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Diagnosis of recent and relapsed cases of human brucellosis by PCR assay

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Abstract

Background: Brucellosis affects human populations in many developing countries including the Middle East, and Latin America where it is still endemic. It has been prevalent in Jordan for years, where 7842 cases of human brucellosis were registered at the Ministry of Health during 10 year-period. This study was initiated by the recent increase in the number of human cases diagnosed in a rural area in the Northern Jordan to help assess the status of the disease in that area. For this purpose blood specimens from brucellosis suspected cases were tested by serology, culture and PCR.

Methods: Peripheral blood specimens from 50 healthy control subjects and 165 seropositive patients having compatible signs and symptoms that were clinically diagnosed to have brucellosis were tested by blood culture, and by PCR. The PCR assay used genus-specific primers from the conserved region of the 16S rRNA sequence, which showed high specificity for the *Brucella* spp.

Results: Diagnosis of *Brucella* was established by PCR in 120 cases (72.7%). All of them were seropositive and 20 were positive by culture. Forty-eight of 58 (82.8%) of the relapsed cases two months after completing the treatment with an increase in the previous serological titers were positive by PCR. The assay has 85.7% positive predicative value, 100% sensitivity and specificity since it correctly identified all cases that were positive by blood cultures, 95.8% by serology and none of the control group was positive.

Conclusions: Results showed that PCR assay can be applied with serology for the diagnosis of brucellosis suspected cases and relapses regardless of the duration or type of the disease without relying on the blood cultures, especially in chronic cases.

Background

Brucellosis continues to be an important zoonosis of economic significance and human suffering. It affects human populations in many developing countries including the Middle East, and Latin America where it is still endemic [1]. The World Health Organization reports an annual incidence of human brucellosis of less than one to 78 cases per 100,000 population in the Middle East, with six coun-

tries reporting an annual total incidence of more than 90,000 cases [2]. Four *Brucella* spp. namely, *Brucella abortus*, *B. canis*, *B. suis* and especially *B. melitensis* are able to cause human infections [3]. Brucellosis is usually transmitted to humans by ingestion of unpasteurized dairy products or by direct contact with infected animals. Occupational disease is contracted by exposure of abattoir workers and veterinarians to infected animals especially

aborted fetuses, fluids, membranes or urine. In developed countries, the incidence of human brucellosis has declined in the last 50 years as a result of infection control measures, and in these countries most cases result from occupational disease, travel-acquired infections, or accidental laboratory exposure through contaminated aerosols [3]. According to the World Health Organization, half a million new human cases each year are reported worldwide [4]. These numbers greatly underestimate the true incidence of human disease [3] because the clinical picture of human brucellosis is extremely variable [5], and because it is under declared to local authorities and misdiagnosed by clinicians.

Brucella spp. are able to cause prolonged morbidity due to the capability of this bacteria to evade the host defense mechanisms by surviving as intracellular organisms. The diagnosis of the disease can be challenging and is frequently delayed or missed because the clinical picture may mimic other infectious and noninfectious conditions [6,7]. Diagnosis can be established by laboratory methods such as serology and blood cultures. Prolonged incubation period, special growth media, and subcultures are required for the isolation of these fastidious, slow growing bacteria. However, cultures are not always positive when other tests are positive [8]. Automated systems have been reported to detect more than 95% of *Brucella melitensis*-positive cultures within seven days of incubation [7]. The technology is lacking in developing countries or rural areas where the disease is prevalent and diagnoses rely mainly on serology. Many serological tests have been used for the diagnosis of human brucellosis such as agglutination tests, indirect immunofluorescence, ELISA. The most commonly used tests are the serum agglutination test (SAT), the Coombs anti-*Brucella* test, the Rose Bengal test, and complement fixation [9]. Each test has its own disadvantages, and the presence of antibodies doesn't always mean an active case of brucellosis, since humans from endemic areas often show weak serological responses [8]. As for other fastidious pathogens, amplification of DNA by PCR offers an alternative way of diagnosis of brucellosis.

