MOLECULAR IDENTIFICATION

Polymorphism at the *dnaK* locus of *Brucella* species and identification of a *Brucella melitensis* species-specific marker

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The *dnaK* gene and surrounding sequences from reference strains of the six *Brucella* species were amplified by the polymerase chain reaction (PCR) with primers chosen according to the published sequence of the *B. ovis* *dnaK* gene and studied for polymorphism with nine restriction endonucleases. The restriction patterns were identical for all species with all restriction endonucleases tested except for *B. melitensis* strain 16M that showed a different pattern with *EcoRV*, consistent with the presence of a single site instead of two for the other *Brucella* species. The absence of the second *EcoRV* site for *B. melitensis* 16M was confirmed by DNA sequence analysis. The second *EcoRV* site in other *Brucella* species was located in a 12-bp segment, which was missing from the published *dnaK* sequence of *B. ovis*, between the stop codon of the *dnaK* gene and its putative transcription terminator sequence. The difference between *B. ovis* strain 63/290 and *B. melitensis* 16M was due to an additional base-pair in *B. melitensis* 16M. Subsequently, 71 other field, vaccinal and reference strains of the six *Brucella* species and their different biovars were studied for restriction fragment length polymorphism (RFLP) of the *dnaK* locus with *EcoRV*. The presence of a unique *EcoRV* site was specific to *B. melitensis* strains. Southern blot analysis of whole genomic DNA digested with *EcoRV* and with the *dnaK* gene used as probe also detected a distinct pattern for *B. melitensis*. These results indicate that both PCR-RFLP and Southern blot analysis of the *dnaK* locus can be used to distinguish *B. melitensis* strains from the other *Brucella* species and may be useful for typing and diagnostic purposes as well.

Introduction

Brucellae are gram-negative facultative intracellular bacteria that can infect man and many species of animals. Six species are recognised within the genus *Brucella*: *B. abortus*, *B. melitensis*, *B. suis*, *B. ovis*, *B. canis* and *B. neotomae* [1]. This classification is mainly based on differences in pathogenicity and in host preference [1]. Distinction between species and biovars is performed by differential tests based on phenotypic characterisation of lipopolysaccharide antigens, phage typing, dye susceptibility, CO₂ requirement, H₂S production and metabolic properties [2].

It has been suggested on the basis of DNA homology (>90% for all species) that *Brucella* is a mono-specific genus [3, 4]. However, Allardet-Servent *et al.* [5] reported that restriction fragment-length polymorphism (RFLP) can be used to distinguish between species within the genus *Brucella* and supported host preference as a basis for classifying the brucellae.

Recently, Fekete *et al.* [6] showed by arbitrarily primed polymerase chain reaction (AP-PCR) that *Brucella* strains can be distinguished according to the banding patterns of their amplified DNA on agarose gels. In addition, Grimont *et al.* [7] showed by Southern blot analysis of *Brucella* DNA, cut by *Bam*HI, with random cloned *Brucella* DNA probes, that the most frequently occurring biovars (*B. melitensis* biovars 1 and 3, *B. abortus* biovars 1 and 3, *B. suis* biovar 2 and *B. ovis*) could be distinguished from each other. Species-specific markers have also been reported for the genes encoding the major 25- and 36-kDa outer-membrane proteins (OMPs) [8, 9]. More recently, Bricker and Halling [10] showed that *B. abortus* biovars 1, 2 and 4, *B. melitensis*, *B. ovis* and *B. suis* biovar 1 can be differentiated by a PCR method exploiting the polymorphism arising from species-specific localisation of the genetic element IS711 in the brucella chromosome.

The heat shock protein (hsp) DnaK belongs to the hsp70 family, which has been highly conserved during evolution [11]. The DnaK proteins possess chaperone, ATPase and initiation of DNA replication functions

[12–17]. Recently, the *dnaK* gene from *B. ovis* has been cloned and sequenced [18]. The deduced amino-acid sequence shares 60–78% homology with that of DnaK proteins from other bacteria [18].

The present study analysed, by use of PCR-RFLP, polymorphism of the hsp *dnaK* locus among 77 *Brucella* reference and field strains representing all species with their different biovars and sought a species-specific marker on the *dnaK* locus of *B. melitensis* strains. *B. melitensis* affects mostly small ruminants and man [19] but may also affect cattle [20].

