

## EVALUATION OF PCR, CULTURE AND SEROLOGY FOR THE DIAGNOSIS OF ACUTE HUMAN BRUCELLOSIS

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**Background:** The diagnosis of brucellosis is frequently difficult to establish. This is not only because clinically, the disease can mimic any infectious and noninfectious disease, but also because the established diagnostic methods are not always successful. In this study, we have tried to evaluate PCR techniques in the diagnosis of brucellosis in comparison to conventional techniques.

**Patients and Methods:** Fifty peripheral blood samples from the following groups were collected: patients with brucellosis (17); patients with febrile illnesses due to factors other than brucella etiology (19); symptomatic occupationally exposed persons (9); and healthy volunteers (5). The last three groups were considered controls. Among the 17 *Brucella* samples, only 14 were obtained before treatment was begun. The samples were tested by serology, using the standard tube agglutination method (STA), blood culture using Bactec machines, and PCR using primer pair to amplify a 223-bp region within a gene coding for a 31-kD *Brucella* antigen. Diagnosis of brucellosis was based on compatible clinical picture in addition to positive blood culture and/or positive serology.

**Results:** Of the 17 blood samples from patients with brucellosis, eight were culture positive for *Brucella* species, and all showed high titer anti-brucella antibodies. Only 14 of them were positive by PCR, and these were the samples submitted before initiation of therapy, representing 100% sensitivity. Among the 33 controls, blood culture was negative for *Brucella* in all of them, while one sample showed high-titer anti-brucella antibodies. The latter was from the febrile illnesses group. PCR-based assay was able to detect four bands in the controls, all of which were from the occupationally exposed asymptomatic group.

**Conclusion:** In view of the several advantages of PCR over the conventional methods for the diagnosis of brucellosis, such as speed, safety, high sensitivity and specificity, the technique might be considered for laboratory diagnosis of brucellosis. However, for the evaluation of asymptomatic highly exposed persons, PCR might be considered complementary to the traditional methods and followed up by serology and/or culture.

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**Key Words:** Brucellosis, PCR.

Brucellosis is a major cause of zoonosis, and an important public health problem in many parts of the world, especially in the Middle East. The disease is endemic in Saudi Arabia.<sup>1</sup> Clinical presentation of the disease is nonspecific, and may be very atypical, therefore, laboratory confirmation by isolation or detection of specific anti-brucella antibodies is essential for confirmation of the diagnosis. However, positive blood cultures occur in 10%-70% of suspected infections,<sup>2</sup> depending on the duration, localization of the infection and the type of *Brucella* species. Furthermore, culturing is time-consuming and

serological tests are better than culture techniques, their specificities are low, especially in endemic areas or in people professionally exposed to *Brucella*. False-positive serological tests may also be caused by other illnesses such as salmonellosis, tularemia, cholera, lupus erythematosus and myeloma,<sup>2</sup> while false-negative results may occur early on in the course of the disease<sup>3</sup> or in case of focal infection.

Because of their potential to detect very small numbers of organisms, PCR-based assays have been applied recently to diagnose many infectious diseases. There are only a few reports on the use of PCR for the diagnosis of human brucellosis from peripheral blood samples.<sup>4,5</sup> Moreover, the advantages of such techniques over the traditional conventional methods have not yet been clearly established. In this report, we compare the traditional diagnostic methods with PCR-based assay for the diagnosis of human brucellosis. To the best of our knowledge, this is the first report of this kind in an endemic Arab area.

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presents a major laboratory hazard, as *Brucella* spp. are class III pathogens. Although the sensitivities of the

TABLE 1. Characteristics of the patients and the control groups.

	Control Group (33)			
	Brucella group (15)	Febrile illnesses	Occupationally exposed group	Healthy volunteers
Number of samples	17*	19	9	5
Positive <i>Brucella</i> culture	8	0	0	0
Blood culture isolates other than <i>Brucella</i>	0	4**	0	0
Positive anti-brucella antibodies <sup>+</sup>	17	1	0	0
Positive serological tests other than <i>Brucella</i>	0	1**	0	0

\* Only 14 samples were collected before treatment; \*\* see text; <sup>+</sup>antibody titers range from 160-2560.

TABLE 2. Comparison of the results of PCR-based assay with those of conventional microbiological techniques for the diagnosis of brucellosis.

	No. of samples	Positive by PCR	Positive by blood culture	Positive by Serology	P-value
Brucella group (15)	14*	14	8	14	<0.001
Sensitivity		100%	57%	100%	
Control group (33)	33	4**	0	1 <sup>+</sup>	<0.001
Specificity		88%	100%	97%	
Corrected Specificity <sup>++</sup>		100%	100%	96%	

\*Only the 14 samples that were collected before treatment were included in the calculation; \*\*all were from the occupationally exposed group; <sup>+</sup>this was from the febrile illness group; <sup>++</sup>calculated after exclusion of asymptomatic occupationally exposed person.

