

Identification of Pathogenic Bacteria in Snapper Fish (*Lutjanus* sp.) from Banda Aceh Waters

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ABSTRACT

Snapper (*Lutjanus* sp.), which is an important marine fish resource, is known to have relatively low movement activity and form relatively small groups. These characteristics make these fish susceptible to various diseases caused by pathogenic bacteria. This study aims to identify the pathogenic bacterial species that contaminate snapper based on 16S rRNA gene analysis. The samples used in this study were the pathogenic bacterial isolates BPK-1 and BPK-2 which had previously been isolated from snapper fish originating from Banda Aceh waters. The bacteria were previously grown in nutrient broth (NB) media, then DNA genome from the bacteria was isolated using Presto™ Mini g DNA Bacteria Kit, followed by PCR amplification of 16S rRNA gene. PCR products were sequenced and nucleotide sequence data were analyzed by bioinformatics software to construct phylogenetic tree. The 16S rRNA gene sequences of the two isolates BPK-1 and BPK-2 were two different bacterial species. BPK-2 isolate has similarities with *Aeromonas* sp. with a similarity percentage of 96.69%. While the BPK-1 isolate has similarities with *Aeromonas salmonicida* with a similarity percentage of 85.88%, so it is suspected that it is a novel species that needs further research.

Keywords: *Aeromonas* sp., pathogenic bacteria, snapper fish, 16S rRNA gene

1. INTRODUCTION

Aceh waters is known a water area that has great potential in the marine and fisheries sector [1], including Banda Aceh waters which have very diverse fishery resources [2]. One of the marine products that has important economic value is snapper (*Lutjanus* sp.) [3]. This fish is a demersal fish, has a stable life cycle, relatively low mobile activity, and forms a relatively small group. This property causes snapper to be susceptible to various diseases [4].

Fish disease frequently can be caused by contamination or infection of pathogenic bacteria, such as *Vibrio* sp. (*V. parahaemolyticus*, *V. alginolyticus*, *V. anguillarum*, and *V. marinus*) [5], *Aeromonas* sp., *Pseudomonas* sp. and *Enterococcus* sp. [6]. These pathogenic bacteria can cause vibriosis, fish infection, and fish mortality [5].

Therefore, in this research, we focused on the fish pathogenic bacteria which are a potential risk in snapper fish. One way to identify pathogenic bacterial species in snapper fish (*Lutjanus* sp.) is by using 16S rRNA gene analysis. The 16S rRNA gene is a subunit component of the prokaryotic 30S ribosome, which is present in all prokaryotes and is commonly used as a molecular marker because it has advantages and strengthens its use as an identification tool [7]. Identification of the 16S rRNA gene from a microbe begins with the isolation of pure cultured bacterial genomic DNA, followed by amplification using the Polymerase Chain Reaction (PCR) technique and sequencing to obtain sequence nucleotides which are then further analyzed for phylogenetic tree construction [8,9]. Dewi *et al.* [10] reported that partial sequencing of the 16S rRNA gene

can be used to identify pathogenic bacteria in fish and shrimp.

Previous research by Muslikha *et al.* [11], also reported the cause of Motile Aeromonas Septicemia (MAS) disease in catfish (*Clarias sp.*) by using the 16S rRNA gene, but so far no research has identified pathogenic bacteria in snapper from Banda Aceh waters based on 16S rRNA gene analysis. This research is important to study that done by research team which previously isolated pathogenic bacteria in snapper to obtain pure isolates (BPK-1, BPK-2) and Gram staining, and the two isolates had been tested for their ability to produce hemolysin.

2. MATERIALS AND METHODS

2.1. DNA Isolation of Pathogenic Bacteria of *Lutjanus sp.*

The genomic DNA from the pathogenic bacteria of snapper fish was isolated by using a modified Presto™ Mini g DNA Bacteria Kit (Geneaid). A total of 1.5 ml of pathogenic bacterial isolates growing on NB media were put into a microtube then centrifuged at a speed of 14000 x g for 1 minute, after settling the supernatant was discarded. Furthermore, 180 µl of GT buffer was added and vortex for 1 minute, then the suspension was put into a microtube and incubated at 37°C for 10 minutes (in the incubation process, the tube is reversed once every 3 minutes).

