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1 Real-time PCR for identification of *Brucella* spp: a comparative study of IS711, *bcs*p31 and *per*
2 target genes

3

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24

24 **ABSTRACT**

25 Culture is considered as the reference standard assay for diagnosis of *Brucella* spp. in humans and
26 animals but it is time-consuming and hazardous. In this study, we evaluated the performances of
27 newly designed real-time PCR assays using Taqman® probes and targeting the 3 following specific
28 genes: (i) the insertion sequence *IS711*, (ii) *bcs31* and (iii) *per* genes for the detection of *Brucella*
29 at genus level. The real-time PCR assays were compared to previously described conventional PCR
30 assays targeting the same genes. The genus-specificity was evaluated on 26 *Brucella* strains,
31 including all species and biovars. The analytical specificity was evaluated on a collection of 68
32 clinically relevant, phylogenetically related or serologically cross-reacting micro-organisms. The
33 analytical sensitivity was assessed using decreasing DNA quantities of *Brucella ovis*, *B. melitensis*
34 bv. 1, *B. abortus* bv. 1 and *B. canis* reference strains. Finally, intra-assay repeatability and inter-
35 assay reproducibility were assessed. All *Brucella* species DNA were amplified in the three tests.
36 However, the earliest signal was observed with the *IS711* real-time PCR, where it varied according
37 to the *IS711* copy number. No cross-reactivity was observed in all three tests. Real-time PCR was
38 always more sensitive than conventional PCR assays. The real-time PCR assay targeting *IS711*
39 presented an identical or a greater sensitivity than the two other tests. In all cases, the variability
40 was very low. In conclusion, real-time PCR assays are easy-to-use, produce results faster than
41 conventional PCR systems while reducing DNA contamination risks. The *IS711*-based real-time
42 PCR assay is specific and highly sensitive and appears as an efficient and reproducible method for
43 the rapid and safe detection of the genus *Brucella*.

44

45 **Keywords:** *Brucella*, real-time PCR, *bcs31*, *per*, *IS711*, genus-specific identification.

46

46 **INTRODUCTION**

47 Brucellosis is a bacterial zoonosis of worldwide importance, and of major public health and
48 economic significance (Godfroid et al., 2005; Pappas et al., 2006). The genus *Brucella* belongs to
49 the α -proteobacteria group and consists of eight recognized species: *B. abortus*, *B. melitensis*,
50 *B. suis*, *B. ovis*, *B. canis*, *B. neotomae* and the strains recently discovered in marine mammals and in
51 common vole (*Microtus arvalis*) and published under the respective species names of *B. ceti*,
52 *B. pinnipedialis* and *B. microti* (Foster et al., 2007; Scholtz et al., 2008). Diagnosis is usually based
53 on serological tests and/or cultivation. Serological assays are rapid, sensitive and easy to perform,
54 but lack specificity due to cross-reactions with other bacteria, particularly with *Yersinia*
55 *enterocolitica* O:9, that result from O chains antigenic similarity (Young et al., 1991; Wrathall et
56 al., 1993; Garin-Bastuji et al., 1999; Godfroid et al., 2002; Nielsen et al., 2004). Therefore, culture
57 remains the “gold standard” for definitive diagnosis, especially in free areas where the positive
58 predictive value of serological tests is very low. However, the zoonotic nature of most *Brucella*
59 species is a potential hazard for laboratory personnel and cultures must be performed in well-
60 equipped laboratories with highly skilled personnel. Moreover, the process from clinical sample to
61 final diagnosis remains long, and requires a minimum amount of viable *Brucella* in the specimen.
62 Therefore, PCR-based methods that are simpler, faster, less hazardous and usually more sensitive
63 have been developed (Bricker, 2002) for *Brucella* detection, especially those using as targets the
64 16S rRNA (Romero et al., 1995; Hermann and De Ridder, 1992; O’Leary et al., 2006), and the
65 *Brucella* Cell Surface 31 kDa Protein (*bcs₃₁*) genes (Baily et al., 1992; Da Costa et al., 1996),
66 which are highly conserved in the genus *Brucella*. However, with these targets, cross-reactivity has
67 been observed with *Ochrobactrum anthropi* and *O. intermedium* (Da Costa et al., 1996; Hermann
68 and De Ridder, 1992; Romero, 1995; Navarro et al., 1999), which is genetically close to the genus
69 *Brucella* (Velasco et al., 1998). The perosamine synthetase (*per*) gene, involved in O-chain
70 biosynthesis, is another highly conserved gene (Cloeckaert et al., 2000) recently used as a PCR

71 target for *Brucella* identification (Lübeck et al., 2003; Bogdanovich et al., 2004). The insertion
72 sequence IS711 (Halling et al., 1993), also called IS6501 (Ouahrani et al., 1993) is highly
73 conserved in the genus *Brucella* but the insertion location as well as the copy number vary
74 according to each species (Ouahrani et al., 1993; Halling et al., 1993). Besides conventional PCR
75 assays, some authors have developed real-time PCR targeting either the IS711, or *bcs*31, or *per*
76 genes to detect *Brucella* (Matar et al., 1996; Bogdanovich et al., 2004; Debeaumont et al., 2005;
77 Queipo-Ortuno et al., 2005; Navarro et al., 2006; Al Dahouk et al., 2007). This method is more
78 rapid, easier to perform, limits the DNA contamination and enables quantification of the specific
79 PCR products. The aim of this study was to design, optimise and evaluate real-time PCR assays for
80 *Brucella* spp. detection by targeting IS711, *bcs*31, or *per* genes. Analytical sensitivity and
81 specificity were compared to those of conventional PCR assays already published and targeting the
82 same genes.

