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RAPID IDENTIFICATION OF *FRANCISELLA TULARENSIS* BY A FLUOROGENIC ENZYME IMMUNOASSAY

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Y.M. Siddiqui, R.E. Fulton, M.H. Knodel and A.R. Bhatti

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ABSTRACT

A highly sensitive fluorogenic enzyme-linked immunosorbent assay (FELISA), which utilizes nitrocellulose membranes as solid phase support and a fluorogenic substrate to indicate the antigen, has been adapted for the rapid identification of *Francisella tularensis*. Multiple samples were assayed in approximately 6 h by this method. The sensitivity achieved in a four layer "sandwich" assay format was 10 femtogram mL^{-1} of outer membrane protein and 100 colony forming units mL^{-1} of *Francisella tularensis* whole cells. The assay was highly specific for the detection of homologous and heterologous strains of *Francisella tularensis* while unrelated bacteria, including *Brucella* strains, exhibited no cross-reactivity.

Key words: *Francisella tularensis*; outer membrane protein; rapid identification; fluorogenic enzyme immunoassay, nitrocellulose membranes

RÉSUMÉ

Une méthode FELISA hautement sensible qui fait appel à des membranes de nitrocellulose comme support de la phase solide et à un substrat fluorogène pour révéler que l'antigène a été adapté pour l'identification rapide de *Francisella tularensis*. De nombreux échantillons ont été testés à l'aide de cette méthode en l'espace d'environ 6 heures. La méthode dans sa version "sandwich" à quatre couches a permis d'obtenir une sensibilité de 10 femtogrammes par mL^{-1} dans le cas de la protéine de la membrane externe et de 100 UFC dans le cas des cellules entières de *Francisella tularensis*. La méthode s'est révélée hautement spécifique pour la détection des souches homologues et hétérologues de *Francisella tularensis*, tandis qu'on n'a pas observé de réaction croisée avec des bactéries non apparentées, y compris les souches de *Brucella*.

Mots clés: *Francisella tularensis*; protéine de la membrane externe; FELISA; immunotitrage enzymatique; membranes de nitrocellulose.

INTRODUCTION

Francisella tularensis is a gram-negative bacterium which causes tularemia, an acute, infectious zoonotic disease with clinical manifestations which may range from ulceroglandular, pneumonic or typhoidal, depending upon the route of infection. The organism can gain entry through skin abrasions, conjunctivae, by ingestion or inhalation and a very small dose (10 - 50 organisms) is infectious. These features, which make this organism exceptionally dangerous, account for its potential as a biological warfare (BW) agent.

Detection and identification of *F. tularensis* has been achieved by a number of methods including immunofluorescence (1,2) and agglutination (3). The latter technique requires high titered antiserum and a high concentration of bacterial cells. Within a clinical setting, the organism may be grown in sufficient quantity to meet these requirements but, in a BW scenario, the quantity of bacteria disseminated in air or water is likely to be very low and more sensitive methods are required. Enzyme-linked immunosorbent assay (ELISA), a highly sensitive method, has been used to detect sonicated cultures of *F. tularensis* (4,5), phenol extracts of *F. tularensis* (6) and *F. tularensis* whole cells (7).

In this study, we have adapted the fluorogenic ELISA (FELISA), described by Fulton et al. (8), for the detection and identification of *F. tularensis*. We have compared the sensitivity of an "indirect" FELISA in which antigens, consisting of *F. tularensis* whole cells or outer membrane protein (OMP) extracted from whole cells, were absorbed directly on nitrocellulose membranes, to that achieved in a "sandwich" format which utilized antibody to *F. tularensis* to capture the antigens. The sensitivity achieved in the "sandwich" format was 10 femtogram (fg) mL^{-1} of OMP and 100 CFU mL^{-1} of *F. tularensis* whole cells.

MATERIALS AND METHODS

Chemicals and Reagents

Alkaline phosphatase-labelled goat anti-mouse IgG and goat anti-rabbit IgG, bovine serum albumin fraction V (BSA) 4-methylumbelliferyl phosphate (4-MUP), Freund's incomplete adjuvant, and diethanolamine (DEA) were purchased from Sigma Chemical Company (St. Louis, Mo). Polyoxythylene sorbitan monolaurate (Tween-20) was purchased from Bio-Rad Laboratories, (Richmond, Ca). Ammonium sulfate and magnesium chloride were purchased from J.T. Baker (Phillipsburg, NJ) and phosphate buffered saline (PBS) tablets (Dulbecco A) were obtained from Oxoid Canada Ltd. (Ottawa, Ont.). Rabbit *F. tularensis* antiserum (Sylvana), for use in the agglutination test and in the FELISA, was purchased from GIBCO/BRL (Burlington, Ont.). For use as capture antibody in the "sandwich" FELISA, this antiserum was purified by ammonium sulfate precipitation by standard techniques (9) and dialysed at 4°C against several changes of PBS.

Preparation of Formalinized Bacterial Cultures

i. *Francisella tularensis*

The live vaccine strain (LVS) of *F. tularensis* was obtained from Dr. F. Jackson, Department of Microbiology, Faculty of Medicine, University of Alberta (Edmonton, Alta). The strain was originally grown in cysteine glucose broth (CGB) (Difco Laboratories, Detroit, Mi), supplemented with 1.5% agar.

For the preparation of OMP and formalinized whole cells, cultures were grown on a chemically defined synthetic medium (10)

containing 1.5% agar. After overnight incubation at 37°C in an atmosphere of 5% CO_2 in air, the bacterial cells were dislodged from the medium surface by agitation with saline and sterile glass beads. A 0.1% inoculum of these cells was used to prepare starter broth cultures, which were incubated for 18 h at 37°C in an atmosphere of 5% CO_2 in air in a New Brunswick environmental shaker (Fisher Scientific, Calgary, Alta.). Each of two 500 mL Erlenmeyer flasks, containirg 100 mL of synthetic medium, was then inoculated with 0.1 mL of an 18-h starter culture and incubated for 18 h at 37°C, as described above. To determine viable cell counts (CFU mL^{-1}), culture aliquots were diluted in tryptic soy broth (TSB) and the dilutions inoculated in triplicate on plates containing synthetic medium supplemented with 1.5% agar. After overnight incubation at 37°C in an atmosphere of 5% CO_2 in air and 60% relative humidity (RH), colonies were counted and the viable cell count was determined to be approximately 2×10^{10} CFU mL^{-1}.

For preparation of formalinized cells, cultures were centrifuged at 15,380 g for 20 min at 4°C in a J2-21 centrifuge (Beckman Instruments, Fullerton, Ca) and each pellet was suspended in 100 ml of physiological saline. Formaldehyde solution was then added to achieve a final concentration of 2.4%. The formalinized cultures were held at 4°C with slight agitation for 48 h, then centrifuged at 15,380 x g for 20 min at 4°C. The resulting pellets were resuspended in 100 mL of fresh 2.4% formalin-saline and incubated with constant stirring at 4°C for 1 wk. To test for viability, aliquots of formalinized bacterial suspensions were inoculated on plates containing synthetic medium supplemented with 1.5% agar and incubated for four days at 37°C in an atmosphere of CO_2 in air and at 60% RH. No colonies of *F. tularensis* were detected. Prior to the FELISA, bacterial suspensions were washed several times in PBS to remove residual formalin and the viability check was repeated.

ii. *Francisella tularensis* Heterologous Strains

Formalin-killed cultures of *F. tularensis* (U.K. designated: 29A, 64, 119, 120, 121, 122, 123, 124, 125, 126) were kindly provided by Dr. A.P. Phillips, Chemical Defence Establishment (Porton, U.K.). Total cell counts mL^{-1}, obtained by counting formalinized cultures in a counting chamber, were determined by Dr. Phillips. Differences between total counts of formalinized cells and viable cell counts (CFU mL^{-1}), taken on young cultures, were minimal and not considered to be significant (Dr. Phillips, personal communication). Therefore, for the purposes of this study, total cell counts were equated with viable cell counts.

