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An IS*711* Element Downstream of the *bp26* Gene Is a Specific Marker of *Brucella* spp. Isolated from Marine Mammals

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DNA polymorphism of the *bp26* gene, coding for a diagnostic protein antigen for brucellosis, was assessed by PCR and restriction fragment length polymorphism analysis using primers to amplify the *bp26* gene with its flanking regions. Surprisingly, whereas PCR performed on DNA of the reference strains of the six recognized *Brucella* species produced a product of the expected size (1,029 bp), PCR performed on DNA of three representative strains from marine mammals (from a seal, a dolphin, and a porpoise) produced a larger product, of about 1,900 bp. Nucleotide sequencing of the 1,900-bp PCR products revealed the presence of an insertion sequence, IS*711*, downstream of the *bp26* gene and adjacent to a Bru-RS1 element previously described as being a hot spot for IS*711* insertion. PCR performed on a large number of field strains from different geographic origins and from marine mammal isolates indicated that the occurrence of an IS*711* element downstream of the *bp26* gene was a feature specific to the marine mammal *Brucella* strains. Thus, this PCR assay is able to differentiate *Brucella* terrestrial isolates from marine mammal isolates and could be applied for diagnostic purposes.

Brucellae are gram-negative, facultative, intracellular bacteria that can infect many species of animals, as well as humans. Six species are recognized within the genus *Brucella*: *B. abortus*, *B. melitensis*, *B. suis*, *B. ovis*, *B. canis*, and *B. neotomae* (8). This classification is mainly based on differences in pathogenicity and host preference (8). The main pathogenic species worldwide are *B. abortus*, which is responsible for bovine brucellosis, *B. melitensis*, the main etiologic agent of ovine and caprine brucellosis; and *B. suis*, which is responsible for swine brucellosis. These three *Brucella* species may cause abortion in their hosts, which results in huge economic losses. *B. ovis* and *B. canis* are responsible for ram epididymitis and canine brucellosis, respectively. For *B. neotomae*, only strains isolated from desert rats have been reported. Distinction between species and biovars is currently performed by differential tests based on phenotypic characterization of lipopolysaccharide antigens, phage typing, dye sensitivity, CO_2 requirement, H_2S production, and metabolic properties (2).

Brucella strains have also been isolated from a great variety of wildlife species, such as bison, elk, feral swine, wild boars, foxes, hares, African buffalo, reindeer, and caribou (9).

The broad spectrum of *Brucella* hosts has recently been enlarged to include marine mammals. A number of recent reports have described the isolation and characterization of *Brucella* strains from a wide variety of marine mammals, such as bottlenose dolphins (*Tursiops truncatus*), common seals (*Phoca vitulina*), harbor porpoises (*Phocoena phocoena*), common dolphins (*Delphinus delphis*), Atlantic white-sided dolphins (*Lagenorhynchus acutus*), striped dolphins (*Stenella caeruleoalba*), hooded seals (*Cystophora cristata*), grey seals (*Halichoerus grypus*), a minke whale (*Balaenoptera acutorostrata*), and an otter (*Lutra lutra*) (3, 5, 10, 13, 18, 22). These strains were identified as brucellae by their colonial and cell morphology, staining characteristics, biochemical activity, agglutination by monospecific antisera, susceptibility to lysis by a *Brucella*-specific bacteriophage, and metabolic profiles. However, their overall characteristics were not assimilable to those of any of the six recognized *Brucella* species. Therefore, it was suggested that they comprise a new species to be called *B. maris* based on the current classification system (18).

It has been shown, on the basis of DNA-DNA hybridization studies, that the genus *Brucella* is a highly homogeneous group (>90% DNA homology for all species), and it has been proposed that this genus should comprise only one genomic species (26). *Brucella* strains isolated from marine mammals also fall into this homogeneous group according to DNA-DNA hybridization (25). Thus, several techniques have been employed to find DNA polymorphisms which would enable the molecular typing of the *Brucella* species and their different biovars (1, 4, 7, 11, 12, 14, 16, 17, 20, 21, 27).

The BP26 protein, also named Omp28, has been previously

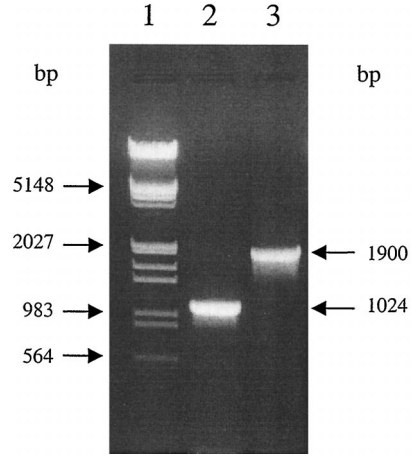

FIG. 1. PCR-amplified *bp26* gene using primers 26A and 26B of *B. melitensis* 16M (lane 2) and seal isolate B2/94 (lane 3) run on a 1% agarose gel. Lane 1, λ DNA *Eco*RI/*Hin*dIII ladder (Appligene, Illkirch, France).

