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Research article

Identification of pathogenic bacteria from eggshell of leatherback sea turtle *(Dermochelys coriacea)* in Lampuuk Beach, Aceh Besar using 16S rRNA gene

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Abstract

Currently, the leatherback turtle population in Indonesia tends to decline. One of the factors that cause turtles' existence is the turtle eggs that fail to hatch because bacteria contaminate them, 80% of the reasons turtle eggs fail to hatch are due to infection by microorganisms found on the eggshell. Turtle eggs have a soft structure and there are pores that function for gas exchange and water absorption. Microorganisms in sand can infect turtle eggs through the pores and cause hatching failure. This study aims to isolate and identify pathogenic bacteria found in leatherback turtle eggshell *(Dermochelys coriacea)* from Lampuuk Beach, Aceh Besar, based on the 16S rRNA gene analysis. Eight eggshell samples from leatherback turtle eggs that failed and hatched were cultured on Nutrient Agar (NA) medium. Then, the samples were inoculated on a blood agar medium for haemolysis test. Molecular identification of the 16S rRNA gene was also carried out to determine bacterial species. A total of five bacterial colonies from leatherback turtle eggshells were successfully isolated consisting of two Gram-negative bacteria (Penyu Belimbing Aceh (PBA) = PBA-1 and PBA-2 and three Gram-positive bacteria (PBA-3, PBA-4, and PBA-5). Based on the haemolysis test, the five bacterial isolates were unable to haemolyze blood. Bacterial DNA of PBA-1 and PBA-2 were successfully isolated and amplified using polymerase chain reaction (PCR) with a DNA target of ~1500 bp. Analysis using BLASTN and phylogenetic tree construction showed that PBA-1 isolate had 98.64% similarity with *Acinetobacter baumannii*, while PBA-2 isolate had 97.82% similarity with *Enterobacter kobei*. Both bacteria are members of the Enterobacteriaceae family. It can be concluded that, there were two pathogenic bacteria can potentially be opportunistic pathogens found in leatherback turtle eggshells that failed or succeeded in hatching, namely *A. baumannii* and *E. Kobei*

Keywords: Eggshell, Enterobacteriaceae, Leatherback turtle, Opportunistic pathogens, Polymerase chain reaction (PCR)

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INTRODUCTION

Turtles are the reptiles that live in the sea and migrate long distances along the Indian Ocean, Pacific Ocean, and Southeast Asia (Parawangsa et al., 2018). There are six types of turtles found in Indonesia out of seven types of turtles globally. The types of turtles found in Indonesia are the green turtle (*Chelonia mydas*), the hawksbill turtle (*Eretmochelys imbricata*), the olive ridley turtle (*Lepidochelys olivacea),* the leatherback turtle (*Dermochelys coriacea*), the flat turtle (*Natator depressus*), and the loggerhead turtle (*Caretta caretta*) (Novitasari et al., 2018). The leatherback turtle is one of the most common turtle species and lays eggs on Lampuuk Beach, Lhoknga District, Aceh Besar Regency, which is one of the turtle nesting beaches in Aceh (Ali et al., 2020).

Data from the World Wildlife Fund for Nature (WWF) shows that in the period 2007-2011, the leatherback turtle population in Indonesia tended to decline so that this species is included in the red list and is internationally included in the endangered species category set by the International Union for The Conservation of Nature (IUCN) (Umma et al., 2020). Universally, leatherback turtles' status concurring to IUCN is recorded as Defenseless, and numerous subpopulations including in Indonesia are critically endangered (WWF, 2023). Eggs that fail to hatch due to bacterial contamination are factors that cause the existence of turtles to be threatened with extinction (Phillott and Parmenter, 2001).

