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Rapid diagnosis of human brucellosis by SYBR Green I-based real-time PCR assay and melting curve analysis in serum samples

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SUMMARY

The aim of this study was to develop a LightCycler-based real-time PCR (LC-PCR) assay and to evaluate its diagnostic use for the detection of *Brucella* DNA in serum samples. Following amplification of a 223-bp gene sequence encoding an immunogenetic membrane protein (BCSP31) specific for the *Brucella* genus, melting curve and DNA sequencing analysis was performed to verify the specificity of the PCR products. The intra- and inter-assay variation coefficients were 1.3% and 6.4%, respectively, and the detection limit was 5 fg of *Brucella* DNA (one genome equivalent). After optimisation of the PCR assay conditions, a standard curve was obtained with a linear range (correlation coefficient = 0.99) over seven orders of magnitude from 10^7 to 10 fg of *Brucella* DNA. The LC-PCR assay was found to be 91.9% sensitive and 95.4% specific when tested with 65 negative control samples and 62 serum samples from 60 consecutive patients with active brucellosis. The assay is reproducible, easily standardised, minimises the risk of infection in laboratory workers, and has a total processing time of <2 h. It could therefore form a promising and practical approach for the rapid diagnosis of human brucellosis.

Keywords BCSP31, brucellosis, molecular diagnosis, PCR, real-time assays, serum samples

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INTRODUCTION

Brucella is one of the world's major zoonotic pathogens and is responsible for enormous economic losses, as well as considerable human disease in endemic areas [1]. The heterogeneous and non-specific clinical symptoms mean that a diagnosis of brucellosis always requires laboratory confirmation [2]. The diagnostic standard remains the isolation of *Brucella* from blood cultures, as well as from other normally sterile body fluids and tissues. However, several studies involving large series of patients with brucellosis have shown that the sensitivity of blood culture is only 70–80%, and that this figure may be much lower in patients with a prolonged clinical course, with focal forms, or who have received antimicrobial therapy previously [3,4]. Furthermore, results of serological tests are difficult to interpret in endemic areas or in the context of permanently exposed individuals.

The high sensitivity and additional advantages afforded by PCR-based assays for the diagnosis of human brucellosis now seem proven [5–8]. Nevertheless, the relative complexity of these techniques has hindered their introduction into clinical laboratories [9]. Unlike classical PCR, real-time PCR is easy to standardise and does not require extensive manipulations, so that the risk of contamination is minimised [10–12]. In order to simplify the molecular diagnosis of human brucellosis so that it can be performed

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by any clinical laboratory, a real-time LightCycler PCR (LC-PCR) assay, based on use of the SYBR Green I DNA-binding fluorophore dye, was developed. This article is the first description of the use of real-time PCR to detect *Brucella* DNA in human clinical samples.

MATERIALS AND METHODS

Clinical samples

Patient samples

The study included 62 serum samples taken from 60 consecutive patients diagnosed with active brucellosis in the Infectious Diseases Units of: Carlos Haya Regional Hospital, Málaga; Virgen del Rocío University Hospital, Seville; and Virgen de la Victoria University Hospital, Malaga, Spain. Two of the patients provided two samples each, one of which corresponded to an initial episode of disease, while the other corresponded to a relapse after concluding anti-brucella treatment (after 3 and 4 months, respectively). In 40 (64.5%) episodes, the diagnosis of brucellosis was established by the isolation of *Brucella* from blood culture; in the remaining 22 (35.5%) cases, diagnosis was based on a compatible clinical picture and the demonstration of specific antibodies at significant titres or seroconversion. Significant titres were considered to be a Wright's test seroagglutination titre of ≥1:160, or an immunocapture-agglutination anti-brucella test (see below) titre of ≥1:320. All the isolates were identified as *Brucella melitensis*.

Negative control group

Control blood samples were obtained from 65 individuals; these comprised 30 patients with febrile syndromes associated with other defined aetiologies that had initially involved a differential diagnosis with brucellosis, 15 asymptomatic patients with a history of brucellosis treated according to standard antibiotic regimens at least 12 months previously, ten asymptomatic subjects with persistently high titres of anti-brucella antibodies who had been exposed in their occupation to *Brucella* infection, and ten healthy individuals (blood donors) with no history of brucellosis or exposure to *Brucella* spp.

