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Research Article

COMPARISON OF SEROLOGICAL TESTS AND PCR FOR DIAGNOSIS OF HUMAN BRUCELLOSIS SUFFERING FROM FEVER

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ABSTRACT

Objective: Brucellosis is an important zoonotic disease throughout the globe and other developing countries. The present study was aimed to compare results of different serological tests and polymerase chain reaction (PCR) for diagnosis of brucellosis in patients suffering from fever in Kolkata and in adjoining districts.

Methods: A total of 2088 serum samples were collected from the patients suffering from fever from January, 2013, to September, 2015. The samples were tested by serological tests, serum tube agglutination test (STAT), Rose Bengal plate test (RBPT), enzyme-linked immunosorbent assay (ELISA) (immunoglobulin M [IgM] and immunoglobulin G [IgG]), and *Brucella* genus-specific PCR.

Results: The study revealed decreasing positive results by STAT (18.43%, N=385), RBPT (12.59%, N=263), IgM ELISA (7.71%, N=161), PCR (4.21%, N=88), and IgG ELISA (1.43%, N=30). When serological tests were compared with PCR, it was found that both STAT and PCR were positive in 84 samples (4.02%), both RBPT and PCR were positive in 65 samples (3.11%), both IgM and PCR were positive in 51 samples (2.44%), and both IgG and PCR were positive in 9 samples (0.43%).

Conclusion: In this cross-sectional study in a zonal population of India, it was found that STAT was the most sensitive test for diagnosis of brucellosis followed by RBPT when compared to PCR test results. Four STAT-negative samples showed positive results in PCR, which were positive by RBPT test. This indicates that if we combine STAT and RBPT for diagnosis of brucellosis, then both sensitivity and specificity of the combined test will increase.

Keywords: Brucellosis, Serum tube agglutination test, Rose Bengal plate test, Enzyme-linked immunosorbent assay, Genus-specific polymerase chain reaction.

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INTRODUCTION

Brucellosis is a widespread zoonosis which causes a significant health problem in both humans and animals. The causative agent of brucellosis is bacteria of genus *Brucella*. It is facultative, gram-negative, non-spore-forming, non-capsulated, intracellular coccobacilli. It infects animals first and then transmits to humans. Among all the species of *Brucella*, *Brucella melitensis* and *Brucella abortus* are the main causative agent of human brucellosis. In India, the other species *Brucella suis* and *Brucella canis* rarely cause human disease [1,2]. Brucellosis is endemic in various countries, particularly in developing countries throughout the globe.

Brucellosis has various clinical symptoms such as prolonged fever, night sweat and weight loss. One of the most important causes of pyrexia of unknown origin is brucellosis. The clinical manifestations of human brucellosis often show similarity with other diseases. Hence, it is necessary to diagnose brucellosis properly. There are several laboratory tests to diagnose brucellosis such as serological tests which include serum tube agglutination test (STAT), Rose Bengal plate test (RBPT), enzyme-linked immunosorbent assay (ELISA), and molecular test such as polymerase chain reaction (PCR) as well as the most difficult *Brucella* blood culture technique. There is no single serological test which can confirm the diagnosis of brucellosis. According to some, PCR is the golden test for brucellosis and also it is used as a predictive marker for the course of the disease [3-6].

The aim of the present study was to compare STAT, RBPT, ELISA, and PCR tests for diagnosis of human brucellosis in fever cases.

METHODS

For this study, permission of the Institutional Ethical Committee had been taken. At the time of investigation, all the patients had been informed about the aim of the study, and written consent had been taken from all of the patients. The study was done at Peerless Hospital and B.K Roy Research Centre in Kolkata, India. A total of 2088 blood samples were collected from the patients suffering from fever. The clinical manifestation of fever cases includes symptoms such as temperature greater than 38.3°C on several occasions within 3 weeks and failure to reach a diagnosis during this period. All the serum samples were stored in −20°C until they were used.

Serological tests

STAT

STAT was performed using a commercial kit [Tulip Diagnostics (p) Ltd. Goa, India]. The kit contains ready to use standardized specific antigen suspensions of *Brucella* having specific reactivity toward antibodies to *B. abortus* and *B. melitensis*. The test was performed according to the company guidelines. At first, 8 test tubes were taken and numbered as 1-8. The test tube labeled with 8 was taken for negative control. The first test tube was filled up with 1.9 ml phenol saline, and other 7 test tubes were filled up with 1 ml phenol saline. Next, 0.1 ml test serum was taken to the first test tube to get 1/20 dilution. After that from first test tube, 1 ml diluted serum sample was transferred to the 2nd test tube, and this process was repeated from the 2nd to 7th test tubes to get 1/40, 1/80, 1/160, 1/320, 1/640, and 1/1280 dilutions. Next, 1 drop (50 µL) of antigen was added to each test tube along with negative control and mixed. After that, all the test tubes were kept in incubator at 37°C for 24 hrs. An antibody titer of 1:160 and above was considered positive for brucellosis.

