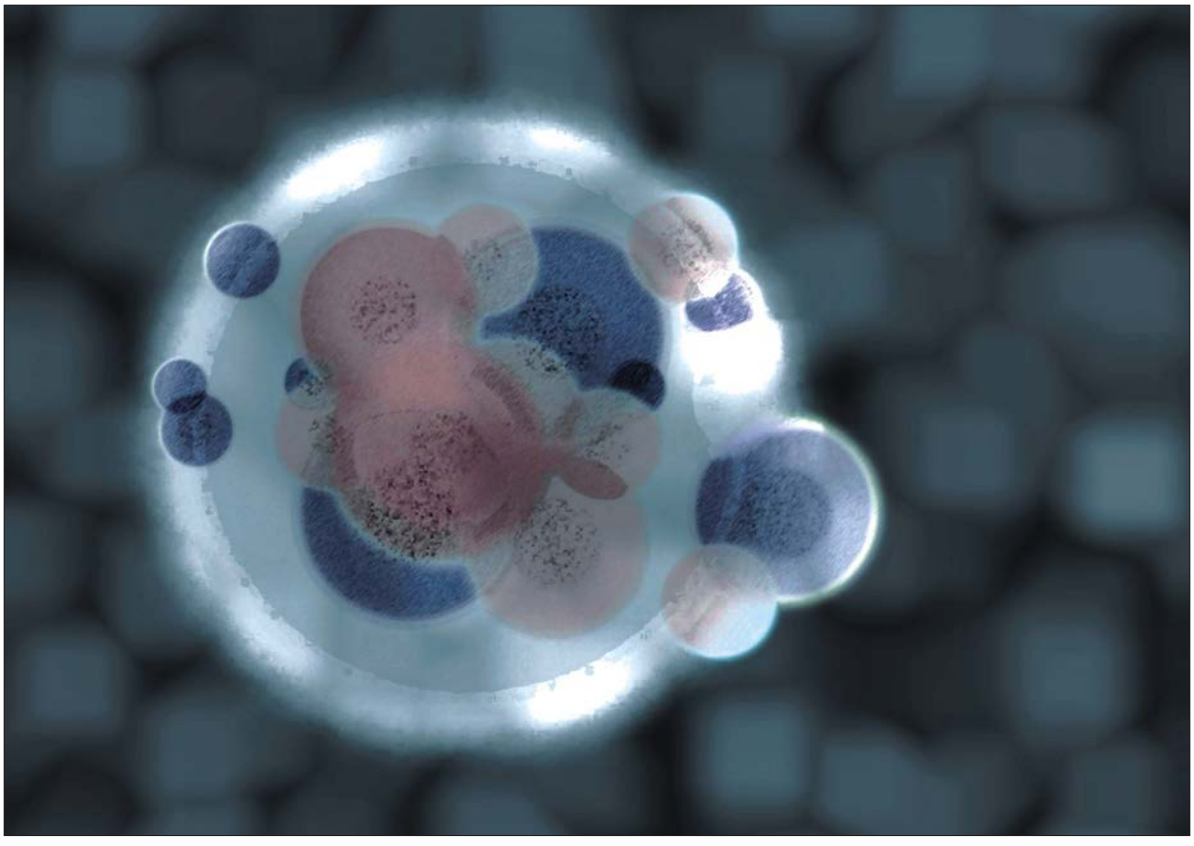

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Research Article

Antigenicity Analysis and Molecular Characterization of Two Outer Membrane Proteins of *Vibrio alginolyticus* Strain VA2 as Vaccine Candidates in Tiger Grouper Culture

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Abstract

Vibriosis is one of the most prevalent fish diseases caused by bacteria from the genus Vibrio. An effective method to counter Vibriosis is vaccination which increases the resistance of the immune system to subsequent pathogen infection. In this study, *Vibrio alginolyticus* strain VA2 isolated from infected tiger groupers was selected for determination of the antigenicity and molecular characterization of its Outer Membrane Proteins (OMPs). Protein profiling of OmpK and OmpW were evaluated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) while their antigenicity were tested by Western Blot. Results of the Western Blot assay revealed three antigenic OMP bands about 23, 31 and 34 kDa. Sequencing results showed that the full length of the target genes OmpK and OmpW of *V. alginolyticus* strain VA2 were 846 and 642 bp, respectively. Multiple alignment analysis showed that both regions of OmpK and OmpW were highly conserved among *Vibrio* species, thus are good candidates for vaccine development. Phylogenetic analysis of OmpK and OmpW showed that most branches in which they were grouped with had bootstrap values higher than 50%, indicating high confidence level of the branches. The antigenic sites of OmpK and OmpW proteins were predicted which were 34 and 27, respectively, thus are good candidates for vaccine development.

Key words: Antigenic, conserved protein, vaccine, antigenicity, cloning, OmpK, OmpW

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Tiger grouper (*Epinephelus fuscoguttatus*) are one of the high valued and important species of the marine aquaculture industry in the Asia Pacific region (Harikrishnan *et al*., 2010; Lin *et al*., 2012; Shapawi *et al*., 2014). However, diseases attacking marine animals including Tiger grouper are becoming a limiting factor in this industry. One of the most prevalent fish diseases is Vibriosis, caused by the bacteria from the genus Vibrio, including *Vibrio alginolyticus* (Cai *et al*., 2010; Harikrishnan *et al*., 2010; Liu *et al*., 2011). Diseases are prevented and treated by various strategies. Aside from the common practices of good disease management, such as practicing strict hygiene, avoiding stress and maintaining a good water quality; antibiotics are often used to treat diseases. Antibiotics can, however, be rendered ineffective due to misuse, resulting to the development of resistant strains. Another alternative strategy would be vaccination which has been shown to elevate the antibody level, induce immune memory and significantly increase the survival of infected fish (Defoirdt *et al*., 2007). *Vibrio alginolyticus* is a Gram-negative marine bacterium from pathogenic *Vibrio* species which is able to infect human and marine animals. As a Gram-negative marine bacterium, the outer membrane, consisting of protein, lipid and sugar (Qian *et al*., 2007) is present in *V. alginolyticus*'s cell wall structure. The Outer Membrane Proteins (OMPs), expressed in Gram-negative bacteria, play an important role in the interaction between the bacteria and the host during infection (Qian *et al*., 2008a; Cai *et al*., 2013) due to its components being easily recognized as foreign substances by the immune defense system of the hosts (Ningqiu *et al*., 2008; Qian *et al*., 2008b). Outer Membrane Proteins (OMPs) are highly immunogenic because of the exposed epitopes on the cell surface (Lun *et al*., 2014).

