

Rapid enzyme-linked immunosorbent assay for the diagnosis of human brucellosis in surveillance and clinical settings in Egypt

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ABSTRACT

Objectives: To optimize and standardize an enzyme-linked immunosorbent assay (ELISA) for rapid diagnosis of human brucellosis in clinical cases identified during a surveillance study for acute febrile illness (AFI).

Methods: Serum samples from patients presenting with AFI at 13 fever hospitals across Egypt between 1999 and 2003 were kept frozen at NAMRU-3 and used in this study. The assay was evaluated in 5 subject groups: brucellosis cases confirmed by blood culture (group I, n=202) 87% positive by standard tube agglutination test (TA), brucellosis cases exclusively confirmed by TA (group II, n=218), blood cultures from AFI cases positive for bacterial species other than *Brucella* (group III, n=103), AFI cases with unexplained etiologies (group IV, n=654), and healthy volunteers (group V, n=50). All members of groups III-V were negative for brucellosis by TA.

Results: Sensitivity and specificity of ELISA for total

specific antibodies were $\geq 96\%$ versus 87% for TA as compared to microbial culture, the current gold standard method for *Brucella* identification. Assessment of *Brucella* antibody classes by ELISA in random subsets of the 5 groups showed significantly high ($p > 0.001$) levels of anti *Brucella* IgG ($\geq 81\%$) and IgM ($\geq 90\%$) in groups I and II only.

Conclusions: The obtained sensitivity and specificity results indicate that our ELISA is more suitable for AFI surveillance and clinical settings than blood culture and TA. The developed assay is also cost-effective, easier to use, faster, and the coated plates can be stocked for at least 8 months, providing a potential for field use and automation.

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Brucellosis is a serious disease endemic to the Middle East, Central and South America, and other parts of the developing world. It is acquired through ingestion of contaminated raw or unpasteurized milk or milk products and through contact with infected animals (example, cattle, goats).^{1,2} A laboratory-based sentinel surveillance conducted in Egypt from 1999 to 2003 identified typhoid fever and brucellosis as the

most common causes of acute febrile illness (AFI).³ A separate population-based surveillance conducted in Fayoum, Egypt, during 2003 revealed that 47% of brucellosis patients were clinically misdiagnosed as having typhoid fever.^{1,3}

Clinically, brucellosis presents as an AFI with few specific signs and can be misdiagnosed or confused with other febrile diseases such as typhoid

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fever, rheumatic fever, spinal tuberculosis, pyelitis, cholecystitis, thrombophlebitis, autoimmune diseases, and tumors.⁴ The gold standard for the diagnosis of brucellosis is the isolation of *Brucella* spp. from blood, bone marrow or other tissues by culture.² However, isolation of *Brucella* spp. from clinical specimens is time-consuming and blood cultures may require an incubation period of up to 6 weeks.⁵ In addition, blood cultures are only positive in 20-53% of the patients,^{2,4} are susceptible to contamination, and the successful isolation of *Brucella* decreases as the disease progresses.¹ The tube agglutination (TA) test is the standard serological method for the diagnosis of human brucellosis. However, the TA test produces low specificity and interpretation of results may be difficult due to cross reactions with *Yersinia* spp. (mainly *Y. enterocolitica*), *Salmonella enterica*, *Francisella tularensis*, *Vibrio cholera* and other bacterial species that share common antigens.⁶ Additional shortcomings of TA are prozone errors and an inability to differentiate immunoglobulin classes during the acute and chronic phases of the disease.⁷ The TA test is also labor-intensive and time-consuming, making it difficult to process large numbers of specimens such as in the context of field or epidemiologic investigations. Although the Rose Bengal test is widely used for brucellosis screening in clinical laboratories, results obtained require confirmation.² The immunofluorescent antibody (IFA), radioimmunoassay (RIA) and Coombs tests are probably more useful than TA, but they are labor-intensive, and lack sufficient sensitivity and specificity,⁸ thus, are not routinely performed in clinical laboratories.^{4,9,10} While previous studies have shown polymerase chain reaction (PCR) as highly sensitive in the diagnosis of brucellosis,^{11,12} the technique is expensive when considering the number of specimens to be tested in epidemiological studies and is poorly suited for laboratories with limited resources.^{13,14}

Enzyme-linked immunosorbent assay (ELISA) has been reported to be superior to both culture and TA for diagnosis,^{6,15-16} and is ideally adapted for screening large numbers of specimens due to its low unit cost and higher sensitivity and specificity estimates.¹⁷ However, application of ELISA for the routine diagnosis of human brucellosis has not gained wide acceptance, possibly due to inconsistent procedures and limitations in interpreting the results.¹⁸ Some commercial ELISA assays rely on the use of reference serum calibrators for the detection of anti-*Brucella* isotypes, making the readings unmatchable

with culture results and irrelevant for use in different epidemiological settings.

