Characteristics of hemolysins from pathogenic bacteria in tropical aquaculture: an in-silico study

Dewi Syahidah
Pathology Laboratory
Institute for Marine Research Aquaculture and Fisheries Extension (IMRAFE)
Email: dewi.syahidah@my.jcu.edu.au

Abstract. Some species of known pathogenic bacteria isolates in tropical aquaculture produces hemolysin. Hemolysin can be identified based on its ability to break down red blood cells in vitro. Some hemolysin is a pore-shaped poison that can damage cell membranes and kill host cells. The character of the 13 sequences of hemolysin protein in several pathogenic bacterial isolates in tropical aquaculture was analysed using the NCBI protein bioinformatics database. The phylogenetic tree was generated, and the analysis was conducted using the base character method (Maximum Parsimony) of Mega 6.06 software. The result showed that there are two big family of hemolysin from the known pathogenic bacteria. The closest characteristics of protein sequences were hemolysin of Streptococcus agalactiae and of S. iniae.

1. Introduction
The world fisheries production has remained stagnant and most of the main fishing areas have reached their maximum potential. On the reverse, the global human population is increasing; thus, the demand for aquatic food products also increases [1], [2], [3]. Sustaining fish supplies from capture fisheries will not be able to meet the growing global demand for aquatic food. Contrastingely, the aquaculture is a great opportunity to supply the demand by aquatic food in most regions of the world; thereby it presents the fastest growing food-producing sector in the world [2], [3], [4], due to increasing of food requirement of healthful protein origin [3].

The development of aquaculture in tropical areas, including in Indonesia is important because in addition Indonesia to being supported by potential data, it also since the condition of Indonesian fishery resources especially capture fisheries, have experienced overfishing in some areas that resulting in a downward trend in the amount of production. To strengthen the capacity and accelerate the process of increasing production, a fishery revitalization program has been carried out cultivation through minapolitan area development activities, superior commodities and capital strengthening aimed at increasing competitiveness through business empowerment cultivation to the community and improving the quality of aquaculture products [5].

The economic potential of fish production is highly dependent on the limiting factors, since several factors have contributed to increase the occurrence of diseases in finfish aquaculture worldwide. Among such factors increased stocking density favors the rapid spread of pathogens, nutritional deficiency; inadequate husbandry management responsible for stress, low water quality and low temperatures can cause debilitation of finfish, favoring the pathogens infection [6].

Currently, the increase annual growth rates of the global population have led to growing of aquaculture industry due to larger global demand for farmed fish for food, fish oil, and fishmeal, putting thereby a strong unsustainable strain on the world’s natural food resources. Nevertheless, diseases have a major impact on global finfish, having significant effects on aquaculture production,
sustainability, and economic viability. Disease impacts are related to the pathogen and can be either unpredictable/sporadic or predictable/regular [7].

One of the main obstacles to the sustainability of aquaculture production is mortality caused by infection with pathogenic microorganisms and degradation of environmental quality. This condition is positively correlated with the increasingly intensive cultivation system being developed [8]. Disease problems constitute significant economic losses in fish production in biomass that consist mainly in mortality, decreased growth and productivity, besides costs of production. Globally, the potential economic loss due to disease outbreaks caused by infection with pathogenic microorganisms is quite significant and has an impact on the amount of production, profit, and sustainability of the cultivation system. Economic loss on the cultivation industry due to disease outbreaks is estimated to reach US$ 9 billion per year [9].

Bacteria are the most common organisms found in the aquatic environment and have high morphological, ecological, and physiological diversity. Most of the pathogenic bacteria in marine fish culture have short rod-shaped cells and are gram-negative. The disease caused generally shows symptoms of septicemia and ulcers [10]. As for some other bacteria vary, among others, have gram-positive properties and have the shape of cocci or rods.