There are recent studies showing the role of the OMPs in protective antigenicity (Qian *et al*., 2008a). Outer membrane protein K (OmpK), common among the species of the family Vibrionaceae, is a receptor for the broad-host-range vibriophage KVP40 in *V. parahaemolyticus* (Ningqiu *et al*., 2008) whilst, OmpW belongs to a small molecular OMPs family common in Gram-negative bacteria. It is involved in transporting small hydrophobic molecules across the bacterial outer membrane (Mao *et al*., 2007) and also functions in osmoregulation of the bacteria which is required to regulate the environmental salt (Qian *et al*., 2007). The present study is focused on evaluating the antigenicity of the OmpK and OmpW proteins of local *V. alginolyticus* strain VA2 isolated from infected grouper and molecular characterization of OmpK and OmpW genes to determine its potential to serve as a vaccine candidate for Tiger grouper culture.

MATERIALS AND METHODS

Bacterial culture and growth conditions: *Vibrio alginolyticus* strain VA2 isolated from diseased tiger grouper (*Epinephelus fuscoguttatus*) was provided by the National Fish Health Research Centre (NaFisH) in Penang, Malaysia. The strain was cultured on Thiosulphate-citrate-bile salts-sucrose (TCBS) agar (Difco, USA) and then maintained in Tryptic Soy Broth (TSB) (MERCK, Germany)+1.5% NaCl containing 20% glycerol at -80°C. *Escherichia coli* TOP10 (Invitrogen, USA) was used as the cloning host to conserve the cloned OMP genes for sequencing and was grown in Luria Bertani (LB) broth and LB agar (Conda, Spain) with ampicillin (50 μg mL^{-1}).

The *V. alginolyticus* VA2 strain was identified based on colony morphology and DNA sequencing using 16S rRNA and Internal Transcribed Spacer (ITS) genes. The DNA extraction was carried out from the overnight culture using the GeneJET Genomic DNA Purification Kit (Thermo Scientific, USA) according to the method recommended by the manufacturer. Extracted genomic DNA was then measured for its DNA concentration by its absorbance at A_{260} nm/A_{280} nm (BioPhotometer plus, Eppendorf, Germany).

Antigenicity analysis of Outer Membrane Proteins (OMPs)

Preparation of formalin killed cells (FKC) of *V. alginolyticus* strain VA2: *Vibrio alginolyticus* strain VA2 was cultured on TCBS agar and in TSB broth+1.5% NaCl and incubated for 16 h at 30°C. The bacterial culture was then harvested by centrifuging at 800 x g for 10 min. The pellets were washed with Phosphate Buffer Saline (PBS) three times and then killed with 0.5% (v/v) formalin. The suspension was incubated for 24 h at 4°C to kill the bacteria. Zero growth on TCBS agar indicates complete bacterial inactivation. The bacterial cell concentration was modulated to 1×10^8 CFU mL^{-1}.

Preparation of antiserum: Rabbit antiserum against *V. alginolyticus* was used to detect the OMP proteins in *V. alginolyticus*. A white rabbit was immunized by 1 mL of the formalin-killed whole cells of *V. alginolyticus* strain VA2, emulsified with Freud's complete adjuvant (Sigma, USA) at ratio 7:3. Booster doses of 1 mL FKC in Freud's incomplete adjuvant (Sigma, USA) at ratio 7:3 respectively were given on day 14 and day 21. On day 28, the rabbit's blood was collected, clotted at room temperature for 1 h and stored at 4°C overnight. The blood was then centrifuged to separate and obtain the rabbit antiserum against *V. alginolyticus* and stored at -20°C.

Isolation and purification of the outer membrane proteins (OMPs): The OMP isolation and purification from *V. alginolyticus* strain VA2 was done according to the method by Gatewood *et al.* (1994) and Sabri (1999) with minor modifications. The OMP protein obtained was stored at -20°C. Protein concentration was determined by Bradford assay.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE): The profiles of outer membrane proteins (OMPs) were examined using SDS-PAGE. Protein sample was mixed with SDS loading buffer with the ration of 1:3 and boiled for 10 min. 4% stacking gel and 10% resolving gels were used to run SDS-PAGE in the Bio Rad Mini PROTEAN Tetra Cell (Bio-rad, USA). After electrophoresis, the gels were stained with coomassie brilliant blue R250 (Fisher Scientific, UK) and destained after a few hours with destaining solution to visualize the protein bands.

Western blotting analysis: The Western blotting analysis was performed according to the standard protocol by Sambrook and Russell (2001). After SDS-PAGE, proteins bands on the polyacrylamide gel were transferred onto a nitrocellulose membrane (Bio-rad, USA) using protein electrotransfer by BioRad Mini-PROTEAN II Cell (Bio-rad, USA) at 100 V for 100 min. After electrotransfer, the nitrocellulose membrane was stained with Ponceau S solution (Sigma, USA) to visualize the protein bands. The membrane was then washed three times (10 min each) with PBS/T (PBS with 0.5% Tween-20) and incubated in blocking buffer (PBS/T+1% BSA) for 90 min. After washing again with PBS/T, the membrane was incubated in polyclonal rabbit antiserum against *V. alginolyticus,* with blocking buffer diluted 1:75 for 18 h in 4°C. After washing again with PBS/T, the membrane was incubated in horseradish-peroxidase-labelled goat anti-rabbit immunoglobin G (IgG) antibodies (Thermo Scientific, USA), diluted 1:1000 in PBS for 2 h. After final washing with PBS/T and TBS, the immunoactivity was analyzed by dipping the membrane in DAB substrate solution (Calbiochem, USA) for colour development. The reaction was stopped by rinsing the membrane with distilled water.

Polymerase Chain Reaction (PCR) of OmpK and OmpW genes: The primers with Ligation Independent Cloning (LIC) extension were used to identify and amplify the full-length Open Reading Frame (ORF) of the OmpK and OmpW genes. The primers were designed according to the sequences of the most conserved regions of OmpK and OmpW from other *Vibrio* species in the GenBank (Table 1).

Polymerase Chain Reaction (PCR) was carried out with a total of 25 µL PCR mixture: 5 µL of 5x taq polymerase buffer, 25 mM $MgCl_2$ solution, 10 mM deoxynucleotide triphosphate (dNTP) mix, 20 µM of each primer, 1.25 U of Taq polymerase (Promega, USA) and 50-100 ng of DNA template. PCR amplification was performed using a mastercycler (Eppendorf, Germany). The amplification conditions used in PCR were: Initial denaturation for 10 min at 94°C; 35 cycles of denaturation for 60 sec at 94°C, annealing for 60 sec at the annealing temperature, extension for 90 sec at 72°C; then 10 min at 72°C for further extension. The annealing temperatures were 52.8°C and 50°C for OmpK and OmpW genes, respectively. The PCR product was then analyzed using electrophoresis on a 1% agarose gel.