Brucellosis has been prevalent in Jordan for years, where 7842 cases of human brucellosis were registered at the Ministry of Health during 10 year-period, from January 1988 to 1997 [10]. Ovine and caprine brucellosis caused by *Brucella melitensis* have been present in sheep and goat flocks in the bedouin sector for years. The number of cases has increased considerably in the mid to late 1980s. The increase might partially be attributed to improved diagnostic methods.

This study was initiated by the recent increase in the number of human cases diagnosed in the health centers in a rural area in the Northern Jordan Badia to help assess the

status of the disease in that area. For this purpose blood specimens from brucellosis suspected cases were cultured and tested with PCR assay using a genus-specific primer pair derived from the conserved region of the 16S rRNA sequence which showed high specificity for the *Brucella* spp. [8]. The results are compared with that of the blood cultures and the Rose Bengal agglutination test.

Methods

Clinical specimens

A total of 215 peripheral blood specimens was collected from patients in four comprehensive health centers over a period of 18 months. Of these blood specimens, 165 were from patients who presented with clinical signs compatible with brucellosis. Clinical diagnosis was made by the physicians in the Badia of Jordan, a rural area in the north east of Jordan inhabited by recently settled Bedouins. These centers receive patients from 40 villages with a population exceeding 12,000 inhabitants. The diagnosis of brucellosis is established based on the presence of compatible signs and symptoms with the demonstration of specific antibodies at significant titer or seroconversion. The physicians are aware of the zoonotic nature of the disease because of the continuous reporting of the disease in sheep, goats and in humans in this area. Sixty (36.4%) of the samples were collected after adequate antibiotic treatment was started.

A questionnaire was completed for each subject at the time of specimen collection to record demographic and other relevant information such as the clinical history, symptoms and physical signs, contact with animals, drinking unpasteurized milk, and homemade dairy products.

The duration of symptoms prior to diagnosis was 1 to 16 weeks. Cases with clinical symptoms less than two months were considered as acute cases, those that lasted more than 6 months before treatment was initiated were considered as chronic cases. A relapse was considered to be either a positive blood culture two months to one year after completing the treatment or the reappearance of compatible symptoms not otherwise explained together with an increase in the previous serological titers. All patients had fever during the course of the disease with other symptoms such as muscle pain, wrist arthritis.

Treatment of recent cases is by a combination of a daily intramuscular injection of one gram of streptomycin for 14 days and doxycycline for a month. In the chronic cases, a combination of doxycycline and rifampin is given to the patient for one month according to the internationally accepted treatment regimens [11]. The treatment was repeated when the symptoms persist and antibody titer was still high two weeks after concluding the treatment.

Control samples

Samples were obtained from 50 subjects, composed of patients randomly selected among patients from the same region attending the same health centers. None of these patients was currently diagnosed or had a history of brucellosis.

The study was approved by the University ethics committee.

Serological techniques

The serological tests were carried out by the laboratories of the health centers. The serologic diagnosis was established by Rose Bengal agglutination test. A titer of 1/160 was considered positive.

Blood cultures

Five to ten ml peripheral blood samples were withdrawn and immediately inoculated under aseptic conditions in broth media (Bloodgrow[®], Medical Wire & Equipment Co. Ltd, Corsham, Wiltshire, England) or diphasic blood culture bottles (Hemoline performance diphasique bioMerieux, Marcy l'Etoile, France). Cultures were incubated at 37°C for 30 days in the presence of 5% CO₂ and were periodically checked for growth. Subcultures on blood agar plates were performed in a blind manner at 10, 20, 30 days, they were recorded as negative after the last negative subculture. *Brucella* spp. were identified using standard methods [12].

DNA extraction

DNA was extracted from blood specimens using a commercial purification system (Wizard Genomic DNA Purification Kit, Promega, Madison, WI) according to the manufacturer's instructions for DNA purification from blood. Final pellets were resuspended in 50 l of TE (10 mM Tris, 1 mM EDTA, pH 7.2).

