Eur J Clin Microbiol Infect Dis (2005) 24: 842–845
DOI 10.1007/s10096-005-0064-0

CONCISE ARTICLE

C. Debeaumont · P. A. Falconnet · M. Maurin

Real-time PCR for detection of *Brucella* spp. DNA in human serum samples

Published online: 8 December 2005
© Springer-Verlag 2005

Abstract Presented here are the results of an evaluation of an in-house real-time PCR assay for the rapid and specific diagnosis of human brucellosis. The assay was based on direct amplification from serum samples of a 169-bp portion of *bcsp31*, a gene found in all *Brucella* species and biovars. Species specificity and selectivity of this real-time PCR assay were evaluated using genomic DNA from 15 *Brucella* strains and 42 non-*Brucella* strains, and the results were 100%. Among 17 culture-proven brucellosis patients, sera from 11 gave a positive amplification signal, corresponding to a sensitivity of 64.7%. In contrast, negative results were obtained for all sera from 60 control patients, corresponding to a specificity of 100%. The results indicate this test is well adapted for definite confirmation of brucellosis cases, when *Brucella* cultures remain sterile and serological tests demonstrate the presence of cross-reacting antibodies against *Brucella* sp. and *Yersinia enterocolitica* O:9 antigens.

Introduction

Brucella species are the etiological agents of brucellosis. Five species have been recognized in the past, according to relative animal host specificity: *B. melitensis* in sheep and goats, *B. abortus* in cattle, *B. suis* in swine, *B. ovis* in sheep, *B. canis* in dogs, and *B. neotomae* in desert rats [1]. More recently, marine mammals have been recognized as an additional animal reservoir for *Brucella* species, and *B. cetaceae* and *B. pinnipediae* are the newly proposed species names associated with cetaceae and pinnipediae, respectively [2, 3].

M. Maurin (✉) · C. Debeaumont · P. A. Falconnet
Laboratoire de Bactériologie, Centre Hospitalier Universitaire,
Faculté de Médecine, Université Joseph Fourier,
BP 217, 38043 Grenoble cedex 9, France
e-mail: mmaurin@chu-grenoble.fr
Tel.: +33-4-76765479
Fax: +33-4-76765912

Diagnosis of human brucellosis remains based upon the isolation of *Brucella* sp. from the blood or other clinical samples of infected patients [4]. However, the sensitivity of culture for detecting *Brucella* is low in patients with subacute or chronic disease, or when an antibiotic therapy has been administered before clinical samples have been collected for *Brucella* culture [4]. Serological techniques are the usual alternative diagnostic methods used when cultures remain negative [5] and, among these, the Wright test is still considered the standard method. The sensitivity of serological tests varies from 65% to 95% [5]. However, low specificity is the major limitation of serological techniques due to serological cross-reactions [5], especially between *Brucella* spp. and *Yersinia enterocolitica* O:9, but also, to a lesser extent, *Francisella tularensis*, *Vibrio cholerae* O:1 (especially in patients vaccinated against cholera), *E. coli* O:157, *Salmonella* O:30, *Afipia clevelandensis*, *Ochrobactrum anthropi*. Thus, PCR-based [6–11] and real-time PCR (rtPCR)-based diagnostic methods [12–15] have been developed in the last two decades to detect *Brucella* DNA in human samples. PCR has proven to be more sensitive than culture in patients with focalized brucellosis [9], and it is particularly useful when an antibiotic therapy has been administered before clinical specimen collection for *Brucella* culture [9]. PCR is also less hazardous than culture for laboratory workers [7]. Our goal was to evaluate a new rtPCR assay for detection of *Brucella* DNA in human serum samples from brucellosis case patients.