Cloning of the OmpK and OmpW genes: The PCR product was purified using the GeneJET Gel Extraction Kit (Thermo Scientific, USA). T4 DNA Polymerase Treatment created 5′ overhangs in the purified PCR products in the presence of dATP. The insert was then ligated into the pET-32 Ek/LIC Vector (Invitrogen, USA). This ligation mixture was then transformed with the Top10 chemically competent *E. coli* cells (Invitrogen, USA) by heat shock method. The bacteria were then incubated in SOC medium (Invitrogen, USA) at 37°C for 1 h with vigorous shaking, followed by plating the bacteria on LB agar containing antibiotic ampicillin (50 µg mL^{-1}). The colonies containing recombinant plasmids were identified by restriction enzyme analysis using enzymes KpnI and HindIII (Fermentas, Lithuania) and confirmed by PCR followed by plasmid sequencing (1st Base, Malaysia).

Bioinformatics analysis of OmpK and OmpW gene: The nucleotides and amino acid sequences were analyzed using Basic Local Alignment Search Tool (BLAST) program, http://blast.ncbi.nlm.nih.gov/Blast.cgi of NCBI, USA (http://www.ncbi.nlm.nih.gov). The sequence identities and similarities of the nucleotide sequence of the recombinant plasmids were compared to the existing ones in the GenBank

Table 1: Primers used in PCR for amplification of OmpK and OmpW of *Vibrio alginolyticus* strain VA2

Primers	Primer sequence (5′-3′)	Expected size (bp)
LIC_OmpK F	**GACGACGACAAG** ATGCGTAAATCACT	846
LIC_OmpK R	**GAGGAGAAGCCCGG** TTAGAACTTGTA	
LIC_OmpW F	**GACGACGACAAG** ATGAAAAAAACAAT	643
LIC_OmpW R	**GAGGAGAAGCCCGG** TTAGAACTTGTA	

F: Forward primer, R: Reverse primer. Bolded are the LIC sequences

Table 2: Strains and accession numbers of OmpK and OmpW protein used for alignment analysis

Strains for OmpK	Accession number	Strains for OmpW	Accession number
V. alginolyticus	AIG20833	*V. alginolyticus*	AIG20834
V. harveyi	ADB92035	*P. damselae*	ABB54458
V. parahaemolyticus	ACK36941	*V. parahaemolyticus*	WP017447044
V. vulnificus	ACK36940	*V. natriegens*	WP020335095
V. cholerae	WP001959278	*V. campbellii*	WP005428268

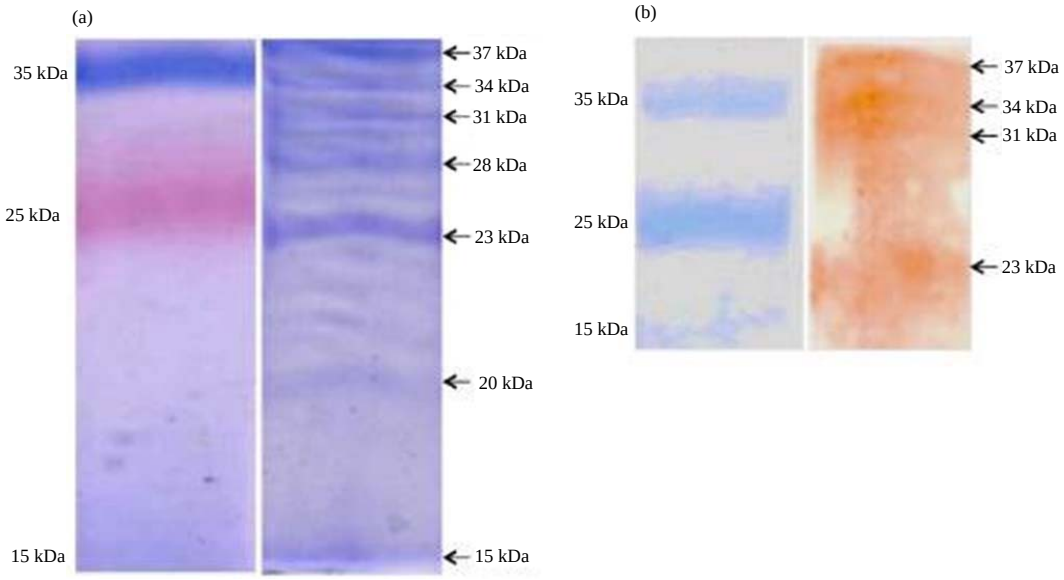

Fig. 1(a-b): (a) SDS-PAGE profiles of OMPs in *Vibrio alginolyticus* strain VA2 stained by Coomassie blue, (b) OMP antigenicity analysis by Western Blot. Membrane stained by DAB substrate solution. Protein ladder used was PageRuler Plus Prestained Protein Ladder (Thermo Scientific, USA)

NCBI database. ClustalW program was used to generate multiple sequence alignment of the OMP proteins (Table 2). Phylogenetic analysis was carried out by constructing phylogenetic trees using the Molecular Genetics Analysis (MEGA) software version 5.2 program, applying the test Neighbor-Joining Tree method and computed with the bootstrap value between 500 or 1000. The EMBOSS program, http://emboss.bioinformatics.nl/cgi-bin/emboss/antigenic was used to predict the antigenic sites in the OMP proteins.

RESULTS

Sodium Dodecyl Sulphate Poly Acrylamide Gel Electrophoresis (SDS-PAGE) and Western blotting analysis of OMPs: Outer Membrane Protein profiling by SDS-PAGE showed seven OMP bands that were clearly visible. The molecular weights were approximately 23 and 28 kDa for the major bands and 15, 20, 31, 34 and 37 kDa for the minor bands (Fig. 1a).

The Western blot analysis with the anti-whole cell of *V. alginolyticus* strain VA2 showed four antigenic OMP bands at approximately 23, 31, 34 and 37 kDa (Fig. 1b).

Cloning and characterization of OmpK and OmpW genes: By PCR and sequencing, the full length of OmpK and OmpW of *V. alginolyticus* strain VA2 sequences were identified which were 846 and 642 bp, respectively (Fig. 2). No bands were observed for the negative control lanes. For OmpK, the amino acid sequence consisted of 281 amino acid residues with a predicted molecular weight of 31.3 kDa. On the other hand, the amino acid sequence of OmpW consisted of 213 amino acid residues with a predicted molecular weight of 31.3 kDa. In restriction enzyme analysis, bands appeared at ~846 bp and ~642 bp (Fig. 3) which were the OmpK and OmpW inserts that had been cleaved from the vector by restriction enzymes *Kpn*I and *Hin*dIII. In addition, there were bands at the top of lanes 2-8 sizing ~5917 bp which were the pET32 Ek/LIC vector, without the insert OmpK or OmpW.