Study of polymorphism by PCR-RFLP has the advantage over Southern blot analysis in being easier to set up and less time-consuming for application to large numbers of samples. Furthermore, PCR combined with the use of species-specific markers to identify the causative agent of disease could be used for epidemiological and diagnostic purposes [21, 22].

Materials and methods

Bacterial strains

The 77 strains used in this study are listed in Table 1. All strains were kept freeze-dried in the *Brucella* Culture Collection, INRA, Nouzilly, France. Cultures were grown on Trypticase Soy Agar (bioMérieux, Marcy l'Etoile, France) supplemented with yeast extract (Difco Laboratories, Detroit, Michigan) 0.1% w/v (TSAYE medium). For fastidious strains (*B. abortus* biovar 2 and *B. ovis*), sterile equine serum (bioMérieux) was added to TSAYE medium to a final concentration of 5% v/v (TSAYES medium). The strains were checked for purity and species and biovar characterisation by standard procedures [2].

DNA preparation

For each strain, a 1-day 37°C culture on a TSAYE or TSAYES slope was harvested with 3 ml of sterile distilled water, and centrifuged at 4500 rpm for 10 min. The pellet was suspended in 567 µl of TE-Na buffer (50 mM Tris, 50 mM EDTA, 100 mM NaCl, pH 8.0); 30 µl of SDS 10% w/v solution and 3 µl of proteinase K 2% w/v solution were then added and held at 37°C for 1 h. The lysed cell suspension was extracted twice with equal volumes of a phenol-saturated solution and then chloroform. Nucleic acids were precipitated by gently mixing the resultant aqueous phase with 2 volumes of cold ethanol. The precipitate was dissolved in 100 µl of TE (10 mM Tris, 1 mM EDTA, pH 8.0). The amount of DNA was measured by electrophoresis of a sample of each preparation through agarose 0.8% gels, and comparison with standard DNA solutions.

Primers

Two 20-mer primers for the *dnaK* gene were obtained from Appligene (Illkirch, France). Primers were chosen to amplify the entire gene with its Shine-Dalgarno and putative transcription terminator sequences according to the *B. ovis*-determined *dnaK* gene sequence [18]. Primers used for amplification of the *dnaK* gene were 70A (5'-TCGAGGTCTTGGCAGTTTGC-3') and 70B (5'-TCCGTTTCATGCAGCGTGAC-3').

Amplification conditions

Amplification reactions were prepared in volumes of 100 µl containing 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 1.5 mM MgCl₂, Triton X-100 0.1%, gelatin 0.2 mg/ml (1 × PCR buffer; Appligene), 200 µM (each) deoxynucleoside triphosphates (dNTP), 1 µM primer, 100 ng of genomic DNA and 2.5 U of *Taq* DNA Polymerase (Appligene). To reduce evaporation, the mixture was overlaid with a drop of mineral oil. The temperature cycling for amplification was performed in a Techne PHC-2 thermocycler as follows: cycle 1, 95°C for 5 min (denaturation), 62°C for 2 min (annealing) and 70°C for 3 min (extension). The next 30 cycles were: 95°C for 1 min (denaturation), 62°C for 2 min (annealing) and 70°C for 3 min (extension). The last cycle was: 95°C for 1 min (denaturation), 62°C for 2 min (annealing) and 70°C for 10 min (extension).

Restriction endonuclease digestion

Restriction endonucleases were chosen according to the *B. ovis*-determined *dnaK* gene sequence. Fig. 1 shows the restriction map of the *dnaK* gene with the restriction endonucleases used. Restriction endonucleases *Ava*II, *Bgl*II, *Hind*III, *Hin*fI, *Kpn*I, *Nco*I and *Pst*I were purchased from Appligene; *Ban*I and *Eco*RV were purchased from Boehringer Mannheim (Germany). Five µl of PCR product were cleaved by 1–5 U of each restriction endonuclease in a reaction volume of 20 µl; the manufacturer's recommended buffer and incubation temperature were used.

Agarose gel electrophoresis

After amplification of the genes, 5 µl of the reaction mixture or 20 µl of each restriction digest were mixed with 1 µl and 2 µl, respectively, of glycerol-dye loading buffer, and the mixture was electrophoresed in agarose gel consisting of standard quality agarose (Appligene) 1 or 1.5% w/v or NuSieve 3:1 agarose (TEBU, Le Perray en Yvelines, France) 2.5% and ethidium bromide (Sigma) 0.5 µg/ml in TBE buffer (89 mM Tris-HCl, 89 mM boric acid, 2 mM EDTA, pH 8.0). The samples were electrophoresed at 20 V overnight on a Apelex ST 1006 electrophoresis unit. Mol. wt DNA marker VI (Boehringer Mannheim) was used as a size standard. A 254-nm UV transilluminator was used to detect the bands.

