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Detection and analysis of hemolysin genes in *Aeromonas hydrophila* isolated from Gouramy (*Osphronemus gouramy*) by polymerase chain reaction (PCR)

Rozi, K Rahayu, D N Daruti

Department of Fish Health Management and Aquaculture, Faculty of Fisheries and Marine, Airlangga University, Surabaya 60115, Indonesia

E-mail: rozi@fpk.unair.ac.id

Abstract. The goal of this study was to detect of *Aeromonas hydrophila* carrying the *hly*A gene in guramy by PCR assay. A total of 5 *A. hydrophila* strains were isolated from gouramy with different location and furthermore genotypic of all *A. hydrophila* strains havedetected by PCR assay for 16S rRNA gene. The primers used in the PCR targeted a 592-bp fragment of the *hly*A gene coding for the hemolysin gene. Particularly *hly*A genes are responsible for haemolysin toxins production in this genus. After gel electrophoresis, the amplicons from representative strains of the *A. hydrophila* were purified using extraction kit and were subjected to the DNA sequencing analysis. The results showed that: (i) the 592bp amplicon of the *hly*A gene was detected in 5/6 of the *A. hydrophila*; (ii) the nucleotide blast results of hemolysin gene sequences of the strains of *A. hydrophila* revealed a high homology of 90-97 % with published sequences, and;(iii) the protein blast showed 95-98 % homology when compared to the published sequences. The PCR clearly identified the haemolysin-producing strains of *A. hydrophila* by detection in *hly*A genes and may have application as a rapid species-specific virulence test.

1. Introduction

Aeromonas hydrophila is a bacterium with the following characteristics: gram-negative, short shaped, aerobic and facultatively anaerobic, non-sporing, motile, has one flagel, and lives in the temperature range of 25-30^{o}C [1]. Chronic infection from these bacteria can cause ulceration, inflammation, and dermal lesions with focal haemorrhages Cipriano [2],where the liver and kidneys are the common target organs during acute septicemia [3]. An infection of this bacteria can also cause symptoms of hemorrhagic diseasesepticemia that has a characteristic wound on the surface of the body, gills, ulcers, abscesses, exopthalmia and belly bloating Austin [4], as well as gastroenteritis, diarrhea and extra intestinal symptoms in humans [5]. This bacterium is very influential in freshwater fish farming and often a disease outbreak can cause levels of high mortality (80 – 100 %) in 1 - 2 weeks. The degree of virulence of *A. hydrophila* can cause fish death depending on the poison generated. Genes Aero and *hly*A play an important role in producing aerolysin and hemolysin toxins in the genus Aeromonas [6].The rapid detection of the hemolysin gene from *A. hydrophila* and their characterization is considered very important so appropriate preventive measures can be undertaken to reduce mortality and losses in fish farming. Given the economic importance it has on the health of aquatic animals and

humans, it is essential that the rapid detection of pathogens and the characterization of these isolates are carried out using molecular techniques. With this in mind, the main purpose of this study is to genetically identify the *A. hydrophila* bacteria and determine the presence of the hemolysin gene in the isolate using PCR assay.

2. Methodology

2.1. Bacteria isolates

A collection of five isolates of *A. hydrophila* was taken from the Microbiology Laboratory of the Faculty of Fisheries and Marine at Airlangga University. The identification of bacteria isolates was also carried out by the Faculty of Fisheries and Marine at Airlangga University through PCR amplification with 16S rRNA and was detected for *A. hydrophila* (Rozi, personal communication), while the *A. hydrophila* ATCC® 35654 ™ strain was used as a control strain

2.2. Extraction of A. hydrophila isolat DNA at the Faculty of Fisheries and Marine, Airlangga University (extraction using the TIANamp-China kit)

