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Mini-review: utilization of *Vibrio parahaemolyticus* virulence coding genes for early detection of acute hepatopancreatic necrosis disease (AHPND)

Mini-review: pemanfaatan gen penyandi virulensi *Vibrio parahaemolyticus* untuk deteksi dini penyakit Acute hepatopancreatic necrosis disease (AHPND)

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ABSTRACT

The ability to track the presence of pathogenic *Vibrio parahaemolyticus*, an AHPND causative agent in shrimp is one of the keys to controlling this infectious disease. A reputable disease diagnosis is appreciated as the ability to track the pathogenic infection when the host abnormality is undetectable due to the low pathogenic cell concentration. This mini-review article discusses the selected virulence encoding genes as molecular markers and the steps of standard validation methods in the application for early detection of AHPND disease. The proper diagnosis method is crucial to prevent the spread of Vibriosis AHPND which significantly results in economic losses for shrimp farmers. In this early warning system, we need a molecular method available for quick detection by applying the tracking tools that can discriminate pathogenic *V. parahaemolyticus* AHPND strain. Several types of potential genes that can be developed into tracking devices for infectious Vibriosis are pathogenic genes encoding the virulence factor. Through several stages of testing the selected virulence encoding genes will be developed into molecular markers. The polymerase chain reaction method and several of its variants have been widely applied using selected molecular markers. Furthermore, the use of molecular markers for the diagnosis of AHPND disease in shrimp must be validated by determining aspects of sensitivity, detection specificity, repeatability consistency, and reproducibility.

Keywords: virulence-encoding gene, *Vibrio parahaemolyticus*, AHPND, diagnose, molecular marker

ABSTRAK

Kemampuan melacak keberadaan patogen *Vibrio parahaemolyticus*, agen penyebab AHPND pada udang adalah salah satu kunci untuk mengendalikan penyakit menular ini. Diagnosis penyakit yang memiliki reputasi baik akan dihargai karena mampu untuk melacak infeksi patogen ketika tanda-tanda abnormalitas pada inang belum terdeteksi karena konsentrasi sel patogen yang masih rendah. Makalah mini-review ini membahas tentang tahap-tahap metode validasi standardalam aplikasi gen penyandi virulensi terseleksi untuk deteksi dini penyakit AHPND. Metode diagnosis yang tepat sangat penting untuk mencegah penyebaran Vibriosis AHPND yang secara signifikan mengakibatkan kerugian ekonomi bagi petambak udang. Dalam sistem peringatan dini ini, diperlukan metode molekular yang tersedia untuk deteksi cepat dengan menerapkan alat pelacak yang mampu membedakan patogen *V. parahaemolyticus* strain AHPND. Beberapa jenis gen potensial yang dapat dikembangkan menjadi alat pelacak penyakit Vibriosis menular adalah gen patogen yang mengkodekan faktor virulensi. Beberapa tahapan pengujian harus dilakukan untuk menjadikan gen penyandi virulensi terpilih sebagai kandidat yang akan dikembangkan menjadi penanda molekular. Metode *polymerase chain reaction* dan beberapa variannya telah banyak diterapkan dengan menggunakan penanda molekular terseleksi. Selanjutnya pemanfaatan penanda molekular untuk diagnosis penyakit AHPND pada udang harus dilakukan validasi dengan menentukan aspek sensitivitas, spesifisitas deteksi, konsistensi pengulangan, dan reprodusibilitas.

Kata kunci: gen penyandi virulensi, *Vibrio parahaemolyticus*, AHPND, diagnosis, penanda molekuler

INTRODUCTION

Pacific white shrimp (*Litopenaeus vannamei*) is economically one of the important export commodities of Indonesian aquaculture. Increasing the production of white shrimp continues to be pursued by revitalizing the unutilized shrimp pond area, as well as by applying intensive aquaculture technology. Several things are still obstacles to achieving the national shrimp production target, both for export needs and domestic shrimp consumption. Various types of infectious diseases due to viral and bacterial infections are still the main obstacles to white shrimp cultivation in Indonesia (Munaeni et al., 2014; Nurhayati et al., 2015; Febrianti *et al*., 2016; Yunarty *et al*., 2016; Utomo *et al*., 2023).

