

Evaluation of Simple and Rapid Specific IgM/IgG Flow Assay and Some Methods Used in Diagnosis of Brucellosis in Gezira and Blue Nile States, Sudan

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Abstract

Brucellosis is a multisystem disease which may present with broad spectrum clinical manifestations which often nonspecific. In Sudan it is one of the febrile illnesses affecting human welfare. In this study, blood samples were collected from 120 individuals who had clinical symptoms of Brucellosis from Gezira and Blue Nile states in Sudan, and 60 apparently healthy individuals as a control. For diagnosis we evaluated the blood culture and three serological tests, Rose Bengal Test (RBT), Serum Agglutination Test (SAT) and the specific IgM and IgG immuno flow assay test. The results obtained revealed that, 12/120 (10%), 7/120 (6.8%) and 6/120(5.0%) were positive for Brucellosis by RBT, SAT and IgM and IgG flow assay test respectively and no correlation between the three tests, while the blood culture was done for 81/120 (67.5%) of the cases, none yielded positive for brucella. This study concluded that the serological methods were superior to bacteriological method for diagnosing Brucellosis, especially IgM and IgG flow assay test and SAT.

Keywords: Brucellosis; Laboratory Testing Methods; Sudan

Introduction

Brucellosis is a widespread zoonosis mainly transmitted from cattle, sheep, swine goats, pigs, camels and dogs through direct contact with blood, placenta, fetuses or uterine secretions or by inhaling airborne agents, and through consumption of contaminated raw animal products (especially unpasteurized milk and soft cheese). In endemic areas, human brucellosis has serious public health consequences. Worldwide, *Brucella melitensis* is the most prevalent species causing human brucellosis [1].

Brucellosis is a multi-system disease with a broad spectrum of clinical manifestations that requires laboratory diagnosis. The isolation of the organism from blood samples or other clinical specimens is a confirmatory diagnosis for Brucellosis, the sensitivity of this technique depends on the stage of illness as well as on other factors. Furthermore, culture does not provide a rapid result and many laboratories in endemic areas do not have culture facilities. So, often the laboratory diagnosis relies on serologic testing methods. A presumptive laboratory diagnosis, the Rose bengal test (RBT) and the standard serum agglutination test (SAT) are widely used as a rapid and simple screening tests but they require confirmation by other tests because false-positive results may occur in endemic areas [2,3]. These agglutination tests are relatively complicated and time-consuming to perform and need to be confirmed by culture and PCR. The developed immune chromatographic lateral flow assays, the Brucella IgM and IgG flow assays, for the detection of Brucella-specific IgM and IgG antibodies, respectively, are much easier and quicker to perform and

are well standardized with sensitivity of 96% with culture- confirmed brucellosis [4]. The flow assay is simply performed by the addition of a drop of serum followed by some running fluid to the sample well of a plastic assay device. The test result is read after 10-15 minutes by visual inspection for staining of the antigen line in the test zone of the assay device. In published research, results show that the flow assays are convenient diagnostic tests for use in endemic areas, a positive result in the IgM and IgG flow assays was obtained in 91% and 97% of the admission sera from adult and pediatric patients with brucellosis respectively [5], Thus Brucella IgM and IgG flow assays would be ideal for use in areas that are endemic for brucellosis and in developing countries.

Human brucellosis is one of the endemic diseases in Sudan; it was reported in Sudan as early as 1908 [6]. In 1950, one case reported from Kassala province among other cases from the Blue Nile province [7]. In a review of the Sudan Medical Records between 1928 and 1937, 102 cases in the Kassala area out of 313 cases from other parts of Sudan [8]. Previous animal studies showed that the prevalence of the disease in cattle, sheep, goats and camels in Kassala province was comparable to that from other parts of Sudan [9]. In Upper Nile region, South Sudan, among small number of school children revealed a 3.7% positivity rate, Melut's hospital patients 5.2% and village children and adults 2.2% [10] Among small sample size of patients presenting with symptoms and signs suggestive of brucellosis in the Gezira area, Central Sudan, were studied. The majority of the patients (76%) were found to have a combined infection of both *Brucella abortus* and *Brucella melitensis* with titers of 1/160 and above [11]. In Kassala State East of Sudan, 187 human sera were collected from occupational contacts, which included

butchers, slaughterhouse workers, milkers and cow attendants, only 1% were found to be reacted positively by Brucellosis [12].

