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RESEARCH OPINIONS IN ANIMAL & VETERINARY SCIENCES

# Cloning and molecular characterization of *omp*31 gene of the Indian isolate of *Brucella melitensis*

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## Abstract

Brucellosis, caused by members of the genus *Brucella*, is an important re-emerging bacterial zoonosis and a significant cause of reproductive losses in animals. The genes encoding for the *Brucella* major *omp*31 has been extensively studied in reference and vaccine strains to determine their role in protection against infection. In this study, native major omps from field isolate of *Brucella melitensis* and *B. abortus* were extracted using N-lauryl sarcosinate. From original 2 gm (wet weight) of *B. abortus* and 1 gm (wet weight) of *B. melitensis* cells, about 5 mg (0.25%) and 2 mg (0.20%) of omp suspended in 1 ml of Tris HCl was recovered, respectively. Analysis of sonicated cells and sarcosyl extracted omps of *B. abortus* and *B. melitensis* revealed the presence of native *omp*31 in field isolate of *B. melitensis* by SDS-PAGE and Western blot, however, the *omp*31 gene was not present in *B. abortus* strain. With the exception of this, there was no any other significant difference in banding pattern and immunoreactivity between the two species both before and after sarcosyl extraction. The western blot analysis performed on native *omp* further confirmed that the *omp*31 protein is one of the major immunodominant proteins in *B. melitensis.* Confirmation of the absence of *omp*31 gene in *B. abortus* was also made by PCR amplification. The *B. melitensis omp*31 gene was PCR synthesized based on its ORF sequence and directly cloned to an entry vector. DNA sequence analysis revealed an open reading frame of 240 codons, and as compared to the consensus sequence of *Brucella omp*31 genes, six nucleotides have been replaced in the field isolate. The predicted sequence of *omp*31 showed a remarkable degree of similarity (97%) to the reported *omp*31 sequences of *Brucella* species and very less significant identity with *omp*s from other bacteria like *Yersinia enterocolitica omp*H, *E. coli* O157 *omp*W, and *Pseudomonas aeruginosa omp*M. Being the major *omp* in *B. melitensis* and *B. ovis* strains, *omp*31 might have a particular usefulness for vaccination against sheep and goat brucellosis.

**Keywords:** *Brucella melitensis,* cloning, *omp*31, sequencing, sheep, vaccine

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## Introduction

Brucellosis is a widespread zoonotic disease that still of veterinarian, public health and economic concern in many developing countries including India (Renukaradhya et al., 2002; Corbel, 2006; Mantur and Amarnath, 2008; Habtamu et al., 2013a; Karthik et al., 2013). The disease is caused by bacteria of the genus *Brucella*, Gram-negative, facultative, intracellular pathogens that can infect primarily of domestic animals, such as cattle, sheep, goats and pigs (Cutler and Whatmore, 2003). Although reported incidence and prevalence of the disease vary widely from country to country, bovine brucellosis caused mainly by *B. abortus* is still the most widespread form of the disease where as small ruminant brucellosis caused by *B. melitensis* is by far the most important clinically apparent disease in humans (Davis, 1990; England et al., 2004). Apart from conventional diagnostics, nowadays molecular tools like of polymerase chain

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reaction (PCR), real time PCR and loop mediated isothermal amplification (LAMP) are revolutionizing the detection of *Brucella* spp (Araj, 2010; Yu and Nielsen, 2010; Habtamu et al., 2013b; Dhama et al., 2013).