Our objective was to develop an ELISA for the rapid and specific detection of total *Brucella* antibodies as an epidemiological tool in disease surveillance settings. Assays for IgG and IgM levels were also standardized in the context of a large-scale surveillance program and tested against a broad spectrum of patient and control groups. Since *B. abortus* and *B. melitensis* are antigenically similar in serological assays, regardless of species or biotype,^{16,19} commercially available *B. abortus* antigen was employed throughout this study.

Methods. Study subjects and blood specimens.

Serum samples were obtained from patients presenting with AFI (n=1177) and seeking care at a network of 13 infectious disease hospitals in Egypt from 1999 to 2003. The standard AFI case definition used for enrollment¹ allowed the admission of any individual with a history of fever ($\geq 38^{\circ}\text{C}$) for 3 or more days without obvious clinical diagnosis such as diarrhea and pneumonia, or with clinical symptoms of typhoid fever, brucellosis, or fever of unknown origin. Two blood samples (5-10 ml each) were collected from each patient. One was immediately injected into a Phase 2TM biphasic blood culture bottle (PML Microbiologicals, Wilsonville, Oregon), and the other was centrifuged at 4000 rpm for 10 min for serum separation and stored at -20°C until used. All specimens were transferred to the US Naval Medical Research Unit-3, Cairo, for bacterial culture and serological testing.

Based on blood culture and TA results, archived serum specimens were grouped as follows: group I consisted of brucellosis cases confirmed by growth in blood culture (n=202), group II were blood culture-negative, but positive for anti-*Brucella* antibodies by TA (titer $>1/320$; n=218), group III consisted of patients infected with bacterial pathogens (as determined by blood culture growth) other than *Brucella* (n=103) and group IV were febrile patients

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with unknown etiology (negative blood cultures and *Brucella* TA serology) (n=654). Group V included healthy volunteers who donated blood samples and had no history of brucellosis or AFI during the previous 6 months (n=50).

Blood cultures. Blood culture bottles were routinely incubated at 35-37°C for up to 21 days only, and were observed daily for signs of microbial growth.^{5,14} Blind subcultures on solid media were also prepared every other day. Recovered colonies were examined by Gram stain and identified by standard methods.¹⁴

Tube agglutination. TA testing was performed on all specimens using a commercially available whole cell *B. abortus* antigen [strain United States Department of Agriculture (USDA) #1119-3, Beckton Dickinson, Maryland (MD)] following the manufacturer's instructions. Briefly, all serum samples were serially diluted up to 1/5120 in normal saline. Subsequently, the antigen was added to all tubes and incubated for 48 hours at 37°C. Agglutination titers were read under indirect light using an agglutination viewer. For endemic areas, a titer ≥ 320 is arbitrarily regarded as positive.²⁰

Enzyme-linked immunosorbent assay. *Brucella* antigen, serum samples and conjugates were optimized using checkerboard titrations with positive and negative serum pools of *Brucella* antibodies from culture positive and healthy samples.²¹ Aliquots (100 μ l) of diluted whole cell *B. abortus* antigen (strain USDA #1119-3, Beckton Dickinson, MD, 2.5 μ l/ml, v/v in carbonate buffer, pH 9.6) were used for coating flat bottom, polystyrene plates (ICN Biomedicals, Ohio). Coated plates were incubated overnight at 4°C, then emptied, dried on paper towels and blocked with 200 μ l/well of 1% bovine serum albumin (BSA) in carbonate buffer for 1 hour at 37°C. A proportion of duplicate plates were stored at -20°C after blocking in order to determine the reliability of plates prepared in advance. Plates were washed twice with phosphate buffered saline containing 0.1% Tween-20 [phosphate buffered saline tween (PBST)] using an ELISA washer (ELx 50, auto strip washer; Bio-Tek Instruments, Inc, Winooski, Vermont).

Determination of total antibody titers. All serum samples were added to the plates in PBST containing 0.1% BSA at dilutions similar to those of TA (1:160-1:5120). Plates were incubated at 37°C for 1 hour and thoroughly washed 4 times using an ELISA washer as previously described. Peroxidase labeled goat anti-human immunoglobulin conjugate [Sigma-Aldrich Chemical Company, St. Louis, Missouri (MO)] diluted 1:20,000 in PBST with 0.1% BSA was added, and

allowed to react for 30 minutes at 37°C. Plates were then washed 4 times and O-phenylenediamine (OPD) substrate (Sigma-Aldrich Chemical Company, St. Louis, MO) was added and incubated for 30 minutes at ambient temperature in the dark. The reaction was stopped by the addition of 1N H₂SO₄ (50 μ l/well) and the developed color was read using a Titertek Multiscan reader (Labsystems, Helsinki, Finland) at 492 nm.

