
Rapid Diagnosis of Brucella Epididymo-Orchitis by Real-Time Polymerase Chain Reaction Assay in Urine Samples

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Purpose: We studied the diagnostic yield of a real-time polymerase chain reaction assay in urine samples for the rapid diagnosis of brucella epididymo-orchitis compared to that of conventional microbiological techniques.

Materials and Methods: We used an SYBR® Green I LightCycler® based real-time polymerase chain reaction to retrospectively study 10 urine samples from patients with Brucella epididymo-orchitis. The assay amplifies a 223 bp sequence of a gene that codes for the synthesis of an immunogenetic membrane protein specific for Brucella genus (BCSP31). After amplifying this 223 bp sequence we performed melting curve analysis to verify the specificity of polymerase chain reaction products.

Results: Brucella melitensis was isolated from blood cultures in 9 cases (90%). Wright's seroagglutination was negative or inconclusive in 30% of cases. Brucella was isolated from urine in only 1 case, whereas real-time polymerase chain reaction assay in urine was positive in 9 (90%). Also, results were available in 4 hours, whereas mean time to availability of the final blood culture results was 5.8 days (range 4.5 to 7).

Conclusions: SYBR® Green I LightCycler® based real-time polymerase chain reaction assay in urine samples is highly sensitive and specific, and easy to perform. It could provide the clinician with results in less than 5 hours. The technique could be a practical and useful tool for the rapid diagnosis of genitourinary complications of human brucellosis.

Key Words: testis, brucellosis orchitis, epididymitis, polymerase chain reaction

Brucellosis is a worldwide zoonosis caused by intracellular, gram-negative coccobacilli of the genus Brucella. Brucellosis remains endemic in many countries and it is responsible for enormous economic losses as well as considerable human morbidity.¹ Like tuberculosis, human brucellosis is a systemic infection with a wide clinical spectrum. From 20% to 40% of patients with brucellosis present with focal complications that can affect any organ or system.² Infections of the genitourinary system are the second most common complication after those involving the locomotor system. Epididymo-orchitis is the most frequent genitourinary complication of brucellosis. It affects 2% to 20% of men with brucellosis and it may present at any time during the course of the disease.³

The diagnosis of focal forms of brucellosis is sometimes difficult because the yield of conventional cultures in non-blood samples is as low as 10% to 40% of all cases.⁴ Moreover, because Brucella species is a slowly growing pathogen, cultures require prolonged incubation, which can at times lead to excessive delays in diagnosis. Furthermore, serolog-

ical diagnosis lacks adequate specificity in areas where the disease is endemic and its results in some slowly evolving focal forms are difficult to interpret.⁵ For these reasons and bearing in mind that diagnostic delay is closely associated with the incidence of focal complications of brucellosis any improvement in the efficacy of the techniques used to diagnose focal complications of brucellosis would be clearly beneficial.

PCR technology provides better results than conventional microbiological techniques for the diagnosis of primary infection and relapse as well as for the focal complications of brucellosis.⁶ PCR assays in urine samples have proved to be more rapid and sensitive than conventional microbiological methods for different genitourinary and systemic infections.^{7,8}

Real-time PCR is a new, automated amplification technique that quantitatively monitors PCR products as they accumulate during thermal cycling. Since this probe does not require post-amplification handling, much more rapid assays are possible.^{9,10} We describe an SYBR® Green I LightCycler® based real-time PCR assay in urine samples for the rapid diagnosis of brucella epididymo-orchitis. We compared its diagnostic yield to that of conventional microbiological techniques.

PATIENTS AND METHODS

Patients

From January 1996 to December 2004, 10 consecutive patients with Brucella epididymo-orchitis were diagnosed,

Submitted for publication November 7, 2005.

Supported by F. I. S. Grant PI02/0957, Red Temática para la Investigación en Brucelosis. Instituto de Salud Carlos III (ISCIII) Grant G03/204 and funds from Consejería de Salud, Junta de Andalucía Grant 152/04.

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treated and followed at the Infectious Diseases Unit, Carlos Haya University Hospital, Málaga, Spain. The diagnosis of brucellosis was established according to 1 of 2 criteria, including 1) isolation of *Brucella* species in blood, or any other body fluid or tissue sample, or 2) the presence of a compatible clinical picture together with the demonstration of specific antibodies at significant titers or seroconversion. Significant titers were considered to be Wright's seroagglutination 1/160 or greater or immunocapture agglutination test 1/320 or greater. Epididymo-orchitis was diagnosed in patients with scrotal enlargement, swelling and pain not due to other causes.