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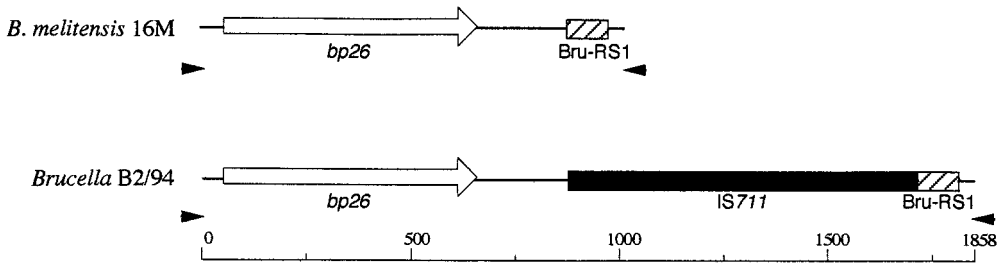

FIG. 2. Schematic view deduced from nucleotide sequencing of the *bp26* gene and flanking regions of *B. melitensis* 16M and seal isolate B2/94. Arrowheads indicate the locations of the primers used for PCR.

TABLE 1. *Brucella* reference, vaccine, and field strains from terrestrial mammals used in this study

Species	Biovar	Strain[a]	Host or source	Geographic origin
B. abortus	1	544 (ATCC 23448; BCCN R4)[b]	Cattle	England
	1	B19 (BCCN V1)[c]	Cattle	United States
	1	99S (BCCN R20)	Cattle	United States
	1	2308 (BCCN R23)	Cattle	United States
	1	BCCN 92-73	Cattle	France
	1	BCCN 94-44	Human	Algeria
	1	BCCN 95-19	Chamois	France
	1	BCCN 95-50	Cattle	Argentina
	1	BCCN 95-51	Cattle	Argentina
	1	BCCN 95-55	Cattle	Costa Rica
	1	BCCN 96-62	Cattle	Italy
	2	86/8/59 (ATCC 23449; BCCN R5)[b]	Cattle	England
	3	Tulya (ATCC 23450; BCCN R6)[b]	Human	Uganda
	3	BCCN 91-90	Cattle	Greece
	3	BCCN 92-25	Cattle	France
	3	BCCN 92-104	Cattle	Guinea
	3	BCCN 93-15	Cattle	Spain
	3	BCCN 93-26	Dromedary	Soudan
	3	BCCN 94-18	Cattle	France
	3	BCCN 94-19	Cattle	France
	3	BCCN 94-63	Cattle	France
	3	BCCN 95-7	Cattle	France
	3	BCCN 95-12	Cattle	France
	4	292 (ATCC 23451; BCCN R7)[b]	Cattle	England
	5	B3196 (ATCC 23452; BCCN R8)[b]	Cattle	England
	6	870 (ATCC 23453; BCCN R9)[b]	Cattle	Africa
	9	C68 (ATCC 23455; BCCN R11)[b]	Cattle	England
	R	45/20 (BCCN V2)[c]	Cattle	England
	R	RB51 (BCCN V5)[c]	Cattle	United States
B. melitensis	1	16M (ATCC 23456; BCCN R1)[b]	Goat	United States
	1	Rev.1 (BCCN V4a)[c]	Goat	Mexico
	1	BCCN 75-478	Sheep	Israel
	1	BCCN 87-92	Human	United States
	1	BCCN 88-42	Sheep	Israel
	1	BCCN 90-61	Sheep	South Africa
	1	BCCN 92-70	Human	France
	1	BCCN 92-106c	Unknown	Algeria
	1	BCCN 93-2	Human	France
	1	BCCN 93-4	Human	France
	1	BCCN 94-37	Human	France
	1	BCCN 96-28	Sheep	Israel
	2	63/9 (ATCC 23457; BCCN R2)[b]	Goat	Turkey
	3	Ether (ATCC 23458; BCCN R3)[b]	Goat	Italy
	3	BCCN 83-198	Human	Spain
	3	BCCN 90-112	Cattle	Greece
	3	BCCN 92-80	Sheep	Spain
	3	BCCN 92-118	Human	Tunisia
	3	BCCN 94-16	Cattle	France
	3	BCCN 95-30	Sheep	Italy
	3	BCCN 95-36	Goat	Italy

