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Usefulness of a quantitative real-time PCR assay using serum samples to discriminate between inactive, serologically positive and active human brucellosis

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ABSTRACT

Diagnosis of brucellosis can be difficult in certain scenarios where conventional microbiological techniques have important limitations. The aim of this study was to develop a LightCycler Quantitative PCR assay in serum samples to discriminate between active and past brucellosis. In total, 110 serum samples from 46 brucellosis patients and 64 controls, including persons who had recently been treated for brucellosis, asymptomatic persons exposed to brucellosis, and patients with febrile syndromes involving a differential diagnosis with brucellosis, were studied. *Brucella* spp.-specific sequences of the PCR primers and probe were selected from the gene encoding an immunogenic membrane protein of 31 kDa (BCSP31). The analytical sensitivity was 1×10^1 fg of *Brucella* DNA. The mean threshold cycles for brucellosis patients and controls were 31.8 ± 1.7 and 35.4 ± 1.1, respectively ($p < 0.001$). The best cut-off for bacterial DNA load was 5×10^3 copies/mL. At this cut-off, the area under the receiver operating characteristic curves was 0.963 (95% CI 0.920–1.005), with a sensitivity of 93.5% and a specificity of 98.4%. Under the assay conditions, the LightCycler Quantitative PCR in serum samples seems to be highly reproducible, rapid, sensitive and specific. It is therefore a useful method for both the initial diagnosis and the differentiation between past and active brucellosis.

Keywords Brucellosis, diagnosis, PCR, quantitative PCR, relapses

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INTRODUCTION

Brucellosis remains the most common zoonotic infection worldwide, representing a major form of disease in humans [1]. *Brucella* is an intracellular pathogen and is able to elude fusion by phagolysosomes, thereby enabling it to survive and even multiply within cells of the mononuclear phagocyte system [2]. This intracellular survival determines the characteristic clinical profile of the disease, i.e. a fluctuating course and a tendency for there to be focal complications and relapses [3].

Clinically, brucellosis is highly polymorphic, affecting any organ or system [4]. Despite correct treatment, a high percentage of patients continue to have non-specific symptoms that are difficult to interpret, with no clinical or microbiological evidence of relapse [5].

To date, none of the conventional microbiological methods has proved to be efficient for the post-treatment follow-up of brucellosis. Blood cultures lack sensitivity in the diagnosis of relapses, and serological tests are of little use, as antibodies, both IgG and IgM, may be present for many months after effective therapy. Additionally, interpretation of serological tests is difficult in patients from endemic areas and those who are professionally exposed to the disease [6,7].

To overcome certain limitations of conventional microbiological techniques, PCR-based assays have been proposed as a useful tool for the diagnosis of human brucellosis [8,9]. Different studies have shown that PCR methods are more sensitive than cultures and more specific than serological tests,

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both for the diagnosis of acute forms and for focal complications of the disease [10,11].

Current real-time PCR techniques involve a faster amplification process and enable quantification of the bacterial DNA load, which could be useful for post-therapeutic follow-up of the infection. However, recent studies have reported the prolonged persistence of DNAaemia in treated patients with a favourable course and no evidence of relapse [12,13]. A LightCycler Quantitative PCR assay (LC Q-PCR) for serum samples was therefore developed, and its capacity to discriminate between active and past brucellosis was evaluated.

PATIENTS AND METHODS

Study population

From April 2002 to March 2007, serum samples were collected from 46 patients who received a diagnosis of brucellosis in the Infectious Diseases Service of Carlos Haya University Hospital, Malaga, Spain.

