Conference Paper

IDENTIFICATION THE GENE NUCLEOTIDE SEQUENCE OF OUTER MEMBRANE PROTEIN AEROMONAS HYDROPHILLA BACTERIA FROM EAST JAVA LOCAL ISOLATES USING POLIMERASE CHAIN REACTION

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Abstract

Aeromoniasis disease or known Ulcer Diseases caused by Aeromonas hydrophila which in fish or shrimp often occurs in some embankment or in the Aquarium can cause sign patches – patches of bleeding can even cause economic loss and death if not promptly treated with treatment that accompanied the good sanitation. This disease affects many farmed freshwater fish in East Java. Previous research has managed to do the characterization of antigenic protein originating from the OMP (outer membrane protein) Aeromonas hydrophilla. Because it is felt necessary to do DNA sequensing encoder protein in question. To achieve these goals then do some exploratory research laboratoric methods, namely: identification of the bacterium Aeromonas hydrophilla fish from suspect infection by PCR, gel electrophoresis and Sequencing of gene fragments of the agarosa OMP A. hydrophilla isolates of East Java. The results obtained from this research is the result of agarosa gel electrophoresis in DNA chromosome Aeromonas hydrophila bacteria cells is carried out at a temperature of annealing 54°C position 400 bp and the results of the sequencing primer that is used produces a sequence of nucleotides is around 355 nucleotides.

Keywords: Aeromonas hydrophilla, agarosa gel electrophoresis, DNA sequencing.

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1. Introduction

Aeromoniasis has been recognized as an important pathogen agent in freshwater fish farming for more than a decade. Aeromonas hydrophila infection in particular, and led to many losses on farmers cultivating fish. Diseases caused by these organisms

can be manifested in various ways such as septicemia, basal haemmoragic ulceration, asymptom-exophthalmoses, septicemia and others.

The control problem of the disease through antibiotic treatment is not always successful and can be harmful to the environment because the resistant bacteria can be selected and resistance can be transferred to human pathogens and fish or other harmful bacteria. Therefore, the development of vaccines to protect fish against disease bacteria will be important. However, the high antigenic diversity among isolates of a. hydrophila is a major obstacle. A number of virulence factors can contribute to the overall virulence of bacteria. These include extracellular products (ECP), the S-layer and adhesin. The structure of the bacteria a. hydrophilla is unique unlike other gram-negative bacteria. The outer surface of this bacteria have villi and flagella, consists of two components that have been identified as potential virulence factors i.e. outer membrane protein (OMP) and lipopolysaccharide (LPS).

OMP is a gram-negative bacterial antigens potentially can directly induce specific humoral immune response lymphocyte β cells i.e. so that faster trigger the formation of antibodies. This protein plays a role in showing the nature of the specific molecular weight immunogenic [16].

A. Hydrophilla East Java local isolates have the nature and character of molecules with different isolates from other countries. The influence of the environment such as climate, weather and temperature can cause a difference or change in properties of each strain of bacteria. This research aims to find and characterize the gene protein a. Encoder Hydrophilla isolates. The sequence of the gene protein. Encoder hydrophilla local isolates obtained further homologed with strains from abroad (GenBank). The difference and homology this nukleotide layout will affect the nature of imunogenicity and this information can be used to manufacture the vaccine material sub unit. For the purposes of the examination of the initial steps of molecular biology through the isolation of chromosomal DNA. Based on the background of the research above, can be formulated several problems as follows: Do bacterial protein DNA hydrophilla Aeromonas isolates East Java locale can be identified by PCR (Polymerase Chain Reaction) and how does the nucleotide sequence Aeromonas hydrophilla bacterial isolates locally East Java?

2. Material and Method

2.1. Sample Aeromanas hydrophila

Research samples takes form local East Java isolates bacterial culture of A. hydrophilla research results a year earlier that had the cultured in the laboratory of Microbiology, Faculty of veterinary medicine, Universitas Airlangga Surabaya. Furthermore, the total DNA isolation is performed by means of a. hydrophila is dissolved with PBS as much as 10% then the suspension included in eppendorf tubes and added with a solution of DNAzol 1000 µl mixed using a vortex and stored 2-15 minutes at room temperature. Mix the disentrifuge suspension for 10 minutes at a temperature of 4°C to speed 10,000 rpm.