Specimen Collection and Processing

Blood cultures were processed in a BACTEC™ 9240 according to the usual techniques. Incubation was maintained for 15 days. Blind subcultures were performed on chocolate agar and *Brucella* agar (Biomedics, San Sebastian de Los Reyes, Madrid, Spain) after 7 and 15 days. These subcultures were incubated at 37C in a 5% to 10% CO₂ atmosphere for 7 days.

Urine samples were cultured onto blood and chocolate agar medium, MacConkey agar and *Brucella* agar. Plates were incubated in a 10% CO₂ atmosphere at 37C for at least 7 days. If growth appeared, the suspected colonies were identified by colonial morphology, Gram staining, oxidase, catalase and urease tests, and positive agglutination with specific antiserum were done. Serological tests were all performed according to previously described techniques.^{11,12}

DNA Extraction

Urine samples were maintained at -20C until processing. DNA was extracted by boiling from 2 ml urine, placed in a 2 ml microcentrifuge tube and centrifuged for 15 minutes at 15,000 × gravity. Supernatant was eliminated. The pellet was resuspended in 200 µl sterile water and centrifuged for 10 minutes at 15,000 × gravity. Supernatant was again eliminated. The pellet was resuspended in 60 µl sterile water and subjected to boiling in a water bath for 10 minutes. It was then cooled on ice and centrifuged for 10 seconds at 15,000 × gravity to remove any drops from the top of the lip before storing at -20C until use. The template DNA suspension (2 µl) was used for PCR.

Real-Time PCR With SYBR® Green I

Primers were selected from the conserved region of the gene that encodes an immunogenic membrane protein of 31 kDa of *Brucella abortus* specific to the *Brucella* genus. The pair of 21 nucleotide primers B₄ (5' tgg ctc ggt tgc caa tat caa 3') and B₅ (5' cgc gct tgc ctt tca ggt ctg 3') (Tib Molbiol, Berlin, Germany) were used in the amplification process. PCR amplifications were performed in capillary tubes with a 20 µl final volume in a LightCycler® instrument. Reaction mixtures contained 2 µl LightCycler® FastStart DNA master mixture for SYBR® Green I, 0.5 µM of each primer, 4 mM MgCl₂ and 2 µl DNA template. All capillaries were sealed and then centrifuged at 500 × gravity for 5 seconds before amplification in a LightCycler® instrument. Following polymerase activation at 95C for 10 minutes 45 thermocycles were run with 10 seconds of denaturation at 95C, 10 seconds of annealing at 60C and 9 seconds of extension at 72C. The temperature transition rate was 20C second⁻¹ for all steps. The double strand PCR product was measured during the

72C extension step by detecting fluorescence associated with the binding of SYBR® Green to the product.

Fluorescence curves were analyzed with LightCycler® software, version 3.5. Melt curve analysis was performed immediately after the amplification protocol under certain conditions, including 0 seconds at 95C (hold time on reaching temperature), 15 seconds at 71C and 0 seconds at 95C. Temperature change rates were 20C seconds⁻¹ except for the final step, which had a temperature change rate of 0.1C second⁻¹. The peak melting temperature attained represented the specific amplified product.

Positive controls were included in all tests. They comprised serial dilutions of *B. abortus* B-19 DNA from 10⁴ to 10 fg. Negative controls containing all elements of the reaction mixture except template DNA were also included. To guarantee the reliability of results all samples were processed in duplicate. To prevent contamination universal precautions were exercised and 1-way flow of DNA extraction and amplification was used. To avoid potential subjectivity the *Brucella* infection status of each patient was unknown during the PCR assay.

The test was only considered positive if the signal from the amplified fragment was clearly visible in each sample. To study the specificity of the technique all samples from patients with brucellosis were paired with an equal number of samples from controls with urinary tract infection, including *Escherichia coli* in 4, *Klebsiella pneumoniae* in 2, *Proteus mirabilis* in 2, *Citrobacter freundii* in 1 and *Pseudomonas aeruginosa* in 1.

RESULTS

Mean age in the 10 patients included was 38.4 years (range 21 to 69). Five patients lived in rural areas, 6 had usual contact with sheep or goats and the other 4 had consumed unpasteurized dairy products. Eight patients had epididymo-orchitis during the initial infection and the other 2 had it during a relapse of brucellosis. The mean duration of symptoms ± SD before diagnosis was 30.2 ± 16.2 days (range 5 to 51). Epididymo-orchitis was unilateral in all cases, including on the right side in 3 and on the left side in 7. All patients had fever, scrotal swelling and testicular pain. Table 1 lists other relevant clinical data.

