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Molecular characterization of *Vibrio harveyi* virulence-associated serine protease and outer membrane protein genes for vaccine development

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Abstract

Vibrio harveyi is the most pathogenic species associated with the infection in a wide range of marine species in aquaculture industries. A few virulence-associated genes have been discovered in *V. harveyi*. This study reports the cloning, sequence analysis and phylogenetic study of serine protease (VHS) and outer membrane protein (OMP) from a pathogenic *V. harveyi*, which isolated from a local outbreak of diseased tiger grouper. Molecular identification revealed that VHS and OMP consist of 1368 and 816 base pairs and encoded for mature peptides of 429 and 251 amino acids, respectively. The amino acids sequence identities of VHS was 100% similarity with protease of *V. harveyi* and OMP was 99% of membrane protein of *V. harveyi*, as compared to published sequence. Phylogenetic analysis and conserved domain search proposed that VHS is a serine endoprotease DegQ and OMP is an OmpK type. Signal peptide, transmembrane β-barrel and subcellular localization have supported the findings whereby demonstrated VHS belonged to periplasmic serine protease DegQ, composed one β-barrel and two α-barrels region. OMP displayed six β-barrels and twelve α-barrels suggesting it is belonged to outer membrane integral membrane protein, specifically act as a porin type outer membrane protein. Prediction of antigenic sites revealed that VHS composed 62 sites and OMP have 36 antigenic sites, assuming that they can provoke immune response of the infected hosts. In conclusion, it is strongly suggests that both genes can be potentially used for developing an effective live-attenuated vaccine candidate against vibriosis and further be applied in aquaculture industries.

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Introduction

Aquaculture sectors has established and fish remains among the most traded food commodities worldwide. In 2012, about 200 countries reported involved in exportation of fish and fishery products. Asia as a whole has been producing more farmed fish than wild catch since 2008, and its aquaculture share in total production reached 54 percent in 2012, with Europe at 18 percent and other continents at less than 15 percent. (FAO, 2014). Infection by pathogenic *vibrios* can lead to severe epizootics disease termed as vibriosis. Vibriosis is one of the major disease problems in aquaculture farming industries (Sarjito *et al.*, 2009). Among *Vibrio* species, *V. harveyi* is considered as a significant opportunistic pathogen that can infect a wide range of marine species, including both vertebrates and invertebrates (Nancy and Owens, 2013; Qin and Yan, 2010; Won and Park, 2008; Austin and Zhang, 2006). Common practice to control the vibriosis mainly depends on the application of antibiotics (Defoirdt, 2014; Zhou *et al.*, 2013; Plant and LaPatra, 2011). Unfortunately, a frequent use of antibiotics in aquaculture will lead to a resistance development in the pathogen and residual accumulation in tissue (Plant and LaPatra, 2011; Zhang *et al.*, 2007). Despite its long application success in preventing the diseases, usage of antibiotics to control this infection is no longer effective. In addition, as the health technical issues concerning a major aspects in food supply demand has become increased, usage of antibiotics in fish treatment are forbidden in some countries such as Europe, US and Japan (Ma *et al.*, 2008). This will restricted the expansion of export potential values and might results in a negative impacts for fish and fishery products industries.

Therefore, vaccination is one of the alternative for disease prevention, can be regarded as insurance policies and now recognized as a viable strategy for disease prevention in aquaculture industry (Plant and LaPatra, 2011). In fact, the application of vaccines has been found tremendously reducing the amount of drugs and chemicals in fish production (Shoemaker *et al.*, 2009). Currently, vaccines that commercially applied for use in fish treatment are mostly a killed attenuated vaccine and a DNA recombinant protein (Plant and LaPatra, 2011; Cheng *et al.*, 2010; Shoemaker *et al.*, 2009). However, this type of vaccines have several limitations such as their possibility to cause a non-target effects due to unspecific mutations of unknown target genes in killed attenuated vaccine, as well as the limitation of vaccination procedures by DNA recombinant protein which administered by injection (Frey, 2007). Thus, as an alternative, live attenuated vaccines provide more attractive vaccine strategy compared to other types of bacterial vaccines. There is live-attenuated vaccine that has successfully patented (Ma *et al.*, 2008) and some constructed mutants are being tested for the potential in developing multivalent fish vaccines (Zheng *et al.*, 2012; Zhao *et al.*, 2011). However more intention is being focused on *V. anguillarum* strains, none attempts have been made towards *V. harveyi*. With respect to this matter, development of live attenuated vaccine candidates against *V. harveyi* is a great promising prospect to cure vibriosis in fish. Unfortunately, its virulence mechanisms are still not fully understood. In order to further develop *V. harveyi* as a live attenuated vaccine candidate, it is vital to investigate its virulence-associated properties and their roles in pathogenicity mechanisms.

As described in previous researches, the pathogenicity of *V. harveyi* were reported to be related to a number of factors including the secretion of extracellular products containing substances such as proteases, haemolysins and lipases, phospholipase, outer membrane protein and adhesive factors (Cheng *et al.*, 2010; Sun *et al.*, 2009; Ningqiu *et al.*, 2008; Zhang *et al.*, 2008; Zhang *et al.*, 2007; Austin and Zhang, 2006). Few researches also have been emphasized on the roles of protease and outer membrane protein as a common virulence associated genes in the pathogenesis of *Vibrio* species (Defoirdt, 2014; Cheng *et al.*, 2010; Li *et al.*, 2010; Ningqiu *et al.*, 2008; Zhang *et al.*, 2008; Zhang *et al.*, 2007). In fact, it has been described that the major protein of OMP such as OmpK is widely distributed among *Vibrio* and

Photobacterium species (Zhang *et al.*, 2007). Therefore, this study aims to identify and characterize the virulence-associated serine protease and outer membrane protein genes from a local pathogenic *V. harveyi* strain. Identification of both virulence-associated genes was done by employing a molecular approach. Characterization of each genes were determined by using computational predictions based on the prediction of signal peptide, transmembrane protein, subcellular localization, antigenic properties and other related important properties. Data obtained from this study will be used to ascertain their potential to be exploited as a live-attenuated vaccine candidate against vibriosis caused by *V. harveyi*.

Materials and methods

Bacterial strains and growth conditions

V. harveyi used in this study kindly provided from National Fish Health Research Centre (NaFisH), Penang, Malaysia, which the strain previously isolated from diseased tiger grouper. This pathogenic strain was maintained and grown in Thiosulphate-citrate-bile-salts-sucrose (TCBS) (Oxoid, US) agar and Tryptone soya broth (TSB) (Oxoid, US) with addition of 1% (w/v) NaCl at 37°C. Strain *Escherichia coli* DH5α was grown in LB broth, Lennox (Difco, US) at 37°C and used as a host for general cloning purposes. LB agar, Miller (Merck, Germany) was also used for the purpose of plating the bacterial cells.

PCR amplification of VHS and OMP genes

Genomic DNA was extracted from bacterium genome by using *EasyPure*®Bacteria DNA kit according to the protocol outlined in the manual (Transgene Biotech Corporation, Beijing). The PCR reaction was performed by using *EasyTaq*®DNA Polymerase (Transgene Biotech Corporation, Beijing). The PCR component consists of 10X EasyTaq®buffer (5µl), 2.5mM dNTPs (4µl), 5U/µl *EasyTaq*®DNA Polymerase (0.5µl), 20µM primers (1µl each), crude DNA template (2µl) and a sterile distilled water added to make a 50µl PCR mixture. The primers used for each gene was as listed in Table 1.

Amplification procedures were carried out in a thermal cycler (DNA Engine Peltier Thermal Cycler Bio-Rad, UK). PCR amplification was performed at initial denaturation 94°C for 2 minutes, followed by 30 cycles consisting of denaturation at 94°C for 30 seconds, annealing temperature at 56°C (VHS gene) and 55°C (OMP gene) for 1 minute and extension at 72°C for 1 minute. In the last cycle, the final extension will be at 72°C for 5 minutes. Detection of the amplified products was analyzed by gel electrophoresis using 1X TAE buffer. The electrophoresis were carried out by using 0.8% agarose supplemented with RedSafe™Nucleic acid staining solution (Intron Biotechnology, Korea) which acts as a non-mutagenic fluorescence reagent for detecting nucleic acid in agarose gels and run at 80V for 45 minutes. The gels were visualized and photograph by using Gel Logic 1500 Imaging System Kodak (Kodak, US).

Cloning, transformation and screening of positive clones for encoded genes

The amplicons obtained from PCR amplification were resolved, isolated and purified by using *EasyPure*® Quick Gel Extraction kit (Transgene Biotech Corporation, Beijing) and cloned into TOPO®TA Cloning kit (Invitrogen, Carlsbard, CA). Ligation process was performed by adding a ratio of 3:1 concentration of the purified PCR product to a pCR 2.1-TOPO®TA vector, followed by incubation at room temperature for 20 minutes, as outlined by the instruction's manual. Transformation of the ligated cells was performed by adding ligation mixture (2µl) into the *E. coli* DH5α competent cells. The mixture was then incubated on ice for 5 minutes followed by heat-shock procedure at 42°C for 30 seconds without shaking and immediately transferred into ice. The LB broth (250µl) was added into the vial, incubated at 37°C and horizontally shaken for one hour.

Then, transformed cells (aliquot of 30, 50 and 100µl) were pipetted and spread onto three different LB agar plate. The LB agar plate was previously prepared and supplemented with 50µg/ml kanamycin and 40µl of X-gal (40µg/ml stock) for the purpose of blue-white screening. The positive recombinant clones were

screened and selected based on blue white screening prior PCR colony. Only white colonies that are able to grow on the LB-kanamycin medium were selected and screened by using PCR colony. The positive clones were then grown in LB broth supplemented with kanamycin proceeded with plasmid purification by using InnuPREP Plasmid Rapid kit (Analytik Jena, Germany). The purified plasmids were sent for commercial DNA sequencing (First Base Laboratories, Malaysia) by using universal primers of M13F (-20) and M13R-pUC(-26) under Sanger protocol.

