Short Communication

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Large-scale evaluation of a single-tube nested PCR for the laboratory diagnosis of human brucellosis in Kuwait

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A single-tube nested PCR assay identifying a 52 bp fragment from the genus-specific *Brucella* IS711 gene was used prospectively in clinical practice for the diagnosis of human brucellosis in Kuwait. Patients with suspected brucellosis and with other infections were investigated as clinically indicated but in all of them culture, serology and PCR for *Brucella* were carried out. Out of 263 suspected cases of brucellosis, diagnostic tests were positive in 199, serology was positive in 199 and culture in 89, while the *Brucella* PCR was positive in 193 (sensitivity 96.98 %, 95 % confidence interval 94.5–99.5 %). Chronic brucellosis, involving symptoms for more than 1 year, was diagnosed in 49 out of these 193 patients. Diagnoses in four out of the six patients with positive serology but negative PCR and culture were non-*Brucella* bacterial meningitis and viral meningitis. False-negative PCR results were possible in the remaining two; both had been on long-term antibiotics for previously diagnosed brucellosis but their adherence may have been questionable, allowing a relapse. The PCR was negative in 244 patients with other infections (specificity 100 %) and in 180 control subjects with negative *Brucella* culture and serology. PCR results were available within 24 h of sample receipt, providing diagnostic information rapidly. The PCR is expensive and technically demanding and may not be appropriate for all cases. Future studies will help define how it may best be used. For example *Brucella* and tuberculous meningitides can be very similar, *Brucella* PCR may allow prompt distinction between the two, avoiding the need for prolonged empirical treatment for both.

Introduction

Brucellosis remains an important cause of persisting febrile illness in many countries in the world, particularly those of the Arabian peninsula (Madkour, 2001). Diagnosis should ideally be based on demonstrating the organism, but reports from a range of endemic areas draw attention to the relatively low frequency of isolation of the causative organism (Mousa *et al.*, 1988). The BACTEC system increases rates of isolation with shorter times to positivity (Bannatyne *et al.*, 1997) but serological testing has been the mainstay of laboratory diagnosis for many years, with the tube agglutination test and its variants most widely used, and complement fixation, the Coombs test and, more recently, ELISAs used to greater or lesser extents (Young, 2001). While *Brucella* is an organ-

ism of relatively low pathogenicity, causing morbidity but low mortality, it is highly infectious and laboratory infections are all too commonly reported (Al-Aska & Chagla, 1989).

PCR for amplification of specific DNA sequences that identify organisms has been applied successfully in the *Brucella* genus (Baily *et al.*, 1992; Bricker & Halling, 1994; Ouaharani *et al.*, 1996). Clinical applications in human disease are reported (Matar *et al.*, 1996; Queipo-Ortuno *et al.*, 1997; Morata *et al.*, 2003). We have described previously (Al-Nakkas *et al.*, 2002) a single-tube nested PCR for diagnosis of brucellosis in venous blood samples using primers from the genus-specific IS711 sequence present in varying copy number across all members of the genus *Brucella*. Our initial study (Al-Nakkas *et al.*, 2002) looked at small numbers of human cases. In the present study we report the results of

Abbreviation: CI, confidence interval.

application of this PCR prospectively in diagnosis of human cases of brucellosis in an endemic country, in other infections and in a further group of healthy controls.

Methods

Patients. Four hundred and forty-three adult patients attending the emergency clinic of the Kuwait Infectious Disease Hospital or the medical services of other hospitals in Kuwait were studied prospectively; these included 263 patients with clinically diagnosed brucellosis and 180 patients whose admitting diagnoses were conditions other than brucellosis. In addition 180 healthy adults, consisting of hospital personnel and relatives of patients, were also studied. Patients and healthy adults gave verbal or written consent to participation in these studies, which were approved by the Ethics Committee of the London School of Hygiene and Tropical Medicine. For all patients a history was taken, physical examination performed and a clinical diagnosis made. All were then investigated as indicated clinically under the supervision of the attending physician. In all cases samples were also taken for *Brucella* serology, *Brucella* culture and *Brucella* PCR. The final diagnosis was determined from patients' clinical records.

Case definition. The case definition for a final diagnosis brucellosis was a history of exposure to domestic animals known to harbour the organism, most often goats and camels, or consumption of unpasteurized milk or milk products, together with an appropriate history of persisting fever, and symptoms and physical signs compatible with this infection, plus positivity for one or more of *Brucella* agglutination (SAT) at $\geq 1/160$, *Brucella* ELISA and *Brucella* blood culture. Compatible signs and symptoms included hepatosplenomegaly, lymph gland enlargement and localization typical of this infection, for example spondylitis, arthritis or epididymo-orchitis.

Laboratory investigations. Blood counts, chemical pathology tests, serology, blood cultures, etc., were processed using standard laboratory protocols.