The *A. hydrophila* isolate was grown on Triple Soya Broth medium (TSB) for 24 hours in about 10 ml bacterial culture suspension in a density of 10^8 cells / ml. The culture was poured into the microtube and centrifuged at 3000 rpm for three minutes, the supernatant was discarded as much as possible until the pellet remained, then added with an ATL buffer at 200 µl, 20 µl proteinase K, and then incubated at 56^0C in a water bath for 10 min.Supernatant was vortexed for 15 seconds and then added 200 µl buffer, then divotex again, furthermore 200 µl of absolute ethanolwas added, and vortexed once more. The solution was then placed into the spin column and centrifuged at 8000 rpm for 1 minute. Next, the filter was moved to a new spin column and added with 500 µl of the AW1 buffer and then centrifuged at 8000 rpm for 1 minute. Afterwards, the filter was transferred to a new spin column with the addition of 500 µl of the AW2 buffer, centrifuged at 14.000 rpm for 3 minutes, transferred to a new micro tube and then added with 200 µl of the AE buffer, incubated for 1 minute at room temperature, and centrifuged at 8000 rpm for 1 minute. The filter results are stored at a temperature of - 70^oC, while the DNA template supernatant containing bacteria is used directly on specific PCR to detect hemolysin (*hly*) genes.

2.3. Amplification of hlyA gene (GoTaq® Green Master Mix, Promega)

A solution of 6.5 µl dH2O and 12.5 µl Green mix is placed in a microtube, then mixed until homogeneous. The primer used is the oligonucleotide of each 2 µl forward primer (5'-GGCCGGTGGCCCGAAGATACGGG-3') and primary reverse (5'-GGCGGCGCCGGACGAGACGGGG-3') with the final concentration of 0.1 µM and 2 µl DNA templates. The PCR program for DNA amplification is as follows: initial denaturation at 95°C for 5 min followed by 30 cycles at 95°C for 2 min, 55°C for 1 min and 72°C for 1 min. 7 min final extension at 72°C. The PCR results from various treatments were electrophoresed on agarose 1.5 % agar (0.75 g dissolved into TAE buffer 50 ml) and was boiled in the microwave. After warming the agarose and adding 2 µl of EtBr solution with a concentration of 10 mg/ mL, the agarose was then poured into an electrophoresis mold. After the agarose hardened, 0.8 µl of PCR product mixed with 0.2 µl loading dye was inserted to wells 2, 3, 4 and so on. The first well was filled with 0.5 µl of molecular marker. Electrophoresis is run with 100 voltages for 30 minutes, after which the gel is lifted and observed above the UV transilluminator, then documented.

2.4. Purification and bioinformatics analysis

Purification of the PCR sample product was done with microclean. 100 µl of PCR product and 100 µl of microclean (1: 1) was mixed to the homogeneous sample product using a pipette. Incubation was done for 5 minutes at room temperature, and centrifuged at 13.000 rpm for 5 minutes. The supernatant solution is removed, vortexed until the remaining supernatant is completely clean, then the pellet is resuspended with TE of 30 µl andstored at -20°C. The purification and sequencing of *A. hydrophila*

DNA was performed with the automatic sequencer ABI PRISM 377 DNA (Applied Biosystem, USA). Then it was run through the BLAST program (NCBI: http://www.ncbi.nlm.nih.gov/BLASTi/. The philogenetic tree was constructed and similarity analysis was conducted with the program version of MEGA 7.

3. Result and Discussion

3.1. Detection of hemolysin gene fragments

The DNA PCR results of the six isolates of *A. hydrophila* on pure cultures using the primer olygonucleotide positively detected hemolysin genes. Overall detection of *hly*A gene fragments showed that 5/6 of the isolates (83.3 %) contained the hemolysin gene in the *A. hydropila* and showed a molecular weight of 592 bp. (figure 1)