iii. Unrelated Bacteria

The following Gram-negative and Gram-positive bacteria were used in this study. Gram negative: *Salmonella typhi* ATCC 19430, *Escherichia coli* 152, *Serratia marcescens* ATCC 13880, *Aerobacter aerogenes* A1, *Neisseria gonorrhoeae* LCDC 73049, *Neisseria meningitidis* DRES 03, *Yersinia pestis* ATCC 19428, and *Pseudomonas aeruginosa* ATCC 14209; Gram positive: *Bacillus subtilis var niger* ATCC 9372. Each of these cultures was grown by rehydrating a vial of lyophilized cells in 10 mL of TSB and incubating overnight at 37°C in an atmosphere of 5% CO_2 in air. One mL of each of these cultures was then inoculated into separate flasks, each containing 100 mL of TSB and incubated in an environmental shaker incubator in an atmosphere of 5% CO_2 in air and at 60% RH for approximately 24 h at 37°C. Viable cell counts (CFU mL^{-1}) were determined for each of the strains by inoculating appropriate dilutions of the bacterial cultures onto TSA plates and incubating overnight, as previously described.

Cultures were inactivated by the addition of formaldehyde to give a final formalin concentration of 2.4%, then incubated at 4°C with slight agitation for two days. Viability tests were performed by inoculating TSA plates with aliquots of each of the formalinized bacterial suspensions. Cultures were centrifuged at 7,750 g for 15 min at 4°C and washed several times with PBS before suspending the respective pellets in the original volume (100 mL) of PBS.

Yersinia pestis was grown under identical conditions, except at a temperature of 27°C.

Formalinized preparations of two strains each of *Brucella abortus*, *Brucella suis* and *Brucella melitensis* were kindly provided by Dr. L. Forbes, Animal Pathology Laboratory, Agriculture Canada (Saskatoon, Sask.). The CFU mL^{-1} counts for each of the strains were determined by Dr. Forbes.

Preparation of *Francisella tularensis* OMP

The OMP of *F. tularensis* was prepared using a modification of procedures described by Ormsbee et al. (1) and Prochazka and Dubanska (12). Five L of chemically defined media were inoculated with 50 mL of an 18-h culture of *F. tularensis*, prepared as previously described, and incubated for 18 h in a shaker incubator at 37°C in an atmosphere of 5% CO_2 in air. The culture was centrifuged at 15,380 g for 20 min at 4°C and the pellet (approximately 13 g in wet weight) was suspended in 250 mL of sterile glass distilled water. Sufficient absolute ether was then added to bring the final volume to 500 mL. This suspension was stirred overnight at room temperature in a fume hood and, on the following morning, was

centrifuged at 6,670 g for 60 min at 4°C. The cell pellet was discarded and the aqueous phase was recentrifuged at 15,380 g for 15 min. In order to remove any traces of ether, the resulting supernate was dialysed for three days against several changes of glass distilled water.

Solid ammonium sulfate was added to the supernate at 4°C to achieve a final concentration of 2.3 M and the mixture was incubated, with continuous slow stirring, overnight at 4°C. The solution was then centrifuged at 9,950 g for 30 min at 4°C and the resulting pellet dissolved in 25 mL of 15 mM Tris-HCl buffer, pH 8.0 and dialysed for 24 h against four changes of the same buffer. The precipitation step was repeated using 3.0 M ammonium sulfate (final concentration). After overnight stirring at 4°C, the solution was centrifuged at 9,950 g for 30 min at 4°C. The resulting pellet was dissolved in 10 mL of 15 mM Tris-HCl buffer, pH 8.0, containing 15 mM NaCl and dialysed for 48 h against frequent changes of the same buffer. The dialysate was slightly brown and cloudy but a clear supernate was collected after centrifugation at 15,380 g for 1 h at 4°C. This supernate was the source of soluble OMP used for immunological characterizations.

Immunization of Mice

Hyperimmune ascites fluids against *F. tularensis* OMP and whole cells were produced using a modification of methods described by Brandt et al. (13), Russel et al. (14) and Chiewsilp and McCown (15). Briefly, the protocol for production of ascites to whole cells was as follows. Five wk-old BALB/C female mice (Charles River, St. Constant, Que.) were injected intraperitoneally (i.p.) with a 1:1 suspension of formalinized cells of *F. tularensis* (approximately 10^8 CFU) in incomplete Freund's adjuvant. Prior to

injection, the formalinized cells were washed thoroughly in PBS, to eliminate any traces of formalin. Animals were subsequently boosted, i.p., at 1 wk intervals for a period of 4 wk with a 1:1 suspension of formalinized cells of *F. tularensis* (approximately 10^9 CFU) in Freund's incomplete adjuvant. The serum antibody titer was determined by an "indirect" FELISA (described below) in which antigen (OMP or whole cells), immobilized on nitrocellulose solid phase supports, was used to capture antibody in dilutions of mouse serum. Once a satisfactory and stable antibody titer had been obtained (approximately after 5 wk), the mice were administered, i.p., S-180 mouse sarcoma cells (American Type Culture Collection, Rockville, Md), to initiate the production of ascites fluids. Approximately 1 wk following injection of sarcoma cells, ascites fluids were harvested by paracentesis.

Immune ascites fluids to OMP were produced in a similar manner. Mice were injected, i.p., with a 1:1 mixture of 100 µg of OMP and adjuvant. Booster doses, however, were administered without adjuvant.

Immunoglobulins were purified from hyperimmune ascites fluids by a procedure described by Volk et al. (16).

Protein Estimation

Because of their high degree of comparability, two methods of protein determination, a micro-Lowry procedure (17, 18) and a bicinchoninic acid (BCA™) protein assay (Pierce Chemical Company, Rutherford, Il) were used interchangeably.

Immunoassay Plates

Millititer™-HA immunoassay plates and the Millititer™ filtration system were purchased from Millipore Corporation (Bedford, Mas). The bottom surface of the wells of these 96-well polystyrene plates is constructed of nitrocellulose membrane with a pore size of 0.45 μm.

Immunoassay Procedures

Immunoassay procedures were carried out using either a three layer "indirect" format or a four layer "sandwich" format. In the "indirect" format, antigen to be detected was applied directly to the nitrocellulose membrane solid phase, whereas, the "sandwich" format utilized antibody immobilized on the solid phase to capture antigen. In both methods, the presence of antigen was detected with unlabelled specific antibody, prepared in an animal species different from that used for preparation of the capture antibody (19) and indicated with enzyme-labelled anti-species antibody and a fluorogenic substrate.

Prior to use, the wells of immunoassay plates were washed three times with PBS, pH 7.4, in a Millititer™ filtration system. All incubations with immune reagents were accomplished at 37°C in a volume of 50 μL. Prior to and during the immunoassay procedures, a volume of 200 μL of PBS was used for washing the wells and removing unbound reagents. Immunoreagents were diluted in blocking buffer consisting of PBS, containing 2% BSA and 0.1% Tween-20, with the exception that, capture antibody in the "sandwich" format and antigen (OMP or whole cells) in the "indirect" format were prepared in coating buffer (0.05M carbonate - bicarbonate buffer, pH 9.6, containing 0.02% sodium azide).

Each of the reagents was standardized prior to the performance of sensitivity studies. To determine the optimal concentration of capture antibody required for saturation of nitrocellulose membranes for the "sandwich" FELISA, rabbit anti-*F. tularensis* antibody (diluted in coating buffer) was titrated with the optimal working dilution (1:1000) of alkaline phosphatase-labelled anti-rabbit IgG. After the washing step, substrate (4-MUP) was added and the relative fluorescence was determined. The optimal working dilutions of the detector and indicator antibodies were determined by checkerboard titration.