Because the Sudan is a grazing and pastoral country the brucella became a health problem.

No records or clear data about the incidence of human brucellosis in Sudan, misdiagnosis and confusing with commonly occurring malaria, cross reacting antigens making interpretation of serological tests very difficult. And as mentioned above, the isolation by culturing is difficult and time consuming and RBT or SAT test is not always useful when they are interpreted as a single test, their sensitivity and specificity are variable and no cut off titre value is established. Hence, the evaluation of IgM and IgG flow assays was evaluated in this study in comparing to other routine serological tests as well as culture.

Materials and Method

Study area

The study was carried out in Gezira and Blue Nile States in Sudan, which are known as agricultural and pastoral states and the livestock mainly are cattle, sheep, goats and camels with some pigs in the southern areas of Blue Nile State. Most of the population of the two states are farmers or nomads.

Study Design and Population

This is a multi center prospective sectional analytical case control study. The patients enrolled in this study were 120 adult males and females with high clinical suspicion (symptoms and signs) for brucellosis, and 60 apparently healthy individuals.

Laboratory Methods

In this study four methods for diagnosis of brucellosis in humans were used: - Blood culture [13,14], RBT and SAT [15-17] and the specific IgM and IgG flow assay test [4].

Blood Culture (Castaneda Method)

6 ml of the patient blood delivered aseptically into two culture bottles media. The cap of CO₂ media bottle left loosen in a candle Jar, the candle lighted with the lid covered. The two media bottles were incubated at 37°C. The media observed for growth at the solid phase every 3 days and the broth titled (when there is no obvious growth) to run the cultured broth on the solid media. When there is growth a pure colony was subcultured on blood agar, Maconkey and serum dextrose agar for further identification and biochemical reactions. Media incubated up to six weeks and discarded if there is no growth and the result reported as negative.

Serology

6 ml of blood were delivered into a clean, dry plain container and serum was separated immediately after clotting.

RBT

Different dilutions of serum: 5, 10, 20, 40 and 80 on the tile in two rows. One drop of well mixed Brucella abortus and Brucella melitensis antigens (Cromatest Company, batch 02109, Spain) was added to each serum volume. The serum and antigen were mixed well and rotated in

cycler for two minutes. Agglutination was looked for within two minutes. Positive and negative controls were used. Results were rated negative when agglutination was absent and 1+ to 4+ positive according to the strength of the agglutination.

SAT

The SAT was performed by preparing two-fold serial dilutions of the serum sample starting at a dilution of 1:20 in the wells of a microtitre plate and the addition of an equal volume of stained Brucella abortus antigen MM101 (Linear Chemicals, Barcelona, Spain). The mixtures were incubated for 24 hours at 37°C and read by visual inspection. A titre 1:160 was considered consistent with brucellosis.

Two rows of 8 tubes for each prepared in a rack. 1.9 ml phenol saline dispensed in the first tube and 1 ml in each of the other 7 tubes. 0.1 ml of unknown serum added to the first tube and mixed well, and one ml is transferred to the second tube and mixed well. The procedure was repeated till the 7th tube and one ml discarded. The 8th tube was left as control.

One drop of well mixed Brucella abortus antigen was added to each one of 8 tubes, including the control Tube. All tubes were shaken well and incubated for 48 hours at 37°C. All tubes were read for agglutination and the highest titre was reported. Positive and negative controls were used and treated in the same manner.

