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Identification of *Brucella* spp. by Using the Polymerase Chain Reaction

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The application of two synthetic oligonucleotides as probes and as primers in the polymerase chain reaction is presented for a specific, sensitive, and quick identification of *Brucella* spp. The specific oligonucleotide sequences were chosen on the basis of a 16S rRNA sequence alignment between *Brucella abortus* and *Agrobacterium tumefaciens*.

Brucellosis is a very widespread and economically important zoonosis which is also infectious for humans (9). The genus *Brucella*, consisting of gram-negative bacteria, forms together with *Ochrobactrum anthropi* and *Phyllobacterium* an rRNA cluster, closely related to the *Agrobacterium-Rhizobium* cluster (1). For the identification of *Brucella* spp., mainly bacteriological and immunological detection techniques are being used. These tests are time-consuming and often give false-positive results. Quick and reliable identification methods are being developed, mainly based on DNA hybridization. The automation of the polymerase chain reaction (PCR) renders this technique very promising for bacterial identification (8). A diagnostic test for detecting *Brucella* spp. in cattle was developed by using a 43-kDa outer membrane protein gene of *Brucella abortus* as a target for the PCR (3). The specificity of the PCR method was tested on microorganisms associated with cattle. Because diagnostic methods using rRNA sequences as a target have been shown to be very reliable for different kinds of samples, we apply two primers from the 16S rRNA of *B. abortus* in the PCR.

The 16S rRNA sequence of *B. abortus* (2) was aligned with those of *Agrobacterium tumefaciens*, *Escherichia coli*, *Bacillus brevis*, and *Pseudomonas testosteroni*. Of these bacterial species, *A. tumefaciens* is most closely related to *B. abortus* with a 16S rRNA homology of 85%. The sequence differences are mainly clustered in two domains from which two sequences, Ba148-167F (5'TGCTAATACCGTATGTG CTT3') and Ba928-948R (5'TAACCGCGACCGGGATGTC AA3'), were chosen (F, forward primer; R, reverse primer). The alignment of the two sequences is shown in Table 1. Ba148-167F and Ba928-948R differ, respectively, 12 and 8 nucleotides from the corresponding *Agrobacterium tumefaciens* sequence.

Both oligonucleotides were tested for specific hybridization with the different *Brucella* species and its relatives (Table 2) by the dot blot hybridization method (Bio-Rad). The hybridization was carried out in a 3× SSC-containing solution (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) with a 5' ^{32}P-labelled probe (7). The bacterial chromosomal DNA was prepared by using the method of Flamm et al. (4). Both probes hybridize specifically with the different *Brucella* species when a hybridization temperature of 45°C was followed by a washing temperature of 50°C. It is not unexpected that both oligonucleotides, though they are derived from variable regions of the rRNA sequence, are able to recognize all the *Brucella* species. The genus *Brucella* forms a very tight cluster, with RNA homologies close to 100% (1). The species tested from the taxonomic neighborhood of *Brucella*:*Ochrobactrum anthropi*, previously known as CDC group Vd (5), the genus *Phyllobacterium* and members of the *Agrobacterium-Rhizobium* cluster, do not hybridize with both probes. Only for the Ba148-167F probe is a very weak signal obtained with the *Ochrobactrum anthropi* DNA. This signal is not unexpected because *Ochrobactrum anthropi* is the closest neighbor of *Brucella* spp. We can assume that when the genetically very closely related species do not hybridize, the other bacterial species will not hybridize either. These results lead to the conclusion that both probes Ba148-167F and Ba928-948R are useful for specific identification of *Brucella* spp.