Relative fluorescence of the enzyme-substrate reaction product was measured directly on Millititer™-HA plates by a MicroFluor fluorometer (Dynatech Laboratories, Alexandria, Va) fitted with 365 nm and 450 nm filters for excitation and emission, respectively. Results were considered positive if the mean fluorescence reading was equal to or greater than two standard deviations above the mean readings of the negative control. This is equivalent to a 95% confidence limit.

"Indirect" FELISA

After the plates had been washed with PBS and the bottom surfaces blotted dry, 50 µL of log dilutions of antigen (OMP or whole cells), suspended in coating buffer, were added to the wells and the plates were incubated overnight at 4°C. Excess or unbound antigen was removed by washing the wells with PBS and the remaining active sites were blocked by incubating plates with blocking buffer (200 µL per well) at 37°C for 1 h. Wells were washed once with PBS and plates were reincubated with fresh blocking buffer for 1 h. This step was carried out two times. The wells were washed once with PBS, then mouse

hyperimmune anti-*F. tularensis* immunoglobulins, diluted 1:2000 in blocking buffer, were added to the wells and the plates incubated for 1 h. Wells were washed three times with PBS and the detecting antibody (alkaline phosphatase-labelled anti-mouse IgG), diluted 1:1000 in blocking buffer, was added and the plates incubated for 1 h. The wells were washed six times with PBS containing 0.05% Tween-20 and, after the plate bottom had been blotted dry, 200 µL of substrate (4-MUP), at a concentration of 10^{-4}M in 10% DEA buffer, pH 9.8, was added to each well. Plates were incubated at room temperature in the dark and the relative fluorescence was measured at 5, 10 and 15 minute intervals following the addition of substrate.

"Sandwich" FELISA

Wells were washed and dried, as previously described, then incubated overnight at 4°C with 50 µL of the optimal dilution (20 µg mL^{-1} in coating buffer) of rabbit anti-*F. tularensis* antibody. Wells were then washed with PBS and blocked with blocking buffer, as previously described. Fifty µL of serial log dilutions of antigen (OMP or whole cells), prepared in blocking buffer, were added to triplicate wells and the plates were incubated at 37°C for 1 h. Subsequent steps were as described for the "indirect" FELISA.

RESULTS

Optimum Concentration of Capture Antibody

The optimum concentration of capture antibody to saturate nitrocellulose membranes for the "sandwich" FELISA was determined by titrating log dilutions of rabbit anti-*F. tularensis* antibody with the optimal dilution of phosphatase-labelled anti-rabbit IgG.

Fluorescence counts increased with the addition of capture antibody to a concentration plateau, beyond which, the counts did not increase with further additions of capture antibody (Fig. 1). The optimum concentration of capture antibody required to saturate the solid phase was determined from the curve to be 20 μg mL^{-1}.

Sensitivity of "Sandwich" FELISA

Capture antibody-sensitized and blocked immunoassay plates were challenged with log dilutions of *F. tularensis* OMP and whole cells, respectively. By this procedure, the detection limit for OMP was determined to be 10 fg mL^{-1} (500 ag per test volume) (Fig. 2). The lower limit of concentration of *F. tularensis* whole cells detected was 100 CFU mL^{-1} (5 CFU per test volume) (Fig. 3).

Sensitivity of "Indirect" FELISA

Log dilutions of *F. tularensis* OMP and whole cells, respectively, were immobilized directly on nitrocellulose membranes and the detection limits for each determined. The lower limit of detection for OMP was 10 ng mL^{-1} (500 pg per test volume) (Fig. 4). The lower limit of test sensitivity for *F. tularensis* whole cells was 10^6 CFU mL^{-1} (50,000 CFU per test volume) (Fig. 5). The "indirect" procedure was thus 10^6 times less sensitive than the "sandwich" procedure for detection of OMP and 10^4 times less sensitive for the detection of whole cells.

Specificity of "Sandwich" FELISA

i. Heterologous Strains of *F. tularensis*

The specificity of the "sandwich" FELISA was investigated by challenging the system with the homologous and 10 heterologous strains

of *F. tularensis* whole cells (formalinized). The OMP for these strains was not available for testing. The homologous and all 10 heterologous strains were positive in the assay (Fig 6).

ii. Unrelated Bacteria

The specificity of the "sandwich" FELISA was further investigated by challenging the system with formalinized whole cells of unrelated bacteria (eight Gram-negative and one Gram-positive). A plot of the ratio of positive to background fluorescence counts indicated that the homologous bacterium (*F. tularensis*) was positive in the assay while unrelated bacteria reacted only at baseline control level (Fig. 7).

It has been reported that *F. tularensis* and the *Brucella sp.* share common cross-reacting antigens (2,3). To determine whether *F. tularensis* and the *Brucella sp.* cross-reacted by FELISA, two strains each of three *Brucella sp. (abortus, suis* and *melitensis)* were used to challenge the "sandwich" FELISA. The FELISA was specific for *F. tularensis* and did not detect any cross-reacting antigens in the Brucella strains tested (Fig. 8).

DISCUSSION

In this paper, we have presented an adaptation of the FELISA, originally described by Fulton et al. (8), for the rapid detection and identification of *F. tularensis*. The technique utilizes nitrocellulose membranes as solid phase support to achieve high-capacity protein binding, a high energy fluorogenic substrate for enhancement of sensitivity, and microtiter assay format for convenience.

The use of nitrocellulose membranes for the adsorption of proteins was introduced over two decades ago (20). Since surface proteins (glycoprotein or nucleoprotein) adhere to nitrocellulose, this membrane has been used to concentrate viruses (21). As reported by Towbin et al. (22) and Newmann and Wilson (23), proteins can be electrophoretically transferred to nitrocellulose membranes from polyacrylamide gels, a technique known as Western blotting. A dot blot technique, developed by Hawkes et al. (24), established that proteins could be efficiently detected when spotted directly on nitrocellulose. In addition, a number of investigators have recently reported the use of nitrocellulose membrane in immunoassays (8, 25, 26, 27, 28). The major advantage in the use of nitrocellulose as solid phase in immunoenzymatic techniques is its high adsorptive capacity for proteins. Close to 100% of the applied protein sample has been shown to bind to nitrocellulose membranes. By contrast, conventional plastic microtiter wells bind protein inefficiently (less than 8% of that applied) (29). The enhanced binding of proteins to nitrocellulose can, in part, be attributed to the large surface area available for adsorption. Whereas adsorption to standard polystyrene microtiter wells occurs on the surfaces only, adsorption on nitrocellulose membranes occurs both on and within the membrane pore matrix, thus providing an extremely large surface area for attachment.

The use of fluorogenic substrates for detection of macromolecular antigens by immunoassay techniques has also been documented (30, 31, 32, 33, 34, 35). The high sensitivity of fluorometric compared to colorimetric methods was recognized as early as 1948 (36) and it has been reported that a theoretical 100-1000-fold increase in sensitivity can be achieved using fluorometric rather than colorimetric detection methods (30, 37, 38). For example, 4-MUP, the substrate used in this study, is hydrolyzed by alkaline phosphatase to 4-methylumbelliferone

which is detectable at a concentration of 10^{-8}M; by comparison, p-nitrophenol phosphate, a chromogenic substrate, is hydrolyzed by the same enzyme to its product (p-nitrophenol) which is detectable at a concentration limit of 10^{-5}M (30).

