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5 **Identification of novel DNA fragments and partial sequence of a genomic island specific**
6 **of *Brucella pinnipedialis***

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29 **Abstract**

30 Since the 1990s, *Brucella* strains have been isolated from a wide variety of marine
31 mammals and were recently recognized as two different species i.e. *B. pinnipedialis* for
32 pinniped isolates and *B. ceti* for cetacean isolates. The aim of this study was to identify
33 specific DNA fragments of marine mammal *Brucella* strains using a previously described
34 Infrequent Restriction Site-PCR (IRS-PCR) method but with three new couples of restriction
35 enzymes applied on a larger panel of marine mammal *Brucella* isolates (n=74) and one human
36 isolate from New Zealand likely from marine mammal origin. This study revealed five DNA
37 fragments specific of *Brucella* strains isolated from marine mammals. Among them two new
38 DNA fragments were specific of *B. pinnipedialis* but were not detected in hooded seal
39 isolates. DNA fragment I identified in the previous IRS-PCR study and fragment VI of this
40 study were located on a cloned and sequenced 6 kb *SacI* fragment. Its nucleotide sequence
41 revealed that it is likely part of a putative genomic island. Sequence analysis showed that it
42 carries four ORFs coding for putative metabolic functions. Although hooded seal isolates are
43 classified within *B. pinnipedialis* it was shown in this study that they do not carry this
44 genomic island and this raises the question about their evolutionary history within *B.*
45 *pinnipedialis*.

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54 1. Introduction

55 Brucellae are Gram-negative, facultatively, intracellular bacteria that can infect many
56 species of animals and man. Six species were initially recognized within the genus *Brucella*:
57 *B. abortus*, *B. melitensis*, *B. suis*, *B. ovis*, *B. canis*, and *B. neotomae* (Corbel et al., 1984;
58 Moreno et al., 2002). This classification is mainly based on differences in pathogenicity, host
59 preference, and phenotypic characteristics (Alton et al., 1998; Corbel et al., 1984; Moreno et
60 al., 2002). Three additional species have been recently included in the genus *Brucella* i.e. *B.*
61 *ceti* and *B. pinnipedialis* isolated from marine mammals, with cetaceans (dolphin, porpoise,
62 and whale species) and pinnipeds (various seal species) as preferred host respectively, and *B.*
63 *microti* isolated from the common vole (Foster et al., 2007; Scholz et al., 2008).

64 At the molecular level, evidence for two marine mammal *Brucella* species i.e. *B. ceti*
65 and *B. pinnipedialis* has been firstly provided by study of DNA polymorphism at the *omp2*
66 locus (Cloekaert et al., 2001). This was further confirmed by an infrequent restriction site-
67 PCR (IRS-PCR) method, taking into account the higher number of IS711 elements in the
68 genome of marine mammal isolates compared to terrestrial mammal *Brucella* species (Bricker
69 et al., 2000; Clavareau et al., 1998; Cloekaert et al., 2003). IRS-PCR revealed four specific
70 DNA fragments useful for the detection and identification of marine mammal *Brucella*
71 isolates (Cloekaert et al., 2003). Interestingly to date three human cases with *Brucella*
72 infections presumably of marine mammal origin, according to specific molecular markers
73 cited above, have been published and may point towards a zoonotic potential of these marine
74 mammal *Brucella* species (Sohn et al., 2003; McDonald et al., 2006).

75 In the present study we used IRS-PCR with three new couples of restriction enzymes
76 on a larger panel of marine mammal *Brucella* isolates (n=74) to identify novel DNA
77 fragments specific of these marine mammal isolates.

78

79 2. Materials and methods

80 2.1. Bacterial strains

81 The marine mammal *Brucella* strains studied are listed in Table 1. Most isolates were
82 from individual stranded dead animals over the period from 1994 to 2005. Isolates from
83 hooded seals in Norway were from apparently healthy animals and caught in their natural
84 habitat (Tryland et al., 2005). Terrestrial mammal *Brucella* strains used in this study were
85 those of the previous IRS-PCR study (Cloeckeaert et al., 2003). Culture conditions of these
86 strains were those described previously by Cloeckeaert et al. (2003).

87

88 2.2. IRS-PCR

89 IRS-PCR was performed as described previously by Cloeckeaert et al. (2003) with
90 some modification, using the previous couple of restriction enzymes *PstI/SalI* and three new
91 couples of restriction enzymes: *BglII/SalI*, *BamHI/SalI*, *EcoRI/SalI*.