Continued on following page

TABLE 1—*Continued*

Species	Biovar	Strain[a]	Host or source	Geographic origin
	3	BCCN 96-32	Sheep	Israel
	3	BCCN 96-142	Human	France
	3	BCCN 96-146	Human	Algeria
B. suis	1	1330 (ATCC 23444; BCCN R12)[b]	Swine	United States
	1	BCCN 95-13	Human	New Caledonia
	1	BCCN 96-138a	Human	Argentina
	1	BCCN 98-21	Human	France
	1	BCCN 98-43	Unknown	Argentina
	2	Thomsen (ATCC 23445; BCCN R13)[b]	Swine	Denmark
	2	BCCN 93-75	Swine	Spain
	2	BCCN 93-80	Swine	Spain
	2	BCCN 94-2	Boar	Belgium
	2	BCCN 94-9	Hare	France
	2	BCCN 94-11	Boar	France
	2	BCCN 97-59	Swine	France
	2	BCCN 97-100	Swine	France
	2	BCCN 97-107	Swine	France
	2	BCCN 98-9	Boar	France
	3	686 (ATCC 23446; BCCN R14)[b]	Swine	United States
	4	40 (ATCC 23447; BCCN R15)[b]	Reindeer	Former USSR
	5	513 (BCCN R21)*	Wild rodent	Former USSR
B. ovis		63/290 (ATCC 25840; BCCN R17)[b]	Sheep	Africa
		Reo 198 (BCCN R22)	Sheep	United States
		BCCN 76-247	Sheep	France
		BCCN 76-250	Sheep	France
		BCCN 91-66	Sheep	Spain
		BCCN 91-70	Sheep	Spain
		BCCN 91-208	Sheep	Spain
		BCCN 91-264	Sheep	Argentina
		BCCN 91-266	Sheep	Argentina
		BCCN 97-41	Sheep	Argentina
		BCCN 98-47	Sheep	Argentina
B. canis		RM6/66 (ATCC 23365; BCCN R18)[b]	Dog	United States
		D519 (BCCN C1)	Dog	Madagascar
		Hoy 1066 (BCCN C3)	Dog	United States
		BCCN 87-62	Dog	Canada
		BCCN 87-65	Dog	Canada
		BCCN 96-104	Dog	Romania
		BCCN 96-121	Dog	France
		BCCN 97-60	Dog	Argentina
B. neotomae		5K33 (ATCC 23459; BCCN R16)[b]	Desert rat	United States

[a] ATCC, American Type Culture Collection; BCCN, *Brucella* Culture Collection, Nouzilly, France.
[b] Reference strain.
[c] Vaccine strain.

identified as an immunodominant antigen in *Brucella* infections of cattle, sheep, and humans (6, 19, 23). In the present study, DNA polymorphisms of the *bp26* gene, coding for this protein, were assessed by PCR-restriction fragment length polymorphism analysis. Primers were designed to amplify the entire *bp26* gene, with its flanking regions, based on the *bp26* nucleotide sequence of *B. melitensis* 16M (GenBank accession no. U45996) (6). The primers used were 26A (forward primer; 5′ GCCCCTGACATAACCCGCTT 3′) and 26B (reverse primer; 5′ GAGCGTGACATTTGCCGATA 3′). PCR was performed on extracted DNAs as described previously (7, 27). Briefly, amplification reaction mixtures were prepared in volumes of 100 μl containing 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100 (1× PCR buffer; Promega, Charbonnieres, France), a 200 μM each concentration of deoxynucleoside triphosphate, a 1 μM concentration of each primer, 100 ng of genomic DNA, and 5 U of *Taq* DNA polymerase (Promega). The temperature cycling for the amplification was performed in a GeneAmp PCR system 9600 thermocycler (Perkin-Elmer) as follows: cycle 1 was 94°C for 5 min (denaturation); the next 30 cycles were 58°C for 1 min (annealing), 70°C for 1 min 30 s (extension), and 94°C for 1 min (denaturation); and the last cycle was 58°C for 1 min (annealing) and 70°C for 10 min (extension). The PCR products were run on 1% (wt/vol) agarose gels containing 0.5 μg of ethidium bromide per ml.

