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# *Identification of Pathogenic Bacteria in Blood Cockle (Anadara granosa) using 16S rRNA Gene*

**Meutia Srikandi Fitria[1*], Aprilia Indra Kartika[2], Ana Hidayati Mukaromah[2], Nurfi Ismatul Unasiah[2]**

[1] Department of Medical Laboratory Technology, Faculty of Nursing and Health Science, Universitas Muhammadiyah Semarang, Indonesia
[2] Magister Study Program of Medical Laboratory Science, Universitas Muhammadiyah Semarang, 50273, Indonesia

**Abstract:** Blood cockle (*Anadara granosa*) is one of the marine resources in Indonesia that contains protein. Processing of blood cockles that are not perfect or raw will be contaminated with pathogenic bacteria that live in the waters. Pathogenic bacteria cause foodborne disease, which is a disease in humans caused by food. Several bacterial pathogens that cause foodborne disease are *Escherichia* sp. *Pseudomonas* sp., and *Vibrio* sp. Pathogenic bacteria in blood cockle should be identified using 16S rRNA as molecular identification. Samples were isolated using BAP, HIA, and BHI media. Bacteria from BHI media were isolated. Isolation DNA was isolated using the phenol-CIAA method. The DNA isolates were amplified by the PCR method based on the 16S rRNA target gene, then visualized the DNA with 2% agarose gel electrophoresis and sequencing. Bacterial colonies produced from BAP media for isolates BVA1, BVA9, and BVA10 were ß-hemolysis. Visualization of hemolytic bacterial DNA in blood cockle culture amplified about 1500 bp. Whereas the results of the sequencing analyzed by BLAST on the NCBI database and the Mega X program for BVA1 and BVA10 isolates showed similarity to *Vibrio* sp. bacteria, whereas BVA9 isolates showed similarity to Bacterium whose species still unknown. The conclusion showed that blood cockle had close simililarity with with *Vibrio* sp. and Bacterium.

**Keyword:** *Anadara granosa;* Pathogenic bacteria*;* 16S rRNA gene

## INTRODUCTION

Pathogenic bacteria are the cause of food poisoning in both raw and processed foods, one of which is blood cockle (*Anadara granosa*). It is one type of shellfish has a high economic value and generally as a source of seafood in Southeast Asia region especially in Indonesia[1]. Blood cockle (*A. granosa*) have high protein content and potential to be contaminated with pathogenic bacteria due to the way they consume food is a filter feeder that is filtering water to acquire food. The presence of pathogenic bacteria can cause foodborne diseases[2]. Identification of pathogenic bacteria is required to find out what type of bacteria that live in blood cockle and to provide information concerning the hygiene of blood cockle when consumed raw. Microbiological identification of bacterial is known as the gold standard for detecting pathogenic bacteria, however it takes a long time[3]. Therefore, the molecular identification is needed. Molecular identification was

Corresponding author.
*E-mail address*: meutia@unumus.ac.id (Meutia Srikandi Fitria)
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carried out by sequencing analysis of the 16S rRNA gene, this method has high level of sensitivity, specificity and takes a less time[4]. The 16S rRNA gene is a marker gene for bacterial identification. The 16S rRNA gene used as a universal primer used in PCR (Polymerase Chain Reaction) and the nucleotide sequence can be determined through sequencing[5].

Several bacteria that cause foodborne disease such as *Escherichia* sp., *Pseudomonas* sp. and *Vibrio* spp. Whereas blood cockle reported to have bacterial contamination, such as those from the species of *Klebsiella* spp. and *Bacillus* spp, *E. coli*, *Pseudomonas* spp., *Staphylococcus* spp., and *Micrococcus* spp. Bacterial infections in humans are often associated with improper processing or raw consumption[6]. Bacterial identification from blood cockle found in the south coast of Thailand are have contamination with *V. parahaemolytticus*[7]. The study regarding in Tanah Merah Kupang Tengah state that blood cockle had *V. harveyi* contamination[8]. Another study showed that blood cockle were contaminated with *Salmonella* sp. and *Vibrio* sp[9]. In addition to bacteria in blood cockle, endoparasite and microplastics were also discovered[10,11].

The study related to the identification of pathogenic bacteria that cause foodborne disease in *A. granosa* has limited research. It is needed to do research it because it can help related to the correct food processing and hygiene of the food. Therefore, it is necessary to conduct research on the molecular identification of pathogenic bacteria in *A. granosa* by sequencing analysis of the 16S rRNA gene.