Materials and methods

Reference strains and human isolates of *Brucella* and non-*Brucella* species used in the present study are listed in Table 1. *Brucella* spp. were grown on Columbia agar plates supplemented with 5% sheep blood (bioMérieux, Marcy l'Etoile, France) in a biosafety level 3-equipped laboratory. DNA extraction from pure cultures of *Brucella* and non-*Brucella* strains was performed using QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). DNA concentration and

Table 1 *Brucella* and non-*Brucella* strains used in the present study

Species (biovar)	Strain	Species (biovar)	Strain
Brucella strains from AFSSA[a]			
B. melitensis (bv1)	16M (ATCC 23456)	*B. melitensis* (bv1)	02-5794
B. abortus (bv1)	544 (ATCC 23448)	*B. melitensis* (bv1)	02-6501
B. suis (bv1)	1330 (ATCC 23444)	*B. melitensis* (bv3)	02-5259
B. canis	RM6/66 (ATCC 23365)	*B. melitensis* (bv3)	02-5451
B. ovis	63/290 (ATCC 25840)	*B. melitensis* (bv3)	02-6475
Brucella strains from Grenoble's Hospital[b]			
B. melitensis (bv3)	CHG1	*B. melitensis*	CHG4
B. melitensis (bv3)	CHG2	*B. melitensis*	CHG5
B. melitensis	CHG3		
Acinetobacter baumanii	Human isolate	*Neisseria meningitidis* B	Human isolate
Afipia clevelandensis	ATCC 49720	*Ochrobactrum anthropi*	CIP 103947
Agrobacterium tumefaciens	CIP 102 118	*Ochrobactrum anthropi*	CIP 103949
Alcaligenes faecalis	CIP 6080 T	*Ochrobactrum anthropi*	CIP 103950
Bacillus pumilus	Human isolate	*Pasteurella multocida*	Human isolate
Bartonella henselae	Human isolate	*Proteus mirabilis*	Human isolate
Burkholderia cepacia	Human isolate	*Proteus vulgaris*	Human isolate
Clostridium perfringens	Human isolate	*Pseudomonas aeruginosa*	ATCC 27853
Enterobacter aerogenes	P 99	*Rhizobium leguminosarum*	CIP 106 959
Enterococcus faecalis	ATCC 29212	*Salmonella enteritidis*	Human isolate
Escherichia coli	ATCC 35218	*Serratia marcescens*	Human isolate
E. coli O157H7	Human isolate	*Shigella sonnei*	Human isolate
Francisella tularensis	Human isolate	*Staphylococcus aureus*	ATCC 25923
Haemophilus influenzae[b]	ATCC 49247	*Staphylococcus epidermidis*	ATCC 1228
Klebsiella oxytoca	Human isolate	*Stenotrophomonas maltophilia*	Human isolate
Klebsiella pneumoniae	CF 104	*Streptococcus agalactiae*	Human isolate
Listeria monocytogenes	Human isolate	*Streptococcus pneumoniae*	ATCC 6303
Legionella pneumophila	Philadelphia	*Vibrio alginolyticus*	Human isolate
Moraxella catarrhalis	Human isolate	*Yersinia enterocolitica O:9*	CIP 72.5A
Mesorhizobium loti	CIP 107 326	*Yersinia enterocolitica O:9*	CIP 101 776
Neisseria gonorrhoeae	Human isolate	*Yersinia pseudotuberculosis*	CIP 104 897

[a]Strains provided by Dr. B. Garin-Bastuji, of Agence Française de Sécurité Sanitaire des Aliments, Maisons-Alfort, France
[b]Centre Hospitalier Universitaire de Grenoble
ATCC American type culture collection, *CIP* collection de l'Institut Pasteur

purity was measured using an absorbance ratio between 260 and 280 nm.

rtPCR was performed using the Light Cycler instrument (Roche Diagnostics, Mannheim, Germany). The target DNA was a 169-bp portion of the *bcsp31* gene, encoding a 31-kDa surface protein that is found in all *Brucella* species and biovars [7]. Primers and probes were as follows: forward primer BCSP31fw [5'-TCA ATG CGA TCA AGT CGG-3']; reverse primer BCSP31rv [5'-GCA TCC TTA CGC GCA A-3']; hybridization probes 5'-ACG CAG TCA GAC GTT GC-(fluorescein)-3' and 5'-(LCRed 640)-ATT GGG CCT ATA ACG GCA CC-(P)-3'. Each reaction mixture contained 10 pMol of each primer, 4 pMol of each hybridization probe, 100-nMol $MgCl_2$, and 5 µl of DNA extract in (20 µl final volume) Fast Start Master Hybridization Probes reaction mix (Roche Diagnostics).

