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Comparative Study of Enzyme Linked Immunosorbant Assay and Agglutination Tests in the Diagnosis of Human Brucellosis in Baghdad

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Abstract

Background Human infection with Brucella spp. had been able to evoke humeral immune response containing both IgG and IgM.

Objective This study designed to compare results obtained from Rose Bengal Test (RBT), Tube Agglutination Test (TAT) and Enzyme Linked Immunosorbent Assay (ELISA) employ serum are described and compared for the detection of human IgG and IgM anti-brucella antibodies.

Methods Serum samples from 105 subjects were collected. 90 were clinically infected with human brucellosis, and 15 were age and gender matched controls. RBT and TAT are the two screening tests routinely recognized, while the 2-mercaptoethanol test (2ME) is the confirmatory assays currently in use. In order to improve the serological diagnosis of human brucellosis, an indirect IgG, IgM and IgG-IgM ELISA kits were evaluated

Results Totally, 90 cases were positive in RBT, from those only 92% shows positive TAT, and by ELISA there are IgG (27.78%), IgM (14.44%) and (57.78%) were Positive for both immunoglobulins.

Conclusion Although RBT and TAT are widely applied tests, they cannot differentiate acute and chronic states of brucellosis. Our data suggest that IgM ELISA may be a suitable test for diagnosis acute brucellosis.

Keywords Brucellosis, ELISA, Tube Agglutination.

Introduction

Brucellosis may present clinically as acute, as chronic after an acute attack, or as chronic and of insidious onset [(1)]. The serological results may differ, depending on the clinical form and stage of the infection [(2)].

Diagnosis is occasionally confounded because of non-specific clinical manifestations, and is confirmed only if brucella species are recovered from blood, bone marrow or other sites [(3-5)]. Brucellosis is usually associated with an intense humoral response [(6,7)]. Immunoglobulin M (IgM) brucella antibody predominates for the first week of the acute infection, after which the IgG antibody level starts to increase, reaches a peak after a few weeks, and predominates over the IgM antibody level until adequate therapy eliminates the infection [(8)]. Isolation of the microorganism is possible only in a minority of the infected patients in the acute phase of the disease [(9)]. Therefore, in the absence of bacteriological confirmation, a presumptive diagnosis can be made on the basis of a single high rising titer of specific antibodies [(8, 10)].

The routine brucella agglutination test is the most frequently used type of serological test for this purpose; however, it does not differentiate between active and inactive disease because it does not differentiate between IgG and IgM agglutinins [(2)]. Among a variety of serological tests, TAT (Tube Agglutination Test) is the most widely used [(17)]. Evaluation of various ELISA assays for IgG and IgM have shown that these techniques are generally more sensitive and specific than conventional tests, while they are able to distinguish specific antibodies of IgM and IgG classes associated with acute and chronic brucellosis [(11)]. The obtained results are always easily interpreted, since they are specific for single immunoglobulin classes [(1)]. On the other hand, they are not routinely available in developing countries, especially in rural areas [(12)]. Thus, this study designed to compare and evaluate the results obtained from different serological procedures for humoral immune response in human brucellosis.

Methods

Subjects

This study included (90) patients complaining of symptoms and signs commonly associated with Brucella infections. All patients were outpatient visitor to the private clinic in Baghdad during March 2008- March 2009; clinical details at presentation were records. All of the patients living in Abu-Ghraib city, an area in which brucellosis is endemic, many of the inhabitants are farmers raising livestock. They were (50) females and (40) male age range from (4-63) years, sera were collected at the first visit and stored in aliquots at -20 °C till analyzed.

Fifteen apparently healthy individuals were age and sex matched with no history of Brucella infection and Rose –Bengal negative test were selected as control group.

Rose Bengal test (RBT)

A drop (30µ) of undiluted serum was placed on circle of the slid, and then adds the same volume of antigen to the drop of the serum, both drops were mixed and the slide was observed for any agglutination within four minutes would indicate the presence of specific of anti-Brucella antibodies.