The FELISA described in this paper for the detection and identification of *F. tularensis* is an adaptation of the FELISA described by Fulton et al. (8) for detection and identification of Newcastle Disease Virus (NDV). As expected, the lower limits of test sensitivity for the detection of *F. tularensis* OMP correlated well with the detection limits reported for NDV protein. The lowest concentration of both *F. tularensis* OMP and NDV protein detectable by "sandwich" FELISA was 10 fg mL^{-1} (500 ag per test volume). The "indirect" FELISA detected 10 ng mL^{-1} of *F. tularensis* OMP, compared with a detection limit for NDV protein of 100 pg mL^{-1}.

The lower limit of test sensitivity of the "sandwich" FELISA was one million times greater than that of the "indirect" FELISA for the detection and identification of *F. tularensis* OMP. Similarily, the "sandwich" FELISA was 00,000 times more sensitive than the "indirect" FELISA for detection and identification of *F. tularensis* whole cells. The enhanced sensitivity of the "sandwich" format may be attributed to amplification of the fluorescence signal as a result of the use of an additional layer (capture antibody) on the solid phase. Enhanced sensitivity of "sandwich" over "indirect" methods was also observed in the FELISA for NDV (8).

The specificity of the FELISA was evaluated by challenging the "sandwich" assay with heterologous strains of *F. tularensis* and with unrelated bacteria, including strains of *Brucella sp.*

As expected, antisera to the LVS (homologous) cross-reacted by FELISA with other (heterologous) strains of *F. tularensis*, suggesting common epitopes, but did not cross-react with unrelated bacteria, including *Brucella sp.* It has been reported that *F. tularensis* shares somatic antigens with both *B. abortus* and *B. melitensis* and this cross reactivity is observed in agglutination reactions (1,3,4). However, no cross-reactivity was observed between *F. tularensis* (LVS) and *B. suis*, *B. melitensis* or *B. abortus* by "sandwich" or by "indirect" FELISA.

A FELISA utilizing nitrocellulose membranes as solid phase support has been adapted for the rapid identification of *F. tularensis*. The assay was highly sensitive with a detection capability, in the "sandwich" format, of 100 CFU mL^{-1} of whole cells and 10 fg mL^{-1} of extracted OMP. This sensitivity is one million times greater than that typically achieved in conventional ELISA performed on polystyrene supports and with chromogenic enzyme - substrate detection systems (39). The assay was also highly specific for the detection and identification of *F. tularensis* and exhibited no cross-reactivity with unrelated bacteria. Assay procedures were easy to perform and, once plates had been sensitized with capture antibody and blocked with blocking reagent, the remaining steps were completed in approximately 3 h. Tests were performed in commercially procurable 96-well nitrocellulose and polystyrene combination plates for convenient assay of multiple samples. Readings were quantitated in a microprocessor-controlled microfluorimeter, thus eliminating the potential for operator bias.

Fig. 1 DETERMINATION OF OPTIMAL CONCENTRATION OF CAPTURE ANTIBODY ON NITROCELLULOSE MEMBRANES. Varying concentrations of rabbit anti-*F. tularensis* antibody were immobilized on nitrocellulose membranes and titrated fluorometrically with the optimal dilution of phosphatase-labelled goat anti-rabbit IgG. Data points are the mean of triplicate determinations on a single plate. Error bars represent standard deviation of the mean.

Fig. 2 SENSITIVITY OF "SANDWICH" FELISA FOR DETECTION OF *F. TULARENSIS* OMP. Varying concentrations of OMP (10^{-5} to 10^{-15} g mL^{-1}) were titrated by "sandwich" FELISA and fluorescence counts determined. Data points are the mean of triplicate determinations on a single plate. Error bars represent standard deviation of the mean.

Fig. 3 SENSITIVITY OF "SANDWICH" FELISA FOR DETECTION OF *F. TULARENSIS* WHOLE CELLS. Varying concentrations of *F. tularensis* whole cells (10^{9} to 10^{1} CFU mL^{-1}) were titrated by "sandwich" FELISA and fluorescence counts determined. Data points are the mean of triplicate determinations on a single plate. Error bars represent standard deviation of the mean.

Fig. 4 SENSITIVITY OF "INDIRECT" FELISA FOR DETECTION OF *F. TULARENSIS* OMP. Varying concentrations of OMP (10^{-4} to 10^{-12} g mL^{-1}) were titrated by "indirect" FELISA and fluorescence counts determined. Data points are the mean of triplicate determinations on a single plate. Error bars represent standard deviation of the mean.

Fig. 5 SENSITIVITY OF "INDIRECT" FELISA FOR DETECTION OF *F. TULARENSIS* WHOLE CELLS. Varying concentrations of

F. tularensis whole cells (10^9 to 10^1 CFU mL^{-1}) were titrated by "indirect" FELISA and fluorescence counts determined. Data points are the mean of triplicate determinations on a single plate. Error bars represent standard deviation of the mean.

Fig. 6 SPECIFICITY OF "SANDWICH" FELISA: *F. TULARENSIS* HETEROLOGOUS STRAINS. Log dilutions (10^9 to 10^7 CFU mL^{-1}) of the homologous (LVS) and 10 heterologous strains of formalinized *F. tularensis* whole cells were titrated by "sandwich" FELISA and fluorescence counts determined. Negative FELISA control consisted of dilutions of blocking buffer only. Data points are the mean of triplicate determinations on a single plate. Error bars represent standard deviation of the mean.

Fig. 7 SPECIFICITY OF "SANDWICH" FELISA: UNRELATED BACTERIA. Log dilutions (10^{10} to 10^4 CFU mL^{-1}) of the homologous bacterium and nine unrelated bacteria were titrated by "sandwich" FELISA and fluorescence counts determined. Negative FELISA control consisted of blocking buffer only. Fluorescence count (FC) is the mean of triplicate test values on a single plate; background count (BC) is the mean of triplicate negative control values plus two standard deviations.

Fig. 8 SPECIFICITY OF "SANDWICH" FELISA: UNRELATED BACTERIA: *BRUCELLA SP.* Log dilutions (10^6 to 10^4 CFU mL^{-1}) of the homologous bacterium and two strains each of three Brucella *sp.* were titrated by "sandwich" FELISA and fluorescence counts determined. Negative FELISA control consisted of blocking buffer only. Data points are the mean of triplicate determinations on a single plate. Error bars represent standard deviation of the mean.