Table 1. *Brucella* strains studied

Study	Species	Biovar	Strain no.	Host or source	Geographic origin
1	<i>B. abortus</i>	1	544 (ATCC 23448; BCCN R4)*	Cattle	England
2	<i>B. abortus</i>	1	B19 (BCCN VI)†	Cattle	USA
3	<i>B. abortus</i>	1	99S (BCCN R20)	Cattle	USA
4	<i>B. abortus</i>	2	86/8/59 (ATCC 23449; BCCN R5)	Cattle	England
5	<i>B. abortus</i>	3	Tulya (ATCC 23450; BCCN R6)	Human	Uganda
6	<i>B. abortus</i>	4	292 (ATCC 23451; BCCN R7)	Cattle	England
7	<i>B. abortus</i>	5	B3196 (ATCC 23452; BCCN R8)	Cattle	England
8	<i>B. abortus</i>	6	870 (ATCC 23453; BCCN R9)	Cattle	Africa
9	<i>B. abortus</i>	9	C68 (ATCC 23455; BCCN R11)	Cattle	England
10	<i>B. abortus</i>	R	45/20 (BCCN V2)†	Cattle	England
11	<i>B. abortus</i>	R	RB51 (BCCN V5)	Cattle	USA
12	<i>B. melitensis</i>	1	16M ATCC 23456; BCCN R1)*	Goat	USA
13	<i>B. melitensis</i>	1	BCCN 93.4	Human	France
14	<i>B. melitensis</i>	1	BCCN 92.106c	Unknown	Algeria
15	<i>B. melitensis</i>	1	BCCN 93.2	Human	France
16	<i>B. melitensis</i>	1	BCCN 88.41	Sheep	Israel
17	<i>B. melitensis</i>	1	BCCN 88.42	Sheep	Israel
18	<i>B. melitensis</i>	1	BCCN 88.43	Human	Israel
19	<i>B. melitensis</i>	1	BCCN 88.39	Sheep	Israel
20	<i>B. melitensis</i>	1	BCCN 75.477	Dog	Israel
21	<i>B. melitensis</i>	1	BCCN 75.473	Cattle	Israel
22	<i>B. melitensis</i>	1	BCCN 75.478	Cattle	Israel
23	<i>B. melitensis</i>	1	BCCN 88.44	Human	Israel
24	<i>B. melitensis</i>	1	BCCN 76.279	Cattle	Israel
25	<i>B. melitensis</i>	1	BCCN 92.70	Human	France
26	<i>B. melitensis</i>	1	Rev. 1 (BCCN V4a)†	Goat	Mexico
27	<i>B. melitensis</i>	1	H38 (BCCN V3)†	Human	Mexico
28	<i>B. melitensis</i>	1	BCCN 90.61	Sheep	South Africa
29	<i>B. melitensis</i>	1	BCCN 87.92	Human	USA
30	<i>B. melitensis</i>	2	63/9 (ATCC 23457; BCCN R2)	Goat	Turkey
31	<i>B. melitensis</i>	3	Ether (ATCC 23458; BCCN R3)	Goat	Italy
32	<i>B. melitensis</i>	3	BCCN 83.35	Human	Turkey
33	<i>B. melitensis</i>	3	BCCN 83.198	Human	Spain
34	<i>B. melitensis</i>	3	BCCN 90.112	Cattle	Greece
35	<i>B. melitensis</i>	3	BCCN 90.128	Cattle	Greece
36	<i>B. melitensis</i>	3	BCCN 75.472	Cattle	Israel
37	<i>B. melitensis</i>	3	BCCN 75.479	Cattle	Israel
38	<i>B. melitensis</i>	3	BCCN 92.42	Human	Tunisia
39	<i>B. melitensis</i>	3	BCCN 92.80	Sheep	Spain
40	<i>B. melitensis</i>	3	BCCN 92.83	Sheep	Spain
41	<i>B. melitensis</i>	3	BCCN 92.106a	Unknown	Algeria
42	<i>B. melitensis</i>	3	BCCN 92.118	Human	Tunisia
43	<i>B. melitensis</i>	3	BCCN 92.120	Human	Tunisia
44	<i>B. melitensis</i>	3	BCCN 92.121	Human	Tunisia
45	<i>B. melitensis</i>	3	BCCN 93.3	Human	France
46	<i>B. melitensis</i>	3	BCCN 93.1	Human	France
47	<i>B. melitensis</i>	R	B115(BCCN R19)	Goat	Malta
48	<i>B. melitensis</i>	R	BCCN 92.119	Human	Tunisia
49	<i>B. melitensis</i>	R	BCCN 92.116	Sheep	France
50	<i>B. melitensis</i>	R	BCCN 92.117	Sheep	France
51	<i>B. melitensis</i>	R	BCCN 93.5	Human	France
52	<i>B. suis</i>	1	1330 (ATCC 23444; BCCN R12)*	Swine	USA
53	<i>B. suis</i>	1	S2 (BCCN V6)†	Swine	China
54	<i>B. suis</i>	2	Thomsen (ATCC 23445; BCCN R13)	Swine	Denmark
55	<i>B. suis</i>	3	686 (ATCC 23446; BCCN R14)	Swine	USA
56	<i>B. suis</i>	4	40 (ATCC 23447; BCCN R15)	Reindeer	Former Soviet Union
57	<i>B. suis</i>	5	513 (BCCN R21)	Wild rodent	Former Soviet Union
58	<i>B. ovis</i>		63/290 (ATCC 25840; BCCN R17)*	Sheep	Africa
59	<i>B. ovis</i>		Reo 198 (BCCN R22)	Sheep	USA
60	<i>B. ovis</i>		BCCN 76.247	Sheep	France
61	<i>B. ovis</i>		BCCN 76.250	Sheep	France
62	<i>B. ovis</i>		BCCN 91.66	Sheep	Spain
63	<i>B. ovis</i>		BCCN 91.70	Sheep	Spain
64	<i>B. ovis</i>		BCCN 91.208	Sheep	Spain
65	<i>B. ovis</i>		BCCN 91.212	Sheep	Spain
66	<i>B. ovis</i>		BCCN 91.217	Sheep	Spain
67	<i>B. ovis</i>		BCCN 91.264	Sheep	Argentina
68	<i>B. ovis</i>		BCCN 91.266	Sheep	Argentina
69	<i>B. ovis</i>		BCCN 91.268	Sheep	Switzerland
70	<i>B. canis</i>		RM6/66 (ATCC 23365; BCCN R18)*	Dog	USA
71	<i>B. canis</i>		BCCN 87.62	Unknown	Mexico
72	<i>B. canis</i>		BCCN 87.66	Dog	Canada
73	<i>B. canis</i>		BCCN C2	Dog	USA
74	<i>B. canis</i>		BCCN C3	Dog	USA
75	<i>B. canis</i>		BCCN C1	Dog	Madagascar
76	<i>B. canis</i>		BCCN C4	Dog	USA
77	<i>B. neotomae</i>		5K33 (ATCC 23459; BCCN R16)*	Desert wood rat	USA