83

84 MATERIALS AND METHODS

85 Bacterial strains and growth conditions.

86 The *Brucella* strains used in this study are listed in Table 1. They included the reference strains of
87 all *Brucella* species and biovars, the B19, Rev.1 and RB51 vaccine strains, and the S99 strain used
88 for the production of approved diagnostic antigens. Sixty-eight other micro-organisms, including
89 strains either phylogenetically related to *Brucella* (*i.e.* belonging to the α -2 proteobacteria group), or
90 sharing common antigens with *Brucella*, or potentially abortifacient, were used for specificity
91 checking (Table 1).

92 *Brucella* strains were grown on blood agar base n°2 (Oxoid, France) supplemented with equine
93 serum (5%) (BABS) at 37°C with 5% CO₂ for 48-72 h. Strains obtained from the BCCM-LMG
94 bacteria collection were grown on LMG-recommended media (<http://bccm.belspo.be>). Other

95 cultures were grown on 5% horse blood agar (bioMérieux, France) at 37°C for 24h-48h. All strains
96 were stored at -20°C in Tryptcase soy broth (bioMérieux, France) with 10% of glycerol.

97 **DNA extraction and quantification.**

98 Micro-organisms were harvested, washed and resuspended in phosphate-buffered saline (PBS).
99 *Brucella* strains DNA was prepared by the phenol/chloroform method as described previously (Da
100 Costa et al., 1996). The DNA of all other micro-organisms was extracted using either Instagen
101 matrix DNA kit (BioRad, France) for Gram-positive bacteria, or High pure PCR Template
102 Preparation Kit (Roche Diagnostics, France) for gram-negative bacteria, according to the
103 manufacturer's instructions. DNA concentration and purity were spectrophotometrically assessed
104 by reading A_{260} and A_{280} and confirmed by visualization on 1% agarose gel. Then, DNA was diluted
105 to 1 µg/ml in nuclease-free water and stored at -20°C until required for analysis.

106 **Primers and probes.**

107 The Primer Express software (Version 2.0, Applied Biosystems, France) was used for all the
108 oligonucleotide primers and the fluorescent dye-labelled probes designed in this study on the basis
109 of IS711/IS6501, *bcs*31, or *per* sequences available in GeneBank by using multiple sequence
110 alignment (<http://www.ebi.ac.uk/Tools/clustalw2/>) to target a conserved region. All primers were
111 purchased from MWG (MWG Biotech, Germany). The *in silico* specificity was analysed using the
112 Basic Local Alignment Search Tool from the GeneBank database.

113 The characteristics of the primers used for conventional PCR (single and/or nested) targeting either
114 IS711 or *bcs*31 or *per* sequences are given in Table 2. The *bcs*31 primers and single PCR *per*
115 primers have been previously described (Baily et al., 1992; Bogdanovich et al., 2004). By contrast,
116 primers used for *per* nested PCR and IS711 primers were newly designed and chosen according to
117 the following criteria: minimum dimer formation, self-complementarity, and minimum homology
118 with known prokaryotic gene sequences.

119 The *bcs31* and *per* probes were TaqMan MGB™ Probes (Applied Biosystems, France)
120 incorporating a 5' FAM reporter and a minor groove binder group (MGB) with a non-fluorescent
121 quencher (NFQ) at the 3' end. The *IS711* probe was a TaqMan probe (MWG biotech, Germany)
122 labelled at the 5' end with FAM as fluorescent reporter and TAMRA as quenching molecule at the
123 3' end. The characteristics of primers and probes used for real-time PCR are given in Table 2.

124 **Conventional PCR assay.**

125 Amplifications were carried out using 50 µl reaction volumes. The mixture contained 38.4 µl of
126 RNase free water, 5 µl of 10X PCR buffer (Promega, France), 200 µM of dNTP mix, 2 mM of
127 MgCl₂, 1 µM of each primer, 1 U of Taq polymerase (Promega, France) and 1 ng of DNA. The
128 DNA quantities used for the analytical sensitivity corresponded to 10-fold dilutions from 10⁻⁶ to 10⁻
129 ¹⁵ g/ml. For the *IS711* single PCR, reaction mixtures were denatured 5 min at 94°C. Then, the first 5
130 cycles (precycles) were performed as follows: 1 min at 94°C, 1 min primer annealing at 60°C, and
131 1 min primer extension at 72°C. Then, the last 35 cycles were performed with an annealing
132 temperature of 55°C. Then, the extension reaction was continued for another 7 min at 72°C to
133 ensure that the final extension step is complete.

134 The B4/B5 primers, and *bruc1/bruc5* primers targeting *bcs31* and *per* gene respectively were used
135 in the single PCR according to the authors (Baily et al., 1992; Bogdanovich et al., 2004). The
136 *bcs31* nested PCR was carried out as described by Da Costa et al. (1996). The *IS711* and *per*
137 nested PCR were performed for 15 cycles with the same timing steps as described above for the
138 single PCR without precycles.

139 The reaction products were visualized on 1.5% agarose gels containing 1 µg ethidium bromide per
140 ml. To avoid any contamination, reaction mixture preparation, DNA amplification and gel
141 migration were done in separates rooms.

142 **Real-time PCR assay.**

143 Real-time TaqMan® PCR was set up in a final volume of 25 µl with 9.65 µl of RNase free water,
144 12.5 µl of TaqMan® Universal PCR Master Mix (Applied Biosystems, France), each primer and
145 TaqMan® probe at concentrations of 0.3 µM and 0.2 µM, respectively, and 2 ng of DNA template.
146 The reaction mixture was initially incubated for 10 min at 95°C. Amplification was performed for
147 45 cycles of denaturation at 95°C for 15 s, annealing and extension at 60°C for 1 min. The PCR
148 reaction was performed on an AB-prism 7300 (Applied Biosystems, France). Samples were
149 processed in duplicates.