Sequence analysis

The sequencing results obtained from sequencing were analyzed and assembled using bioinformatics analysis to obtain the full length sequence. The possible consensus sequences were assembled by using a CAP3 (Contig Assembly Program version 3) server (http//www.doua.prabi.fr/software/cap3) (Huang and Madan, 1999). The resulting DNA contig were analyzed and evaluated by online program using nBLAST analysis (Basic local Alignment tool) program (http://www.ncbi.nlm.nih.gov/BLAST) to determine the identities and the similarity of the determined nucleotide sequences. The homology searches of nucleotide and protein sequences of VHS and OMP were conducted with BLAST program. Open reading frame was predicted by using the ORF Finder server (http://www.ncbi.nlm.nih.gov/projects/gorf/orfig.cgi). The nucleotide sequence obtained was translated into protein sequence by using EXPASY translate tool server (http://web.expasy.org/cgi-bin/translate/dna_aa).

Putative signal peptides and motif were predicted by SignalP v4.1 server (http://www.cbs.dtu.dk/services/SignalP/) (Petersen *et al.*, 2011). Prediction of the antigenic sites was performed by using EMBOSS-GUI server (http://www.bioinfo.hku.hk/EMBOSS/). Prediction of the topology of β-barrel outer membrane proteins was carried out by using PRED-TMBB server (http://bioinformatics.biol.uoa.gr/PRED-TMBB/) (Bagos *et al.*, 2004). The TMRPres2D version 0.91 (http://bioinformatics.biol.uoa.gr/TMRPres2D) has been used to obtain high quality for the visual presentation of transmembrane protein models from the PRED-TMBB graphical output. Other online sequence database was also been used such as MEROPS (http://merops.sanger.ac.uk/), a database specifically about the peptidase, their substrates and inhibitor (Rawlings *et al.*, 2010) in order to search for the possible similarities in VHS protein sequence. Prediction of protein subcellular localization has been performed by using PSORTb v3.0.2 server (http://www.psort.org/psortb/) (Yu *et al.*, 2010).

Multiple sequence alignment and phylogenetic analysis

Multiple sequence alignment of VHS and OMP protein was generated by using Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/).

Phylogenetic tree was generated by Neighbor-joining algorithm of the MEGA 6.06 software (Tamura *et al.*, 2013). The protein sequences of VHS and OMP were used to construct phylogenetic trees by using a protein sequence retrieved from the Genbank database. Aligned sequences were bootstrapped 1000 times.

Nucleotide sequence accession number

The nucleotide sequences of the VHS and OMP genes have been deposited in the Genbank data library (http://www.ncbi.nlm.nih.gov/Genbank/) under accession number KT266880 and KT266881, respectively.

Results

DNA Cloning and Sequence characterization of VHS and OMP genes

The full length PCR amplification of gene encoding VHS and OMP were successfully obtain using primers listed in Table 1. Based on ORF finder analyses, both genes were found to have one tandem open reading frame. The nucleotide sequence of VHS consisted of an open reading frame of 1368 base pairs in length including the stop codon. The ~~OMP~~ VHS encoded for

a polypeptide of 455 amino acid (Fig. 1(a), Genbank accession no. KT266880). While, the ORF sequence of OMP was consists of 816 base pairs in length including stop codon, encoded for 271 amino acid (Fig. 1(b), Genbank accession no. KT266881).

Similarity analysis indicated that the deduced amino acid of VHS sequence was highly conserved which 100% identity similar to protease of *V. harveyi* (WP_005447751.1) followed by 99% similarities with peptidase of *Vibrio* sp. HENC-01 (WP_009697599). On the other hand, the deduced amino acid of OMP revealed a 99% similarity with membrane protein of *V. harveyi* (WP_017189208.1), membrane protein of *Vibrio* sp OY15 (WP_033905541.1) and OmpK of *V. alginolyticus* (ACI 97457.1).

Table 1. Oligonucleotide primers used for PCR amplification and cloning of *V. harveyi* VHS and OMP genes.

Target gene	Primer Design	Reference sequences
Serine protease (VHS)	Forward primer: 5'-ATGAAAAAACCATTGCTTGCGTTAAC-3' Reverse primer: 5'-TTAGCGGATAACGAGGTAAACCG-3'	EU344975.1 (This study)
Outer membrane protein (OMP)	Forward primer: 5'-ATGCGTAAATCACTTTTAGCTCTTAGC-3' Reverse primer: 5'- TTAGAACTTGTAAGTTACTGCGATGT-3'	(Nehlah *et al.*, 2014)

The putative conserved domains detected by using NCBI conserved domain search program (http://ncbi.nlm.nih.gov/structure/cdd/wrpsb.cgi) showed that VHS was conserved with a trypsin_2 domain (residues 274 to 684) and also processing two PDZ serine protease domain (residues 799 to 1068 and 1129 to 1362) which was homologous to those of serine endoprotease multi-domain. The OMP showed to be conserved to a channel_Tsx at residue 88-813. The OMP was also found to be homologue to a nucleoside specific channel forming protein. Homology similarity of the highest percentage identities of amino acids sequences for both genes are summarized as in Table 2.

Table 2. The homology search of amino acid sequence of VHS and OMP genes.

Target gene	Description	Similarity (%)	Accession number
VHS	Protease (*V. harveyi)*	100%	WP_005447751.1
	Peptidase of *Vibrio* sp. HENC-01	99%	WP_009697599.1
	Serine endoprotease *Deg*Q (*V. harveyi*)	99%	WP_049534663.1
	DegQ-like protein of *V. harveyi*	99%	ACA49815.1
	Serine endoprotease *Deg*Q (*V. campbellii*)	97%	WP_038890836.1
OMP	Membrane protein of *V. harveyi*	99%	WP_017189208.1
	Membrane protein of *Vibrio* sp OY15	99%	WP_033905541.1
	OmpK of *V. alginolyticus*	99%	ACI 97457.1
	Membrane protein (*V. tasmaniensis*)	94%	WP_017106634.1
	Membrane protein (*V. cyclitrophicus*)	94%	WP_016784788.1

Multiple sequence alignment

Multiple sequence alignment was generated by using Clustal Omega server and the output for multiple alignments was visualized by using Boxshade version 3.21 (www.ch.embnet.org/software/BOX_form.html) with a 0.5 threshold. The alignment was shown in Fig. 2(a) VHS and (b) OMP. The highly conserved region was marked with asterisk (*) in the consensus item. The black shaded and grey shaded region represents an identical region and a similar region, respectively. According to the multiple alignment generated from both genes, it was found out that VHS has a

significant highly conserved regions to protease, particularly with serine endoprotease DegQ produced in other strains of *V. harveyi* and *V. campbellii* (Fig. 2a).

This finding demonstrated that the VHS is belonged to serine endoprotease group. In contrast, OMP was been observed to have highly conserved region for 48 amino acids at the beginning of deduced amino acid sequence. However, mostly of the remaining amino acid sequences still showed a similar region (grey shaded), which indicated that OMP gene is significantly belonged to outer membrane protein (Fig. 2b). These results may be probably due to the sequences variation and divergence that normally found in outer membrane protein.

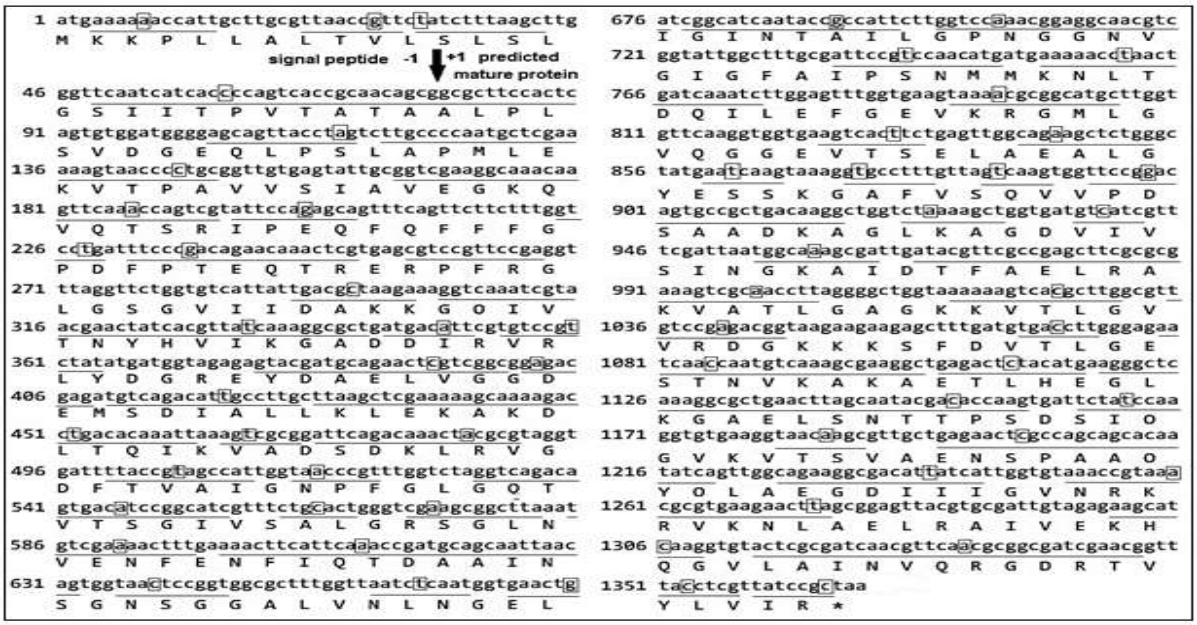

Fig. 1(a). A deduced amino acids of VHS (Genbank accession number: KT266880). The possible antigenic sites indicated by underlined letters, maximum position score of antigenic sites marked in boxes. Prediction of cleavage sites between the signal peptide and mature VHS is indicated by vertical arrow. Numbering of the amino acid residues of the mature VHS starts with +1 and residues of the signal sequence have a negative numbers. The asterisks mark for the stop codon.