The oligonucleotides Ba148-167F and Ba928-948R were applied as primers in the PCR. The PCR was performed on DNA extracts or crude cell lysates by the standard protocol

TABLE 1. Alignment of primer sequences Ba148-167F and Ba928-948R, derived from the 16S rRNA sequence of *B. abortus*

Organism	Ba148-167F (5' → 3')	Ba928-948R (5' → 3')
Brucella abortus	TGCTAATACCG TATGTGCTT	TTGACATCCCGGTCGCGGTTA
Agrobacterium tumefaciens	AATTAATACCG CATACGCCCTAC	TTGACATTCGGGGTTTGGGCA
Escherichia coli	AGCTAATACCG CATAACGTCGCA	TTGACAT CCACGGAAGTTT
Bacillus brevis	TGCTAATACCGGATAGGTTTTTGGATTGCAT GA	TTGACAT CCCGCTGACCG
Pseudomonas testosteroni	AGCTAATACCG CATGAGATCTAC	TTGACAT GGCAGGAACTTA

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TABLE 2. Origin and identity of bacterial strains used

Species	Biovar	Strain	Source[a]	PCR outcome[b]
Brucella abortus	3	Tulya	NIDO	+
Brucella abortus	1	B19	NIDO	+
Brucella abortus	9	AF 268	NIDO	+
Brucella abortus	9	AF 270/3	NIDO	+
Brucella melitensis	1		NIDO	+
Brucella melitensis	3	Ether	NIDO	+
Brucella suis	1		NIDO	+
Brucella ovis		1051	NCTC	+
Brucella canis		10854	NCTC	+
Brucella neotomae		10084	NCTC	+
Ochrobactrum anthropi		3331	LMG	(−)
Phyllobacterium rubiacearum		1 (t1)	LMG	−
Agrobacterium tumefaciens		188	LMG	−
Agrobacterium rhizogenes		152	LMG	−
Rhizobium loti		4284	LMG	−
Bradyrhizobium japonicum		4252	LMG	−
Bacillus lentus		3584	LMG	−
Bacillus firmus		7125	LMG	−
Listeria monocytogenes		38 MB	IHE	−
Listeria welshimeri		81.49	CIP	−
Listeria innocua		80.11	CIP	−
Listeria seeligeri		100.100	CIP	−
Listeria ivanovii		78.42	CIP	−
Lactobacillus casei		57 MB	RZS	−
Streptococcus faecalis		58 MB	RZS	−
Clostridium acetobutylicum		5710	LMG	−
Escherichia coli		60 MB	RZS	−

[a] NIDO, Nationaal Instituut voor Diergeneeskundig Onderzoek (National Institute for Veterinarian Research), Belgium; NCTC, National Collection of Type Cultures, United Kingdom; LMG, Culture Collection of the Laboratory for Microbiology Ghent, Belgium; IHE, Institute for Hygiene and Epidemiology, Belgium; CIP, Collection Nationale de Cultures de Micro-organismes de l'Institut Pasteur (International Collection of the Institute Pasteur), France; RZS, Rijkszuivelstation (Government Dairy Research Station), Belgium.

[b] +, all the test cases are positive; −, all the test cases are negative; (−), in half of the test cases a weak amplification is shown.

described by Innis and Gelfand (6). Denaturation, annealing, and extension were carried out at 95, 50, and 72°C, respectively. In a final reaction mixture of 100 μl, 2 to 3 ng of DNA or 1 μl of a crude cell lysate was used. Crude cell lysates were obtained by sonication of 10^9 bacterial cells in 100 μl of H_2O for 60 s and then by heating for 2 min by 100°C. Before starting the sonication, *Brucella* cells were killed by heating for 2 h at 80°C. The PCR products were analyzed on a 1% agarose gel by the standard protocol (7). The result from the PCR on *Brucella* DNA is shown in Fig. 1. Only one band of the expected size (800 bp) is amplified. The restriction enzymes *Eco*RI, *Rsa*I, and *Sma*I digest this fragment to minor ones of the right size (Fig. 2). Some of the closely related bacterial species were tested together with some species which are commonly found in milk and milk products. The results are listed in Table 2 and shown in Fig. 1. Only the different *Brucella* species do amplify the specific DNA fragment. The other species, including the genetically closely related species, react negatively. The specificity of the amplification was tested on extracted chromosomal DNA and on crude cell lysate with the same results. Each bacterial strain was tested 10 times in independent experiments. Only a small amount of amplified DNA with the *Ochrobactrum anthropi* DNA was seen in about half of the test cases. Higher annealing temperatures did not reproducibly prevent amplification. The confusing results found for *Ochrobactrum anthropi* are probably due to the fact that a very strict standardization of the different PCR conditions (e.g., temperature and salt concentration) is needed for discriminating this bacterial species from *Brucella* spp. *Ochrobactrum anthropi* is most closely related to *Brucella* spp. and, together with the genera *Brucella* and *Phyllobacterium*, forms one rRNA branch (1). Because only *Ochrobactrum anthropi* may show a very weak amplification and all the others, including a *Phyllobacterium* species, do not react, the utility of the method for specific identification of *Brucella* spp. is not affected. The data presented in this article may form the basis for the development of a quick and reliable method for the detection of *Brucella* spp.

