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Molecular characterization and Bioinformatics analysis of 31kDa major outer membrane protein gene of *Brucella melitensis,* Malaysian isolate

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Abstract

Brucellosis research is currently focused on molecular characterization of *omp31* gene from different Brucella species with the ultimate goal of development suitable, safe, and effective vaccine or specific diagnostic test. In this study, the molecular characterization, amino acid variations, protein structure and function of *omp31* from *B. melitensis,* Malaysian isolate was investigated by using Bioinformatics approach. The bacterium was reactivated on selective medium base. Then, the bacterial growth was examined by Gram stain and biochemical tests. The *omp31* gene, encoding a major outer membrane protein, was PCR-amplified by using primers selected according to the *B. melitensis* strain 152 published sequences. The nucleotide and predicted amino acid sequences were analysed, using Bioinformatics software and tools, which include domain analysis, homology search, multiple sequence alignment and constructing phylogenetic tree. The results show the cDNA sequence of *omp31* consists of 690 base pairs, which translated into its open reading frame (ORF) sequence of 229 amino acids in size. From the domain analyses, the *omp31* has beta-barrel structure that acts as a transporter for ions and small molecules, which cannot diffuse across a cellular membrane. Moreover, it has Beta-ketoacyl synthase domain that produces fatty acids of various lengths for use by the organism. The phylogenetic analysis of *omp31* and its homologous sequences revealed that *omp31* is conserved among all the *B. melitensis* strains and it has 100% similarity with *omp31* of *B. melitensis* M28 (Chinese strain). In conclusion, the *omp31* has high sequence homology with published sequences, suggesting that *omp31* of *B. melitensis* is highly conserved. The domain analysis provides further functionality of *omp31,* in addition to its antigenic characteristic. Accordingly, the *omp31* might be an interesting candidate for development of *omp31* based specific diagnostic test against goat Brucellosis caused by *B. melitensis*.

Keywords: *Brucella melitensis; omp*31; Bioinformstics tools; Brucellosis

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Introduction

Brucellosis, especially caused by *Brucella melitensis*, remains one of the most common zoonotic diseases in the world (Seleem et al., 2010; Adone and Pasquali, 2013). *B. melitensis* is a gram-negative, facultative and intracellular bacterium that has the ability to persist and replicate inside the host cells whilst evading its immune system (Celli and Gorvel, 2004). The species of *B. melitensis* possess cell envelope and it is morphologically similar to that of other Gram negative bacteria and it includes cytoplasmic membrane, the periplasmic space, a peptidoglycan layer and an outer membrane (Cloeckaert et al., 1992). The outer membrane proteins of Brucella species were first identified in 1980 by the detergent extraction of the cell envelope and classified according to their apparent molecular mass using SDS-PAGE into three groups. The first group is 88 or 94 kDa, the second group 36–38 kDa (omp2a and omp2b) porin proteins and the third group of 31–34 and 25–27 kDa protein (Winter, 1987). The *omp31* gene has proven useful in differentiating the Brucella species using PCR-RFLP and southern blot hybridization tests with the exception of *B. neotomae,* which could not be differentiated from *B. suis* biovars 1, 3, 4 and 5. It was also revealed that *B. abortus* lacks a large DNA fragment of about 25 kb covering the *omp31* and other genes (Vizcaino et al., 1997). Furthermore, the *omp31* was reported to be highly conserved except for 9 nucleotide substitutions in *B. ovis* compared to that of *B. melitensis*. These nucleotide substitutions resulted in different antigenic properties of *omp31* (Vizcaino et al., 2001). Accordingly, we planned to know the characterization of *omp31* from *B. melitensis,* Malaysian isolate, comparing with the others reported in the gene bank by using Bioinformatics approach, which includes protein structure, function, amino acid variations and evolutionary relationship, with the ultimate goal of developing specific diagnostic test or suitable, safe, and effective subunit vaccine against *B. melitensis.*

Materials and Methods

Bacterial strain

The *B. melitensis* VRI 6530/11, field strain, was obtained from the Biobank at a veterinary research institute, Ipoh, Malaysia. The bacterium was reactivated on selective medium base, containing Brucella selective supplement and inactivated horse serum (OXOID, UK). Bacterial growth on the medium was examined for purity and morphologically characterized using Gram stain. Biochemical tests on the culture, specifically Oxidase, Catalase, Indol, Nitrate, Citrate utilization, Urease, Hydrogen sulphide production and Nutrient gelatine liquefaction were performed according to the standard procedures (OIE, 2012).