There are at least eight most important bacterial diseases in tropical aquaculture, including vibriosis, aeromonas, pseudomonas, flavobacteriosis, streptococcus, clostridiosis, and franciella noatunensis. Almost all-important bacterial pathogens in tropical aquaculture produce a virulent coding gene called hemolysin. Hemolysins are proteins which can be identified based on their lysis ability to break down red blood cells in vitro. Some of hemolysins are toxins in pore forms, which can break cells membranes and kill the host cells [11].

Despite laboratory research on bacterial diseases in tropical aquaculture being focused on the experimental infection as challenge tests of the pathogen into some cultured fish, the characteristics of the hemolysin using in-silico study, which is defined in biology and other experimental sciences is one performed on computer via computer simulation, is still limited. Furthermore, a better understanding of the characteristics of hemolysin from different bacteria will pave the way for future research to overcome diseases in tropical aquaculture. This present study aims to investigate the characteristics of 13 bacterial pathogens in tropical aquaculture using in silico modelling.

2. Methods

The in-silico study begun by collecting references about bacterial pathogens in tropical aquaculture to find the bacterial isolates which pose hemolysin. Following this, the protein sequences of selected hemolysin were collected from NCBI website. The sequences were saved in FASTA using Clustal 2.1 before being aligned and analysed using Mega 6.06 software to generate the phylogenetic trees. Finally, the characteristic of hemolysin can be further explored using RCSB PDB (Research Collaboratory for Structural Bioinformatics Protein Data Bank).

3. Results and discussions

Bacteria are the most common organisms found in the aquatic environment and have high morphological, ecological, and physiological diversity. Most of the pathogenic bacteria in marine fish culture have short rod-shaped cells and are gram-negative. The disease caused generally shows symptoms of septicemia and ulcers [5;10]. As for some other bacteria vary, among others, have gram-positive properties and have the shape of cocci or rods. There are 13 main groups of bacteria from different genera, including Vibrio, Aeromonas, Pseudomonas, Streptococcus, Mycobacterium, Clostridium, and Francisella [11;12]. All the genera containing hemolysin, which is important component during the disease infection.

The previous research on bacterial diseases of cultured finfish remained at laboratory stage, with the scope is very limited to the susceptibility of different finfish to some isolates of bacteria. The information on the relationship between the hemolysin characteristics and the severity level of different isolates is still poorly understood.
Below here are the results of in-silico model of characteristics of hemolysins from different important bacteria isolates could be the first insight to further investigation to fill the gap.

3.1. The protein sequence of the 13 bacteria’s hemolysin

Fig. 1 illustrates the different length of the 13 hemolysin sequences of the selected bacteria isolates, including: *Vibrio anguillarum*, *V. parahaemolyticus*, *V. vulnificus*, *Aeromonas caviae*, *A. hydrophila*, *A. sobria*, *A. veronii*, *A. jandaei*, *Pseudomonas anguilliseptica*, *P. fluorescens*, *Streptococcus agalactiae*, *S. Iniae*, *Mycobacterium fortuitum*, and *M. marinum*, that were collected from NCBI databases, saved as FASTA using Clustal 2.1 software.

![Figure 1. The protein sequences of hemolysin from NCBI in FASTA file](image-url)
3.2. Sequence analyses using Mega 6.06

3.2.1. Sequence alignment

The sequences listed in FASTA file were alignment using Mega 6.06 (see Fig. 2.).

![Sequence alignment using Mega 6.06](image)

**Figure 2.** The sequence alignment from the tested 13 hemolysin using Mega 6.06.

The sequence was trimmed and alignment to find the conserved sequence among the 13 sequences. Following that the result was saved in Mega format for further step in phylogenetic construction.

3.2.2. Phylogenetic trees

There are several methods used to create phylogenetic trees, including Neighbor-Joining, Maximum Parsimony, Maximum Likelihood, and Bayesian. Neighbor-Joining has the advantage that the analysis is carried out relatively quickly, while the weakness of this method is that if there are many sequence differences, the confidence estimate tends to be low. The Maximum Parsimony method has the advantage of analyzing hundreds of sequences quite quickly and the level of confidence is high enough if the sequences are similar. The weakness of the Maximum Parsimony method is that if there is a variation of substance in long branches, the results obtained are not good. The third method of making phylogeny is Maximum Likelihood, which is a method that can present complete phylogenetic data. The weakness of this method is the slow analysis because it depends on the perfection of access and the source of the calculation.