Control serum samples were obtained from 64 subjects. These included 36 asymptomatic patients with a history of brucellosis, treated according to usual antibiotic regimens (13 patients immediately after treatment, five after 2–4 months, five after 4–6 months, and 13 after 6–24 months), 12 asymptomatic but exposed persons from families affected by brucellosis following consumption of unpasteurized cheese or milk, and five asymptomatic persons permanently exposed to *Brucella* infection in their professions (three veterinary surgeons and two abattoir workers) with persistently high titres of anti-*Brucella* antibodies. The 64 subjects also included 11 patients with febrile syndromes of other defined aetiologies, initially involving a differential diagnosis with brucellosis: four with acute cytomegalovirus infection, two with tuberculous vertebral osteomyelitis, and one each with acute human immunodeficiency virus infection, Epstein–Barr virus infection, microscopic polyangitis, giant cell arteritis, and HLA B-27-positive ankylosing spondylitis. Approval from the Institutional Review Board and Medical Ethics Committee was obtained before the start of the study.

In order to guarantee that all the patients with brucellosis had active disease, cases were considered only if they fulfilled one of the following criteria: first, isolation of *Brucella* spp. from blood or any other body fluid or tissue sample; and second, the presence of a typical focal complication of brucellosis (e.g. sacroiliitis, vertebral osteomyelitis or epydidymo-orchitis) together with specific antibodies at significant titres or seroconversion. Significant titres were considered to be a standard tube agglutination test (SAT) score of ≥1/160 and an immunocapture agglutination test score of ≥1/320.

Microbiological studies

Two blood cultures, the rose Bengal test, the SAT and the immunocapture-agglutination test were performed for all patients with active brucellosis, febrile syndromes of other aetiologies or a history of brucellosis, and for subjects permanently exposed to *Brucella*.

Blood samples were incubated in a non-radiometric semi-automatic BACTEC 9240 system (Becton Dickinson, Diagnostic Instrument Systems, Sparks, MD, USA). Blood cultures were processed according to usual techniques. Incubation was maintained for 15 days, with blind subcultures on chocolate agar and *Brucella* agar being performed after 7 and 15 days. These subcultures were incubated at 37°C in a 5–10% CO_2 atmosphere for 3 days. All isolates were identified according to normalized protocols [14]. Serological tests were all performed as previously described [15,16].

DNA extraction from serum samples

Serum samples for PCR were taken at the same time as the blood cultures. Two aliquots of serum were conserved at −20°C until processing. DNA was extracted using the Ultra-Clean DNA-BloodSpin Kit (Mo Bio Laboratories, Carlsbad, CA, USA). Prior to DNA extraction, 200 μL of serum was centrifuged for 15 min at 15 000 *g*. The supernatant was discarded, and the pellet was resuspended with the volume of buffer specified in the manufacturer's instructions. DNA pellets were resuspended in 200 μL of molecular biology-grade water. Finally, DNA was concentrated by adding 8 μL of 5 M NaCl and 400 μL of 100% cold ethanol, mixed, and centrifuged at 15 000 *g* for 5 min. The DNA pellets were brought to a final volume of 40 μL in water, and stored at 4°C until use. Aliquots of 5 μL of the suspension were used for PCR analysis.

Primer and probe design

The *Brucella* spp.-specific sequences of the PCR primers and probe were selected from the conserved region of the gene encoding an immunogenic membrane protein of 31 kDa (BCSP31) specific to the *Brucella* genus, which is present in all known biovars (Gen-Bank M-20404). Primers were designed with Beacon Designer Software (Premier Biosoft International, Palo Alto, CA, USA) and synthesized by Proligo (Sigma-Aldrich Co., Paris, France). The sequences of the primers and the TaqMan probe were as follows: forward primer, Tq1, 5′-TGCCGGAG CCTATAAGGACG-3′; reverse primer, Tq2, 5′-CGAGTGCCTT GCGTGTATCC-3′; and TaqMan probe, Stq, 5′-ACCGACCC TTGCCGTTGCCGC-3′. The Stq TaqMan probe was fluorescence labelled at the 5′-end with 6-carboxyfluorescein phosphoramidite (FAM) as the reporter dye, and at the 3′-end with 5-carboxytetramethylrhodamine (TAMRA) as the quencher. Primer and probe sequences were checked for specificity using a BLAST search, and no significant matches were found.