Phylogenetic analysis

Amino acid sequences used in the construction of phylogenetic tree were selected and retrieved based on the sequences deposited in NCBI database. Construction of the phylogenetic tree was rooted to *Photobacterium* sp (VHS) and *Aliivibrio wodanis* (OMP) as an outgroup references. All positions containing gaps and missing data were eliminated. The phylogenetic tree constructed is depicted in Fig. 3a (VHS) and 3b (OMP). The phylogenetic tree constructed for VHS (Fig. 3a) was divided into three descendent taxa which represented by serine endoprotease DegQ taxon (A), protease DO (DegP) taxon (B) and the serine protease DegQ from an outgroup taxon (C).

The cladogram depicted that VHS gene (shown in boxes) was clustered with serine endoprotease DegQ ancestor. This ancestor was split into two nodes which are serine endoprotease DegQ consisting of species that belong to *Harveyi* clade and the other nodes consisting of a serine endoprotease DegQ from others species that belonged to clade outside than *Harveyi* clade. However, both of these nodes apparently were belong as the closest relatives. Protease DO (*V. tapetis*) in taxon (B), basically referring to DegP, was generated different ancestor compared to serine endoprotease DegQ taxon. However, DegP is actually is a part of HtrA family domain, which sharing a sequence identities with DegQ and DegS.

Fig. 3(b) depicted the phylogenetic tree for OMP gene. The cladogram showed three different taxa have been generated, which represents a taxon A displayed for OmpK cluster groups, followed by taxon B for outgroup outer membrane protein (*Aliivibrio wodanis*) and taxon C showing for an OmpA cluster groups. Taxon A was found to split into two major ancestors, outer membrane protein (OmpK) produced by mostly a marine pathogen and another ancestor which belonged to outer membrane protein that normally produced by a human pathogen. The OMP (indicated by boxes in phylogenetic tree) was clustered in OmpK taxon (A). This suggested that membrane protein for OMP gene is belonged to OmpK type protein.

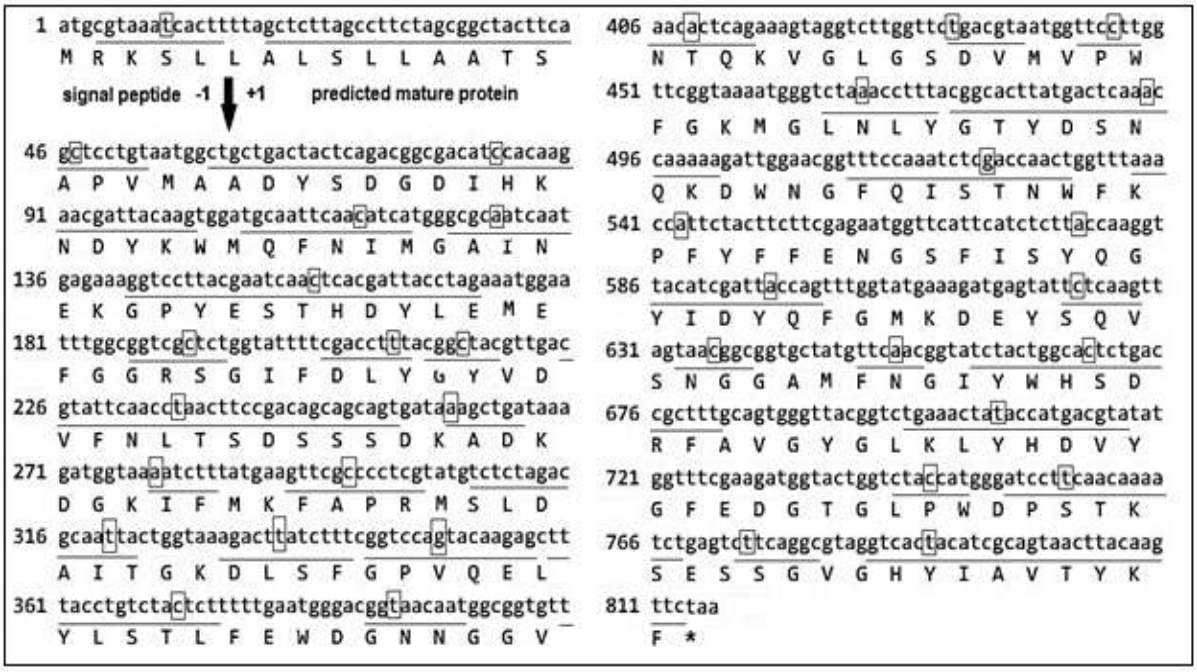

Fig. 1(b). A deduced amino acids of OMP gene (Genbank accession number: KT266881). The possible antigenic sites indicated by underlined letters, maximum position score of antigenic sites marked in boxes. Prediction of cleavage sites between the signal peptide and mature OMP is indicated by vertical arrow. Numbering of the amino acid residues of the mature OMP starts with +1 and residues of the signal sequence have a negative numbers. The asterisks mark for the stop codon.

It also showed that the OMP gene is grouped with the closest relatives of pathogen species that are commonly found in a species that causes diseases in marine fish such as *V. parahaemolyticus*. The taxon B, which represent the outgroup references (*Aliivibrio wodanis*) are clustered together with OmpA produced by *V. parahaemolyticus*. It is assumed that the outer membrane protein sequence used for *Aliivibrio wodanis* is encoded for outer membrane protein type A (OmpA). The taxon (C) represents a group of outer membrane protein A (OmpA) which clearly have a different roles and distinct pathogenesis mechanism compared to OmpK.

Signal peptide, transmembrane topology and protein subcellular localization prediction

Putative signal peptides of VHS and OMP were identified by using the SignalP v4.1 analysis. Both of the genes were found to have a signal peptide in their *N*-terminus region. The cleavage sites were found situated between position 26 and 27 for VHS and position 20 and 21 for OMP. The location of predicted signal peptide and mature protein was indicated in Fig. 1a (for VHS) and 1b (for OMP). The score that determined for maximum cleavage score were at position 27 (VHS) and 21 (OMP). Therefore, prediction of mature protein for VHS encoded for 429 amino acids, started from position 27 to 455 of

deduced amino acid. The OMP encoded for 251 matured amino acids, which begin from position 21 to 271 of its deduced amino acids. Results evaluated also parallel to the outcomes analysed by PSORTb v3.02 and PRED-TMBB.