Morais et al. (2010) and Keene (2012) stated that the bacteria that commonly contaminate turtle eggs are Enterobacteriaceae groups such as *Shigella* sp., *Enterobacter* sp., *Klebsiella* sp., *Escherichia coli*, *Proteus* sp., and *Salmonella* sp. This group of bacteria is a group of bacteria that often contaminates and can produce toxins in eggs, causing eggs to fail to hatch. In a study conducted by Dutton et al. (2013), it was found that there were 77.7% of *Salmonella* sp. isolated from leatherback turtle egg samples from West Indies. The genus *Bacillus* has also been associated with failed hatching of leatherback turtle eggs in the Parque Nacional Marino las Baulas Conservation Park (Hidayat et al., 2014). Based on Hidayat et al. (2014), bacterial isolates from hatched eggshells were identified as *B. cereus*, *Klebsiella* sp. Meanwhile, *B. cereus*, *Klebsiella* sp., *Salmonella* sp. and *Shigella* sp. identified from eggshells that failed to hatch. In addition, Al-Bahry et al. (2009) revealed that *Salmonella typhimurium* penetrated all eggshell layers of green turtles. Sea turtle egg shells can be horizontal bacterial contamination as a secondary route of infection (Poppe et al., 1998), if the turtle eggs laid are mixed with contaminated feces material from the intestinal tract or from other environmental sources (Catalano and Knabel, 1994). Katni et al. (2022) reported *Aeromonas, Enterobacter* spp., *K. pneumonia, Citrobacter freundii, Proteus vulgaris*, *Citrobacter* sp., and *S. enterica* were found in both egg content and eggshell. Al-Bahry et al., (2011) stated that microorganisms on the outer shells had succeeded in penetrating the eggs through the eggshell pores.

Based on personal communication with the turtle conservation in Lampuuk Beach, at least every year, it is found that eggs fail to hatch. Information on the presence of microorganism contamination in turtle conservation and captive areas in Indonesia is still very limited, especially for

leatherback turtles, so it is necessary to research to determine the possibility of contamination of pathogenic bacteria originating from leatherback turtle (*D. coriacea*) eggshells obtained at Lampuuk Beach based on identification using 16S rRNA gene analysis.

Several studies on the identification of marine microorganisms through a DNA molecular approach, especially in Banda Aceh, have not been widely reported. Meanwhile, 16S rRNA gene sequencing for research in Indonesia, especially in fisheries and marine affairs, has been widely carried out. This is because the 16S rRNA gene has several advantages, one of which is having a hypervariable region section that makes it easier to identify the type of bacteria. This method is, in fact, very effective to use because it has high accuracy and does not take time to identify bacteria.

MATERIALS AND METHODS

Sampling and Sample Preparation

This research was conducted at the Laboratory of Veterinary Public Health and Laboratory of Research, Faculty of Veterinary Medicine, Universitas Syiah Kuala, Banda Aceh. The materials used in this study include eggshells leatherback turtle (*Dermochelys coriacea*). A total of eight leatherback turtle eggshells were cleaned of dirt and sand after being obtained from the captivity. Samples of turtle eggshells that hatched (four samples) and those that failed to hatch (four samples), were then put into sterile plastic samples separately. The shells were separated from the contents of the eggs and soaked for 30 minutes in a measuring cup filled with sterile distilled water. The eggshells are then crushed with a mortar and pastel.

Bacterial Isolation and Purification

A total of six test tubes that have been filled with sterile distilled water as much as nine mL. A total 1 g of eggshell that has been mashed and homogenized is inserted into one tube so that a dilution of 10^{-1} is obtained. Then serial dilutions were carried out up to 10^{-6} dilutions. Bacterial isolation was carried out by taking a sample solution from the 10^{-5} and 10^{-6} dilution tubes and then growing them on 0.1 ml Nutrient Agar (NA) solid media with two replications using the spread plate technique and incubating at 37 ºC for 24 hours. After obtaining several types of colonies with different morphology, the isolates were purified using the streak plate technique. The sample plates were then incubated upside down at 37 ºC for 24 hours.

