Determination of the aerolysin gene in Aeromonas hydrophila using the polymerase chain reaction (PCR) technique

G Christy¹, R Kusdawati²* and D Handijatno³

¹ Program Study of Aquaculture, Faculty of Fisheries and Marine, Universitas Airlangga, Surabaya, Indonesia
² Department of Fish Health Management and Aquaculture Faculty of Fisheries and Marine, Universitas Airlangga, Surabaya, Indonesia
³ *Corresponding author: rahayukusdar@gmail.com

Abstract. Aeromonas hydrophila is a bacterium that often causes outbreaks of fish diseases with high mortality rates. A. hydrophila has several virulence factors such as cytotoxin, protease, S-layers, and aerolysin. Aerolysin is an important virulence factor, as it is a marker of a strain of Aeromonas that can be virulent or not. This study aims to prove whether or not there is an aerolysin gene in the A. hydrophila isolates or not. The study was carried out using a PCR test on the A. hydrophila isolates from the Fish Disease and Environmental Examination, Serang Banten (SR isolate) and on the ATCC of A. hydrophila (ATCC Isolate) as a positive control. Biochemical tests and hemolysis in Blood Agar were carried out before the PCR test was carried out. The primer sequence used in the PCR process was “5’-cctatggcctgagcgagaag’-‘3’ for the forward and “5’-cagttccagtcccaccact’-‘3” for the reverse. The results show that the SR isolates have an aerolysin gene. These results show the formation of a band with a length of 430 bp that matches the amplicon target on the SR isolates.

1. Introduction

One of the common bacteria in aquatic ecosystems that acts as a microbial flora for aquatic animals in stable environmental conditions is A. hydrophila. [1]. This bacteria is very influential in relation to the cultivation of freshwater fish and it often causes disease outbreaks with high mortality rates (80 - 100%) in a short period of time (1-2 weeks) [2]. Motile Aeromonas Septicemia (MAS) is one of the diseases caused by A. hydrophila, which causes death in catfish [3].

The virulence level of A. hydrophila depends on the toxins produced. The pathogenicity of the infection of A. hydrophila is due to the production of several virulence factors such as cytotoxins, proteases, S-layers, and aerolysin [4]. Aerolysin is produced by Aeromonas sp., and involves hemolytic and enterotoxic activity [5]. Aerolysin (aerA) has been defined as one of the virulence markers used to identify the pathogenicity of the Aeromonas strain [6].

Bacteria can be identified by conducting biochemical tests based on the biochemical reactions [7]. However, a biochemical test can’t detect the presence of virulent genes present in the isolates. The PCR method is a method that is used to determine the potential for the pathogenic detection of toxin genes in Aeromonas sp. [8]. The PCR techniques can be used to amplify the DNA segments millions of times in just a few hours. This research is intended to detect the presence of an aerolysin gene in A. hydrophila, which is one of the virulence factors. Based on this background, the purpose of this study was to determine whether there is an aerolysin gene in the SR isolates or not, which can be used to determine if the isolate is virulent or not.
2. Materials and method

2.1 Material
The main items used in the study were one isolate of bacteria *A. hydrophila* obtained from the Fish Disease and Environmental Examination, Serang Banten (SR Isolate), Trypticase Soya Agar (TSA) media, Trypticase Soya Broth (TSB) Media, aquades, primary oligonucleotides, dNTP, dH2O (free water nuclei), MgCh, DNA Polymerase tags, 70% alcohols, agarose gels, TBE, DNAzol DNA Kit, Master Mix Promega and aerA genes *A. hydrophila*. Forward Primer 5′ ccatggctgagcagag3′ and reverse 5′ccagtccagtccacca3′ with a DNA target of 430 bp were also used.

2.2 Method
This research was conducted between March to June 2018 at the Microbiology Laboratory of the Faculty of Veterinary Medicine, Airlangga University, and the Microbiology Laboratory in the Faculty of Fisheries and Marine, Airlangga University, Surabaya. The exploratory method was used in this research by obtaining *A. hydrophila* from the Fish Disease and Environmental Examination, Serang Banten (SR Isolate) and conducting biochemical tests and hemolysis activity tests on the Blood Agar. The determination of *aerolysin* genes was done by using PCR techniques. The results of the electrophoresis conducted on the SR isolates was compared with the ATCC (American Type Culture Collection) isolates as a positive control.