92 One microgram of DNA was digested with 10 U of *PstI* (or *EcoRI*, *BglII*, *BamHI*)
93 (Promega) and 10 U of *SalI* (Promega) in 1x buffer H (Promega) for 2 h 30 min at 37 °C in a
94 volume of 15 µl. Sterile distilled water, 2.5 U of T4 DNA ligase (Promega), 1x ligase buffer
95 (Promega), the *PstI* adapter (or *BglII* adapter, *BamHI* adapter, *EcoRI* adapter) (20 pmol) and
96 the *SalI* adapter (20 pmol) were added for a total volume of 25 µl. The mixture was incubated
97 at 16 °C for 2 h and then at 60 °C for 20 min to inactivate T4 DNA ligase. The sample was
98 redigested with 5 U of *PstI* (or *BglII* or *BamHI* or *EcoRI*) and 5 U of *SalI* at 37 °C for 30 min
99 to cleave any restriction sites reformed by ligation, and then was submitted to amplification.
100 Each PCR mixture included 2.5 µl of a 1/10 dilution of template DNA, 0.5 U of *GoTaq* DNA
101 polymerase (Promega), deoxynucleoside triphosphates (200 µM each) (Promega), and the
102 oligonucleotide primers in 1x PCR buffer (Promega). Typically, the oligonucleotides *Ps1* (or

103 BG1) and either PsaIA, PsaIC, PsaIG, or PsaIT were used together as primers. Primer
104 sequences are indicated in Table 2. Amplification was performed in an iCycler thermocycler
105 (BioRad) with an amplification profile that consisted of an initial denaturation step at 94 °C
106 for 5 min, five cycles of denaturation at 94 °C for 30 s, primer annealing at 55 °C for 1 min,
107 and extension at 72 °C for 90 s, and then 30 cycles of denaturation at 94 °C for 30 s, primer
108 annealing at 60 °C for 30 s, and extension at 72 °C for 90 s. All experiments included
109 negative controls which were processed with the samples. The IRS-PCR reaction products
110 were run on 2% (w/v) agarose gels containing 0.5 µg of ethidium bromide per ml.

111 2.3. Specific PCRs

112 Specific IRS-PCR fragments (V, VI) of the marine mammal *Brucella* isolates were
113 gel-purified and their nucleotide sequence was determined to design specific primers. The
114 primers used for specific PCRs of DNA fragments V and VI are listed in Table 2. Moreover,
115 specific PCRs for DNA fragments I, II, III, and IV were performed on all the marine mammal
116 strains of this study. PCR was performed on extracted DNAs as described previously. Briefly,
117 amplification reaction mixtures were prepared in volumes of 100 µl containing 10 mM Tris-
118 HCl (pH 9.0), 50 mM KCl, 1.5 mM MgCl₂, 0.1% Triton X-100 (1 x PCR buffer; Promega), a
119 200 µM concentration of each deoxynucleoside triphosphate, a 1 µM concentration of each
120 primer, 100 ng of genomic DNA, and 5 U of *Taq* DNA polymerase (Promega). The
121 temperature cycling for the amplification was performed in an iCycler thermocycler (BioRad)
122 as follows: cycle 1 was 94 °C for 5 min (denaturation); the next 30 cycles were 62 °C for 30 s
123 (annealing), 70 °C for 30 s (extension), and 94 °C for 30 s (denaturation); and the last cycle
124 was 62 °C for 30 s (annealing) and 70 °C for 10 min (extension). The PCR products were run
125 on 0.8% (w/v) agarose gels containing 0.5 µg of ethidium bromide per ml.

126

127 2.4. Cloning and partial nucleotide sequence of the putative *B. pinnipedialis* genomic island

128 PCR was performed with primers used to amplify fragment I as described previously
129 (Cloeckeaert et al., 2003). The PCR product of 319 bp was purified using the QIAquick PCR
130 purification Kit (Qiagen™). This probe was then labelled with the NEBlot-Phototope kit
131 (Biolabs™), following the manufacturer's protocol.

132 A *B. pinnipedialis* strain B2/94 (reference strain) genomic library was constructed in
133 lambdaGEM-11 *Bam*HI half-site arms (Promega, Madison, Wis.) by following the
134 instructions of the manufacturer. Briefly, *B. pinnipedialis* B2/94 DNA, extracted and purified
135 as described previously by Cloeckeaert et al. (2003), was partially digested for 30 min at 37 °C
136 with *Sau*3AI (Promega) at 0.025 U/μg of DNA, the enzyme concentration giving the highest
137 percentage of fragments ranging from 15 to 23 kb. DNA fragments were ligated with T4
138 DNA ligase (Promega) to lambdaGEM-11 *Bam*HI half-site arms. Recombinant phage DNA
139 was packaged in vitro with the Packagene System (Promega), and the library was titrated by
140 determination of the number of PFU that appeared after infection of *E. coli* KW251 cells
141 (Promega). Recombinant phages were transferred to nitrocellulose filters, and phages were
142 screened by hybridization of the lysis plaques with the probe described above. DNA of a
143 positive phage was extracted from culture supernatants of *E. coli* KW251 cells infected with
144 the phage and cultured until lysis was observed. Phage DNA was then cut with *Sac*I, and
145 restriction fragments were ligated into pGEM-7Zf+ (Promega) cut with *Sac*I. Competent *E.*
146 *coli* JM109 cells (Promega) were transformed with recombinant plasmid DNA as described
147 previously (Vizcaíno *et al.*, 1996), and bacteria were spread on Luria-Bertani (LB) broth-
148 ampicillin (50 μg/ml) plates containing isopropyl-1-thio-β-D-galactopyranoside (IPTG) and
149 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal). *E. coli* JM109 colonies bearing
150 recombinant plasmids were transferred to nitrocellulose, lysed with 10% sodium dodecyl
151 sulfate (SDS), and screened with the specific probe mentioned above. One positive colony
152 was found bearing a plasmid with a *Sac*I insert of about 6 kb. This plasmid was named