Genomic DNA extraction

Total genomic DNA extraction was carried out using a DNeasy Blood & Tissue Kit, QIAGEN, Germany. Briefly, a single colony from the bacterial culture was inoculated into 10 ml of Brucella broth (OXOID), and incubated at 37°C with shaking at 200 rpm for 5-7 days. One ml of the cultured broth was then taken and placed in 2 ml Eppendorf tube, and centrifuged at 8000 rpm for 10 minutes. The supernatant was discarded and the pellet was resuspended in 200 µl PBS. Then, 20 µl of proteinase K was added to the suspension and mixed thoroughly. Two hundred microlitre of buffer AL (lysis buffer) was then added and mixed well to yield a homogeneous solution. The homogeneous solution was incubated at 56°C for 10 minutes. Two hundred microliter of ethanol 96% (v/v) was then added to the suspension, and mixed well by gentle vortexing before applying to a DNeasy column. The genomic DNA was bound in the column by centrifugation at 8000 rpm for 1 minute and the flow-through was discarded. The column was then washed by adding 500 µl of AW1 buffer, centrifuged at 8000 rpm and the flow- through was discarded. Washing was repeated using 500 µl of AW2 buffer, centrifuged at 14000 rpm for 3 minutes and the flow-through was discarded. The genomic DNA was then eluted into 1.5 ml microcentrifuge tube by adding 200 µl of AE buffer. The tube was incubated at room temperature and centrifuged at 8000 rpm for 1 minute.

Measurement of DNA concentration and purity

The concentration and purity of DNA-extracted from the pure bacterial colonies was determined spectrophotometrically by measuring its absorbance at wavelength A_{260} and A_{280} nm. A ratio between 1.7 and 2.0 indicates, the DNA is pure through preparation (Sambrook et al., 1989).

PCR amplification of *omp*31

The specific pair primer of *omp31* gene was designed based on the available nucleotide sequence on the NCBI and GenBank databases and was synthesized by Integrated DNA Technologies, Pte. Ltd, Singapore. The pair primer is forward ^{5}" ATTGCGGATCCAT GAAATCCGTAA^{3}' and reverse ^{5}'GGTACCTCGAG TTAGAACTTGTAGT^{3}. The amplification reaction mixture was prepared in volume of 25 µl, which comprised of 2.5 µl of 10× Top Taq PCR buffer, 5 µl of 5x Q-solution, 1 µl dNTP, 2 µl $MgCl_2$ (25mM), 1 µl TopTaq DNA polymerase, 1 µl from each primers (20 pmol/µl), 2µl of *B. melitensis* genomic DNA and 9.5 µl of nuclease free water. The temperature cycling for the amplification was performed using thermocycler, with initial denaturation 94°C for 5 minutes and for 35 cycles of denaturation (94°C for 1 minute), annealing (49°C for 1 minute) and extension (72°C for 1 minute),

followed by a final extension at 72°C for 10 min. Following amplification, the PCR product was analysed using 1% agarose gel containing 0.5 µg/ml ethidium bromide along with 100 bp DNA ladder (1 St. BASE, Singapore) using 1x TBE electrophoresis buffer.

