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Molecular characterization of *Omp31* gene of Indian field Isolates of *Brucella melitensis*

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ABSTRACT

PCR amplification and sequencing of *Omp31* gene of 19 Indian field isolates of *Brucella melitensis* was performed. The sequence analysis revealed that all 19 isolated had 100% nucleotide sequence identity confirming the fact that the *Omp31* gene of *B. melitensis* is highly conserved and stable. The amino acid sequences also revealed 100% sequence homology with published sequences. The protein structure predicted using the amino acid sequences confirms that this porin protein consists of 8 β barrels and 3 surface exposed loop portions.

Key words: *Brucella melitensis, Omp31* gene sequence

Brucellosis, one of the most important and widespread bacterial zoonosis in the world, is caused by members of the genus *Brucella* (Cutler *et al.* 2005), and human beings are susceptible to *B. melitensis*, *B. abortus*, *B. suis* and *B. canis* of which *B. melitensis* is the most virulent species (Acha and Syzfres 2006, Fugier *et al.* 2007). Man gets infected mainly through ingestion of contaminated milk and unpasteurized dairy products, contact with fluids and tissues from aborted foetuses and aerosol route of transmission (Fugier *et al.* 2007). It is one of the most common laboratory acquired infections (Boschiroli *et al.* 2001). *Brucella* outer membrane proteins (omps) were found to be exposed on the surface of the organism using monoclonal antibodies (Cloeckaert *et al*. 2002). *Brucella* omps induced cellular immunity and gave protection against the infection (Doosti *et al*. 2009). The omps are classified into major groups 2 (*Omp*2a and *Omp*2b) and 3 (*Omp25* and *Omp31)* (Gupta *et al.* 2012). The genes encoding these omps are located on chromosome I of *B. melitensis* (Cloeckaert *et al.* 2002). Diversity of gene encoding the major omp at species, biovars and strain level is of taxonomical and epidemiological interest (Moriyon and Lopez-Goni 1998). The polymorphism of *Omp2* gene was used to distinguish *B. melitensis* Rev1 vaccine strain from *B. melitensis* biovar 3 field strains using restriction enzyme analysis (Gupta *et al.* 2012). The *Omp31* gene was reported to be highly conserved except 9 nucleotide substitution in *B. ovis* compared to that of *B. melitensis*. The nucleotide difference resulted in different antigenic properties of *Omp31* of *B. ovis* (Vizcaino *et al.* 2001). The protein encoded by this gene is a porin protein associated with the peptidoglycan layer of *Brucella* spp. (Moriyon and Lopez-Goni 1998, Cloeckaert *et al.* 2002) and antibodies against it were detected in sheep naturally and experimentally infected with *B. melitensis* (Cassataro *et al.* 2004). *B. melitensis* Omp31 exhibited 34% homology with Omp25 protein of *Brucella* and also with omps of other members of alpha 2 subdivision of *proteobacteria* (Cloeckaert *et al.* 2002). *Brucella* species displayed a high degree of genomic relatedness (Verger *et al.*1985) with speculation of difference in pathogenicity and host preferences mediated through omps (Martín-Martín *et al.* 2009). This could have implication in the use of omps based subunit or recombinant vaccine. In the present study, the *Omp31* gene of 19 Indian field isolates of *B. melitensis* were analysed for the presence of nucleotide variations by PCR amplification and sequence analysis.

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MATERIALS AND METHODS

Brucella melitensis *isolates*: In the present study 19 Indian field isolates of *B. melitensis* available in the *Brucella* laboratory, Division of Veterinary Public Health, Indian Veterinary Research Institute, were used; details are given in Table 1.

Table 1. Details of Indian field isolates of *Brucella melitensis* used in this study and their *Omp31* gene accession numbers