RBPT

Rose Bengal antigen was procured from a commercial company (ID. vet innovative diagnostics, France). The antigen is used for detecting *B. abortus* (bovine)-, *B. melitensis* (ovine)-, or *B. suis* (swine)-specific antibodies in sera. The test was performed according to the company guidelines. 25 µL of Rose Bengal antigen and an equivalent amount of serum were mixed up on a slide. After 4 minutes, if there is slight agglutination, the presence of specific antibodies was demonstrated by the formation of agglutinates that were visible to the naked eye. In the absence of specific antibodies, the mixture remains homogeneous.

ELISA

The *Brucella* ELISA test was performed using a commercial kit (Immunolab immunoglobulin M [IgM] and immunoglobulin G [IgG], Germany), and the procedure of the test was followed by the kit instruction. At first microtiter, wells (which are coated with *Brucella* antigen (*B. abortus* strain, w99) were filled up with 100 µL each of the diluted (1:101) samples along with ready to use standards and controls except one well which was used for the substrate blank. Next, at the room temperature, the plate was incubated for 1 hr. After that, the wells were washed with the buffer solution. Next, 100 µL ready to use conjugate was added to all the wells. Then, the plate was covered with enclosed foil and incubated at room temperature for 30 minutes. After that, the wells were washed with wash buffer, and 100 µL each of the ready to use substrate was pipetted to all wells. Then, the plate was covered with the enclosed foil and incubated at room temperature for 20 minutes in the dark. Finally, 100 µL stop solution pipetted to all the wells to stop the reaction. The plate was then subjected for reading at 450 nm.

Extraction of genomic DNA followed by confirmation with Brucella genus-specific PCR

The genomic DNA extraction of the suspected human serum sample was done by Qiagen blood mini kit (Germany). All the procedures were followed as described by Ghatak *et al*. [7].

PCR was done (Fig. 1) with the reagent of Sigma company, and the primers were BCSP-B4(F)-TGGCTCGGTTGCCAATATCAA, BCSP-B5(R)-CGCGCTTGCCTTTCAGGTCTG, and all the procedures were followed as described by Joshi *et al*. [8].

RESULTS

A total of 2088 samples were tested. The investigation revealed (Fig. 2) decreasing positive results by STAT (18.43%, N=385), RBPT (12.59%, N=263), IgM ELISA (7.71%, N=161), PCR (4.21%, N=88), and IgG ELISA (1.43%, N=30) (Fig. 2). Furthermore, when all the serological tests and PCR tests were compared , it was found that STAT and PCR together showed maximum number of positive cases (Table 1).

It was also found that both STAT and PCR were positive in 23 samples when all RBPT tests were negative. Both RBPT and PCR were positive in 4 samples when all STATs were negative. All the three tests, STAT, RBPT, and PCR, were positive found in 61 cases. It was also found that both IgM and PCR were positive in 47 cases when all IgG were negative. Furthermore, IgG and PCR both were positive in 5 cases when all IgM were negative. All the three tests- IgM, IgG and PCR were positive in 4 cases (Table 2).

Sensitivity and specificity tests

Specificity and sensitivity of all the serological tests were done with respect to PCR for measuring the test efficacy. The sensitivity of a test is defined as it is the capacity of test to detect positive cases or patient with disease who will have a positive result when compared with gold standard. Specificity is the capacity of the test to detect negative or non-diseased people who will have a negative result when compared with gold standard [9,10]. The sensitivity and specificity of all the test results considering PCR as gold standard are given in Table 3 and Fig. 3.

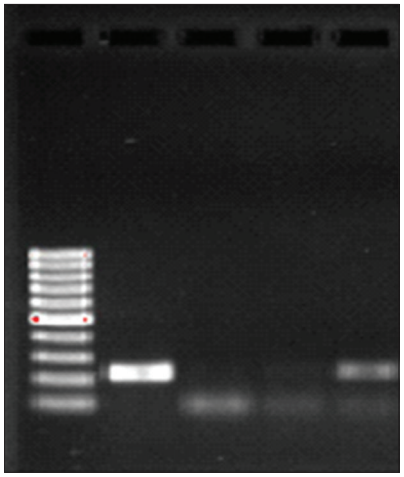

Fig. 1: The gel picture of polymerase chain reaction product. The 1st lane =100 bp ladder. 2nd lane = positive control, 3rd lane = negative control, and 5th lane = sample positive

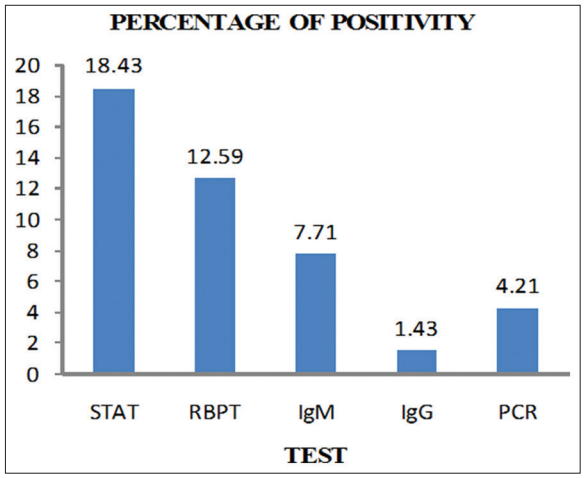

Fig. 2: The percentage of seropositivity and polymerase chain reaction positivity