In the lysis step, 200 µl was added GB buffer to the microtube, then vortexed for 30 seconds, the solution was then incubated at 70°C for 10 minutes (homogenized sample per 3 minutes). At the same time, 30-50 µl the elution buffer was incubated at a temperature of 70°C for 10 minutes. At the DNA binding step, the solution was added with 200 µl of absolute ethanol and then vortexed. Then the solution was transferred to the GD column which had a collective tube attached, then centrifuged using a centrifugator (Mini spin, Eppendorf) at a speed of 14000 xg for 2 minutes. The remaining supernatant in the collective tube is discarded and replaced in the new GD column.

Process washing was carried out in GD column added 400 µl of W1 buffer and centrifuged at a speed of 13000 xg for 2 minutes then the supernatant was removed. A total of 600 µl of W2 buffer was added to the GD column then centrifuged again for 3 minutes. The elution step of the pellet DNA in the GD column was paired with a sterile microtube and 50 µl of elution buffer was added into the matrix column and then left for 15 minutes, centrifuged 14,000 xg for 2 minutes. The genomic DNA were then observed using the electrophoresis method using 1% agarose gel with added fluorosave DNA stain. The visualization of DNA was observed with UV transilluminator and documented with Geldoc 1000 (BIO RAD).

2.2. PCR Amplification of 16S rRNA Gene

The DNA amplification of 16S rRNA gene was performed with polymerase chain reaction (PCR) (BIORAD). The 16S rRNA gene analysis was carried out as follows: the DNA template of 100 ng snapper fish pathogenic bacteria was added to PCR mixture (total volume is 25 ml) contained 12.5 µl of *Taq* polymerase, primers 63*f* (5'-CAG GCCTAACAC ATGCAAGTC-3') and 1387*r* (5'-GGGCGGWGTGTA CAA GGC-3') [12], 10 pmol of each primer, and 7.5 µl nuclease free water. 1 pre-denatured cycle (5 min, 94 °C), followed by 30 cycles of denaturation (1 minute, 94 °C), annealing (1 minute, 55, elongation (1 minute) and post-elongation (10 minutes, 72 °C). The ~ 1300 bp PCR products were separated on a 1% agarose gel with fluorosave DNA stain and observed with UV transilluminator, also documented with Geldoc 1000 (BIORAD).

2.3. Sequencing and Analysis of 16S rRNA Gene Sequencing, Bioinformatics Analysis, and Phylogenetic Tree Construction

The PCR products was directly sequenced in First Base Co., Malaysia, a sequencing service company, using DNA sequencer ABI PRISM 3100. The 16S rRNA sequences data were compared with available database at GenBank on The National Center for Biotechnology Information (NCBI) database (<http://www.ncbi.nlm.nih.gov>) by using the Basic Local Alignment Search Tool Nucleotide (BLASTN) software. The 16S rRNA sequenced data were aligned and phylogenetic tree were constructed using MEGA 5.05 software [13], based on a neighbor-joining tree (NTJ) [14], with a bootstrap value of 1000x.

3. RESULTS AND DISCUSSION

3.1. DNA Profile of Pathogenic Bacteria from *Lutjanus sp.* from Banda Aceh Waters

DNA isolation is a series of processes for separating DNA from other components [15]. The success at this stage will greatly determine the quality of DNA obtained. In this research has successfully isolated DNA from two pathogenic bacteria from snapper fish (Figure 1).

According to Hidayati *et al.* [16], bright and thick bands indicate high DNA isolation while thin bands indicate small isolation results. The size of the genomic DNA obtained by a microorganism is very different depending on the microorganism.

3.2. The 16S rRNA Gene Amplification of Pathogenic Bacteria from *Lutjanus sp.*

Based on the amplification of 16S rRNA gene, the two pathogenic bacteria isolated from *Lutjanus sp.* produced a single band with expected molecular size~ 1300 bp

(Figure 2). The DNA amplification stage functions to duplicate a DNA fragment [17]. Primary pair 63f (5' - CAG GCC TAA CAC ATG C AA GTC - 3) and 1387r (5' - GGG CGG WGT GTA CAA GGC-3) used in this study was successfully used to amplify the two pathogenic bacteria isolated from *Lutjanus* sp.