## MATERIAL AND METHOD

The type of the research is descriptive explorative. The sample of *A. granosa* obtained from Sayung Market, Demak. The research was conducted from Januari to April 2021. Pure bacterial isolation was carried out at Microbiology Laboratory. Therefore, DNA isolation and amplification at Molecular Biologi, Medical Laboratory Technology, University of Muhammadiyah Semarang. Sequencing analysis of 16S rRNA gene was carried out at PT. Genetika Science, Indonesia.

**Research stages**

**Pure Bacterial Isolation of *A. granosa* Culture**

Three grams of *A. granosa* were put in a clean and dry place to be mashed. Then one tablespoon of *A.* granosa was diluted into 5 ml of Physiological NaCl and homogenized. Isolation was carried out by culturing *A. granosa* on Nutrient agar then incubated at 37°C for 24 hours. Later the bacteria were isolated again on BAP media at 37°C for 24 hours. Then hemolysis formed was observed on BAP (Blood Agar Plate) media against a bright light background. Colonies on BAP media which contained a full clear zone and a clear green zone were inoculated on HIA (Heart Infusion Agar) fertilizing media for 24 hours at 37°C then colonies from HIA were cultured on BHI (Brain Heart Infusion) agar for 48 hours at 37°C in the incubator.

**DNA isolation of A. granosa bacteria**

*A. granosa* bacterial colonies from HIA media was implated in 5ml BHI media, then incubated at 37°C for 24 hours. The colonies were centrifuged at 12000 rpm for 10 minutes at 4°C. The pellet required for the isolation was added with 750 µl of lysis buffer then vortexed for a few seconds. Then added 20 µl of proteinase K, the solution shaken for 15 minutes using a rotator. Then the solution incubated at 55°C for 30 minutes. Later centrifuged for 10 minutes at 12000 rpm at 4°C.

The supernatant solution transferred to a 1.5 ml microtube and 700 µl of phenol CIAA was added, stirred slowly for 30 minutes, then centrifuged at 12000 rpm for 10 minutes at 4°C. The top part was transferred to microtube, then in a 1:1 ratio 96% ethanol was added, the solution needed to mix gently until fine threads were visible, then centrifuged at 12000 rpm for 10 minutes at 4°C. The supernatant solution was eliminated from microtube, and the pellet was washed with 70% cold ethanol, then centrifuged at 12000 rpm for 10 minutes at 4°C for three times. The supernatant solution was removed from the pellets then air dried. Then 200 µl of

TE solution was added to dissolve the bacterial DNA so that it could be visible in the 1 % agarose gel electrophoresis.

DNA isolate concentration and purity was measured using nanodrop spectrophotometer. Two µl was pipetted in nanodrop at a 260/280 nm wavelength. The limit ratio of DNA purity is between 1.8 – 2.0.

**Amplification of *A. granosa* bacteria**

The compositon of mixed PCR was 12.5 µl of taq polymerase enzyme, 7.5 µl of Nuclease Free Water, 2µl each of forward and reverse primers, and 1µl of DNA samples were added to the PCR microtube. The PCR microtube is inserted into the PCR, then the temperature is set at the initial denaturation stage (pre-denaturation) at 95°C for 6 minutes, the denaturation stage at 95°C for 30 seconds, then the annealing stage at 55°C for 30 seconds. The extension at 72°C for 2 minutes. The final extension stage at 72°C for 10 minutes and the cooling down stage at 4°C for 6 minutes. The results of PCR amplification and markers were read using 2% agarose gel electrophoresis. PCR amplification using 16S rRNA forward and reverse primers has a target area about 1500 bp.

**Data analysis**

The further processing to the sequencing analysis stage of PCR product is sent to PT Genetics Science Indonesia. The nucleotide sequences obtained were then used for comparison with the sequence data in Gebank through the BLAST (Basic Local Alignment Search Tool) program at the National Center for Biotechnology Information (NCBI), National Institute for Health, USA on the Website https://blast.ncbi.nlm.nih.gov/. The results from the sequencing were aligned and a phylogenetic tree was created using the Mega X program[12].

## RESULTS AND DISCUSSION

Bacterial colonies were cultured on BAP media to see the nature of the bacteria in hemolyzing red blood cells. The bacterial culture of *A. granosa* obtained three colonies of bacteria capable of lysing red blood cells so that it is harmful to humans. The three bacterial colonies (BVA1, BVA9, and BVA10) produced ß-hemolysis colonies with the characteristics of forming a transparent zone around the colony (Figure 1).