2.2. Amplification of DNA using PCR

Set of tools Ready to Go PCR kit from GE Health Care used to perform the process of DNA amplification by PCR technique. First 5 µl of DNA that has been heated are incorporated into the PCR tube bead, then added 2 µl primary forward, 2 reverse primer µl and 16 l µ NFW so the total aqueous amounts to 25 µl. next spindowned and incorporated into the PCR Thermocycle which has been programmed, the initial denaturation for 45 seconds at a temperature of 95°C, annealing for 60 seconds at a temperature of 660C, the extension for 60 seconds at a temperature of 72°C as much as 35 cycles and ends with the final extension for 7 minutes at a temperature of 72°C. Next PCR products inserted into the ice and stored at a temperature of-20°C prior to the process of electrophoresis [3].

The primer used for PCR were as follows:

A pair of forward primer (GCTATGAAAAAACTAAAAATAACTG-3V-5V, nt 423 – 448) and reverse primer (CAGTATAAGTGGGGAAATGGAAAG-3V-5V, nt 1983 – 2007) was designed using special software Oligo 5.0 software (Hirono and Aoki, 1991; HIRONO et al., 1992).

2.3. Electrophoresis Of PCR Results

As many as 5 µl DNA A. Hydrophilla plus 2 µl loading buffer, put in a 2% agarose containing ethidium bromide 1 mg/ml for the running process. The result was divisualisasi with UV light 302 nm wave length. DNA electrophoresis was conducted to find out the length of the amplicon A. Hydrophilla [6].

2.4. PCR Products Purification

PCR product purification is done by QIA quick spin column. Done by mixing Buffer volume 3 QG (Guanidine Thiocyanate) into 1 volume of gel sample (from the results of electrophoresis of PCR products). Then it is heated (incubation) at a temperature of 50°C for 10 minutes. Add 1 volume of gel sample 150 propanol (100 µl). Then move to the QIA quick spin column tube 2 ml. for 1 minute with Sentrifuge speed 12,000 rpm at room temperature. Then discard supernatan and add 500 µl Buffer to QG in the QIA quick spin column and sentrifuge for 1 minute, speed 12,000 rpm at room temperature. Supernatan thrown out then washed by adding Buffer PE 750 µl into the QIA quick spin column then sentrifuge for 1 minute with speed 12,000 rpm at room temperature. Then discard supernatan and sentrifuge again for 1 minute at speeds of 13,000 rpm. Move the QIA quick spin column into a 1.5 ml tube microcentrifuge. add 30 µl Buffer EB, let stand for 1 minute, sentrifuge again for 1 minute with the speed of 10,000 rpm at room temperature. Take supernatant as a result of the pure product.

2.5. Labeling

Done by adding Big Dye Terminator version 1.1 cycle as much as 1.6 µl in 5 µl product purification. Then add the primer as much 1.6 µl and sequenced buffers as much as 6.4 µl is defined by 5 times. Furthermore added ad libitum H_2O 20 µl and mixed results are incorporated into the PCR machine with program: Predenaturasi 96°C temperature for 3 minutes, Denaturation temperature 96°C for 10 seconds as many as 25 cycle, Annealing temperature 50°C for 5 seconds, the temperature of the extension 60°C during 4 minutes and 4°C final Extension

2.6. Precipitation

It aims to eliminate and deposit the remains of dye from product labeling. Add 3 M NaOAcetat 2 µl (± 2xvol) then stir with a vortex and incubated in ice for 10 minutes. Next disentrifuge with 14,000 rpm speed at temperatures above 4°C for 20 minutes. Next pellet 200 µl added ethanol 70%. Sentrifuge again with 14,000 rpm speed at temperatures above 4°C for 5 minutes. Then grab the solution slowly, then the resulting pellets are dried is vacuum drawn into the pump for about 10 minutes, save the DNA in temperature-200 c (Applied Biosystem, 2010).

2.7. Sequencing

Results of precipitation supresan reagent is added to hi-tech DiTM Formamide 25 µl then it is heated at a temperature of 25°C for 2 minutes, and then incubated in ice for 3 minutes. After it was moved into the mikrotube and put into the machine sequencing ABI PRISM 310 GENETIC ANALYSER-Applied Biosystems capillary length 1 5-47 cm × 50 µm which can generate sequences up to 640 nucleotides. Running made during overnight. The results of sequencing data processed using software ClustalW (Mega 5) to get the nucleotide sequencing (Applied Biosystem, 2010)