Determination of IgG and IgM antibody classes. A similar ELISA procedure was used to measure the optical density (OD) of specific anti-*Brucella* IgG and IgM antibodies in random subsets of the various study groups. After coating and blocking the 96-well plates, serum samples were loaded at an appropriate dilution determined by checkerboard titration to be 1:500 for IgG and 1:1000 for IgM. Plates were incubated for 1 hour at 37°C and washed 4 times as previously described. Peroxidase labeled goat anti-human IgG whole molecule (at a dilution of 1:2000 in PBST, 0.1% BSA) or anti-IgM heavy chain (at a dilution of 1:10,000 in PBST, 0.1% BSA) antibodies were used (Sigma-Aldrich Chemical Company, St. Louis, MO) and the plates were incubated for 30 min at 37°C. Washing conditions, OPD substrate and reading of developed OD values were conducted as described above.

Determination of cutoff values. Cutoff values were set at 3 standard deviations above the arithmetical mean of the OD obtained from healthy controls.²² A cutoff point of 0.20 was established for the detection of total antibody and titers were determined as the highest dilutions showing ODs \geq cutoff. For IgG, cutoff value points of 0.25 was established, whereas IgM assay at 0.40.

Validity of ELISA. The ELISA sensitivity and positive predictive values (PPV) were evaluated in comparison to blood-culture confirmed brucellosis (group I). ELISA specificity and negative predictive values (NPV) were evaluated using cases that showed other bacterial pathogens in blood cultures (*Brucella* spp. negative by culture and serology, group III). Calculations and statistical analyses were performed as follows:²²

Sensitivity = [True positive / (True positive + False negative)] \times 100;

Specificity = [True negative / (True negative + False positive)] \times 100;

PPV = [True positive / (True positive + False positive)] \times 100;

NPV = [True negative / (True negative + False negative)] \times 100.

Table 1- Sensitivity, specificity, positive (PPV) and negative predictive values (NPV) of ELISA in the diagnosis of human brucellosis in Egypt, 1999-2003.

Study groups	ELISA *Pos/Total tested	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
I. Culture-confirmed brucellosis cases	196/202	(97)	**N/A	(99)	N/A
II. TA serology-confirmed brucellosis cases	218/218	(100)	N/A	(99)	N/A
III. AFI positive culture for other bacteria	4/103	(4)	(96)	N/A	(94)
IV. AFI unknown etiology	9/654	(1.4)	(98.6)	N/A	(99)
V. Healthy volunteers	0/50	0	(100)	N/A	(89)

*Pos = Positive (ELISA titer of ≥ 320), **Not applicable
TA - tube agglutination, AFI - acute febrile illness

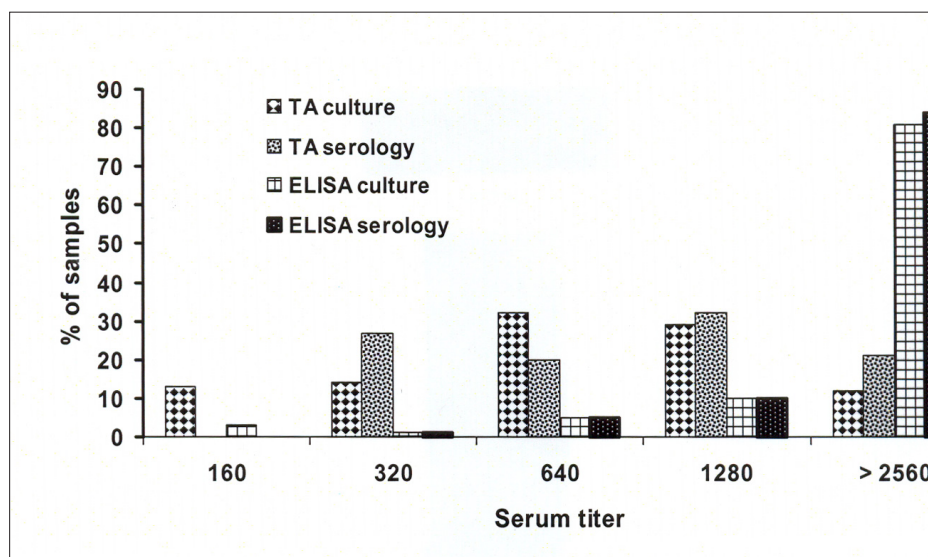


Figure 1- Distribution of tube agglutination (TA) and ELISA titers in brucellosis patients confirmed by culture or serology. Diagnostic titer for brucellosis is ≥ 320 .