The emergence of highly infectious diseases causes high mortality and low growth rates of shrimp in hatcheries and rearing ponds (Yuhana *et al*., 2022). The main bacterial disease that infects white shrimp is vibriosis (Arisa *et al*., 2015; Zubaidah *et al*., 2015; Widanarni *et al*., 2018). Vibriosis has caused mass mortalities in all shrimp stages, from nauplius, zoea, mysis, and post-larvae to adults in rearing ponds. Vibriosis disease in seedling segmentation is often associated with luminous *Vibrio harveyi* or while in the pond rearing stage it is caused by non-luminous *V. parahaemolyticus* (Fuandila *et al*., 2019; Munaeni *et al*., 2020; Nainggolan *et al*., 2020). In previous studies, we have identified the important genes of the pathogens which is very crucial for developing the rapid and early diagnosis tools for the disease detection (Kadriah *et al*., 2011; Kadriah *et al*., 2014; Setiawan *et al*., 2015; Nainggolan *et al*., 2020).

Acute hepatopancreatic necrosis disease (AHPND) or acute hepatopancreatic necrosis syndrome was initially called Early Mortality Syndrome (EMS) (Lee *et al.*, 2015; Tran *et al*., 2013; Dong *et al*., 2017a) in the white shrimp culture, since the shrimp mass mortality occurred in the early period of cultivation (Choi *et al*., 2017; Kumar *et al*., 2020a). The main infectious agent is the special AHPND strain of *V. parahaemolyticus* which causes serious production declines and financial losses in the global shrimp farming industry (Li *et al*., 2017; Kumar *et al*., 2020b). The bacterial species *V. parahaemolyticus* has also been reported as the causative agent of AHPND in white shrimp and tiger prawns (Bondad-Reantaso & Arthur, 2018). In the Decree of the Minister of Marine Affairs and Fisheries Republic of Indonesia, Kepmen (2015) concerning the Determination of Quarantine Types of Pests and Diseases of Fish, Quarantine Group Categories, Carrier, and Their Distribution, it has been stated that the specific strain of *V. parahaemolyticus* that causes EMS/AHPND is one of the quarantined fish disease and pests Category 1 Group.

The presence of AHPND strain of *V. parahaemolyticus* is prohibited from entering and spreading in the territory of the Republic of Indonesia. AHPND is reported to have spread in Asian countries, starting in 2009 in China (Hong *et al*., 2016), spreading in 2010 to Vietnam (Nochiri *et al*., 2014; Tran *et al*., 2013), Malaysia (Joshi *et al*., 2014), then in 2012 it was reported as an epidemic in Thailand (Kondo *et al*., 2014; Kongrueng *et al*., 2014a, b), 2015 was reported in the Philippines (Han *et al*., 2015a; de la Peña *et al*., 2015; de la Peña *et al*., 2017; Chu *et al*., 2016). In 2013 it spread to Latin America, Mexico (Nunan *et al*., 2014; Soto-Rodriguez *et al*., 2015), AHPND resulted in mass mortality in shrimp aged 30 days after stocking (NACA, 2012; NACA, 2013; Karunasagar & Karunasagar, 2018). China has lost 80% of white shrimp production because of this disease.

In 2010 Vietnam suffered an economic loss of 570,000 to 7,200,000 USD (Zorriehzahra & Banaederakhshan, 2015). According to FAO (2013), in 2011 the impact of AHPND attacks on white shrimp cultivation in Malaysia resulted in losses of up to 100 million USD. Thailand was affected by the AHPND attack in 2012 which resulted in losses of up to 109.9 million USD (Songsangjinda & Jaree, 2016). In 2013 the AHPND attack also caused a very significant decrease in the production of white shrimp in the Western Hemisphere, resulting in an economic loss of 118 million USD in Mexico this country (Schryver *et al*., 2014). The AHPND outbreak has not been officially reported to occur in shrimp farming in Indonesia.