Amplicon extraction

QIA quick^{R} Gel extraction kit (QIAGEN, Germany) was used to extract the amplicon of *omp*31 from agarose gel. Briefly, the *omp*31 fragment from the agarose gel was excised with a clean, sharp scalpel. The size of the gel slice was minimized by removing extra agarose. The gel slice was then weighed in a colourless tube and 3 volumes of Buffer QG were added to 1 volume of gel (based on gel weight 100 mg ~ 100 µl). The gel slices were then incubated at 50°C for 10 min. To enhance the gel dissolving, the tube was vortexed every 2–3 min during the incubation period. The QIAquick spin column was placed in a 2 ml collection tube. The dissolved gel was applied to the QIAquick column, and centrifuged for 1 minute at 13,000 rpm, flow-through was discarded and QIAquick column was placed back in the same collection tube. This was repeated with 0.5 ml of Buffer QG added to the QIAquick column and centrifuged for 1 minute at 13,000 rpm. Then the flow-through was discarded and 0.75 ml of Buffer PE added to QIAquick column and centrifuged for 1 minute at 13,000 rpm. The flow-through was discarded and the QIAquick column was centrifuged for an additional 1 minute at 13,000 rpm. QIAquick column was placed into a clean 1.5 ml microcentrifuge tube. Fifty microlitre of Buffer EB (10 mM Tris·Cl, pH 8.5) was added to the center of the QIAquick membrane to elute DNA. It was allowed to stand for 1 minute and then the column was centrifuged at 13,000 rpm for 1 min. The purity of eluted DNA was determined by electrophoresis in 1% agarose gel.

Sequencing and bioinformatics analysis of *omp*31

The eluted DNA was sent for sequencing at MyTACG Bioscience Enterprise Company, Selangor, Malaysia. The nucleotide and predicted amino acid sequences were analysed, using Bioinformatics software and tools, which include:

1. Simple Modular Architecture Research Tool (SMART) and Protein family database (Pfam) version 29.0 were used for domain analysis (Bateman et al., 2004).
2. Basic Local Alignment Search Tool (BLAST) was performed for homology search of *omp*31 gene.
3. T-coffee software was used for constructing a multiple sequence alignment (http://tcoffee.crg.cat/apps/tcoffee/index.html).
4. PHYLogeny Inference Package version 3.69 was used for building Neighbor-Joining (NJ) phylogenetic tree (http://evolution.genetics.washington.edu/phylip.html) (Felsenstein, 1996).

Nucleotide sequence accession number

The *omp31* nucleotide sequence of *B. melitensis* VRI 6530/11, Malaysian isolate, has been deposited in the GenBank under the accession number KU693449.

Results

Nucleotide and protein sequences of *omp31*

The nucleotide sequence of *omp*31 comprises 690 bp and these nucleotides are encoded by 229 amino acids, as depicted in Figure 1.

Domain analysis of *omp*31 protein sequence

Based on Pfam database, one domain exists in the *omp31* protein sequence and its starting from residue 6 to 229 as shown in Table1. This domain belongs to outer membrane protein beta-barrel, a large beta-sheet that twists and coils to form a closed structure in which the first strand is bonded to the last by a hydrogen bond. The function of beta barrel structure is transporting the ions and small molecules that cannot diffuse across a cellular membrane into the bacterium.

In SMART database, three domains were identified; the first is Beta-ketoacyl synthase (PKS_KS) that starts from the residue 2 to 196. It is an enzyme that catalyses the condensation of malonyl-ACP in fatty acid biosynthesis pathway. The second is an autotransporter domain that started from residue 4 to 205. This family of autotransporter domain corresponds to the presumed integral membrane beta-barrel domain. The third domain is a G-patch that starts from residue 66 upto 132 and it is widespread among eukaryotes but absent in archaea and bacteria. All of these domains can be retrieved by using domain ID as shown in Table 1.

Identification of *omp31* homologous genes in protein database

From non-redundant protein sequence database, a total of 100 homologous protein sequences were retrieved from the SmartBLAST analysis. Some sequences were filtered out because they have either truncated or representatives in different Brucella species or other bacterial species. As a result, only 10 out of 100 *omp31* homologous sequences from non-redundant protein sequence database were chosen to be included in the subsequent analysis, and this is as shown in Table 2. The *omp31* protein sequence showed 100% homology with the reported *B. melitensis* strain M28. Whereas, 99% homology was found with *B. melitensis* that accession numbers ACV07678.1, WP004685017.1, ACQ84164.1, AAL54086.1 and ADC95625.1. A 97% homology was found with *B. melitensis* (ADL14702.1) and 64% homology was found with *B. melitensis* with accession numbers AAL51583.1, AEQ09179.1 and WP041596356.1 (Table 2).