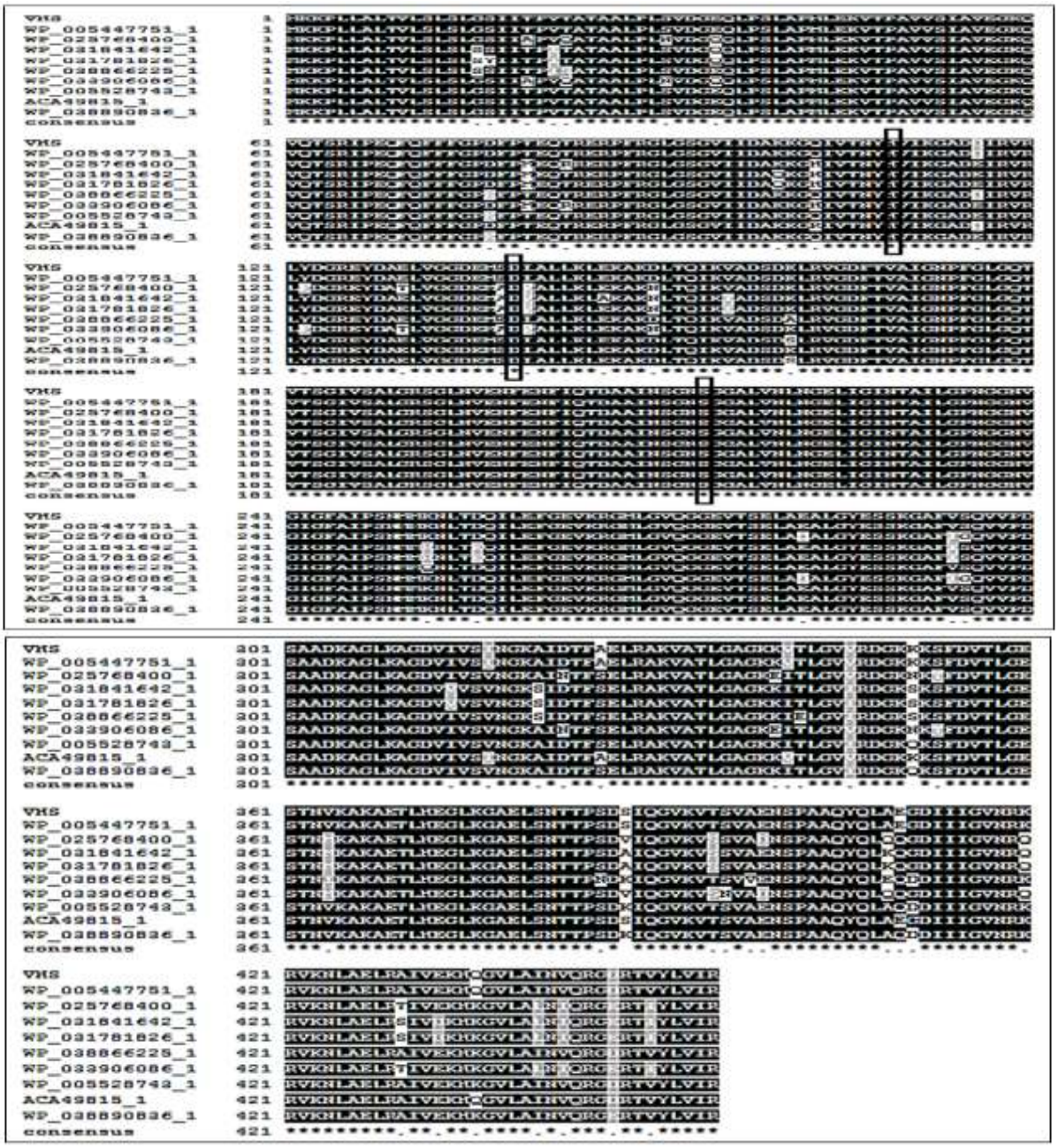

Fig. 2(a). Multiple alignment of deduced amino acid sequence of VHS from *V. harveyi* with other closest similarity with VHS amino acid sequences retrieved from Genbank. Catalytic triad of common serine endoprotease genes has displayed by boxed (D: Aspartate, H: Histidine and S: serine). Amino acid sequence abbreviations are as follows: protease of *V. harveyi* (WP_005447751_1), serine endoprotease DegQ of *V. harveyi* group (WP_025768400_1), serine endoprotease DegQ of *V. parahaemolyticus* (WP_031841642_1), serine endoprotease DegQ of *V. parahaemolyticus* (WP_031781826_1), serine endoprotease DegQ of *Vibrio* (WP_038866225_1), serine endoprotease DegQ of *Vibrio* sp. OY15) (WP_033906086_1), protease of *V. campbellii* (WP_005528743_1), DegQ-like protein of *V. harveyi* (ACA49815_1) and serine endoprotease DegQ of *V. campbellii* (WP_038890836_1). Consensus regions are shaded in black (identical regions) and grey (similar regions).

According to PSORTb v 3.02 outcomes, signal peptide had been detected in both genes which the signal has been found in non-cytoplasmic region. The VHS was predicted to be localized at the periplasmic region with one internal helix found. The periplasmic region was determined to be matched with Protease DO precursor (UniProtKB/Swiss-Prot accession no: P26982.1), which encoded for a stress-response protein in *Salmonella typhimurium* virulence. In contrast, the OMP was found to be localized at the outer membrane, but none of internal helices has been detected. The signal for OMP gene was matched with outer membrane integral membrane protein (UniProtKB/Swiss-Prot accession no: P26982.1), which encoded for OmpK gene produced by *V. parahaemolyticus* that serves as receptor for a broad-host-range vibriophage. These results were consistent with the visualization of PRED-TMBB, whereby showing the signal sequence found to be located extracellularly (Fig. 4).

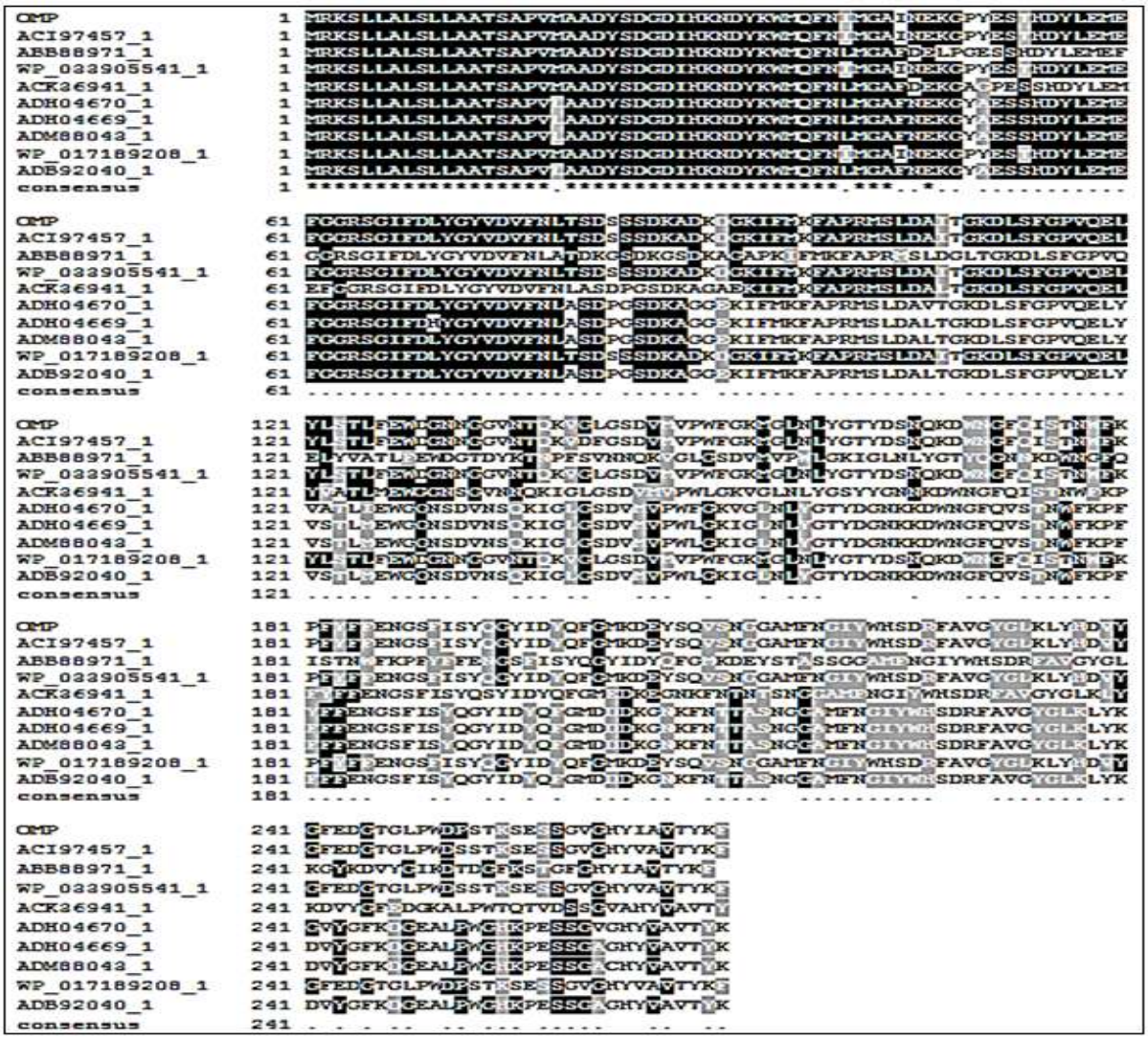

Fig. 2(b). Multiple alignment of deduced amino acid sequence of OMP from *V. harveyi* with other closest similarity with outer membrane protein amino acid sequences retrieved from Genbank. Amino acid sequence abbreviations are as follows: OmpK *V. alginolyticus* (ACI97457_1), outer membrane protein (*V. harveyi*) (ABB88971_1), membrane protein of *Vibrio* sp. OY15 (WP_033905541_1), OmpK of *V. parahaemolyticus* (ACK36941_1), outer membrane protein K of *V. alginolyticus* (ADH04670_1), outer membrane protein K of *V. parahaemolyticus* (ADH04669_1), outer membrane protein K of *V. parahaemolyticus* (ADM88043_1), membrane protein of *V. harveyi* (WP_017189208_1) and outer membrane protein K of *V. parahaemolyticus* (ADB92040_1). Consensus regions are shaded in black (identical regions) and grey (similar regions).

Apart from that, PRED-TMBB analysis was used to predict a topology of β-barrel outer membrane proteins for both genes. The output for graphical visualization of both genes was shown in Fig. 4. The algorithm used was posterior decoding algorithm to locate the transmembrane strands. The graphical output from PRED-TMBB analysis was successfully supported the findings obtained from SignalP v4.1 and PSORTb v3.0.2 analysis. Prediction of the β-barrel membrane protein showed VHS consisted only one β-barrel (region 79 to 90 of VHS deduced amino acids) compared to six β-barrels membrane protein in OMP (region 45 to 54, 77 to 101, 129 to 139, 163 to 171, 202 to 214 and 238 to 262 of OMP deduced amino acid). The transmembrane segments of α-helical membrane in VHS only demonstrated two transmembrane α-helices (region 70 to 78 and 91 to 97 in VHS deduced amino acids), whereas OMP showed twelve transmembrane α-helices in total (region 36 to 44, 55 to 61, 68 to 76, 102 to 114, 118 to 128, 140 to 150, 154 to 162, 172 to 184, 191 to 201, 215 to 223, 227 to 237 and 263 to 270 in OMP deduced amino acids).