2.2.1 Biochemical test identification
The biochemical test was to ensure that the isolates were *A. hydrophila*. The biochemical tests were carried out included TSIA test, indole test, urea test, citrate test and sugar test (glucose test, lactose test and maltose test).

2.2.2 Determination of the aerolysin genes (aerA) through the PCR technique
The DNA Extraction of *Aeromonas hydrophila* was done using DNAZol Direct kit (DN 131).

3. Results and discussion
The identification of the SR isolate was included in the morphological test. This helps to convince the researcher that the bacteria was *A. hydrophila*. The biochemical test continued after the morphological test. This was then followed by the hemolytic activity test and the gene identification test done using the PCR technique.

3.1 Result
3.1.1 Bacteria identification
After the morphological identification of the bacterial isolates, biochemical tests were carried out on the isolates in order to determine the biochemical activity of the bacteria. The tests included SC, O / F, TSIA, MIO, oxidase, sugars (glucose, lactose, maltose) and urea. Furthermore, the results of the biochemical tests were compared to the references of [9] in order to prove that the isolates to be tested were *A. hydrophila*. The biochemical test results can be seen in Table 1.

| Table 1. Biochemical test results of sr and the control and positive isolates |
|---------------------------------|-----------------|-----------------|-----------------|
| Biochemical Tests               | (ATCC)           | SR Isolates     | References [9]  |
| TSIA Media                      |                 |                 |                 |
| - Color                         | K / A            | K / A           | K / A           |
| - H2S                           | -               | -              | +               |
| MIO Media                       |                 |                 |                 |
| - Indol                         | +               | +              | +               |
| - Motility                      | +               | +              | +               |
| SC Media                        | +               | +              | +               |
Based on the results of the biochemical tests, we determined that the SR isolates have a percentage of similarity with a reference [9] of 90%. It can be proven that the SR isolates are *A. hydrophila*. The same percentage of similarity was also seen in the positive control ATCC isolates, which had a 90% similarity; this means that the ATCC isolate is *A. hydrophila* in accordance with the references of [9].

### 3.1.2 Hemolytic Activity Test in the BAP Media (Blood Agar Plate)

The hemolytic activity test aims to test the pathogenicity of the bacteria on humans and animals. Cultures of isolates grown on Blood Agar media were mixed with 5% sheep blood and then incubated for 24 - 48 hours at room temperature. The isolates that are capable of demolishing red blood cells were characterized by the formation of clear zones around the colony. The formation of clear zones shows that the tested bacterial isolates include pathogenic bacteria [10].

The reaction of each isolate to the media of the Blood Agar showed the same results. Both SR and ATCC isolates formed clear zones in incubation after 24 hours. The SR and ATCC isolates included bacteria that are able to lyse blood because there is a parasite zone around the colonies that has the potential to contain the aerolysin gene. The results of the observation showed that there was a change in color in the media from red to clear around the colony. Based on the lysis process, the two isolates included bacteria that lysed the blood perfectly (*β*-hemolysis). The perfect lysis process can be seen from a completely clear zone [10].

### 3.1.3 Aerolysin Gene detection (*aerA*) Aeromonas hydrophila

The positive results of *A. hydrophila* obtained from the biochemical tests and hemolytic activity test were followed up by a PCR test to prove the presence of an aerolysin gene in *A. hydrophila*. This research used “5’-ccatagctgctaggagac-3’” for the forward primer *aerA* gene and “5’-ccagttcagcagcc-3’” for the reverse primer. The electrophoresis results of the PCR process can be seen in Figure 1.

![Electrophoresis of aerolysin genes PCR (aerA) results with an Amplicon Length of 430bp.](image)

**Figure 1.** Electrophoresis of the aerolysin genes PCR (*aerA*) results with an Amplicon Length of 430bp.
3.2 Discussion
Based on the observations in the TSA and MCA media after the gram test, oxidase test and catalase test according to the statement of [9], the results show that the colonies of A. hydrophila had a white turbidity with a circular and convex colony shape. The colonies were grown on the MCA media and incubated for 24 hours at 37°C.