>gene_*omp*31_ nucleotide sequence
1 TTAGAACTTG TAGTTCAGAC CGACGCGAAC AGTGTGGAAA TTGACCTTGC 51 TCTCAAGGAA GCTATTGTCA
ACGTCGACGA GGTTGCGCTT GCCGAGGTCG 101GTGTAGAGGT ATTCCGACTT GAGCGTCCAG TTGTTGTTGA
TGGCATATTC 151AGCACCAGCG CCGAGGGTCC AGCCAGCTTT CGTCTTGTCG GACCACGTGT
201GCAGGGCACT TGCATCATCA CCCAGGTTGA ACGCAGACTT GACCTTACCA 251TAGGCCAGAC
CGCCGGTACC ATAAACCATG AGGCGTTCGG TAGCCGTGTA 301GCCAAGACGG GCACGAACTG TGCCGAACCA
CTCGACCTTG GTTTCAGCTT 351TGCCTTCGAG ACCGCTGGCA CCGGCTGAAA TCGAACCCGT AACGCTCGAT
401CCCTGGAAGT CGGTTTCCGC GCCGAGCACG ACGCCGTTGT CGAGCTGCCA 451GTTGTAACCG
GCCTGAACAC CACCGACGAA GCCGCCAGCT GTTACGTCGA 501GCGAACCGGA AACCTGTTCG TTGTCTTCCT
TGTCAAAGCT AGAAAATGGA 551TGCTTGAACT TGCCGCCTGC GTAACCGGCG TTGATACCGA TATAGCCGCC
601GGTCCACGAG AAGGTGTCAA CAGGAGCAGC AGTAGGGGCG GAAGGTTCAG 651AAACAACCAC
GTCGGCAGCC ATAGCGGACG TGGCGAACAT
>gene_*omp*31_ protein sequence
1 MFATSAMAAD VVVSEPSAPT AAPVDTFSWT GGYIGINAGY AGGKFKHPFS SFDKEDNEQV 61 SGSLDVTAGG
FVGGVQAGYN WQLDNGVVLG AETDFQGSSV TGSISAGASG LEGKAETKVE 121 WFGTVRARLG YTATERLMVY
GTGGLAYGKV KSAFNLGDDA SALHTWSDKT KAGWTLGAGA 191 EYAINNNWTL KSEYLYTDLG KRNLVDVDNS
FLESKVNFHT VRVGLNYKF

Fig. 1: Nucleotide and protein sequences of *omp31*

Table 1: Omp31 domain analyses using Pfam and SMART databases. One domain was identified in Pfam and three domains were found by SMART

Database	Domain ID	Domain description	Positions
Pfam	PF 13605.2	OMP_b-brl Outer membrane protein beta-barrel domain	Start from 6 to 229
SMART	SM000825	PKS_KS Beta-ketoacyl synthase	Start from 2 to 196
	SM000869	Autotransporter	Start from 4 to 205
	SM000443	G_patch	Start from 66 to 132

Construction of multiple sequence alignment for omp31 protein sequence

The alignment of 10 homologous protein sequences with *omp31* of *B. melitensis* VRI 6530/11, gene _id_128 in the alignment, was constructed using T- coffee program that has an average length of 216 amino acids. The minimum length of sequences included in the analysis was 188 amino acids and the maximum length was 229 amino acids. The conserved region, and missing or variation of amino acid residues in the alignment are depicted in Figure 2.

Phylogenetic tree

Neighbor-joining phylogenetic tree for 10 *omp31* homologous protein sequences, from different *B. melitensis* strains, was constructed and it is largely resolved the members of *omp31* protein sequences into clade as depicted in Figure 3. The phylogenetic tree revealed that *omp31* domains are conserved among all the *B. melitensis* strains and it has 100% similarity with *omp31* of *B. melitensis* M28 (Chinese strain) (Figure 3).