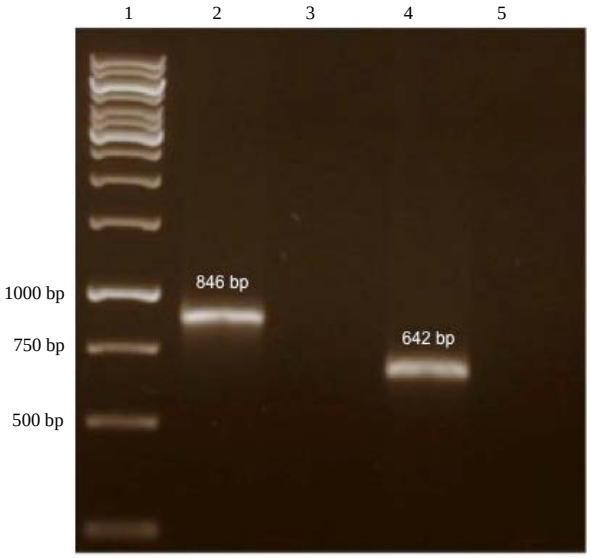

Fig. 2: Gel electrophoresis for Polymerase Chain Reaction (PCR) amplification of OmpK and OmpW genes of *Vibrio alginolyticus* strain VA2, Lane 1: DNA GeneRuler, 1kb DNA Ladder (Thermo Scientific, USA), Lane 2: PCR amplification of OmpK, Lane 4: PCR amplification of OmpW, Lane 3 and 5: Negative control of PCR amplification

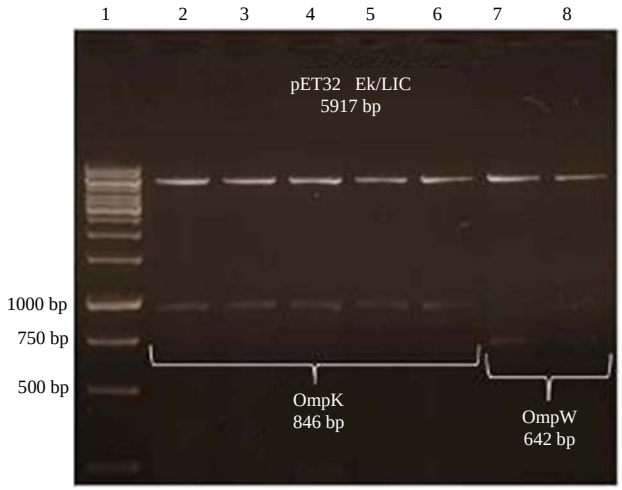

Fig. 3: Restriction enzyme (RE) analysis of recombinant plasmids using enzymes KpnI and HindIII, Lane 1: DNA GeneRuler, 1kb DNA Ladder (Thermo Scientific, USA), Lanes 2-6: The detection of pET32 Ek/LIC-OmpK recombinant plasmid digested with KpnI and HindIII, Lanes 7-8: The detection of pET32 Ek/LIC-OmpW recombinant plasmid digested with KpnI and HindIII. The top bands are the pET32 Ek/LIC vector and the lower bands are the OmpK and OmpW inserts which had been cleaved from the vector

The nucleotide and amino acid sequences for both OmpK and OmpW were deposited in the GenBank database (OmpK nucleotide accession no.: KJ930426; OmpK protein accession No.: AIG20833; OmpW nucleotide accession no.: KJ930427; OmpW protein accession no.: AIG20834).

Using BLASTn in GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi), it was found that the OmpK and OmpW genes from *V. alginolyticus* strain VA2 both have high similarities with the same species and with other *Vibrio* species (Table 3). The nucleotide identities of OmpK were 97% with

Table 3: Strains and GenBank accession numbers with high alignment similarities with OmpK and Ompw of *Vibrio alginolyticus* strain VA2

Strain name	GenBank accession no.	Percentage of similarity (%)
OmpK		
V. alginolyticus OmpK gene	DQ063588	97
V. alginolyticus strain EpGS020803 OmpK gene	GU318323	96
V. harveyi strain BsGR021101 OmpK gene	GU318332	96
V. parahaemolyticus strain 26075(B) OmpK gene	FJ462709	83
V. vulnificus strain 1.1758 OmpK gene	FJ462708	81
OmpW		
V. alginolyticus ATCC 17749	CP006719	99
V. alginolyticus OmpW gene	AY944132	99
P. damselae OmpW gene	DQ251175	91
V. parahaemolyticus strain VPL4-90 OmpW gene	JX555977	91
V. parahaemolyticus UCM-V493	FJ462708	91

Protein ladder used was PageRuler Plus Prestained Protein Ladder (Thermo Scientific, USA). Major protein band is ~45 kDa. OmpK and OmpW proteins appeared as minor bands which are approximately 31.3 and 23.3 kDa, respectively

V. alginolyticus, 96% with *Vibrio harveyi*, 83% with *Vibrio parahaemolyticus* and 81% with *Vibrio vulnificus*. As for OmpW, the identities were 99% with *V. alginolyticus* and 91% with *Photobacterium damselae* and *V. parahaemolyticus*. Multiple alignment analysis of the amino acid sequences of OmpK and OmpW from different *Vibrio* species showed that the amino acids were also highly conserved (Fig. 4).

Phylogenetic tree analysis showed that there were two main groups, G1 and G2. For OmpK, only *V. parahaemolyticus* strain FJ8 was in G2 while the rest of the strains were in G1. The *V. alginolyticus* strain VA2 was grouped with *V. alginolyticus* TrHS020801, EpGS020803 and *V. harveyi* BsGR021101 with a bootstrap value of 50%. They are similar to *V. alginolyticus* OmpK gene DQ063588 with a bootstrap value of 100%. As for OmpW, the OmpW gene of *V. alginolyticus* strain VA2 was clustered together with 3 other *V. alginolyticus* strains which are AY944132, GQ891116 and DQ075316, with a bootstrap value of 56%. The similarity of *V. alginolyticus* strain VA2 and strain AY944132 was 99% (Fig. 5).

Using the EMBOSS program, the antigenic sites of OmpK and OmpW protein sequences were predicted. The EMBOSS predicted 34 and 27 antigenic sites for OmpK and OmpW proteins respectively (Fig. 6).

DISCUSSION

There are some critical features for a molecule to be used in vaccine development. For instance, they should have high conservation between members of the same species, expressed on the surface of pathogens so that they are easily recognized by antigen presenting cells and should be immunogenic (Khushiramani *et al*., 2007). Because bacterial outer membrane proteins (OMPs) possess these features, they are a suitable vaccine candidate.

In this study, the OMPs of *V. alginolyticus* strain VA2 were isolated and purified using the sodium lauryl-sarcosinate method and the protein profiles were analyzed by SDS-PAGE. The profile of OMPs showed minor bands including the 15, 20, 31, 34 and 37 kDa bands while the 23 and 28 kDa were the major bands with higher intensity (Fig. 1a). The presence of major and minor bands shows that the OMP profiles are diversed. Two of these molecular weights revealed by SDS-PAGE, namely the 23 and 31 kDa were in agreement with the expected protein sizes of OmpW and OmpK, respectively by previous studies. The 23 kDa band is possibly the OmpW protein as the OmpW protein is predicted to be 23.3 kDa (Cai *et al*., 2013). On the other hand, the 31 kDa band is possibly the OmpK protein as the OmpK protein is predicted to be 31.3 kDa (Qian *et al*., 2008b).