Control of brucellosis has commonly been performed by vaccination of animals using live attenuated *Brucella* strains; *B. abortus* S19 and RB51 for cattle, and *B. melitensis* Rev1 for small ruminants (Blasco, 1997). However, it has been reported that these vaccines have several drawbacks due to their capacity to produce underlying infections in animals and humans and to elicit antibodies against lipopolysaccharide (LPS), which interfere in the differential diagnosis between vaccinated and naturally infected animals (Moriyon et al., 2004). Thus, tests based on anti-LPS antibodies give false positive results because of its cross reactivity with other Gram negative bacteria like *Yersinia enterocolitica* O:9, *Escherichia coli* O157, *Pseudomonas aeruginosa,* and *Salmonella* species (Corbel, 1985; Weynants et al*.,* 1996). Therefore, the development of an effective vaccine which would avoid the drawbacks of live vaccine has been a challenge for many years. *Brucella* surface molecules, non-lipopolysaccharide, are good candidates for such a vaccine. Currently, interests in the *Brucella* major outer membrane proteins (*omp*s) have been stemmed from their potential usefulness as immunogenic and protective antigens (Zygmunt et al., 1994; Vizcaino et al., 2004). These proteins have been shown to be surface exposed by the use of monoclonal antibodies (MAbs) and techniques such as enzyme-linked immunosorbent assay (ELISA), immunoelectron microscopy and flow cytometry (Cloeckaert et al*.,* 2002).

The availability of recombinant *Brucella omp*s could be of great importance to determine their role in protection against infection. The genes coding for the *Brucella* major *omps* have been designated as *omp2a* and *omp2b* for the 36-38 kDa or group 2 proteins (Marquis and Ficht, 1993), *omp25* for the 25-27 kDa (Cloeckaert et al*.,* 1996) and *omp31* for the 31-34 kDa *omp* or group 3 proteins (Vizcaino et al*.,* 1996). Cloning of the *Brucella* species *omps* is found to be an interesting approach for the determination of their potential usefulness for the development of subcellular vaccines. Antibodies against *omp*31 have been detected in sheep naturally and experimentally infected with *B. melitensis* and have shown to protect mice against *B. melitensis* and *B. ovis* challenges (Zygmunt et al., 1994; Vizcaino et al*.*, 2004). These features have made *omp*31 as a potential and interesting candidate for the development of a subcellular vaccine against sheep and goat brucellosis.

## Materials and Methods

### Bacterial Strains and Vector

Field isolates of *Brucella melitensis* and *B. abortus,* isolated and identified using conventional and molecular techniques in the study period, were used for extraction of outer membrane protein, and cloning and sequencing of *omp*31 gene. *Escherichia coli* DH5α cells were obtained and revived from the repository of Division of Bacteriology and Mycology, IVRI. Other bacterial strains used in this study are listed in Table1. A T/A cloning vector pTZ57R/T (Fermentas, USA) was used for cloning and sequencing of the amplified gene.

**Table 1: Bacterial stains used in the study**

| Bacterium | Strain | Source |
|---|---|---|
| *B. melitensis* | 16M | Veterinary Public Health Division, IVRI |
| *B. melitensis* | Field strain | Sharanpur District, Nurud, UP |
| *B. abortus* | S19 | Division of Biological Product, IVRI |
| *B. abortus* | Field strain | IVRI |
| *E. coli* | DH5α | Bacteriology & Mycology Division, IVRI |

### Preparation and Harvesting of Cells

The field isolates of *B. melitensis* and *B. abortus* were propagated in test tubes containing 10 ml each of *Brucella* broth medium. The growth was checked for purity before being used for inoculation. About 5-6 ml of the suspension was then transferred into flasks containing 500 ml each of *Brucella* broth medium and incubated at 37$^{o}$C for 72 hours. The purity was again checked by microscopic examination and any flask showing contamination was discarded. The cell suspension was then inactivated by pressure steaming in autoclave by releasing the pressure cap for 1 hour at 90-100$^{o}$C. After cooling to room temperature, they were transferred into 50 ml tubes and centrifuged at 6,000 rpm for 25-30 minutes to settle down the cells. The supernatant was discarded and the pellet was washed twice with PBS (pH 7.2). The harvested cells were used for the downstream process or stored at -20$^{o}$C till use.