153 pMZ4501. The *SacI* insert was sequenced by the GENOME express™ society (Meylan,
154 France).

155

156 2.5. Nucleotide accession numbers

157 The nucleotide sequence of the novel *B. pinnipedialis* DNA fragment V and the partial
158 sequence of the *B. pinnipedialis* genomic island have been deposited in GenBank under
159 accession numbers EU373600 and AY174073 respectively.

160

161 3. Results and discussion

162 3.1. Identification of specific DNA fragments of *B. pinnipedialis*

163 IRS-PCR was performed on 18 terrestrial mammal reference (species and biovar)
164 strains and 74 marine mammal strains and one human isolate from New Zealand likely from
165 marine mammal origin (McDonald et al., 2006). Three new couples of restriction enzymes
166 were tested relative to the previous IRS-PCR study: *BglII/SalI*, *BamHI/SalI*, *EcoRI/SalI*. The
167 screening of 50 new marine mammal isolates and the use of these different couples of
168 restriction enzymes revealed five DNA fragments specific to *Brucella* isolated from marine
169 mammals (Fig 1). Nucleotide sequencing of the fragment identified by the couple of
170 restriction enzymes *SalI/EcoRI* with the primers PSalG and BG1 corresponded to fragment II
171 with portion of IS711 identified in the previous IRS-PCR study (Fig. 1) (Cloeckert et al.,
172 2003). Furthermore, the fragment identified by the couple of restriction enzymes *SalI/BglII*
173 with primers PSalA and BG1 corresponded to fragment III of this previous IRS-PCR study.
174 The other fragment identified by the couple of restriction enzymes *SalI/BglII* with the primers
175 PSalG and BG1, revealed that it corresponded to the *B. ceti* B1/94 *bp26* gene with portion of
176 IS711 found downstream of *bp26* (AF242533) (Cloeckert et al., 2000). Fragments II and III
177 corresponded to part of genes encoding the *B. ceti* D-β-hydroxybutyrate dehydrogenase

178 (AY174070) and the *B. ceti* cytochrome B561 like protein (AY174071) respectively. Thus in
179 the present IRS-PCR study, we confirmed two fragments previously identified, containing
180 part of the IS711 element.

181 The two other DNA fragments, identified respectively by the restriction enzymes
182 *SalI/BamHI* with primers PSaIG and BG1 and by the restriction enzymes *SalI/PstI* with
183 primers PSaIC and PS1, of 305 bp and 780 bp were specific of *B. pinnipedialis* strains
184 identified in this study and were named V and VI respectively. Nucleotide sequencing of
185 fragment V revealed nucleotide and amino acid sequence identity of 96 % with an
186 hypothetical protein from *Ochrobactrum anthropi* (GenBank accession number
187 YP_001371022). Nucleotide sequencing of fragment VI revealed a deduced amino acid
188 sequence with an amino acid sequence identity of 76 % to an aldehyde dehydrogenase from
189 other *Brucella* species (GenBank accession number YP_001257642). Specific PCRs were
190 performed with primers designed on the basis of nucleotide sequences of fragments V and VI.
191 Like for the IRS-PCR results these DNA fragments were confirmed to be absent in all
192 terrestrial mammal and cetacean strains studied (Table 1). Interestingly both fragments were
193 shown to be present in all pinniped (seal and otter) isolates except in hooded seal isolates
194 (Table 1). Nevertheless, hooded seal isolates belong likely also to *B. pinnipedialis* according
195 to other characteristics like their oxidative metabolism profiles and their *omp2a* and *omp2b*
196 gene characteristics (Jacques et al., 2007; Tryland et al., 2005). Fragments V and VI were also
197 confirmed to be present in the human isolate from New Zealand.