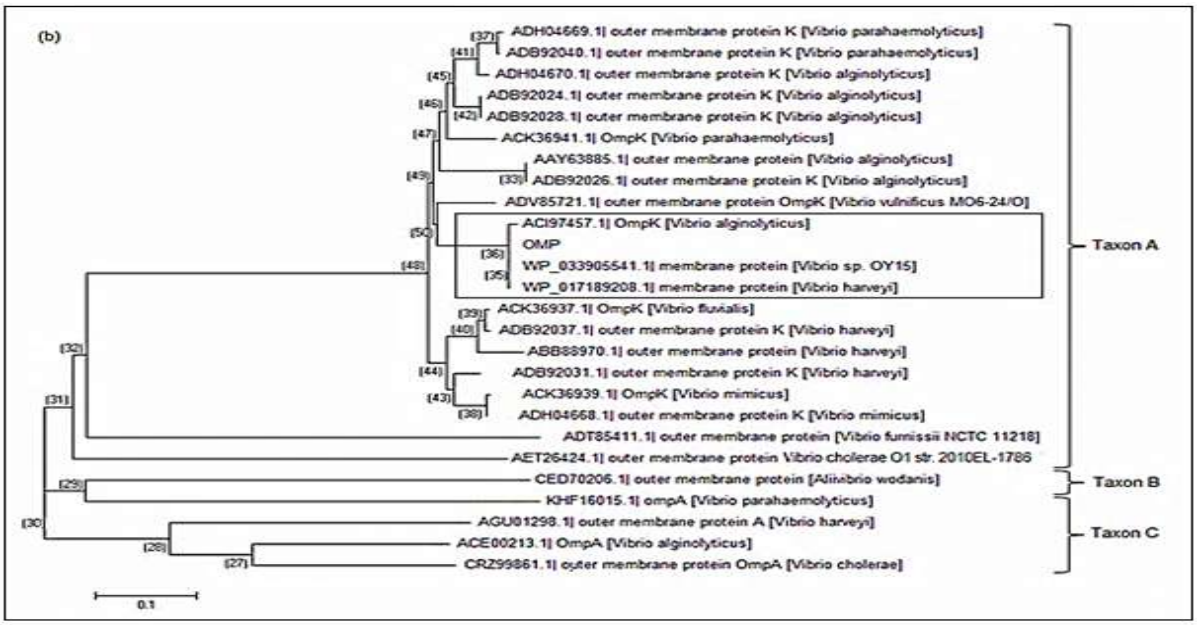

Fig. 3(b). Phylogenetic tree based on the comparative analysis of amino acid sequences of OMP with other known outer membrane protein of amino acid sequences, respectively. Aligned sequences were bootstrapped 1000 times and the numbers at the forks indicate the bootstrap proportions. Amino acid sequence abbreviations are as follows: OmpK of *V. alginolyticus* (ACI97457.1), outer membrane protein of *V. furnissii* NCTC 11218 (ADT85411.1), OmpK of *V. fluvialis* (ACK36937.1), outer membrane protein OmpK of *V. vulnificus* MO6-24/O (ADV85721.1), outer membrane protein of *Aliivibrio wodanis (*CED70206.1), membrane protein of *Vibrio* sp. OY15 (WP_033905541.1), outer membrane protein of *V. cholerae* O1 str. 2010EL-1786 (AET26424.1), OmpK of *V. parahaemolyticus* (ACK36941.1), outer membrane protein K of *V. harveyi* (ADB92037.1), outer membrane protein K of *V. harveyi* (ADB92031.1), OmpK of *V. mimicus (*ACK36939.1), outer membrane protein of *V. alginolyticus* (AAY63885.1), outer membrane protein K of *V. mimicus* (ADH04668.1), outer membrane protein K of *V. alginolyticus* (ADH04670.1), outer membrane protein K of *V. parahaemolyticus* (ADH04669.1), outer membrane protein of *V. harveyi* (ABB88970.1), membrane protein of *V. harveyi* (WP_017189208.1), outer membrane protein K of *V. parahaemolyticus* (ADB92040.1), outer membrane protein K of *V. alginolyticus (*ADB92024.1), outer membrane protein K of *V. alginolyticus* (ADB92028.1), outer membrane protein K of *V. alginolyticus* (ADB92026.1), outer membrane protein A of *V. harveyi* (AGU01298.1), OmpA of *V. alginolyticus* (ACE00213.1), ompA of *V. parahaemolyticus* (KHF16015.1) and outer membrane protein OmpA of *V. cholera* (CRZ99861.1).

Discussion

A putative serine protease and outer membrane protein was successfully cloned from a pathogenic *V. harveyi*. Strong evidence showed that VHS and OMP are belonged to serine endoprotease DegQ and outer membrane protein K (OmpK), respectively. Conserved domain database showed VHS was conserved with trypsin and PDZ serine protease domain. These findings also aligned with MEROPS database which showed that this gene belonged to peptidase family S1 that contains serine endopeptidases. As noted by Polgár (2005), the family S1 contains the catalytic triad of Histidine (H), Aspartate (D) and Serine (S) with the catalytic type is a serine. PDZ domains is a short forms derived from combination of first letter between PSD95 (post synaptic density protein), DlgA (Drosophila disc large tumor suppressor) and ZO1, a mammalian tight junction protein.

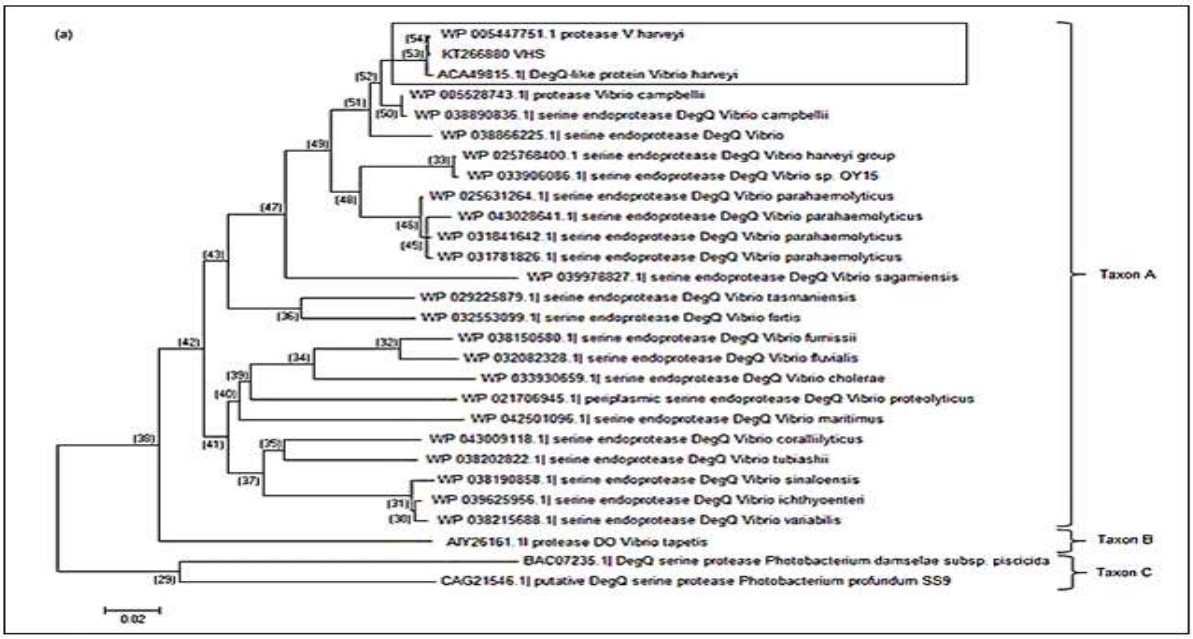

Fig. 3(a). Phylogenetic tree based on the comparative analysis of amino acid sequences of VHS with other known serine protease amino acid sequences. Aligned sequences were bootstrapped 1000 times and the numbers at the forks indicate the bootstrap proportions. Amino acid sequence abbreviations are as follows: protease of *V. harveyi* (WP_005447751.1), serine endoprotease DegQ of *V. harveyi* group (WP_025768400.1), serine endoprotease DegQ of *V. parahaemolyticus* (WP_031841642.1), serine endoprotease DegQ of *V. parahaemolyticus* (WP_043028641.1), serine endoprotease DegQ of *V. parahaemolyticus* (WP_031781826.1), serine endoprotease DegQ of *Vibrio* (WP_038866225.1), serine endoprotease DegQ of *Vibrio* sp. OY15 (WP_033906086.1), serine endoprotease DegQ of *V. furnissii* (WP_038150580.1), serine endoprotease DegQ of *V. fluvialis* (WP_032082328.1), serine endoprotease DegQ of *V. tasmaniensis* (WP_029225879.1), serine endoprotease DegQ of *V. sagamiensis* (WP_039978827.1), serine endoprotease DegQ of *V. maritimus* (WP_042501096.1), serine endoprotease DegQ of *V. sinaloensis* (WP_038190858.1), serine endoprotease DegQ of *V. cholera* (WP_033930659.1), protease of *V. campbellii* (WP_005528743.1), serine endoprotease DegQ of *V. parahaemolyticus* (WP_025631264.1), protease DO of *V. tapetis* (AIY26161.1), serine endoprotease DegQ of *V. coralliilyticus* (WP_043009118.1), serine endoprotease DegQ of *V. ichthyoenteri* (WP_039625956.1), DegQ-like protein of *V. harveyi* (ACA49815.1), serine endoprotease DegQ of *V. variabilis* (WP_038215688.1), periplasmic serine endoprotease DegQ of *V. proteolyticus* (WP_021706945.1), serine endoprotease DegQ of *V. fortis* (WP_032553099.1), serine endoprotease DegQ of *V. tubiashii* (WP_038202822.1), DegQ serine protease of *Photobacterium damselae* subsp. *piscicida* (BAC07235.1), putative DegQ serine protease of *Photobacterium profundum* SS9 (CAG21546.1), serine endoprotease DegQ of *V. campbellii* (WP_038890836.1).

Detailed examination of enzymatic analysis by Zhang *et al.* (2008), demonstrated that the recombinant $DegQ_{Vh}$ protein expressed in and purified from *E. coli* was an active serine protease whose activity required the integrity of the catalytic site and the PDZ domains. Similarly, catalytic triad residues of VHS were also found to be conserved when compared to others serine protease representing different *Vibrio* strains (Fig. 2a). This finding has confirmed that deduced amino acids sequence of VHS resembles a similar protein function and structural protein formation including its catalytic triad arrangement towards other compared sequences. Zhang *et al.* (2008) has stated that the serine endoprotease DegQ was a part of HtrA (high temperature requirement A) family member, together with DegP (also known as DO) and DegS, which share a relatively high level of sequence identity. HtrA proteins possess the dual function of protease and chaperon and can switch roles according to the input of environmental stimuli (Spiess *et al.*, 1999).