IgG & IgM Flow Assay

The device opened, labelled with the specific antigen IgG or IgM and test number. 10 micro litre of patient serum was added to the sample hole. 130 micro litre of buffer was added immediately to the sample in the test hole. The control was looked for first to ensure the validity of the test. Test samples read & reported if positive in crosses, (+, ++,+++ or ++++) according to the intensity of the colour of the red line or negative if no colour developed.

Ethical Approval

Ethical approval for this study was obtained from the ethical committee of the University of Gezira and permission from the health authorities in both states.

The participants were asked to participate in this study after appropriate informed consent was obtained.

Statistical analysis

The statistical analysis of the collected data was performed by means of SPSS (Statistical Software Package of Social Sciences, version 10). Descriptive methods were used to calculate frequencies and proportions in groups. Chi-square is used to detect the significance of the test.

Results

In this study, 180 subjects were enrolled from Wad Medani Teaching Hospital in Gezira State and Eldamazin Teaching Hospital in Blue Nile State, 120 were suspected to have brucellosis (78 from Blue Nile State and 42 from Gezira State) and 60 as control. The age of the selected subjects was from 15–70 years, there were more females 108/180 (60%) than males 72/180 (40%).

Table 1 revealed that the tested sera by RBT was positive for brucella antibodies in titres ranging from 1/20–1/1280 and the control sera showed positive in titers ranging from 1/20–1/160, while SAT was positive for burucella antibodies in titters ranging from 1/20–1/640 and the control sera showed positive only in titre of 1/20 and 1/40.

Titer	RBT		SAT	
	Test	Control	Test	Control
1/20	69/120 (57.5%)	48/60 (80.0%)	106/120(88.3%)	59/60 (98.3%)
1/40	12 /120(10.0%)	6 /60(10.0%)	6 /120(5.0%)	1/60 (1.7%)
1/80	17/120 (14.2%)	2 /60(3.3%)	1 /120(0.8%)	-
1/160	10/120 (8.3%)	4 /60(6.7%)	4/120 (3.3%)	-
1/320	10/120 (8.35)	-	2/120 (1.7%)	-
1/640	1 /120(0.8%)	-	1 /120(0.8%)	-
1/1280	1 /120(0.8%)	-	-	-

Table 1: Shows the comparison between ranges of titers of RBT & SAT (Suspected Positive and Control Sera). Key : SAT (Serum Agglutination Test RBT (Rose Bengal Test)

In Table 2, the brucella IgM flow assays test was positive in 4/12 (33.3%) of the RBT positive sera and 4/7 (57.1%) of the SAT positive sera, while the tested sera were negative in 108/108(100%) of RBT and in 113/113 (100%) of SAT in negative sera. In Table 3 the brucella IgG flow assay tested sera was positive in 2/12 (16.7%) of the RBT positive sera and 2/7 (28.6%) of the SAT positive sera, while tested sera were negative in 108/108 (100%) of RBT and in 113/113 (100%) of SAT in negative sera.

Tested sera (n=120)	Brucella IgM flow assay					
	-ve	+	++	+++	++++	Positivity%
RBT- positive (n=12)	8	2	1	1	-	33.3%
SAT- positive (n=7)	3	2	1	1	-	57.1%
RBT-negative (n=108)	108	-	-	-	-	0.0%
SAT-negative (n=113)	113	-	-	-	-	0.0%

Table 2: Shows staining intensity of the Brucella IgM flow assay according to the results of RBT and SAT

Tested sera (n=120)	Brucella IgG flow assay					
	-ve	+	++	+++	++++	Positivity%
RBT-positive (n=12)	10	1	1	-	-	16.7%
SAT-positive (n=7)	5	1	1	-	-	28.6%
RBT-negative (n=108)	108	-	-	-	-	0.0%
SAT-negative (n=113)	113	-	-	-	-	0.0%

Table 3: Shows staining intensity of the Brucella IgG flow assay according to the results of RBT and SAT

As indicated in Table 4, 12/120 (10%), 7/120 (6.8%) and 6/120 (5.0%) were positive for Brucellosis by RBT, SAT and IgM and IgG flow assays respectively, while the blood culture was done for 81/120 (67.5%) of the cases, none yielded positive for brucella.