150 **Analytical Sensitivity.**

151 Sensitivity was determined by testing decreasing DNA quantities of *B. ovis* 63/290, *B. melitensis*
152 16M, *B. abortus* 544 and *B. canis* RM6/66 (10-fold dilutions from 10⁻⁶ to 10⁻¹⁵ g/ml). These strains
153 were chosen due to their high (*B. ovis*), and low (*B. melitensis*, *B. abortus* and *B. canis*) copy
154 number of IS711: 38, 7, 7 and 6 respectively, as previously described (Ouahrani et al, 1993).

155 **Analytical Specificity.**

156 The specificity of the different primers and probes was first assayed *in silico* by using BLAST
157 software in order to avoid non-specific amplification. Analytical specificity was then evaluated on
158 the 68 non-*Brucella* micro-organisms listed in Table 1. In conventional PCR, 1 ng of *B. ovis* DNA
159 was used as a positive control. In real-time PCR, an inhibition control (mixture containing both the
160 pathogen DNA and 0.2 ng of *B. ovis* DNA) was simultaneously tested for each organism. Standard
161 curves were generated using a 10-fold dilution series of DNA ranging from 0.2 ng to 0.2 fg for the
162 TaqMan® assay and were used to determine the cycle threshold (C_T) values and the lower limit of
163 detection of the respective assay. For all PCR assays, the negative control consisted in sterile water
164 instead of DNA template.

165 **Repeatability and reproducibility of the real-time PCR assay.**

166 Seventy-two samples were amplified in the same run for the intra-assay repeatability and 15
167 samples were amplified in a single run on 4 consecutive days for the inter-assay reproducibility. The
168 respective coefficients of variation (CVs) were then calculated.

169

170 **RESULTS**

171 **Amplification of the IS711, *bcs*31 and *per* genes in *Brucella* reference strains.**

172 DNA amplification of the three sequences was obtained with all the *Brucella* species and biovars
173 listed in Table 1. In real-time PCR, for all *Brucella* strains tested, a low variation in C_T values was
174 observed for *bcs*31 and *per* gene targets when the same quantity of DNA was used as template in
175 the assays (C_T : 14.49-17.57 with 20 ng of *Brucella* DNA). By contrast, in the real-time PCR
176 targeting the IS711, the observed C_T varied according to the *Brucella* species and biovars (Table 3).
177 Moreover, for the same DNA amount, the earliest detection was observed for *B. ovis* 63/290 and the
178 latest for *B. melitensis* 16M biovar 1 with a difference of C_T of 4.88 (Table 3). A maximum
179 difference of C_T of 2.23 was found between *bcs*31 and *per* genes (ΔC_T *bcs*/per, table 3) with an
180 earlier detection for the *bcs*31-based PCR. However, the C_T observed with IS711 was equal or
181 lower than the one observed with either *bcs*31 or *per*-based PCR, whatever the *Brucella* species.
182 The difference of C_T observed between IS711 and *bcs*31 (ΔC_T IS/*bcs*, table 3) was 0.18-2.35,
183 0.41-2.50, 0.76-3.52, and 4.81 for *B. abortus*, *B. melitensis*, *B. suis* and *B. ovis* respectively (Fig. 1;
184 Table 3). Results obtained with DNA from both *B. pinnipedialis* and *B. ceti* presented a
185 ΔC_T IS/*bcs* of 4.44 and 5.71 respectively (Table 3).

186 **Specificity of conventional and real-time PCR assays.**

187 No amplification products were observed in real-time PCR, whatever the target, with any of the
188 non-*Brucella* micro-organisms tested. In conventional PCR, none of the non-*Brucella* strains
189 studied were detected when using the IS711 and *per* gene primers. However, DNA from *O.*
190 *intermedium* LMG 3301 and LMG 3306 strains were amplified when using B4/B5 or

191 Bruc887/Bruc1457 *bcs*31 primers in single PCR. Nevertheless these cross-reactions were not
192 confirmed in nested PCR (data not shown).

193 **Sensitivity of conventional and real-time PCR assays.**

194 The respective lower limit of detections of conventional and real-time PCR assay observed with 10
195 fold-serial dilutions of *B. abortus*, *B. melitensis*, *B. ovis* and *B. canis* DNA are given in Table 4. In
196 all cases, the real-time PCR was more sensitive than the conventional PCR. In conventional PCR
197 assays, the lower limit of detection was identical for *bcs*31 and *per* targets. However, a 50-fold
198 higher sensitivity for *B. canis* RM6/66 and *B. melitensis* 16M DNA and a 500-fold higher
199 sensitivity for *B. abortus* 544 and *B. ovis* 63/290 were observed for these targets in real-time PCR.
200 For the IS711 target, a 10-fold maximum variation was observed according to the species tested,
201 with a 50-500 higher sensitivity for the real-time PCR. Finally, the sensitivity of the IS711 target
202 was identical or 10 times higher than the sensitivity of the two other targets, in real-time as well as
203 in conventional PCR (Table 4).

204 **Repeatability and reproducibility of the real-time PCR assay.**

205 Since IS711 appeared as the best candidate for routine brucellosis diagnosis, evaluation of the
206 repeatability and the reproducibility was only done for this target on samples consisting of 0.02 ng
207 of *B. ovis* 63/290 DNA. The C_T values intra-assay and inter-assay CVs were 1.06% and 4.17%
208 respectively (data shown).