198

199 3.2. Identification of a putative genomic island specific of *B. pinnipedialis*

200 A 5853 bp *SacI* fragment comprising the *B. pinnipedialis*-specific fragment I was
201 cloned and sequenced. A search in databases using blastn revealed that this sequence was
202 novel. Nucleotide sequencing revealed that its overall G+C content was lower i.e. 53 % than

203 the *Brucella* overall G+C content which is 57.3 % (Lavigne et al., 2005). The lower G+C
204 content and the fact that this DNA fragment is absent in all other *Brucella* species including
205 four *Brucella* species for which the genome sequences are available (*B. abortus*, *B. melitensis*,
206 *B. suis*, and *B. ovis*) indicates that it is likely part of a genomic island. Four ORFs were
207 identified in this fragment (Fig 1C). ORF1 (G+C content of 51.7 %) codes for a putative
208 membrane protein with 35 % amino acid sequence identity to that of *Oceanicola batsensis*
209 HTCC2597 (GenBank accession number EAQ04147.1), ORF2 (G+C content of 53 %) codes
210 for a putative enoyl-CoA hydratase with 46 % amino acid sequence identity to that of
211 *Burkholderia xenovorans* LB400 (GenBank accession number ABE36201.1), ORF3 (G+C
212 content of 54.5 %) codes for a putative aldehyde dehydrogenase highly similar to that of other
213 *Brucella* species and *Ochrobactrum anthropi* (73 % amino acid sequence identity; GenBank
214 accession number AAX75972.1), and ORF4 (G+C content of 54 %) codes for a
215 deoxycytidylate deaminase-related protein from *Stigmatella aurantiaca* (33 % amino acid
216 sequence identity; GenBank accession number EAU69662). The genes were named *bpiM*,
217 *bpiE*, *bpiA*, and *bpiD* respectively (Fig. 1C).

218 Among the two novel IRS-PCR DNA fragments identified for *B. pinnipedialis*,
219 fragment VI was identified in the almost 6 kb nucleotide sequence as part of *bpiA*. Fragment
220 V was not found within this sequence. However, the fact that strains positive for fragments I
221 and VI are also positive for fragment V, and an identical size of fragments obtained by
222 Southern blot of *Xba*I digested DNA with probes of fragment I and V (data not shown),
223 suggest that fragment V could also be part of this genomic island.

224 *Brucella* genome sequencing have revealed the existence of several genomic islands
225 ranging from 3.8 to 44.1 kb in size. Thus, nine genomic islands, called GI-1 to GI-9, have
226 been identified to date in the *Brucella* sequenced genomes (Paulsen et al., 2002; Rajashekara
227 et al., 2004; Vizcaino et al., 2004). All genomic islands display a different G+C content to the

228 overall G+C content of *Brucella* species. Most of them were likely acquired by horizontal
229 transfer (Rajashekara et al., 2004). According to the nucleotide sequence the *B. pinnipedialis*-
230 genomic island could be a metabolic island but further sequencing is required to identify the
231 putative functions of this island and also to identify more specific features of genomic islands
232 like presence of genes involved in integration, excision, and transfer. This will be reached
233 with the genome sequencing of the *B. pinnipedialis* reference strain which is underway.

234 Although hooded seal isolates are considered as *B. pinnipedialis* strains, they do not
235 carry this genomic island. Possibly acquisition of this genomic island in *B. pinnipedialis* may
236 be posterior to separation between hooded seal isolates and other seal isolates and therefore
237 hooded seal isolates could form an ancestor inside the *B. pinnipedialis* species. This
238 hypothesis is currently being investigated in our laboratory by analyzing IS711 distribution
239 and *omp2a* and *omp2b* gene diversity. Preliminary results indicate that hooded seal isolates
240 carry less IS711 elements in their genomes than the *B. pinnipedialis* reference strain
241 (unpublished results). There are also less *omp2a*-specific nucleotides at the 3' end of their
242 *omp2b* genes compared to that of the *B. pinnipedialis* reference strain indicative of a lesser
243 extent of *omp2b* gene conversion to *omp2a* (unpublished results). These preliminary data
244 conforsts the hypothesis of hooded seal isolates being ancestors inside the *B. pinnipedialis*
245 species.

246

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249 strains of marine mammal origin.

250

251

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- 317
- 318

319 **FIGURE LEGENDS**

320 Fig 1. A. IRS-PCR patterns of *Brucella* strains identifying the five DNA fragments specific of
321 marine mammal *Brucella* strains. Lane A, IRS-PCR pattern of *B. ceti* strain B1/94 obtained
322 with restriction enzymes *SalI/EcoRI* and primers PsaG and BG1; lane B, IRS-PCR pattern of
323 *B. ceti* strain B1/94 obtained with restriction enzymes *SalI/BglIII* and primers PsaA and BG1;
324 lane C, IRS-PCR pattern of *B. ceti* strain B1/94 obtained with restriction enzymes *SalI/BglIII*
325 and primers PsaG and BG1; lane D, IRS-PCR pattern of *B. pinnipedialis* strain B2/94
326 obtained with restriction enzymes *SalI/PstI* and primers PsaC and PS1; lane E, IRS-PCR
327 pattern of *B. pinnipedialis* strain B2/94 obtained with restriction enzymes *SalI/BamHI* and
328 primers PsaG and BG1. Lane M: molecular mass marker. The specific DNA fragments 1, 2,
329 3, 4 and 5 for which the nucleotide sequence has been determined are indicated by arrows.