MCA media is a selective and differential medium used for the isolation and identification of bacteria that have the ability to grow in lactose fermentation. MCA contains bile salts, peptone, neutral red, crystal violet and lactose. Bile salts and violet crystals function to inhibit the growth of Gram negative bacteria in the media. Lactose is a source of carbohydrates that can be fermented by bacteria and neutral red. The red colonies showed a pH <6.8 and the colorless colonies showed a pH> 6.8 [11].

The biochemical test showed that the A. hydrophila isolates have the characteristics of producing catalase, oxidative, fermentative and lactose fermentation [12]. The results of the study were in accordance with the biochemical test results on both isolates and when compared with the reference from [9]; it had a 90% similarity percentage, therefore it can be concluded that the SR and ATCC isolates are A. hydrophila.

The process of the pathogenic bacteria A. hydrophila invading the host's body is preceded by the attachment of the bacteria to the host's surface skin by utilizing pili, flagella and hooks to move and attach itself firmly to the outer layer, which consists of scales protected by a substance called chitin. During this process, A. hydrophila produces chitinase enzymes which play a role in degrading the chitin layer so then bacteria can easily enter the host. In addition to utilizing bacterial chitinase A. hydrophila also secretes other enzymes such as lecithinase in an effort to enter the bloodstream [13]. A. hydrophila contain an ecsoenzyme encoded by lipase, nuclease, and serine protease genes and also contains an exotoxin in the form of aerolysin [12].

The Aera and hlyA gene are the main genes that are responsible for producing aerolysin toxins and hemolysin, where aerolysin is an extracellular protein produced by certain strains of A. hydrophila-soluble, hydrophilic, with hemolytic and cytolytic properties. The mechanism of the aerolysin toxins in bacteria A. hydrophila involves binding to specific glycoprotein receptors on the surface of the eukaryotic cells before entering into the fat layer and forming holes. The Aerolysin toxins that form holes pass into the bacterial membrane as an apreprotoxin containing peptides. These toxins can attack epithelia cells and cause gastroenteritis [2].

The PCR results from the SR isolates indicate that there is an aerolysin gene in the bacteria. The ribbon that was formed was in the range of 430 bp in the third well, which contained SR isolates. This shows that the isolates studied were SR isolates of species A. hydrophila, which were virulent according to [14]. Species A. hydrophila has four virulent genes, namely nuclease, aerolysin, serine and protease.

4. Conclusion
Based on the research that has been carried out, it can be concluded that the SR isolates from the Fish Disease and Environmental Examination Unit, Serang Banten, contain aerolysin genes and this proves that A. hydrophila is a virulent species.

5. References
[1] Cipriano R C, and Bullock G C 2001 Fish Diseases Leaflet 66, 2-8.
[2] Lukistyowati I and Kurniasih 2012 Journal Veterinary 13, 43-50.
[3] Sukenda L, Jamal D, Wahjuningrum A, and Hasan 2008 J. of Indo Aqua, 7, 159-169.
[4] Rahman M H, Kawai K and Kusuda R. 1997 J. of Fish Pathol 32, 163-168.
[5] Xu X J, Ferguson M R, Popov V L, Houston C W, Peterson J W and Chopra A K. 1998 J. Infect. Immunol, 66, 3501-3509
[6] Zhang D, Pridence J W, and Klesius P H 2013 J Vet Microbiol 165, 478-482.
[7] Murray 2005 Microbiology Textbook. EGC. Jakarta. Medical Book Publishers
[8] Chang Y C, Wang J Y, Selvam A, Kao S C, Yang S S and Chihshih D Y 2002 J. Food Prot 71, 2094-2099.
[9] Austin B and Austin D A. (1993). Bacterial Fish Pathogens. In Disease in Farmed and wild fish. England. Ellis Horwood Ltd.: 732.
[10] Difco 2009 Manual of Microbiological Culture Media. Zimbro MJ, Power DA, Miller SM, Wilson GE, Johnson JA (Eds). 2nd ed. Maryland: Becton, Dickinson and Co.
[11] Hendrix C, and Sirois M 2007 Laboratory Procedure for Veterinary Technician. Fifth Edition. Canada: Mosby Elsevier: 114-140.
[12] Muslikha, Pujiyanto S, Jannah S N, and Novita H 2016 J. of Bio 5, 1-7
[13] Mangunwardoyo W, Eni C, and Tepy U 2010 J Sci. Phar 2, 57-63.
[14] Nam I Y and Kiseong J 2007 J. Microbiol 4, 297-304.