209

210 **DISCUSSION**

211 In the last years, several authors have evaluated the sensitivity and the specificity of real-time PCR
212 assays for the detection of *Brucella* (Bricker and Halling, 1994; Redkar et al., 2001; Bricker et al.,
213 2002; Newby et al., 2003; Probert et al., 2004).

214 In the present study, we compared the sensitivity and the specificity of three targets, *i.e.* *per*, *bcs*31
215 and IS711 genes in real-time PCR, in comparison with conventional PCR. To our knowledge, no

216 previously published real-time PCR presented primers based exclusively on the IS711 gene, while
217 this sequence is specific and its copy number could allow improving the sensitivity of the PCR.
218 Detection of the three targets from *Brucella* DNA confirms the presence of the IS711, *bcs31* and
219 *per* genes in all *Brucella* species and biovars. There was no detectable difference in sensitivity of
220 PCR assays between *Brucella* species and biovars using *bcs31* and *per* primers. By contrast, the
221 IS711 detection depends on its copy number among *Brucella* species and biovars. Nevertheless, the
222 ΔC_T IS/bcsp of *Brucella* species is not really significant. To be relevant, ΔC_T IS/bcsp-based
223 *Brucella* species identifications should be investigated and validated on a more substantial number
224 of strains of the same species and sub-species in a further work. Differences of C_T values obtained
225 between *bcs31* and *per* real-time PCR assays are assumed to be related to primer performance,
226 since each gene is theoretically present in equal quantities in the prepared DNA.
227 A greater sensitivity was observed for the IS711 detection by real-time PCR, the highest being
228 observed with *B. ovis* 63/290 DNA, probably due to its high copy number in the genome
229 (38 copies). *B. canis* RM6/66 DNA, *B. melitensis* 16M and *B. abortus* 544 DNA presented the same
230 results while 6-7 IS711 copies are present in their respective genome (Table 4).
231 The real-time PCR assays were evaluated with a variety of other bacterial species and were
232 highlighted as being specific; neither micro-organism phylogenetically related, nor pathogens
233 known to share common epitopes with *Brucella* showed a cross-reaction. Interestingly, no
234 amplification occurred with real-time *per* primers from *Y. enterocolitica* O:9 and *E. coli* O:157,
235 two organisms that contain the *per* gene, thus indicating the specificity of the selected target
236 sequence (Bogdanovitch et al., 2004). The two sets of real-time PCR IS711 primers presented in
237 this study were selected among many pairs after intensive testing. This careful design of the IS711
238 primers allowed a significant improvement of the specificity of the assay since no cross-reaction
239 was observed, notably with *Ochrobactrum* species, the closest phylogenetically relative of *Brucella*
240 (Velasco et al., 1998; Yanagi et al., 1993).

241 The specificity of conventional (single and nested) PCR assays presented similar results to those
242 reported by Da Costa et al. (1996).

243 A 5'-nuclease IS711 PCR assay was tested by Probert et al. (2004) and Redkar et al. (2001) who
244 evaluated the detection limit of the assay. These authors reported a result approximately identical to
245 ours, with respective detections limits of 150 fg for *B. abortus* and *B. melitensis* DNA and 250 fg
246 for *B. abortus*, *B. melitensis*, *B. ovis* and *B. suis*. Both PCR assays share an IS711-primer and a
247 species-specific primer in order to differentiate *Brucella* species, which could explain these low
248 results. Nevertheless, the IS711 real-time PCR assay described by Newby et al. (2003), based on the
249 same principle, was able to detect 7.5 fg of *B. abortus* DNA. Bogdanovitch et al. (2004) described a
250 detection limit of 2,000 fg for *B. abortus*, *B. melitensis* and *B. suis* DNA and 200 fg for *B. neotomae*
251 and *B. ovis* DNA with a *per* gene-based real-time PCR. Al Dahouk et al. (2007) evaluated a real-
252 time PCR based on the B4/B5 primers described by Baily et al. (1992) and compared it to real-time
253 PCRs established by Bogdanovitch et al. (2004), Newby et al. (2003), Probert et al. (2004) and
254 Redkar et al. (2001). As little as 18 fg of *B. abortus* DNA could be detected in this study using the
255 primers described by these authors, Bogdanovitch et al. (2004) excepted. The *B. melitensis* DNA
256 tested presented a detection limit of 16 fg with the primers designed by Probert et al. (2004) and
257 Redkar et al. (2001). The hybridization probes used in this last study allowed to avoid an
258 amplification of the *O. anthropi* DNA tested, which confirms the great advantage of using a probe
259 in real-time PCR (Newby et al., 2003).

260 The detection limit of the *per* real-time PCR assay in this work was markedly improved in
261 comparison to the real-time PCR published by Bogdanovitch et al. (2004). Moreover, the insertion
262 sequence IS711-based sensitivity reached was higher than previous works for the detection of the
263 genus *Brucella*. Combining these observations with our data, we could suggest the sensitive IS711
264 real-time PCR presented in this study for the detection of the genus *Brucella*.

265 In conclusion, real-time PCR appears to offer several advantages over conventional PCR: it is less
266 labour-intensive, faster and it is a closed system with no need of post-PCR handling, preventing
267 DNA contamination. Therefore, the use of the IS711-based TaqMan® real-time PCR assay appears
268 promising due to its high sensitivity for the safe, rapid and specific detection of the genus *Brucella* in
269 clinical samples, while the two other targets could be used as confirmatory tools, in order to
270 optimise the diagnostic specificity. However, protocols should be carefully validated on
271 representative numbers of *Brucella*-infected and -free samples before being implemented in routine
272 diagnosis in animal and human brucellosis.

273

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282

283 **Conflicts of interest Statement:** The authors have declared that no conflict of interest exists.

