

Molecular typing of *Brucella* field strains isolated in Italy

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Summary

Brucella field strains were identified using molecular techniques. A polymerase chain reaction (PCR) method, based on amplification of the insertion sequence IS711, was used to identify the isolates at species level. Subsequently, restriction fragment length polymorphism analysis of the *omp2a* and *omp2b* genes was used to assign the *Brucella* species to the different biovars. A total of 248 field strains were processed and complete agreement was obtained with the species/biovar identifications made by conventional bacteriological methods. PCR-based tests were more rapid and proved valuable in overcoming some of the drawbacks of conventional methods.

Keywords

Biovars, Brucellosis, Italy, Molecular techniques, Polymerase chain reaction, Restriction fragment length polymorphism.

Introduction

The genus *Brucella* classically includes six species, namely: *B. melitensis*, *B. abortus*, *B. ovis*, *B. canis*, *B. suis* and *B. neotomae* (4). Each species has been subdivided further into biovars. On the basis of the homology of DNA-DNA hybridisation, it has been suggested that *Brucella* be considered a monospecific genus, represented by the species *B. melitensis* which includes various biovars (7, 8). To more fully understand epidemiological information from the Italian disease eradication programme, it was essential to identify the *Brucella* strains that affect both livestock and wildlife during outbreaks. The current official diagnostic method is the isolation of bacterial colonies from host tissues, milk or vaginal exudates, followed by characterisation (1, 5). However, the conventional method used to identify *Brucella* strains is not only time-consuming, but also poses a hazard for laboratory staff when manipulating infectious agents. New molecular methods, such as amplification of the insertion sequence (IS711) (2) and restriction fragment length polymorphism analysis (RFLP) of the *omp2a* and *omp2b* genes (3), have been developed to identify *Brucella* strains at the genetic level. The use of these methods overcomes the drawbacks of conventional methods. The AMOS (*abortus melitensis ovis suis*) PCR assay is a multiplex PCR designed to detect the IS711 insertion elements in the four following *Brucella* species: *B. abortus*; *B. melitensis*, *B. ovis* and *B. suis*. The copy number and distribution of the IS711 insertion elements are species-specific in the *Brucella* chromosome. The PCR assay

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exploits the variability of the sequences flanking the IS711 elements by using specific primers which hybridise to these variable regions (2).

The *omp2a* and *omp2b* gene sequences encode the major outer membrane proteins (OMPs) of *Brucella*. Due to intra-specific differences in the sequences, digestion of the amplified *omp* genes, with a battery of restriction enzymes, yield different patterns according to the biovars (3). Most *Brucella* species and some biovars can be identified and differentiated with the correct restriction enzymes/*omp* gene combinations. To characterise the Italian field strains of *Brucella*, the authors assessed the performance of the above-mentioned PCR-based methods, comparing them with standard bacteriological methods.

Materials and methods

Reference strains

The DNAs extracted from all the *Brucella* reference strains were provided by the Central Veterinary Laboratory in Weybridge (United Kingdom).

Bacterial isolates

A collection of 248 *Brucella* field strains, isolated and typed in Italy from humans and animals between 2001 and 2003, were stored using the Microbank™ system and grown on Trypcase soy broth, supplemented with 0.6% (w/v) yeast extract and 5% (v/v) sterile equine serum. The strains were monitored for purity and species and biovars were typed using standard bacteriological procedures (1, 5).

DNA preparation

Brucella cells were harvested by centrifugation of 1 ml of broth; DNAs were then extracted using standard phenol-chloroform methods (6) and maintained at -20°C until use.

Species identification

The AMOS-PCR assay described by Bricker and

Halling (2) was used to amplify the insertion sequence IS711, with slight modifications of the original procedure. Briefly, the samples were tested in a final volume of 50 µl PCR reaction mixture containing the following:

- 5 µl of 10x PCR buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl: Applied Biosystems)
- 2.5 mM MgCl₂ (buffer II: Applied Biosystems)
- 200 µM each dNTP
- 1 µM IS711 specific primer
- 0.2 µM each of the *B. abortus*, *B. melitensis*, *B. ovis* and *B. suis* specific primer
- 2.5 U AmpliTaq polymerase (Applied Biosystems)
- 5-10 ng of DNA template.

Amplifications were performed for 40 cycles in a thermal cycler (Applied Biosystems Gene Amp PCR system 9700) with denaturation at 95°C, annealing at 60°C, and then an extension step at 72°C (each for 30 sec). Cycling was preceded by a 10 min denaturation step at 95°C and was followed by a final 7 min extension at 72°C. Amplicons were checked by fluorescence after electrophoresis in a 1.5% agarose in the presence of ethidium bromide (2 µg/ml).