From the Western blotting analysis, the polyclonal rabbit antiserum reacted with four of the OMP protein bands which are 23, 31, 34 and 37 kDa (Fig. 1b). The rabbit antiserum recognized these OMP proteins, showing that they are antigenic, able to induce immune response towards *V. alginolyticus*. The 23 and 31 kDa bands were postulated to be OmpW and OmpK proteins, respectively. There had been many previous studies showing that OMPs are considered as highly immunogenic (Maiti *et al*., 2009). In a study of Maiti *et al*. (2012), two OMPs from *Aeromonas hydrophila,* namely Aha1 and OmpW were shown to be antigenic. OmpW was considered a major antigen in bacterial infections and shown to be immunogenic in *V. cholera* (Kurupati *et al*., 2006). Qian *et al*. (2007) also reported the OmpW from *V. alginolyticus* to be immunogenic as it induced high antibody production in vaccinated large yellow croaker. Li *et al*. (2010) characterized OmpK as an immunogenic antigen. The OmpK of *V. harveyi*, *V. alginolyticus* and *V. parahaemolyticus* were able to give significant protection against the respective pathogens in large yellow croakers (Li *et al*., 2010). Meanwhile, Qian *et al*. (2008b) showed that OmpK from *V. alginolyticus* had immunoreactivity. Large yellow croakers vaccinated with OmpK were significantly protected against *V. alginolyticus* infection. The immunoreactivity results of this study agree well with the

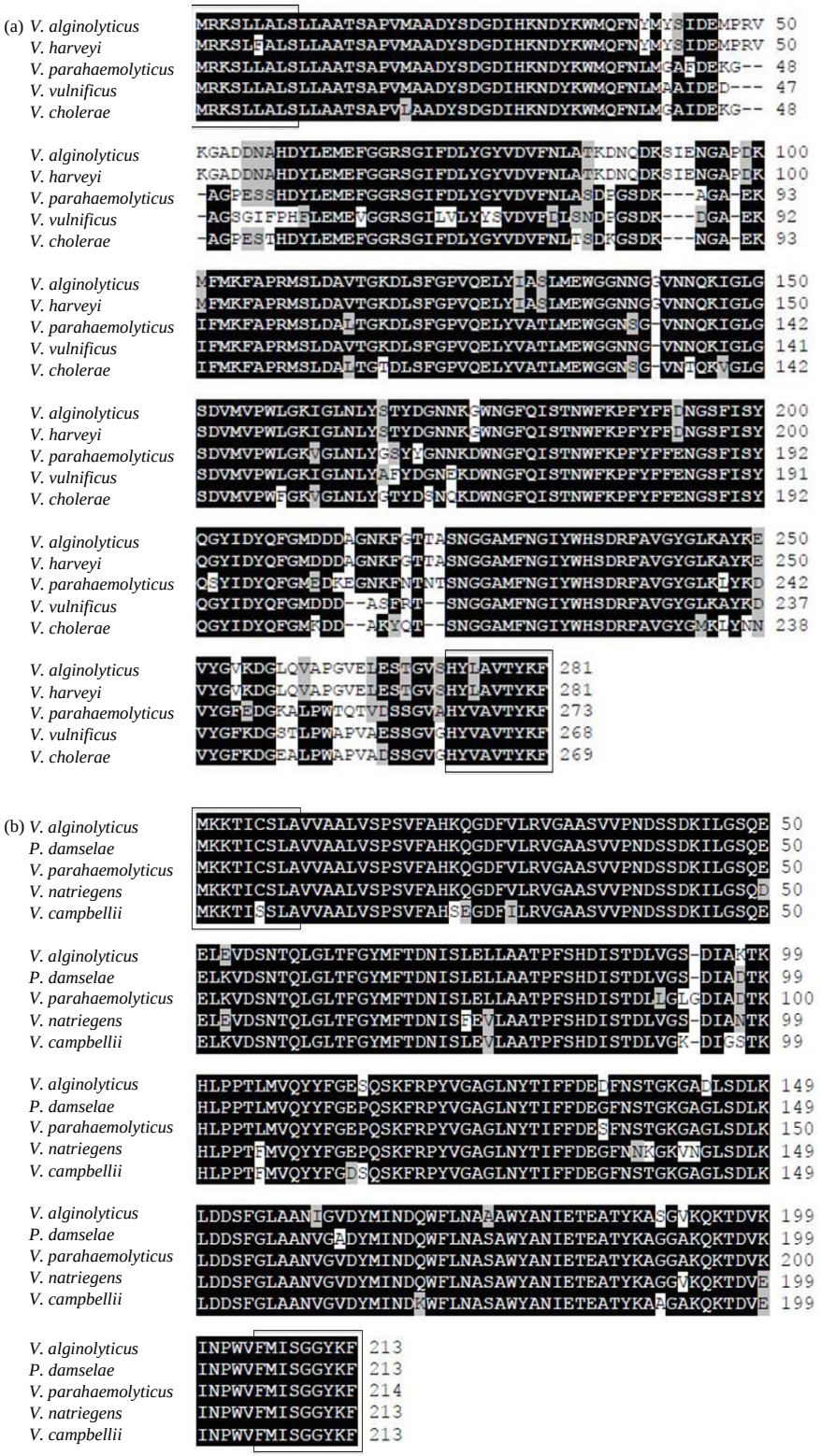

Fig. 4(a-b): Multiple alignment analysis of the amino acid sequence of (a) OmpK and (b) OmpW from *Vibrio alginolyticus* and other strains (listed in Table 2). ClustalW program was used to align the amino acid sequences. Black boxes indicate identical amino acid residues while gray boxes indicate similar residues. The rectangles show the gene portion used to design primers