284

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386 **FIGURES CAPTIONS**

387

388 **Fig. 1.** IS711 real-time PCR amplification curves of *B. ovis* 63/290 (A); *B. melitensis* Ether (B);

389 *B. suis* 513 (C); and *B. abortus* 544 (D) genomic DNA (20 ng).

390 *The fluorescence axis is in logarithmic scale and the data are baseline-subtracted.*

391

392 **Fig. 2.** IS711 (A), *bcp31* (B) and *per* (C) amplification curves of *B. ovis* 63/290 genomic DNA. A

393 10-fold dilutions series (from 0.02 ng to 0.002 fg) was used as a template.

394 *The fluorescence axis is in logarithmic scale and the data are baseline-subtracted.*

395

395

TABLE 1. *Brucella* species and biovars and non-*Brucella* organisms included in this study.

Strains	Reference ^g	Source ^h
<i>Brucella</i> species ^a		
<i>B. melitensis</i> biovar 1 16M	ATCC 23456	Afssa Lerpaz
<i>B. melitensis</i> biovar 1 Rev.1	EDQM reference strain	Afssa Lerpaz
<i>B. melitensis</i> biovar 1 53H38 ^b	-	Afssa Lerpaz
<i>B. melitensis</i> biovar 2 63/9	ATCC 23457	Afssa Lerpaz
<i>B. melitensis</i> biovar 3 Ether	ATCC 23458	Afssa Lerpaz
<i>B. melitensis</i> biovar 3 115 ^b	-	Afssa Lerpaz
<i>B. abortus</i> biovar 1 544	ATCC 23448	Afssa Lerpaz
<i>B. abortus</i> biovar 1 B19 ^c	-	Afssa Lerpaz
<i>B. abortus</i> biovar 1 S99 ^d	-	Afssa Lerpaz
<i>B. abortus</i> biovar 1 RB51 ^e	-	Afssa Lerpaz
<i>B. abortus</i> biovar 2 86/8/59	ATCC 23449	Afssa Lerpaz
<i>B. abortus</i> biovar 3 Tulya	ATCC 23450	Afssa Lerpaz
<i>B. abortus</i> biovar 3 Afssa Lerpaz ^f	Field strain	Afssa Lerpaz
<i>B. abortus</i> biovar 4 292	ATCC 23451	Afssa Lerpaz
<i>B. abortus</i> biovar 5 B3196	ATCC 23452	Afssa Lerpaz
<i>B. abortus</i> biovar 6 870	ATCC 23453	Afssa Lerpaz
<i>B. abortus</i> biovar 9 C68	ATCC 23455	Afssa Lerpaz

<i>B. suis</i> biovar 1 1330	ATCC 23444	Afssa Lerpaz
<i>B. suis</i> biovar 2 Thomsen	ATCC 23445	Afssa Lerpaz
<i>B. suis</i> biovar 3 686	ATCC 23446	Afssa Lerpaz
<i>B. suis</i> biovar 4 40	ATCC 23447	Afssa Lerpaz
<i>B. suis</i> biovar 5 513	NCTC 11996	Afssa Lerpaz
<i>B. neotomae</i> 5K33	ATCC 23459	Afssa Lerpaz
<i>B. ovis</i> 63/290	ATCC 25840	Afssa Lerpaz
<i>B. canis</i> RM6/66	ATCC 23365	Afssa Lerpaz
<i>B. pinnipedialis</i>	NCTC 12890	Afssa Lerpaz
<i>B. ceti</i>	NCTC 12891	Afssa Lerpaz

Serologically cross-reacting bacteria

<i>Escherichia coli</i> O157:H7	Field strain	Afssa Lerqap
<i>Francisella tularensis</i> subsp. <i>holarctica</i>	ATCC 6223	Afssa Lerpaz
<i>Pasteurella multocida</i>	NCTC 12177	Afssa Lerpaz
<i>Salmonella enterica</i> subsp. <i>enterica</i> serovar Urbana (group N)	Field strain	Afssa Lerqap
<i>Xanthomonas maltophilia</i>	Field strain	Afssa Lerqap
<i>Yersinia enterocolitica</i> O: 9	NCTC 11174	Afssa Lerpaz

Phylogenetically related bacteria

<i>Agrobacterium radiobacter</i>	LMG 140	BCCM
<i>Agrobacterium rhizogenes</i>	LMG 150	BCCM
<i>Agrobacterium tumefaciens</i>	LMG 43.1	BCCM
<i>Agrobacterium vitis</i>	LMG 8750	BCCM
<i>Ochrobactrum intermedium</i>	LMG3301	BCCM
<i>Ochrobactrum intermedium</i>	LMG 3306	DM-UN
<i>Ochrobactrum anthropi</i>	LMG3331	BCCM
<i>Phyllobacterium myrsinacearum</i>	LMG 1.1	BCCM
<i>Rhizobium tropici</i>	LMG 9503	BCCM
<i>Sinorhizobium meliloti</i>	LMG 6133	BCCM