Bacteriological and serological techniques

Two blood cultures, as well as a range of serological tests, including the rose Bengal plate agglutination test, Wright's seroagglutination test and an immunocapture-agglutination test (see below), were performed for all patients with active brucellosis, febrile syndromes of other aetiologies, or a history of brucellosis. Blood cultures were processed in a semi-automatic BACTEC 9240 system (Becton Dickinson, Sparks, MD, USA). *Brucella* isolation and identification were by techniques described previously [13]. All isolates were sent to the National Brucellosis Reference Laboratory in Valladolid, Spain, for definitive identification and biotyping. The rose Bengal agglutination test and Wright's seroagglutination test were performed as described previously [14], and the immunocapture-agglutination test (Brucellacapt; Vircell SL, Sante Fé, Spain) was performed according to the manufacturer's instructions [15].

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 from 200 μL of serum, placed in a 0.5-mL microcentrifuge tube, and centrifuged for 15 min at 15 000 *g*. The supernatant was discarded and the pellet was resuspended in 200 μL of sterile water and centrifuged for 10 min at 15 000 *g*. The supernatant was again discarded and the pellet was resuspended in 40 μL of sterile water, placed in a boiling water bath for 10 min, cooled on ice, and then centrifuged for 10 s at 15 000 *g* before being stored at −20°C until use. Aliquots (2 μL) of the suspension (template DNA) were used for PCR analysis.

Real-time PCR with SYBR Green I

Primers were designed from the conserved region of the gene encoding an immunogenic membrane protein of 31 kDa (BCSP31) of *Brucella abortus* that is specific to the *Brucella* genus and present in all known biovars [16]. The 21-mer primers used in the amplification process were forward primer B_4 (5′-TGGCTCGGTTGCCAATATCAA-3′) and reverse primer B_5 (5′-CGCGCTTGCCTTTCAGGTCTG-3′) (Tib Molbiol, Berlin, Germany) [17]. PCRs were performed in 20-μL final volumes in capillary tubes in a LightCycler instrument (Roche Diagnostic, Mannheim, Germany). Reaction mixtures contained 2 μL of LightCycler FastStart DNA mastermix for SYBR Green I (Roche Diagnostic), 0.5 μM each primer, 4 mM $MgCl_2$ and 2 μL of template DNA. All capillaries were sealed, centrifuged at 500 *g* for 5 s, and then amplified in a LightCycler instrument, with activation of polymerase (95°C for 10 min), followed by 45 cycles of 10 s at 95°C, 10 s at 60°C, and 9 s at 72°C. The temperature transition rate was 20°C/s for all steps. Double-stranded PCR product was measured during the 72°C extension step by detection of fluorescence associated with the binding of SYBR Green I to the product. Fluorescence curves were analysed with LightCycler software v. 3.5.

Melting curve analysis was performed immediately after the amplification protocol under the following conditions: 0 s (hold time) at 95°C, 15 s at 71°C and 0 s (hold time) at 95°C. Temperature change rates were 20°C/s, except in the final step, which was 0.1°C/s. The melt peak generated represented the specific amplified product. The crossing point (C_p) was defined as the maximum of the second derivative from the fluorescence curve.

Positive controls were included in all tests and comprised serial dilutions of *B. abortus* B-19 DNA from 10^3 fg to 5 fg; negative controls were also included and contained all the elements of the reaction mixture except template DNA. All samples were processed in duplicate. Universal precautions and one-way flow of DNA extraction and amplification were used to prevent contamination. To avoid potential observer bias, the status of each patient concerning *Brucella* infection was unknown during the PCR assay.

Sequencing of LC-PCR product

In order to confirm the identities of the amplified fragments, the LC-PCR products were sequenced at the Laboratorio de Secuenciación de Sistemas Genómicos (Valencia, Spain). The ABI PRISM BigDye Terminator Cycle sequencing reaction kit v. 3.0 (Applied Biosystems, Madrid, Spain) was used for the

sequencing reactions. Sequence analysis was performed by capillary electrophoresis in an ABI PRISM model 3100 automated sequencer (Applied Biosystems).

Statistical analysis

Data were analysed using SPSS v. 10.0 for Windows (SPSS Inc., Chicago, IL, USA). Student's *t*-test was used for comparison of means, and chi-square and Fisher's tests were used for comparison of proportions. Non-parametric Spearman correlation coefficients were used to assess the association between continuous variables. All tests were two-tailed, and p values < 0.05 were accepted as statistically significant. To evaluate the assay precision, intra- and inter-assay coefficients of variation (CV) were calculated. The sensitivity, specificity, positive and negative predictive values, and likelihood ratios were calculated by two-by-two analysis.