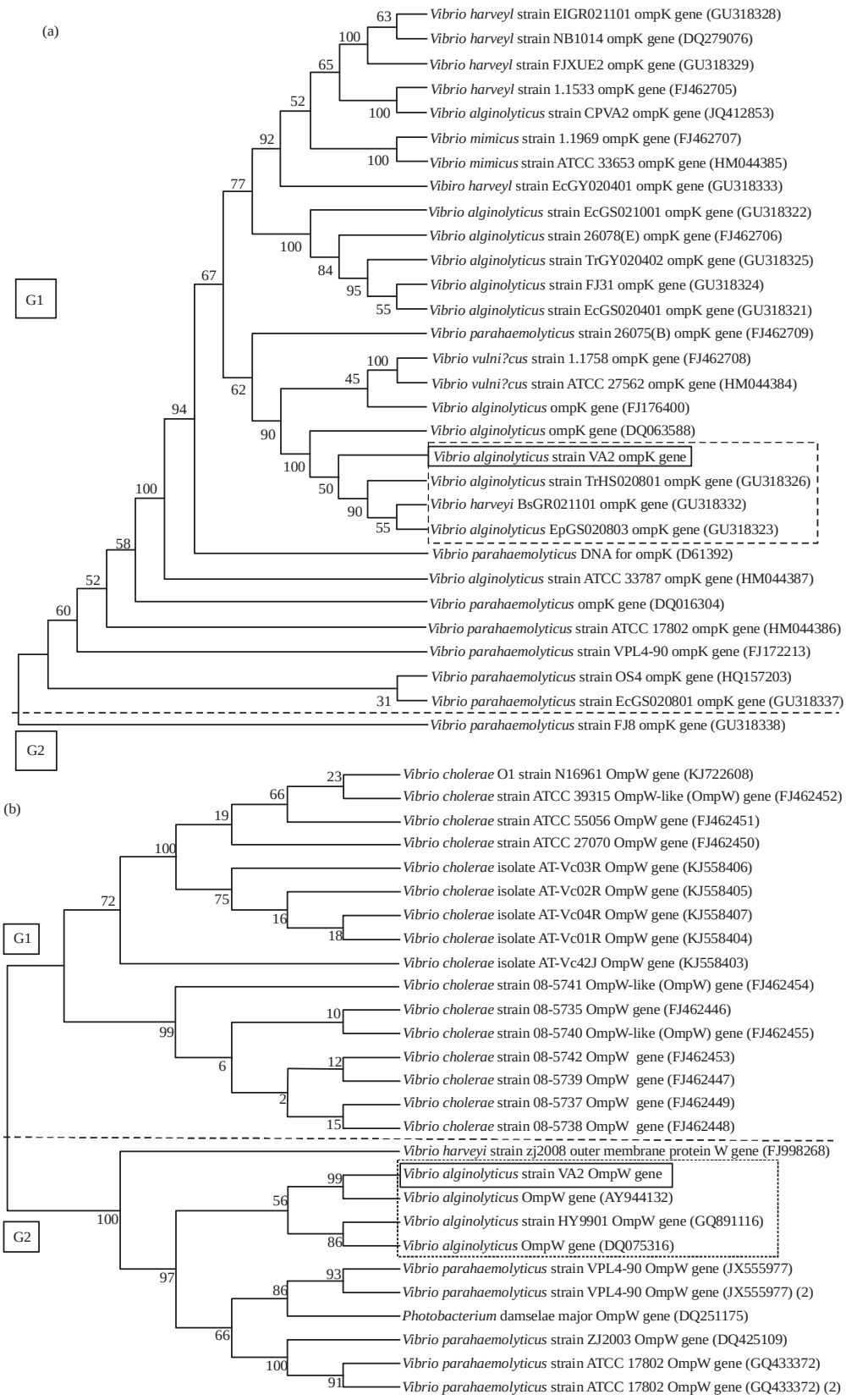

Fig. 5(a-b): Phylogenetic trees for (a) OmpK and (b) OmpW. Bootstrap analysis was performed using the neighbor-joining method. The bootstrap values are shown at the forks

(a)

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1    ATG CGT AAA TCA CTT TTA GCT CTT AGC CTT CTA GCG GCT ACT TCA GCT CCA GTT ATG GCG   60
1     M   R   K   S   L   L   A   L   S   L   L   A   A   T   S   A   P   V   M   A   20

61   GCA GAT TAC TCT GAC GGC GAT ATC CAC AAA AAC GAT TAC AAG TGG ATG CAG TTC AAC TAC  120
21    A   D   Y   S   D   G   D   I   H   K   N   D   Y   K   W   M   Q   F   N   Y   40

121 ATG TAC TCA ATT GAT GAA ATG CCA CGA GTT AAA GGC GCT GAC GAC AAC GCT CAC GAT TAC  180
41    M   Y   S   I   D   E   M   P   R   V   K   G   A   D   D   N   A   H   D   Y   60

181 CTA GAA ATG GAA TTT GGC GGT CGC TCT GGT ATT TTC GAC CTA TAC GGT TAC GTA GAC GTA  240
61    L   E   M   E   F   G   G   R   S   G   I   F   D   L   Y   G   Y   V   D   V   80

241 TTT AAC CTA GCG ACT AAA GAT AAC CAA GAT AAG TCA ATC GAG AAC GGC GCT CCT GAC AAG  300
81    F   N   L   A   T   K   D   N   Q   D   K   S   I   E   N   G   A   P   D   K  100

301 ATG TTC ATG AAA TTT GCA CCA CGT ATG TCT CTA GAT GCA GTA ACT GGT AAA GAC CTA TCT  360
101   M   F   M   K   F   A   P   R   M   S   L   D   A   V   T   G   K   D   L   S  120

361 TTC GGT CCA GTT CAA GAG CTA TAC ATT GCA TCT CTA ATG GAA TGG GGT GGT AAC AAC GGT  420
121   F   G   P   V   Q   E   L   Y   I   A   S   L   M   E   W   G   G   N   N   G  140

421 GGT GTA AAC AAC CAG AAA ATC GGT TTA GGT TCT GAT GTA ATG GTT CCT TGG TTA GGT AAA  480
141   G   V   N   N   Q   K   I   G   L   G   S   D   V   M   V   P   W   L   G   K  160

481 ATC GGC CTA AAC CTG TAC AGC ACT TAC GAT GGC AAC AAC AAA GGT TGG AAC GGT TTC CAA  540
161   I   G   L   N   L   Y   S   T   Y   D   G   N   N   K   G   W   N   G   F   Q  180

541 ATC TCG ACT AAC TGG TTC AAA CCA TTC TAC TTC TTC GAC AAT GGT TCA TTC ATT TCT TAC  600
181   I   S   T   N   W   F   K   P   F   Y   F   F   D   N   G   S   F   I   S   Y  200

601 CAA GGT TAC ATT GAT TAC CAA TTC GGT ATG GAT GAT GAT GCT GGT AAC AAA TTT GGT ACT  660
201   Q   G   Y   I   D   Y   Q   F   G   M   D   D   D   A   G   N   K   F   G   T  220

661 ACC GCG TCA AAC GGT GGT GCA ATG TTC AAC GGC ATT TAC TGG CAC TCA GAT CGC TTC GCG  720
221   T   A   S   N   G   G   A   M   F   N   G   I   Y   W   H   S   D   R   F   A  240

721 GTT GGT TAC GGC CTG AAA GCA TAC AAA GAA GTA TAT GGT GTA AAA GAC GGT CTA CAA GTC  780
241   V   G   Y   G   L   K   A   Y   K   E   V   Y   G   V   K   D   G   L   Q   V  260