Biovar identification

Amplifications of the *omp2a* and *omp2b* genes were performed as described by Cloeckaert *et al.* (3) with slight modifications of the reaction conditions. The PCR reaction was performed in 50 µl total volume, containing the following:

- 5 µl of 10x PCR buffer
- 2.5 mM MgCl₂
- 200 µM each dNTP
- 1 µM of each specific primer
- 2.5 U AmpliTaq polymerase
- 5-10 ng DNA template.

Amplifications were performed as for the AMOS-PCR assay. Restriction analyses of the amplicons were performed using a battery of four endonucleases with esanucleotide recognition sites (*Pst*I, *Sty*I,

EcoRI and *KpnI*), two endonucleases with pentanucleotide (*HinfI*) and tetranucleotide (*TaqI*) recognition sites, respectively. In particular, the *omp2a* gene was cleaved with *HinfI*, *PstI* and *StyI*, while *omp2b* was digested with *EcoRI*, *HinfI*, *KpnI* and *TaqI*. Digestions were performed in accordance with the instructions of the manufacturer. Fragments were run on a 3% agarose gel and viewed under UV light after ethidium bromide staining. Each restriction morph was designated by a capital letter.

Results

Species identification

Amplification of the insertion sequence IS711 using the AMOS-PCR yielded amplicons of different sizes in the different *Brucella* species (Fig. 1). However, as described by Bricker *et al.* (2), individual

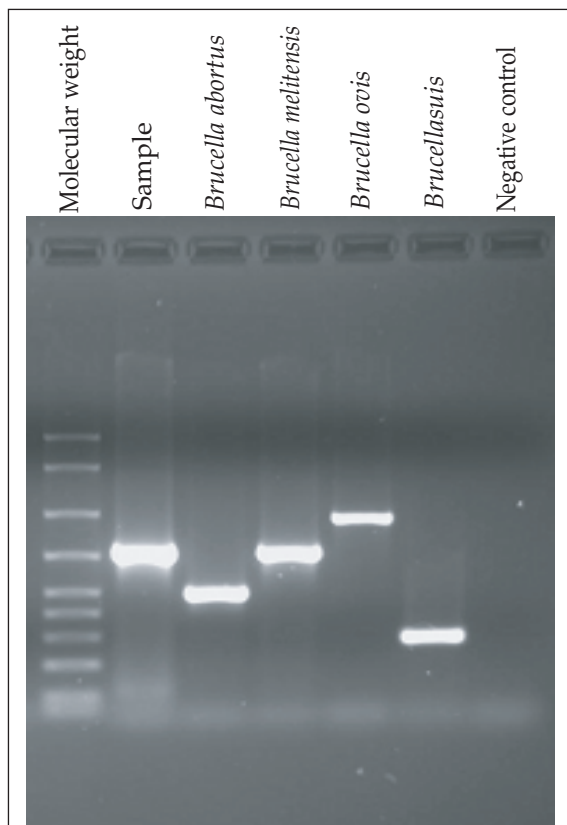


Figure 1
AMOS-PCR products amplified from field isolates of *Brucella* sp.

biovars within a species could not be differentiated. In particular, species discrimination was determined including *B. abortus* biovars 1, 2 and 4, *B. melitensis* biovars 1, 2 and 3, *B. ovis* and *B. suis* biovar 1. As expected, no PCR products were obtained for *B. abortus* biovars 3, 5, 6 and 9 or for *B. suis* biovars 2, 3, 4 and 5 (2).

Biovar identification

RFLP patterns resulting from the correct combination of the *omp* genes and restriction enzymes (Tables I and II) differentiated most biovars within the

Table I
Restriction patterns of the *omp2a* gene.

Brucella species	Biovar	Pattern	Restriction pattern		
			HinfI	PstI	StyI
<i>B. abortus</i>	1,2,4	A	P1		
<i>B. abortus</i>	3,5,6,9	B	P2		
<i>B. melitensis</i>	1	C	P3		
<i>B. melitensis</i>	1,2,3	B	P2		
<i>B. suis</i>	1,3,4	D	P3	P2	
<i>B. suis</i>	2	E	P3	NC	
<i>B. suis</i>	5	F	P4	NC	

NC= not cut

Table II
Restriction patterns of the *omp2b* gene.

Brucella species	Biovar	Pattern	Restriction pattern			
			EcoRI	HinfI	KpnI	TaqI
<i>B. abortus</i>	1,2	A	P1			
<i>B. abortus</i>	3,4,5,6,9	B	P2			
<i>B. melitensis</i>	1,3	B	P1			
<i>B. melitensis</i>	1,2,3	E	P3			
<i>B. suis</i>	1,5	B	P1	P1		
<i>B. suis</i>	2	F	P3	NC		
<i>B. suis</i>	3,4	G	P1	NC		

NC= not cut.