There are two approaches of constructed phylogenetic trees used in this study, namely Maximum Parsimony (MP) (Fig. 4.) and Maximum Likelihood (ML) (Fig. 5.).
3.2.3. First approach

**Figure 3.** Phylogenetic tree of the 13 hemolysin sequences using the maximum parsimony method in mega 6.06 with 100 bootstraps.

3.2.4. Second approach

**Figure 4.** Phylogenetic tree of the 13 hemolysin sequences using the maximum likelihood (ML) method in mega 6.06 with 100 bootstraps.

The MP method (Fig. 3.) divides the 13 hemolysins tested into 2 families. The first family of hemolysin are from *Vibrio anguillarum*, *V. parahaemolyticus*, *V. vulnificus*, *Aeromonas caviae*, *A. sobria*, *A. veronii*, *A. jandaei*, *Pseudomonas anguilliseptica*, and *P. fluorescens*, *Mycobacterium fortuitum*, and *M. marinum*, whereas hemolysins from *Streptococcus agalactiae*, *S. Iniae* are the second group. The outgroups are hemolysin from *Streptococcus agalactiae* and from *S.
iniae. Although 5 of the bootstrap below 70 (circled), this method is more accurate than ML. The ML (Fig. 4.) also divides the 13 hemolysins tested into two families. The first family consists of *Vibrio anguillarum*, *V. parahaemolyticus*, *V. vulnificus*, *Aeromonas caviae*, *A. hydrophila*, *A. veronii*, *A. jandaei*, *Pseudomonas anguilliseptica*, *P. fluorescens*, *Streptococcus agalactiae*, *S. Iniae*, *Mycobacterium fortuitum*, and *M. marinum*, whereas hemolysin from *Aeromonas sobria*. The bootstrap value below 70 is very high and some are even zero (circled).

3.3. Characteristic of hemolysin using RCSB PDB

Fig. 5. Structure information of hemolysin

Fig 5. illustrates the main page of RCSB PDB (Research Collaboratory for Structural Bioinformatics Protein Data Bank) browser. The information of hemolysin can be abundantly downloaded by typing “hemolysin” in the destined box. More information on the structure of the protein can be seen. More information on the organisms which pose hemolysin, including a fish pathogenic bacterium, *Vibrio vulnificus*. The uni-prot molecule names are also presented. In addition, the taxonomy, and the experimental methods are described.

Fig. 6. demonstrates three codes of hemolysins of *Vibrio vulnificus*, 4OWJ, 4OWL and 4OWK, submitted to RCSB PDB. More information on each characteristic can be tracked.

Fig. 7. shows an example of 3D structure, annotation, similarities of the 4OWJ hemolysin. The figure can be rotated as the cursor is clicked on the drawing. More information on 4OWJ be downloaded.
Figure 6. Structure summary of 4OWJ from *V. vulnificus*

Figure 7. 3D view of 4OWJ hemolysin from *V. vulnificus*
The study group who submitted the report of *V. vulnificus* to RSCB PDB, Kaus et al (2014) described Pore-forming toxins (PFTs) are a class of pathogen-secreted molecules that oligomerize to form transmembrane channels in cellular membranes. Determining the mechanism for how PFTs bind membranes is important in understanding their role in disease and for developing possible ways to block their action. *Vibrio vulnificus*, an aquatic pathogen responsible for severe food poisoning and septicemia in humans, secretes a PFT called *V. vulnificus* hemolysin (VVH), which contains a single C-terminal targeting domain predicted to resemble a β-trefoil lectin fold. The X-ray crystal structure of the VVH lectin domain solved to 2.0Å resolution reveals a heptameric ring arrangement of the oligomeric form of the related, but inactive, lectin from *Vibrio cholerae* cytolysin. Structures bound to glycerol, N-acetyl-d-galactosamine, and N-acetyl-d-lactosamine outline a common and versatile mode of recognition allowing VVH to target a wide variety of cell-surface ligands. Sequence analysis considering the structural and functional data suggested that VVH may represent an earlier step in the evolution of *Vibrio* PFTs [13].