330 B. Schematic representation of the nucleotide sequences of specific DNA fragments 1, 2, 3, 4
331 and 5 from marine mammal *Brucella* isolates identified in this study with the new couples of
332 restriction enzymes. Arrows indicate location of the primers used for specific PCRs II, III, V
333 and VI (see also Table 2). BMEI0268 and BMEII1073 are annotations according to the
334 sequenced genome of *B. melitensis* 16M and correspond respectively to D- β -hydroxyburate
335 dehydrogenase and cytochrome B561 genes (GenBank accession numbers NC 003317 and
336 NC 003318).

337 C. Schematic view and annotation of the 5853 bp partial sequence of the *B. pinnipedialis*-
338 specific genomic island according to homology in databases. Plain arrow represents a gene
339 coding for a putative membrane protein; Striped arrows represent genes coding for putative
340 enzymatic proteins. BpiM: putative membrane protein; BpiE: putative enoyl-CoA hydratase;
341 BpiA: putative aldehyde dehydrogenase; BpiD: deoxycytidylate deaminase related-protein.

Table 1. Marine mammal *Brucella* strains studied

Species	Strain	Host or source	Latin name	Geographic origin	Specific PCRs ^a						
					I	II	III	IV	V	VI	
<i>B. pinnipedialis</i>	B2/94 ^b	Common seal	<i>Phoca vitulina</i>	Scotland	+	-	-	-	+	+	
	M2466/93/4	Common seal	<i>Phoca vitulina</i>	Scotland	+	-	-	-	+	+	
	M445/99/2	Common seal	<i>Phoca vitulina</i>	Scotland	+	-	-	-	+	+	
	M13/01/1	Common seal	<i>Phoca vitulina</i>	Scotland	+	-	-	-	+	+	
	M292/94/1	Common seal	<i>Phoca vitulina</i>	Scotland	+	-	-	-	+	+	
	M336/94/1	Common seal	<i>Phoca vitulina</i>	Scotland	+	-	-	-	+	+	
	M339/94/2	Common seal	<i>Phoca vitulina</i>	Scotland	+	-	-	-	+	+	
	M972/94/1	Common seal	<i>Phoca vitulina</i>	Scotland	+	-	-	-	+	+	
	M514/96/4	Common seal	<i>Phoca vitulina</i>	Scotland	+	-	-	-	+	+	
	M449/02/2	Common seal	<i>Phoca vitulina</i>	Scotland	+	-	-	-	+	+	
	M621/99/2	Grey seal	<i>Halichoerus grypus</i>	Scotland	+	-	-	-	+	+	
	M194/00/1	Grey seal	<i>Halichoerus grypus</i>	Scotland	+	-	-	-	+	+	
	M2375/94/3	Grey seal	<i>Halichoerus grypus</i>	Scotland	+	-	-	-	+	+	
	M89/03/05	Porpoise	<i>Phocoena phocoena</i>	Scotland	+	-	-	-	+	+	
	M192/00/1	Minke whale	<i>Balaenoptera acutorostrata</i>	Scotland	+	-	-	-	+	+	
	M1771/94/1	Otter	<i>Lutra lutra</i>	Scotland	+	-	-	-	+	+	
	22a-2	Hooded seal	<i>Cystophora cristata</i>	Norway	-	-	-	-	-	-	
	23a-1	Hooded seal	<i>Cystophora cristata</i>	Norway	-	-	-	-	-	-	
	24a-2	Hooded seal	<i>Cystophora cristata</i>	Norway	-	-	-	-	-	-	
	25a-1	Hooded seal	<i>Cystophora cristata</i>	Norway	-	-	-	-	-	-	
	30a-1	Hooded seal	<i>Cystophora cristata</i>	Norway	-	-	-	-	-	-	
	17a-1	Hooded seal	<i>Cystophora cristata</i>	Norway	-	-	-	-	-	-	
	37a-1	Hooded seal	<i>Cystophora cristata</i>	Norway	-	-	-	-	-	-	
	38g-1	Hooded seal	<i>Cystophora cristata</i>	Norway	-	-	-	-	-	-	
	39a-1	Hooded seal	<i>Cystophora cristata</i>	Norway	-	-	-	-	-	-	
	53c-1	Hooded seal	<i>Cystophora cristata</i>	Norway	-	-	-	-	-	-	
	M163/99/10	Hooded seal	<i>Cystophora cristata</i>	Scotland	-	-	-	-	-	-	
	M603/99/7	Hooded seal	<i>Cystophora cristata</i>	Scotland	-	-	-	-	-	-	
	02/611	Human	<i>Homo sapiens</i>	New Zealand	+	-	-	-	+	+	
	<i>B. ceti</i>	B1/94 ^b	Porpoise	<i>Phocoena phocoena</i>	Scotland	-	+	+	-	-	-
		M1570/94/1	Porpoise	<i>Phocoena phocoena</i>	Scotland	-	+	+	-	-	-
		M1661/94/2	Porpoise	<i>Phocoena phocoena</i>	Scotland	-	+	+	-	-	-
		M39/94/1	Porpoise	<i>Phocoena phocoena</i>	Scotland	-	+	+	-	-	-
M854/98/8		Porpoise	<i>Phocoena phocoena</i>	Scotland	-	+	+	-	-	-	
M1747/98/3		Porpoise	<i>Phocoena phocoena</i>	Scotland	-	+	+	-	-	-	
M499/99/10		Porpoise	<i>Phocoena phocoena</i>	Scotland	-	+	+	-	-	-	
M12/00/3		Porpoise	<i>Phocoena phocoena</i>	Scotland	-	+	+	-	-	-	
M23/03/4		Porpoise	<i>Phocoena phocoena</i>	Scotland	-	+	+	-	-	-	
M165/03/6		Porpoise	<i>Phocoena phocoena</i>	Scotland	-	+	+	-	-	-	
M78/05/2		Porpoise	<i>Phocoena phocoena</i>	Scotland	-	+	+	-	-	-	
M40/05/1		Porpoise	<i>Phocoena phocoena</i>	Scotland	-	+	+	-	-	-	
M291/03/2		Porpoise	<i>Phocoena phocoena</i>	Scotland	-	+	+	-	-	-	
M93/04/3		Porpoise	<i>Phocoena phocoena</i>	Scotland	-	+	+	-	-	-	
M234/05/2		Porpoise	<i>Phocoena phocoena</i>	Scotland	-	+	+	-	-	-	
M199/04/2		Porpoise	<i>Phocoena phocoena</i>	Scotland	-	+	+	-	-	-	
M58/05/1		Porpoise	<i>Phocoena phocoena</i>	Scotland	-	+	+	-	-	-	
M38/04/3		Porpoise	<i>Phocoena phocoena</i>	Scotland	-	+	+	-	-	-	
M51/04/2		Porpoise	<i>Phocoena phocoena</i>	Scotland	-	+	+	-	-	-	
M493/99/1		Porpoise	<i>Phocoena phocoena</i>	Scotland	-	+	+	-	-	-	
M103/99/1		Porpoise	<i>Phocoena phocoena</i>	Scotland	-	+	+	-	-	-	
M615/99/2		Porpoise	<i>Phocoena phocoena</i>	Scotland	-	+	+	-	-	-	