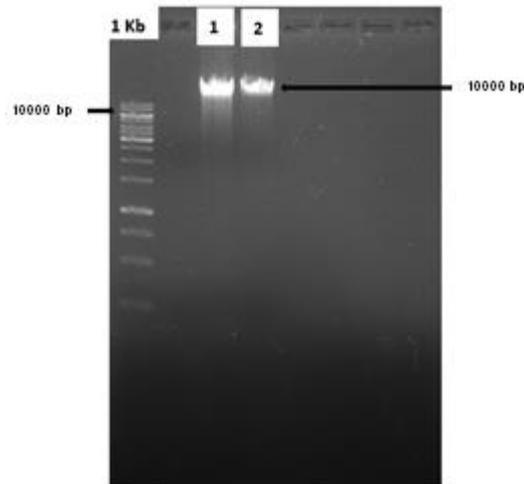


Figure 1 The genomic DNA of the pathogenic bacteria isolated from *Lutjanus* sp. origin from Banda Aceh waters. 1 Kb: DNA Marker; Lane 1: BPK-1 isolate; Lane 2: BPK-2 isolate.

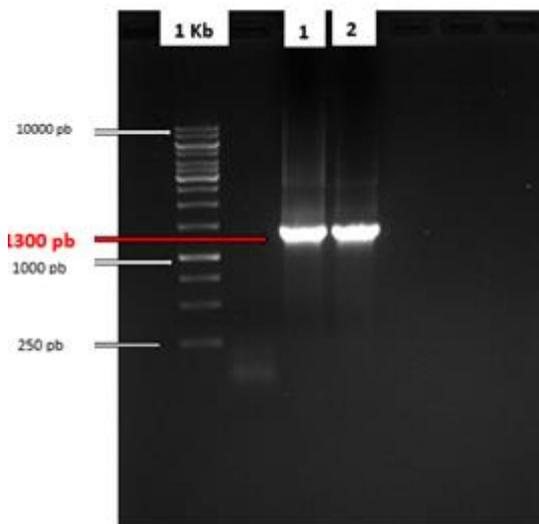


Figure 2 The PCR amplification product of 16S rRNA gene of pathogenic bacterial isolates from *Lutjanus* sp. on 1% agarose gel. 1 Kb: DNA Marker; Lane 1: BPK-1; Lane 2: BPK-2.

Primers play an important role in the amplification process, especially in recognizing and marking DNA fragments to be amplified. The 63F primers were designed in such a way to amplify the 16S rRNA gene in most bacterial strains and the 1387R primers were used for amplification of the 16S rRNA gene in most bacterial domains [12].

3.3. Homology of 16S rRNA Sequences from Pathogenic Bacteria Isolated from *Lutjanus* sp.

DNA sequencing is a process for determining the nucleotide sequence of DNA fragments which aims to identify a nucleotide data obtained from the sequencing results in the form of a chromatogram format consisting of forward and reverse primers which will be analyzed through the Bioedit application.

The data analysis in this study used the Local Alignment Search Tool (BLASTN) software application, which is a bioinformatics tool related to the use of biological sequence databases. The similarity of the DNA sequence of the pathogenic bacteria of snapper (*Lutjanus* sp.) from Banda Aceh waters with the DNA sequence that was deposited in Genbank can be seen through the alignment of nucleotide data from NCBI (<http://www.nlm.nih.gov>) with the sample DNA sequence. Based on the BLASTN bioinformatics analysis, the DNA sequences of both BPK-1 and BPK-2 isolates did not show any similarities, which were distinguished between Table 1 and Table 2.

Table 1. Percent similarity the sequences of the 16S rRNA pathogenic bacterial isolate BPK-1 compared with references strain sequences in Genbank

References strain (Genbank)	Query cover	% Similarity	Access No.
<i>Aeromonas salmonicida</i> strain SRW-OG1	98%	85.88%	MT525253.1
<i>Aeromonas salmonicida</i> strain GSI4 16S	98%	85.88%	MG711833.1
<i>Aeromonas salmonicida</i> strain S13 16S	98%	85.88%	KC210757.1
<i>Uncultured bacterium</i> clone AA_31 16S	98%	85.88%	JX120470.1
<i>Aeromonas salmonicida</i> AST1 16S strain	98%	85.88%	JN051353.1

Table 2. Percent similarity the sequences of the 16S rRNA pathogenic bacterial isolate BPK-2 compared with references strain sequences in Genbank