781 GCT CCA GGT GTT GAG CTA GAA TCG ACT GGT GTA AGC CAC TAC CTA GCT GTA ACT TAC AAG  840
261   A   P   G   V   E   L   E   S   T   G   V   S   H   Y   L   A   V   T   Y   K  280

841 TTC TAA 846
281   F   *  282
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(b)

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1    ATG AAA AAA ACA ATC TGC AGT CTA GCA GTG GTT GCT GCA CTC GTG TCA CCA AGT GTT TTC   60
1     M   K   K   T   I   C   S   L   A   V   V   A   A   L   V   S   P   S   V   F   20

61   GCT CAT AAA CAA GGT GAC TTC GTT CTT CGT GTT GGT GCG GCG TCT GTC GTT CCA AAT GAC  120
21    A   H   K   Q   G   D   F   V   L   R   V   G   A   A   S   V   V   P   N   D   40

121  AGC AGT GAT AAG ATT CTT GGT TCT CAA GAA GAG TTA GAA GTT GAC TCA AAT ACG CAG CTT  180
41    S   S   D   K   I   L   G   S   Q   E   E   L   E   V   D   S   N   T   Q   L   60

181  GGT TTG ACG TTT GGC TAC ATG TTC ACA GAC AAC ATC AGT TTA GAG CTT CTA GCA GCA ACA  240
61    G   L   T   F   G   Y   M   F   T   D   N   I   S   L   E   L   L   A   A   T   80

241  CCA TTC AGC CAC GAC ATT TCG ACA GAT TTG GTT GGT AGT GAT ATC GCG AAA ACC AAA CAT  300
81    P   F   S   H   D   I   S   T   D   L   V   G   S   D   I   A   K   T   K   H  100

301  TTA CCA CCA ACG CTA ATG GTG CAG TAC TAT TTT GGC GAG TCT CAA AGT AAG TTC CGT CCA  360
102   L   P   P   T   L   M   V   Q   Y   Y   F   G   E   S   Q   S   K   F   R   P  120

361  TAC GTT GGT GCA GGT CTG AAC TAC ACC ATA TTC TTT GAT GAA GAT TTC AAT AGT ACG GGT  420
121   Y   V   G   A   G   L   N   Y   T   I   F   F   D   E   D   F   N   S   T   G  140

421  AAA GGC GCT GAC CTG TCA GAT TTG AAA TTA GAT GAT TCA TTC GGT CTA GCA GCG AAT ATT  480
141   K   G   A   D   L   S   D   L   K   L   D   D   S   F   G   L   A   A   N   I  160

481  GGT GTG GAT TAT ATG ATC AAC GAT CAA TGG TTC CTC AAC GCC GCT GCG TGG TAC GCA AAC  540
161   G   V   D   Y   M   I   N   D   Q   W   F   L   N   A   A   A   W   Y   A   N  180

541  ATT GAG ACA GAA GCA ACT TAT AAA GCA AGT GGA GTG AAG CAA AAA ACC GAC GTC AAA ATT  600
181   I   E   T   E   A   T   Y   K   A   S   G   V   K   Q   K   T   D   V   K   I  200

601  AAC CCT TGG GTA TTT ATG ATC AGC GGC GGT TAC AAG TTC TAA  642
201   N   P   W   V   F   M   I   S   G   G   Y   K   F   *  214
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Fig. 6(a-b): Antigenicity analysis of (a) OmpK and (b) OmpW gene sequences using EMBOSS. The gene sequence was submitted to http://emboss.bioinformatics.nl/cgi-bin/emboss/antigenic to analyze its antigenicity. The sequences underlined are the predicted antigenic sites in the genes

previous findings that OMPs are antigenic. However, Mao *et al*. (2007) found that the Omp TolC from *V. parahaemolyticus* may have less possibility to elicit antibody response. Immunization of fish with recombinant TolC elicited high antibody titre but the post-infection serum of survived fish failed to recognize the protein. This may be due to insufficient antibody response to be detected by the immune system.

Molecular characterization of OMPs has been investigated by many researchers. In this study, the sizes of the full length ORF of OmpK and OmpW of *V. alginolyticus* strain VA2 were 846 and 642 bp, respectively (Fig. 2). The PCR products of OmpK and OmpW were successfully cloned into the vector pET-32 Ek/LIC (Fig. 3).

The analysis of BLASTn showed that the OmpK and OmpW genes from *V. alginolyticus* strain VA2 both have high similarities with the same species and with other *Vibrio* species, showing that they are highly conserved among the *Vibrio* species (Fig. 4a, b). It is crucial for a vaccine antigen to be conserved between species so that the immune response to the antigen will work effectively against different strains of the species (Zhang *et al*., 2007), suggesting their suitability as vaccine candidates. A study by Qian *et al*. (2008a) and Hamod *et al*. (2012) showed that the OmpK and OmpW proteins were highly conserved. As a result, the conserved OmpK was able to give significant protection to vaccinated Indian major carp *Labeo rohita* against *V. anguillarum*, in which the antibody level against OmpK in the vaccinated fish was elevated and 67.8% Relative Percentage of Survival (RPS) of immunized fish showed high efficacy protection (Hamod *et al*., 2012). A study done by Nandi *et al*. (2000) showed that the OmpW protein was conserved in *V. cholera* strains, suggesting that the OmpW gene can be targeted for the identification of *V. cholera* strains using PCR.

For phylogenetic analysis of OmpK (Fig. 5a), most of the bootstrap values were more than 50% for every branch which indicates high confidence level of the branches formed. The OmpK gene of *V. alginolyticus* strain VA2 was not directly grouped with any other strains. This could be caused by changes in the nucleotide sequences between strains. However, it is grouped with 3 other strains which were similar 100% to the references strain *V. alginolyticus* OmpK gene DQ063588 which proofed that they have high similarity sequences. Although OmpK related proteins are present in the *Vibrio* species, there is variation in the nucleotide and amino acid sequences (Maiti *et al*., 2009). This can be seen from the distance between *V. alginolyticus* and other *Vibrio* species. As for OmpW, some of the bootstrap values in G1 were below 50% but in G2, all of the branches are more than 50% (Fig. 5b). This shows high confidence level because of the high nucleotide sequence similarity between the strains. The OmpW gene of *V. alginolyticus* strain VA2 was in G2, with 99% similarity with strain *V. alginolyticus* AY944132.

The antigenicity of OmpK and OmpW protein sequences predicted using EMBOSS showed high antigenicity values (Fig. 6a, b) indicating that both OmpK and OmpW proteins are potentially very antigenic and might be able to elicit immune response in vaccinated Tiger grouper. This result is in agreement of the antigenicity analysis by Western blot.

CONCLUSION

In conclusion, such antigenic conserved proteins, OmpK and OmpW have all the protective attributes to be used as vaccine candidates against infection by *V. alginolyticus*. Following this study, a protective efficacy will be evaluated in Tiger grouper and the antibody response of Tiger grouper against *V. alginolyticus* will be analyzed.