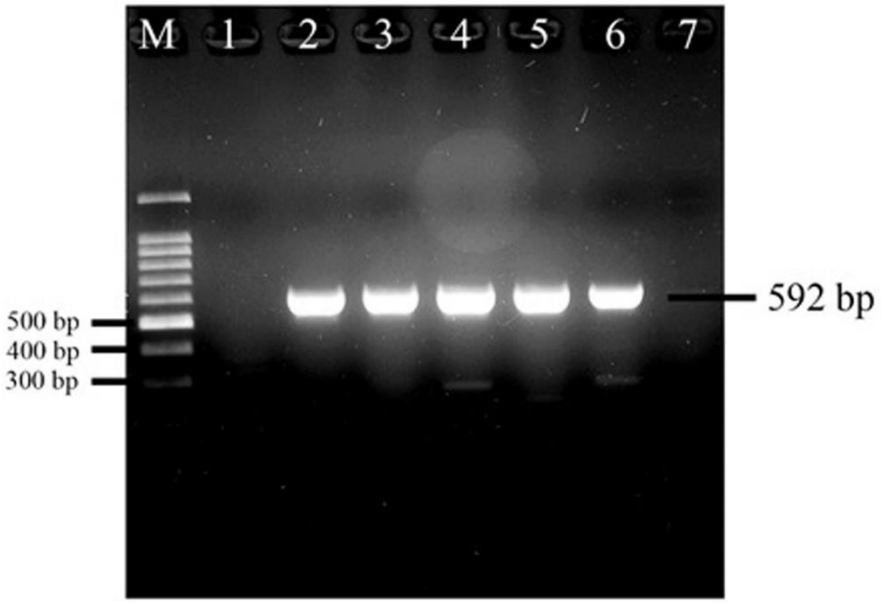

Figure 1. Detection and identification of *A. hydrophila* hemolysin gene by amplification of fragments in the PCR assay. M: indicates the 100 bp ladder as a marker, lane 1: the control negative, lane 2: the control ATCC® 35654™, lane 3: isolate AHI1, lane 4: isolate AHI2, lane 5: isolate AHI3, lane 6: isolate AHI4, lane 7: isolate AHI5, respectively.

3.2. Homology of hemolysin gene

The hemolysin gene sequencing results were compared with the GenBank database from the National Center for Biotechnology Information (NCBI) using the BLAST program (http://www.ncbi. nlm.nih.gov/). The homology of the nucleotide sequence of the hemolysin gene sequence of *A. hydrophila* showed a high homology between 90 % - 95 %. The prominent protein concentrations of *A. hydrophila*, showed 95 % - 98 % homology (data not shown) when compared with sequences published in the GenBank database of the National Center for Biotechnology Information (NCBI).

3.3. Phylogenetic tree gene hemolysin

The phylogenetic tree constructed using the neighboring joining method showed five isolates as *A. hydrophila* strains. However, there are several comparative isolates showing different genotypes. Phylogenetically, the relationships of the five isolates tested were divided into three groups. The firstgroup contained was adjacent to the *A. hydrophila*hemolysin gene, while the second group

consisting of five isolates have a relationship to one isolate controlled with the *A. hydrophila* ATCC 35654 strain. The third group of the five isolates tested was clustered separately (figure 2)

Table 1. Homology levels of hemolysin sequence of *A. Hydrophila*.

Isolates	Identity closest to the GenBank reference	Accession Number	% Identities
AH1 JA_*hly*A	*A. hydrophila* strain JNC1007 haemolysin (*hly*A) gene, partial cds	JQ003206.1	95 %
AH2 SA_*hly*A	*A. hydrophila* strain DWCG102 haemolysin (*hly*A) gene, partial cds	JF738031.1	95 %
AH3 SU_*hly*A	*A. hydrophila* strain SDDLAH/09 hemolysin gene, partial cds	FJ972621.1	96 %
AHI4 SR_*hly*A	*A. hydrophila* gene for hemolysin, complete cds, strain:AH085	AB206039.1	97 %
ATCC _*hly*A	*A. hydrophila* strain CHS-3 haemolysin gene, partial cds	KF483996.1	90 %

Based on the literature, an *A. hydrophila* infection is acute when marked by the very quick death of the fish with the appearance of signs of clinical infection such as: exophthalmia, red patches on the skin, accumulation of fluid in the abdominal pouch, flatulence, bleeding gills and injury to the dermis, and separated scales [20]. According to Kamiso [7] *A. hydrophila* can cause the death of 90-100 % in a short time. Research by Daskalov [8] also shows that *A. hydrophila* is widely distributed through food, drinking water and the environment, and is a pathogen that causes hemorrhagic diseases, zoonotic diseases, and food-borne infections in freshwater fish. Several factors of virulence and pathogenicity are involved with the presence of different exotoxins e.g., haemolysin, enterotoxins and cytotoxins and exoenzymes e.g., proteases and lipases [9]. The two major groups of haemolysins such as extracellular haemolysin and aerolysin [10] are produced by *A. hydrophila*.