Probability for significance of differences among variables was determined by Student's t-test using Microsoft Excel, a Windows Software Package.

Results. Characteristics of patients. Enrolled brucellosis patients had an average fever duration of 10 days (range 3-90 days), temperatures ranging from 38-40°C, an average age of 28 years (range 3-60 years), and were predominately male (71%).

Total Brucella antibodies by ELISA. Assay sensitivity. Serum samples from 97% (196/202) of patients with blood cultures positive for *Brucella* spp. were also positive by ELISA for total specific antibodies (Table 1); the majority (79%) were reactive at dilutions $\geq 1:2560$ (Figure 1). Among this culture-positive and ELISA-positive group, only 87% (175/202) also tested positive by TA (titer ≥ 320). All

sera in the blood-culture-negative but TA-confirmed group (group II; TA titer ≥ 320) were reactive by ELISA (Table 1) with only 1% (2/218) of samples demonstrating weak positive reactions. The average ELISA ODs obtained on serum from blood culture- and TA-confirmed cases were significantly higher than those of healthy controls ($p < 0.001$). However, ODs were not significantly different among the various control groups (III, IV and V) and all three of these patient sample groups were negative by TA. Relative to blood culture, the PPV of ELISA for total antibody was 99% for subject groups I and II (Table 1). There was no significant difference between freshly prepared or pre-coated and pre-blocked plates that were frozen during the study period up to eight months.

Assay specificity. The overall assay specificity was 98.4% (Table 1). Of the 807 serum samples tested, a total of 13 samples from either group III (4/103; 4%; specificity 96% and NPV 94%) or group IV (9/654, 1.4%; specificity 98.6 % and NPV 99%) were weakly positive (OD range=0.2–0.4) by ELISA. None of the group V samples were reactive (specificity 100% and NPV 89%).

Detection of IgG and IgM using ELISA. Based on OD values obtained from the healthy control group (group V), values for positive reactions were estimated to be 0.25 for IgG and 0.40 for IgM. *Brucella*-specific IgG was reactive in groups I (88%) and II (81%) at relatively lower proportions than IgM (96% and 92%, respectively). A significant difference ($p < 0.001$) was noted between the isotype ODs of culture or TA confirmed cases and those of other groups III, IV and V. Generally, cases with low IgG titers were elevated for IgM and vice versa. Combining results from the 2 isotype assays, a sensitivity rate of 100% was obtained (compared to culture and TA positive cases). While specific IgG was not detected in any of the control groups (III, IV and V), IgM was reactive in 4% (4/99) of group III cases. When patients were evaluated in relation to their fever duration (prior to admission or initiation of antimicrobial therapy), it was found that 59% of confirmed brucellosis cases had fever for 2-6 days, 25% for 7-15 days, 5% for 16-22 days and 11% for 23 days or more. In all brucellosis patients, IgG and IgM were detectable in all brucellosis patients regardless of the fever duration or disease onset. Interestingly, IgM was elevated in 92% of the short fever (≤ 7 days) cases and in 100% of the long fever (≥ 30 days) cases. In contrast, IgG was relatively high in 85% of the cases, irrespective of the differences in fever duration.

Discussion. Brucellosis is a disease with significant public health and economic impacts for many developing countries, including Egypt. To better define the burden of this disease and its risk factors, epidemiologic studies performing accurate and efficient laboratory-based confirmatory testing on large numbers of serum specimens are a necessity. However, the current diagnostic standards for brucellosis based on blood culture and agglutination methods are labor-intensive, time-consuming and impractical in disease surveillance settings.