RESULTS

Analytical sensitivity

The LC-PCR assay successfully amplified 10 fg of *Brucella* DNA in a ten-fold serial dilution of a pure culture of *B. abortus* B-19 (Fig. 1A). The detection limit of the method in 200 µL of serum sample spiked with serial dilutions of *B. abortus* B-19 was one bacterial cell (equivalent to 5 fg of DNA). Fig. 1B shows the correlation between log DNA concentrations and the C_p values. Amplicon-specific signals with melting temperatures of 88.16 ± 0.05°C are shown in Fig. 2. The melting curve revealed no presence of primer-dimers.

Reproducibility

The intra-assay variability was determined by amplifying, in quadruplicate, dilutions of a pure culture of *B. abortus* B-19, equivalent to 100, 50, 10 and 5 fg of *Brucella* DNA. C_p values obtained for the same dilutions on seven different days were used to determine the inter-assay variability. The mean CV for intra-assay repetitions was 1.3%, with CV values of 0.9%, 1.9%, 0.9% and 1.4% for the samples with 100, 50, 10 and 5 fg of *Brucella* DNA, respectively. The inter-assay CV was 6.45% for the entire group of samples, and was 2.6%, 6.3%, 8.7% and 8.1% for the samples with 100, 50, 10 and 5 fg of *Brucella* DNA, respectively.

Detection of *Brucella* spp. in serum samples

Of the 62 samples from patients with brucellosis, 57 (91.9%) were positive in the LC-PCR assay and

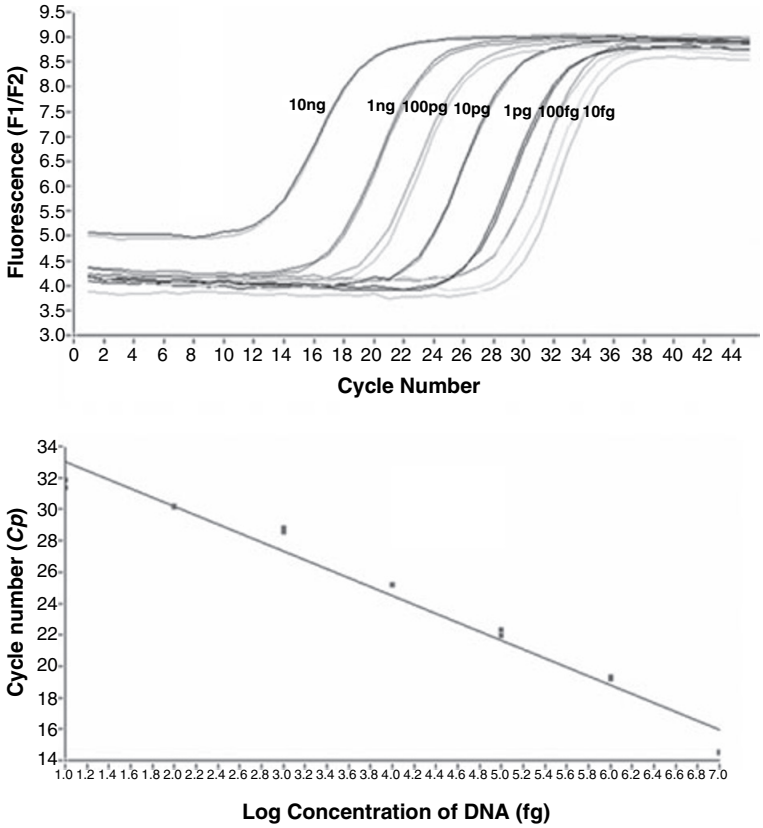

Fig. 1. Examples of LightCycler (LC) printouts showing detection of *Brucella* DNA by the LC-PCR assay. (A) Fluorescent signal related to cycle number on a panel of ten-fold dilutions between 10^7 and 10 fg of *Brucella abortus* B-19 DNA. *Brucella* DNA samples were amplified in duplicate for 45 cycles. (B) Logarithmic standard curve of (A). C_p values are plotted against decreasing concentrations of *Brucella* DNA. The slope is -2.89 cycles/$\log_{10}$ and the correlation coefficient is 0.99.