### Extraction of Outer Membrane Proteins

Cells of *B. melitensis* and *B. abortus,* harvested previously, were used for extraction of native outer membrane proteins (*omp*s) following the method described by Verstreate et al*.* (1982) with some modifications. Sediment bacterial cells approximately about 2 g of *B. abortus* and 1 g of *B. melitensis* were pooled and suspended at 1 g (wet weight) per 20 ml of Tris-HCl buffer (10 mM, pH 7.5), and 1 mg each of DNase and RNase (Sigma Chem. Co.) added per 100 ml. Disruption of the cells was performed by applying 20 jerks of 2 minutes each at 10 micron with 1 minute

alternative gap in MSE Soniprep 150 Sonicator. To maintain the low temperature, the tube containing cells were placed in ice throughout the entire process. The disrupted suspension was centrifuged at 6,000 rpm for 20 min at 4°C and the supernatant was re-centrifuged at 12,000 rpm at 4°C for 30 minutes to sediment the gross particles. The resulted supernatant was then ultracentrifuged at 100,000 x g for 90 minutes to pellet the crude membrane. The pellet was resuspended in 10 mM Tris HCl (pH 7.5) containing 10 mM HEPES with 2% N-Lauryl sarcosinate (Sigma Chem. Co.) and incubated at room temperature for 1 hour to solubilise the cytoplasmic membrane proteins. Clumps were broken up by pipetting up and down several times. The *omps* were then transferred into a 5 ml ultracentrifuge tube and pelleted at 100,000 x g for 60 minutes at 4°C. The pellet was washed twice and resuspended in 1 ml of Tris-HCl (10 mM, pH 7.5) and stored at -20°C till use.

**SDS-PAGE and Immunoblotting**

The extracted native *omp*s were subjected to electrophoresis on 10% SDS-PAGE according to the protocol described by Laemmli (1970) with slight modifications. The electrophoresed native outer membrane proteins were electrotransferred to a nitrocellulose membrane in a semi-dry transblot system (ATTO, Japan) following the protocol described by Coligan et al. (1994 ) with minor modifications. Hyper immune sera raised in rabbit against *B. melitensis* biovar 1(16M) (kindly provided by Division of Veterinary Public Health Laboratory, IVRI) was used as primary antibody during immunoblot analysis to confirm the presence of the major *omp*31.

**PCR Amplification of *omp31* Gene**

DNA extracted from *B. melitensis* and *B. abortus* field isolates, reference and vaccine strains by CTAB method as described by Wilson (1990), were used to amplify *omp*31 gene encoding an outer membrane protein 31 (*omp*31) using custom synthesized primer obtained from Sigma-Aldrich, USA (*omp*31 F: 5' GGATCC ATG AAG TCC GTA ATT TTG 3' and *omp31* R: 5' AAG CTT TTA GAA CTT GTA GTT CAG 3' with *Bam*HI and *Hind*III restriction sites at 5' end, respectively). The PCR was performed in a total volume of 25 µl with the reaction mixture containing 10x PCR buffer with 15 mM $MgCl_2$, 10 mM of each dNTPs, 3U/µl of Taq DNA polymerase (Baglore Genei, India), 10 pMol primers (Sigma-Aldrich, USA), and 90 µg/ml of template DNA. PCR programme was performed with initial denaturation at 94°C for 10 min followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 56°C for 1 min, extension at 72°C for 1 min and a final extension of 72°C for 10 min. The PCR amplified product(s) was analyzed by 1.2% agarose gel containing 0.5 µg/ml ethidium bromide along with 100 bp DNA ladder (MBI-Fermentas, USA) using 1x TAE electrophoresis buffer.