The EMBL accession numbers of the 16S rRNA sequences are as follows: *Brucella abortus*, X13695; *Agrobac-*

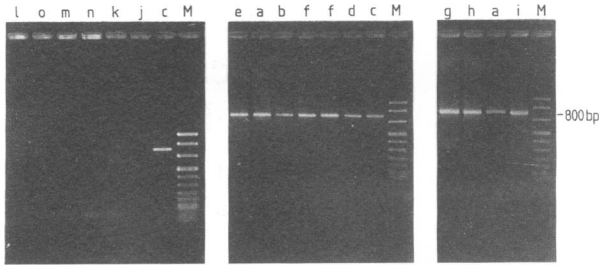

FIG. 1. PCR amplification with Ba148-167F and Ba928-948R as primers. Ba148-167F and Ba928-948R are derived from the 16S rRNA molecule of *Brucella abortus*. The amplified fragment of 800 bp is indicated. M, Molecular weight marker (p UCBM2-1-*Hpa*I + pUCBM21-*Dra*I-*Hin*dIII). The different bacterial species from which the DNA is analyzed are indicated by a letter above each lane. a, *B. abortus*, biovar 3; b, *Brucella melitensis*; c, *Brucella suis*; d, *Brucella ovis*; e, *Brucella canis*; f, *Brucella neotomae*; g, *Brucella melitensis*; h, *Brucella abortus*, biovar 9; i, *Brucella abortus*, biovar 1; j, *Ochrobactrum anthropi*; k, *Phyllobacterium rubiacearum*; l, *Agrobacterium tumefaciens*; m, *Agrobacterium rhizogenes*; n, *Rhizobium loti*. The references of these strains are summarized in Table 2.

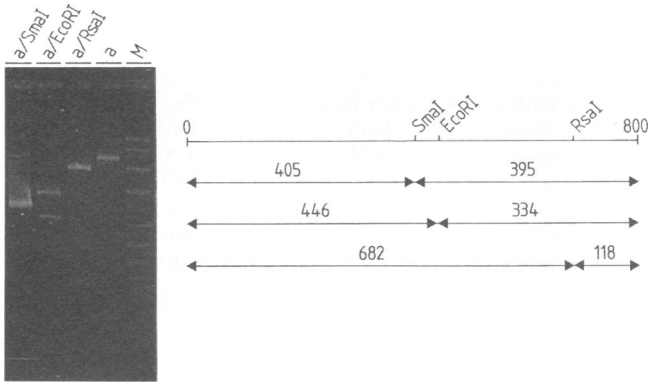

FIG. 2. The restriction enzyme fragment pattern obtained after digestion of the PCR-amplified DNA. At the left side, the analysis of the PCR-amplified fragment with restriction digests is presented. Ba148-167F and Ba928-948R are used as primers. The restriction enzymes used are indicated above the lanes. a, *Brucella abortus*; M, molecular weight marker (pUCBM21-*Hpa*I + pCBM21-*Dra*I-*Hin*dIII). The schematic representation of the target fragment is presented at the right side. The 800-bp fragment of the 16S rRNA of *B. abortus* from position 148 to 948 is presented as a full line. The location of the restriction sites and the length of the digested fragments are indicated.

terium tumefaciens, M11223; *Escherichia coli*, J01695; *Bacillus brevis*, M10111; and *Pseudomonas testosteroni*, M11224.

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