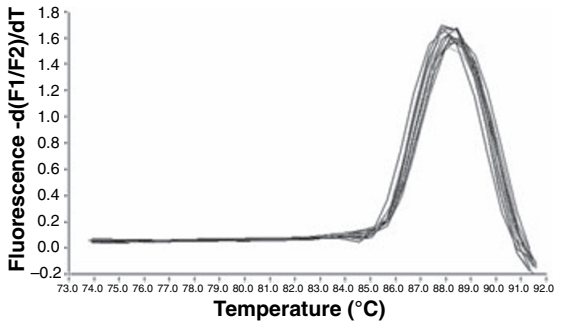

Fig. 2. Examples of LightCycler (LC) printouts showing melting curves of the amplified fragments generated by the LC-PCR assay. Specific signals had melting temperatures of 88.16 ± 0.05°C.

five (8.1%) were negative. Four of the five patients with false-negative results had positive blood cultures. In three of the five (4.8% of the total sample), the LC-PCR was positive following a second extraction and amplification of the same serum sample. The melting temperature for all *Brucella*-positive samples was consistent with that produced by the standard curve. The identity of the PCR product was confirmed by determining the nucleotide sequence of the amplified fragment, which matched exactly the *B. abortus* (BCSP31) DNA sequence described previously [5].

In the negative control group, 62 (95.4%) samples failed to produce exponential fluorescence curves during the LC-PCR, but three (4.6%) yielded a positive LC-PCR assay result. Two of these three samples, considered to be false-positive, were from patients (a farmer and a shepherd) who had habitual contact with sheep and goats during their work, while the third was from a female who sometimes ate unpasteurised cheese.

Overall, the sensitivity and specificity of the serum LC-PCR assay were 91.9% and 96.4%, respectively. The positive and negative predictive values, and positive and negative likelihood ratios, were 95.0%, 92.5%, 19.9% and 0.08%, respectively. The mean C_p values ranged from 29.9 ± 3.3 cycles for patients with active brucellosis to 35.0 ± 4.2 cycles for the three negative control patients who yielded a positive LC-PCR assay result (p 0.013).

DISCUSSION

The advent of the LightCycler and real-time PCR has provided an opportunity to develop an assay that meets the requirements for rapid diagnosis. This technology offers a significant improvement for the detection of infections with *Brucella* spp. In addition, the use of sealed capillary tubes in the LightCycler format, combined with the absence of post-amplification manipulation of PCR products, reduces significantly the risk of contamination resulting from amplicon carryover.

PCR analysis of several blood fractions, including whole blood, mononuclear leukocytes and serum, has been used to identify *Brucella* DNA in patients with brucellosis [5,8,18]. Because members of the *Brucella* genus are facultative intracellular pathogens, and the inoculum found in patients with brucellosis is normally very low [19], most studies of the diagnostic yield of PCR assays in human brucellosis have been undertaken with whole blood samples. Although this type of sample has the advantage of providing the maximum possible number of target bacteria, the presence of potential PCR inhibitors makes the assay more technically difficult to perform [20].

Multiple reasons justify the use of a simple SYBR Green I dye format to provide the fluorescent signal. First, the 223-bp target amplified is very specific to the *Brucella* genus and is present in all its biovars. Second, melting curve analysis of the amplified DNA allows clear distinction of the specific products from artefacts, such as primer-dimers, which are also minimised by the FastStart step included in the procedure. Third, treatment of *Brucella* infection is the same, irrespective of the causative species of *Brucella*. Fourth, this approach is simpler and less costly, since many fluorescent labels, rather than a single molecule, are incorporated into the amplified fragment [21,22].

The analytical sensitivity of the LC-PCR assay was higher than those of conventional PCR procedures, followed by dot-blot hybridisation and PCR-ELISA, as 5 fg of bacterial DNA was amplified successfully. Such amounts of DNA can be expected in any blood sample from a patient with active brucellosis. The precision of the assay, with an intra-assay variation of <1.5% and an inter-assay variation of < 6.5%, was similar to or lower than that of other PCR assays based on similar principles [23], while the diagnostic yield was 91.9%, i.e., far higher than that for blood cultures [3,4]. Melting curve analysis increased the specificity of the assay by confirming that a

positive fluorescence signal obtained during the real-time PCR was associated with an amplified product with a characteristic T_m. Melting curve analysis showed that all *Brucella*-positive specimens and positive controls returned the same T_m, indicating that the LightCycler primer sequences were highly conserved.