Brucella species identified by the AMOS assay. For example, *B. melitensis* biovar 3 can be distinguished by first using the digestion of the *omp2a* gene with *PstI*, which excludes biovar 1 if the specific pattern P3 is obtained (Fig. 2), then using the digestion of *omp2b* with *HinfI*, which excludes biovar 2 if the specific pattern P1 is obtained.

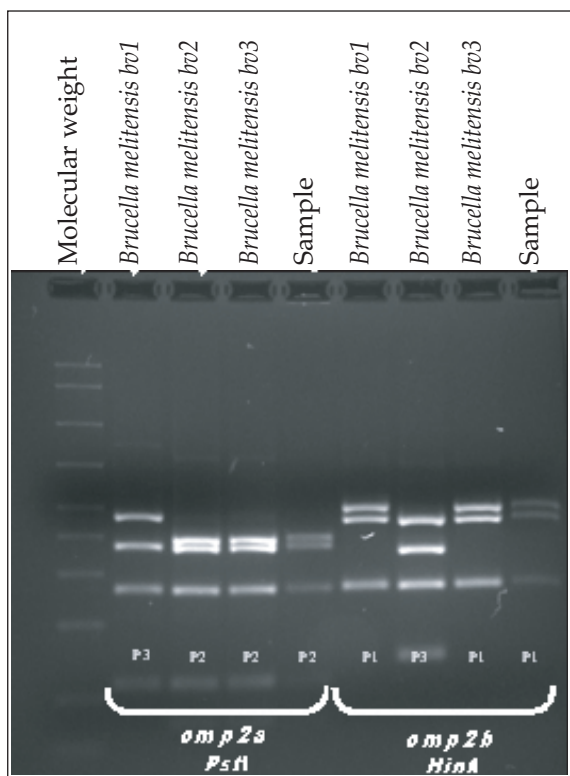


Figure 2
Example of restriction patterns in biovars of *Brucella melitensis* field isolates: *omp2a* and *omp2b* amplicons digested with PstI and HinfI, respectively (P1, P2 and P3 as in Tables I and II)

Biovars of the *B. abortus* species are genetically very close (7, 8). As expected, some of them (biovars 3, 5, 6 and 9) showed identical restriction patterns. As a consequence, not all biovars can be differentiated individually.

A total of 248 *Brucella* field isolates were processed with the PCR-based and RFLP analyses as described above. A combination of AMOS-PCRs and RFLPs

of the *omp* genes identified the isolates as follows (Table III):

- 152 as *B. melitensis* biovar 3
- 23 as *B. abortus* biovar 1
- 39 as *B. abortus* group, biovars 3, 5, 6 and 9
- 3 as *B. ovis*
- 30 as *B. suis* biovar 1
- 1 as *B. suis* biovar 2.

Complete agreement was found between molecular methods and conventional bacteriological procedures in the characterisation of the field strains examined at the species and biovar levels.

Discussion and conclusions

The molecular approach adopted in this study has several advantages when compared to bacteriological techniques. One of the drawbacks of the standard methods is the long duration of the process, during which at least two weeks elapse between the collection of the clinical specimen and the final identification of the species/biovars. Moreover, due to the complexity of the tests (the subjectiveness of some characteristics, such as colony morphology), these procedures should be performed by highly skilled personnel in an authorised laboratory. Finally, the large number of characteristics examined and the minor differences between some species and biovars may produce conflicting data and complicate the interpretation. Thus, the results might not be always conclusive. In contrast, molecular techniques identify *Brucella*

Table III
Distribution of *Brucella* biovars identified in field isolates from some domestic and wild species, and humans.

<i>Brucella</i> species and biovars	Sheep/Goat	Cattle	Buffalo	Pigs	Wild boar	Human	Total
<i>B. melitensis</i> biovar 3	113	28	5	-	-	6	152
<i>B. abortus</i> biovar 1	-	20	3	-	-	-	23
<i>B. abortus</i> biovar 3,5,6,9	-	39	-	-	-	-	39
<i>B. ovis</i>	3	-	-	-	-	-	3
<i>B. suis</i> biovar 1	-	-	-	1	29	-	30
<i>B. suis</i> biovar 2	-	-	-	-	1	-	1

strains within a working day. Furthermore, the RFLP analysis is relatively easy to perform and provides objective results that are easy to interpret. Moreover, the analysis of results of molecular methods is not affected by the presence of contaminants and, as there is no need for live organisms, it is considered much safer for laboratory staff in comparison to the bacteriological methods.

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