Discussion

Sequencing of *omp31* from *B. melitensis* VRI 6530/11 strain revealed a 690 bp encoded by 229 amino acids that exhibiting typical features accounting for its outer membrane localization. In addition, the C-terminal end of the *omp31* protein sequence is a phenylalanine that seems to be essential for the efficient translocation of proteins across the bacterial outer membrane (Struyve et al., 1991).

From the domain analyses, the *omp31* has one domain belonging to the beta-barrel, which acts as a transporter for ions and small molecules (Schirmer, 1998; Wimley, 2003). We also noted Beta-ketoacyl synthase domain, which produce fatty acids of various lengths for use by the organism in energy storage and creation of cell membranes. These functions of *omp31* are an essential for cell survival (Witkowski et al., 2002; Khandekar et al., 2003). A search for homology between *omp*31 gene and other sequences reported in the gene bank revealed 100%, 99%, 99%, 99%, 99%, 99%, 97%, 64%, 64% and 64% sequence similarities with *B. melitensis* M28 (ADZ67646.1), *B. melitensis* (ACV07678.1), *B. melitensis* (WP004685017.1), *B. melitensis* (ACQ84164.1), *B. melitensis* bv. 1 str. 16M (AAL54086.1), *B. melitensis* bv. 1 str. M5 (ADC95625.1), *B. melitensis* (ADL14702.1), *B. melitensis* bv. 1 str. 16M (AAL51583.1), *B. melitensis* NI (AEQ09179.1) and *B. melitensis* (WP041596356.1) respectively. These findings are in agreement with others who reported the predicted sequence of *omp*31 showed a remarkable degree of similarity (97%) to the reported *omp*31 sequences of *Brucella* species (Vizcaino et al., 1996; Cloeckaert et al., 2002; Habtamu et al., 2013). The predicted amino acid sequence of *omp*31 revealed a length of 229 amino acids in which amino acids such as glutamine (Q), glycine (G), aspartic acid (D) and threonine (T), have been replaced by aspartic acid (D), serine (S) and alanine (A) at 60, 70, 84 and 93 amino acid positions, respectively, as compared to the reported sequences of *Brucella omp*31 proteins (Fig. 2). The substitutions in amino acids can

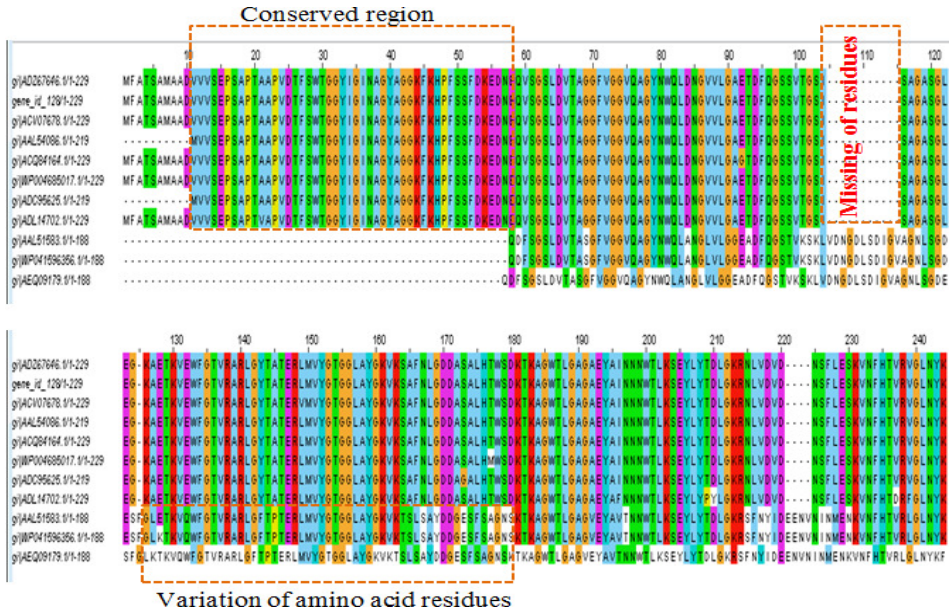

Fig. 2: Multiple sequence alignment of *omp31* protein sequence. The alignment consists of 10 sequences from different *B. melitensis* strains. Numbers at the up indicate amino acid positions. Information about the sequences is given in the Table 2.