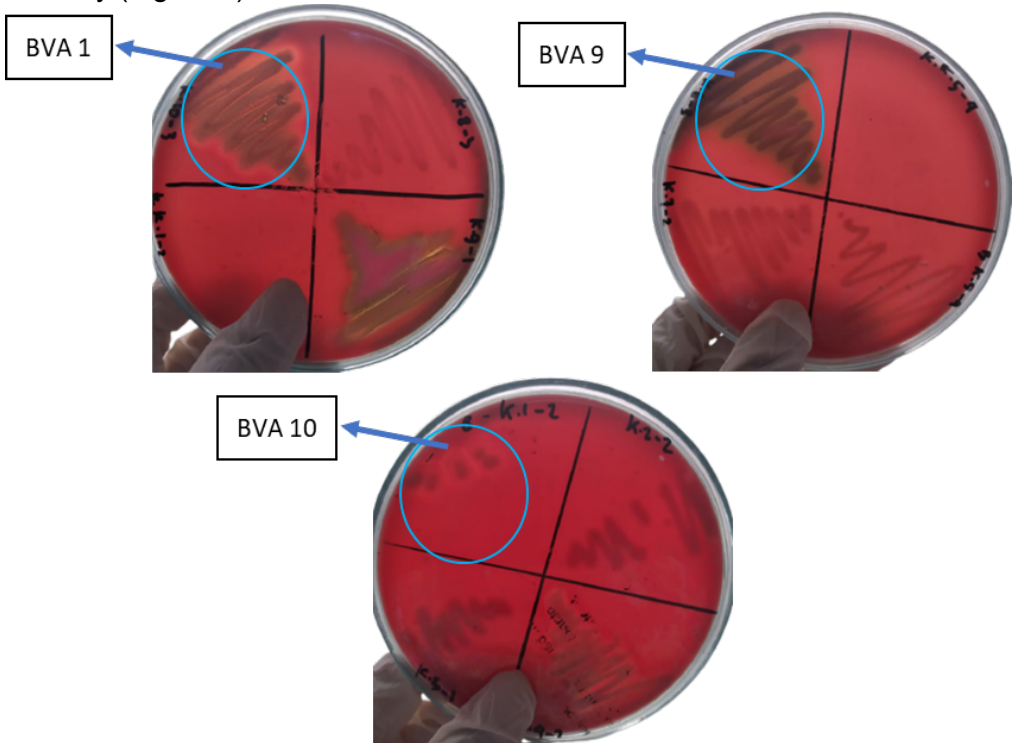


Figure 1. Bacterial colonies of *A. granosa* isolates in BAP media

Each colony was taken, and gram stain was performed to see the characteristic of the bacteria. The three colonies such as isolates BVA1, BVA9, and BVA10 were Gram-negative bacteria. They were basil or rod-shaped and the color were red. ß-hemolysis bacteria have the ability to multiply faster than α-hemolysis. The production of enterotoxin from α-hemolysis and ß-hemolysis, can lead to pathogenicity. ß-hemolysis strains can last longer than α-hemolysis[13].

Each bacterial colony was inoculated on HIA media, then instilled on BHI media for DNA isolation. DNA isolation was conducted using the phenol-CIAA method and then the 16S rRNA gene was amplified by the PCR method. The PCR product of each isolate then visualized with 2% agarose electrophoresis (Figure 2).

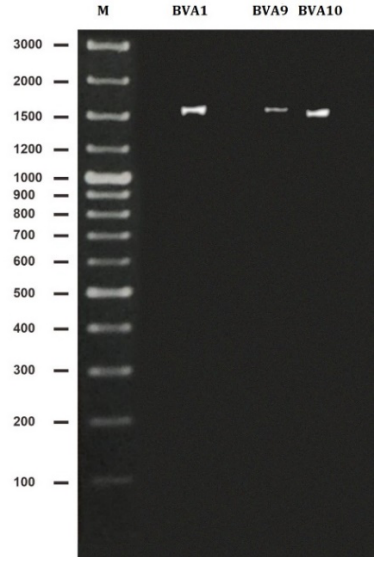


Figure 2. Visualization of the PCR product of *A. granosa* bacterial isolates

BVA1, BVA9, and BVA10 isolates had PCR products about 1500 bp due to the 16S rRNA gene has a size of about 1550 base pairs and about 500 bases at the end is a hypervariable region[4]. The hypervariable region in the 16S rRNA gene is used to identify bacteria or determine the characteristics of a bacteria[5]. The PCR products of the three isolates (BVA1, BVA9, and BVA10) were then sequenced.