This study provided initial knowledge for the substances of a hemolysin from an aquatic pathogen that might pose similarities to that of other bacterial pathogens. This also means that it is possible to imitate ways of treating bacterial diseases from pathogenic bacteria of other organisms.

4. Conclusion
This in-silico study is the first report to show the initial information on the characteristics of some important bacterial hemolysin. Both approach of the constructed phylogenetic trees showing that the 13 bacteria isolates are grouped into two big families. The closest characteristics of protein sequences were hemolysin of *Streptococcus agalactiae* and of *S. iniae*. There are some important points to consider from this study. First, the characteristics of the examined hemolysin should be further analysed using Omics and the reports can be submitted to the RSCB PDB database. Second, trials to experimentally injected of the different family of bacteria isolates into cultured finfish should be conducted. In addition, the modelling obtained from this in-silico study pave the way to find out the severity level of two families of bacteria based on their hemolysin sequences. A more important consideration is, the opportunity to overcome bacterial diseases in tropical aquaculture is widely open.

References
[1] Food and Agriculture Organization of the United Nations-FAO 2014 *The state of world fisheries and aquaculture: opportunities and challenges*. Roma
[2] Bueno G W, Ostrensky A, Canzi C, Matos F T, Roubach R 2015 Implementation of aquaculture parks in Federal Government waters in Brazil. *Review in Aquaculture* 7:1–12
[3] Kato H C A, Freitas A A 2015 Panorama of the aquaculture expansion of aquaculture and the fish consumption in Brazil. *Journal of Fish Science* 9:81–84
[4] Subasinghe R P, Soto D, Jia J 2009 Global aquaculture and its role in sustainable development. *Review in Aquaculture* 1:2–9
[5] Novriadi R, Purnomowati R, Yunianto D, Santosa J 2014 *Penyakit ikan laut di Indonesia*. Kementerian Kelautan dan Perikanan. Direktorat Jenderal Perikanan Budidaya. Direktorat Kesehatan Ikan dan Lingkungan. 37 p.
[6] Martins M L, Onaka E M, Moraes F R, Bozzo F R, Paiva A M F C, Goncalves A 2002 Recent studies on parasitic infections of freshwater cultivated fish in the state of São Paulo, Brazil. *Acta Science* 24:981–985
[7] Shinn A J, Pratoomyot J, Bron J, Paladini G, Brooker E, Brooker A 2015 Economic costs of protistan and metazoan parasites to global mariculture. *Parasitology* 142:196–270
[8] Subasinghe R P, Bueno P B, Phillips, M J, Hough C, McGladdery S E, Arthur J R 2001 *Aquaculture development, health and wealth*. In aquaculture in the third millennium. technical proceedings of the conference on aquaculture in the third millennium (Subasinghe R P et al., eds), pp. 167-191. Bangkok and FAO, NACA
[9] Cao L, Wang W, Yang Y, Yang C, Yuan Z, Xiong S, Diana J 2007 Environmental impact of aquaculture and countermeasures to aquaculture pollution in China. *Environmental Science in Pollution Research* **14**: 452 – 46

[10] Irianto A 2005 *Patologi Ikan Teleostei*. Gadjah Mada University Press. Yogyakarta.

[11] Haenen O 2017 Major bacterial diseases affecting aquaculture. Presentation slides. Aquatic AMR Workshop I: 10-11 April 2017, Mangalore, India

[12] Austin B, Austin DA 1999 Bacterial fish pathogens, Diseases of farm and wild fish. 3rd (revised) edition. Springer-Praxis, Goldaming

[13] Kaus K, Lary J W, Cole J L, Olson, R 2014 Glycan specificity of the Vibrio vulnificus hemolysin lectin outlines evolutionary history of membrane targeting by a toxin family. *Molecular Biology* **15**: 2800-2812