3. RESULTS AND DISCUSSION

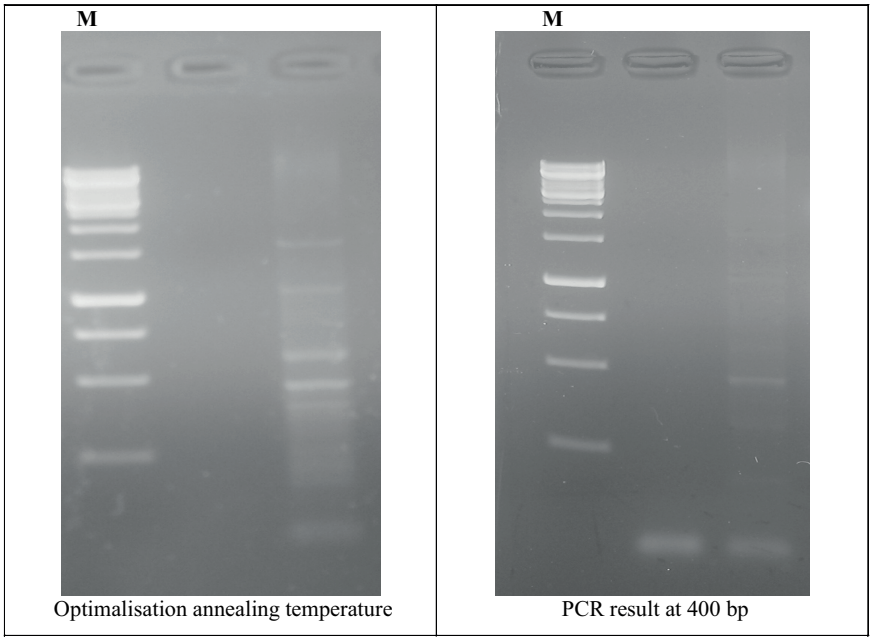
3.1. Sample Aeromanas hydrophila

Research samples in the form of the bacterium *Aeromonas hydrophila* isolates came from East Java local fish Aeromoniasis sufferers who collected from the region of Sidoarjo, Blitar and Malang. The type of fish used as samples is also the largest commodities of the region namely gurame, catfish and carp. *Aeromonas hydrophila* study on isolates propagated from local media stock and cultured on Tryptic Soy Agar solid media and incubated during 18-24 hours.

3.2. Electrophoresis of PCR Results

Degradation of bacterial cells is performed with the Sonication technique. A. hydrophila from solid media washed with PBS and centrifuged with 8,000 rpm speed for 15 minutes. Furthermore the pellets A. hydrophila reconstituted with 1 ml of PBS and then sonicated at 20 Hz for 4 × 4-minute time interval 2 minutes.

After the breaking of the bacterial cell wall is then performed DNA purification, to separate the DNA from the RNA molecule and the protein as well as contaminants of cell debris. Standard methods using for the identification, separation and purification of DNA fragments using gel electrophoresis is agarosa. Gel electrophoresis of DNA migration through agarosa is influenced by the size and conformation of the DNA molecule, agarosa concentration, temperature and electrical current. Agarosa gel electrophoresis results on the DNA chromosome Aeromonas hydrophila bacteria cell can be seen in the image below.