Tube Agglutination Test (TAT)

Test was performed according to Hausler and Koontz(13) using a standardized suspension of Brucella organisms prepared from *B. abortus*, the 2ME test was performed identically except that 2ME was added to each test tube to a final concentration of 0.05 M, and 0.85% saline was used to dilute the antigen rather than 0.85% saline containing 0.5% phenol. (13-15).

- A 0.9-ml volume of 0.85% saline (containing 0.1 M 2ME for the 2ME test, or 0.5% phenol for the TAT test) was added to a test tube, and 0.5 ml of the same solution was added to four more tubes. Also, 0.5 ml of the same solution was added to an antigen control tube, and 0.75 ml was added to a reading standard tube.
- Two fold serum dilutions beginning with 1:80 were formed by adding 0.1 ml of serum to tube 1, followed by sequential mixing and removal of 0.5 ml of the mixture to the subsequent tube and 0.5 ml of final tube was discarded, The antigen suspension was added in to tubes 1 through 5 and to the antigen standard tube, and 0.25 ml of this suspension was added to the reading standard tube. The reading standard tube was used to simulate 50% clearing of the antigen suspension after the agglutination reaction.
- The final serum dilutions in tubes 1 to 5 were 1:80 to 1:1280, the rack containing the test tubes was shaken 10 times to mix the antigen suspension and serum and incubated at 37 °C for 20 minute, each tube was examined without mixing or centrifugation for agglutination.

Enzyme linked immunosorbent assay (ELISA)

The test kit is provided by (Biocheck®), diluted patients serum,control and calibrater is added to the purified Brucella antigen coated on the surface of microwells, plate incubated for 1hr.at 37°C then the excess non reacted sera removed through three cycle of washing with washing buffer.

While the reacted sera detected by adding 100µ of diluted Horse radish peroxidase (HRP) diluted conjugated secondary antibody, the conjugate used for two ELISA plates were (HRP-anti human IgG for detection of human IgG and HRP-anti human IgM for detection of human IgM), after incubation for 30 min. plate washed with washing buffer and 100µ of substrate Tetramethylbenzidine (TMB) reagent added then incubate in dark place for 30 min at 37°C, reaction will be stopped by addition 100µ stopping solution(H_2SO_4) and read at 450nm. All results above the cut-off value (10 IU/Ml) considers positive.

Statistical analysis

All data were presented as frequency and percentage of categorical data and Chi-square test used for comparison of the results. Cut-off value was measured by calculation the upper limit of the 99% confidence interval, which calculated by the calculation of the mean of the (OD-values) of controls group readings (M) and the standard deviation (SD) and the standard error (SE) [16]. Cut-off value = M + 2.57(SD × SE).

Results

Comparative results of three different methods were used in the diagnosis of human brucellosis. Rose Bengal test considered as a gold standard agglutination test in the diagnosis of human brucellosis, the results showed that 90 sera from the patients with brucellosis showed a positive and 15 negative.

Also all samples were tested by Tube agglutination Test (TAT), A good correlation when Rose Bengal test were compared with the Tube agglutination Test, there were 83 (92%) positive sera and only 7 (8%) considered negative when Ab titer ≤80. All RBT negative sera were also negative for TAT as in table 1.

Table 1. Comparative result between positive and negative Rose Bengal Test with Tube Agglutination Test results

Rose Bengal Positive (90)			Rose Bengal Negative (15)		
TAT Negative 7 (8%)			TAT Negative 15 (100%)		
Titer	No.	Percentage	Titer	No.	Percentage
No agglutination 1:80	3 4	43% 57%	No agglutination	15	100%
Total	7	100%	Total	15	100%
TAT Positive 83 (92%)			TAT Positive 0 (0%)		
1:160 1:320 1:640 1:1280	17 32 23 11	20% 39% 28% 13%			
Total	83	100%	0	0	(0%)

With any positive result for RBT and TAT, 2-mercaptoethanol is performed and the results showed that only (16 patients were positive) and the 74 were negative, this negative results was also seen in 15 negative sera.

For IgG and/or IgM, as tested by ELISA, showed positive results in accordance with the other assays. For RBT positive sera, IgG ELISA (27.78%) showed positive results, IgM (14.44%) and (57.78%) were Positive for both immunoglobulins. While, sera from RBT negative always gave negative results for IgM and only 2 sera were positive for IgG as in table 2.