The results of the sequencing were compared with the data in Genbank through the BLAST program and then aligned and made a phylogenetic tree with the Mega X program. Based on the results from the Mega X program, three phylogenetic trees were obtained from the three isolates. BVA1 and BVA10 isolates obtained phylogenetic tree as follows (Figures 3 and 4).

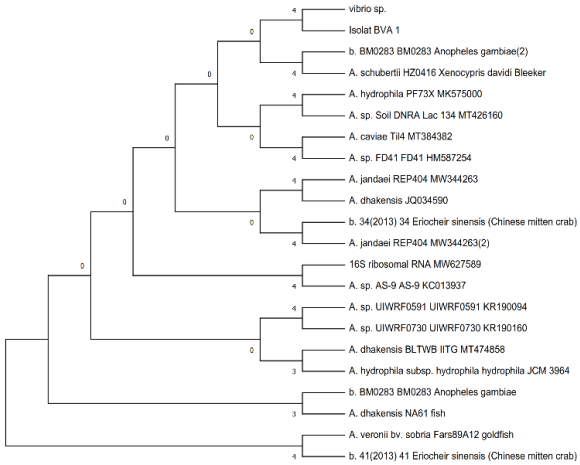


Figure 3. Phylogenetic tree analysis of BVA1 isolates that were similar to *Vibrio* sp.

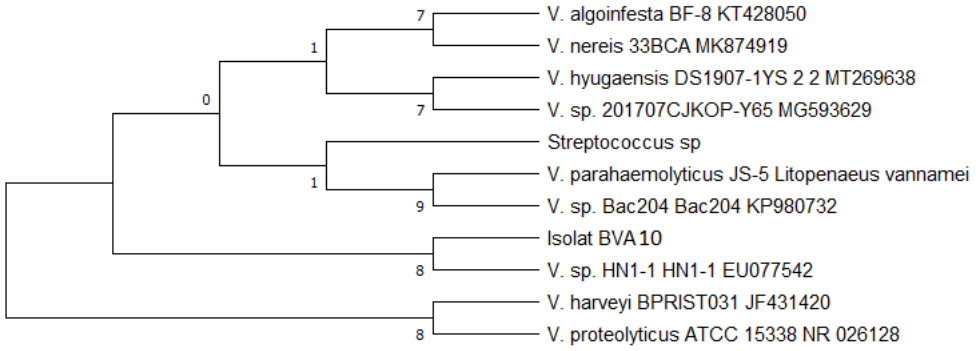


Figure 4. Phylogenetic tree analysis of isolates BVA10 has similarities with *Vibrio* sp.

Based on the results of the phylogenetic tree analysis (Figures 3 and 4), it was shown that the isolates BVA1 and BVA10 had similarities with *Vibrio* sp. It is Gram-negative bacteria that are single-celled with short curved (comma) or straight rods. The length of the *Vibrio* sp. is about 1.4-50 nm and the wide is about 0.3-1.3 nm, motile and have polar flagella[14]. The genome of *Vibrio* sp is divided into two chromosomes, which are formed by recombination and horizontal gene transfer (HGT; acquisition of genetic material by transfer from another organism). Although these pathogens may be diverse in genomic, the bacteria came from aquatic and marine environments. *Vibrio* sp. prefer warm environments and salty waters[15]. The presence of *Vibrio* sp. in traditional seafood processed products should be a concern because these products are ready to eat and can be a source of disease caused by food[16]. It responsible for most human diseases caused by the natural microbiota of aquatic environments and seafood. The most common bacterial pathogenic species are *Vibrio cholerae*, *Vibrio parahaemolyticus*, *Vibrio vulnificus*, and *Vibrio alginolyticus*[9].