ATCC, American Type Culture Collection, Rockville, MD; BCCN, Brucella Culture Collection, Nouzilly (France).

*Reference strains.

†Vaccine strains.

Fig. 1. Restriction map of the PCR-amplified *dnaK* gene, according to the gene sequence determined from *B. ovis* by Cellier *et al.* [18]. The coding sequence is shown by a shaded rectangle under the restriction map. Restriction sites are indicated as follows: Av, *AvaII*; B, *BanI*; Bg, *BglII*; EV, *EcoRV*; H, *HindIII*; Hf, *HinfI*; K, *KpnI*; N, *NcoI*; P, *PstI*.

Southern blot

PCR-amplified *dnaK* gene from *B. melitensis* 16M was purified by the GeneClean procedure (Bio 101, La Jolla, CA, USA) and labelled with the random priming PolarPlex kit (Millipore, Bedford, MA, USA) for use as probe in the Southern blot hybridisation. Whole genomic DNA (2–5 µg) from *Brucella* strains was digested with *EcoRV* and run on SeaKem agarose (FMC, Rockland, ME, USA) 0.8% gel in TBE buffer. The fragments were transferred to a nylon Immobilon-S membrane (Millipore) by vacuum transfer by the VacuGene System (Pharmacia LKB Biotechnology, Uppsala, Sweden). Hybridisation and detection reactions were performed according to the PolarPlex protocol. Biotinylated Lambda/*HindIII* and Phi X 174/*HaeIII* digests (Millipore) were used as mol. wt DNA markers.