PCR assay

The Brucella DNA-Detect PCR Kit (Vita-Tech International Inc., CAN), including the reagents and oligonucleotide primers designed for the direct amplification of the genus *Brucella*, was used for the detection of *Brucella* spp. in blood samples. The primers used in the kit were genus-specific primer pair designed to amplify a highly conserved region within the 16S rRNA of the genus *Brucella* [8].

The size of the amplification products was a 905 fragment of the rRNA gene. The reaction mixture contained 5 µl of 10 × PCR buffer, 1 µl of the primer mix, 2 µl of dNTP mix (5 mM each), 3 µl of 25 mM MgCl₂, 1 unit of Taq DNA polymerase, 5 µl of sample DNA in a volume of 50 µl. The same mixtures were used with the positive control provided by the kit. The mixture with no DNA sample, and DNA

free water were used as negative controls to monitor contamination.

The reaction was performed in a thermal cycler (Gene Amp PCR System 9700, Perkin Elmer, Norwalk, Con.). The cycling conditions were an initial denaturation at 95°C for 5 min, template denaturation at 95°C for 30s, annealing at 54°C for 90s, and primer extension at 72°C for 90s for a total of 35 cycles, with a final extension at 72°C for 6 min. A sample was considered positive when the size of the DNA band matched with that of the positive control (905 bp). All standard precautions recommended for prevention of contamination with DNA and amplicons were undertaken. Twelve microliters of the PCR product were run by electrophoresis in a 2% agarose gel in 1 × TBE buffer (Promega, Madison, Wis.), and gels were stained with ethidium bromide (2 µg/ml).

PCR amplification products were detected by visualization of the bands under UV light.

Statistical analysis

The means, ranges, percentages of positive samples, specificity, sensitivity and positive predictive value of PCR were calculated.

Results

Clinical data

The mean age of the patients was 46 years (range, 6 to 86 years). Of these patients, 58 (35.2%) were males and 107 (64.8%) were females. The information recorded in the questionnaire indicated that, out of 165 patients included in this study, 66 (40%) had contact with animals (e.g., sheep and goats) either raised nearby where they live or at work (e.g., farmers). Seventy-four (44.9%) acquired their infections by consuming unpasteurized milk or homemade dairy products (e.g., soft cheese), 32 (19.4%) acquired their infection possibly from contact with animals as mentioned above, 24 (14.5%) acquired their infection possibly from one of these two sources.

The other 35 (21.2%) patients acquired their infection from unknown sources, since they either drink pasteurized milk, do not drink milk, or drink powder milk, and had no homemade cheese. Two of the patients drink camel milk.

The mean duration of symptoms before diagnosis of the brucellosis was 35 days (range 10–60 days) in 140 (84.8%) of the cases (Table 1). The duration for the other 25 (15.2%) was more than 60 days. Fifty-eight (35.2%) of the previously treated patients had symptoms suggestive of a relapse within a year after concluding treatment. These patients had symptoms consistent with brucellosis with an increase in antibody titers and no other

Table 1: Epidemiological, clinical, and serological results of 165 cases of human brucellosis.

Characteristics	Values
No. of patients studied	165
Male: Female	1:1.85
Mean age in years (range)	45.5 (5–86)
Clinical and laboratory diagnosis:	
Mean duration of the chronic cases in years (range)	8.5 (1–16)
No. of patients with fever	165/165 (100%)
No. of patients with muscle pain and anorexia	140/165 (84.8%)
No. of relapsed patients	58/165 (35.2%)
No. of positives in Rose Bengal agglutination test (1/160)	140/165 (84.8%)
No. of patients with positive blood cultures	20/165 (12%)
No. of patients with positive PCR	120/165 (72.7%)
No. of relapsed patients with positive PCR	48/58 (82.8%)

Table 2: Comparison of the PCR results with blood culture and serology for the 165 cases

Result of tests	Sero ^a , Cult ^b		Sero, Cult		Sero, Cult		Sero, Cult		Total
	+	+	+	-	-	+	-	-	
PCR +	20		100		0		0		120
PCR -	0		20		0		25		45
Total	20		120		0		25		165

^a Serology: Rose Bengal agglutination test (titer, \$/160) ^b Cult: blood culture

infections. They complied with the treatment according to the information provided in the questionnaire.