Along with the incidence of AHPND disease in shrimp culture in the Asian region, some studies reported an increasing prevalence of vermiform such as gregarine in shrimp hepatopancreas (HP) and midgut had been detected (Sriurairatana *et al*., 2014; Sirikharin *et al.*, 2015). Infected shrimp produce high amounts of elongated, webbed white stool, a phenomenon known as WFS (white feces syndrome) or white stool syndrome. At the rearing stage in the shrimp pond, Vibriosis threatens the health of the shrimp in the early days

of culture (DOC). At the age of rearing AHPND infects postlarvae in the first 20-30 days after being reared in ponds, causing mortality often to reach 100%.

The target organs of pathogens causing AHPND are mainly intestinal related tissues and therefore cause clinical symptoms such as pale, shrinking, damaged hepatopancreas, empty stomach and empty intestines. Initially, AHPND was called Early Mortality Syndrome (EMS), but after the discovery of the unique strain of *V. parahaemolyticus* that causes this disease, the name of the disease was changed to acute hepatopancreatic necrosis or AHPND (Sirikharin *et al*., 2015; Lee *et al*., 2015). The application and availability of molecular markers as an early detection tool are very important for disease diagnosis so that mass shrimp mortality and further economic losses can still be avoided. Proper diagnosis and treatment methods are needed to control and prevent further outbreaks. Flegel and Lo (2013) identified and released specific primer pairs for strains of *V. parahaemolyticus* isolates associated with AHPND.

AHPND-specific strain of *V. parahaemolyticus* is responsible for and is the causative agent of AHPND disease in shrimp. This strain of *V. parahaemolyticus* has virulence factors in the form of a toxin that is expressed as thermostable direct hemolysin (tdh), TDH-related hemolysin (trh) and two types III secretion systems (T3SS) (Makino *et al*., 2003). The result of the expression of this virulence coding gene is the synthesis of a toxin protein that is damaging to the digestive organs of shrimp. One of the efforts to control the spread of infectious diseases in shrimp can be done by focusing attention on the occurrence of disease transmission and the factors that cause it. This article summarizes the basic principles of AHPND Vibriosis disease in shrimp, disease transmission, as well as the basic selection principles, development, and validation steps of selected virulence coding genes for the appropriate, a standardized diagnostic method.

Pathogenicity mechanism and the virulence encoding genes in *V. parahaemolyticus* strain AHPND

The important steps of microbial pathogenicity are as follows: cells use motility organelles and other means to attach, enter the cell and spread within the host; attach (attachment) to the target cell on the host; attack/damage host cells, compete for iron and other nutrients; against the host's innate immune defenses such as phagocytosis and complement systems and avoid adaptive immune defenses (Fierer *et al*., 2017). Pathogen cells must come into physical contact with a host cell before they can attach to the cell and refuse to be removed from the body. Motile bacteria can use flagella and their chemotaxis to swim through mucus towards mucosal epithelial cells. Due to their thinness, their internal flagella enter the lymph vessels and blood vessels and spread to other parts of the body.

One of the host's innate immune defenses is the ability to physically remove pathogenic bacteria from the body. Pathogenic bacteria are able to evade this mechanism by producing pili, cell wall adhesive proteins, and/or a biofilm-forming and protective capsule that allows the bacteria to adhere more firmly to host cells. At the tip of the stem of the pilus pathogenic bacteria is also equipped with an adhesive structure that has a shape that corresponds to a specific receptor on the host cell for initial attachment (Fierer *et al*., 2017). Some pathogenic bacteria produce a molecule called invasin which activates the cytoskeletal machinery of the host cell allowing the entry of pathogens into the cell by phagocytosis.

Pathogens enter vulnerable host cells that provide a supply of nutrients, and even protect bacteria from complement, antibodies, and other defense molecules. Some pathogens are capable of attacking phagocytic cells, neutralizing their killing ability, and turning them into safe sites for bacterial replication. The ability of a pathogen to thrive is directly related to its ability to compete successfully with host tissues and other normal flora for absorbing limited nutrients. Pathogens can compete by synthesizing certain nutrient transport systems or cell wall components that are able to bind to the limiting substrate and transport it into the cell.

