

Prevalence of Brucellosis among Febrile Negative Malaria Patients by PCR in Northern Kordofan State, Sudan

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Received date: April 11, 2017; Accepted date: August 09, 2017; Published date: August 12, 2017

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Abstract

Background: Diagnosis of Brucellosis is still facing some obstacles; to date it depends on conventional diagnosis based on blood culture and serological methods. PCR method now days offers the hope of rapid diagnosis.

Aim: To determine the prevalence of brucellosis among malaria negative febrile patients in Northern kordofan State, Sudan.

Methods: In this cross sectional study, one hundred blood samples were collected from febrile malaria negative patients of different ages from North kordofan State during the period from April to May 2016, and examined by Rose Bengal Plate Test (RBPT), ELISA and nested PCR methods.

Results: The results showed 40 samples were positive by RBPT method 52 samples were positive by ELISA and 81 sample positive by nested PCR.

Conclusion: There was high prevalence of brucellosis among the study population as detected by the three tests. Further studies using various diagnostic methods should be considered to determine the prevalence of human brucellosis at the national level.

Keywords: *Brucella*; PCR; ELISA; RBPT; Negative malaria patients; Sudan

Introduction

Brucellosis is one of the most common zoonotic diseases worldwide [1]. The disease is caused by *Brucella* spp. Four of 6 nomen species of the genus *Brucella* are pathogenic for humans, i.e., *B. melitensis* (transmitted from sheep and goats), *B. abortus* (from cattle and other bovidae), *B. suis* (from pigs), and *B. canis* (from dogs) [2].

Mediterranean basin (Portugal, Spain, Southern France, Italy, Greece, Turkey, North Africa), Mexico, South & Central America, Eastern Europe, Asia, Africa, the Caribbean and the Middle East are listed by CDC as high risk areas for brucellosis [3]. Even though countries like UK, Australia, New Zealand, Japan and Canada have been declared *Brucella* free, half a million new cases of human brucellosis are annually reported worldwide [4,5].

The disease primarily presents as fever of unknown origin with multiple clinical signs and symptoms. Patients regularly suffer serious focal complications such as spondylitis, neurobrucellosis or *Brucella* endocarditis [6]. The clinical features and presentation of human brucellosis overlap with many other infectious and noninfectious diseases [7] such as typhoid fever, rheumatic fever, spinal tuberculosis,

pyelitis, cholecystitis, thrombophlebitis, autoimmune disease, and tumours [8,9]. The clinical picture is not specific and laboratory testing should support the diagnosis. An accurate diagnosis is important, as therapeutic failure and relapse, a chronic course, and sometimes severe complications such as bone and joint involvement are characteristic of the disease [10].

Presumptive diagnosis of brucellosis can be made by the use of several serological tests to *Brucella* antibodies, but the "gold standard" remains isolation and identification of the bacterium. However, cultural examinations are time-consuming, hazardous and not sensitive and can only detect the organism in 10%-70% of suspected infections (Moyer and holcomb, 1995). Despite the vigorous attempt for more than one century to come up with a definitive diagnostic technique for brucellosis, diagnosis still relies on the combination of several tests to avoid false negative results [11]. A large number of different tests have been used for the serological diagnosis of brucellosis thus demonstrating the lack of an ideal technique. The sensitivity of these serological tests ranges from 65% to 95%, but their specificity in areas of endemicity is low, because of the high prevalence of antibodies in the healthy population. Moreover, most serological tests can produce cross-reactions with other bacteria, and they also have important limitations during the early phases of the disease, in persons whose occupations involve exposure to *Brucella* species, in patients with a recent history of brucellosis, and in patients who

undergo relapse [12,13]. Cultural methods are as well difficult because the *Brucellae* are facultative intracellular pathogens and their numbers found in patients are normally low [14]. Most studies of PCR assays involving human brucellosis have been undertaken with whole blood samples and in these studies different extraction methods, the use of different primer pairs, different target genes and different amounts of DNA were applied. Whole blood was used as clinical specimen in all the studies, but the authors differed in the amount of human DNA that should be added to each PCR assay [15,16]. Recently, Zerva et al. [17] used serum instead of whole-blood samples for the diagnosis of human brucellosis by PCR. Serum offers several advantages for nucleic acid amplification methods since inhibition by anticoagulants, hemoglobin, human DNA, or any other substances present in blood but not in serum is alleviated.

The excellent sensitivity reported by Matar and Morata, using the primers B4/B5, in the diagnosis of human brucellosis has not been reproduced by any other group [17,18].

Material and Methods

Data collection

Ethical approval for this study was obtained from EL Neelain University and informed written consent was obtained regarding data and blood samples collection from each patient. Only patients who agreed to participate were enrolled in this study.

Inclusion and exclusion criteria

The study included malaria negative febrile patient who has contact with animal and/or consume raw milk product. Febrile malaria positive patients were excluded.

