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Sero-Prevalence and Molecular Detection of Brucellosis among Febrile Patients in West Darfur State, Sudan

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Abstract

Brucellosis is a worldwide zoonosis with a high degree of morbidity in humans. It was formerly known as Mediterranean fever, Malta fever or undulant fever. The aim of this study to determine the prevalence of brucellosis among febrile negative malaria patients consume raw milk in West Darfur State, Sudan. In this cross sectional study, one hundred and half blood samples were collected from febrile patients and examined by Rose Bengal (RBPT), ELISA, SAT and PCR methods. The results showed 55 samples were positive by RBPT method, 67 samples were positive by SAT, 72 samples were positive by ELISA and 110 sample positive by nested PCR. Deficiency of awareness of brucellosis with prevailing routine habit of consumption raw milk and close contact with infected animals can function as mean of infection to human beings, molecular methods and could be a useful tool for the detection of *Brucella* spp.

Keywords: *Brucella*; PCR; ELISA; SAT; RBPT; Febrile patients; Sudan

Introduction

Brucellosis is a global zoonosis infectious disease [1]. It is caused by a member of genus *Brucella* that have the ability for persistence in the host cells and replicate, is related with their ability to cause a persistent infection and to inhibition the immunity. Various *Brucella* species affect sheep, goats, cattle, deer, elk, pigs, dogs and humans [2]. The disease was also reported in camels [3,4] and in marine mammals [5]. Transmission to humans occurs through different routes: the ingestion of unpasteurized milk and dairy products; direct contact with infected animal tissues; or accidental ingestion, inhalation or injection of cultured *Brucella*. Brucellosis is currently thought to be a possible biological weapon as it is highly contagious and air born transmission of the agent. [6]. Symptoms of brucellosis are not pathognomonic [7]. The disease in animals is characterized by bacteremia followed by localization of the organism in the reticuloendothelial tissues, reproductive organs and sometimes joints. Lesions of the reproductive tract of the pregnant female in cattle, sheep and goats may result in death and abortion of the fetus. *Brucella* also causes lesions in the male reproductive organs in cattle, sheep, goats and dogs and also bursitis in horses [8]. In human the most common clinical symptoms are fever (78%), arthralgia (65%), myalgia (47%) and back pain (45%) [9]. As 78% patients with brucellosis suffers from fever, it is a diagnostic challenge in malaria-endemic areas. Hepatomegaly and splenomegaly are reported in 23% and 26% patients, respectively [9]. Life-threatening focal complications are endocarditis and neurobrucellosis but the overall case fatality is less than 1% [10,11]. Severe complications of brucellosis infection are not rare, with 1 case of endocarditis and 4 neurological cases per 100 patients as reported by Dean et al. [9]. It is also reported by Dean et al. [9] that one in 10 men suffers from epididymo-orchitis. Poor diagnosis and treatment may result in complications like osteoarticular (sacroilitis, spondylitis, peripheral arthritis and osteomyelitis), dermal (erythematous papular lesions, purpura, dermal cysts), genitourinary (orchiepididymitis, glomerulonephritis and renal abscess), respiratory (pleural effusions and pneumonia), cardiovascular (endocarditis), and neurologic disorders (peripheral neuropathies, meningoencephalitis, transient ischemic attacks, psychiatric manifestations and cranial nerve compromise) resembling many other infectious and non-infectious diseases [10,12]. The economic and public health impact of brucellosis remains of concern in developing countries [13]. The disease poses a barrier to trade of animals and animal products, an impediment to free animal movement [14].

The diagnosis of human brucellosis is usually based on the isolation of *Brucella* spp. from blood, tissue specimens, body fluids and bone marrow, the serological tests for the detection of anti-*Brucella* spp. antibodies and the molecular methods for the detection of *Brucella* spp. DNA [15]. In countries where brucellosis is enzootic (i.e., present in animal reservoirs), human confirmed cases are based on clinical symptoms associated with positive serology without attempts to isolate *Brucella* spp. [16]. Serological testing is fast, non-hazardous and more sensitive than culture and therefore preferred in routine clinical practice. The PCR is more sensitive than blood cultures and more specific than serological tests [17]. The analytical sensitivity can be further increased by using real-time PCR assays, which can detect as few as five bacteria per reaction [18,19]. Moreover, real-time PCR enables high-throughput screening of clinical samples.