Gram Staining

The test bacteria were inoculated on the object glass and fixed over the fire. Then the smear of bacteria was dipped in a crystal violet for 1 minute and then dipped again with lugol for 1 minute. The preparate was washed with 95% alcohol for 30 seconds and rinsed with running water. The bacterial preparate was flooded into a container containing safranin dye for 30 seconds, then rinsed using running water. The bacterial smear was dried and dripped with immersion oil, observed at 1000 x magnification under a microscope.

Hemolysis Test

This test is performed to observe the ability of bacteria to hemolyze red blood cells. Bacterial colony isolates were grown on Blood Agar (BA) media at 37 °C for approximately 18-24 hours and the hemolytic properties were observed. There are three types of hemolysis, namely alpha hemolysis, beta hemolysis, and gamma hemolysis. Alpha hemolysis is a partial lysis or partial lysis of red blood cells, beta hemolysis is a complete lysis of red blood cells and hemoglobin, gamma hemolysis is that there is no hemolysis because there is no color change in the media (Buxton, 2005). After the hemolysis test was carried out and the results were obtained, the bacterial isolates were grown into the Nutrient Broth (NB) media to continue the DNA isolation step.

Isolation of Pathogenic Bacterial DNA in Leatherback Turtles (*Dermochelys coriacea*)

Isolation of the DNA of pathogenic bacteria in this study using Presto ™ Mini g DNA Bacteria Kit (Geneaid), modified. A total of 1.5 ml of isolates of pathogenic bacteria that grew on NB media were put into a microtube and then centrifuged (Minispin, Eppendorf) at 14,000 x g for 1 minute, after precipitate the flow-through was discarded. Furthermore, the addition of 180 µl of buffer GT and vortexed 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 was inverted every 3 minutes).

In the lysis step, 200 µl GB buffer was added to the microtube, then vortexed for 30 seconds, the solution was then incubated at 70 °C for at least 10 minutes to guarantee the sample lysate is clear, and during incubation samples were inverted every 3 minutes). At this time, pre-heat the required 30-50 µl of Elution Buffer at 70 ºC for 10 minutes. The next step is DNA binding, 200 µl of absolute ethanol was added to the sample lysate and then vortexed immediately. Next, the sample lysate was transferred to the GD column in a 2 ml collection tube attached, then centrifuged using a centrifugator (Mini spin, Eppendorf) at 14000 x g for 2 minutes. Discard the 2 ml Collection Tube containing the flow-through then place the GD Column in a new 2 ml Collection Tube.

The washing process is carried out by added 400 µl of W1 buffer and centrifuged at 14000 x g for 2 minutes then the flow-through was discarded. A total of 600 µL of W2 buffer was added to the GD column and then centrifuged again for 3 minutes at 14000 x g . The last step is DNA elution, the dried GD column was paired with a 1.5 ml sterile microtube and added 50 µL of pre-heated elution buffer into the center of the column matrix and allowed to stand for 15 minutes, then centrifuged at 14000 x g for 2 minutes to elute the purified DNA. The results of bacterial DNA isolation were then observed by electrophoresis method using 1% agarose gel, 80 Volt, for 60 minutes. The agarose gel was observed on a UV transilluminator and documented using Geldoc 1000 (BIO RAD).

16S rRNA Gene Amplification of Pathogenic Bacteria from Leatherback Turtles Eggshells

16S rRNA gene of pathogenic bacteria from leatherback turtle eggshell was amplified by using PCR. The amplification of this gene uses a pair of primers 27f (5' – AGAGTTTGATCMTGGCTCAG – 3) and 1492r (5' – TACGGYTACCTTGTTACGACTT – 3') (Yakimov et al., 2001). In a total

volume of 25 µL of the PCR reaction, the composition of the PCR reaction consisted of (PCR mix containing 12.5 µL of taq polymerase, 10 pmol of primer 27*f* and 1492*r* 1 µL, genomic DNA 3 µL and nuclease free water (NFW) 7.5 µL. The PCR conditions used were pre-denaturing cycle (5 minutes, 94 ºC), followed by 30 cycles of denaturation (1 minute, 94 ºC), annealing (1 minute, 55 ºC), elongation (1 minute, 72 ºC) and post – elongation (10 min, 72 ºC). The PCR results were electrophoresed using 1% agarose gel with fluorosave DNA stain.

