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

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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 form North kordofan State during the period from April to May 2016, and examined by Rose Bengal (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 holocomb, 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 anti-human IgM conjugate was added to each well and incubated for 1 h at 37°C. After washing for three times, 100 µl of pre-diluted samples, positive and negative controls (1:100) were added to appropriate wells and incubated for 1 h at 37°C. 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 (2NH₄SO₄) 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 Satured 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: PCR was performed and the test was carried out in a total volume of 25 µl in the first PCR reaction, containing 5 µl of DNA mixed with 20 pmol of each primers outer primers; forward p1 (5’ AAT ACA CTG TGG TTT TAT GGG CCC 3’) and reverse p6 (5’ CCA TTG CTG GTC TTT TCA ACC ACA GGT 3’) (for detection of the bscp31 gene that codes for the 31 KDa antigen). The amplification was done by 35 cycles of PCR reaction (denaturation at 94°C for 1 min, annealing at 49°C for 60 sec and extension at 72°C for 10 min). The second round of the nested PCR was done with 5 µl of PCR product of the first round using 20 pmol of each of the inner primers; forward B4 (5’ TGG CTC GGT TGC CAA TAT CAA 3’) and reverse B5 (5’ CGC GCT TGC CTT TCA GGT CTG 3’) (nucleotide sequences 1498-1525 and 1600-1576 respectively) byapplying another touchdown PCR (denaturation at 94°C for 30 sec, annealing at 51°C for 60 sec and extension at 72°C for 6 min). The amplicons were resolved and screened using 1.5% agarose gel electrophoresis method.

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
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].

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.

<table>
<thead>
<tr>
<th>Test</th>
<th>Positive</th>
<th>Negative</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBPT</td>
<td>40 (40%)</td>
<td>60 (60%)</td>
<td>100</td>
</tr>
<tr>
<td>ELISA</td>
<td>52 (52%)</td>
<td>48 (48%)</td>
<td>100</td>
</tr>
<tr>
<td>PCR</td>
<td>81 (81%)</td>
<td>19 (19%)</td>
<td>100</td>
</tr>
</tbody>
</table>

Kappa value = 0.43

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**

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References

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