Non-*Brucella* organisms potentially abortifacient

<i>Campylobacter fetus</i> subsp. <i>fetus</i>	LMG 6442	BCCM
<i>Candida albicans</i>	Field strain	Afssa Lerpaz
<i>Coxiella burnetii</i> *	Nine Mile	Afssa-Lerpra
<i>Coxiella burnetii</i> *	Field strain 1	Afssa-Lerpra
<i>Coxiella burnetii</i> *	Field strain 2	Afssa-Lerpra
<i>Coxiella burnetii</i> *	Field strain 3	Afssa-Lerpra
<i>Listeria monocytogenes</i>	NCTC 7973	Afssa Lerpaz
<i>Listeria innocua</i>	Field strain	Afssa Lerpaz
<i>Salmonella enterica</i> subsp. <i>enterica</i> serovar <i>Abortusovis</i>	Field strain	Afssa Lerpaz
<i>Yersinia pseudotuberculosis</i>	ATCC 29833	Afssa Lerpaz
Other non- <i>Brucella</i> organisms		
<i>Acinetobacter baumannii</i>	Field strain	Afssa Lerpaz
<i>Aeromonas hydrophila</i>	Field strain	Afssa Lerpaz
<i>Afipia broomeae</i>	LMG 18885	BCCM
<i>Bacillus brevis</i>	Field strain	Afssa Lerqap
<i>Bacillus subtilis</i>	ATCC 6633	Afssa Lerpaz
<i>Bordetella bronchiseptica</i>	CIP A 34	Afssa Lerpaz
<i>Brevibacillus</i> sp.	Field strain	Afssa Lerqap
<i>Campylobacter jejuni</i>	LMG 8841	BCCM
<i>Campylobacter sputorum</i> biovar <i>faecalis</i>	LMG 6617	BCCM

<i>Citrobacter freundii</i>	Field strain	Afssa Lerpaz
<i>Corynebacterium bovis</i>	Field strain	Afssa Lerpaz
<i>Corynebacterium pseudotuberculosis (ovis)</i>	CIP 102968 T	Afssa Lerpaz
<i>Enterobacter aerogenes</i>	Field strain	Afssa Lerpaz
<i>Enterococcus faecalis</i>	ATCC 19433	Afssa Lerpaz
<i>Klebsiella pneumoniae</i>	Field strain	Afssa Lerpaz
<i>Lactobacillus saerimneri</i>	LMG 22087	BCCM
<i>Listeria ivanovii</i>	Field strain	Afssa Lerpaz
<i>Listeria seeligeri</i>	Field strain	Afssa Lerpaz
<i>Listeria welshimeri</i>	Field strain	Afssa Lerpaz
<i>Mycobacterium avium</i>	Field strain	Afssa Lerpaz
<i>Mycobacterium bovis</i>	Field strain	Afssa Lerpaz
<i>Mycobacterium paratuberculosis</i>	ATCC 7912	Afssa Lerpaz
<i>Mycobacterium tuberculosis</i>	ATCC H37Rv	Afssa Lerpaz
<i>Proteus mirabilis</i>	CIP 103181	Afssa Lerpaz
<i>Pseudomonas aeruginosa</i>	ATCC 27853	Afssa Lerpaz
<i>Rhodococcus equi</i>	Field strain	Afssa Lerpaz
<i>Salmonella bodjonegoro</i>	Field strain	Afssa Lerpaz
<i>Salmonella typhimurium</i>	NCTC 12484	Afssa Lerpaz
<i>Serratia marcescens</i>	Field strain	Afssa Lerpaz

<i>Staphylococcus aureus</i>	ATCC 6538	Afssa Lerpaz
<i>Staphylococcus epidermidis</i>	Field strain	Afssa Lerpaz
<i>Staphylococcus haemolyticus</i>	Field strain	Afssa Lerpaz
<i>Streptococcus agalactiae</i>	Field strain	Afssa Lerqaz
<i>Streptococcus bovis</i>	Field strain	Afssa Lerpaz
<i>Streptococcus dysgalactiae</i>	Field strain	Afssa Lerqaz
<i>Streptococcus equi</i> subsp. <i>Equi</i>	Field strain	Afssa Lerpaz
<i>Streptococcus equi</i> subsp. <i>zooepidemicus</i>	Field strain	Afssa Lerpaz
<i>Streptococcus equisimilis</i>	Field strain	Afssa Lerpaz
<i>Streptococcus faecium</i>	Field strain	Afssa Lerpaz
<i>Streptococcus pneumoniae</i>	Field strain	Afssa Lerpaz
<i>Streptococcus pyogenes</i>	Field strain	Afssa Lerpaz
<i>Yersinia enterocolitica</i> O:47	Field strain	Afssa Lerpaz

396 * Pure DNA directly provided by Afssa- Lerppra, Sophia-Antipolis, France.

397 (-): no reference.

398 All *Brucella* strains were from the *Brucella* Afssa Culture collection, Maisons-Alfort, France^a;

399 some strains were originally provided by INRA, Nouzilly, France^b; USDA, NVSL, Ames, USA^c;

400 VLA, Weybridge UK^d and CITA, Saragossa, Spain^e.

401 ^f: used by Afssa as internal positive control in bacteriology.

402 ^g: ATCC, American Type Culture Collection (USA); NCTC, National Collection of Type Cultures

403 (UK); EDQM, European Directorate for the Quality of Medicines (Strasbourg, France) ; CIP,

404 Collection de l'Institut Pasteur (Paris, France) ; LMG, Laboratory of Microbiology Gent Bacteria

405 Collection (University of Gent, Belgium).

406 ^hBCCM, Belgian Co-ordinated Collections of Micro-organisms (University of Gent, Belgium);
407 Afssa Lerpaz, Laboratoire d'Etudes et de Recherches en Pathologie Animale et Zoonoses (Unité
408 Zoonoses Bactériennes) and Afssa Lerqap, Laboratoire d'Etudes et de Recherches sur la Qualité des
409 Aliments et sur les Procédés agroalimentaires (Maisons-Alfort, France); Afssa Lerpra, Laboratoire
410 d'Etudes et de Recherches sur les Petits ruminants et les Abeilles (Sophia-Antipolis, France); DM-
411 UN, Departamento de Microbiología-Universidad de Navarra (Pamplona, Spain).