## Materials and Methods

A total of 50 peripheral blood samples were obtained from 33 controls and from 15 consecutive patients with brucellosis diagnosed in the Infectious Disease Department at Dammam Central Hospital and Maternity and Children Hospital, over a six-month period. There were eight males and seven females, aged between 6 and 65 years. One patient gave three samples (accounting for a total of 17 samples), corresponding to initial infection, convalescent phase and suspected relapse. Two additional samples from the patients with brucellosis could only be obtained after treatment was begun during their follow-up in the outpatient clinic.

All patients had fever with arthralgia and/or backache, with a mean duration of symptoms of about four months (range 1-13). They all gave a positive history of animal contact or ingestion of unpasteurized dairy products. In eight of these patients, brucellosis was confirmed by culture and high titer anti-brucella antibodies that were performed at the time of diagnosis. For the others, diagnosis was based on compatible clinical picture and high-titer antibodies. High titer was defined as a titer of  $\geq 1:160$  by the standard tube agglutination (STA) method. The 33 control blood samples (Table 1) were obtained from the following subgroups: 1) 19 patients with febrile illnesses due to factors other than *Brucella* etiology (four cases of bacteremia due to *Salmonella*, *Staphylococcus*

*aureus*, *Staphylococcus epidermidis* and *Pseudomonas* and 15 cases of backache and/or arthralgia with or without fever in which no particular pathogen could be isolated). In all these patients, blood cultures for *Brucella* were negative, while repeated serological tests using febrile agglutinins showed a titer of *Salmonella paratyphoid* of 1:320 in one patient and a titer of 1:160 for *Brucella* in another one. The latter was a sickler presenting with backache due to vaso-occlusive crisis with no history of exposure to animals or animal products. Repeated serology in this patient did not show titer increment of the anti-brucella antibody. 2) Nine asymptomatic patients who were occupationally exposed to *Brucella*, with or without history of brucellosis in the previous 2 to 4 years. Two of them showed low-titer anti-*Brucella* (1:40) antibodies on repeated occasions. 3) Five healthy volunteers with no history of brucellosis or exposure to animals. From all the patients and controls, three specimens were collected simultaneously and were sent for culture, serology and molecular methods. The majority of the specimens were processed within 24 hours.

## Microbiological and Serological Techniques

Blood cultures were processed with BACTEC 9240 and BACTEC NR660 (Becton Dickinson, USA), incubated for six weeks and subcultured weekly. Suspected colonies were identified by colonial morphology, gram-staining and standard biochemical and serological methods; typing was not available. Serological tests were done using standard tube agglutination method<sup>3</sup> and SAS Febrile Antigens (USA).

## Sample Processing for PCR

DNA was prepared using the method described by Miller et al.<sup>6</sup> with a slight modification. Briefly, a minimum of 2 mL of blood collected in a citrated tube was resuspended in 5 mL erythrocyte lysis solution (5 mM MgCl<sub>2</sub>, 320 mM sucrose, 12 mM Tris HCl, 1% Triton X-100 [pH 7.5]), mixed and centrifuged at 15,000 Xg for 2 minutes. Treatment with erythrocyte lysis solution was repeated, followed by treatment with deionized water until the leukocyte pellets lost all reddish coloring. Template DNA was obtained from the leukocytes by adding 400  $\mu$ L of nucleic lysis buffer (60 mM NH<sub>4</sub>Cl, 24 mM Na<sub>2</sub> EDTA [pH 8]), sodium dodecyl sulfate (1%) and proteinase K 1 mg/mL, followed by mixing and incubation for 2 h at 55°C or overnight at 37°C with slow shaking. A quantity of 100  $\mu$ L of NaCl (6 M) was then added, followed by centrifugation at 15,000 Xg for 10 min. The supernatant containing total DNA was transferred to a fresh tube and two volumes of cold ethanol was then added and shaken until the DNA precipitated, which was then collected by fishing, using hooks and dissolved in 30  $\mu$ L double-distilled water. DNA, which was not tested immediately, was stored at -70°C until the time of the assay. The

concentration and purity of the DNA were determined spectrophotometrically by reading  $A_{260}$  and  $A_{280}$ .