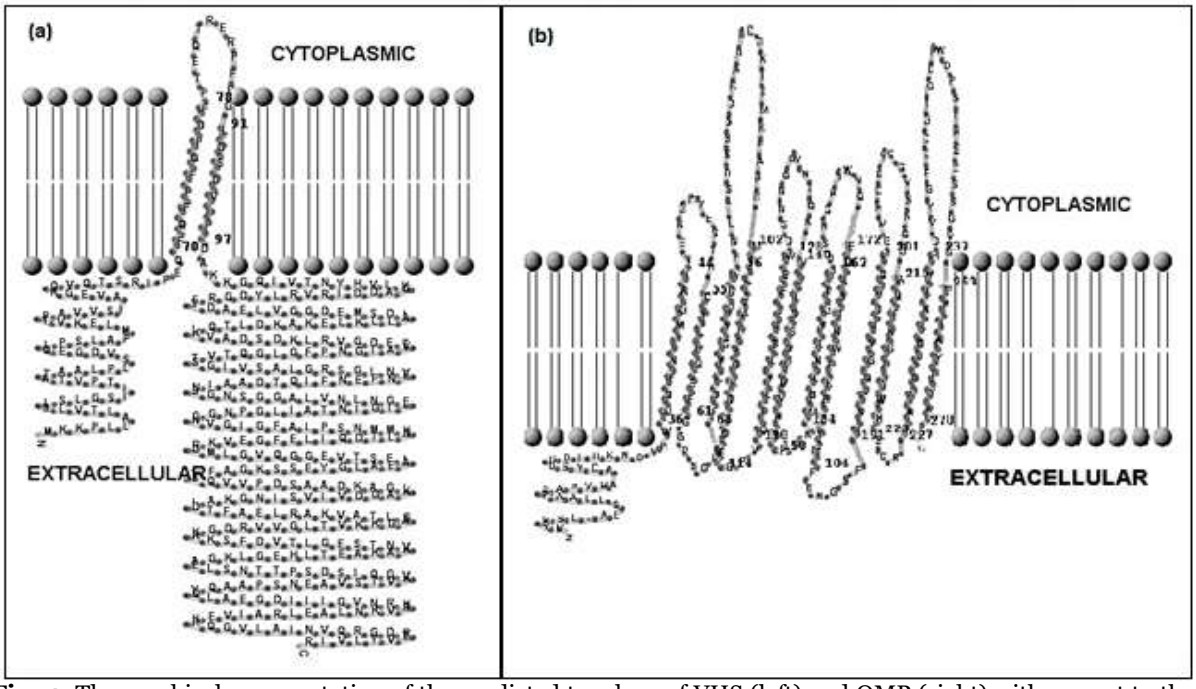

Fig. 4. The graphical representation of the predicted topology of VHS (left) and OMP (right) with respect to the lipid bilayer. The VHS and OMP sequence are shown in the one-letter amino acid code.

DegQ and DegS protein were found located next to each other on the chromosome in *E. coli*, transcribed independently and 36% identical to DegP. However, coding sequence of DegQDegS is physically separated from the DegP cluster (Waller and Sauer, 1996). In addition, analysis on the available genome sequences of the *Vibrio* species by Zhang *et al.,* (2008) revealed that *V. harveyi*, *V. parahaemolyticus*, *V. vulnificus*, *V. cholerae*, and *V. fischeri*, all possess only two members of HtrA family proteins, which are DegS and the DegQ counterpart. They also demonstrated that the purified recombinant $DegQ_{Vh}$ was a protective immunogen that could confer protection upon Japanese flounder fish against infection by *V. harveyi*. On reflection, for this study, it is reasonable to focus on serine endoprotease DegQ to be further investigated for their potential as vaccine candidates.

On the other hand, putative OMP was found to be conserved with outer membrane protein K (OmpK) that connected to channel_Tsx protein, a nucleoside-specific channel-forming protein. Tsx channel represents for a nucleoside specific outer membrane (OM) transporter of Gram negative bacteria. This structure is believed to provide a mechanism for nucleoside transport across the bacteria outer membrane (Ye and van-den Berg, 2004). As mentioned by Nikaido (2003), these channels are

divided into three (general porins, substrate specific transporters and active transporter), depending on mode of transport. Furthermore, OmpK is widely distributed among species of the family Vibrionaceae and serves as the receptor for broad-host-range vibriophage KVP40 (Mao *et al.*, 2007; Inoue *et al.*, 1995). Therefore, it can be suggested that OMP gene in this study is responsible to regulate the secretion systems that played an important role in transportation and delivery systems of virulence factors in *V. harveyi.*

In the pathogenesis, outer membrane of Gram-negative pathogenic bacteria is forms a protective permeability barrier around the cells and serves as a molecular filter for hydrophilic substances. These roles are normally carried out by channels present in outer membrane in order to mediate the transport of nutrients and ions across the membrane into the periplasm (Ye and van-den Berg (2004). Despite of their roles, the components of the outer membrane is easily recognized as foreign substances by immune defense systems of hosts (Ningqiu *et al.*, 2008; Kawai *et al.*, 2004). This study gives a credit for outer membrane protein to act as good antigenic properties due to its ability in inducing protective immunity (Kawai *et al.*, 2004; Rahman and Kawai, 2000; Lutwyche *et al.*, 1995). A number of researchers have reported that recombinant OmpK of *V. harveyi* and *V. parahaemolyticus* can protect hosts from infection by virulent *V. harveyi* and *V. parahaemolyticus* (Li *et al.*, 2010; Ningqiu *et al.*, 2008; Mao *et al.*, 2007; Zhang *et al.*, 2007).

As pointed out by Li *et al.* (2010), orange-spotted groupers (*Epinephelus coioides*) which were vaccinated with recombinant OmpK were protected with a RPS value of 100%. These results indicated that the OmpK is an effective vaccine candidate against *V. harveyi* in Orange-spotted groupers. Even though OMP only showed less conserved regions in comparison to other sequences (Fig. 2b), this result can be acceptable because there is no report of sequence variations in OmpK genes for *Vibrio* species that could affected its protective levels (Li *et al.*, 2010). Previous study on the OMP profiles of 32 *Vibrio* type strains by Zhang, *et al* (1997) also showed that the major OMP profiles of different *Vibrio* species had considered heterogenicity, with major OMP ranging between 54kDa, 43kDa and 27kDa, but no common major OMPs in all *Vibrio* strains had been found (Qin and Yan, 2010).

Phylogenetic studies depicted that VHS (Fig. 1a) clusters together with serine endoprotease DegQ producers whilst OMP (Fig. 1b) formed cluster together with OmpK producers. Interestingly, both genes were shown to be clustered into the nodes which mainly consists species that belong to *Harveyi* clade. These results are in a good agreement with Vanmalae *et al.* (2015). They were demonstrated that *Vibrios* belonging to the *Harveyi* clade are important pathogens of a large number of marine animals in the aquaculture industries. There are 142 species have been described in the Vibrionaceae family of bacteria, classified into seven genera: *Aliivibrio*, *Echinimonas*, *Enterovibrio*, *Grimontia*, *Photobacterium*, *Salinivibrio* and *Vibrio*. Up to date, these genera were divided into 25 distinct clades, including one super clade proposing for *Salinivibrio-Grimonti-Enterovibrio* clade (Sawabe *et al.*, 2013). The more surprising correlation is VHS and OMP genes are closely related to *Harveyi* clade together with other noted species in this clade such as *V. alginolyticus*, *V. campbellii, V. parahaemolyticus and V. sagamiensis.*

Signal peptide analyses which revealed that signal peptide was presence in both genes are important to localize the signal towards the secretory pathway of newly synthesized proteins. The presence of signal peptide in VHS is found to be consistent and significant reported in MEROPS database. Majority family S1 endopeptidase will enter the secretory pathway and have an *N*-terminal signal peptide. Typically, the entire gene is proposed to be synthesized as precursors with an *N*-terminal extension that is cleaved to form the active enzyme. Previous study by Zhang *et al.* (2008), reported that prediction of putative signal peptide of serine endoprotease DegQ were identified at the *N*-terminus

of ORF1368 with the most likely cleavage sites situated between the positions A26 to A27. This finding was similar with our finding which determined the signal peptide also situated in between position 26 to 27 (Fig. 1a).

In comparison, OMP gene has displayed a signal peptide with cleavage site in between position 20 to 21. Previous research findings had reported a cloned OmpK that resembles a signal peptide in between position 20 to 21 (Ningqiu *et al.*, 2008; Zhang *et al.*, 2007). This finding was also consistent with Bannwarth and Schultz (2003) which described that outer membrane proteins are produced with the *N*-terminal signal sequence. This will directs the nascent polypeptide through the translocon in the inner membrane to the periplasmic space. Then, the signal sequence is removed during translocation and the native protein is folded and inserted into the outer membrane (Bannwarth and Schultz, 2003). Without the sequence of signal peptide, probably the gene will be expressed as inclusion bodies (Ningqiu *et al.*, 2008; Bannwarth and Schultz, 2003). As reported by Ningqiu (2008), it was found that the gene encoding for OmpK was expressed as inclusion bodies without the sequence of the signal peptide.