DNA sequencing

The dideoxy chain termination method of Sanger *et al.* [23] was employed with the SequiTherm™ Cycle Sequencing Kit (Epicentre Technologies, Madison, WI, USA) according to the manufacturer's protocol. PCR-amplified *dnaK* genes from *B. ovis* and *B. melitensis* were purified from an agarose gel with the GeneClean kit (Bio 101) and used as templates, and primer 70B was used for annealing in the sequencing reaction. The

temperature cycling for the amplification was performed in a Techne PHC-2 thermocycler as follows: cycle 1, 95°C for 5 min (denaturation), 50°C for 30 s (annealing) and 70°C for 1 min (extension). The next 30 cycles were: 95°C for 30 s (denaturation), 50°C for 30 s (annealing) and 70°C for 1 min (extension).

Results

PCR-RFLP

Fig. 1 shows the restriction map of the PCR-amplified *dnaK* locus comprising the ribosome binding site, open reading frame and transcription terminator sequences, according to the DNA sequence determined from *B. ovis* [18], with restriction endonucleases *AvaII*, *BanI*, *BglII*, *EcoRV*, *HindIII*, *HinfI*, *KpnI*, *NcoI*, *PstI* used for RFLP study. The PCR-amplified *dnaK* loci from the six *Brucella* species reference strains – *B. abortus* 544 (biovar 1), *B. melitensis* 16M (biovar 1), *B. suis* 1330 (biovar 1), *B. ovis* 63/290, *B. canis* RM6/66 and *B. neotomae* 5K33 – showed identical restriction patterns with *AvaII*, *BanI*, *BglII*, *HindIII*, *HinfI*, *KpnI*, *NcoI* and *PstI*, which corresponded to restriction patterns of the published *B. ovis dnaK* sequence (Fig. 2, and data not shown). A distinct restriction pattern of the *dnaK* locus from *B. melitensis* 16M was observed with *EcoRV*; there was a single *EcoRV* site 'EcoRV (1767)' instead of two *EcoRV* sites for *dnaK*

Fig. 2. Electrophoretic analysis of PCR-amplified *dnaK* genes of *B. ovis* 63/290 (lanes 2, 4, 6, 8, 10, 12, 14, 16, 18) and *B. melitensis* 16M (3, 5, 7, 9, 11, 13, 15, 17, 19) digested with *AvaII* (2 and 3), *BanI* (4 and 5), *BglII* (6 and 7), *EcoRV* (8 and 9), *HindIII* (10 and 11), *HinfI* (12 and 13), *KpnI* (14 and 15), *NcoI* (16 and 17), *PstI* (18 and 19); lane 1, DNA mol. wt marker VI (Boehringer, Mannheim) 2176, 1766, 1230, 1033, 653, 517, 453, 394, 298, 234, 220, 154 bp.

genes of the other *Brucella* species (Fig. 2). However, the *EcoRV* pattern for the *dnaK* gene of *B. ovis* 63/290 did not correspond to that of the published *B. ovis* *dnaK* nucleotide sequence [18]. According to the published nucleotide sequence [18], only one *EcoRV* site is present at the *dnaK* locus of *B. ovis* at the same position as that of *B. melitensis*. Therefore, study by PCR-RFLP of the *dnaK* locus with *EcoRV* was extended to a large number of *Brucella* reference, vaccinal and field strains from different geographic origins and hosts, listed in Table 1. The presence of the unique *EcoRV* site in the *dnaK* locus was specific to *B. melitensis* strains. All other *Brucella* strains, including *B. ovis* strains, showed the presence of two *EcoRV* sites in their *dnaK* sequences as shown for that of *B. ovis* 63/290 in Fig. 2.

Southern blot analysis

Southern blot analysis of whole genomic DNA from *Brucella* species digested with *EcoRV* was performed with the PCR-amplified *dnaK* gene as probe to confirm the specific pattern of *B. melitensis*. A single band, positioned at *c.* 2300 bp, was detected for *EcoRV*-digested DNA from *B. abortus* 544, *B. suis* 1330, *B. ovis* 63/290, *B. canis* RM6/66 and *B. neotomae* 5K33 (Fig. 3), whereas two bands, one at the same position as the band of the former strains and a second less strongly stained band of higher mol. wt (around 3000 bp), were detected for the type strains of the three *B. melitensis* biovars – *B. melitensis* 16M (biovar 1), *B. melitensis* 63/9 (biovar 2) and *B. melitensis* Ether (biovar 3) (Fig. 3).