We developed and evaluated an ELISA for the diagnosis of brucellosis. Since both *B. abortus* and *B. melitensis* are reported to express serologically indistinguishable antigens,¹⁶ commercially available whole cell suspensions of *B. abortus* were employed

as coating antigens throughout this study, to ensure consistency and reproducibility. These antigens can be used to detect specific antibodies against a range of *Brucella* species, with the exception of *B. canis* and *B. ovis*.¹⁶ Although all cultured *Brucella* isolates in Egypt are *B. melitensis*,²³ our pilot studies showed no significant differences in duplicate TA or ELISA tests using both *B. abortus* or *B. melitensis* and our ELISA results were compared to a range of culture-confirmed, *Brucella* positive and negative human sera. The developed ELISA assay was highly sensitive (97%) in detecting the presence of total anti-*Brucella* antibodies (PPV=99%). Only 3% of the culture confirmed cases showed lower OD values than the cutoff, with titers of $< 1/160$. A possible explanation for this is the low or immature immune response among these patients, since their fever had started 3 to 10 days earlier. These cases may still be diagnosed as having brucellosis given the presence of suggestive clinical findings and the necessity for demonstrating a rising titer. The sensitivity of the TA test was only 87% in culture-positive cases. This may be explained by the observation that ELISA is sensitive to 0.05 ng of antibody, whereas the agglutination test is not sensitive to < 500 ng.²⁴ In addition, TA has been shown to be insensitive to non-agglutinating antibodies.^{7,20} Approximately 80% of the samples showed titers ≥ 2560 in ELISA compared to TA ($\leq 20\%$ at the same dilution, Figure 1), which suggests a higher confidence in interpreting the results using this method (OD₄₉₂ values ≥ 0.20 ; corresponding to titers ≥ 320).

The OD values of the serology confirmed group (TA positives) were parallel to those of the culture positive group, indicating a similar pattern of antibody response, regardless of the recoverability of *Brucella* from blood cultures. Failure to isolate *Brucella* from blood cultures of group II patients may be due to the low sensitivity of the culture technique, undulating septicemia or prior antimicrobial therapy. False-positives in this group are unlikely since our case definition excluded all febrile cases showing explicit systematic manifestations. The high marginal titer required for positive cases (> 320 for both TA and ELISA) also contributes to the elimination of cross-reactions from previous infections with antigenically related pathogens.

The ELISA developed in this report was highly specific for *Brucella* as demonstrated by control groups III-V. A small percentage of samples from groups III (4%) and IV (1.4%) showed weak ELISA reactivity (range=0.20-0.40) despite the repeated negative *Brucella* results by TA and blood culture. This may be due to the presence of low background

titers in this population since brucellosis is endemic in Egypt. No reactivity was observed in samples from group V. Specific IgM was detected in 4% of group III patients, contrary to IgG, which was not found in any of the control groups. The detection of IgM may be due to the less specific nature of this antibody, despite the relatively high dilutions and cutoffs used.

It may be useful to screen acute sera for both IgG and IgM since our results show those isotypes to be reactive in patients who had fever for only 2-6 days (85% for IgG and 93% for IgM). Despite a slight trend for IgG to express higher titers as the duration of fever increased, IgM was reactive in some cases that had fever ≥ 90 days, suggesting that early and late phases of brucellosis do not present 2 distinct immunological entities.² In an earlier study, IgM has been reported in 33% of cases with protracted symptoms of brucellosis.⁴ In addition, for cases showing marginal immune responses, the demonstration of a rising antibody titer may be necessary to confirm the disease. This was not feasible in our study and PCR was not conducted due to its reported low specificity compared to the TA test.¹² As to brucellosis patients, the sensitivity of our ELISA to immunoglobulin classes (IgG and IgM) was higher than that reported in previous studies (96% for group I and 92% for group II).^{16,20,25-26} However, the minor decrease in the sensitivity among Group II patients may be related to the concentration of each isotype at different disease phases. Overall, the observed discrepancies may be due to differences in patient populations studied, nature of test constituents, reagents and ELISA formats.

When considering the difficulties associated with AFI diagnosis by PCR or bacterial culture (technical complexity and high cost), ELISA appears to be more suitable for the diagnosis of human brucellosis.^{4,17} Our results are unique in suggesting the use of this assay for measuring total *Brucella* antibody titers in AFI patients, whether in clinical or epidemiological settings (98.6% specificity, with PPV and NPV $\geq 94\%$). The method can also provide specific titers for *Brucella* antibody isotypes at sensitivities higher than those reported for other assays. The obtained specificity rates are superior to those reported from commercial ELISA assays that require reference serum calibrators and cannot be used for screening the general AFI populations due to socio-epidemiologic reasons⁶ and for being much cheaper. It would be of value to compare the performance of these methods in further laboratory testing. Unlike culture, which requires 3 to 21 days, or TA, which requires 1-2 days, our ELISA assay permits the screening of a large number of specimens in a few hours. The assay is cost-effective

with 5 ml of *Brucella* antigen sufficient for testing 3,200 clinical samples at 6 dilutions. In preliminary experiments, pre-coated and sealed ELISA plates have been used after stored frozen for >8 months and the procedure could be easily automated.

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