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

The amplified DNA was sent to the sequencing service company 1st BASE Co, Malaysia, according to the standard DNA sequencer protocol (ABI PRISM 3100). Sequence data were compared with data from GenBank in The National Center for Biotechnology Information (NCBI) database, using the Basic Local Alignment Search Tool Nucleotide (BLASTN) program. For nucleotide matching and construction of the 16S rRNA gene phylogenetic tree, MEGA 5.05 software was used (Tamura et al., 2011). Based on the neighbor-joining tree (NTJ) (Saitou and Nei, 1987), with a boostrap value of 1000x.

RESULTS

A total of five bacterial isolates were successfully isolated and purified from all eight eggshell samples, with morphological diversity shown on NA media (Figure 1). Colony morphology and Gram staining are presented in Table 1 and Figure 2. Based on the observations in Table 1, it was found that of the five bacterial isolates, four of them were round with circular edges and only one was irregular type, colony size obtained ranged from 1.0 mm to 5.0 mm, and three isolates were white, one isolate was cream and one isolate was red. Based on the results of Gram staining (Figure 2), it is known that the three bacterial isolates belonged to the Gram-positive group of bacteria and two of them were Gram negative bacteria (Figure 2).

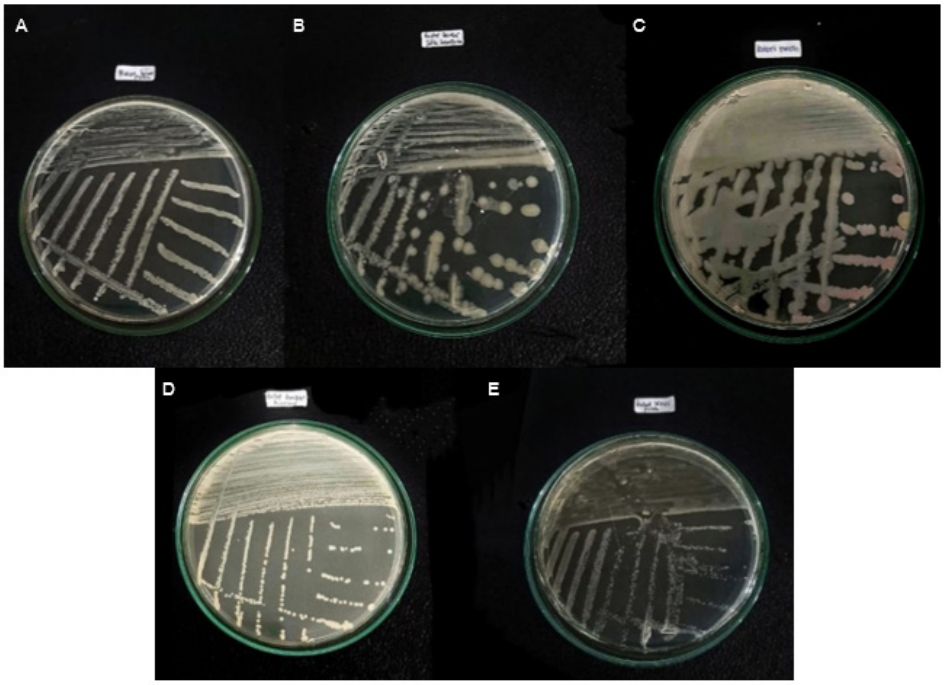


Figure 1 Morphology of bacterial isolates isolated from leatherback turtle eggshells on NA media. A= PBA-1 isolate, B= PBA-2 isolate, C= PBA-3 isolate, D= PBA-4 isolate, and E= PBA-5 isolate.