It has also been reported that signal peptide is important to understand and analyzed the sorting and localization of the protein as some secreted proteins and membrane proteins have been synthesized in a form of precursor peptide called the *N*-terminal. This *N*-terminal normally consists of about 15 to 25 amino acid sequences (Li *et al.*, 2013). As described by Kazemian *et al.* (2014), the signal peptide is structured as such that at the *N*-terminus a positively charged n-region is located. The length of residues in the *n*-region varies from 1 to 12 residues and is followed by the hydrophobic region, '*h*-region', of 7 to15 residues. After the *h*-region another 3 to 8 residues long polar and uncharged *c*-region is positioned, where the cleavage point is located. Besides, prediction of signal peptide region in *N*-terminus of the sequences is important to be done in predicting a membrane protein topology.

With respect to integral membrane proteins, it can be divided into two distinct classes based on fold mechanism of transmembrane segments, represents by α-helical bundle class and the β-barrel class (Zou *et al.*, 2010; Schulz, 2002). It has also been described that signal peptides and *N*-terminal β-barrels regions usually possess a hydrophobic region (Petersen *et al.*; 2011; Nielsen *et al.,* 1997). However, transmembrane β-helices region have longer hydrophobic regions and do not have cleavage sites compared to signal peptide region that is shorter approximately consists of 7 to 15 residues (Kazemian *et al.*, 2014; Petersen *et al.*, 2011). This transmembrane protein regions are normally contains one or more hydrophobic segments and it easily discriminated from non-membrane proteins (Chou and Elrod, 1999). Identification of β-barrel transmembrane protein is important due to these regions have been found exclusively present in the outer membrane Gram negative prokaryotes (Schulz, 2002).

A comparison of the two results revealed that VHS composed of one β-barrel and two units of α-barrel whilst OMP has six β-barrel and twelve α-barrel structure (Fig. 4). This evidence suggests that both genes consist of transmembrane protein, which assumed that this protein can function on both sides of the bilayer or transport molecules across them. Surprisingly, VHS was predicted to be localized in periplasmic region which contrary to OMP that predicted to be localized in outer membrane integral membrane protein. However, different localization of the protein is usually associated with different biological functions (Chou and Elrod, 1999). Therefore, this results may explained by the fact that VHS and OMP have two different roles in virulence activity of *V. harveyi*.

It is apparent that VHS demonstrated to be associated with periplasmic serine protease (DegQ) based on prediction of subcellular location by using PSORTb v3.0.2 analysis. This result was also further support by the findings from homology search with MEROPS database. According to MEROPS database, members of this family are located in the periplasm and have

separable functions as both protease and chaperone. It was also described that DegQ members have a trypsin domain and two copies of a PDZ domain. These descriptions are also consistent with the BLASTp and conserved domain search. The chaperone function is dominant at low temperatures, whereas the proteolytic activity is turned on at elevated temperatures. It can thus be suggested that VHS played important roles to protect the bacteria from thermal and other stresses, and may be important for the survival of bacterial pathogens.

Whereas, OMP displayed a unique protein structure and this showing strong evidence that OMP is structurally belong to the porin. These results agree with the findings observed by Zhang *et al.* (2007), in which the simulation of three-dimensional structure of OmpK showed a presence of 12 anti-parallel β-sheets (α-barrel regions) which organized the β-cylinder and six surface-exposed loops (β-barrel regions), with five short periplasmic turns. It was also demonstrated that the membrane-spanning anti-parallel β-strands connected by short periplasmic turns and surface-exposed loops will made up a pore inside the cylinder. This result also consistent with pSORTb v 3.02 analysis whereby showing OMP belonged to outer membrane integral membrane protein. It can therefore be assumed that OMP played roles in the cell plasma membrane and played roles as sensors of external signals, transferring information across the membrane and allowing the cell to change its behavior in response to environmental cues.

Taken together, the findings also supported by the results obtained which predicted both genes possess an antigenic region (Fig. 1a-b). This antigenic region (also known as epitope) is important for vaccine development. In accordance with the present result, previous studies have demonstrated that the OmpK is a conserved protective antigen among *V. harveyi*, *V. alginolyticus* and *V. parahaemolyticus* species (Li *et al.,* 2010). Research findings by Qin and Yan (2010) also showed that five proteins of the OMP extracted from a pathogenic *V. harveyi* TS-628, displayed a strong protein reaction in western blot analysis. Their results were demonstrated that OmpK are major immunogenic antigens of *V. harveyi*. Thus, the OmpK has a strong possibility to be applied as a versatile vaccine candidate to combat the vibriosis. It also proposed that the potential antigenic regions in a protein will elicit similar antibodies and have the ability to generate a possible cross-reactivity.

In summary, we have successfully cloned and molecularly analyzed the virulence associated serine protease and outer membrane protein genes from locally isolated pathogenic *V. harveyi*. It is clearly demonstrated that both genes played the important roles in the pathogenesis routes. VHS is believed to involve in the thermal resistant properties or as a chaperone for proteolytic activity whilst OMP as a porin channel which probably played role in secreting a virulence-associated protein, particularly during the infection in hosts. As the conclusion, this study revealed that VHS and OMP have the effective capacity and potential to be capable in protecting fish species against vibriosis caused by *V. harveyi*. In future, development of live-attenuated vaccine candidate will be further extended by employing both of these genes.

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References

Austin B, Zhang XH. 2006. *V. harveyi*: a significant pathogen of marine vetebrates and invertebrates. Letters in Applied Microbiology **43,** 119-124.
http://dx.doi.org/10.1111/j.1472-765X.2006.01989.x

Bagos PG, Liakopoulos TD, Spyropoulos IC, Hamodrakas SJ. 2004. PRED-TMBB: A web server for predicting the topology of β-barrel outer membrane proteins. Nucleic Acids Research **32,** W400-W404.
http://dx.doi.org/10.1093/nar/gkh417

Bannwarth M, Schulz GE. 2003. The expression of outer membrane proteins for crystallization. Biochimica et Biophysica Acta **1610,** 37-45.
http://dx.doi.org/10.1016/S0005-2736(02)00711-3

Cheng S, Zhang WW, Zhang M, Sun L. 2010. Evaluation of the vaccine potential of a cytotoxic protease and a protective immunogen from a pathogenic *V. harveyi* strain. Vaccine **28(4),** 1041-1047.
http://dx.doi.org/10.1016/j.vaccine.2009.10.122

Chou KC, Elrod DW. 1999. Prediction of membrane protein types and subcellular locations. Proteins **34,** 137-153.
http://dx.doi.org/10.1002/(SICI)10970134(19990101)34:1<137::AID-PROT11>3.0.CO;2-O

Defoirdt T. 2014. Virulence mechanisms of bacterial aquaculture pathogens and antivirulence therapy for aquaculture. Reviews in Aquaculture **6(2)**, 100-114.
http://dx.doi.org/10.1111/raq.12030

Finlay BB, Falkow S. 1997. Common themes in microbial pathogenicity revisited. Microbiology and Molecular Biology Reviews **61,** 136-169

Food and Agriculture Organization of the United Nations (FAO). 2014. World Review of Fisheries and Aquaculture Topic: Status and Trends. In: The State of World Fisheries and Aquaculture 2014: Opportunities and challenges, Rome, 223 pp.
http://www.fao.org/3/a-i3720e.pdf

Frey J. 2007. Biological safety concepts of genetically modified live bacterial vaccines. Vaccine **25(30)**, 5598-5605.
http://dx.doi.org/10.1016/j.vaccine.2006.11.058

Huang X, Madan A. 1999. CAP3: A DNA sequence assembly program. Genome Research **9**, 868-877.
http://dx.doi.org/10.1101/gr.9.9.868

Inoue T, Matsuzaki S, Tanaka S. 1995. Cloning and sequence analysis of *Vibrio parahaemolyticus ompK* gene encoding a 26-kDa outer membrane protein, OmpK, that serves as receptor for a broad-host-range vibriophage, KVP40. FEMS Microbiology Letters **134,** 245-249.
http://dx.doi.org/10.1111/j.15746968.1995.tb07945.x

Kawai K, Liu Y, Ohnishi K, Oshima S. 2004. A conserved 37 kDa outer membrane protein of *Edwardsiella tarda* is an effective vaccine candidate. Vaccine **22(25-26),** 3411–3418.
http://dx.doi.org/10.1016/j.vaccine.2004.02.026

Kazemian HB, Yusuf SA, White K. 2014. Signal peptide discrimination and cleavage site identification using SVM and NN. Computers in Biology and Medicine **45,** 98-110.
http://dx.doi.org/10.1016/j.compbiomed.2013.11.017

Li N, Yang Z, Bai J, Fu X, Liu L, Shi C, Wu S. 2010. A shared antigen among *Vibrio* species: Outer membrane protein-OmpK as a versatile Vibriosis vaccine candidate in Orange-spotted grouper (*Epinephelus coioides*). Fish and Shellfish Immunology **28(5-6)**, 952-956.
http://dx.doi.org/10.1016/j.fsi.2010.02.010

Li ZH, Tang ZX, Fang XJ, Zhang ZL, Shi LE. 2013. Bioinformatics analysis of a non-specific nuclease from *Yersinia enterocolitica* subsp. *palearctica*. Computational Biology and Chemistry **47,** 207-214.
http://dx.doi.org/10.1016/j.compbiolchem.2013.09.003

Lutwyche P, Exner MM, Hancock REW, Trust TJ. 1995. A conserved *Aeromonas salmonicida* porin provides protective immunity to rainbow trout. Infection and Immunity **63(8),** 3137-3142.

Ma Y, Zhang Y, Zhao D. 2008. Polyvalent attenuated live vaccine for preventing and curing vibriosis of cultivated fish. Patent No: US2008/0274136 A1.