Isolate No.	Host	Year of isolation	Place of isolation	Accession No.
BME_57/97_VPH	-	1997	Unknown	*JN185700*
BME_32_VPH	Man	2004	Bijapur	*JN185701*
BME_187_VPH	Man	2006	Belgaum	*JN185702*
BME_16_VPH	Man	2007	Belgaum	*JN185699*
BME_28_VPH	Man	2007	Belgaum	*JN185703*
BME_36c_VPH	Man	2008	Bannerghatta	*JN185704*
BME_36b_VPH	Man	2008	Bannerghatta	*JN185705*
BME_39_VPH	Woman	2008	Dharwad	JN185707
BME_36a_VPH	Man	2008	Bangalore	*JN185706*
BME_51_VPH	Man	2009	Belgaum	*JN185708*
BME_52_VPH	Woman	2009	Belgaum	*JN185709*
BME_53_VPH	Man	2009	Belgaum	*JN185710*
BME_55/VPH	Man	2009	Dharwad	*JN185711*
BME_56_VPH	Man	2009	Vellore	*JN185712*
BME_67/VPH	Man	2009	Belgaum	*JN185713*
BME_69_VPH	Man	2009	Belgaum	*JN185714*
BME_70/VPH	Man	2010	Dharwad	*JN185715*
BME_72_VPH	Man	2010	Belgaum	*JN185716*
BME_73_VPH	Woman	2010	Belgaum	*JN185717*

Isolation of genomic DNA: The isolates were cultured on glycerol dextrose agar (GDA) slants for 48 h at 37°C. A loopful of the culture was used for genomic DNA isolation using DNeasy blood and tissue kit as per the manufacturer's recommendations. The quality of the extracted DNA was analysed by 0.8% agarose gel electrophoresis and the DNA were stored at –20°C untill used.

PCR amplification of Omp31 *gene:* Primers specific for the *Omp31* gene *B. melitensis* were designed based on the available nucleotide sequences on the NCBI and GenBank database and got synthesized commercially. The details of the primers are: forward primer (5'- 3'): ATG ACC CAC CCA AGA TAC AAG, reverse primer (5'- 3'): GGC CTT TCC CGT TTC CAG TTC. Polymerase chain reaction (PCR) was initially standardized with DNA extracted from standard *B. melitensis* 16M. Later, the PCR amplification of *Omp31* gene of 19 *B. melitensis* Indian field isolates was carried out. The reaction mixture (25 µl) consisted of 2.5µl of 10× PCR assay buffer, 2.5µl of dNTP mixture (2mM each), 1.5µl of $MgCl_2$ (25mM), 1 µl of each primers (10 pmol/µl), 0.3µl Taq DNA polymerase (3U/µl) and 5µl of genomic DNA and 11.2µl of nuclease free water. Amplification was performed using gradient thermocycler for 30 cycles, with denaturation at 94°C for 60 sec, annealing at 56°C for 45 sec, and extension at 72°C for 60 sec followed by a final extension at 72°C for 5 min. Following amplification, the products were analysed for the presence of a single band of desired molecular weight on 1.5% agarose gel in the presence of ethidium bromide (0.5 µg/ml) and documented gel documentation system.

Sequencing of Omp31 *gene:* PCR amplification of *Omp31* from all 19 Indian field isolates of *B. melitensis* was performed in four, 50 µl reaction mixtures to get 200µl amplicon volume using the protocol described above. The amplified product were mixed with 6× gel loading dye and subjected to electrophoresis in 0.7% agarose gel. The amplicons were cut out from the gel and the amplicons were eluted using gel extraction kit as per the maufracuturer's recommendations. The final elution was done in 50 µl elution buffer and stored at –20°C until further use. The purified product was checked for presence of PCR product by agarose gel electrophoresis and the products were got directly sequenced commercially.

Nucleotide and amino acid analysis: The nucleotide sequence obtained was analysed using Editseq, SeqMan, SeqBuilder and Megalign of DNA Star programme and sequence homology with reported sequences was checked using basic local alignment search tool (NCBI - BLAST). The amino acid sequence analysis was carried out with Megalign and Protean programme (DNA Star software) and PRED-TMBB online tool for trans-membrane protein structure prediction (http: //biophysics.biol.uoa.gr/PRED-TMBB/).

RESULTS AND DISCUSSION

PCR amplification of Omp31 gene: The *Omp31* gene from all 19 *B. melitensis* Indian field isolates was amplified by PCR and all were found to produce the specific amplicon of 788bp as analysed by agarose gel electrophoresis (Fig. 1).

