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Identification of *Brucella* isolated from goats using *Pst* I site polymorphism at *Omp2* gene loci

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ABSTRACT

In present study *Brucella* was isolated from different target tissues of goats. The isolated brucellae cultures were primarily characterized using classical biotyping methods. For molecular typing of *Brucella* isolates we used, PCR-RFLP, which relies on DNA polymorphism. We used the *Omp2* gene of *Brucella* as a locus of two nearly homologous repeated copies that differ slightly among *Brucella* species and biotypes in presence or absence of the *Pst* I site to differentiate between them. Our results revealed that DNA fragments obtained from *B. melitensis* 16M strain isolated from goats and *B. melitensis* Rev1strain produced three bands, an intact 282-bp fragment from the amplified *omp2a* gene that lacks the *Pst I* site and two smaller fragments of 238 and 44 bp, the product obtained from digestion of the *omp2b* amplified fragment. In contrast *B. melitensis* biovar 3 produced only two smaller fragments from both genes (*omp2a* and *omp2b*), a 238-bp fragment and a 44-bp fragment. Because of the existing *Pst* I site polymorphism between *Brucella* strains, the test may distinguishes between different strains of *B. melitensis* Rev1 vaccine strain from *B. melitensis* biovar 3 field strains in less than 12 hours. This method can be used in clinical samples directly.

Key words: *Brucella melitensis,*PCR-RFLP,*Omp2* gene

Brucellosis mainly affects the reproductive tract of goats and sheep, and cause abortion in these animals. Globally, despite the remarkable results achieved by the majority of industrialized countries, where bovine brucellosis has been eradicated or controlled, ovine-caprine brucellosis remains a problem and is prevalent where goats and sheep are kept (Blasco *et al.* 1994). Major OMPs (Outer Membrane Protein) from *Brucella* have been classified into two groups, viz. group 2 having closely linked (*Omp 2a* and *Omp 2b*) which are 36 and 38 kDa in size respectively and group 3 (Omp 25 and Omp 31) which are 31–34 kDa in size respectively (Cloeckaert *et al*. 2002). Group 2 encodes porin proteins with a high degree (85%) of homology (Ficht *et al.* 1989). Interestingly, *Omp2a* is not expressed in lab grown *B. abortus*, while *Omp 2b* is expressed.

The composition of the *omp2* locus from two gene copies with differences in their *Pst I* restriction endonuclease sites was used to establish an epidemiologic fingerprint for the *omp2* gene in the *Brucella* strain. For *Brucella melitensis*, the identification at the biovar level is currently performed by four main tests: CO_2 requirement, production of H_2S, dye (thionin and basic fuschin) sensitivity, and agglutination with monosecific A and M antisera. Routine typing tests, if properly carried out, will identify the majority of *Brucella* cultures as a particular biovar of one of the established species (Alton *et al.* 1988) but for confirmation molecular typing is needed.

The *omp2* gene exists as a locus of two nearly homologous repeated copies that differ slightly among *Brucella* spp. and biotypes (Ficht *et al.* 1988). In the present study, we used this information to amplify a 282-bp fragment and performed PCR-RFLP analysis of *Pst* I site polymorphism of *omp2* loci of brucellae in order to evaluate this as a tool for differentiation of strains of *Brucella melitensis*.

MATERIALS AND METHODS

Isolation of Brucella melitensis from goats: For isolation of *Brucella melitensis* 65 samples (milk 20, vaginal swab 15, aborted fetus 06, fetal stomach content 06, uterus 05 and supramammary lymph nodes 05) were processes. Samples from goats were processed and cultured on Brucella agar enriched with 5% horse serum under sterile laboratory conditions (Alton *et al*. 1988). These plates were incubated at 37° C for 48 hours under micro-aerophilic conditions and were regularly monitored for bacterial growth. Simultaneously, *Brucella melitensis* 16M, Rev-1 and biovar-3 standard strains were revived (Microbiology lab,

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CIRG, Makhdoom). For harvesting of bulk culture, warm normal saline solution (NSS) was poured on the bacterial growth and the growth was scraped with sterile swab and collected in a flask for further use.

Classical biotyping: Classical biotyping of *Brucella* was done by growth characteristic, biochemical, antigenic and phage typing as per the methods described by Alton *et al.* (1988).

Extraction of genomic DNA: Genomic DNA was extracted as per the method of Sambrook *et al*. (1989). For visualization, the DNA sample was electrophoresed in 0.8% agarose gel in Tris acetate EDTA (TAE) buffer. The extracted DNA was purified using DNA purification kit (Promega) as per manufacturer's instructions.