Sample collection

A total of 100 blood samples were collected from febrile malaria negative patients who have contact with animal and/or consume raw milk product from Northern Kordofan State during the period from April to May 2016. Collected blood samples were centrifuged at 5000 rpm for 5 min to obtain the serum. The serum was immediately stored at -20°C until used.

Serological methods

Rose bengal plate agglutination: The Rose Bengal plate agglutination test (RBPT) antigen (produced by the Central Veterinary Research Laboratories Khartoum, Sudan) was used. For this test 30 µl of plain serum is dispensed on a white glossy ceramic tile and mixed with an equal volume of RBT antigen.

Definite clumping/agglutination were considered as positive reaction, whereas no clumping/agglutination were considered as negative for RBPT.

In house Indirect ELISA for *Brucella abortus* IgM antibody: This in-house Elisa test was developed by one of the authors (EEM) at the Department of Medical microbiology, Faculty of Medical Laboratory Science, El Neelain University, Khartoum, Sudan. The anti IgM conjugate was obtained from a commercial (VIRCELL, S.L. Pza., Spain)

A hundred µl of *Brucella abortus* antigen diluted in coating buffer was added to each well of the 96-wells ELISA plates and incubated

overnight at 4°C. After washing for three times with a washing buffer, 200 µl of blocking buffer was added to each well and incubated for 1 h at 37°C. Following washing for three times, 100 µl of pre-diluted samples, positive and negative controls (1:100) were added in to appropriate wells and incubated for 1 h at 37°C. After washing for three times 100 µl of anti-human IgM conjugate was added to each well and incubated for 1 h at 37°C. After three more washes, 100 µl of substrate solution Tetramethylbenzidine (TMB) and Hydrogen Peroxide (H₂O₂) was added to each well. The reaction mixture was then incubated for 20 min at room temperature in the dark. 100 µl of stop solution (2N H₂SO₄) was then added to each well. Finally, the optical density was measured using micro-plate reader instrument (Asys Expert Plus, Austria) at 450 nm. The mean absorbance (O.D) for each set of duplicate controls and samples was calculated.

Polymerase chain reaction (PCR)

DNA Extraction: DNA extraction was done by Saturated sodium chloride method as mentioned by Miller et al. 300 µl of blood samples were resuspended in 1.5 ml Eppendorf's tube with 1000 µl red cell lysis buffer (RCLB), mixed well and centrifuged at 5000 rpm for 10 min, Supernatant was discarded and 300 µl of white cell lysis buffer (WCLB) was added, 10 µl of 10% SDS and 20 µl of protein's K solution were then added and the mixture was incubated for 1 h at 65°C. Then 100 µl of 6M NaCl was added followed by 200 µl of cold chloroform and centrifuged at 18000 rpm for 6 min supernatant containing the DNA was then transferred to a new tube and absolute ethanol was added and centrifuged at 14000 rpm for 5 min. The supernatant was then discharged and the pellet was washed with 600 µl 70% ethanol and centrifuged at 6000 rpm for 5 min, the ethanol was discarded and the pellets were resuspended in 100 µl DW, and stored at -20°C until used [19].

Nested PCR: Four primers for the nested PCR were synthesized according to Agricultural Industry Criteria of the People's Republic of China (NY/T 1467e2007) [8]. These were Bp1 (50-CGT GCC GCA ATT ACC CTC-30), Bp2 (50-CCG TCA GCT TGG CTT CGA-30), Bp3 (50-GAT GCT GCCCGC CCG ATA A-30) and Bp4 (50-GCA CCG AGC GAG CCT TGA AA-30). Amplifications were carried out over two rounds of PCR in a Mastercycler Personal (Biometra, Germany) in volumes of 50 µl containing 1 PCR buffer, 2 mM MgCl₂, 200 mM each deoxynucleoside triphosphate, 1 mM each primer, 200e400 ng of genomic DNA and 2.5 U of DNA polymerase. Using the primers of Bp1 and Bp2, the first PCR amplification consisted of an initial denaturation step at 95 C for 5 min, followed by 35 cycles of denaturation at 94 C for 1 min, annealing at 49 C for 1 min, elongation at 72 C for 1 min, and concluded with a final extension step for 10 min at 72 C. Using the Bp3 and Bp4 primers and the first-round PCR product as template, the second amplification proceeded through a total of 20 cycles comprising denaturation at 94 C for 30 s, annealing at 51 C for 1 min, primer extension at 72 C for 1 min and a final extension of 6 min at 72 C. The second round PCR products were electrophoresed on a 1% agarose gel, and the target amplicon was 419 bp.

Data analysis: The data were analyzed using the statistical package for the social sciences (SPSS version 20). Cross tabulation-Chi-square and Kappa values were used to compare the different test.