The PCR profile was as follows: initial template denaturation at 95°C for 8 min, and 45 cycles of template denaturation at 95°C for 10 s, primer annealing at 57°C for 10 s, and primer extension at 72°C for 8 s, with a temperature transition rate of 20°C/s for all steps. A negative control (purified PCR-grade water) and a positive control (*B. melitensis* 16M genomic DNA) were included in all PCR assays.

A *B. melitensis* 16M genomic DNA suspension was prepared in sterile distilled water and titrated spectrophotometrically. Assuming a molecular mass for the *B. melitensis* genome of approximately 3 fg of DNA [7, 12], this suspension contained approximately 6×10^5 genome copies per 5 µl DNA extract. For each PCR assay, tenfold serial dilutions (10^{-1} to 10^{-7}) of this external standard were run in parallel with serum samples to be tested, and the log of the concentration of each dilution series was plotted versus the cycle number at which the fluorescent signal increased above a threshold value (Ct value). The slope of the standard curve generated allowed calculation of the

reaction efficiency (e) according to the following equation: $e=10^{-(1/\text{slope})}$. Intra-assay and inter-assay reproducibility of DNA titration were assessed by calculation of variation coefficients. The analytical sensitivity of the rtPCR assay was defined as the minimum *B. melitensis* DNA concentration that could be amplified in ≥90% of ten independent experiments.

In order to evaluate the influence of serum or blood on the efficiency and analytical sensitivity of our rtPCR assay, serum or blood samples from volunteers were spiked with the *B. melitensis* 16M DNA suspension and used for rtPCR testing.

As a laboratory associated with the French Reference Laboratory for *Brucella* at AFSSA (Agence Française de Sécurité Sanitaire des Aliments, Maisons-Alfort, France), we receive serum samples from suspected cases of human brucellosis for diagnostic expertise. We selected 17 serum samples from 17 patients with culture-proven brucellosis for this study. Control samples included 30 sera from blood donors, and sera from five patients with systemic lupus erythematosus, five patients with Epstein Barr virus infection, five patients with cytomegalovirus infection, five patients with acute *Coxiella burnetii* infection, five patients with Lyme disease, and five patients with *Bartonella henselae* (cat-scratch disease) infection. Serum samples were tested for the presence of antibodies against *Brucella* and *Y. enterocolitica* O:9 antibodies (Table 2), and for the presence of *Brucella* sp. DNA by rtPCR (DNA was extracted from 200-µl samples).

Results and discussion

Using genomic DNA extract, our rtPCR protocol amplified the expected 169 bp amplicon from the 15 *Brucella* strains, but not from the 42 non-*Brucella* species. Interestingly, *O. anthropi* DNA was not amplified, whereas cross-amplification has been previously reported with *bcsp*31-targeting PCR assays [6, 10–12]. Thus, species specificity and selectivity of our rtPCR assay were 100%. When using *B. melitensis* 16M DNA suspension in sterile distilled water, statistical analysis revealed high reproducibility of the standards, with intra-assay and inter-assay variances ranging from 0.0022 to 0.075 and from 0.0027 to 0.12, respectively. Reaction efficiency was 1.95.

A linear regression was found between the log of *B. melitensis* 16-M genome copies and Ct values ($y=-3.43x+39.26$, $R^2=0.99$), over five orders of magnitude (from 7.14×10^5 down to 71.4 genome copies/5 µl DNA extract). The analytical sensitivity was found to be seven genome copies (~21 fg DNA) per 5 µl of DNA extract. When using DNA templates prepared from serum or blood samples spiked with *B. melitensis* 16 M, reaction efficiencies were 1.95 and 1.98, respectively. The equation for the linear regression line for the standard curves generated and its corresponding R^2 value were, respectively, $y=-3.44x+35.2$, $R^2=0.99$ and $y=-3.035x+39.01$, $R^2=0.99$. A linear regression of over five orders of magnitude was found, from 6×10^5 down to 60 genome copies/5-µl DNA extract. The analytical sensitivity was found to be six genome copies

Table 2 Results of rtPCR and serological test for the 17 patients with brucellosis as proven by culture of a *Brucella* strain from blood (patients 1–14) or synovial fluid (patient 15)