Overall, PCR amplification of *hly*A gene fragments showed that 5/6 of the *A. hydrophila* isolates (83.3%) showed a haemolysin gene with a molecular weight of 592 bp. (figure 1). Chacón et al. stated in 2003 [29] a similar observation was in earlier studies on the *A. jandaei* type strain where it presented the aerolysin/hemolysin gene. Pathogenicity of enteric bacteria is produced by cytotoxin [30]. Based on the analysis of phylogenetic trees of the hemolysin gene (figure 2), it shows that the isolates AH1 JA_*hly*A, AH2 SA_*hly*A, AH3 SU_*hly*A, AHI4 SR_*hly*A and ATCC _*hly*ABK1 were the same isolates because they were in one cluster. Both isolates were closely related to refractory strains *A. hydrophila* strains JNC1007 *A. hydrophila* strains DWCG102 haemolysin (*hly*A) gene, partial cds, *A. hydrophila* strains SDDLAH / 09 hemolysin gene, partial cds, *A. hydrophila* gene for hemolysin, complete cds, strains: AH085, and *A. hydrophila* strains CHS-3 haemolysin gene, partial cdshaemolysin (*hly*A) gene, partial cds with a 90-97 % equivalent percentage. In phylogenetic trees, all AH1 JA_*hly*A, AH2 SA_*hly*A, AH3 SU_*hly*A, AHI4 SR_*hly*A and ATCC _*hly*ABK1 isolates were the same isolates. The five isolates are in a branch with the closest species of 26 *A. hydrophila* strains with 97 percent equality percentage with the strain of reference strain of *Vibrio parahaemolyticus, Sterptococcussuis* and *E. coli.*

The virulence rate of *A. hydrophila* which can cause fish mortality depends on the toxicity produced. The Aero and *hly*A genes are responsible for producing aerolysin and hemolysin toxins in the Aeromonas genus [6]. Aerolisin is an extracellular protein produced by some soluble *A.hydrophila*strains, a hydrophilic protein having hemolytic and cytolytic properties. Aerolysin binds

to specific glycoprotein receptors on the surface of eukaryotic cells before entering into the fat layer and forming a hole. The aerolysin poison that forms a hole passes into the bacterial membrane as a peptide-containing preprotoxine. The toxin can attack epithelia cells and cause gastroenteristis [6]. Haemolysin produced by extracellular products (ECPs) of bacteria become a bacterial defense factor against the defenses of the host blood because it is able to lysis blood cells. The bacteria can survive the blood flow to spread throughout the body of the host cells as well as towards the target organs. Bacteria also have the factor of pathogenicity in the form of enzymes found in ECPs, including caseinase, gelatinase, amylase, lipase, phospolipase, chitinase, collagenase, elastase, hyaluronidase, and proteinases that are able to decipher the complex compounds into simpler compounds, so the bacteria can easily break through the host cell [11].