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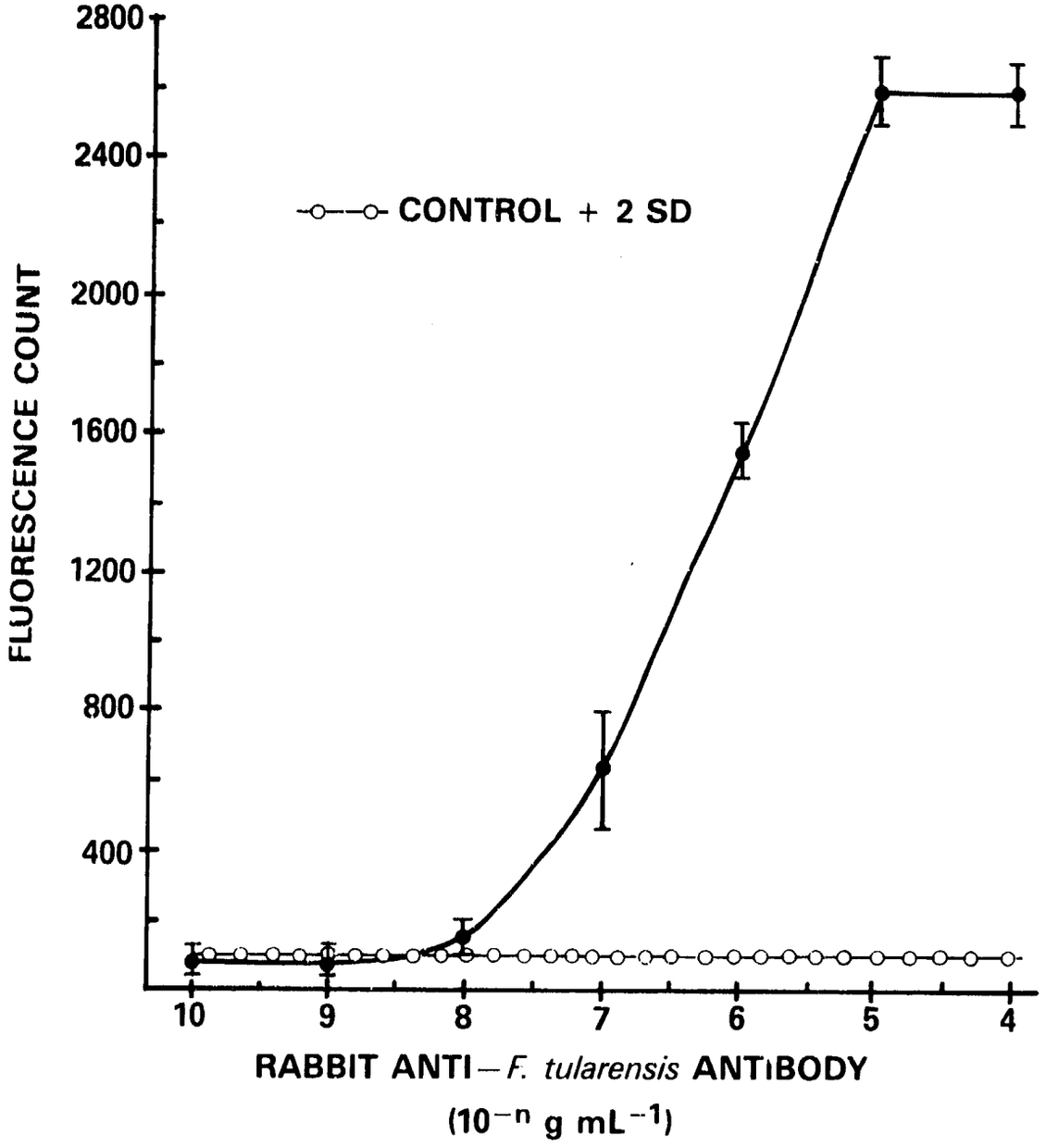
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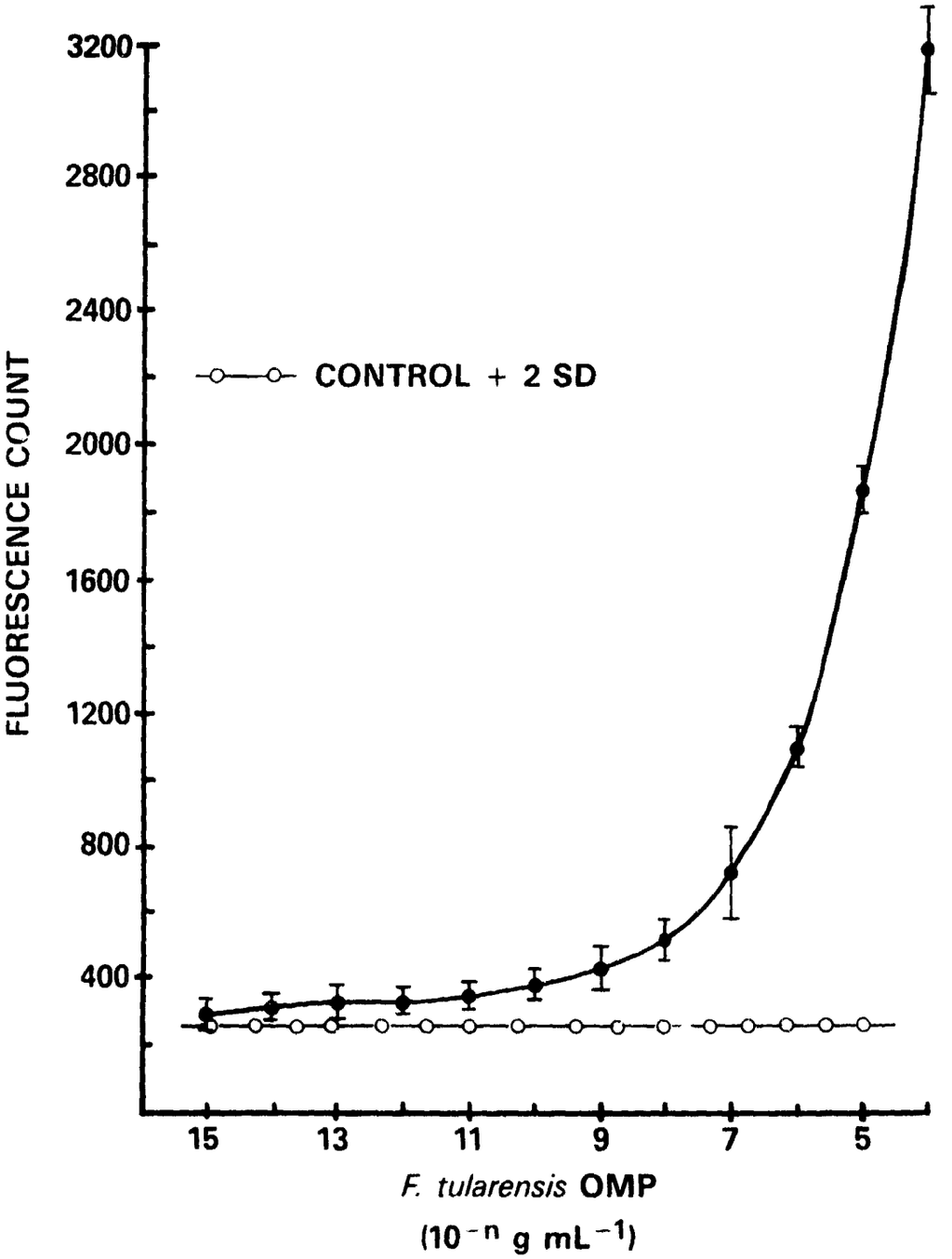
RABBIT ANTI–F. tularensis ANTIBODY
(10⁻ⁿ g mL⁻¹)