The existence of five (8.1%) false-negative results, some in patients with positive blood cultures, was surprising. Although some of these false-negatives could result from the presence of inhibitors in the sample, this is unlikely. Zerva *et al*. [18], using a similar volume of serum, found 6% false-negative results by conventional PCR methods. The small reaction volume used in the LC-PCR systems poses a potential disadvantage compared to conventional PCR, since only a small volume of template can be added to the mastermix. The use of very small volume samples from patients with low concentrations of circulating brucellae could result in an absence of target DNA in the sample tested. Three of the patients who yielded an initial negative result were positive following a second extraction from the same serum sample. These findings suggest that increasing the sample volume, or performing a second test for borderline results, may increase the sensitivity of the assay. In either case, if the assay is to be used regularly for routine diagnostic purposes, it would be advisable to include an internal amplification control in the assay to monitor PCR inhibition.

The assay was considered to have good specificity. Of the three negative control samples with apparent false-positive results, two were from patients who were exposed continuosly to *Brucella* spp. during their employment, and the other was from a patient who consumed unpasteurised cheese. Since the B4 and B5 primers used have been shown to be highly specific for *Brucella* spp. [24], *Brucella*-specific DNA might have been present in these samples. Therefore, these patients could either be false-positive or have oligosymptomatic or asymptomatic self-limiting forms of infection [25,26]. Nevertheless, these findings suggest that caution is needed in the interpretation of a positive result from an individual who is exposed regularly to *Brucella*. The high sensitivity of PCR-based methods enables the amplification of possible remnants of circulating DNA in asymptomatic subjects, although these have no clinical relevance [10].

Although the present results show that the mean C_p values of the patients with brucellosis were significantly lower than those of the patients considered to be false-positive, the quantification method used in this study is not very precise, as there is a large overlap between the C_p values of both groups. The preparation and use of recombinant plasmids may enable a DNA threshold to be defined which correlates with active infection.

Overall, the results of this study agree with those reported previously [5–7,18,27]. Nevertheless, before the molecular diagnosis of brucellosis can become a clinical reality, studies aimed at harmonising the extraction and amplification protocols are required, as well as verification of inter-laboratory reproducibility. However, the LC-PCR assay described in this study could be a practical and useful tool for the rapid diagnosis of human brucellosis. It is highly sensitive and specific, is easy to perform and could provide results to a clinician in < 2 h. In addition, the risks to laboratory personnel associated with handling the microorganism are minimised [28].

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REFERENCES

1. Corbel MJ. Brucellosis: an overview. *Emerg Infect Dis* 1997; **3**: 213–221.
2. Young EJ. An overview of human brucellosis. *Clin Infect Dis* 1995; **21**: 283–289.
3. Colmenero JD, Reguera JM, Martos F *et al*. Complications associated with *Brucella melitensis* infection: a study of 530 cases. *Medicine (Baltimore)* 1996; **75**: 195–211.
4. Yagupsky P. Detection of brucellae in blood cultures. *J Clin Microbiol* 1999; **37**: 3437–3442.
5. Queipo-Ortuño MI, Morata P, Ocón P, Manchado P, Colmenero JD. Rapid diagnosis of human brucellosis by peripheral-blood PCR assay. *J Clin Microbiol* 1997; **35**: 2927–2930.
6. Morata P, Queipo-Ortuño MI, Reguera JM, García-Ordoñez MA, Pichardo C, Colmenero JD. Posttreatment follow-up of brucellosis by PCR assay. *J Clin Microbiol* 1999; **37**: 4163–4166.
7. Morata P, Queipo-Ortuño MI, Reguera JM, García-Ordoñez MA, Cardenas A, Colmenero JD. Development and evaluation of a PCR-enzyme-linked immunosorbent assay