#### DNA Amplification

The primers B4 (5'-TGGCTCGGTTGCCAATATCAA-3') and B5 (5'-CGCGCTTGCCTTTCAGGTCTG-3'), described previously by Bailey et al.,<sup>7</sup> were used to amplify a target sequence of 223-bp within a gene code for the production of a 31-kDa membrane protein specific to the genus *Brucella*.<sup>8</sup> PCR was performed using a protocol described elsewhere.<sup>4</sup> Briefly, a 50  $\mu$ L volume reaction mixture containing 10 mM tris-HCl (pH 8.4), 50 mM KCl, 1 mM  $MgCl_2$ , 200  $\mu$ M each deoxyribonucleotide triphosphate (dATP, dGTP, dTTP, dCTP, Pharmacia LKB Biotechnology, Uppsala, Sweden), 1.5 U of Taq polymerase (Perkin-Elmer Cetus Co., Norwalk Conn., USA), oligonucleotide B4 and B5 (100 nM each; Gulf Biotech, Riyadh, Saudi Arabia) and 2-4  $\mu$ g of total DNA extracted or 150 ng from the positive control was processed in a thermocycler (Perkin-Elmer 9600). The cycling conditions consisted of initial denaturation at 93°C for 5 min., followed by 35 cycles of 60 sec. of template denaturation at 90°C, 30s of primer annealing at 60°C, and 60 sec. of primer extension at 72°C and final extension at 72°C for 7 min. Specificity of the assay was examined by testing *E. coli* (ATCC No. 25922) DNA as targets. DNA free control was also included to monitor contamination.

Each sample was tested at least in duplicate. Generally recommended procedures were used to avoid contamination. The products (10-18  $\mu$ g from each reaction mixture) were analyzed by agarose gel (1.5%) electrophoresis at 160 v for 15 min., stained with ethidium bromide and photographed on a UV Tran illuminator. The presence of a clear-cut band was considered as a positive result. The sensitivity of the assay was tested by decreasing the amount of target DNA in the reaction mixture through serial dilutions of total DNA of one positive case and processing, as described above.

#### DNA Extraction from Culture

Positive control with genomic DNA isolated from a suspension of *Brucella* spp. was extracted using a modified alkaline lysis method described by Bernboim and Doly (1979) and Ish-Horowicz and Burke 1981.<sup>9</sup> Briefly, colonies of *Brucella* spp. were harvested from chocolate agar, killed in 67% methanol, and pelleted by centrifugation at 15,000 Xg for 3 min. after addition of distilled water. The supernatant was drained away completely to keep the pellet dry as much as possible. The pellet was then resuspended in 100  $\mu$ L of solution I (50 mM glucose, 25 mM tris-Cl [pH 8], 10 mM EDTA), followed by the addition of 200  $\mu$ L of solution II (0.2 N NaOH, 1% SDS), mixing and storage on ice. Finally 150  $\mu$ L of solution III (5 MK acetate 60 mL, glacial acetic acid 11.5 mL,  $H_2O$  28.5 mL) was added, tubes were mixed thoroughly and centrifuged at 15,000 Xg for 5 min. at 4°C,

the supernatant was discarded and the DNA extracted as described above.

## Results

Of the 17 blood samples obtained from patients with brucellosis, eight were positive by culture (Table 1), with a mean detection time of 7 days (range 4-14). All the samples showed high-titer antibrucella antibodies, including the three samples submitted after treatment. PCR was positive only in the 14 samples which were collected before treatment during active infection and the one case of relapse, thus representing 100% sensitivity. PCR was negative in the remaining three samples that were submitted after treatment. Samples containing *E. coli* DNA and DNA free target were negative in all assays. Among the 33 controls, 29 had negative results by PCR (Table 2), and the four false-positive cases were all from the occupationally exposed group. These were asymptomatic herdsmen, one with a history of febrile illness and treatment two years previously. None had a positive blood culture or detectable antibrucella antibodies, thus accounting for 88% specificity. The dilution method showed that as little as 30 fg of total DNA can give a visible band.

## Discussion

The high endemicity of brucellosis in the Arabian Peninsula has stimulated many researchers to research many of its aspects, such as epidemiology and clinical features. However, there have only been a few reports covering the diagnostic issues. This has led others<sup>10</sup> to call for expanding research, particularly on the use of new technology to address unsettled diagnostic issues relating to brucellosis. (We report the results of PCR as compared to serology and culture methods for the diagnosis of brucellosis). The PCR is currently used for the diagnosis of many infectious diseases. The publication of Queipo-Ortuno et al.<sup>4</sup> provided the basis for this study. Our PCR-based assay was able to identify all 14 samples submitted during active infection, which gave a sensitivity of 100%. This high sensitivity is probably related to the ability of the PCR-based assay to detect as little as 30 fg of total DNA. This dilution method also showed that the assay could be used with a wide range of target concentration. Romero et al.<sup>11</sup> found that the lowest detection limit using DNA from culture isolates is 80 fg. Despite the different source of DNA that we used, our assay was sensitive enough to detect *Brucella* genome from 30 fg of total DNA. The three samples which were submitted after completing treatment were negative by PCR, indicating the potential