M195/03/10	Porpoise	<i>Phocoena phocoena</i>	Scotland	-	+	+	-	-	-
7763/2	Bottlenose dolphin	<i>Tursiops truncatus</i>	France	-	+	+	-	-	-
M452/97/2	Common dolphin	<i>Delphinus delphis</i>	Scotland	-	+	+	-	-	-
M870/97/1	White-beaked dolphin	<i>Lagenorhynchus albirostris</i>	Scotland	-	+	+	-	-	-
M997/94/2	White-sided dolphin	<i>Lagenorhynchus acutus</i>	Scotland	-	+	+	-	-	-
M2438/95/1	White-sided dolphin	<i>Lagenorhynchus acutus</i>	Scotland	-	+	+	-	-	-
M2788/97/1	White-sided dolphin	<i>Lagenorhynchus acutus</i>	Scotland	-	+	+	-	-	-
M181/97/1	White-sided dolphin	<i>Lagenorhynchus acutus</i>	Scotland	-	+	+	-	-	-
M2/00/1	White-sided dolphin	<i>Lagenorhynchus acutus</i>	Scotland	-	+	+	-	-	-
M187/00/1	White-sided dolphin	<i>Lagenorhynchus acutus</i>	Scotland	-	+	+	-	-	-
M52/01/1	White-sided dolphin	<i>Lagenorhynchus acutus</i>	Scotland	-	+	+	-	-	-
B202R	Minke whale	<i>Balaenoptera acutorostrata</i>	Norway	-	+	+	-	-	-
M490/95/1	Common seal	<i>Phoca vitulina</i>	Scotland	-	+	+	-	-	-
B14/94	Common dolphin	<i>Delphinus delphis</i>	Scotland	-	-	-	+	-	-
M644/93/1	Common dolphin	<i>Delphinus delphis</i>	Scotland	-	-	-	+	-	-
M9/02/01	Striped dolphin	<i>Stenella coeruleoalba</i>	Scotland	-	-	-	+	-	-
M13/05/1	Striped dolphin	<i>Stenella coeruleoalba</i>	Scotland	-	-	-	+	-	-
M40/95/1	Striped dolphin	<i>Stenella coeruleoalba</i>	Scotland	-	-	-	+	-	-
M2194/94/1	Striped dolphin	<i>Stenella coeruleoalba</i>	Scotland	-	-	-	+	-	-
M642/99/2	Striped dolphin	<i>Stenella coeruleoalba</i>	Scotland	-	-	-	+	-	-
M656/99/1	Striped dolphin	<i>Stenella coeruleoalba</i>	Scotland	-	-	-	+	-	-
M22/02/01	Striped dolphin	<i>Stenella coeruleoalba</i>	Scotland	-	-	-	+	-	-
M654/99/1	Striped dolphin	<i>Stenella coeruleoalba</i>	Scotland	-	-	-	+	-	-
M61/05/1	White-sided dolphin	<i>Lagenorhynchus acutus</i>	Scotland	-	-	-	+	-	-