Nucleotide sequencing and analysis: The amplicon were purified and got sequenced as described above, the sequences obtained were aligned for homology with reported *Omp31* gene sequence of *B. melitensis* to identify the *Omp31* gene specific 609bp sequence. The 609bp sequence so obtained for each isolate was aligned with reported *B. melitensis*

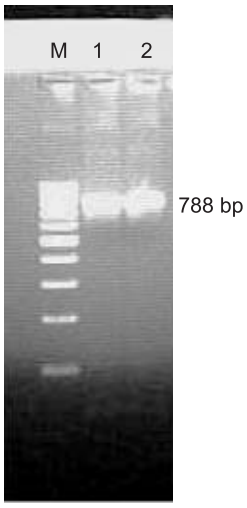

Fig. 1. Agarose gel electrophoresis of PCR amplified *Omp31*gene of *B. melitensis* field isolates. Lane M: 100 bp DNA Marker; Lane 1&2: *Omp31*amplified product of 788 bp.

Per cent identity

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	
1		100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	99.5	1 BME_16_VPH.seq
2	0.0		100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	99.5	2 BME_28_VPH.seq
3	0.0	0.0		100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	99.5	3 BME_32_VPH.seq
4	0.0	0.0	0.0		100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	99.5	4 BME_36a_VPH.seq
5	0.0	0.0	0.0	0.0		100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	99.5	5 BME_36b_VPH.seq
6	0.0	0.0	0.0	0.0	0.0		100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	99.5	6 BME_36c_VPH.seq
7	0.0	0.0	0.0	0.0	0.0	0.0		100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	99.5	7 BME_39_VPH.seq
8	0.0	0.0	0.0	0.0	0.0	0.0	0.0		100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	99.5	8 BME_51_VPH.seq
9	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	99.5	9 BME_52_VPH.seq
10	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	99.5	10 BME_53_VPH.seq
11	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	99.5	11 BME_55_VPH.seq
12	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	99.5	12 BME_56_VPH.seq
13	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	99.5	13 BME_57_97_VPH.seq
14	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	99.5	14 BME_67_VPH.seq
15	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		100.0	100.0	100.0	100.0	100.0	100.0	100.0	99.5	15 BME_69_VPH.seq
16	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		100.0	100.0	100.0	100.0	100.0	100.0	99.5	16 BME_70_VPH.seq
17	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		100.0	100.0	100.0	100.0	100.0	99.5	17 BME_72_VPH.seq
18	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		100.0	100.0	100.0	100.0	99.5	18 BME_73_VPH.seq
19	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		100.0	100.0	100.0	99.5	19 BME_187_VPH.seq
20	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		100.0	100.0	99.5	20 BME_ ATCC 23457_NC_012441.1.seq
21	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		100.0	99.5	21 BME_ M5-90_CP001851.1.seq
22	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		99.5	22 BME_ M28_CP002459.1.seq
23	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0		23 BME_ Rev.1_NZ_ACEG01000065.1.seq
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	

Divergence

Fig. 2. Nucleotide sequence pair distances of *Omp31*gene of Indian field isolates of *B. melitensis* with reported *B. melitensis* sequences using Clustal W method.

Omp31 gene sequences and all 19 *Omp31* gene sequences were found to have 100% sequence homology among them and with the reported *B. melitensis* (*B. melitensis* ATCC 23457, *B. melitensis* M5-90 and *B. melitensis* M28) *Omp31* gene sequences and 99.5% identity with *B. melitensis* Rev1 *Omp31* (Fig. 2). The 19 sequences were submitted to GenBank and their accession numbers are given in Table 1. Although the isolates used in this present study were obtained over a period 13 years (1997–2010) in spite, such a long term circulation between human and animal population, the sequence homology obtained indicated that the *Omp31* gene is highly conserved and stable which is remarkable. Our finding confirmed the earlier reports of Vizcaino *et al.* (2001) that the *B. melitensis Omp31* gene is highly conserved and it is also supported by the earlier reports of Gee *et al.* (2004) who have reported that the strong nucleotide identity of *Omp31* gene of *B. melitensis* and other *Brucella* species may be due to the high degree of genetic relatedness of these species. Phylogenetic mapping analysis of the 19 Indian field isolates of *B. melitensis* with other reported *B. melitensis Omp31* gene sequences showed that all 19 sequences has a close phylogenetic origin (Fig. 3). The observations about similarity among the *Omp31* of Indian isolates compared to