Table 2. Comparative result of ELISA test results in positive Rose Bengal Test cases.

Titer	Positive RBT cases (90)		
	IgG	IgM	Both (IgG and IgM)
1:16	4 (16%)	3 (23%)	2 (4%)
1:32	5 (20%)	1 (8%)	7 (13%)
1:64	11 (44%)	5 (38%)	24 (46%)
1:128	4 (16%)	4 (31%)	13 (25%)
1:256	1 (4%)	-	6 (11%)
Total	25 (100%)	13 (100%)	52 (100%)

The results of ELISA IgG and IgM showed disagreement with Rose Bengal test 58.26% and 66.96%respectively, while, ELISA results for both IgG and IgM Showed agreement with RBT results (58.26%) see table 4, thus it is becomes clear that both immunoglobulins gives rising titer when they measured by ELISA test and 100% of them were RBT positive (in acute brucellosis) with 100% sensitivity as seen in table 3.

Table 3. The sensitivity, specificity of TAT and ELISA compared with Latex

Test	Latex	
	Sensitivity	Specificity
TAT	100%	100%
ELISA IgG	93%	87%
ELISA IgM	100%	100%
ELISA both	100%	100%

Table 4. Agreement and disagreement between Rose Bengal test and ELISA.

Test	Result	RBT			Agreement	Disagreement
		Positive	Negative	Total		
ELISA IgG	Positive	25	2	27	33.04%	58.26%
	Negative	65	13	78		
	Total	90	15	105		
ELISA IgM	Positive	13	0	13	24.35%	66.96%
	Negative	77	15	92		
	Total	90	15	105		
ELISA both	Positive	52	0	52	58.26%	33.04%
	Negative	38	15	53		
	Total	90	15	105		

Discussion

In the absence of a positive culture, the diagnosis of brucellosis rests on the demonstration of specific antibodies. A variety of serologic tests have been applied to brucellosis, of which TAT is the most widely used [17]. In the TAT test a single serum titer of 1/160 or greater is considered significant [18]. However, early in infection lower titers may be present; therefore, it is important to obtain both acute and convalescent-phase sera. Sometimes agglutination can be masked at low dilutions of serum. If the diagnosis of brucellosis cannot be achieved by TAT because of the low titer of antibodies and the presence of blocking antibodies, Brucella IgG-specific and IgM-specific ELISA test systems have been shown to be an acceptable alternative to TAT for the diagnosis of subacute and chronic brucellosis. The detection of specific immunoglobulins by a single, simple and rapid test is a major advantage of ELISA [19].

Kostoula et al reported that ELISA appears to be more sensitive than the tube agglutination test for the diagnosis of human brucellosis, because this method detects specific IgG, IgM and IgA antibodies [20]. Gazapo et al stated that ELISA IgG and IgM positivity are helpful for epidemiological evaluations, whereas some false positive results can be obtained in classical tube agglutination tests due to the cross reactivity between Brucella spp. and Salmonella spp., Vibrio cholera and Yersinia bacteria [19]. As is well known, incomplete antibodies are commonly seen in subacute and chronic brucellosis, and so ELISA is recommended by some authors as a susceptible test for the diagnosis of such cases and it was asserted that ELISA could detect incomplete antibodies [21,22].

In the present study, ELISA was demonstrated to be more accurate when compared with TAT, indeed, 2 from 15 cases that were negative with TAT were revealed to be positive with ELISA for IgG, however, For RBT positive sera, IgG ELISA (27.78%) showed positive results, IgM (14.44%) and (57.78%) were Positive for both immunoglobulins., therefore, we concluded that it is advisable to perform both IgG and IgM ELISA technique in order to achieve higher accuracy.

The results allow us to conclude that both agglutination based tests we tested are useful as screening tests for the diagnosis of human brucellosis. In addition, ELISA is more reliable than conventional methods because of the sensitivity and the possibility of distinguishing between specific IgG and IgM immunoglobulins.

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