Table 1: Both seropositive and PCR positive cases

Seropositive+PCR positive	n (%)
STAT+PCR	84 (4.02)
RBPT+PCR	65 (3.11)
IgM+PCR	51 (2.44)
IgG+PCR	9 (0.43)

STAT: Serum tube agglutination test, RBPT: Rose bengal plate test, PCR: Polymerase chain reaction, IgM: Immunoglobulin M, IgG: Immunoglobulin G

Table 2: Positivity patterns of PCR with other serological tests

STAT positive	RBPT negative	PCR positive	23 (1.10%)
STAT negative	RBPT positive	PCR positive	4 (0.19%)
STAT positive	RBPT positive	PCR positive	61 (2.9%)
IgM positive	IgG negative	PCR positive	47 (2.25%)
IgM negative	IgG positive	PCR positive	5 (0.23%)
IgM positive	IgG positive	PCR positive	4 (0.19%)

RBPT: Rose Bengal plate test, PCR: Polymerase chain reaction, IgM: Immunoglobulin M, IgG: Immunoglobulin G, STAT: Serum tube agglutination test

DISCUSSION

Brucellosis shows overlapping clinical manifestation with other diseases. Diagnostic problems arise due its similar O-antigen side chain

Table 3: Sensitivity and specificity of serological tests when compared to PCR test

Tests	PCR		Sensitivity (%)	Specificity (%)
	Positive	Negative		
STAT				
Positive	84	301	95.45	84.95
Negative	4	1699		
Total	88	2000		
RBPT				
Positive	65	198	73.86	90.10
Negative	23	1802		
Total	88	2000		
STAT+RBPT (dual positive)				
Positive	61	186	100	90.06
Negative	0	1687		
Total	61	1837		
IgM				
Positive	51	110	57.95	94.50
Negative	37	1890		
Total	88	2000		
IgG				
Positive	9	21	10.22	98.95
Negative	79	1979		
Total	88	2000		
IgM+IgG (dual positive)				
Positive	4	3	11.11	99.84
Negative	32	1872		
Total	36	1875		

PCR: Polymerase chain reaction, IgM: Immunoglobulin M, IgG: Immunoglobulin G, RBPT: Rose Bengal plate test, STAT: Serum tube agglutination test

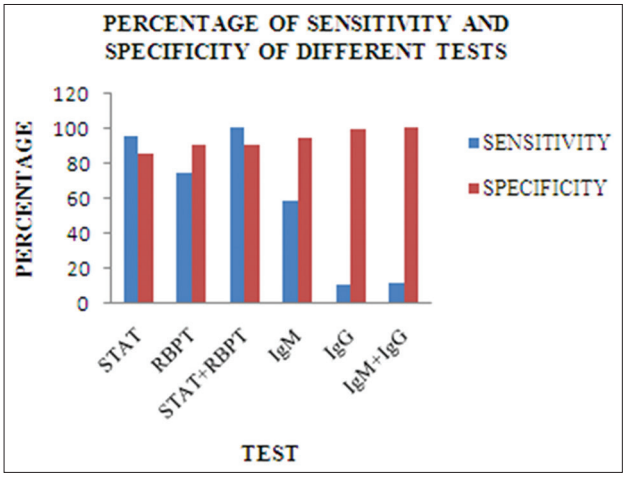

Fig. 3: The comparison among sensitivity and specificity of all the serological tests with respect to polymerase chain reaction

of lipopolysaccharide with *Salmonella* spp., *Escherichia coli* O116 and O157, *Pseudomonas maltophilia, Yersinia enterocolitica* O: 9, and some other microbes [11]. The present study was done by several serological tests and PCR, and the tests were compared for their sensitivity and specificity for diagnosis of brucellosis taking PCR as gold standard test. Further analysis was done to find more sensitive and specific tests for brucellosis diagnosis.

As clinical presentation of brucellosis is nonspecific and shows very complex clinical manifestation, a battery of tests may be ideal for diagnosis of brucellosis. Therefore, we had done all the serological tests and PCR, and then, serological tests were compared with PCR for finding the most sensitive and specific test for the diagnosis of brucellosis. From the result, we found that combination of STAT and RBPT showed the highest sensitivity as well as high specificity. Although the combination of IgM and IgG ELISA showed the highest specificity (99.84%), it was low sensitive than STAT with RBPT dual positive cases. Furthermore, Metri *et al.* [12] reported RBPT has a close relation with STAT in the diagnosis of human brucellosis.

CONCLUSION

From this study, it can be concluded that in case of diagnosis of agglutinating antibodies, we can combine both (STAT and RBPT) tests for highest sensitivity and followed by diagnosis of non-agglutinating antibodies through ELISA test for the highest specificity.

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