The diagnosis of brucellosis was established by isolating *Brucella* in blood cultures in 9 cases. In the remaining case the diagnosis was clinical-serological. All strains isolated were identified as *Brucella melitensis*. Mean time to availability of the final blood culture results was 5.8 days (range 4.5 to 7). The rose Bengal test was positive in all cases.

TABLE 1. Clinical features in patients with *Brucella epididymo-orchitis*

| | No. Pts |
|--------------------------|---------|
| Fever | 10 |
| Testicular pain | 10 |
| Scrotal swelling | 10 |
| Chills | 10 |
| Sweating | 8 |
| Constitutional symptoms* | 7 |
| Hepatomegaly | 4 |
| Arthritis | 4 |
| Splenomegaly | 1 |

* Two or more of anorexia, asthenia, malaise or weight loss.

Wright's seroagglutination titers were above 1/160 in 7 cases, 1/80 in 1 and negative in the remaining 2, whereas immunocapture agglutination titers were higher than 1/320 in all.

In only 30% of cases was the leukocyte count 10,800/ml or greater. Four patients had abnormal urinalysis, including 3 with 8 to 15 leukocytes per high power field, while 1 had leukocyturia and proteinuria 650 mg daily. Table 2 lists other relevant laboratory data.

Brucella was isolated from urine in only 1 case, whereas real-time PCR assay in urine was positive in 9. In each of the 9 cases *Brucella* specific amplicons could be easily distinguished by the characteristic melting temperature (see figure). Real-time PCR was negative in all control samples from patients with urinary tract infection.

The patient in whom real-time PCR was negative was a farmer who had right epididymo-orchitis 6 weeks in duration and in whom *B. melitensis* was isolated in blood culture. However, urine culture was negative.

All patients were treated with 100 mg doxycycline twice daily for 2 months plus 1 gm streptomycin sulfate intramuscularly 4 times daily for the first 2 to 3 weeks. After treatment all patients were followed for 6 months and all were discharged from treatment as asymptomatic.

DISCUSSION

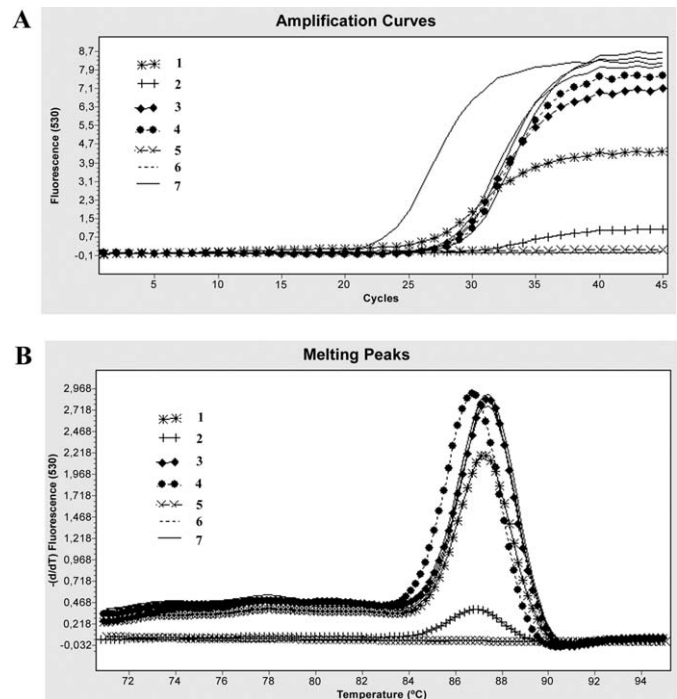
Brucellosis remains a public health problem in many areas of the world, especially in the Mediterranean basin, the Middle East, India, Mexico, and Central and South America.¹³ The incidence of the disease in the United States is low, although only an estimated 4% to 10% of cases are in fact recognized. From 1973 to 1992, 426 cases of human brucellosis were reported in California.¹⁴ Brucellosis should be included in the differential diagnosis of acute scrotum, granulomatous epididymo-orchitis and testicular mass in areas where the disease is endemic.¹⁵

Although epidemiological, clinical and laboratory data may suggest *Brucella* epididymo-orchitis,^{16,17} confirmation is required with microbiological techniques. Prior studies showed that the percent of patients with *Brucella* epididymo-orchitis who had a positive blood culture was 14% to 69%.^{3,16} Although 90% of the patients in our study had positive blood cultures, the mean time to availability of blood culture results was 6 days. Despite the advances in sensitivity and time to detection resulting from the introduction of new semiautomatic systems for processing blood cultures