Table 2: The homologous results of *omp31* protein sequence among *B. melitensis* strains that reported in the Gene bank and NCBI

No.	*B. melitensis* strain	Protein name	Gene bank and NCBI number	Sequence identity (%)
1	*B. melitensis* M28	Outer membrane 31KDa	ADZ67646.1	100%
2	*B. melitensis*	Outer membrane 31KDa	ACV07678.1	99%
3	*B. melitensis*	Hypothetical protein	WP004685017.1	99%
4	*B. melitensis*	Outer membrane 31KDa	ACQ84164.1	99%
5	*B. melitensis* bv. 1 str. 16M	31 kDa outer-membrane immunogenic protein precursor	AAL54086.1	99%
6	*B. melitensis* bv. 1 str. M5	31 kDa outer-membrane immunogenic protein precursor	ADC95625.1	99%
7	*B. melitensis*	Outer membrane 31KDa	ADL14702.1	97%
8	*B. melitensis* bv. 1 str. 16M	31 kDa outer-membrane immunogenic protein precursor	AAL51583.1	64%
9	*B. melitensis* NI	Outer membrane protein	AEQ09179.1	64%
10	*B. melitensis*	Membrane protein	WP041596356.1	64%

be used for differentiating Brucella species. These findings are in agreement with others who reported the *omp31* gene is an interesting gene for differentiating of Brucella species by using PCR-RFLP and southern blot hybridization tests. Exception of *B. neotomae,* which could not be differentiated from *B. suis* biovars 1, 3, 4 and 5. It was also revealed that *B. abortus* lacks a large DNA fragment of about 25 kb covering the *omp31* and other genes (Vizcaino et al., 1997). Moreover, the presence of conserved region in amino acid sequences in all the different strains of *B. melitensis* can support the development of *omp31* based specific primer for diagnosis of goat Brucellosis (Singh et al., 2013). The phylogenetic analysis also showed that *omp31* from *B. melitensis* VRI 6530/11, local isolate, was arranged in the same cluster with the others *omp31* sequences (Fig. 3). The strong similarity of *omp*31 genes among *B. melitensis* may be due to the high degree of genetic relatedness among these species (Gee et al., 2004). We also observed that *omp31* shows close homology (100%) to the *omp31* of *B. melitensis* M28, Chinese strain, this indicates a common ancestral evolutionary origin of these two isolates. It is known that geographical location plays an important role in the relationship between the bacteria, the isolates from similar regions tending to be more closely related (Liò and Goldman, 1998).

Conclusion

Based on Bioinformatics approach, the *omp31* has high sequence homology with published sequences, suggesting that *omp31* gene of *B. melitensis* is highly conserved. The domain analysis provides further functionality of *omp31,* in addition to its antigenic characteristic. Accordingly, the *omp31* might be an interesting candidate for development of *omp31* based specific diagnostic test for goat Brucellosis that caused by *B. melitensis.*

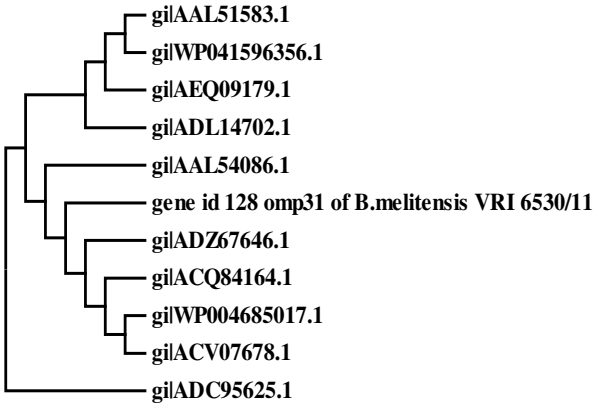

Fig. 3: Neighbor-Joining (NJ) phylogenetic tree of *omp31* protein sequences. Tree was viewed by using MEGA4 software. *B. melitensis* different strains are indicated on the tree branches.

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