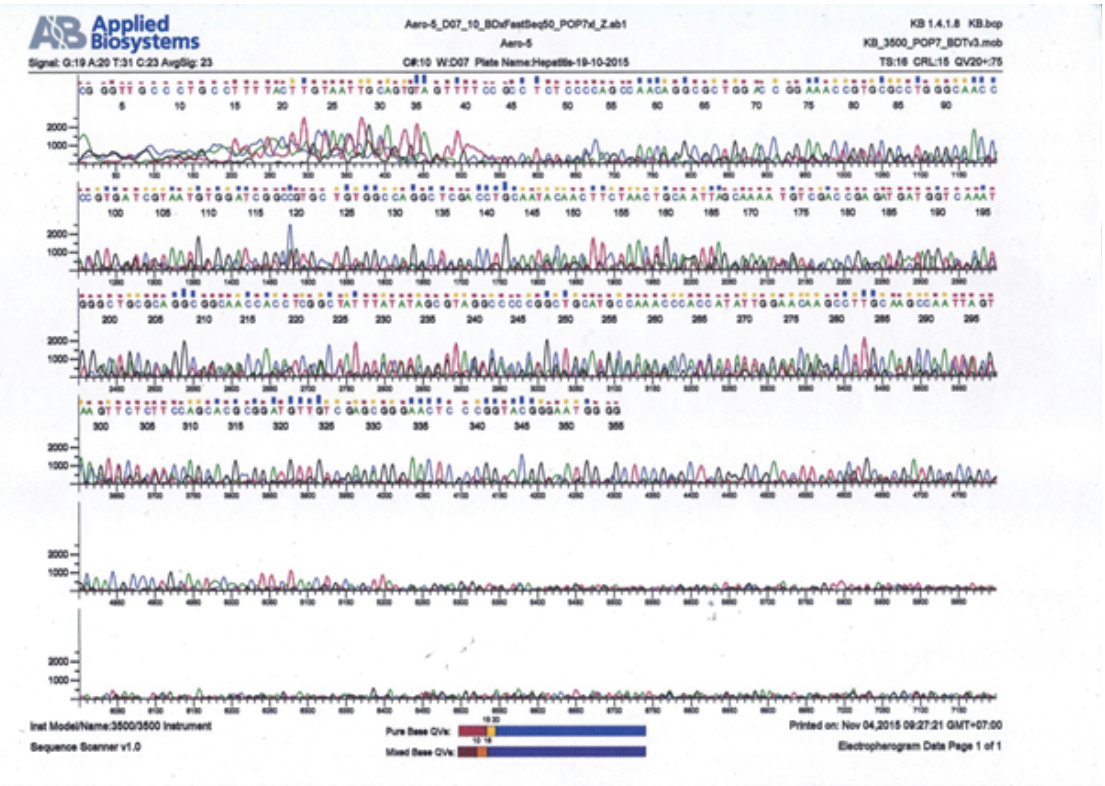
Optimalisation annealing temperature | PCR result at 400 bp

According to Fatchiyah et al (2011) on DNA PCR reactions required specific primer, prints, a DNA polymerase enzyme termostabil, Mg2 + ion bufer PCR and gene cycler. Efficient amplification results are between 100–400 BP. amplification Results more than 1000 bp likely the process is less efficient because a long product susceptible to enzyme inhibitor that affects the work of DNA polymerase.

Electrophoresis of PCR observations showed that a pair of primer used for the amplification of the PCR process generates panjang nukkleotida has not been as expected i.e. bp 1008 (Thangaviji et. al, 2012). The Ribbon which looks at the results of electrophoresis of PCR samples were below the position marker 500 BP. bp Calculation samples obtained results 400 basepairs in length which is bp obtained from the sample amplification.

3.3. PCR Product Sequencing

Sequensing performed to figure out the sequence of nucleotides in the DNA chain. Next PCR results Amplicon sequenced by using the sequencer ABI PRISM 310 Genetic Analyser-Applied Biosystem.



The results of the sequencing primer that was used and then processed and combined by using the software Manager's Cloned so that data obtained by the order of the nucleotide sequencing results as follows:

CGGGTTGCCCTGCCTTTTACTTGTAATTGCAGTGTAGTTTTCCGCCTCTCCCCAGCCAACAGGCGCTGGACCGG
AAACCGTGCGCCTGGGCAACCCCGTGATCGTAATGTGGATCGGCCGTGCTGTGGCCAGGCTCGACCTGCAATAC
AACTTCTAACTGCAATTAGCAAAATGTCGACCGAGATGATGGTCAAATGGGCTGCGCAGGCGGCAACCACCTCG
GCTATTTATATAGCGTAGGCCCCCGGCTGCATGCCAAACCCACCATATTGGAACAAGCCTTGCAAGCCAATTAG
TAAGTTCTCTTCCAGCACGCGGATGTTGTCGAGCGGGAACTCCCGGTACGGGAATGGGG

3.4. Nucleotide sequence of the gene fragments of Aeromonas hydrophilla isolates East Java

Study on sequencing amplikon "PCR using machine ABI PRISM 310 Genetic Analyser-Applied Biosystem can only produce a maximum 500 nucleotide sequence. On the process of reading the end of DNA strands less maximum unreadable. The machine is not working optimally so that the results obtained are only readable sequence of nucleotides is around 355. Therefore in this study is only a partial nucleotide sequence of a gene fragment of Aeromona hydrophila isolates East Java can be unreadable.

4. CONCLUSIONS

Agarosa gel electrophoresis Results on the DNA chromosome Aeromonas hydrophila bacteria cells is carried out at a temperature of annealing 54°C position 400 bp. And the results of the sequencing of the primer that is used produces a sequence of nucleotides is around 355 nucleotides.

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