^a PCRs using specific primers designed from the nucleotide sequences of IRS-PCR fragments I, II, III, IV (Cloekaert et al., 2003), V and VI (this study) specific to marine mammal *Brucella* isolates (see Fig. 1).

^b Reference strain.

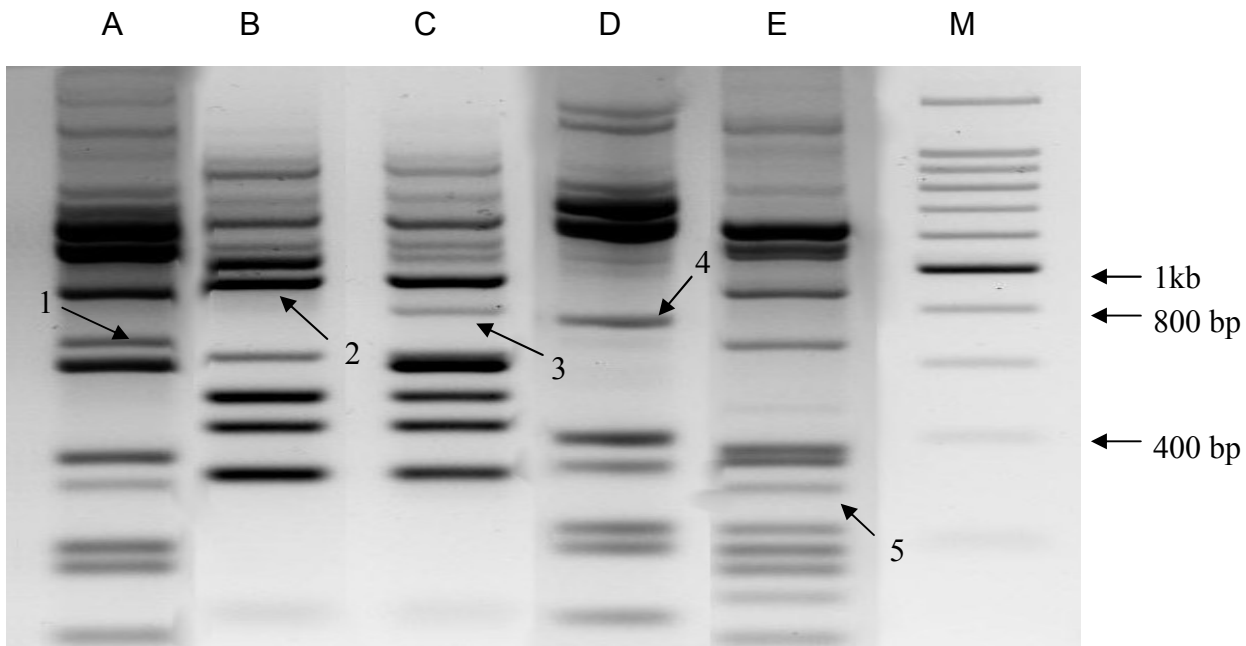
Table 2. Primers and adapters used in this study