Diagnostic tests for brucellosis. *Brucella* SATs were performed in the Serology Laboratory at the Infectious Diseases Hospital using *Brucella* A and M antigens in the Brucelloslide test kit (bioMérieux). A titre of 1/160 or greater was considered positive (Lulu *et al.*, 1988). *Brucella* ELISA serology was performed in the Department of Microbiology, Faculty of Medicine, University of Kuwait using Immunotab ELISA kits (Serion) detecting anti-*Brucella* IgG, IgM and IgA antibodies in the patients' serum. For culture, venous blood was inoculated into aerobic and anaerobic bottles and incubated in a BACTEC 9240 Fluorescent Instrument System incubator for 3 weeks for signs of growth. Positive bottles were then subcultured into serum dextrose agar (Oxoid) and any organisms resembling *Brucella* isolated were speciated using sera detecting A and M antibodies to *Brucella abortus* and *Brucella melitensis*, respectively (S A Scientific).

***Brucella* PCR.** The *Brucella* PCR was described in detail previously (Al-Nakkas *et al.*, 2002). Briefly EDTA blood samples were processed in a class III safety cabinet using the method described by Kawasaki (1990) for safe extraction of DNA. The assay used oligonucleotide primers (R & D Systems) from the IS711 DNA sequence, with outer primers of 24–25 nucleotides spanning a 325 base pair segment and inner primers of 16–17 nucleotides, yielding a 52 bp final product. Conditions for the PCR were those previously noted (Al-Nakkas *et al.*, 2002) using a Hybaid Combi TR2 or System 9600 (Perkin Elmer) thermal cycler. Duplicate samples with appropriate positive and negative controls were used at all stages. *Brucella* PCR assays were carried out by one of us (A. Al-Nakkas) without knowledge of serology and blood culture results.

Because primer-dimer complexes formed at room temperature can be

extended to yield false positive responses in the first cycle of the PCR reaction as the reaction mixture warms to the denaturation temperature, a hot-start protocol was used to dissociate primer-dimers before PCR cycling began. The two sets of primers were placed in reaction tubes and overlaid with a wax cap. The remaining components, nucleotide bases, Taq polymerase, test or control materials, etc., were layered over the wax cap. The wax cap melts at 60 °C and so any primer-dimer complexes would have dissociated during heating to 60 °C when the wax cap melts to allow mixing of the reaction components. Biotin-digoxigenin labelling of primers allowed binding of positive reaction products to avidin-lined wells of microtitre plates and their detection by anti-digoxigenin antibody linked to peroxidase, developing a blue colour reaction by adding 3,3',5,5'-tetramethylbenzidine read visually or by an automated ELISA reader.

Results that corresponded in the duplicate assays were read as positive or negative. Where there were ambiguous results, the PCR was repeated on the same extracts. If the result remained ambiguous, then no designation was made for that sample without repeating the sample preparation step. Final PCR results were then compared with those of serology and blood culture.

Results and Discussion

Two hundred and sixty-three patients were thought on clinical grounds to have brucellosis at admission. This diagnosis was supported by laboratory data in 199 patients and active brucellosis was the final diagnosis in 195 patients. Alternative infections were identified in four patients with positive *Brucella* serology but negative culture and PCR; these are discussed below. Diagnoses in the 64 cases classified as not having brucellosis were tuberculosis (14), typhoid (9), meningitis not due to *Brucella* (28), rheumatoid arthritis (10) and septic arthritis (3).

Among patients with positive responses for *Brucella*, 89 were both serology and culture positive while 110 were serology positive only. Eighty-five of the isolates were *B. melitensis* and four were *B. abortus*. Among 180 other febrile patients the diagnoses made were chickenpox (83), malaria (59), hepatitis (33), measles (3) and mumps (2). In these cases tests for *Brucella*, serology and culture were uniformly negative.

The *Brucella* PCR was positive in 193 out of 199 patients (97 %) in whom other tests for brucellosis were positive (Table 1). Forty-nine of these 193 PCR-positive patients had clinical features suggesting infection for more than 1 year, indicating chronic infection, of whom four had positive culture. The PCR was negative in 180 patients with other infections that were recognized by the admitting clinicians and in the 64 non-*Brucella* cases from the group initially admitted with the clinically suspected diagnosis of brucellosis. One hundred and eighty control subjects had negative PCR. The test was useful for detecting patients with a wide range of disease durations.

Final diagnoses in the six patients with positive serology and negative culture and PCR are listed in Table 2. All had meningitic presentations and all had previously had chronic brucellosis. In four, alternative causes for their current presentation were found (meningococcal meningitis, 2; pneumococcal meningitis, 1; and viral meningitis, 1). Each

Table 1. *Brucella* PCR results

Results from 443 patients presenting with febrile illness, including patients with a clinical diagnosis of brucellosis and those with diagnoses other than brucellosis.