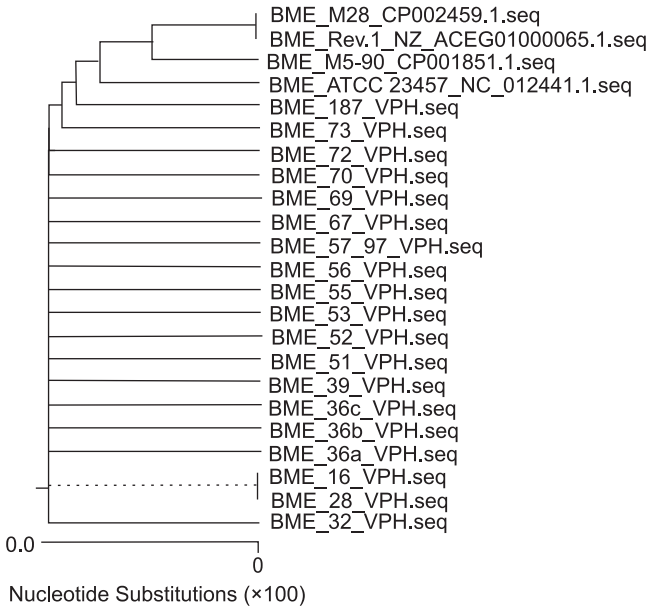

Fig. 3. Phylogenetic tree constructed for *Omp31*gene nucleotide sequences of Indian field isolates of *B. melitensis* with reported *B. melitensis Omp31*gene sequences by Clustal W method.

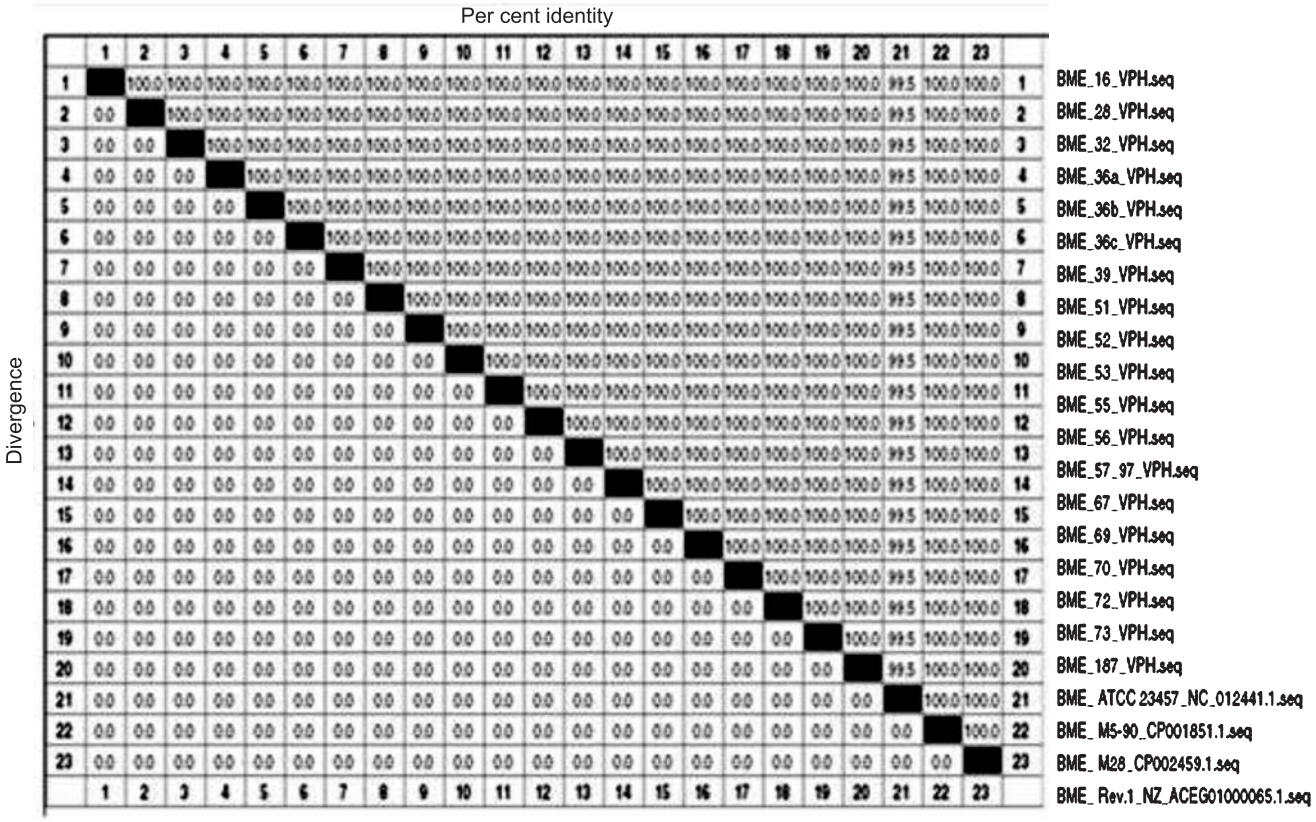

Fig. 4. Amino sequence pair distances of Omp31of Indian field isolates of *B. melitensis* with published *B. melitensis* Omp31amino acid sequences using Clustal W method.