**Cloning**

The purified PCR product was ligated into pTZ57R/T Cloning Vector (Fermentas, USA) by T/A cloning strategy. The ligation reaction was set for 10 µl final volume in a 200 µl PCR tube as per the method prescribed by the manufacturer (InsT/Aclone™ PCR Product Cloning Kit, Fermentas, USA) and transformation of competent *E. coli* DH5α cells with ligation product was performed following the method described by Sambrook and Russell (2001) with slight modification. The transformed cell suspension was then plated on LB agar plates containing ampicillin (100 µg/ml), X-gal (40 µg/ml) and IPTG (30 µg/ml). These plates were incubated at 37ºC for 14 to 16 hours. The recombinant clone(s) harboring plasmid DNA with inserts were screened based on their ampicillin resistance, production of white colonies in X-gal containing LB agar plate, colony and plasmid PCR, and release of insert by RE digestion using *Bam*HI (G GATCC) and *Hind*III (A AGCTT) enzymes.

**Nucleotide Sequencing**

The recombinant clones were subcultured for overnight in LB medium with ampicillin (100 µg/ml) and was used for stab culture in LB agar slants. Subsequently, clones were sent for sequencing at DNA sequencing facility, Chromous Biotech, Banglore, India. The nucleotide and predicted amino acid sequences obtained were analyzed by homology search with other *omp*31 genes and gene products using Basic Local Alignment Search Tool (BLAST) and with Simple Modular Architecture Research Tool (SMART) program (http://smart.embl-heidelberg.de/). Further analysis of the sequence was carried out by aligning it with other *omp*31 genes of *Brucella* and other species using DNAStar programme.

## Results and Discussion

The native outer membrane protein of *B. abortus* and *B. melitensis* was extracted with N-lauryl sarcosinate after physical disruption of the cells by sonication. From original 2 g (wet weight) of *B. abortus* and 1 g (wet weight) of *B. melitensis* cells, about 5 mg (0.25%) and 2 mg (0.20%) of *omp* suspended in 1 ml Tris HCl (10 mM pH 7.5) was recovered, respectively.

Sonicated cells and sarcosyl extracted *omps* of *B. abortus* and *B. melitensis* were analyzed by SDS-PAGE and western blotting. The contrast between sonicated cells and sarcosyl extracted *omps,* after analysis by SDS-PAGE and Coomassie brilliant blue R-250 staining showed the presence of the major protein of

interest, *omp*31 (31kDa) in *B. melitensis* and its absence in *B. abortus* (Fig. 1). Similarly, western blot analysis of the electrophoresed proteins revealed the presence of 31 kDa in *B. melitensis* and its absence in *B. abortus* (Fig. 2). With the exception of this, there was no any other significant difference in banding pattern and immunogenic reactivity between the two species in both before and after sarcosyl extraction step described previously.

Moreover, PCR amplification of *omp*31 gene (723 bp) from previously extracted genomic DNA using specific oligonucleotide primers (Ashrafzadeh et al., 2009) confirmed the presence of this gene in *B. melitensis* and its absence in *B. abortus*. Similar findings have been reported previously by Vizcaino et al. (1997, 2001), Anon (2001), Salhi et al. (2003), Cassataro et al. (2004), Cutler et al. (2005) and Whatmore (2009).

Consequently, the amplified product obtained as a single band of 723 bp in *B. melitensis*, was used for cloning and sequencing of the gene. To clone *B. melitensis omp*31, the amplified product was ligated in pTZ57R/T cloning vector and transformed into competent *E. coli DH5α* cells. The recombinant plasmids harbouring the insert were confirmed by restriction digestion using *Bam*HI and *Hind*III restriction enzymes resulting in release of insert identical in size with amplified products as shown in agarose gel electrophoresis (Fig. 3).

The recombinant plasmid designated as pTZ57 *OMP*31 was used for nucleotide sequencing by di-deoxy chain termination method using universal M13 primers (Fig 4). Sequences have been published on NCBI gene database or Genbank (Accession No. HM543515) and analysed using Basic Local Alignment Search Tool (BLAST), Simple Architecture Research Tool (SMART) and MegAlign DNAStar Software programs. The search was done with the reported gene sequences available in Genbank, EMBL and DDBJ sequences using the non redundant database.