Iron (siderophore) is an essential nutrient for the growth of both pathogenic and host cells. Both pathogenic and host bacteria synthesize compounds capable of binding siderophores (Fierer *et al*., 2017). In addition, the pathogen is able to fight shrimp innate immune defenses such as phagocytosis and the body's complement pathway. AHPND pathogenesis system involves the Quorum Sensing mechanism.

The density of bacterial cells in the environment has been a factor triggering the level of expression of virulence factors in several types of pathogenic bacteria, this is known as the quorum sensing system. Bacterial quorum

sensing (QS) is the ability at the cellular level of bacteria to detect the total density/population of cells in their environment. In this system, biochemical communication occurs between cells and is related to gene regulation processes in response to increased cell density by synthesizing, releasing and detecting signaling molecules called autoinducers (AI) (Defoirdt *et al*., 2004; Defoirdt *et al*., 2008). In general, autoinducers are classified into three types, namely acyl homoserine lactone (AHL), auto-inducing peptides (AIP), and autoinducer 2 (AI-2) (Huang *et al*., 2016).

Gram-negative pathogenic bacteria generally have autoinducer compounds in the form of AHL. The quorum sensing signal molecule produced by *V. parahaemolyticus* is N-acyl homoserin lactones (AHL) in the form of 3-oxo-C6-HSL (Vinoj *et al*., 2014). Signal molecules produced by bacteria after reaching the quorum can then activate regulatory proteins as inducers of virulence-related gene expression. Pathogenic *V. parahaemolyticus* possess virulence factors that enable them to invade target organs in shrimp.

The unique *V. parahaemolyticus* strain AHPND has a group of tdh (thermostable direct hemolysin) genes, and trh (TDH-related hemolysin) which encodes a toxin protein. The tdh and trh genes are specific fragment sequences of the virulence plasmid (pAP1) that are only found in the *V. parahaemolyticus* strain AHPND. These two genes are responsible for and encode the A/B Pir toxin that causes damage to the hepatopancreas and causes death in shrimp (Han *et al*., 2015a; Bondad-Reantaso, 2016). Dong *et al*. (2017a, b) observed the Pirvp toxin protein (consisting of PirAvp and PirBvp toxin subunits) which is homologous to the binary toxin *Photorhabdus* spp. on insects. Wang *et al*. (2015) classified the virulence factors of *V. parahaemolyticus* including hemolysin (TDH and TRH toxin) form pore on cells.

Other factors are urease, two type III secretion systems and two type VI secretion systems. These two secretion systems cause both cytotoxicity in cultured cells and enterotoxicity in the animal host. Previous study (Matsuda *et al*., 2012) also reported that TDH is associated with bacterial pathogenesis. One is a hemolytic capability.

TDH binds to the membranes of blood or host cells, and forms a pore on the membrane surface, ultimately leading to the permeation of the colloids on cells. The other enzymatic activity which is necessary for cytotoxicity. TDH causes cells toxicity and forms a channel in the cell membrane which induces an increase in the extracellular Ca concentration and Cl secretion (Matsuda *et al*., 2012). When the osmotic pressure of the cell exceeds the upper limit for cell self-regulation, morphological changes were occur in the cell, resulting in cell abnormality and death.

T3SS1 secretion system is essential for systemic infection, whereas T3SS2 is associated with the enterotoxicity of *V. parahaemolyticus* (Ham & Orth, 2012). The T6SSs, novel recently identified systems are important for the adhesion of *V. parahaemolyticus* to cells and are also involved in intracellular mobility. T6SS1 has antibacterial activity under warm conditions, enhancing the environmental fitness of *V. parahaemolyticus* (Salomon *et al*., 2013b). A recent study (Wang *et al*., 2020) reported that the fatality of the AHPND strain of *V. parahaemolyticus* is due to the presence of pVA1 plasmid which is capable of expressing the major virulence factor of the PirA*vp*/PirB*vp* toxins.