reference strains could be in agreement to the development of *Omp31* based vaccine for protection against *Brucella* infection as was demonstrated by Cassataro *et al.* (2005).

Amino acid sequence analysis: The amino acid sequences were predicted based on the Omp31 nucleotide sequences obtained for all 19 *B. melitensis* Indian field isolates. These were also aligned with reported Omp31 amino acid sequence of *B. melitensis* ATCC 23457, *B. melitensis* M5-90, *B. melitensis* M28 and *B. melitensis* Rev1 *strains*. Here also 100% homology was found with *B. melitensis* ATCC 23457, *B. melitensis* M5-90 and *B. melitensis* M28 Omp31 amino acid sequences while *B. melitensis* Rev1 Omp31 showed 99.5% homology (Fig. 4). This finding is similar to that of earlier reports of Vizcaino *et al.* (1996) and Cloeckaert *et al.* (2002). The phylogenetic analysis also showed that all 19 isolates were arranged in the same cluster with other reported *B. melitensis* Omp31 amino acid sequences (Fig. 5). The secondary structure of the Omp31 protein of *B. melitensis* (BME_16_VPH) was predicted using PRED-TMBB online tool which showed that the Omp31 porin protein consisted of eight β barrels and 3 surface exposed loop portions with the N terminal surface exposed loop portion having more surface exposed area than others as previously described by Vizcaino *et al.* (2001) and Cloeckaert *et al.* (2002)**.** The hydrophobicity and antigenic index profiles of Omp31 of *B. melitensis* calculated by using protean programme (DNA star software), approximately 34% of the whole protein was hydrophobic and a predominant of hydrophilic regions were

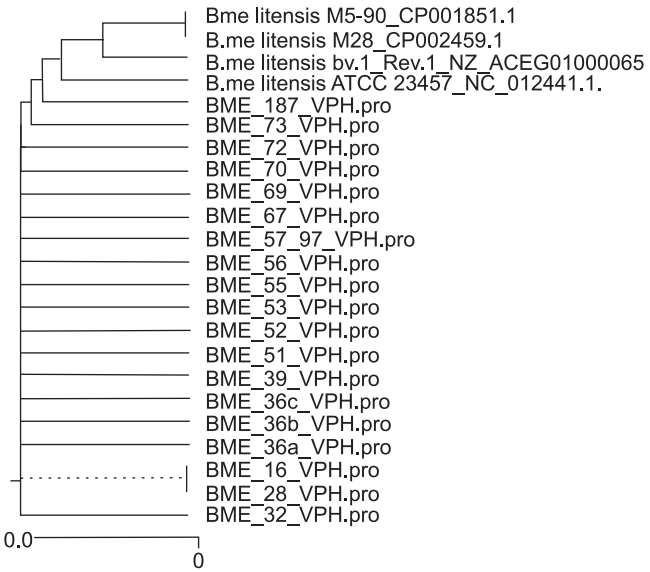

Fig. 5. Phylogenetic tree constructed for Omp31 amino acid sequences of Indian field isolates of *B. melitensis* with reported *B. melitensis* Omp31 amino acid sequences by Clustal W method.

distributed over the surface of the Omp31 protein**.** The antigenic index assay showed that the antigenic portions of Omp31 are distributed almost throughout the protein and the amino acids in the regions between 50–90, 120–142 and 160–190 could be highly antigenic (Fig. 6; available as supplementary file).

The nucleotide sequences of *Omp31* gene of 19 Indian field isolates of *B. melitensis* showed 100% sequences homology between them and with published sequences suggesting that *Omp31* gene of *B. melitensis* is highly conserved and is remarkably stable. The *Omp31* gene could be further exploited for development of a subunit vaccine alone or in conjunction with some other suitable gene product.

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