reactions. Previous experiments demonstrated that brucellae could be identified accurately with specific labeled antiglobulin. Conversely, it was possible to titrate brucella serum antibody by fluorescent antibody techniques. Preliminary experiments showed that either inhibition tests or indirect fluorescent antibody tests gave observable end points with varying dilutions of unlabeled serum tested with phenol-killed brucellae. In general, highest titters were demonstrated in the agglutination test and the lowest by indirect fluorescent antibody tests. Titters obtained by inhibition tests were similar to agglutinin titers. No positive reactions were demonstrated with sera lacking brucella antibody, with perhaps one exception in which a 1:10 dilution of *P. tularensis* rabbit antiserum reacted in the agglutination test.

These experiments indicated that either inhibition or indirect fluorescent antibody tests might be utilized as rapid methods for demonstrating brucella antibody titers in serum of man or lower animals. If standard methods of incubation (48 hr) are used for agglutination tests, the fluorescent antibody tests offer the important advantage of decreased time required to obtain positive results.

**DISCUSSION**

A laboratory diagnosis of brucellosis normally requires many days or weeks. The organism grows slowly on culture media and frequently requires increased CO$_2$ tension for primary isolation. Although it can be identified with a high degree of accuracy by agglutination tests, heavy cell concentrations are necessary to demonstrate a positive reaction. The presence of contaminants and environmental debris seriously affect the reading of such tests. Brucella antibody can be demonstrated in the serum of brucellosis patients by agglutination tests; however, a 48-hr incubation
The present study describes several conditions whereby fluorescent antibody techniques may be used to detect and identify either the brucellae or their serum antibody. It was possible to prepare fluorescein-labeled antibrucella globulin which stained organisms in smears made from suspensions of B. abortus, B. melitensis, and B. suis. Complete cross-reactions occurred among the 3 species, and these were analogous to those demonstrated by agglutination tests. The specificity of fluorescent antibody reactions for brucellae also was established by the facts that (i) inhibition of the reaction was demonstrated only with brucella antiserum, (ii) adsorption of antibody with brucellae removed homologous staining reactions, and (iii) staining was absent among other bacterial species in the direct test.

It was demonstrated that brucellae can be detected with fluorescent antibody in smears made from suspensions containing as few as \(2.5 \times 10^3\) brucellae per ml, whereas suspensions containing at least \(6.0 \times 10^5\) brucellae per ml were necessary to demonstrate positive agglutination reactions. The fact that massive bacterial and environmental debris did not affect seriously the sensitivity of the reactions is decidedly advantageous. This higher degree of sensitivity of the fluorescent antibody test made it possible to detect and identify individual brucellae in artificially prepared unknowns within 1 hr. Identification by cultural isolation and agglutination tests required at least 72 to 96 hr, provided agglutination tests were read after incubation for 2 hr rather than 48 hr. The results of fluorescent antibody tests were obtained without enrichment, whereas conventional tests were dependent upon enrichment and usually isolation of the organism in pure culture.

Brucellae were not detected by fluorescent antibody in 4 out of 10 unknowns to which they had been added. In each case the total number of organisms present approached the borderline of sensitivity of the technique as demonstrated in pure cultures. It is possible that the substrate of the unknown prevented effective removal and concentration of the organisms. In 7 of 10 unknowns containing brucellae, the organisms were not detected by conventional procedures, even though methods of concentration were similar to those employed for fluorescent antibody tests. In no instance were false positive results of either test reported in the study of 37 unknowns.

The sensitivity and rapidity of fluorescent antibody techniques indicated that they may be useful in detecting brucellae in either environmental or clinical materials.

It was possible to demonstrate brucella antibody in rabbit antiserum and in human serum obtained from patients with confirmed brucellosis by agglutination tests or by inhibition or indirect fluorescent antibody tests. Highest titers were demonstrated by agglutination and the lowest, by indirect fluorescent antibody tests. Inhibition test titers were similar to, but slightly lower than, agglutinin titers. Specificity of inhibition and indirect fluorescent antibody tests appears to be of the same level as that of agglutination tests. Thus, either kind of test, with proper controls, may serve to identify not only the brucella antibody in serum, but also the brucellae themselves.

The use of fluorescent antibody tests to titrate brucella antiserum may have practical value in that reactions could be obtained earlier than those obtained by agglutination tests.

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**SUMMARY**

Conditions are described for preparing antigens and antisera and for performing three kinds of fluorescent antibody tests for detecting brucellae and their antibodies in various kinds of materials. The sensitivity and specificity of the fluorescent antibody tests were compared with that of cultural and agglutination techniques. It was demonstrated that positive fluorescent antibody reactions were demonstrable with smears of suspensions containing as few as \(2.5 \times 10^3\) viable or nonviable brucellae per ml. The presence of massive bacterial or environmental contamination did not appear to affect the sensitivity or
specificity of the tests. Demonstration of positive agglutination reactions required suspensions containing no less than 6.0 × 10^6 cells per ml. Higher serum antibody titer were obtained by agglutination and fluorescent antibody inhibition tests than by indirect fluorescent antibody tests.

REFERENCES