Patient no.	Sex/age (y)	*Brucella* strain isolated	rtPCR (genome copies/5 µl DNA extract)	Serology			
				Wright test[a]	Brucella IFA[b]		*Y. ent.*O:9[c]
					IgM	IgG	IgG
1	M 28	*B. melitensis* bv3	Positive (48)	>2560	2560	1280	>6400
2	M 41	*B. abortus* bv3	Positive (25)	ND	640	640	ND
3	F 46	*B. melitensis* bv3	Positive (82)	80	20	20	400
4	F 33	*B. melitensis* bv2	Positive (183)	640	320	80	3200
5	H 58	*B. melitensis* bv3	Positive (36)	1280	640	640	1600
6	H 26	*B. melitensis* bv3	Positive (32)	ND	ND	ND	ND
7	H 61	*B. melitensis* bv3	Positive (58)	160	160	640	400
8	F 56	*B. abortus* bv3	Positive (44)	40	20	80	<100
9	F 11	*B. melitensis* bv2	Positive (650)	640	640	640	800
10	H 69	*B. suis* bv2	Positive (8)	1,280	1,280	160	800
11	H 26	*B. melitensis* bv1	Positive (256)	1,280	>1,280	160	1,600
12	F 29	*B. melitensis* bv3	Negative	320	160	160	800
13	H 59	*B. melitensis* bv3	Negative	320	160	160	800
14	H 57	*B. abortus* bv3	Negative	320	<20	160	400
15	H 34	*B. melitensis* bv3	Negative	1,280	<20	1,280	800
16	F 59	*B. melitensis* bv3	Negative	20	20	80	<100
17	F 55	*B. melitensis* bv3	Negative	<20	<20	320	100

[a] Wright test (J2L Elitech, Labarthe Inard, France)
[b] *Brucella* IFA: immunofluorescence assay using a *B. abortus* suspension as antigen (Becton Dickinson, Pont-de-Claix, France)
[c] *Y. ent.*O:9 IFA: microplate agglutination test using *Yersinia enterocolitica* O:9 suspension as antigen (BioRad, Marnes la Coquette, France)

(~18-fg DNA) per 5 μl of DNA extract for serum samples, but 60 genome copies (~180-fg DNA) per 5 μl of DNA extract for blood samples. Limits of detection of *Brucella* DNA extracted from human blood samples varying from 250 fg down to 10 fg have been reported in the literature [8, 10, 12, 15].

The 17 brucellosis patients (10 men/seven women; mean age, 46 years; age range, 11–69 years) presented with clinical manifestations compatible with acute or subacute brucellosis, e.g., asthenia (7/17), high fever (6/17), sweats (5/17), myalgia (4/17), arthritis (3/17), orchiepididymitis (2/17), spondylitis (1/17), sacroiliitis (1/17), or aortic aneurysm infection (1/17).

Species and biovar determination of isolated strains was performed at AFSSA (Table 2). Fifteen of the 17 serum samples gave a strong positive reaction in the plate agglutination test. *Brucella* and *Y. enterocolitica* O:9 antibody titers are presented in Table 2. Eleven of the 17 serum samples tested with rtPCR gave a positive result, corresponding to a sensitivity of 64.7% (95% confidence interval, 42–87.4%). Bacterial titers ranged from approximately 25 to 650 genome copies per 5 μl of DNA extract (Table 2). In the literature, sensitivities and specificities of PCR and rtPCR assays targeting the *bcsp31* gene to detect *Brucella* DNA in human blood or serum samples have varied from 58% to 100% and from 91.9% to 96.5%, respectively [8, 10, 14]. Interestingly, most patients with a positive rtPCR test result showed high IgM antibody titers for *Brucella* spp. (median, 640; range, 20–2,560 versus median,<20; range,<20–160 for rtPCR-negative patients), whereas IgM-type antibodies are usually found early in the course of brucellosis [5]. The low sensitivity we found may be partly explained by inadequate preservation of many serum samples (>3 months at −20°C) before rtPCR testing. A higher sensitivity may be expected with freshly collected samples.