TABLE 2. *Brucella* primers and probes sequences used for amplification by conventional PCR and real-time PCR

PCR	Primer sequence			PCR products size (bp)	Location	Reference
	Forward primer (5'→3')	Probe (5'→3')	Reverse primer (5'→3')			
IS711 Single PCR	IS313: ctggctgatacggcgactttgaa	n.p. ^a	IS639: ggaacgtgttgattgaccttgat	350	313/639	Hénault et al., 2000
IS711 Nested PCR	IS340: gtcctcattgatagcaccatcg	n.p.	IS576: taagtgatcggcacataggctgc	260	340/576	
<i>bcs31</i> Single PCR	B4: tggctcggttgccaataca	n.p.	B5: cgcgcttgcccttcaggctctg	224	788/991	Baily et al., 1992

<i>bosp31</i>						
Single	Bruc887:	n.p.	Bruc1457:	594	887/1457	Da Costa et al., 1996
PCR	tttatgatggcaagggcaaggtgg		cactatcgagcttgatgagcttgc			
<i>bosp31</i>						
Nested	Bruc968:	n.p.	Bruc1404:	460	968/1404	
PCR	aggatgcaaacatcaaatcggctc		cgtgtatcctcgtccagagaacc			
<i>per</i>						
Single	bruc1:	n.p.	bruc5:	367	325/668	Bogdanovich et al., 2004
PCR	cggtttatgtggactctctcg		cagtattctcgtgtaggcgaagta			
<i>per</i>						
Nested	Per51:	n.p.	Per261:	231	51/261	This study
PCR	gtgcgactggcgattacaga		gccttcaccggctgtaattgt			

<hr/>						
<i>IS711</i>						
real	IS421:	ISTq:	IS511:	178	421/438/511	This study
time	cgctcgcgcggtgat	FAM- acgaccaagctgcatgctgttgcgatg-	cttgaagcttgcggacagtcacc			
PCR		TAMRA				
<hr/>						
<i>bcp31</i>						
real	BCSP1163:	BCSPTq:	BCSP1199:	55	1163/1183/1199	This study
time	tctttgtggcgctatcc	FAM-acgggcgcaatct-MGB- NFQ	ccgttcgagatggccagt			
PCR						
<hr/>						
<i>per</i>						
real	Per525:	PerTq:	Per575:	68	525/552/575	This study
time	gttagtttctttgggaacaagacaa	FAM- tacgaccggtgaaggcgggatgMGB- NFQ	gaggattgcgcgctagca			
PCR						
<hr/>						

413 ^an.p.: no probe

TABLE 3. Comparison of C_T values in the real-time PCR assay

<i>Brucella</i> strain	biovar	IS711 C_T	<i>bcs</i> 31 C_T	<i>per</i> C_T	ΔC_T IS/ <i>bcs</i> p ^c	ΔC_T <i>bcs</i> p/ <i>per</i> ^d	IS711 copy number	Reference
<i>B. melitensis</i> 16M ^a	1	17.16	17.57	18.30	0.41	0.73	7	AE008917 / AE008918 ^f
<i>B. melitensis</i> 63/9 ^a	2	14.12	16.62	17.08	2.50	0.46	10	Ouahrani et al., 1993
<i>B. melitensis</i> Ether ^a	3	16.11	17.54	18.08	1.43	0.54	9	Ouahrani et al., 1993
<i>B. melitensis</i> Rev.1 ^a	1	15.87	17.31	17.96	1.44	0.65	n.d. ^e	n.d.
<i>B. melitensis</i> 53H38 ^a	1	13.93	15.87	16.66	1.94	0.79	n.d.	n.d.
<i>B. melitensis</i> 115 ^a	3	13.01	14.85	15.48	1.84	0.63	n.d.	n.d.
<i>B. abortus</i> 544 ^a	1	15	16.69	17.26	1.69	0.57	7	Ouahrani et al., 1993
<i>B. abortus</i> 86/8/59 ^a	2	13.19	15.22	15.78	2.02	0.56	7	Ouahrani et al., 1993
<i>B. abortus</i> Tulya ^a	3	13.66	14.87	15.84	1.20	0.97	6	Ouahrani et al., 1993

<i>B. abortus</i> AFSSA ^a	3	12.79	15.11	15.92	2.32	0.81	6	Ouahrani et al., 1993
<i>B. abortus</i> 292 ^a	4	14.04	15.14	15.95	1.10	0.81	7	Ouahrani et al., 1993
<i>B. abortus</i> B3196 ^a	5	14.19	15.13	15.97	0.95	0.84	7	Ouahrani et al., 1993
<i>B. abortus</i> 870 ^a	6	14.88	15.06	15.87	0.18	0.81	8	Ouahrani et al., 1993
<i>B. abortus</i> C68 ^a	9	12.59	14.73	15.71	2.14	0.98	6	Ouahrani et al., 1993
<i>B. abortus</i> B19 ^a	1	15.97	17.24	17.77	1.27	0.53	n.d.	n.d.
<i>B. abortus</i> S99 ^a	1	12.79	15.14	16.03	2.35	0.89	n.d.	n.d.
<i>B. suis</i> 1330 ^b	1	18.47	19.23	19.49	0.76	0.26	7	AE014291 / AE014292 ^f
<i>B. suis</i> Thomsen ^a	2	12.24	15.14	16.36	2.90	1.22	13	CP000911 / CP000912 ^f
<i>B. suis</i> 686 ^a	3	12.44	14.49	16.72	2.04	2.23	6	Ouahrani et al., 1993
<i>B. suis</i> 40 ^a	4	12.60	14.74	15.47	2.14	0.73	6	Ouahrani et al., 1993
<i>B. suis</i> 513 ^a	5	13.43	16.95	17.54	3.52	0.59	n.d.	n.d.