Serology

One hundred and forty (84.9%) of these patients were positive by Rose Bengal agglutination test with titers ranging between 1/160 and 1/1280 units. The 25 clinical cases who were seronegative were not included in the group of certainly infected patients.

The number of patients who were tested for *Brucella* antibodies in that area during the study was 1050, they had symptoms consistent with brucellosis, but only 452 (43%) were positive at the time they were tested.

Blood cultures

Brucella was isolated in blood cultures from 20 of the 165 (12%) blood specimens who didn't receive antibiotic treatment at the time the specimen was collected. Isolates were identified by standard methods as *B. melitensis*. These samples were positive by both PCR and serology.

PCR assay

One hundred and twenty (72.7%) of the 165 samples were positive by PCR. These patients had symptoms for

more than one month to several years and were all positive by serology; recent infections of less than one month were negative by PCR. The DNA bands that appeared in the acute cases were brighter than those of the chronic cases.

The PCR was negative on conclusion of the treatment for 25 of the 45 negative patients. The other twenty negative patients were suspected cases who were recently diagnosed based on signs and symptoms. They were still negative by serology and by culture and hence, these patients could not be considered as true infections (Table 2).

Forty-eight (82.8%) of the 58 relapsed cases were positive by PCR and by serology. The positive predicative value (e.g., the proportion of true positive among all positives) of the assay was 85.7% based on the serology results.

Control samples

None of the 50 subjects was positive by serology or by PCR.

The sensitivity of the PCR assay is 85.7% compared to serology and the specificity is 100% since all the control subjects were negative.

Discussion

Although brucellosis is a notifiable disease in many countries, official figures do not fully reflect the number of cases reported annually, and the true incidence has been estimated to be between 10 and 25 times higher than what the reported figures indicate [13].

The increase in the incidence of human cases was linked to the lambing season in Jordan [10,14]; these reports are in agreement with the findings of the current study. Bedouins are at higher risks of contracting brucellosis, due to the lifestyle, environmental and social conditions of this population [15].

Diagnosis of human brucellosis based on the clinical picture alone is not definitive since the symptoms mimic other diseases and is fairly nonspecific. The isolation of the organism in culture or detection by molecular methods is necessary to confirm the clinical diagnosis. There are few studies concerning the use of PCR technique in animals [16,17], the studies of human cases are limited and the number of patients included was small [3,18,19]. The present work studied the potential use of a single-step PCR assay as a rapid test for the diagnosis of acute, chronic infections, and a relapse in previously treated patients. Patients were also studied by means of blood cultures, Rose Bengal agglutination. In many laboratories the serological diagnosis of human as well as animal brucellosis is based on a Rose Bengal test, which was improved from being a screening test to be a titrable one [20].

One of the genetic targets frequently used for strain identification and strain phylogeny is the rRNA operon, particularly the 16S rRNA gene used in this study. These genes are highly conserved and diverge very slowly. The DNA sequences from separate species within a genus will differ by only a few percent. Sequence identity among 16S rRNA sequences is typically interpreted as indicating a single species [21]. A PCR assay with primers derived from the 16S rRNA sequence of *Brucella abortus* was developed [8]. The specificity and high sensitivity of this assay provide a valuable tool for the diagnosis of brucellosis.

The PCR results confirmed the clinical diagnosis in 120 (72.7%) patients, the results obtained by serology in 120/140 (85.7%) and by blood cultures in 20/20 (100%). It correctly diagnosed 48/58 (82.8%) of the relapse cases who were identified based on the symptoms and the increase in the antibody titer after completing the treatment. It can reduce the time needed for blood cultures and its limitations caused by the low number of bacteria in the blood especially in chronic and focal type of the disease [22]. The assay was able to detect *Brucella* DNA in cases of acute, chronic and relapsed brucellosis. The DNA bands in chronic cases were weak most probably due to low num-

bers of *Brucella* in the peripheral blood. The same results were reported by a previous study in serum samples obtained from staff of cattle breeding centers [23].