Mao Z, Yu L, You Z, Wei Y, Liu Y. 2007. Cloning, expression and immunogenicity analysis of five outer membrane proteins of *Vibrio parahaemolyticus* zj 2003. Fish and Shellfish Immunology **23(3)**, 567-575.
http://dx.doi.org/10.1016/j.fsi.2007.01.004

Mims CA, Nash A, Stephen J. 2001. Vaccines and How They Work, In: Mims' Pathogenesis of Infectious Disease (Fifth Edition), USA, Academic Press.392-415.

Nancy BS, Owens L. 2013. Virulence changes to *Harveyi* clade bacteria infected with bacteriophage from *Vibrio owensii*. Indian Journal of Virology **24(2),** 180-187.
http://dx.doi.org/10.1007/s13337-013-0136-1

Nehlah Rosli, Ina-Salwany Md Yasin, Murni Karim, Nur-Nazifah, Siti Zaharah Abdullah. Molecular characterization and antigenicity of outer membrane protein (OMP) of *Vibrio alginolyticus*, isolated from diseased Tiger Grouper (*Epinephelus fuscoguttatus*). In Proceeding of the 9th Symposium on Diseases in Asian Aquaculture (DAA9), 24 - 28 November 2014, Ho Chi Minh City, Vietnam.

Nielsen H, Engelbrecht J, Brunak S, Von-Heijne G. 1997. Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. Protein Engineering **10(1)**, 1-6.
http://dx.doi.org/10.1093/protein/10.1.1

Nikaido H. 2003. Molecular basis of bacterial outer membrane permeability revisited. Microbiology and Molecular Biology Reviews **67(4)**, 593-656.
http://dx.doi.org/10.1128/MMBR.67.4.593-656.2003

Ningqiu L, Junjie B, Shuqin W, Xiaozhe F, Haihua L, Xing Y, Cunbin S. 2008. An outer membrane protein, *OmpK* is an effective vaccine candidate for *V. harveyi* in Orange-spotted grouper (*Epinephelus coioides*). Fish and Shellfish Immunology **25(6),** 829-833.
http://dx.doi.org/10.1016/j.fsi.2008.09.007

Petersen TN, Brunak S, Von-Heijne G, Nielsen H. 2011. SignalP 4.0: Discriminating signal peptides from transmembrane regions. Nature Methods **8(10),** 785-786.
http://dx.doi.org/10.1038/nmeth.1701

Plant KP, LaPatra SE. 2011. Advances in fish vaccine delivery. Developmental and Comparative Immunology **35(12)**, 1256-1262.
http://dx.doi.org/10.1016/j.dci.2011.03.007

Polgár L. 2005. Review: The catalytic triad of serinepeptidases. Cellular and Molecular Life Sciences
62(19), 2161-2172.
http://dx.doi.org/10.1007/s00018-005-5160-x

Qin YX, Yan QP. The antigenicity of the flagellin, outer membrane proteins and lipopolysaccharide of *Vibrio harveyi*. In Bioinformatics and Biomedical Technology (ICBBT), 2010 International Conference on Bioinformatics and Biomedical Technology. 16-18 April 2010. Chengdu, China. 292-295 p.
http://dx.doi.org/10.1109/ICBBT.2010.5478956

Rahman MH, Kawai K. 2000. Outer membrane proteins of *Aeromonas hydrophila* induce protective immunity in goldfish. Fish and Shellfish Immunology **10(4),** 379-382.
http://dx.doi.org/10.1006/fsim.1999.0245

Rawlings ND, Barret AJ, Bateman A. 2010. MEROPS: The peptidase database. Nucleic Acids Research **38**, D227-233.
http://dx.doi.org/10.1093/nar/gkp971

Sarjito Radjasa OK, Sabdono A, Prayitno SB, Hutabarat S. 2009. Phylogenetic diversity of the causative agents of Vibriosis associated with groupers

fish from Karimunjawa Islands, Indonesia. Current Research in Bacteriology **2(1),** 14-21. http://dx.doi.org/10.3923/crb.2009.14.21

Sawabe T, Ogura Y, Matsumura Y, Feng G, Rohul Amin AKM, Mino S, Nakagawa S, Sawabe T, Kumar R, Fukui Y, Satomi M, Matsushima R, Thompson FL, Gomez-Gil B, Christen R, Maruyama F, Kurokawa K, Hayashi T. 2013. Updating the *Vibrio* clades defined by multilocus sequence phylogeny: proposal of eight new clades, and the description of *Vibrio tritonius* sp. nov. Frontiers in Microbiology **4(414),** 1-14. http://dx.doi.org/10.3389/fmicb.2013.00414

Schulz GE. 2002. The structure of bacterial outer membrane proteins. Biochimica et Biophysica Acta **1565(2)**, 308-317. http://dx.doi.org/10.1016/S0005-2736(02)00577-1

Shoemaker CA, Klesius PH, Evans JJ, Arias CR. 2009. Use of modified live vaccines in aquaculture. Journal of the World Aquaculture Society. **40(5),** 573-585. http://dx.doi.org/10.1111/j.1749-7345.2009.00279.x

Spiess C, Beil A, Ehrmann M. 1999. A temperature-dependent switch from chaperone to protease in a widely conserved heat shock protein. Cell **97**, 339-347. http://dx.doi.org/10.1016/S0092-8674(00)80743-6

Sun K, Zhang WW, Hou JH, Sun L. 2009. Immunoprotective analysis of *VhhP2*, a *Vibrio harveyi* vaccine candidate. Vaccine **27(21),** 2733-2740. http://dx.doi.org/10.1016/j.vaccine.2009.03.012

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. 2013. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Molecular Biology and Evolution **30(12)**, 2725-2729. http://dx.doi.org/10.1093/molbev/mst197

Vanmalae S, Defoirdt T, Cleenwerck I, De Vos P, Bossier P. 2015. Characterization of the virulence of *Harveyi* clade *Vibrios* isolated from a shrimp hatchery *in vitro* and *in vivo*, in a brine shrimp (*Artemia franciscana*) model system. Aquaculture **435**, 28-32. http://dx.doi.org/10.1016/j.aquaculture.2014.09.015

Vázquez-Juárez RC, Barrera-Saldaña HA, Hernández-Saavedra NY, Gómez Chiarri M, Ascencio F. 2003. Molecular cloning, sequencing and characterization of omp48, the gene encoding for an antigenic outer membrane protein from *Aeromonas veronii*. Journal of Applied Microbiology **94(5),** 908-918. http://dx.doi.org/10.1046/j.1365-2672.2003.01928.x

Waller PRH, Sauer RT. 1996. Characterization of *degQ* and *degS*, *Escherichia coli* genes encoding homologs of the DegP protease. Journal of Bacteriology **178(4),** 1146-1153.

Won KM, Park SI. 2008. Pathogenicity of *Vibrio harveyi* to cultured marine fishes in Korea. Aquaculture **285(1-4),** 8-13. http://dx.doi.org/10.1016/j.aquaculture.2008.08.013

Ye J, van den Berg B. 2004. Crystal structure of the bacterial nucleoside transporter Tsx. The EMBO Journal **23(16)**, 3187-3195. http://dx.doi.org/10.1038/sj.emboj.7600330

Yu NY, Wagner JR, Laird MR, Melli G, Rey S, Lo R, Dao P, Sahinalp SC, Ester M, Foster LJ, Brinkman FSL. 2010. PSORTb 3.0: Improved protein subcellular localization prediction with refined localization subcategories and predictive capabilities for all prokaryotes. Bioinformatics **26(13),** 1608-1615. http://dx.doi.org/10.1093/bioinformatics/btq249

Zhang W-W, Sun K, Cheng S, Sun L. 2008. Characterization of $DegQ_{Vh}$, a serine protease and a protective immunogen from a pathogenic *Vibrio harveyi* strain. Applied and Environmental Microbiology **74(20)**, 6254-6262.

http://dx.doi.org/10.1128/AEM.00109-08

Zhang C, Yu L, Qian R. 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*). Journal of Applied Microbiology **103(5),** 1587-1599.
http://dx.doi.org/10.1111/j.1365-2672.2007.03386.x

Zhang X, Robertson P, Austin B, Xu H. 1997. Comparison of outer membrane protein profiles of *Vibrio* sp. Acta Microbiologica Sinica **37(6),** 449-454.

Zhao Y, Liu Q, Wang X, Zhou L, Wang Q, Zhang Y. 2011. Surface display of *Aeromonas hydrophila* GAPDH in attenuated *Vibrio anguillarum* to develop a noval multivalent vector vaccine. Marine Biotechnology **13(5)**, 963-970.
http://dx.doi.org/10.1007/s10126-010-9359-y

Zheng Z, Xiao Y, Wu H, Wang Q, Xiao J, Zhang Y, Liu Q. 2012. Different approaches to expressing *Edwardsiella tarda* antigen GAPDH in attenuated *Vibrio anguillarum* for multivalent fish vaccines. Journal of Fish Diseases **35,** 569-577.
http://dx.doi.org/10.1111/j.1365-2761.2012.01381.x

Zhou Z, Pang H, Ding Y, Cai J, Huang Y, Jian J, Wu Z. 2013. VscO, a putative T3SS chaperone escort of *Vibrio alginolyticus*, contributes to virulence in fish and is a target for vaccine development. Fish and Shellfish Immunology **35(5)**, 1523-1531.
http://dx.doi.org/10.1016/j.fsi.2013.08.017

Zou L, Wang Z, Wang Y, Hu F. 2010. Combined prediction of transmembrane topology and signal peptide of β-barrel proteins: Using a hidden Markov model and genetic algorithms. Computers in Biology and Medicine **40(7),** 621-628.
http://dx.doi.org/10.1016/j.compbiomed.2010.04.006