Surprisingly, whereas PCR performed on DNA of the reference strains of the six recognized *Brucella* species produced a product of the expected size (1,029 bp), PCR performed on DNA of three representative strains from marine mammals (a seal, a dolphin, and a porpoise) produced a larger product, of about 1,900 bp (Fig. 1). The nucleotide sequences of the 1,900-bp PCR products of the three marine *Brucella* strains (B2/94, B1/94, and B14/94) were determined, and they re-

TABLE 2. Marine mammal sources of *Brucella* strains used in this study

Latin name	Common name	*Brucella* strain	Geographic origin
Balaenoptera acutorostrata	Minke whale	B202R	Norway
Cystophora cristata	Hooded seal	M2006/94/6	Scotland
Delphinus delphis	Common dolphin	B14/94, M644/93/1, M452/97/2	Scotland
Halichoerus grypus	Grey seal	M2375/94/3	Scotland
Lagenorhynchus acutus	White-sided dolphin	M997/94/2, M2438/95/1, M18/96/1, M181/97/1, M2788/97/1	Scotland
Lagenorhynchus albirostris	White-beaked dolphin	M870/97/1	Scotland
Lutra lutra	Otter	M1771/94/1	Scotland
Phoca vitulina	Common seal	B2/94, M2357/93/1, M2466/93/4, M2533/93/1, M292/94/1, M336/94/1, M339/94/2, M972/94/1, M490/95/1, M514/96/4	Scotland
Phocoena phocoena	Porpoise	B1/94, M1068/91/2, M39/94/1, M1570/94/1, M1661/94/2, M515/96/2, M854/98/8, M1747/98/3	Scotland
Stenella caeruleoalba	Striped dolphin	M2194/94/1, M40/95/1	Scotland
Tursiops truncatus	Bottlenose dolphin	7763/2	France

vealed the presence of an insertion sequence, IS*711*, downstream of the *bp26* gene (Fig. 2). Interestingly, the IS*711* element was found adjacent to a Bru-RS1 element described as being a hot spot for IS*711* insertion (15). Bru-RS1 is a repeated palindromic DNA element of 103 bp which is highly conserved among brucellae and found more than 35 times in the *Brucella* genome (15). Such a Bru-RS1 element was previously described to occur downstream of the *bp26* gene of *B. melitensis* 16M (19). Insertion of the IS*711* element resulted in duplication of the nucleotides TA (data not shown) at the target site, as previously described for *B. ovis* (16). To assess whether the occurrence of an IS*711* element downstream of the *bp26* gene was specific to *Brucella* strains isolated from marine mammals, PCR with primers 26A and 26B was performed on a large number of field strains of *Brucella* from different geographic origins and a large number of the recent isolates from different marine mammals (Tables 1 and 2). All terrestrial isolates, including *B. ovis* strains for which a higher number of IS*711* copies have been described (16, 17, 21), showed a PCR profile in an agarose gel with a band of size of 1,029 bp, whereas PCR on all marine mammal isolates showed the typical 1.9-kb band, implying the presence of the IS*711* element (data not shown). The *bp26* gene by itself, as shown by restriction fragment length polymorphism analysis with different restriction enzymes (*Alu*I, *Cla*I, *Eco*RII, *Eco*RV, *Hae*II, *Hae*III, *Hin*fI, *Pst*I, *Sau*3A, *Sty*I, and *Taq*I) and nucleotide sequencing, did not appear to be useful for molecular typing purposes and thus must be rather conserved among brucellae (data not shown). Only a few differences were observed in the *bp26* gene, and these were in *B. abortus* strains (data not shown).

IS*711* elements, also known as IS*6501* (21), have been described as useful targets for molecular characterization of *Brucella* species and biovars based on the number and distribution of IS*711* copies within the bacterial genomes (4, 16, 17, 21, 24). IS*711*-based fingerprints were described as stable, species specific, and to some extent biovar specific. *B. ovis* has been shown to carry a larger number of IS*711* copies than the other *Brucella* species (16, 17, 21). Recently, it has been shown that *Brucella* strains isolated from marine mammals have more copies of IS*711* than all classical species except *B. ovis* (3). Bricker et al. (3) cloned one of these IS*711* elements and its flanking regions to develop a PCR assay which would be specific for strains isolated from marine mammals. However, they obtained an amplification product of the expected size for all marine mammal isolates and not for the classical *Brucella* species and biovars, except for *B. ovis*. The PCR assay of our study with primers 26A and 26B, although developed by chance, has the advantage of discriminating between all terrestrial isolates, including *B. ovis*, and the marine mammal isolates. This simple PCR assay could have several uses in the future, such as possibly tracing these marine mammal strains if they are transmitted to livestock.

Nucleotide sequence accession numbers. The nucleotide sequences of the genes from *bp26* to IS*711* have been deposited in GenBank under accession numbers AF242532, AF242533, and AF242534.

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