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REFERENCES

Cai, S.H., Y.S., Lu, Z.H. Wu, J.C. Jian, B. Wang and Y.C. Huang, 2010. Loop-mediated isothermal amplification method for rapid detection of *Vibrio alginolyticus*, the causative agent of vibriosis in mariculture fish. Lett. Applied Microbiol., 50: 480-485.

Cai, S.H., Y.S. Lu, J.C. Jian, B. Wang and Y.C. Huang *et al*., 2013. Protection against *Vibrio alginolyticus* in crimson snapper *Lutjanus erythropterus* immunized with a DNA vaccine containing the *ompW* gene. Dis. Aquat. Organ., 106: 39-47.

Defoirdt, T., N. Boon, P. Sorgeloos, W. Verstraete and P. Bossier, 2007. Alternatives to antibiotics to control bacterial infections: Luminescent vibriosis in aquaculture as an example. Trends Biotechnol., 25: 472-479.

Gatewood, D.M., B.W. Fenwick and M.M. Chengappa, 1994. Growth-condition dependent expression of *Pasteurella haemolytica* A1 outer membrane proteins, capsule and leukotoxin. Vet. Microbiol., 41: 221-233.

Hamod, M.A., M.S. Nithin, Y.N. Shukur, I. Karunasagar and I. Karunasagar, 2012. Outer membrane protein K as a subunit vaccine against *V. anguillarum*. Aquaculture, 354-355: 107-110.

Harikrishnan, R., C. Balasundaram and M.S. Heo, 2010. Molecular studies, disease status and prophylactic measures in grouper aquaculture: Economic importance, diseases and immunology. Aquaculture, 309: 1-14.

Khushiramani, R., S.K. Girisha, I. Karunasagar and I. Karunasagar, 2007. Cloning and expression of an outer membrane protein ompTS of *Aeromonas hydrophila* and study of immunogenicity in fish. Protein Exp. Purif., 51: 303-307.

Kurupati, P., B.K. Teh, G. Kumarasinghe and C.L. Poh, 2006. Identification of vaccine candidate antigens of an ESBL producing *Klebsiella pneumoniae* clinical strain by immunoproteome analysis. Proteomics, 6: 836-844.

Li, N., Z. Yang, J. Bai, X. Fu, L. Liu, C. Shi and S. Wu, 2010. A shared antigen among Vibrio species: Outer membrane protein-OmpK as a versatile Vibriosis vaccine candidate in orange-spotted grouper (*Epinephelus coioides*). Fish Shellfish Immunol., 28: 952-956.

Lin, Y.H., H.Y. Lin and S.Y. Shiau, 2012. Estimation of dietary pantothenic acid requirement of grouper, *Epinephelus malabaricus* according to physiological and biochemical parameters. Aquaculture, 324-325: 92-96.

Liu, H., Q. Wang, Q. Liu, X. Cao, C. Shi and Y. Zhang, 2011. Roles of Hfq in the stress adaptation and virulence in fish pathogen *Vibrio alginolyticus* and its potential application as a target for live attenuated vaccine. Applied Microbiol. Biotechnol., 91: 353-364.

Lun, J., C. Xia, C. Yuan, Y. Zhang, M. Zhong, T. Huang and Z. Hu, 2014. The outer membrane protein, LamB (maltoporin), is a versatile vaccine candidate among the Vibrio species. Vaccine, 32: 809-815.

Maiti, B., P. Raghunath, I. Karunasagar and I. Karunasagar, 2009. Cloning and expression of an outer membrane protein OmpW of *Aeromonas hydrophila* and study of its distribution in *Aeromonas* spp. J. Applied Microbiol., 107: 1157-1167.

Maiti, B., M. Shetty, M. Shekar, I. Karunasagar and I. Karunasagar, 2012. Evaluation of two outer membrane proteins, Aha1 and OmpW of Aeromonas hydrophila as vaccine candidate for common carp. Vet. Immunol. Immunopathol., 149: 298-301.

Mao, Z., L. Yu, Z. You, Y. Wei and Y. Liu, 2007. Cloning, expression and immunogenicty analysis of five outer membrane proteins of *Vibrio parahaemolyticus* zj2003. Fish Shellfish Immunol., 23: 567-575.

Nandi, B., R.K. Nandy, S. Mukhopadhyay, G.B. Nair, T. Shimada and A.C. Ghose, 2000. Rapid method for species-specific identification of *Vibrio cholerae* using primers targeted to the gene of outer membrane protein OmpW. J. Clin. Microbiol., 38: 4145-4151.

Ningqiu, L., B. Junjie, W. Shuqin, F. Xiaozhe, L. Haihua, Y. Xing and S. Cunbin, 2008. An outer membrane protein, *OmpK*, is an effective vaccine candidate for *Vibrio harveyi* in Orange-spotted grouper (*Epinephelus coioides*). Fish Shellfish Immunol., 25: 829-833.

Qian, R., W. Chu, Z. Mao, C. Zhang, Y. Wei and L. Yu, 2007. Expression, characterization and immunogenicity of a major outer membrane protein from *Vibrio alginolyticus*. Acta Biochimica Biophysica Sinica, 39: 194-200.

Qian, R., Z. Xiao, C. Zhang, W. Chu, Z. Mao and L. Yu, 2008a. Expression and purification of two major outer membrane proteins from *Vibrio alginolyticus*. World J. Microbiol. Biotechnol., 24: 245-251.

Qian, R.H., Z.H. Xiao, C.W. Zhang, W.Y. Chu and L.S. Wang *et al*., 2008b. A conserved outer membrane protein as an effective vaccine candidate from *Vibrio alginolyticus*. Aquaculture, 278: 5-9.

Sabri, M.M.Y., 1999. Immunology of the outer membrane proteins of *Pasteurella haemolytica* A2, A7 and A9 in sheep. Master Thesis, Universiti Putra Malaysia.

Sambrook, J. and D.W. Russell, 2001. Molecular Cloning: A Laboratory Manual. 3rd Edn., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY., USA., ISBN-13: 9780879695774, Pages: 2344.

Shapawi, R., I. Ebi, A.S.K. Yong and W.K. Ng, 2014. Optimizing the growth performance of brown-marbled grouper, *Epinephelus fuscoguttatus* (Forskal), by varying the proportion of dietary protein and lipid levels. Anim. Feed Sci. Technol., 191: 98-105.

Zhang, C., L. Yu and R. Qian, 2007. Characterization of OmpK, GAPDH and their fusion OmpK-GAPDH derived from *Vibrio harveyi* outer membrane proteins: Their immunoprotective ability against vibriosis in large yellow croaker (*Pseudosciaena crocea*). J. Applied Microbiol., 103: 1587-1599.