Plasmid construction

The amplification product of 140 bp from *Brucella abortus* B-19 obtained with primers Tq1 and Tq2 was purified from the agarose gel using the gel extraction kit QIAquick (QIAGEN, Hilden, Germany), according to the manufacturer's instructions. The purified PCR amplicon was cloned into PCR2.1-TOPO and transferred into *Escherichia coli* DH-5 alpha using the TOPO-TA cloning kit (Invitrogen, Barcelona, Spain). The plasmid DNAs were purified using the QIAprep Spin Miniprep (QIAGEN), linearized by digestion with *Eco*RI (Roche, Barcelona, Spain), and sequenced to confirm the identity of the inserted bacterial DNA (Laboratorio de Secuenciación de Sistemas Genómicos, Valencia, Spain). The absorbance of the DNA solution was measured three times at 260 nm in a

NANODROP ND-1000 spectrophotometer (Nano Drop Technologies, Inc., Wilmington, DE, USA), and dilutions of plasmid DNAs were used as standards for subsequent PCR analysis.

Standard curve construction

Plasmid standards containing 2×10^4 to 2×10^0 copies per reaction were prepared by diluting the purified plasmid in water.

All standards were amplified in quadruplicate. The standard curve was generated and exported using the LightCycler software v.4.0. It was subsequently loaded as an external standard curve after each run and calibrated, with a calibrator/positive control being included in all runs.

LC Q-PCR

PCRs were performed in 20-μL final volumes in capillary tubes in a LightCycler instrument (Roche Diagnostic, Mannheim, Germany). Reaction mixtures contained 4 μL of LightCycler FastStart DNA Master^{PLUS} HybProbe (Roche Diagnostic), each primer at 0.6 μM, Taqman probe at 200 nM, and between 50–100 ng of template DNA.

All capillaries were sealed, and centrifuged at 500 *g* for 5 s, and the DNAs were amplified in the LightCycler, with activation of polymerase (95°C for 10 min), followed by 45 cycles of 10 s at 95°C, 20 s at 60°C, and 6 s at 72°C. The temperature transition rate was 20°C/s for all steps. The concentration of *Brucella* DNA present in the clinical samples was calculated by LightCycler software. The crossing point (Cp or threshold cycle) was defined as the maximum of the second derivative from the fluorescence curve. All runs included a negative water control and calibrator/positive control. The positive control contained 2×10^4 copies of the plasmid per reaction. All samples were processed in duplicate and, to be considered positive, both replicates had to be positive. The final bacterial DNA load was taken to be the mean value of the two separate samples. To prevent contamination, universal precautions were taken and one-way flow of DNA extraction and amplification was used. To avoid potential observer bias, the status of each patient, with respect to *Brucella* infection, was unknown during the PCR assay. PCR analyses were considered to be negative for *Brucella* DNA if the Cp values exceeded 45 cycles.

Statistical analysis

The Student *t*-test and/or Mann–Whitney test were used to compare continuous variables, and the χ^2 test or Fisher test to compare categorical variables. A p-value of <0.05 was considered to be statistically significant. The diagnostic accuracy was assessed by calculating the area under receiver operating characteristic (ROC) curves. Sensitivity, specificity, positive and negative predictive values, likelihood ratios (LRs) and 95% CIs were calculated using the Twobytwo 1.0 analyzer program.

RESULTS

Analytical sensitivity

The analytical sensitivity of the LC Q-PCR was initially determined by amplifying ten-fold serially diluted plasmid DNA with a detection limit of 2×10^0 copies per reaction. The assay showed a linear quantitative range over five orders of magnitude from 2×10^5 down to 2×10^0 copies per reaction, with a linear regression equation of $C_p = -3.32 \log$ (copy no.) + 36.93, correlation coefficient (R^2) value of 0.99, and a PCR efficiency (E) of 2.0.