However, there are approximate ~90 open reading frames (ORFs) encoding important functional proteins. More importantly, those ORFs are functionally very crucial in pathogenicity mechanisms such as toxin secretion, plasmid maintenance, and a post-segregational killing (PSK) system of the host's cells. In addition, ORFs also contain genes necessary for transmission processes and drug resistance, gene transposition, as well as bacterial conjugation. AHPND disease that was previously attributed only to *V. parahaemolyticus*, now it has been reported that the causative agents are diverse *Vibrio* species.

The mechanism of horizontal gene (HGT) through plasmid conjugation has caused the spread of the AHPND virulence factor among *Vibrio* spp. such as *V. harveyi*, *V. campbelii* (Dhar *et al*., 2019), *V. owensii* (Xiao *et al*., 2017; Liu *et al*., 2018), and *V. punensis* (Restrepo *et al*., 2018). Other gene products of type II and III secretion systems in the pVA1 plasmid may associate with the toxic effects of among those *Vibrio* sp. Genes encoding anti-restriction proteins are important for bacterial resistance against the drugs as the curative agents for AHPND disease in shrimp. Therefore the positive amplification of PCR targeting the PirA*vp*/PirB*vp* toxins (Dong *et al*., 2017b) are no more directly associated specifically with the infection of *V. parahaemolyticus*.

The subunits of PirAB toxin as the secreted by *Vibrio parahaemolyticus* known to have high

homology and function with *Bacillus thuringiensis* Cry toxin. Both toxins are reported capable of inducing the pore formation in host epithelial cells (Almanza-Martínez *et al*., 2020; Brito *et al*., 2019). The damaged plasma membrane resulted a permanent protein pores that unstabilized cellular homeostasis. After structural alignment, the PirAB toxin caused cell death as previously described in the action mechanism of Cry.

In their study, Almanza-Martínez *et al.* (2020) reported that α-amylase-like protein is identified as possible binding receptor protein of PirAB-toxin from *V. parahaemolyticus* in Pacific white shrimp *Litopenaeus vannamei*. The application of a specific blocking agent from bioactive compounds may become a key factor to inhibit the virulence determinants of the pathogenic cells. The specificity of the attachment of PirB*vp* toxin to shrimp hepatopancreatic epithelial cells has resulted in acute damage and necrosis of hepatopancreatic tubules. There is an important role of the specific cellular receptor of the PirB*vp* toxin subunit in this target organ, as has been reported by de los Santos *et al.* (2022) i.e. the presence of two glycoprotein molecules measuring 60 and 70 kDa (Gc60 and Gc70).

These two glycoprotein subunits play a crucial role in the pathogenesis mechanism of AHPND strains which can be detected in the epithelial cells of shrimp hepatopancreas. AHPND strain with acute virulence has PirB*vp* toxin in the form of lectin in its pathogenesis mechanism which functions to recognize glycoprotein receptors. Endoglycosidase as an agent that can eliminate the binding of the shrimp hepatopancreatic receptor with the toxin molecule of PirB*vp*. The virulence factor of AHPND strain *V. parahaemolyticus* is known to be the PirAB*vp* toxin subunit (Kumar *et al*., 2021).

Gu *et al*. (2022) reported the highest increase in protein expression in response to the challenge of VPAHPND, which was identified as LvFABP. More detailed sequencing analysis revealed strong homology between LvFABP and lipocalin/cytosolic fatty acids. In the target organ of hepatopancreas, the interaction of PirB*vp* virulence factor with LvFABP as a receptor that binds is required so that it plays a very important role in the subsequent cellular pathogenesis process. This study has been successfully confirmed by Western blotting, by in vitro analysis for protein-protein interactions and by challenge assays through in vivo experiments.

Utilization of virulence genes to be developed as an early warning system tool with PCR-based methods

The polymerase chain reaction (PCR)-based method is an enzymatically in-vitro process amplifying the specific target sequence of the pathogenic DNA. This is a fast, sensitive, accurate laboratory diagnostic method when compared to other conventional such as culture-dependent methods. Some advantages of this method, i.e., PCR specifically detect only one target molecule in complex population mixtures without the need of culturing them and reducing the time for analyzing to conventional biochemical assays. Detection medium due to the complexity and variability need of the nutrients of diverse pathogen isolates.