<i>Brucella</i> result	PCR positive	PCR negative	Total
Culture+/serology+	89	0	89
Culture-/serology+	104	6	110
Culture-/serology-	0	244*	244
Total	193	250	443

*This group included patients admitted with a clinical diagnosis of brucellosis shown to be negative on laboratory testing for this infection ($n = 64$) and those admitted with initial diagnoses other than brucellosis ($n = 180$).

of these had previously had chronic brucellosis with symptoms lasting 8–10 months and had been symptom-free after prolonged antibiotic treatment for 7–11 months. The remaining two had been on prolonged antibiotic therapy; one presented during this study, while notionally still taking tetracycline and co-trimoxazole, with fever and worsening muscle and joint pains and with serological tests becoming more strongly positive (Table 2) and the other presented with a relapse of symptoms while on 7 months of the same antibiotics, again serology became more strongly positive. Thus two of these six results were false negative PCR results.

The sensitivity of the test was 96.98 % (95 % CI, 94.5–99.5) if it is assumed that negative *Brucella* PCR results in all six patients with positive *Brucella* serology results were true false negative results. If, however, it is accepted that only two of the six were false negative results the sensitivity is 98.97 % (95 % CI, 97.5–100) and the specificity is 100 %.

We have applied single-tube nested PCR for the diagnosis of human brucellosis in clinical practice in an endemic area and have obtained satisfactory results with good sensitivity and specificity when compared with serological testing. The PCR gave results as good as serological testing with the added advantage of identifying the presence of the organism and

was superior to culture, which was positive in only 89 out of 195 of the total number of *Brucella* cases. Positive results were obtained for patients with symptoms of varying durations, with 49 out of 193 having chronic disease. This provides an indication that PCR can be of value in the laboratory diagnosis of chronic infections. The *Brucella* DNA sequences detected by PCR in culture negative cases may represent intracellular organisms incapable of replicating because of factors such as prior antibiotic therapy, which can sterilize peripheral blood, preventing identification of organisms by culture (Bannatyne *et al.*, 1997).

In developing this test we showed that it gave positive results over five *Brucella* species, *B. melitensis* (two biovars), *B. abortus* (four biovars), *B. suis* (six biovars), *B. canis* and *B. ovis* (one biovar each) (Al-Nakkas *et al.*, 2002), because the IS711 gene is genus specific. PCR using *Brucella* insertion sequence DNA can be made species-specific by characterization of flanking sequences (Ouaharani *et al.*, 1996) though in the context of the Arabian Peninsula the dominant species is *B. melitensis* (Madkour, 2001) as our culture results have shown. Culture of the organism will remain important for monitoring the species present and antimicrobial sensitivities although thus far resistance to the commonly used agents has not been an important consideration, perhaps because multi-drug therapy has been the rule. A species-specific PCR would allow surveillance to ensure that the species present in an area is remaining relatively constant.

Ensuring high standards of performance with the PCR technique is complex and technically demanding and it is reasonable to consider what its place is when serology using either the traditional SAT or more recent ELISA techniques yield satisfactory results that correlate with clinical evaluations. In endemic areas where the history is typical and examination and investigation does not suggest an alternative diagnosis, positive serology may well be satisfactory for management in resource-limited settings. Culture should be attempted but is often negative. PCR may well prove to be of more use in identifying the cause of localized disease such as arthritis and meningitis in *Brucella* endemic areas where routine culture is negative and tuberculosis is particularly prominent in the differential diagnosis. In this study we have shown the applicability of single-tube nested PCR for diag-

Table 2. Final diagnoses in six patients with positive *Brucella* serology and negative culture

Reference no.	Clinical diagnosis	Final diagnosis	PCR	SAT	ELISA IgM	ELISA IgG	Culture
129	Chronic brucellosis	Meningococcal meningitis	Neg	Neg	Neg	132	Neg
136	Chronic brucellosis	Pneumococcal meningitis	Neg	Neg	Neg	133	Neg
174	Chronic brucellosis	Viral meningitis	Neg	Neg	Neg	185	Neg
204	Chronic brucellosis	Brucellosis	Neg	1/160	Neg	614*	Neg
320	Chronic brucellosis	Meningococcal meningitis	Neg	Neg	Neg	125	Neg
346	Chronic brucellosis	Brucellosis	Neg	1/160	Neg	697†	Neg

*Seven months earlier ELISA IgG 196.

†Eight months earlier ELISA IgG 227.

nosis of brucellosis from venous blood samples. In future studies the range of specimens examined will be increased to include cerebrospinal fluid, joint fluid, synovial biopsy material, lymph gland sampled by biopsy and fine needle aspiration, and bone marrow. The application of the *Brucella* PCR assay for samples from tissues that have shown granulomatous pathology would be valuable to help define further the role of this diagnostic test.

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