Amplification of outer membrane protein 2 (Omp 2) gene

Primer synthesis: Based on the available nucleotide sequences on the NCBI and Gen Bank database primers for amplification of *Omp2b* gene, were designed using DNA Star 'software'. The primers were:

Forward primer: 5'-TGGAGGTCAGAAATGAAC-3,

Reverse: 5'- GAGTGCGAAACGAGCGC-3

PCR amplification: PCR amplification was performed by the method of Mullis and Faloona (1987). A typical reaction mixture contained 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% (wt/vol) Triton X-100, 0.2 mg of bovine serum albumin (fraction IV) per ml, and 10 mM Tris-HCl (pH 8.5). Each reaction mixture was supplemented with 100 mM each of the four deoxyribonucleotides, 100 ng of sample DNA, and each oligonucleotide primer (1µM). PCR was performed as follows: 35 cycles of PCR, with 1 cycle consisting of 20 s at 95°C for DNA denaturation, 1 min at 50°C annealing, and 1 min at 72°C for polymerase-mediated primer extension. At the end a final extension at 72°C for 7 min was given. Ten microliters of the amplified product was analyzed by electrophoresis in 1.5% agarose gels in TEA buffer (20 mM Tris-acetate, 1 mM EDTA pH 8.0).

Restriction analysis of Omp2 *gene:* Restriction enzyme *Pst I* was used as per the manufacturer's instructions. The digested DNA was separated by electrophoresis 1.5% agarose gels (w/v in Tris-acetate buffer). DNA fragments were visualized by staining with ethidium bromide (1.5 g/ml).

RESULTS AND DISCUSSION

Isolates of Brucella*:* A total of 65 samples were processed for isolation of Brucella. Out of this a total of 16 suspected *Brucella* isolates were isolated.

Identification of Brucella organisms: A combination of growth characteristics, biochemical serological and bacteriological methods usually enable *Brucella* to be correctly identified.

Morphology and staining: All the 16 suspected *Brucella* isolates were gram-negative, cocco-bacilli, usually arranged singly. The morphology was fairly constant in 10 isolates, however 6 isolates showed pleomorphic character. True capsule was not detected in any of 16 isolates.

Growth characteristics: In most of the cases the growth was smooth, clear, pale-honey-colored appearance. The colonies producing haemolysis on blood agar or lactose fermentation on Mac Conkey agar were eliminated from further consideration as *Brucella*.

Antigenic characteristics: Out of 16 *Brucella* isolates, 6 were with smooth surface antigens and reacted in agglutination with antisera prepared against smooth *Brucella* culture. Out of the 06 isolates, one was originated from fetal membranes, three were from stomach content and two were of vaginal swabs. Rest 10 isolates did not reacted with antisera. These 10 isolates were either may be rough *Brucella*

Table 1. Species and biovar differentiation of the species of the genus *Brucella* isolated from goats

Suspected *Brucella* isolates	Source	Growth characteristics					Monospecific sera				Phage typing						Interpretation
		Urea	H_2S	CO_2	BF	TH	A	M	R	Ac	Tb	Wb	BK_2	Fi	Iz	R/C	
P1	Fetal membrane	++	-	-	+	+	-	+			NL	NL	CL	NL	PL	NL	*Brucella melitensis* 16M
P2	Stomach content	++	-	-	+	+	-	+			NL	NL	CL	NL	PL	NL	*Brucella melitensis* 16M
P3	Stomach content	++	-	-	+	+	+	+			NL	NL	CL	NL	PL	NL	*Brucella melitensis* biovar *3*
P4	Stomach content	++	-	-	+	+	+	+			NL	NL	CL	NL	PL	NL	*Brucella melitensis* biovar *3*
P5	Vaginal Swab	++	-	-	+	+	+	+			NL	NL	CL	NL	PL	NL	*Brucella melitensis* biovar *3*
P6	Vaginal Swab	++	-	-	-	+	-	-			NL	NL	CL	NL	PL	NL	*Brucella variant*

BF, Basic fuchsin at 200µl/ml (1/50,000 w/v); TH, thionin at 20µl/ml (1/50,000 w/v); CL, confluent lysis; PL, partial lysis; NL, no lysis; Plq, plaques; NL Some lytic activity observed, but not considered true lysis.

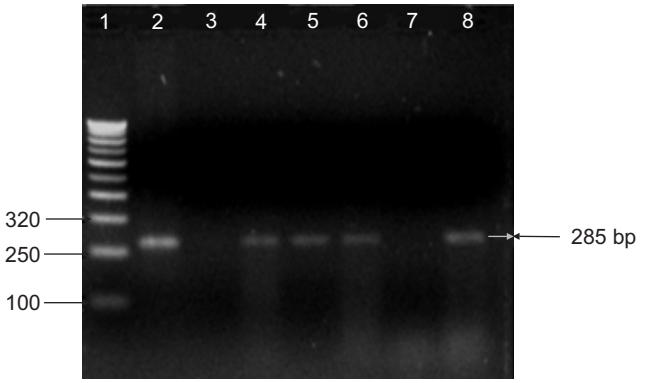

Fig. 1. Agarose gel electrophoresis of PCR-amplified *omp2* gene of *Brucella melitensis* strains. Lane 1: Standard DNA marker; lane 2: positive control;lane 3, negative control; lane 4, 5, 6, 8: *Omp 2* amplified product of 282 bp.