Fig. 1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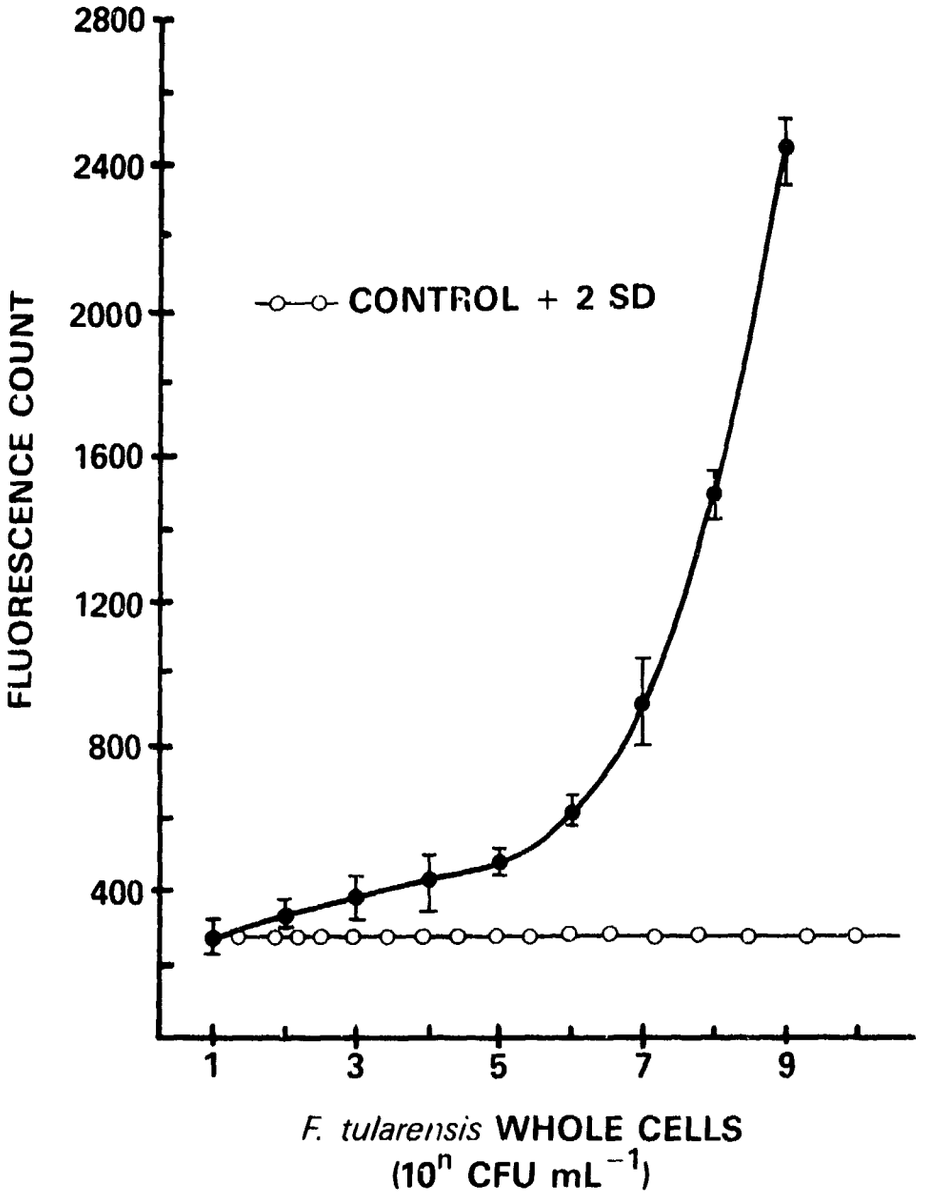
F. tularensis OMP
(10⁻ⁿ g mL⁻¹)

Fig. 2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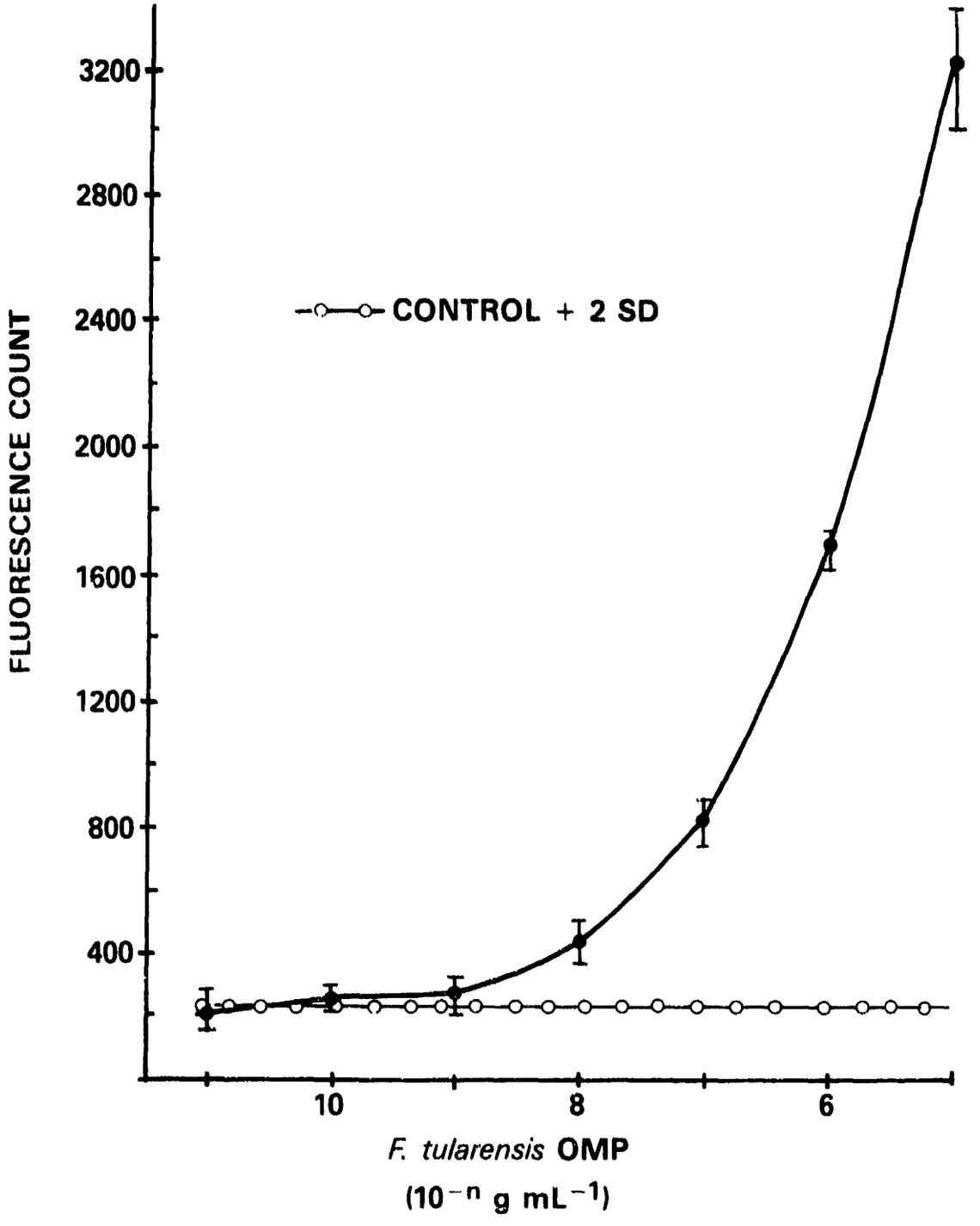
F. tularensis WHOLE CELLS
(10^n CFU mL^-1)