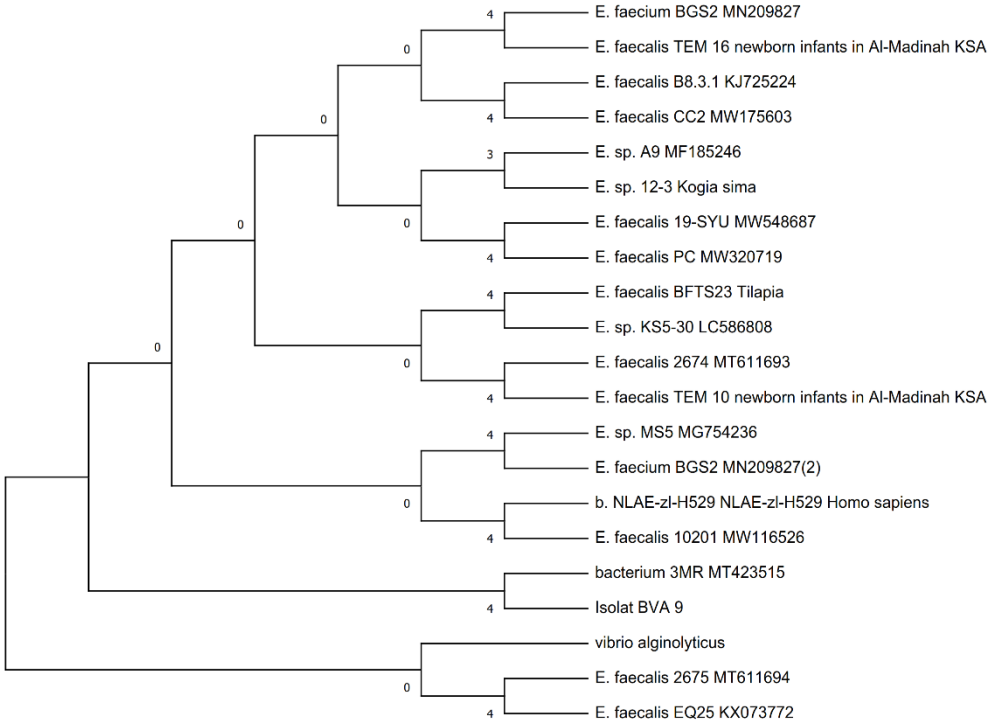


Figure 5. Phylogenetic tree analysis of BVA9 isolates were similar to Bacterium.

The sequence of BVA9 isolates were similar to Bacterium 3MR MT423515 because the sequence of BVA9. Bacterium 3MR MT423515 is a type of bacteria whose species has not been decided. This bacterium was also discovered by Gao from China, but the journal about Bacterium has not been published[17].

*A. granosa* is a species that lives in muddy coastal waters, but it can also be found in mangroves and seagrass. Bacterial contamination in the cultivation of blood clams is influenced by human activities around. Human activities can produce household waste that pollutes waters if it contains pathogenic bacteria, so it can affect the quality of blood cockle because they are filter feeders (filtering water to get food)[18].

The foodborne disease is closely related to bacterial pathogenic factors, the ability to attack tissues, the speed of pathogen proliferation, colonization, and the host's defense system against pathogens. The hemolytic activity of bacteria makes bacterial defense factors attack the host's defenses by lysing the host's blood cells. Bacteria that can survive will enter the bloodstream so that they spread to all host body cells and target organs[19]. Target organs can be animal organs such as blood cockles or from humans. *Vibrio* sp. can infect animals and humans due to imperfect food processing.

The presence of *Vibrio* sp. in aquaculture waters such as blood cockles and other seafood are also influenced by physical and chemical parameters. The chemical parameters that affect are the levels of ammonia and organic matter in cultivation. Another studies showed that poor physical and chemical parameters are the cause of the abundance of *Vibrio* sp. in vaname shrimp rearing water[20,21].

## CONCLUSION

The bacterial isolate BVA1 and BVA10 in *A. granosa* were similar to *Vibrio* sp. Therefore isolate bacteria BVA9 had similarity with Bacterium. *Vibrio* sp. has often been found in aquacultured seafood, while for Bacterium the species has not yet been determined. Blood cockle with imperfect processing may still contain *Vibrio* sp. and can cause foodborne disease.

## AUTHOR'S CONTRIBUTION STATEMENT

Meutia Srikandi Fitria carried out the DNA extraction, PCR and compiling publication manuscript, Aprilia Indra Kartika performed analyzing sequencing data, Ana Hidayati Mukaromah measured the concentration and purity DNA, Nurfi Ismatul Unasiah carried out the bacteria culture, NA, BAP, and BHI.

## FUNDING INFORMATION

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## DATA AVAILABILITY STATEMENT

The utilized data to contribute to this investigation are available from the corresponding author on reasonable request.

## DISCLOSURE STATEMENT

The views and opinions expressed in this article are those of the authors and do not necessarily reflect the official policy or position of any affiliated agency of the authors. The data is the result of the author's research and has never been published in other journals.

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