Nucleotide sequence analysis of *B. melitensis omp*31 gene showed 99% homology to *B. melitensis* strains (183, 63/9, 152, 293 and Ether), *B. suis* strain 1330 *omp*31 genes. A similar homology of 98.9% and 98.6% was found with *B. canis* strain RM6/66 and *B. melitensis* strain Rev1 *omp*31 gene complete DNA sequence, respectively. The strong nucleotide identity of *omp*31 genes between the field isolate *B. melitensis* and other *Brucella* species may be due to the high degree of genetic relatedness of these species (Gee et al., 2004).

A search for homologies of *omp*31 gene with other sequenced *Brucella omp* genes revealed 60.7%, 27.4%, 25.2%, 24.5% and 21.3% identity with *B. abortus* 544 *omp*31b, *B. abortus* S19 and *B. suis* 7 *omp*25, *B. melitensis* 16M *omp*2b, *B. melitensis* 019 *omp*2a and *B.*

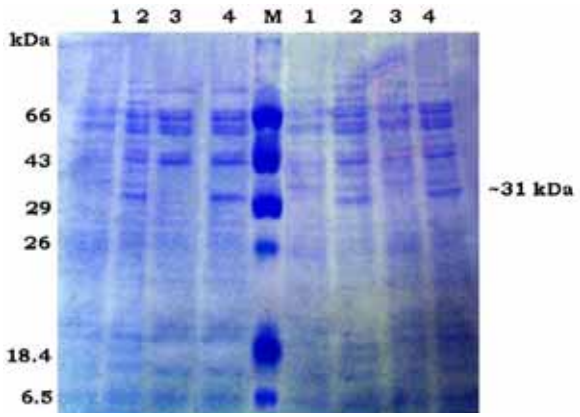


**Fig. 1: Analysis by SDS-PAGE and Coomassie brilliant blue R-250 staining of sonicated cells and sarcosyl extracted *OMP*s. Lane M: Pre-stained Protein molecular weight marker; Lane 1 & 2: Sonicated *B. abortus* and *B. melitensis* cells, respectively; Lane 3 & 4: Extracted *OMP*s of *B. abortus* and *B. melitensis*, respectively.**

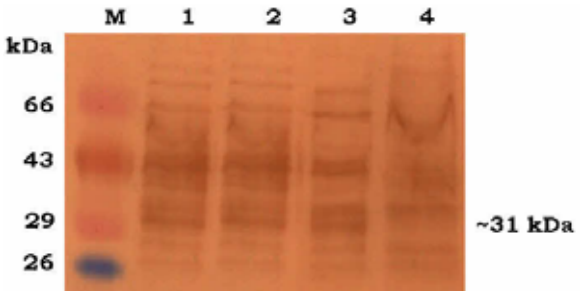


**Fig. 2: Immunoblot analysis of sonicated cells and *OMP*s after SDS-PAGE and transfer to nitrocellulose membrane. Lane M: Pre-stained protein molecular weight marker; Lane 1 & 2: Sonicated *B. melitensis* cells; Lane 3 & 4: Extracted *B. melitensis* and *B. abortus OMP*s, respectively.**

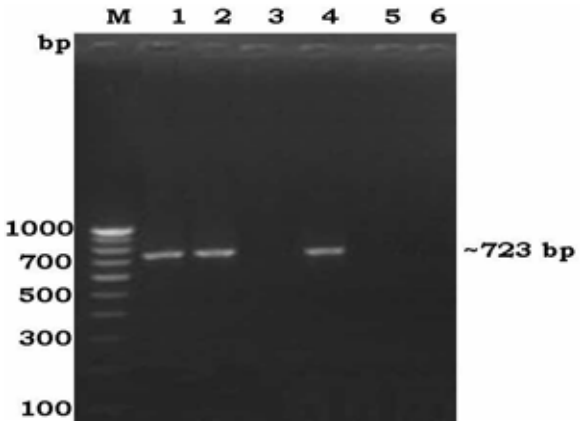


**Fig. 3: PCR amplification of *B. melitensis omp31* gene. Lane M: 100bp DNA ladder; Lane 1&2: *B. melitensis omp*31 fragment (723bp); Lane 3: Negative control; Lane 4: Positive control (*B. melitensis* 16M *omp31*); Lane 5&6: *B. abortus* (no *omp31*).**