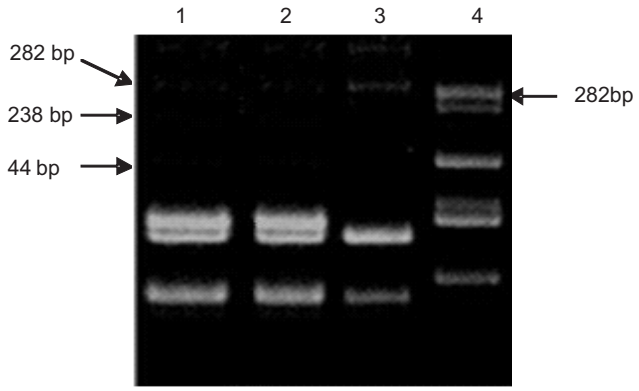

Fig. 2. Agarose gel electrophoresis of restriction digested (*Pst I*) *omp2* PCR product fragments from isolated *Brucella* strains. Lane 1, *B. melitensis* strain 16M; lane 2, *B.melitensis* Rev1; lane 3, *B.melitensis* biovar 3; lanes 4, Standard DNA marker.

or variants of *Brucella* (Table 1).

Biochemical tests: Brucella cultures were oxidative rather than fermentative in metabolism. All the 6 smooth *Brucella* were oxidase and urease positive. (Table 1). The cultures of *Brucella melitensis* were catalase and oxidase positive, and did not require supplementary CO_2 for growth. They did not produce H_2S more than a trace. They hydrolysed urea and grew in the presence of basic fuschin.

Classical biotyping of Brucella *cultures:* Only the 6 out of 16 were further processed for species identification after its confirmation and species identification was done based on two main sets of properties: lysis by phages and oxidative metabolic profile on selected amino acid and carbohydrate substrates. The characteristics of all 6 *Brucella* isolates as revealed by the routine typing tests (Table 1). In present study important phages like Tb, Wb, Bk2, Fi, Iz were used for phage typing.

Molecular typing: The *PstI* digestion pattern of the *omp2* amplified gene fragments resembled that of strain 16M, the prototype strain for virulent *B. melitensis* biovar 1, and that of the vaccine strain Rev.1 (Fig. 2). In contrast, the *PstI* digestion profile of the *omp2a* gene amplified fragments from all other isolates depicted a reproducible and conserved pattern that was different from that shown for strains 16M and Rev.1 (Fig. 2). This suggests that a genetic link might be established between the prototype strain 16M and the vaccine strain Rev.1.

We used the *omp2* gene which is present as a locus of two nearly homologous repeated copies that differ slightly among *Brucella* species and biotypes in presence or absence of the *Pst 1* site to differentiate between them. The PCR test was performed with all 6 *B. melitensis* biovars isolated from goats.

The PCR was first performed to test the specificity of *Brucella* and when ever there was a single band with a expected size of 282 bp was obtained only then *Brucella* DNA was used as a template for RFLP. The results revealed that DNA fragments obtained from two isolates from goats identified as *B. melitensis* 16M strain produced three bands, an intact 282-bp fragment from the amplified *omp2a* gene that lacks the *Pst I* site and two smaller fragments of 238 and 44 bp, the product obtained from digestion of the *omp2b* amplified fragment. This pattern resembled with standard strains *B. melitensis* 16M and Rev-1. In contrast, other 3 isolates were characterized as *B. melitensis* biovar 3 produced only two smaller fragments from genes (*omp2a* and *omp2b*), a 238-bp fragment and a 44-bp fragment. This pattern was also resembled with pattern obtained with standard biovar-3 strain (Fig. 2).

The earlier results obtained by Cloeckaert *et al.* (1995) confirmed these data, showing that *B. melitensis* isolates were split between those with a single *PstI* site located in the *omp2b* gene and those with two *PstI* sites, one in *omp2a* and one in *omp2b*.

The results obtained from these tests showed that two isolates identified as *B. melitensis* 16M and 3 isolates were identified as *Brucella melitensis* biovar 3 on the basis of classical and molecular biotyping). Besides the 282- and 238-bp DNA bands, *B. melitensis* 16M produced an additional identical smaller fragment, which was calculated to be 44-bp. It was calculated that the two small bands (44 and 238 bp) together were of same size as the uncut DNA. In a comprehensive study, Meyer (1984) had shown that unlike *B. abortus*, *B. melitensis* lacked plasticity in the features characterized by the conventional biotyping methods. Our data indicated that in contrast to these findings, *B. melitensis* has undergone genetic diversions in a pattern similar to that previously shown to occur in other *Brucella* spp.

It was concluded that a total of 16 suspected *Brucella* isolates were isolated and on morphological, biochemical and molecular characterization it was found that the two isolates identified as *B. melitensis* 16M strain and 3 isolates were identified as *Brucella melitensis* biovar 3. Sixth one was the variant of *Brucella* spp. Because of the existing *Pst I* site polymorphism between *Brucella* strains, the test

may distinguishes between different strains of *B. melitensis* in less than 12 hours. This method can be used in clinical samples directly.

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