References strain (Genbank)	Query cover	% Similarity	Access No.
<i>Aeromonas</i> sp. strain L4 16S	99%	96.69%	MT516456.1
<i>Aeromonas</i> sp. strain A16OP9 16S	99%	96.69%	MN519576.1
<i>Aeromonas salmonicida</i> subsp.	99%	96.69%	MK881070.1
<i>Aeromonas salmonicida</i> strain K12 16S	99%	96.69%	MK548520.1
<i>Aeromonas salmonicida</i> strain K6 16S	99%	96.69%	MK548516.1

From Tables 1 and Table 2, it can be seen that the 16S rRNA gene sequences of BPK-1 and BPK-2 with the references strain (Genbank) are *Aeromonas* sp. has a similarity percent of 85.88% and 96.69%, respectively. The E-value is an important predictive value for the sequence as measured by the E-value statistic which shows the number 0 indicating that the sample sequence is homologous to the sequence from Genbank. The E-value was obtained in this research is 0.0. The Genbank sequence consists of a maximum score, total score, query cover and maximum identity. Maximum score and total score are characterized for the Genbank sequences that are the most similar. The e-value is close to 0.0 and the maximum identity is close to the maximum value, which is 100%. The two sequences do not have homology, so the two sequences do not have an evolutionary or related relationship. In addition, the homology of 16S rRNA gene sequences < 97% indicated as a novel species [18].

3.4. Phylogenetic Tree of 16S rRNA Gene Pathogenic Bacteria of *Lutjanus* sp.

The 16S rRNA gene amplification are frequently used in molecular identification and construction of phylogenetic relationship among prokaryote. The phylogenetic tree construction was made based on alignment of DNA sequences using the neighbor join tree method with 1000x bootstrap value in MEGA 5.05

software. The results of the phylogenetic tree analysis are shown in Figure 3.

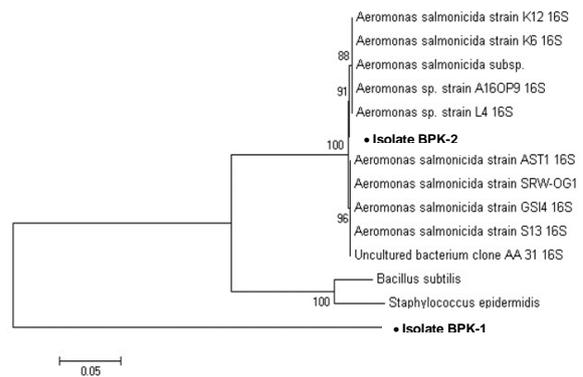


Figure 3 Phylogenetic tree of the 16S rRNA gene pathogenic bacteria (BPK-1 and BPK-2) isolated from *Lutjanus* sp. origin from Banda Aceh waters with 1308 nucleotide

Based on 16S rRNA gene analysis in this research, it is reported here that bacterial isolate BPK-2 was closely related to *Aeromonas* sp., while bacterial isolate BPK-1 was separated from cluster of *Aeromonas* sp. and *Aeromonas salmonicida* and indicated as a novel species with <97% maximum identity, E-value 0.0, and it belonged to *Aeromonas salmonicida* (Figure 3). However, this assumption must be proven by further investigation using the polyphasic method on these isolates, so that it can be continued to state the newness of a species. *Aeromonas salmonicida* is one species of the genus *Aeromonas* which is pathogenic and very dangerous to fish [19].

4. CONCLUSION

Based on the results of the study, it can be concluded that the pathogenic bacteria of snapper from Banda Aceh waters (isolates BPK-1 and BPK-2) were successfully isolated and amplified using the 16S rRNA gene. Then from the results of the analysis using BLASTN and phylogenetic tree construction isolates BPK-1 and BPK-2 were different types of bacteria. BPK-2 isolate has similarities with *Aeromonas* sp. with 96.69% similarity percent. Meanwhile, the BPK-1 isolate is thought to have a chance as a new species because it only has a similarity percentage of 85.88%, but it must be investigated further.

AUTHORS' CONTRIBUTIONS

WES and MNS conceptualized dan designed this research. The research was carried out by WBS, WES, MNS, and SFS. WES, WBS, and TZH analyzed the data and result. WES, MNS, TRF, MH, HV, TZH, MZR, and WBS drafted, revised, and finalized the manuscript. All authors read and approved the final manuscript.

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