TABLE 2. Hematological and biochemical findings in patients with *Brucella* epididymo-orchitis

| | Mean ± SD |
|--|---------------|
| Leukocytes (cells/ml) | 8,913 ± 2,525 |
| Hemoglobin (gm %) | 10.9 ± 1.2 |
| Erythrocyte sedimentation rate (mm/hr) | 57.1 ± 33.6 |
| Alanine aminotransferase (IU/l) | 42 ± 19 |
| Aspartate aminotransferase (IU/l) | 76 ± 839 |
| Alkaline phosphatase (IU/l) | 173 ± 118 |
| γ-Glutamyl transferase (IU/l) | 185 ± 173 |
| Total protein (gm/l) | 66.2 ± 6.5 |
| Alfa-2 globulin (gm/l) | 9.6 ± 1.8 |
| Adenosine deaminase (U/l)* | 23.7 ± 13.4 |
| C-reactive protein (mg/l) | 141 ± 70 |

* Performed in 8 patients.



Amplification curves show detection of *Brucella* DNA by LC-PCR in urine samples. Fluorescent signal evolution was related to cycle number. 1 to 4, patients 1 to 4 with brucellar epididymo-orchitis, respectively. 5, patient with brucellosis but without epididymo-orchitis. 6, negative control. 7, positive control. Melting curves show peaks of amplified fragments generated by LC-PCR. Specific signals had mean melting temperature of 87.28 ± 0.28 C in patients and 87.39 ± 0.03 C in controls.

Brucella isolation often requires a lengthy incubation. Moreover, it should not be forgotten that *Brucella* species are class III pathogens that frequently cause infection in laboratory workers.

With rapid diagnosis and correct medical treatment the prognosis of *Brucella* epididymo-orchitis is favorable. However, when diagnosis is delayed, the disease often leads to abscesses, which may require orchidectomy.^{3,16}

The yield of urine culture for the diagnosis of *Brucella* epididymo-orchitis was low in our study with results similar to those of others.^{3,17} However, our real-time PCR assay in urine samples was positive in 90% of cases. In each PCR positive case *Brucella* specific amplicons could be easily distinguished by the characteristic melting temperature.

Unlike classic PCR, real-time PCR is easy to standardize and it does not require extensive manipulation. The advent of LightCycler® technology has enabled the development of a PCR assay that meets the requirements of rapid diagnosis. In addition, using sealed capillary tubes in the LightCycler® format combined with the absence of post-amplification manipulation of PCR products significantly decreases the risk of contamination due to amplicon carryover.

Urine has been shown to be a good sample for the rapid molecular diagnosis of different systemic and genitourinary infections for which conventional microbiological diagnosis proves difficult.⁶ The results of this study show that the yield of our real-time PCR assay in urine samples is far superior to that of conventional culture for the diagnosis of *Brucella* epididymo-orchitis. The greater sensitivity is because PCR is able to amplify intramacrophage pathogens or

pathogens that are damaged or nonviable as a result of previous treatment, which would be impossible to isolate in conventional cultures. Real-time PCR was negative in all control samples, in keeping with the specificity of the primers used. Previous studies have demonstrated the high specificity of B4 and B5 primers in a wide panel of microorganisms, including most usual uropathogens.¹⁸

The presence of 1 patient with brucellosis who had negative real-time PCR warrants comment. *Brucella* DNA may not have been present in the urine sample or our real-time PCR assay may not have detected DNA in the sample from this patient. However, given the high analytical sensitivity of the technique (10 fg *Brucella* DNA, representing 2 microorganisms), the latter possibility is unlikely in a patient with epididymo-orchitis. A further possible explanation for this false-negative result could be the presence of inhibitors in the sample. Urea at concentrations above 50 mM can completely inhibit Taq polymerase.¹⁹ However, the 2 dilutions and the boiling process to which the sample was submitted before amplification also make this possibility unlikely. A much more probable cause would be the prolonged storage of the sample before testing, especially considering that 1 or 2 freeze-thaw cycles could decrease the availability of DNA for PCR.²⁰

CONCLUSIONS

Real-time PCR assay in urine samples is highly sensitive and specific, and easy to perform. It could provide the clinician with results in less than 4 hours. In addition, it avoids the risks to laboratory personnel associated with handling the microorganism and the sample can be stored at -20°C until processing. Thus, real-time PCR assay of urine samples could be a practical and useful tool for the rapid diagnosis of genitourinary complications of human brucellosis.

ACKNOWLEDGMENTS

Ian Johnstone assisted with the English translation.

Abbreviations and Acronyms

PCR = polymerase chain reaction

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