Table 1 Colony morphology characteristics and Gram staining of bacteria from leatherback turtle eggshells

Isolates code	Type	Size	Pigmentation	Cell shape	Gram stain
PBA-1	Circular	3.0 mm	Cream	Coccobacil	Negative
PBA-2	Irregular	4.0 mm	White	Streptococcus	Negative
PBA-3	Circular	5.0 mm	Red	Coccus	Positive
PBA-4	Circular	1.0 mm	White	Diplococcus	Positive
PBA-5	Circular	2.2 mm	White	Coccus	Positive

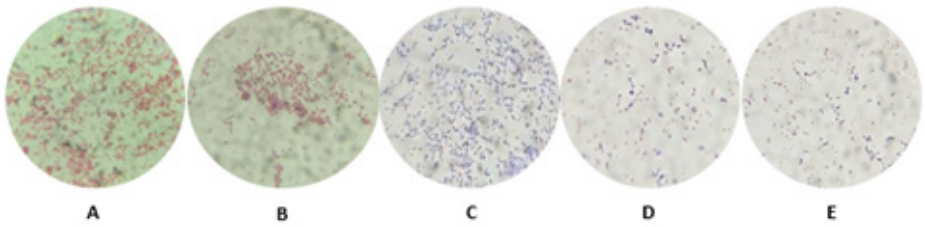


Figure 2 The results of Gram staining of bacterial isolates from leatherback turtle eggshell under a microscope with 1000x magnification. A= PBA-1 isolate, B= PBA-2 isolate, C= PBA-3 isolate, D= PBA-4 isolate, and E= PBA-5 isolate.

Based on the hemolysis test on blood agar media (Figure 3 and Table 2), it was found that the five isolates were non-hemolytic. This is because the color of the red blood cell colonies does not change (gamma hemolysis), it can be seen from Figure 3. This shows that there is no hemolysin enzyme. Table 2 shows that the five bacterial isolates from leatherback turtle eggshells could not hemolyze blood.

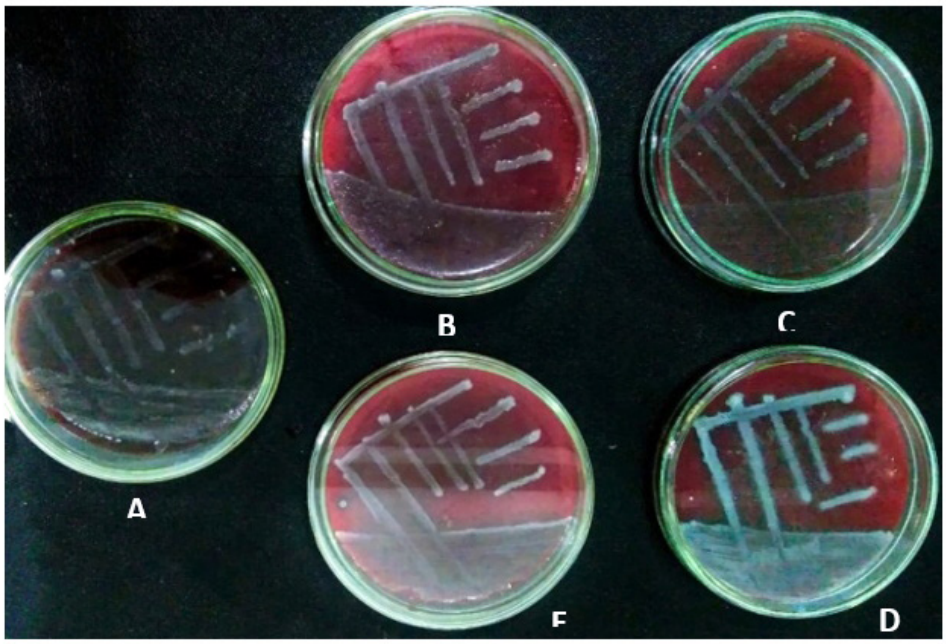
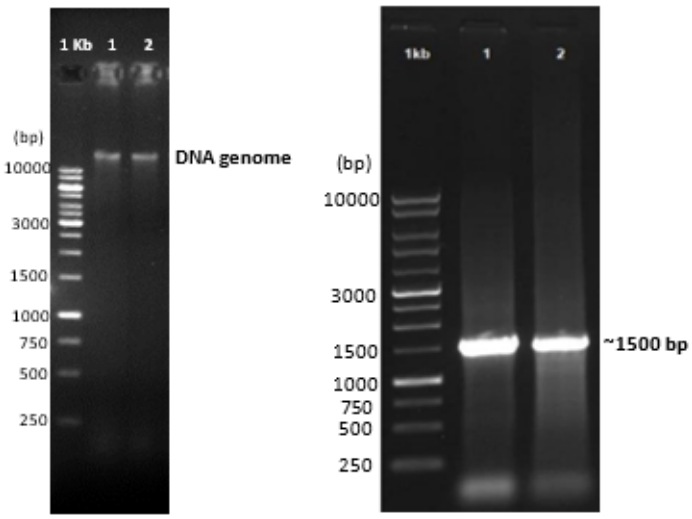