Fig. 3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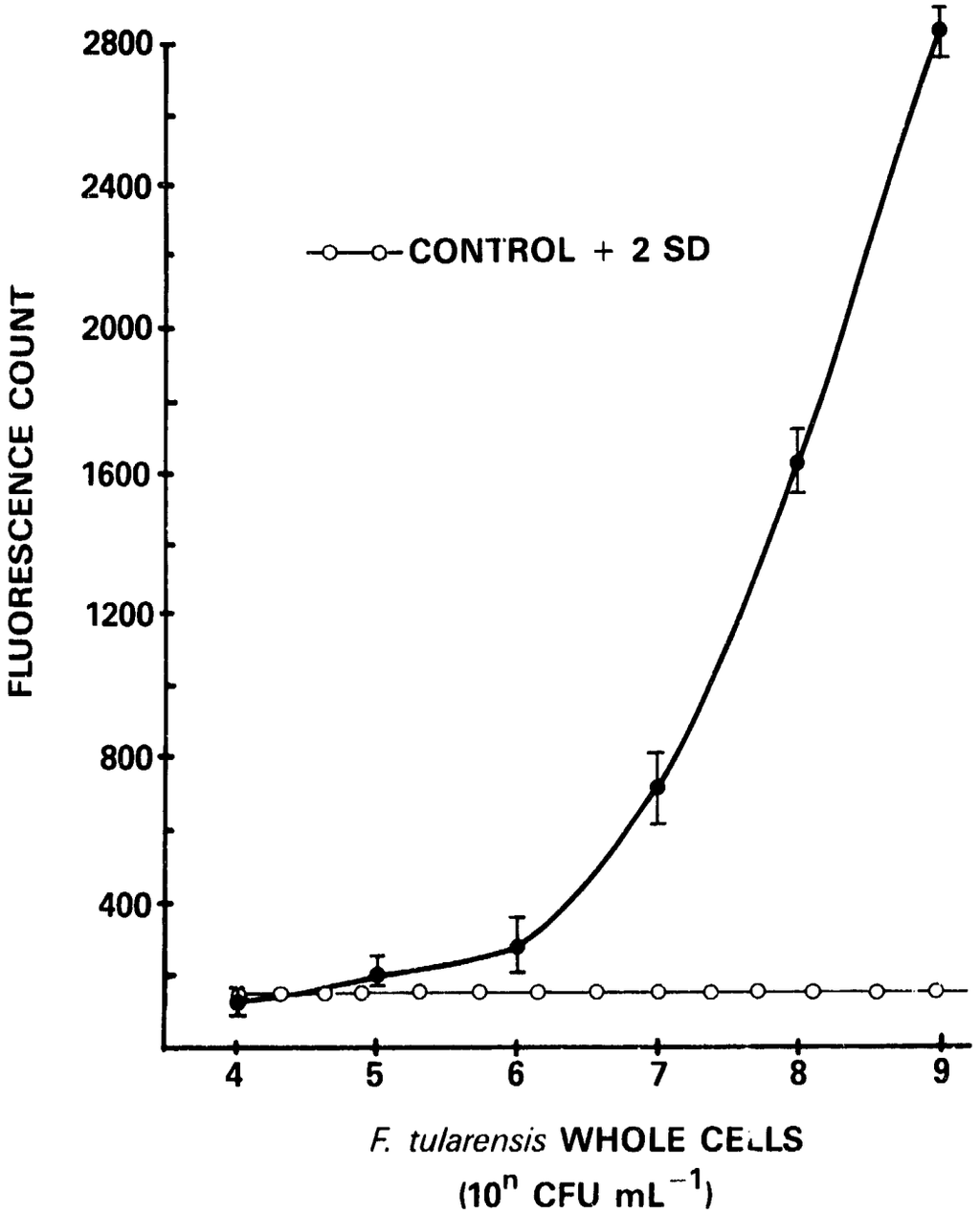
F. tularensis OMP
(10⁻ⁿ g mL⁻¹)

Fig. 4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F. tularensis WHOLE CELLS
(10^n CFU mL^-1)

Fig. 5

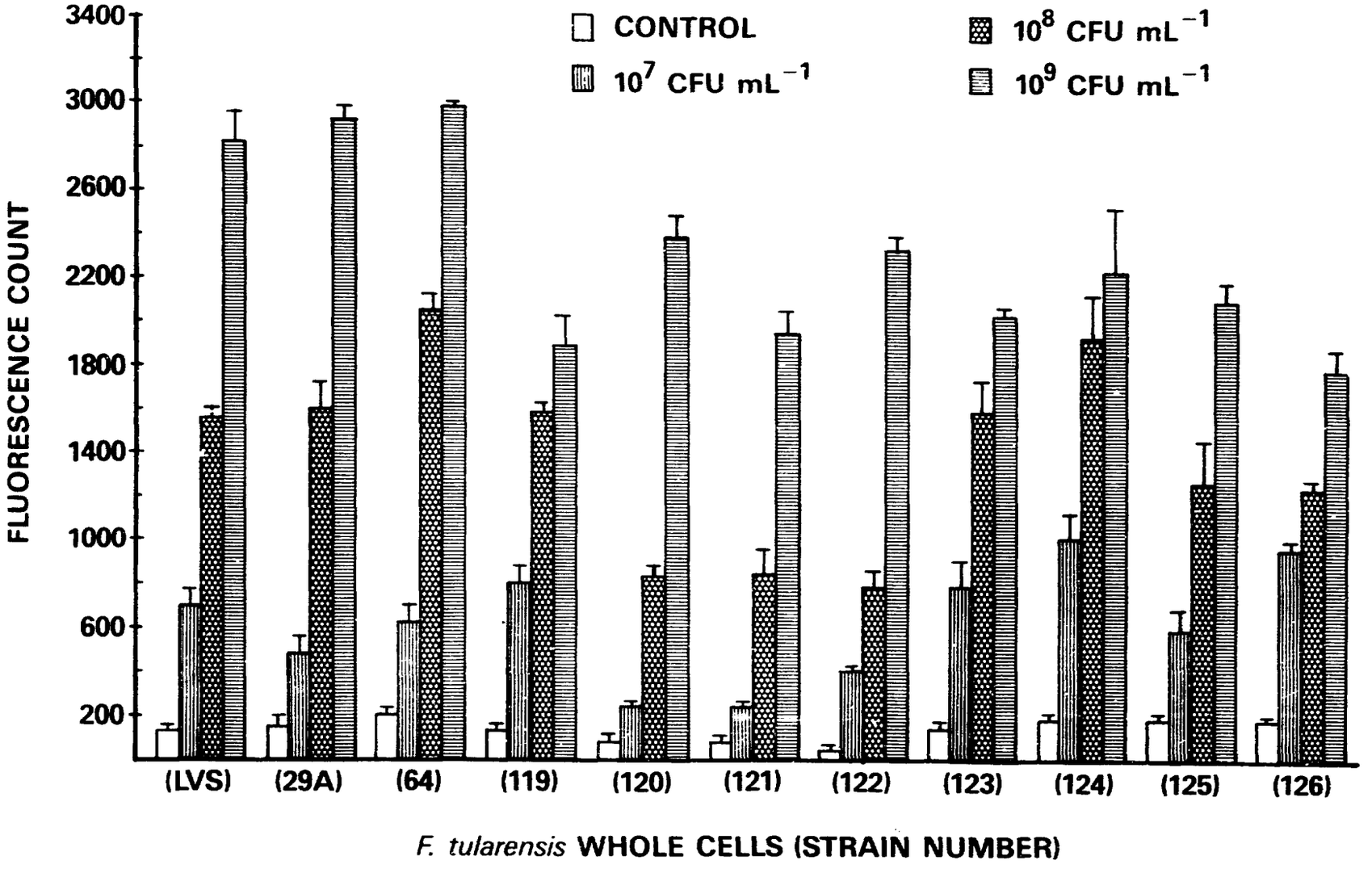
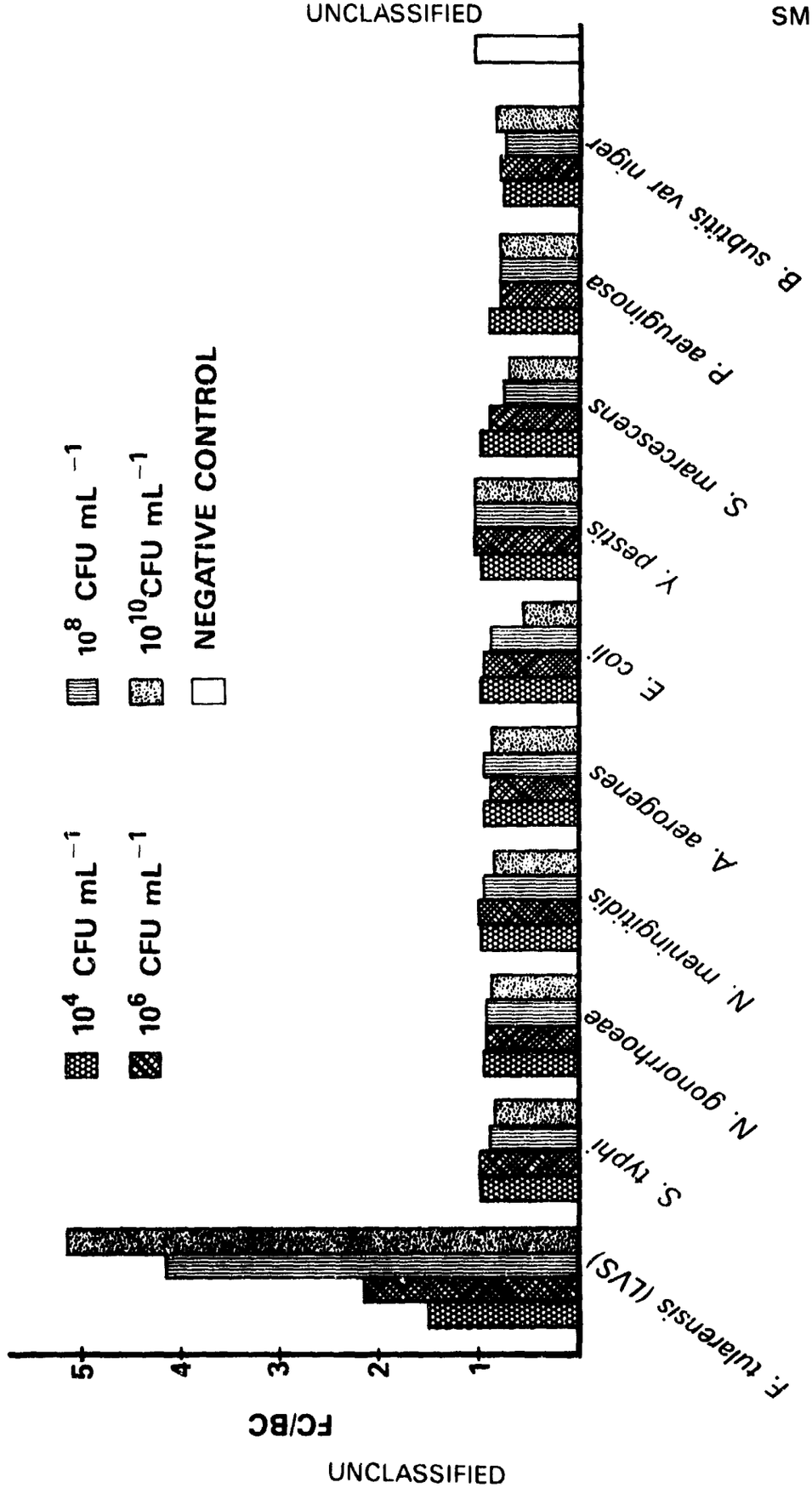