No. of cases	Tested methods	Positive	Percentage of positive	Negative	Percentage of negative
120	RBT	12	10.0%	108	90.0%
120	SAT	7	5.8%	113	94.2%
120	IgG/IgM	6	5.0%	114	95.0%
81	Culture	0.0	0.0%	81	100.0%

Table 4: Shows the performance of the four methods for diagnosis of Brucellosis

Discussion

In this study, 81 cases were subjected to blood culture and none yielded positive for brucella. This can be explained by the fact that brucella organisms are generally very difficult to be isolated in culture similar to that reported by Maichomo et al. in 1998 [18]. Also the random use of extensive antibiotics by the Sudanese individuals may inhibit and suppress its growth in vitro. Because the definitive diagnosis of brucellosis by blood culture is difficult and of a very low sensitivity it was not possible to be established as a gold standard for this study, so this study depended on the correlation and agreement between the serological diagnostic tests. As documented in Table 4, there was no correlation or agreement between the three performed serological tests with significant different between them (P=0.001), complete correlation between these tests is unlikely, because they detect the presence of antibodies to brucellosis in different immunological processes and in different ways as indicated in Table 1 the comparison between RBT and SAT results.

In the comparison between RBT, SAT and IgG / IgM immuno flow assay results, the RBT was never negative when the other two tests were positive. All tests disagreements occurred when the RBT was positive and the other two tests were negative.

RBT can be used as a rapid screening test, but requires confirmation with a more specific test. In routine clinical settings, SAT is often used for confirmation with titres above a certain threshold value being considered consistent with active brucellosis as reported before by Kerr et al. 1986 [19]. SAT still remains the test of choice in diagnosis in the presence of appropriate signs and symptoms, a presumptive diagnosis of brucellosis is usually defined as standard tube agglutination titre of 1/160 or greater as evaluated before in 1986 [19] and in 1992 [10] and create a base line titre for RBT that is equal to or more than 1/320, and this in agree with that of reported by Al Dahouk in 2003 [17].

For SAT, 7 cases (5.8%) gave positive results, the liters were equal or more than 160 and in this test the use of phenol saline is very important because the test needs long incubation period.

As showed in Table 2 and 3, the brucella IgM /IgG flow assays tested was positive for 6(50.0%) out of the 12 RBT positive samples and also 6(85.7%) of the 7 RBT positive samples that were confirmed by SAT. The details of the 6 IgM /IgG flow assay were as follows: IgM gave four

positive samples and IgG gave two positive were recent or acute cases and the 2 IgG positives were past or chronic cases.

The results of the IgM immune flow assays indicated acute and sub-acute stages of brucellosis in most patients. They showed 4 patients with specific IgM, in which the reactions were rated as moderately strong. One gave three crosses, one gave two crosses and the last two gave one cross for each, where the IgG immune flow assay was lower and gave two crosses and one cross which may indicate relapsing or reinfection. A similar result has been observed by Zeytinoğlu A et al. [21] in turkey, who noticed that the brucella IgM positive cases with high staining intensity were consistent with acute and sub-acute stages and brucella IgG high staining intensity consistent with relapsed or chronic cases. The low frequency of Brucellosis revealed by this study gives general idea. But it may not reflect the true prevalence. The finding of this study is in agreement with that published by Elansary et al. in Kassala [12] in that human brucellosis is not a major health problem in Sudan and it is in contrast to that reported by Hasan irmak after investigating sera of suspected individuals in endemic area in Turkey [5]. This study concluded that the serological methods were superior to cultural methods for diagnosis of brucellosis. Using IgM / IgG immuno flow assay test for diagnosis of brucellosis especially in rural areas and further researches in blood cultures for diagnosis are recommended.

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