When DNA templates prepared from serum spiked with serial dilutions of *B. abortus* B-19 cells were used, the analytical sensitivity was found to be 2×10^0 genomic copies per reaction (equivalent to 1×10^1 fg of DNA). A linear regression of over five orders of magnitude was found, from 2×10^5 down to 2×10^0 copies per reaction. The equation for the linear regression line for the standard curve generated and its corresponding E and R^2 values were $C_p = -3.23 \log$ (copy no.) + 35.63, $R^2 = 0.99$ and $E = 2.0$.

Reproducibility

Intra-assay variability was determined by amplifying, in quadruplicate, diluted plasmid DNA, equivalent to 2×10^4 to 2×10^0 copies per reaction. Cp values obtained for the same dilutions on five different days were used to determine the inter-assay variability. The mean coefficients of variation for intra-assay and inter-assay repetitions were 0.8% and 1.5%, respectively.

Patient characteristics

Of the 46 patients with brucellosis, 28 (60.9%) were men and 18 (39.1%) women. The mean age was 42.5 ± 16.4 years (range, 15–81 years). The mean duration of the symptoms prior to diagnosis was 7.3 ± 11.1 weeks (range, 1–65 weeks).

Twenty-eight patients (60.9%) had fever with no apparent focus, and 18 (39.1%) had focal complications (eight with vertebral osteomyelitis, six with orchiepididymitis, two with meningoencephalitis, and one each with sternoclavicular arthritis and liver abscess).

Blood cultures were positive in 32 patients (69.6%). All isolated strains were identified as *Brucella melitensis*. The SAT and the immunocapture-agglutination test yielded titres within the diagnostic range in 67.4% and 89.1% of cases, respectively. Both tests had negative results or yielded titres below the diagnostic range in 10.8% of cases.

Forty-four (95.7%) patients with brucellosis had a positive LC Q-PCR result vs. five (7.8%) of the 64 control patients. The patients with a false-negative LC Q-PCR result were two male shepherds (24 and 57 years old) with a febrile syndrome and from whose blood cultures *B. melitensis* was isolated. Four of the five controls with a positive LC Q-PCR result were patients with brucellosis who had just completed treatment with doxycycline plus streptomycin, and the fifth was a veterinary surgeon involved in the caprine vaccination and sacrifice campaigns, who, for 10 days, had fever and arthralgia that were self-limiting with symptomatic treatment. The blood cultures from this patient were always negative, and he remained asymptomatic over the following 12 months; the two LC Q-PCR controls during this period were negative. Thus, qualitatively, the sensitivity, specificity, positive predictive value and negative predictive value of the LC Q-PCR assay were 95.7%, 92.2%, 89.8% and 96.7%, respectively, with a positive LR of 12.2 (95% CI 5.3–28.5) and negative LR of 0.05 (95% CI 0.01–0.18).

Quantification of bacterial DNA load in clinical samples

The mean threshold cycles for the brucellosis patients and controls were 31.8 ± 1.7 and 35.5 ± 1.1, respectively ($p < 0.001$). The mean bacterial DNA load for patients with brucellosis was 8.2×10^6 copies/mL (range, 4.1×10^3 to 3.2×10^8 copies/mL) vs. 3.3×10^3 copies/mL (range, 6.0×10^0 to 2.1×10^5 copies/mL) for the controls.

Among the 46 brucellosis patients, 43 (93.5%) had a bacterial DNA load of $>10^5$ copies/mL, whereas none of the four controls with a positive LC Q-PCR result after treatment had a bacterial DNA load of $>4 \times 10^3$ copies/mL.