Serological tests to detect *Brucella* spp. were negative for all 30 serum samples from blood donors. Among sera from the 30 non-brucellosis patients, none tested positive with the plate agglutination test or the Wright test, while one sample from a systemic lupus erythematosus patient displayed anti-*Brucella* IgG and IgM titers of 160, and another sample from a Lyme disease patient displayed an IgG antibody titer of 80. The *Brucella* BCSP31 rtPCR test was negative for sera from all 60 control patients not infected with *Brucella* spp., corresponding to a specificity of 100%.

In conclusion, we report the use of a *bcsp-31* gene-targeting rtPCR test that detected *Brucella* DNA in serum samples from bacteremic brucellosis cases. This test will allow us to confirm diagnosis in patients with suspected brucellosis but negative blood cultures. It may also serve as a rapid and safe method for identifying *Brucella* strains isolated from human or animal samples.

Acknowledgement This work was partly supported by a public health service grant from Direction Régionale de la Recherche Clinique (2005).

References

1. Sarinas PSA, Chitkara RK (2003) Brucellosis. Sem Resp Infect 18:168–182
2. Cloeckaert A, Grayon M, Grepinet O, Boumedine KS (2003) Classification of *Brucella* strains isolated from marine mammals: infrequent restriction site-PCR and development of specific PCR identification tests. Microbes Infect 5:593–602
3. Cloeckaert A, Verger JM, Grayon M, Paquet JY, Garin-Bastuji B, Foster G, Godfroid J (2001) Classification of *Brucella* spp. isolated from marine mammals: polymorphism at the *omp*2 locus. Microbes Infect 3:729–738
4. Yagupsky P (1999) Detection of *Brucellae* in blood cultures. J Clin Microbiol 37:3437–3442
5. Young EJ (1991) Serologic diagnosis of human brucellosis: analysis of 214 cases by agglutination tests and review of the literature. Rev Infect Dis 13:359–372
6. Casanas MC, Queipo-Ortuno MI, Rodriguez-Torres A, Orduna A, Colmenero JD, Morata P (2001) 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 20:127–131
7. Matar GM, Khneisser IA, Abdelnoor AM (1996) Rapid laboratory confirmation of human brucellosis by PCR analysis of a target sequence on the 31-kilodalton *Brucella* antigen DNA. J Clin Microbiol 34:477–478
8. Queipo-Ortuno MI, Morata P, Ocon P, Manchado P, Colmenero JD (1997) Rapid diagnosis of human brucellosis by peripheral-blood PCR assay. J Clin Microbiol 35:2927–2930
9. Morata P, Queipo-Ortuno MI, Reguera JM, Miralles F, Lopez-Gonzales JJ, Colmenero JD (2001) Diagnostic yield of a PCR assay in focal complications of brucellosis. J Clin Microbiol 39:3743–3746
10. Morata P, Queipo-Ortuno MI, Reguera JM, Garcia-Ordonez MA, Gardenas A, Colmenero JD (2003) Development and evaluation of a PCR-enzyme-linked immunosorbent assay for diagnosis of human brucellosis. J Clin Microbiol 41:144–148
11. Baily GG, Krahn JB, Drasar BS, Stoker NG (1992) Detection of *Brucella melitensis* and *Brucella abortus* by DNA amplification. J Trop Med Hyg 95:271–275
12. Newby DT, Hadfield TL, Roberto FF (2003) Real-time PCR detection of *Brucellaabortus*: a comparative study of SYBR green I, 5'-exonuclease, and hybridization probe assays. Applied Environ Microbiol 69:4753–4759
13. Probert WS, Schrader KN, Khuong NY, Bystrom SL, Graves MH (2004) Real-time multiplex PCR assay for detection of *Brucella* spp., *B. abortus*, and *B. melitensis*. J Clin Microbiol 42:1290–1293
14. Queipo-Ortuno MI, Colmenero JD, Baeza G, Morata P (2005) Comparison between lightCycler real-time polymerase chain reaction (PCR) assay with serum and PCR-enzyme-linked immunosorbent assay with whole blood samples for the diagnosis of human brucellosis. Clin Infect Dis 40:260–264
15. Redkar R, Rosa S, Bricker B, DelVecchio V (2001) Real-time detection of *Brucella abortus*, *Brucella melitensis* and *Brucella suis*. Mol Cell Probes 15:43–52