Results

Out of the 100 samples tested, 81 sample were positive by PCR; 52 sample positive by ELISA; 40 positive by RBPT (Table 1), and 4 samples were negative in all the tests. ELISA detected 4 samples as positive that were negative in the rest of the tests. There were 4 samples that were positive by ELISA and RBPT but negative in the PCR. Three samples negative by PCR were positive in the serological tests while 49 samples that were negative by serological tests were positive in PCR. There were 26 ELISA and PCR positive samples that were negative in the RBPT and 22 samples were positive in all the test. The agreement between I-ELISA and RBPT (0.48) Table 4, between I-ELISA and PCR (0.37) Table 2, and between RBPT and PCR (0.43) Table 3.

Test	Positive	Negative	Total
RBPT	40 (40%)	60 (60%)	100
ELISA	52 (52%)	48 (48%)	100
PCR	81 (81%)	19 (19%)	100

Table 1: Detection Frequencies of brucellosis infection in malaria negative febrile patient from north kordofan State using RPBT ,ELISA, and PCR.

Test	PCR +ve	PCR -ve	Total
ELISA Positive (+ve)	46	7	53
ELISA Negative (-ve)	30	9	39
Total	76	16	62
Kappa value=0.37			

Table 2: Cross tabulation of PCR and ELSA results.

Test	PCR +ve	PCR -ve	Total
Rose bengal Positive (+ve)	32	8	40
Rose bengal Negative (-ve)	50	10	60
Total	82	18	100
Kappa value =0.43			

Table 3: Cross tabulation of PCR and Rospengal results.

Test	ELISA +ve	ELISA -ve	Total
Rose Bengal Positive (+ve)	23	14	37
Rose bengal Negative (-ve)	29	26	55
Total	52	40	92
Kappa value=0.48			

Table 4: Cross tabulation of ELISA and Rose Bengal results.

Discussion

Symptoms and signs of human brucellosis are not specific [13,20-22]. Hence, exact diagnosis of brucellosis cannot just be based on clinical symptoms, because it presents similar symptoms to other diseases such as malaria, typhoid and leptospirosis. Therefore isolation of organism in culture or identification of organism by serological and molecular methods for confirming clinical diagnosis is necessary [23,24].

Our results indicated high incidence of brucellosis in patients with fever in north kordofan state, irrespective of the test used. This is hardly surprising since this State is rich In animal wealth especially camels which are known to have high prevalence of *brucella* infection [25].

Our results also showed that PCR revealed the highest sensitivity in detecting *brucella* infection (81%) indicating that most of our patients were at the acute phases of the disease, it also indicate that PCR is probably the method of choice for diagnosis of brucellosis in the feverish patients in endemic areas [26].

Kapaa analysis of ours results revealed highest agreement (0.48) between RBPT and the in- house IgM ELISA. This is plausible as both tests are capable of detecting IgM antibodies which are the first antibodies to appear in early stages of infection.

Kappa analyses also showed moderate correlation between RBPT and PCR (0.43) and lower correlation between ELISA and PCR (0.37) this could be attributed to the fact that RBPT is known as highly sensitive test that could detect both IgM and IgG antibodies while our ELISA was only designed to detect IgM antibodies.

The results also validate the locally developed in-house ELISA as a good diagnostic test that identified (52%) of the patients as suffering from brucellosis compared to RBPT (40%). It would be interesting to compare this ELISA with other serological tests such as standard tube agglutination test (SAT) and complement fixation test (CFT) it also of interest to investigate the ability of these tests to monitor such patients after receiving treatment especially the use of PCR to confirm recovery from infection.

Our result also showed few sample that were positive by ELISA and RBPT tests but negative by PCR. This may indicate that the level of bacteremia in these samples might have been lower than the detection threshold of PCR.

Finally, our findings strongly suggest that patients with brucellosis in N. Kordofan were likely to leave the hospital without the specific treatment for brucellosis. This agrees with recent findings that showed that clinicians in Kenya continue to treat febrile patients for presumptive malaria, resulting in missed opportunities to accurately detect and treat other causes of fever [11,27]. The results also highlight the usefulness of nested PCR as a complementary assay to both ELISA and RBPT as a diagnostic approach in diagnosis of acute brucellosis and the need to establish national and regional reference laboratories with facilities for performing nested PCR assay [28,29].

Conclusion

Definitive diagnosis of brucellosis remains a difficult task. No single test is perfect, clinical history coupled with combination of two or more tests reduces diagnostic errors. The combination of serological and molecular is a good diagnostic criterion. Independent of the disease stage, PCR techniques are more sensitive and more specific

than serological tests. Establishment of Molecular methods could be a useful tool for detection of *Brucella* spp.

Acknowledgments

Staff members of the virology Department, Central laboratory are highly acknowledged for their help and provision.

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