<i>B. neotomae</i> ^a	14.04	17.35	17.01	3.30	-0.34	8	Bricker et al., 2000
<i>B. ovis</i> 63/290 ^a	12.28	17.09	18.39	4.81	1.3	38	CP000708 / CP000709 ^f
<i>B. canis</i> RM6/66 ^a	16.02	17.06	17.63	1.04	0.57	6	CP000872 / CP000873 ^f
<i>B. pinnipedialis</i> ^a	12.06	16.50	17.46	4.44	0.96	> 25	Bricker et al., 2000
<i>B. ceti</i> ^b	13.25	18.96	19.21	5.71	0.25	> 25	Bricker et al., 2000

415

416 ^a C_T values were obtained from amplification of 20 ng of *Brucella* DNA; ^b C_T values were obtained from amplification of 2 ng of *Brucella* DNA;

417 ^c ΔC_T IS/bcsp is the difference between the IS711 C_T and the *bcs31* C_T ; ^d ΔC_T bcsp/per is the difference between the *bcs31* C_T and the *per* C_T ;

418 ^f Accession number from GeneBank; ^e not defined.

419

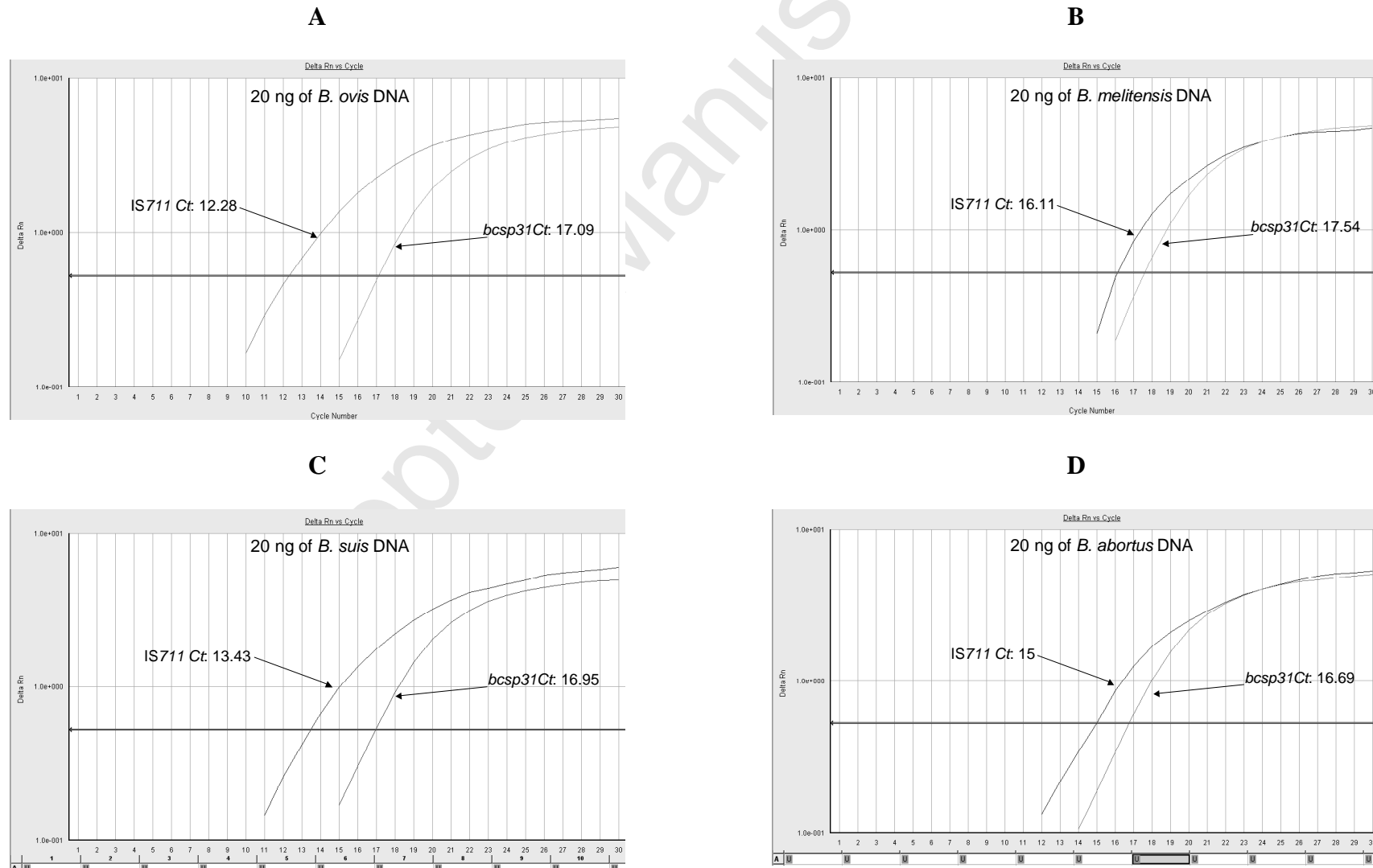
420

421

TABLE 4. Comparison of conventional and real-time PCR assays lower limit of detection (fg)

<i>Brucella</i>	IS711 copy number	Conventional PCR			Real-time PCR		
		IS711	<i>bcp31</i>	<i>per</i>	IS711	<i>bcp31</i>	<i>per</i>
<i>B. canis</i> RM6/66	6	100	1000	1000	2	20	20
<i>B. abortus</i> 544	7	100	1000	1000	2	2	2
<i>B. melitensis</i> 16M	7	1000	1000	1000	2	20	20
<i>B. ovis</i> 63/290	38	100	1000	1000	0.2	2	2

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19 **FIGURE 1.**

20 **FIGURE 2.**