DNA sequence analysis

The presence of the second *EcoRV* site located at the 3' end of the PCR-amplified *dnaK* gene of *B. ovis* 63/290 was confirmed by DNA sequence analysis. This *EcoRV* site was located outside the *dnaK* open reading frame, between the stop codon and the putative transcription terminator sequence, in a 12 bp segment that was missing in the published *dnaK* sequence of *B. ovis* [18] (Fig. 4). A supplementary base-pair (Fig. 4) occurred in the 12-bp segment of *B. melitensis* 16M, explaining the lack of the *EcoRV* site in this species and its specific pattern seen in PCR-RFLP and Southern blot analysis.

Fig. 4. Location of the *EcoRV* polymorphic site by DNA sequence analysis of PCR-amplified *dnaK* of *B. ovis* 63/290 and *B. melitensis* 16M. The *EcoRV* site was located between the stop codon and putative transcription terminator sequence of *dnaK*. The position number 2378 corresponds to that of the *B. ovis* *dnaK* sequence published by Cellier *et al.* [18]. The 12 bp segment missing in this sequence is underlined. The *EcoRV* site is double underlined. The absence in *B. ovis* 63/290 of the additional base-pair present in *B. melitensis* 16M is indicated by a point.

Fig. 3. Southern blot analysis of *Brucella* DNA cut with *EcoRV*. PCR-amplified *dnaK* gene of *B. melitensis* 16M was used as probe. Lane 1: DNA mol. wt marker, fragment sizes are 23130, 9416, 6557, 4361, 2322, 2027, 1353, 1078, 872 bp; 2: *B. abortus* 544 (biovar 1) DNA; 3: *B. melitensis* 16M (biovar 1) DNA; 4: *B. melitensis* 63/9 (biovar 2) DNA; 5: *B. melitensis* Ether (biovar 3) DNA; 6: *B. suis* 1330 (biovar 1) DNA; 7: *B. ovis* 63/290 DNA; 8: *B. canis* RM6/66 DNA; 9: *B. neotomae* 5K33 DNA.

Discussion

Polymorphism at the *dnaK* locus among *Brucella* species was studied by PCR-RFLP and Southern blot analysis. As expected from the point of view of conservation of DnaK protein among different genera and the fact that *Brucella* constitutes a homogeneous group according to DNA-DNA hybridisation [3], the *dnaK* gene seemed to be highly conserved among *Brucella* species. More gene polymorphism was detected for, instance, in the major *Brucella* 25- and 36-kDa (porin) OMP genes [8, 9] which are probably more variable in relation to host environment. Nevertheless, the species-specific polymorphism found with *EcoRV* at the *dnaK* locus of *B. melitensis* strains is interesting for typing, epidemiological and diagnostic purposes and may be complementary to other species-specific markers previously detected in other genes [8, 9]. It is interesting to note that these species-specific markers, found for instance at the *dnaK* locus

in the present study, are due to an additional single base-pair in *B. melitensis*, or in the case of the *omp25* gene [8] due to one single nucleotide substitution, and that these markers are remarkably conserved within species. However, the simultaneous use of several species-specific markers must still be considered on a large number of strains before one or more of them can routinely be used for typing purposes.

The result of the presence of an additional base-pair at the *dnaK* locus of *B. melitensis* compared with the other *Brucella* species may contribute, as with previous studies focused on *Brucella* gene polymorphism [8,9], to the understanding of the phylogeny of *Brucella* spp. Indeed, if the excision was considered to have occurred in the other *Brucella* species, *B. melitensis* might represent the progenitor species of the others. This idea is also supported by the study of *omp2b* porin gene polymorphism by PCR-RFLP, in which hypothetical *Brucella* phylogenetic networks were established based on single mutational events [8].

Although a single species concept may be considered for *Brucella* on the basis of DNA-DNA homology [3], it is still important for epidemiological purposes to distinguish between the defined *Brucella* species on the basis of their differential host preference. *B. melitensis* strains are most often isolated from small ruminants like sheep and goats, but in some areas where *B. melitensis* is highly prevalent, infection of cattle, which are more usually infected by *B. abortus*, has also been reported [20]. Consequently, it is important to know the causative agent of infection for establishing prophylactic and eradication schemes. The contribution of molecular biology to the identification of species-specific markers and methods like PCR may greatly facilitate and hasten the identification of *Brucella* strains.

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