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  1  ATGAAATCCG  TAATTTTGGC  GTCCATCGCC  GCTATGTTCG  CCACGTCCGC  TATGGCTGCC
 61  GACGTGGTTG  TTTCTGAACC  TTCCGCCCCT  ACTGTTGCTC  CTGTTGACAC  CTTCTCGTGG
121  ACCGGCGGCT  ATATCGGTAT  CAACGCCGGT  TACGCAGGCG  GCAAGTTCAA  GCATCCATTT
181  TCTAGCTTTG  ACAAGGAAGA  CAACGAACAG  GTTTCCGGTT  CGCTCGACGT  AACAGCTGGC
241  GGCTTCGTCG  GTGGTGTTCA  GGCCGGTTAC  AACTGGCAGC  TCGACAACGG  CGTCGTGCTC
301  GGCGCGGAAA  CCGACTTCCA  GGGATCGAGC  GTTACGGGTT  CGATTTCAGC  CGGTGCCAGC
361  GGTCTCGAAG  GCAAAGCTGA  AACCAAGGTC  GAGTGGTTCG  GCACAGTTCG  TGCCCGTCTT
421  GGCTACACGG  CTACCGAACG  CCTCATGGTT  TATGGTACCG  GCGGTCTGGC  CTATGGTAAG
481  GTCAAGTCTG  CGTTCAACCT  GGGTGATGAT  GCAAGTGCCC  TGCACACGTG  GTCCGACAAG
541  ACGAAAGCTG  GCTGGACCCT  CGGCGCTGGT  GCTGAATATG  CCTTCAACAA  CAACTGGACG
601  CTCAAGTCGG  AATACCTCTA  CCCCTACCTC  GGCAAGCGCA  ACCTCGTCGA  CGTTGACAAT
661  AGCTTCCTTG  AGAGCAAGGT  CAATTTCCAC  ACTGATCGCT  TCGGTCTGAA  CTACAAGTTC
721  TAA