The best cut-off for LC Q-PCR bacterial DNA load in serum samples was 5×10^3 copies/mL. At this cut-off, 43 of the 46 patients with active brucellosis were correctly classified, with sensitivity of 93.5% (95% CI 86.3–100%) and specificity of 98.4% (95% CI 95.4–100%). The positive and negative LRs were 59.8 (95% CI 8.5–418.9) and 0.07 (95% CI 0.02–0.20), respectively. Determination of the maximum specificity required raising the cut-off to 2.2×10^5 copies/mL, at which level the sensitivity fell to 76.1% (95% CI 63.8–88.4%). Fig. 1 shows the ROC curves for LC Q-PCR bacterial DNA load in serum samples. Table 1 shows the diagnostic yield of the LC Q-PCR in different situations.

DISCUSSION

One of the most difficult problems in diagnosing human brucellosis concerns the presence of individuals who are serologically positive and are either asymptomatic or oligosymptomatic. Human brucellosis shows a marked tendency to relapse after conclusion of treatment [17–19]. Even with the correct treatment, the incidence of relapse in cases of brucellosis remains high,

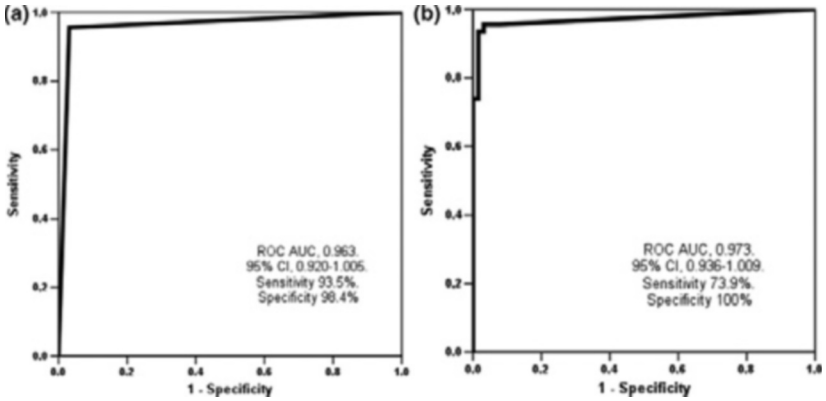

Fig. 1. Area under the receiver operating characteristic curves (ROC AUC) for testing the ability of the LC Q-PCR to distinguish active from inactive brucellosis. ROC curves were constructed by plotting sensitivity and 1-specificity corresponding to each cut-off value for the *Brucella* DNA load. (a) Considering a cut-off of 5×10^3 copies/mL. (b) Considering a cut-off of 2.2×10^5 copies/mL to achieve a specificity of 100%.

Table 1. Diagnostic yield: comparison between blood cultures and LC Q-PCR in serum samples

	Sensitivity	Specificity	PPV (%)	NPV (95% CI)	Positive LR	Negative LR
Blood cultures	69.6 (56.3–82.9)	100	100	78.8 (68.9–88.7)	ND[a]	0.30 (0.20–0.47)
LC Q-PCR, all samples (cut-off ≥ $5x10^3$ copies/mL)	93.5 (86.3–100)	98.4 (95.4–100)	97.7 (93.3–100)	95.5 (90.4–100)	59.9 (84–418.9)	0.07 (0.02–0.2)
LC Q-PCR, excluding outlier[b] (cut-off ≥ 5×10^3 copies/mL)	93.5 (86.3–100)	100	100	95.5 (90.4–100)	ND[a]	0.07 (0.02–0.19)
LC Q-PCR, all samples (cut-off ≥ 2×10^5 copies/mL)	73.9 (62.1–86.6)	100	100	84.2 (76.0–92.4)	ND[a]	0.26 (0.16–0.42)

PPV, positive predictive value; NPV, negative predictive value; positive LR, positive likelihood ratio; negative LR, negative likelihood ratio.
[a] Not done for mathematical reasons (division by zero).
[b] Veterinary surgeon with self-limiting symptoms.

ranging from 5% to 23% in the largest series reported [20–24].

As with other infections caused by fastidious microorganisms, molecular techniques have proven to be more efficient than conventional methods in specific clinical cases of *Brucella* infection.