Primers	Restriction enzyme	Adapter sequence (5' to 3')	Oligonucleotide sequence (5' to 3')	Annealing temp (°C)
Primers and adapters for IRS-PCR				
	<i>SalI</i>	Sal1: PO ₄ -TCGATACTGGCAGACTCT AX2: GCCAGTA	Pas1A: AGAGTCTGCCAGTATCGACA Psa1T: AGAGTCTGCCAGTATCGACT Psa1C: AGAGTCTGCCAGTATCGACC Psa1G: AGAGTCTGCCAGTATCGACG	55 55 55 55
	<i>PstI</i>	PS1: GACTCGACTCGCATGCA AH2: TCGGAGT	PS1: GACTCGACTCGCATGCA	55
	<i>EcoRI</i>	BG1: GACTCGACTCGCA ER2: AATTTGCGAGT	BG1: GACTCGACTCGCA	55
	<i>BglII/BamHI*</i>	BG1: GACTCGACTCGCA BG2: GATCTGCGAGT	BG1: GACTCGACTCGCA	55
Primers for specific PCRs				
Fragment I			I1: AGGTAICTTTCATTTCGTTCCGG I2: GCGACTCAGATGATCCCACC	58 58
Fragment II			II1: GCGGCTGGACGGCAGAATAA II2: AATGGACAGCGGTTTCATGCC	58 58
Fragment III			III1: GCAAATCCCAGTTGGAAATG III2: TGCTCATCTGTAAGGCTTCG	58 58
Fragment IV			IV1: GCTGGAGGATTTCCTTCTTG IV2: CGGAAGATATGCTTCGATCC	58 58
Fragment V			V1: AGGGGCTGGATGGCTTGTGTT V2: CGGATAGAGAAGATGCGGTC	58 58
Fragment VI			VI1: TCCAGCTCTGAAGGAGATC VI2: CAGATCCGGTTTGGACATAA	58 58

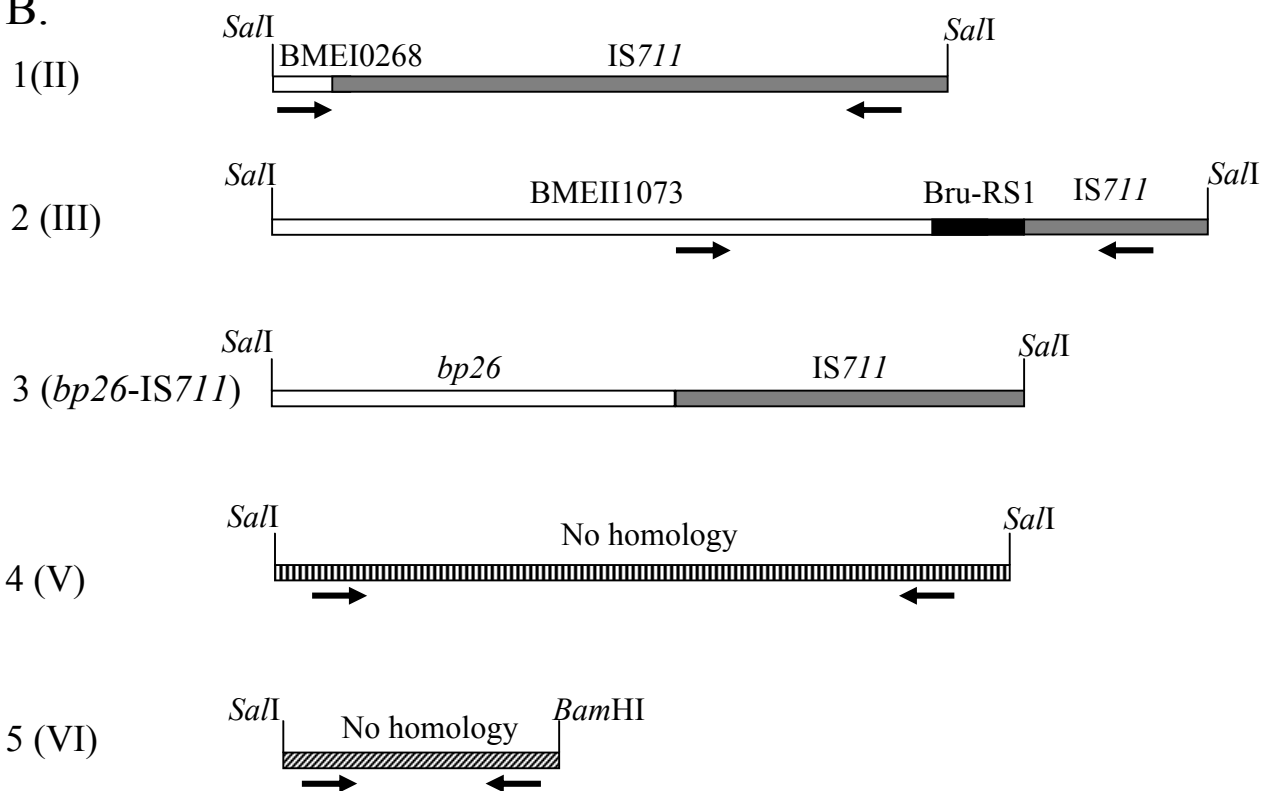
* Adapter and primer sequences are the same for both enzymes *BglII* and *BamHI*.

Fig. 1

A.



B.



C.