MKSVILASIAAMFATSAMAADVVVSEPSAPTVAPVDTFSWTGGYIGINAGYAGGKFKHPFSSFDKEDNEQVSGSLDVTA
GGFVGGVQAGYNWQLDNGVVLGAETDFQGSSVTGSISAGASGLEGKAETKVEWFGTVRARLGYTATERLMVYGTGG
LAYGKVKSAFNLGDDASALHTWSDKTKAGWTLGAGAEYAFNNNWTLKSEYLYPYLGKRNLVDVDNSFLESKVNFHT
DRFGLNYKF
```

**Fig. 4: Nucleotide sequence of *B. melitensis omp31* and its predicted amino acid sequence**

*melitensis* Rev1 *omp*28, respectively. Low percentage homology was found with other bacteria *omp* gene sequences such as *Y. enterocolitica* cationic *omp*H 21.6%, *E. coli* O157: H7 *omp*W 22.4%, P. *aeruginosa ompM* 21.6%, *Mycobacterium bovis* strain AN5 *omp*A 24.1%, and *Mannheimia haemolytica* strain A7 *omp*31 24.8%; however a little more significant similarity (29.2%) was observed with *Rhizobium leguminosarum* bv. viciae strain VF39SM *RopB* gene sequence encoding a 22kDa outer membrane protein. The predicted amino acid sequence of *B. melitensis omp*31 revealed a length of 240 amino acids in which amino acids such as alanine (A), isoleuocine (I), threonine (T), aspartate (D) and valine (V) have been replaced by valine, phenylalanine (F), proline (P), tyrosine (Y), Aspartate and phenylalanine at 32, 195, 208, 209, 232 and 234 amino acid positions, respectively, as compared to the reported sequences of *Brucella omp*31 proteins (Fig. 5).

Similarities of 97.1% identity with *B. melitensis* strains (152 and 183) and *B. suis* strain 1330 *omp*31 were observed, while *B. canis* strain RM6/66 and *B. melitensis* strains (293, Ether and Rev 1) were found to be 97.6% similar with the field isolate of *B. melitensis omp*31.

According to a report by Vizcaino et al. (1996), *omp*31 revealed a significant predicted amino acid sequence homology (34.3%) with *omp*25 of *B. abortus* 544. Later, Salhi et al. (2003) showed around 67% amino acid sequence identity of *omp*31 with its counterpart *omp*31b and about 30% with *omp*25 in *B. melitensis* and *B. suis* strains. Similar findings were observed in the present study showing 61.8% and 28.6% amino acid sequence identity of *B. melitensis omp*31 with its counterpart *omp*31b and *omp*25 in *B. abortus* 544 and *B. suis* strain 63/290, respectively.

Moreover, comparison of amino acid sequence of *omp*31 with sequenced *omp*s from other bacteria such as *Y. enterocolitica* cationic *omp*H, *E. coli* O157: H7 *omp*W, *P. aeruginosa omp*M, *M. bovis* strain AN5 *omp*A, and *M. haemolytica* strain A7 *omp*31 using sequence pair distance revealed less significant similarity of 12.7%, 10%, 13.7%, 12.9% and 18.6%, respectively. However, *B. melitensis omp*31 amino acid sequence similarity with a 22kDa *omp*, designated as RopB, of *R. leguminosarum* bv. viciae strain VF39SM showed higher identity (25.1%). A related sequence homology (35.2%) has been reported previously by Roest et al. (1995) with the same protein (22kDa *omp*) in *R. leguminosarum* bv. viciae strain 248. The similarity found between the *Brucella* spp. and *R. leguminosarum* bv. viciae 248 proteins is not surprising, as hybridizations of rRNAs and of DNAs have shown the close phylogenetic origin of *Brucella* and bacteria of the *Agrobacterium-Rhizobium* complex (de Ley et al., 1987).

According to the hydrophobicity and antigenic index profiles of *omp*31, calculated by using Protean DNAStar software (Kyte and Doolittle, 1982), it could be inferred that the *omp*31 protein has putative surface exposure regions randomly distributed throughout the protein. The protective epitope recognized by MAbs could be located within a region covering the most hydrophilic part (51-74 amino acid sequence spanning) of the predicted protein that probably constitutes a surface-exposed portion of *omp*31. Although it has not been clearly defined, the epitope localization in this hydrophilic region would be in accordance with previous reports by Bowden et al. (1995) and Cloeckaert et al. (1990) showing that this epitope is surface exposed and highly accessible to antibodies.

In conclusion, findings of the present study revealed that *omp*31 gene encoding 31 kDa outer membrane protein found in all reported species of *Brucella* (except in *B. abortus*) was also present in Indian isolate of *B. melitensis* and the protein encoded by this gene found to have surface-exposed portion that would be highly accessible to antibodies. The western

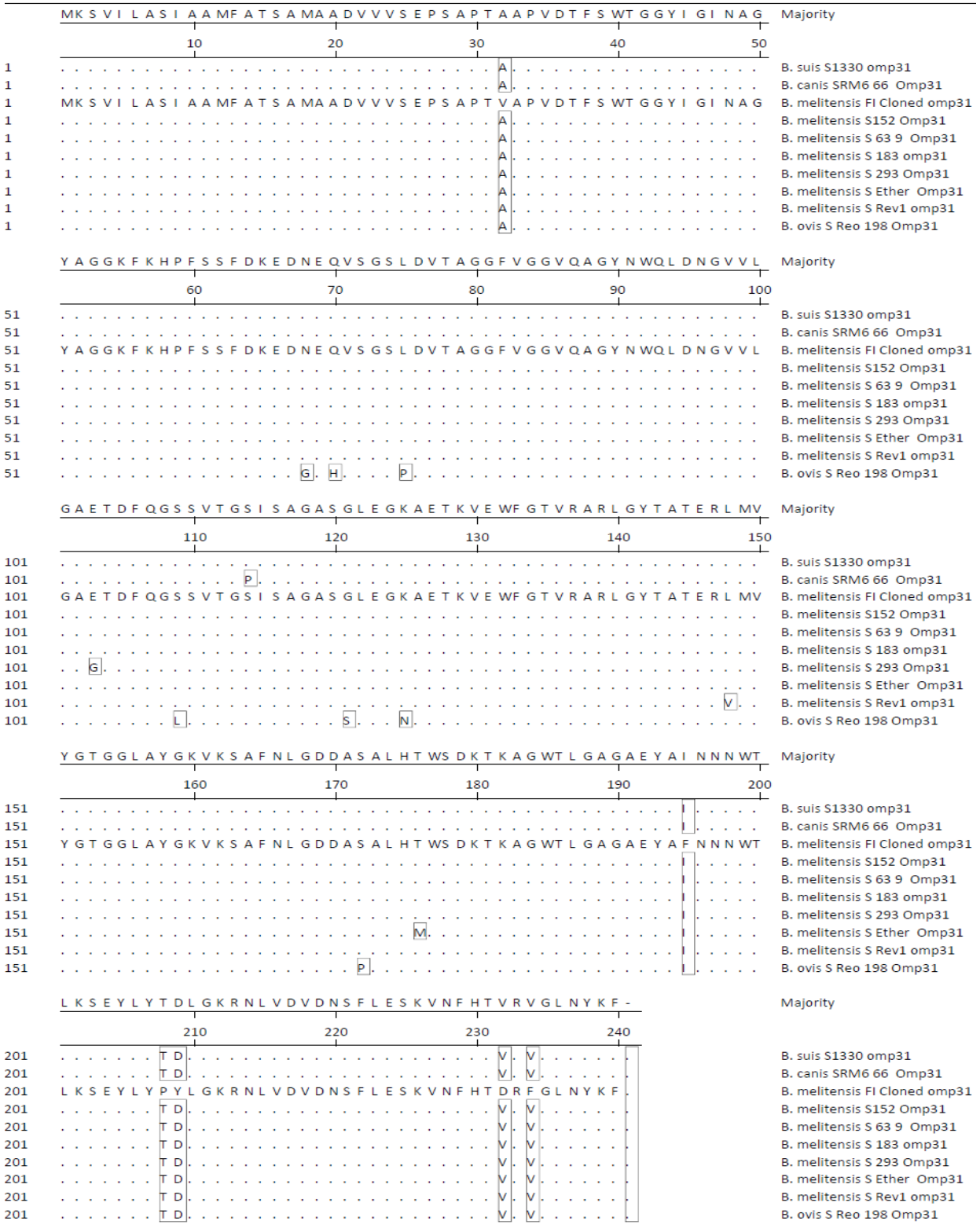


**Fig. 5: Amino acid alignment using Clustal V method: C*omp*arison of deduced amino acid sequences of *B. melitensis Omp*31 with some reported *Brucella Omp*31**

blot analysis performed on native *omp* further revealed that the *omp*31 protein is one of the major immunodominant proteins in *B. melitensis* as previously described by Cloeckaert et al. (1990) and Bowden et al. (1995), and might have a particular usefulness for vaccination against sheep and goat brucellosis and for developing new generation vaccines (Dhama et al., 2008; Vahedi et al., 2011). Recombinant proteins can also be used for the diagnosis of brucellosis by ELISA (Chaudhuri et al., 2010). This study is a preliminary step in production of an effective vaccine against *B. melitensis* using *omp* 31 protein. Immunogenicity study of the *omp* 31 protein was the keen interest of this study.

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