However, the relative complexity of these techniques, the lack of standardized procedures and, occasionally, socio-economic limitations in the most affected countries have hindered the development and implementation of these techniques [25].

Very few studies have examined the possible usefulness of PCR-based methods for discriminating between active and past brucellosis, and some of these have important biases that make interpretation of the results difficult [13].

In a previous study using a single-step conventional PCR in whole blood samples, our group reported that 96.4% of the patients had a negative PCR result upon conclusion of treatment [26]. Similar results have been communicated by others [8].

In the present study, the sensitivity and specificity of the LC Q-PCR were 95.7% and 92.2%, respectively. Thus, qualitatively considered, the diagnostic yield of the LC Q-PCR is clearly better than that of conventional microbiological methods. Similar findings have recently been reported by Navarro *et al*. [12], who amplified a specific sequence of *B. melitensis*. Unfortunately, the control group in that study included only healthy blood donors. Therefore,no conclusions could be drawn concerning the utility of the LC Q-PCR in other scenarios where it is necessary to eliminate the presence of active brucellosis [12].

Previous studies have reported that 2–5% of patients with recent brucellosis, or asymptomatic subjects permanently exposed to the disease, may have a positive PCR result with persistently negative blood cultures [27]. Hypothetically, in these subjects, bacteraemia, if it exists, should be very low and closely associated with the amount of circulating DNA.

The LC Q-PCR technology described here allows quick, sensitive and reproducible measurement of the bacterial DNA load. This load was significantly higher in the patients with active brucellosis than in the controls for whom PCR results were positive. These results are especially important in relation to the rigorous criteria for the control group.

Determining the ROC curves is a validated way of measuring the diagnostic accuracy of a test or the discriminative power of a prediction rule [28]. The cut-off value that maximized the sum of sensitivity and specificity for discrimination between active and past brucellosis was 5×10^3 copies/mL. With this value, the area under the ROC curve for active brucellosis was 0.963. Thus, considering the entire sample, this cut-off would ensure 93.5% sensitivity and a 97.7% positive predictive value in cases of active brucellosis.

Evidence exists that some highly exposed persons, i.e. veterinary surgeons who undertake vaccination campaigns, abattoir workers, and herdsmen who assist their animals in giving birth, may have self-limiting infections after inoculation [29]. This may be the explanation for the high bacterial DNA load in one of our control samples. The repeatedly negative PCR results over the following months support this possibility. If we consider that this is what happened in the only person in the control group who had an LC Q-PCR result with a bacterial DNA load higher than 10^3 copies/mL, and thus exclude this person from the overall analysis, the sensitivity and positive predictive value of this cut-off would be 93.5% and 100%, respectively.

Using similar technology, Navarro *et al*. consistently detected *B. melitensis* DNA in blood samples of patients with brucellosis throughout treatment and follow-up, despite apparent recovery from infection [12]. The heterogeneity of the

treatment used in that study and the high rate of relapse reported (seven of 18 patients, 38.8%) are completely ouside the norm of usual clinical practice; this may explain, in part, the discrepancy.

Another possible explanation is the manner of interpreting the results, as Navarro *et al.* considered a result to be positive if just one of three replicates was positive [12]. The fact that the DNA load in the present study was higher than that reported by Navarro *et al.* is clearly related to the type of sample used and to the efficiency of the method. Navarro *et al.* used whole blood samples, and their PCR mixture contained 400 ng of human DNA, both of which can have a negative effect on the efficiency of the amplification process.

Serum could be a better sample, as it reduces inhibition by haemoglobin, human DNA, or any other substance present in whole blood, and does not require red blood cell lysis, washings by centrifugation, or measurement and adjustment of isolated DNA [30].

In conclusion, the LC Q-PCR using serum samples appears to be a useful method, not only for the initial diagnosis of brucellosis, but also for the differentiation between active and past brucellosis.

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TRANSPARENCY DECLARATION

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