Materials and Methods

Sample collection

This was a health facility based descriptive cross-sectional study. A total of 150 blood samples were collected from febrile negative malaria patients consume raw milk in Wester Darfur State during the period

from August to November 2017. 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.

Exclusion criteria

The study excluded Febrile positive malaria patients.

Ethical consideration

This study was approved by the ethical committee of International University of Africa, Faculty of Medical Laboratory Sciences and verbal consent was obtained from each patient enrolled in this study.

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 25 µl of plain serum is dispensed on a white glossy ceramic tile and mixed with an equal volume of RBT antigen. Agglutination was considered as positive reaction, whereas no agglutination was considered as negative for RBPT.

Serum agglutination test (SAT): The SAT antigen was prepared and standardized in Division of *Brucella* research in Veterinary Research Institute (VRI) Soba, the antigen was diluted 1:120 using phenol saline. According to Buxton et al. The test was read by examining the tubes against a black background with light coming from behind the tubes. A positive reaction is one in which the serum-antigen mixture is clear and agglutinated antigen appears at the bottom of the tube. Gentle shaking does not disrupt the floculi. This is a complete agglutination and is recorded as ++++. In partial agglutination serum-antigen mixture is partially clear and gentle shaking does not disrupt the floculi, this was recorded as +++ or ++. Some sedimentation as + and no clearing as negative reaction.

Indirect ELISA IgM antibody: iELISA was performed according to Limet et al. [20] using B. abortus biotype 1 (Weybridge 99) S-LPS as antigen.

Molecular Detection

DNA extraction

DNA extraction was done by following SDS and Proteinase K extraction method [21]. 300 µl of blood samples were suspended 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 10 µl of protein's K solution were then added and the mixture was incubated for 1 h at 65°C. Then 100 µl of 6 M NaCl was added followed by 200 µl of cold chloroform and centrifuged at 11000 rpm for 6 min supernatant containing the DNA was then transferred to a new tube and absolute ethanol was added and centrifuged at 11000 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 purified DNA was dissolved in100 µl TE buffer and stored at -20°C until tested by PCR.

Polymerase chain reaction (PCR): PCR was performed and the test was carried out using following primers: forward: 5'-GACGAACGGAATTTTTCCAATCCC-3' and reverse: 5'-TGCCGATCACTTAAGGGCCTTC

AT -3'. Each reaction was performed in total volume of 25 µl, containing 5 µl master mix (Solis Bio dyne master mix), 2 µl of primer, 5 µl of DNA and 13 µl of distilled water. Reactions were performed using PCR machine under the following cycling conditions 110°C, 95°C for 5 min; 35 cycle were 95°C for 1 min, 65 °C for 1min, 72°C for 1 min extension at 72°C for 7 min. 5 µl of the PCR product was analyzed by gel electrophoresis in 2% Agarose, and stained with 0.15% Ethidium bromide and the product was visualized by using UV gel documentation system. The expected size of *B. abortus* DNA amplicon is 494 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

Serological testing of the 150 samples found 55 (36.6%) samples positive by RBT; 67 (44.6%) samples positive by SAT and 72 (48%) positive by I-ELISA for presence of antibodies against *Brucella*, whereas, molecular testing (73.3%) sample were positive by PCR (Table 1). There were 9 samples that were positive by SAT but negative in RBPT. There were 9 samples that were positive by ELISA, SAT and RBPT but negative in the PCR. While 62 samples that were negative by serological tests were positive in PCR There were 22 SAT and PCR positive samples that were negative in the RBPT and 48 samples were positive in all the test. The agreement between RBPT and PCR (0.34) (Table 2), between SAT and Rose-Bengal (0.83) (Table 3), between I-ELISA and RBPT (0.71) (Table 4) and between I-ELISA and PCR (0.54) (Table 5).

Test	Positive samples	Negative samples	Total
Rose-Bengal Test	55	95	150
SAT	67	83	150
ELISA	72	78	150
PCR	110	40	150

Table 1: Frequencies of brucellosis among febrile patient in Wester Darfur State, Sudan by using RPBT, SAT, ELISA and PCR.