Fig. 6


F. tularensis (LVS)
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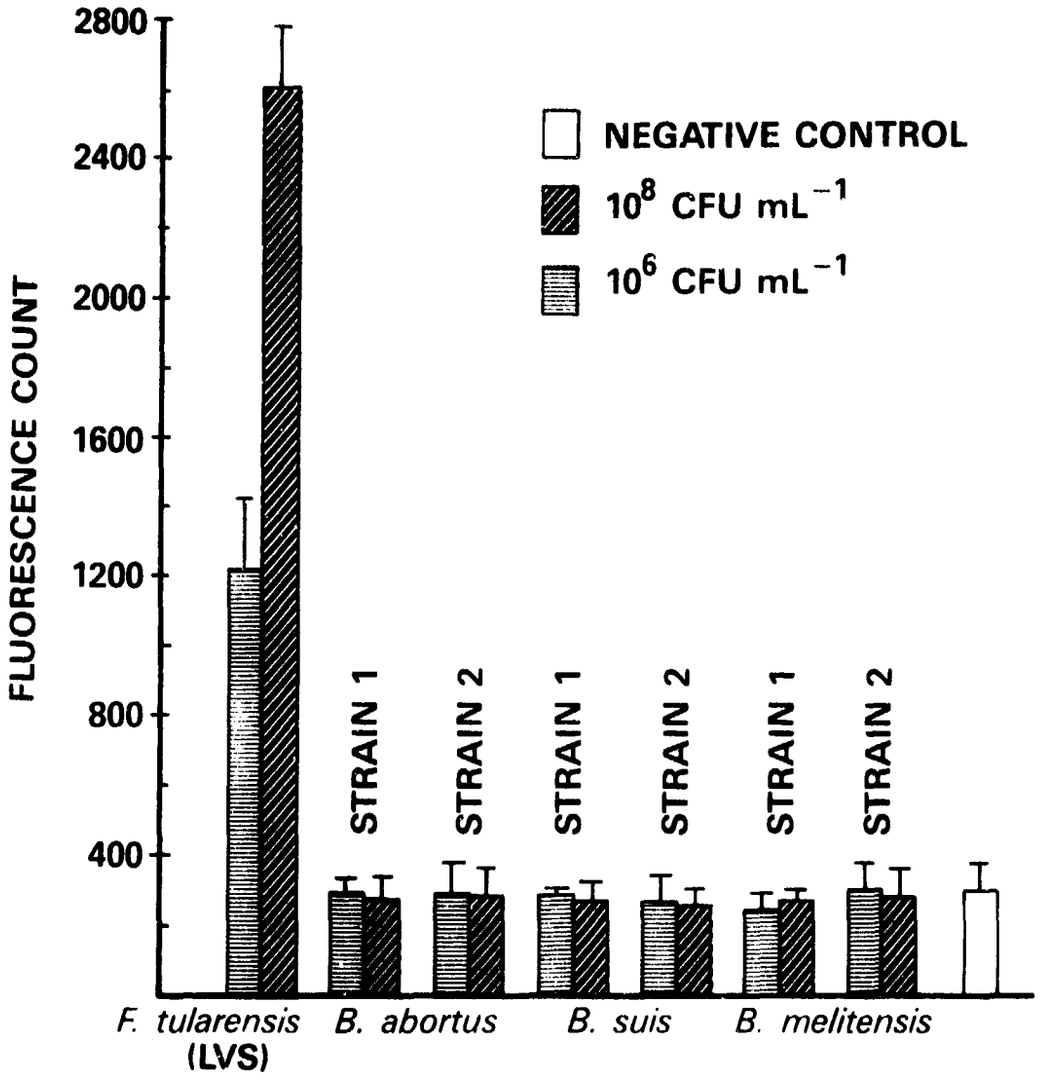
N. gonorrhoeae
N. meningitidis
A. aerogenes
E. coli
Y. pestis
S. marcescens
P. aeruginosa
B. subtilis var niger
10^4 CFU mL^{-1}
10^6 CFU mL^{-1}
10^8 CFU mL^{-1}
10^{10}CFU mL^{-1}
NEGATIVE CONTROL
FC/BC
1
2
3
4
5

Fig. 7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FLUORESCENCE COUNT
NEGATIVE CONTROL
10^8 CFU mL^-1
10^6 CFU mL^-1
STRAIN 1
STRAIN 2
STRAIN 1
STRAIN 2
STRAIN 1
STRAIN 2
F. tularensis (LVS)
B. abortus
B. suis
B. melitensis

Fig. 8

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Y.M. Siddiqui, R.E. Fulton, A.R. Bhatti and M.H. Knodel

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A highly sensitive fluorogenic enzyme-linked immunosorbent assay (FELISA), which utilizes nitrocellulose membranes as solid phase support and a fluorogenic substrate to indicate the antigen, has been adapted for the rapid identification of Francisella tularensis. Multiple samples were assayed in approximately 6 h by this method. The sensitivity achieved in a four layer "sandwich" assay format was 10 fg mL^{-1} of outer membrane protein and 100 colony forming units mL^{-1} of Francisella tularensis whole cells. The assay was highly specific for the detection of homologous and heterologous strains of Francisella tularensis while unrelated bacteria, including Brucella strains, exhibited no cross-reactivity.

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Francisella tularensis, outer membrane protein; rapid identification; fluorogenic enzyme immunoassay; nitrocellulose membranes