usefulness of such a technique for confirming cure. The numbers involved in this technique were too small to recommend it as a method of follow-up, and are only part of an ongoing study. Similar findings are needed for firm conclusions to be drawn. Although the sensitivity of the serological methods using agglutination assay was also 100% (Table 2), there was obvious limitation in distinguishing established cure from active infection, as the antibody titers were persistently high in the three samples submitted in convalescent phases during the study period. This result is in agreement with the findings of a previous report,<sup>12</sup> and confirms the fact that serological follow-up of patients by agglutination tests does not always correlate suitably with the clinical outcome. If only agglutination tests had been considered in the three patients, it would have been difficult to establish cure unless several samples some few weeks apart had been taken to demonstrate declining antibody titer. The sensitivity of blood culture was only 57% (8/14) and there was a delay of about one week before results were issued, while PCR-based assays were able to give the results on the same day.

Although the apparent specificity of serological tests is superior to the PCR (97% vs. 88%), the corrected specificity of PCR, as calculated after exclusion of the occupational group, was higher (100%). No amplification signal was detected in the samples from febrile illnesses group despite the fact that there were four different microbial isolates in some of them, including *Salmonella*, which is known to share antigenic components with *Brucella*.<sup>3</sup> While the serological test showed one false-positive case among the febrile group, representing 97% specificity, other serological tests that measure antibodies against specific *Brucella* proteins such as ELISA were found not to cause the problem of cross-reaction with other gram-negative bacteria.<sup>12</sup>

The use of newer serological ELISA techniques was not included in our comparative study. These tests are generally more sensitive, as they are based on primary interaction, provide a profile of immunoglobulin classes for the diagnosis of acute and chronic brucellosis,<sup>13</sup> might be useful markers for active brucellosis,<sup>12</sup> and may readily be standardized by the use of purified, smooth lipopolysaccharide antigen and monoclonal antibody.<sup>14</sup> Nevertheless, agglutination tests remain the standard methods against which other tests must be compared.<sup>15</sup>

For statistical analysis, we excluded the occupationally exposed group because it is unlikely that these tests are requested in asymptomatic individuals. We believe that the ability of PCR technique to detect four bands in otherwise asymptomatic but heavily exposed persons is a reflection of the extreme sensitivity of this technique, which is known to compromise the specificity of any assay.<sup>16</sup> The clinical significance of positive results for such a highly sensitive method remains unclear.<sup>17</sup> Queipo-Ortuno et al.<sup>4</sup> did not find a single positive case in their control group, perhaps

due to the difference in exposure and protective measures in the two groups. The intermittent exposure of these people might have led to a localized infection or represent a very early stage of the disease not sufficient to produce detectable antibodies,<sup>16</sup> however, the possibility of inadequately treated or self-limited past infection<sup>18</sup> cannot be excluded. In either case, follow-up of these patients, both clinically and by serology or culture, is highly recommended for early diagnosis and treatment. We have advised these patients to report any symptoms and to repeat serology every 2-3 months.

The results of statistically significant tests applying hypothesis testing<sup>19</sup> are also shown in Table 2. Both the sensitivity and specificity of PCR was significantly greater than that of culture and serology, respectively ( $P < 0.001$ ).

Because *Brucella* spp. are intracellular parasites, relapse is not an uncommon complication. The diagnosis of relapse is even more difficult than the diagnosis of initial infection, as the patient may remain seropositive for a year or longer,<sup>3</sup> and negative blood culture is not uncommon in chronic infection.<sup>2</sup> In this report, there was a clear band in the single patient presenting with symptoms of suspected relapse after five months of completing treatment. His blood culture was negative and serology showed persistent high titer antibrucella antibodies. This was a finding in a single case of relapse, and we are in the process of collecting more cases before valid conclusions can be made about the significance of PCR in diagnosing relapse.

In conclusion, the PCR-based assay has several advantages over the current microbiological methods for the diagnosis of brucellosis, including speed, safety, high sensitivity and specificity, therefore, it should be considered. For evaluation of asymptomatic, occupationally exposed persons, the traditional methods might be superior, especially if the cost is taken into consideration. Nevertheless, the information provided by PCR should be considered complementary to the results of conventional methods for the time being. Further researches on a large number of similar cases are necessary to establish the suitability of each method.

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