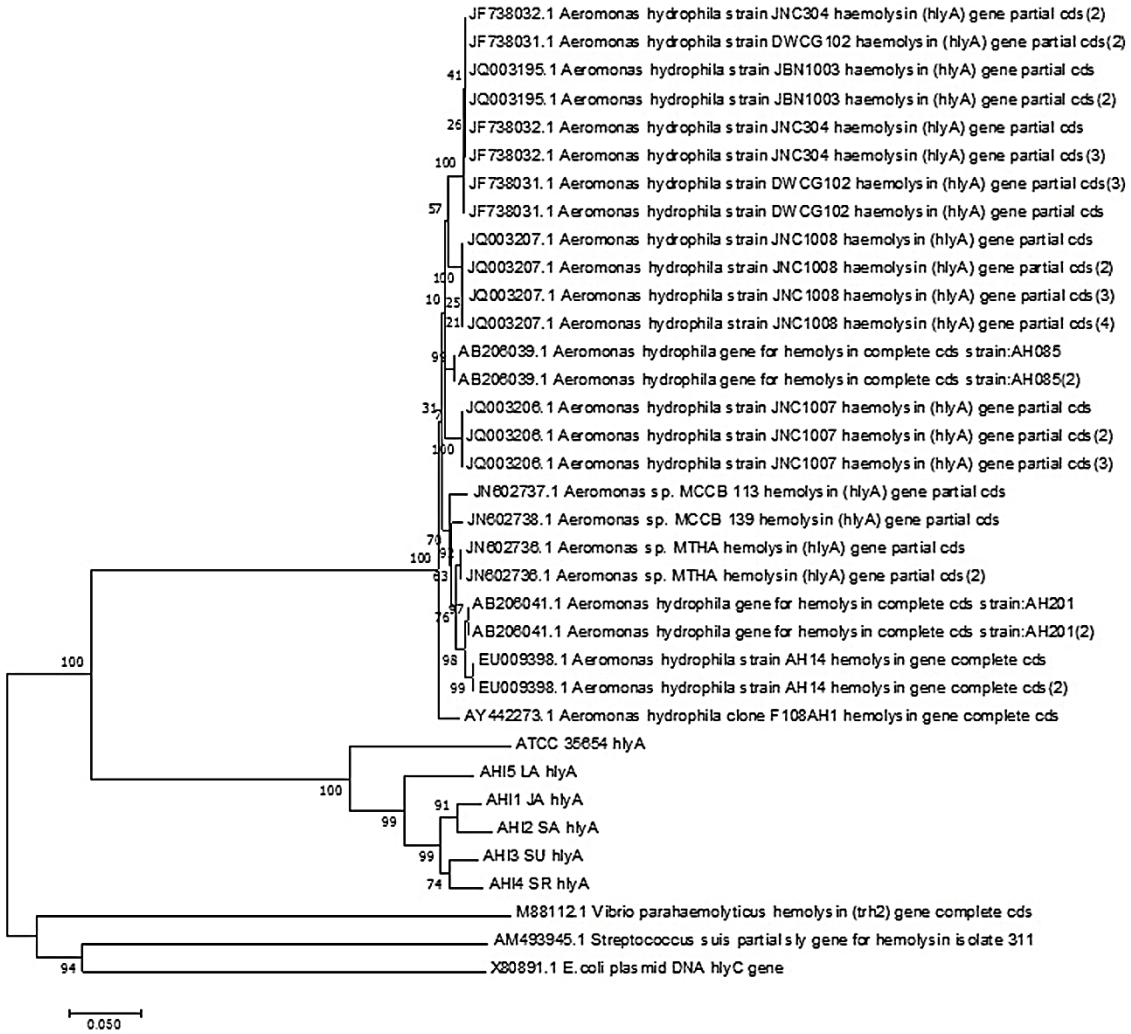

Figure 2. Phylogenetic relatedness based on nucleotide sequence hemolysin gene using the Neighbor-Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the p-distance method and are in the units of the number of base differences per site. Evolutionary analyses were conducted in MEGA7.

The presence and frequency of the hemolysin gene (*hly*A) in the Aeromonas strains in the study was consistent with previous PCR surveys [12] that detected *hly*A in all strains including the A. hydrophila species. In this study, the *hly*A gene is widespread. On the contrary, the study of Pollard *et al.* [13] and Lior and Johnson [14] showed only detectable aerA genes in hemolytic, cytotoxic and enterotoxic A. hydrophila but not in A. veronii biotype of sobria and A. caviae. The difference number of strains and the lack of a reliable analysis of the negative strains of PCR, can explain this anomaly. When genotypes of known virulent strains positive of detectable from PCR Wong [15], then all A. hydrophila isolates with *hly*A and aerA genotypes are virulent. These isolates also exhibit hemolytic and cytotoxic activity. Since the aerA gene and hemolysin are found in most isolates from these infection [16].

Other hemolytic/ cytotoxic factors associated with virulence have not been described. It is possible to detect the Aeromonas virulence genes according to characterization into three main groups of virulence markers (ieaerolism, hemolism, and enterotoxin) during a single PCR amplification of clinical, environmental or food isolates, as promoted by Kingombe *et al.* [17]. In this study, the application of this method to detect Aeromonas virulence genes in food and environmental samples has been shown to offer an attractive alternative to the rapid screening of potentially lethal aeromonad in food and the environment.

4.Conclussion

A. hydrophila is a threat to gourami in Indonesia. Further research needs to be done to determine the pathogenesis of these bacteria and their relationship with host cells. Research on the regional distribution of bacteria and analysis of nucleotide sequences in virulent isolates should also be undertaken to develop DNA vaccines that are strain-specific to these infections.

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