Twenty of the forty-five cases that were negative by PCR were recently diagnosed cases of brucellosis (< one month). These cases were negative both by serology and by blood cultures, indicating that they are true negatives. The other 25 samples were negative by the three methods, which might be due to the absence of infection, the possibility of temporary or episodic lack of bacteremia in brucellosis patients, the stage or nature of the disease, or the treatment that lowers the number of bacterial cells in the peripheral blood beyond detection. The therapy can modify PCR, culture, and serology results and therefore these cases should constitute a category different from those where samples were taken before therapy.

Several factors were reported to inhibit PCR in a blood specimen such as the high concentrations of leukocytes DNA and heme compounds [19].

The sensitivity of the PCR assay was 100% based on the results of the 20 patients who were positive by both serology and blood culture and 85.7% based on the number of patients who were positive by serology alone. The specificity of the assay was 100% since none of the 50 control blood samples was positive by PCR. The threshold sensitivity for the PCR assay was reported to be from 80 ng to 0.08 fg [8] as determined by testing serial dilutions of *B. abortus* 2308 DNA detected after 40 cycles of amplification. Similar findings with respect to the amount of *Brucella* DNA detected by PCR have been obtained by using primer sequences from the genes encoding a 43-kDa outer membrane protein [24] and a 31-kDa *B. abortus* antigen [18]. It was suggested that 60 to 100 fg of bacterial DNA is equivalent to 20 cells [25]. The importance of this finding is based on the fact that low numbers of circulating *Brucellae* are present in focal and chronic forms of the disease that are difficult to detect by culture and leads to a suboptimal recovery rate of *Brucella* from blood.

The PCR test proved to be useful in the early detection of relapses as was reported by another [18]. study [26]. The diagnosis of these relapses is difficult by conventional methods. Relapses after concluding a correct treatment are known to occur in brucellosis [3,27]. This problem is related to the ability of *Brucella* spp. to evade some of the basic mechanisms of the host's immune system [26]. The relapse rate of 32.5% of the patients after a combined treatment could be explained in part by lack of compliance in some patients to the full course of treatment. This is common in such rural communities where Bedouins show up for treatment several weeks after having symptoms and discontinue treatment once the fever

subsides. It might be also explained by the fact that *Brucella* organisms localize in the reticuloendothelial system after surviving the intracellular mechanisms by phagocytes and polymorphonuclear leukocytes [3,28]. A combination regimen of two or three drugs is more effective than a single-drug regimen. The relapse rate may be as high as 50% in cases of single-drug regimens [28,29]. Longer courses of therapy are required to cure relapses and focal forms of the disease such as meningitis and osteomyelitis. The positive PCR results obtained in the 48 (82.8%) of the relapse cases in this study is especially important indicating that the assay could be a useful tool to confirm a relapse in cases of a treated brucellosis. However, due to the extremely high sensitivity of the technique, the ability to amplify the DNA of non-viable, non-culturable *Brucella* DNA from dead or phagocytized cells especially the remains of DNA present in the circulating mononuclear cells in certain patients who have concluded successful-treatment [26] should be considered when interpreting the results.

The criteria to indicate a cure of brucellosis is not definite, since negative blood cultures do not exclude the presence of the disease. Another study reported recovering the bacteria in 53.4% to 95% blood cultures of patients but successful isolation of the organism decreased over time [7]. Other specimens from bone marrow and liver tissue or lymph nodes may improve recovery rates [3], but these specimens are invasive and are not always feasible. Antibodies may remain elevated for a long time after conclusion of the treatment [30], which was noticed in some of the chronic cases in this study that were negative by PCR.

Conclusion

The results of this study are in agreement with several previous studies [8,18,22,26] that reported on the usefulness of PCR and recommended its use for the diagnosis of initial episodes, focal complications, post treatment follow-up, and relapses.

The peripheral-blood-based PCR assay described is highly sensitive and specific, easy to perform, and could provide results to a clinician in less than 8 h. In addition, it avoids the risks to laboratory personnel associated with handling the microorganism.

Competing interests

None declared.

Author contributions

LN carried out all the experimental work related to this study.

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