However the clinical microbiology analyses continues to rely on culture-based methods, including isolation and characterization of the isolates phenotypically by conducting biochemical tests. At the beginning of the use of biomolecular methods to detect AHPND only with conventional PCR, namely with AP1 and AP2 primers (Flegel & Lo, 2013) and then AP3 primers (Sirikharin *et al*., 2015). Han *et al.* (2015b) made a breakthrough in designing VpPirA real-time PCR primers to detect AHPND and compared them with single step AP3 primers in conventional PCR. The sequence of *VpPirA* Forward Primer was 5′-TTGGACTGTCGAACCAAACG-3′ and the Reverse primer was 5′-GCACCCCATTGGTATTGAATG-3′. Those *VpPirA* primer pairs resulted in a quantitative real-time PCR being more sensitive, specific and efficient than conventional AP3 single step PCR primers.

Verification and validation of PCR methods for early diagnosis/detection of AHPND in shrimp

The method validation is a crucial step in the quality assurance process based on PCR technique. Steps in validation determine the accuracy, sensitivity, and specificity of a test method that has been developed based on selected primer pairs. To achieve the validation process, optimization and standardization of reagent concentrations and PCR conditions must be carried out which involves the temperature and time (ramp time) of the denaturation process, primer annealing and primer elongation. Likewise, the estimated interpretation of the results of the analysis and diagnosis on testing (OIE 2018a, b).

VpPirA primer (Han *et al*., 2015b) has been designated by OIE (Office International des Epizooties) as a primer and standard probe for the real-time PCR method in testing PirA toxin that causes AHPND need to be internally validated for widely used as standard method. Therefore, several analytical parameters that must be considered in the verification and validation of diagnostic methods as previously mentioned according to the European Medicines Agency (1995) are based on the following criteria: Precision, is the level of conformity between the test results measured through the spread of the average procedure of a method carried out repeatedly on a homogeneous sample. Accuracy, is the level of closeness of the analysis results to the actual analyte content and is expressed as a percent recovery. The detection limit, is the lowest concentration of analyte that can be detected.

Analytical sensitivity is the ability to detect or test the analyte sensitively the minimum concentration of pathogens that can be detected in the sample. This is to prevent false negative results from being declared negative even though they are false, because they are unable to amplify target DNA below the detection limit concentration. The benefits of analytical sensitivity testing determine the limit of detection (LoD), the lowest amount of DNA that can still be detected in the test sample. The quantitation limit, is the lowest analyte concentration that can show a good level of precision and accuracy.

Analytical specificity, is the ability to detect or test the analyte accurately against other components in the sample. The benefit of analytical specificity testing is to test that the selected primer application does not cross-react with non-target DNA (to prevent false positive results, the PCR result is positive, even though it is wrong, because it amplifies non-target DNA sequences). Linearity and range of detection, is the success of a method to obtain test results proportional to the analyte concentration in the specified range. Ruggedness, is the level of repeatability of the test results of a method from the same sample under normal test conditions. Robustness, is the capacity of a method not to be affected by the smallest method variation.

The validity of *Vibrio parahaemolyticus* AHPND (Vp AHPND) strain detection in whiteleg shrimp (*Litopenaeus vannamei*) in Indonesian shrimp culture was successfully performed employing the real-time PCR method. This step was carried out through several stages including the testing of specificity, sensitivity, repeatability, reproducibility, and sampling field tests in shrimp ponds (Nainggolan *et al*., 2020). The analytical performance test showed that the application of real-time PCR method using *VpPirA* primer was effective in detecting Vp AHPND strain and showed a sensitivity up to 10 copies µL. The cut off value qPCR method of the primer *VpPirA* is at Cycle threshold (Ct) value of 39. The real-time PCR method using *VpPirA* primer also has good repeatability and reproducibility values in detecting Vp AHPND strain. All analytical performance testings of the qPCR method meet the standard requirements to detect Vp AHPND strain. The result of field tests for all whiteleg shrimp samples from the shrimp ponds, however, revealed negative infection of Vp AHPND. This study suggested that real-time PCR is effective and valid for early detection of *V. parahaemolyticus* AHPND strain in shrimp.

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