Test	PCR Positive	PCR Negative	Total
Rose-Bengal positive	40	15	55
Rose-Bengal negative	65	30	95
Total	105	45	150
Kappa value=0.34			

Table 2: Cross tabulation of PCR and Rose-Bengal results.

Test	SAT Positive	SAT Negative	Total
Rose-Bengal positive	36	9	45
Rose-Bengal negative	42	22	64
Total	78	31	109
Kappa value=0.83			

Table 3: Cross tabulation of SAT and Rose-Bengal results.

Test	ELISA Positive	ELISA Negative	Total
Rose-Bengal positive	38	18	56
Rose-Bengal negative	34	37	71
Total	72	55	127
Kappa value=0.71			

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

Test	PCR Positive	PCR Negative	Total
ELISA positive	76	13	89
ELISA negative	19	18	37
Total	95	31	126
Kappa value=0.54			

Table 5: Cross tabulation of PCR and ELISA results.

Discussion

The diagnosis of brucellosis remains as one of the most challenging tests of medical knowledge and clinical acumen of the physicians. Brucellosis is diagnosed either by isolation of *Brucella* organisms in culture or by a combination of serological tests and clinical findings consistent with brucellosis. Isolation of the *Brucella* organism is the definitive means of diagnosis, but in practice it is difficult due to the early tissue localization and the exacting culture requirements of the organism. In practice, blood cultures are positive in 10-30% of brucellosis and the remainder is diagnosed serologically. Symptoms and signs of human brucellosis are not specific [22-24]. Isolation of organism in culture or identification of organism by serological and molecular methods for confirming clinical diagnosis is necessary [25,26]. The RBPT is usually used for brucellosis screening, its high sensitivity, ease and speed of use, as well as its low cost have made it very popular in hospital emergency departments for the diagnosis of febrile syndromes [27]. However, this test result must also be approved by another test.

Serum agglutination test (SAT)

The SAT has been used extensively for brucellosis diagnosis and, although simple and cheap to perform, its lack of sensitivity and specificity mean that it should only be used in the absence of alternative techniques. In each set of tests, a positive control serum calibrated against the International Standard for *B. abortus* antiserum (ISABS) must be included. ELISA is a rapid, sensitive and specific assay providing a profile of immunoglobulin classes in the diagnosis of acute and chronic brucellosis; therefore, it is useful for mass screening and could be considered the method of choice for the serological diagnosis of the named disease [28]. PCR assay for the diagnosis of human brucellosis, appeared to be a more sensitive technique than microbiological methods, not only for the diagnosis of a first episode of infection, but also for the early detection of relapses [29].

The present study revealed that the overall sero-prevalence of brucellosis in patients with fever in north Darfur state was 36.6%, 44.6%, 48% and 73.3 with RBPT and SAT, ELISA and PCR respectively. Our results also showed that PCR revealed the highest sensitivity in detecting *Brucella* infection (73.3%) indicating that most of our patients were at the acute phases of the disease, it also indicates that PCR is probably the method of choice for diagnosis of brucellosis in the feverish patients in endemic areas.

The prevalence of human brucellosis found in this study is slightly higher than reported by Abdelhady R. The prevalence (36%) based on RBPT in this study was less than that obtained earlier in some herds (60%). Clinicians in continue to treat febrile patients for presumptive malaria, resulting in missed opportunities to accurately detect and treat other causes of fever [30,31]. The results also highlight the usefulness of PCR as a complementary assay to ELISA, SAT and RBPT as a diagnostic approach in diagnosis of acute brucellosis. The magnitude of human *Brucella* infection can serve as a barometer of the prevalence of the disease in domestic animals. Eradication of brucellosis in animals is the key to prevention in humans. Nevertheless, public health education assumes an important role in preventing the transmission of brucellosis from animals to humans.

Conclusion

The risk of spread the disease due to uncontrolled movement of animals, poor hygiene and management conditions and free sale of infected animals in the markets cannot be overlooked. Deficiency of awareness of brucellosis with prevailing routine habit of consumption raw milk and close contact with infected animals can function as mean of infection to human beings. However, detection of pathogen should be used clinical history coupled with combination of two or more tests reduces diagnostic errors. molecular methods and could be a useful tool for the detection of *Brucella* spp.

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