for diagnosis of human brucellosis. *J Clin Microbiol* 2003; **41**: 144–148.
8. Bricker BJ. PCR as a diagnostic tool for brucellosis. *Vet Microbiol* 2002; **90**: 435–446.
9. Fredricks DN, Relman DA. Application of polymerase chain reaction to the diagnosis of infectious diseases. *Clin Infect Dis* 1999; **29**: 475–486.
10. Kami M, Fukui T, Ogawa S *et al.* Use of real-time PCR on blood samples for diagnosis of invasive aspergillosis. *Clin Infect Dis* 2001; **33**: 1504–1512.
11. Kuoppa Y, Boman J, Scott L, Kumlin U, Eriksson I, Allard A. Quantitative detection of respiratory *Chlamydia pneumoniae* infection by real-time PCR. *J Clin Microbiol* 2002; **40**: 2273–2274.
12. Harder TC, Hufnagel M, Zahn K *et al.* New LightCycler PCR for rapid and sensitive quantification of parvovirus B19 DNA guides therapeutic decision-making in relapsing infections. *J Clin Microbiol* 2001; **39**: 4413–4419.
13. Moyer N, Holcomb PLA. Brucella. In: Murray PR, Baron EJ, Pfaller MA, Tenover FC, Yolken RH, eds. *Manual of clinical microbiology*, 6th edn. Washington, DC: American Society for Microbiology, 1995; 382–386.
14. Alton GG, Jones LM, Pietz DE. *Laboratory techniques in brucellosis*, 2nd edn. Geneva: World Health Organization, 1975.
15. Orduña A, Almaraz A, Prado A *et al.* Evaluation of an immunocapture-agglutination test (Brucellacapt) for serodiagnosis of human brucellosis. *J Clin Microbiol* 2000; **38**: 4000–4005.
16. Mayfield JE, Bricker BJ, Godfrey H *et al.* The cloning, expression, and nucleotide sequence of a gene coding for an immunogenic *Brucella abortus* protein. *Gene* 1988; **63**: 1–9.
17. Baily GG, Krahn JB, Drasar BS, Stoker NG. Detection of *Brucella melitensis* and *Brucella abortus* by DNA amplification. *J Trop Med Hyg* 1992; **95**: 271–275.
18. Zerva L, Bourantas K, Mitka S, Kansouzidou A, Legakis NJ. Serum is the preferred clinical specimen for diagnosis of human brucellosis by PCR. *J Clin Microbiol* 2001; **39**: 1661–1664.
19. Gamazo C, Vitas AI, Lopez-Goñi I, Diaz R, Moriyon I. Factor affecting detection of *Brucella melitensis* by BACTEC NR730, a nonradiometric system for hemocultures. *J Clin Microbiol* 1993; **31**: 3200–3203.
20. Queipo-Ortuño MI, Garcia-Ordoñez MA, Colmenero JD, Morata P. Hydrogen peroxide improves the efficiency of a peripheral blood PCR assay for diagnosis of human brucellosis. *Biotechniques* 1999; **27**: 248–252.
21. Newby DT, Hadfield TL, Roberto FF. Real-time PCR detection of *Brucella abortus*: a comparative study of SYBR green I, 5′-exonuclease, and hybridization probe assays. *Appl Environ Microbiol* 2003; **69**: 4753–4759.
22. Shu PY, Chang SF, Kuo YC *et al.* Development of group- and serotype-specific one-step SYBR green I-based real-time reverse transcription-PCR assay for dengue virus. *J Clin Microbiol* 2003; **41**: 2408–2416.
23. Costa JM, Pautas C, Ernault P, Foulet F, Cordonnier C, Bretagne S. Real-time PCR for diagnosis and follow-up of *Toxoplasma* reactivation after allogeneic stem cell transplantation using fluorescence resonance energy transfer hybridization probes. *J Clin Microbiol* 2000; **38**: 2929–2932.
24. Casañas MC, Queipo-Ortuño MI, Rodriguez-Torres A, Orduña A, Colmenero JD, Morata P. Specificity of a polymerase chain reaction assay of a target sequence on the 31-kilodalton *Brucella* antigen DNA used to diagnose human brucellosis. *Eur J Clin Microbiol Infect Dis* 2001; **20**: 127–131.
25. Coghlan JD, Longmore HJ. The significance of *Brucella* antibodies in patients in a rural area. *Practitioner* 1973; **211**: 645–652.
26. Poole PM. A 6-year survey of human brucellosis in a rural area of north-western England and north Wales. *Postgrad Med J* 1975; **51**: 433–440.
27. Vrioni G, Gartzonika C, Kostoula A, Boboyianni C, Papadopoulou C, Levidiotou S. Application of a polymerase chain reaction enzyme immunoassay in peripheral whole blood and serum specimens for diagnosis of acute human brucellosis. *Eur J Clin Microbiol Infect Dis* 2004; **23**: 194–199.
28. Staszkiewicz J, Lewis CM, Colville J, Zervos M, Band J. Outbreak of *Brucella melitensis* among microbiology laboratory workers in a community hospital. *J Clin Microbiol* 1991; **29**: 287–290.