Figure 3 The results of the hemolysis test of bacterial isolates from leatherback turtle eggs on blood agar media after incubation at 37 ºC for 24 hours. A= PBA-1 isolate, B= PBA-2 isolate, C= PBA-3 isolate, D= PBA-4 isolate, and E= PBA-5 isolate.

Table 2 The results of the hemolysis test of bacterial isolates from leatherback turtle eggs

Isolates code	Hemolysis test
PBA-1	*Gamma hemolysis (γ)* - Non hemolysis
PBA-2	*Gamma hemolysis (γ)* - Non hemolysis
PBA-3	*Gamma hemolysis (γ)* - Non hemolysis
PBA-4	*Gamma hemolysis (γ)* - Non hemolysis
PBA-5	*Gamma hemolysis (γ)* - Non hemolysis

The molecular identification process consists of three stages, namely extraction, amplification and electrophoresis. From the five bacterial isolates, two bacteria from the Gram-negative group were selected with isolate codes PBA-1 and PBA-2 and planted on NB media. The success of DNA isolation was indicated by the presence of a single thick and intact band at the top of the marker as shown in Figure 4a.



(A) (B)

Figure 4 The total genomic DNA (A) and PCR amplification of 16S rRNA gene (B) from bacterial isolates isolated from leatherback turtle eggshell were electrophoresed on from with 1% gel agarose. Marker= 1 Kb, 1= PBA-1, 2= PBA-2.

The amplified DNA was then continued with the electrophoresis stage as a qualitative test to measure the process of DNA concentration obtained from the PCR process using universal primer 27*f* and 1492*r* for the 16S rRNA gene. The results of electrophoresis can be seen in Figure 4b.

Based on the BLASTN bioinformatics analysis, the DNA sequences of PBA-1 and PBA-2 isolates did not show similarities, the data are presented in Table 3. The results of the molecular analysis based on the 16S RNA gene sequence in Table 3 show that the PBA-1 isolate has similarities with the *Acinetobacter baumannii* strain ATCC 17978 (accession number CP053098.1) with a similarity level of 98.64% and PBA-2 isolates showed a similarity with bacteria *Enterobacter kobei* strain BLR45 (accession number MW624688.1) with a similarity level of 97.82%. Both isolates showed very good results.

Table 3 BLASTN results of bacterial isolates from leatherback turtle eggshell

Isolates code	References strain	E. value	Percentage similarity (%)	No. accession
PBA-1	*Acinetobacter baumannii* strain ATCC 17978	0.0	98.64	CP053098.1
PBA-2	*Enterobacter kobei* strain BLR45	0.0	97.82	MW624688.1

The results of the phylogenetic tree analysis are shown in Figure 5. Based on Figure 5, it can be seen that PBA-1 and PBA-2 isolates of leatherback turtle bacteria from Lampuuk beach have no kinship relationship because the two isolates are separated between clusters. PBA-1 and PBA-2 isolates were Gram negative bacteria. The results of the construction of the PBA-1 isolate phylogenetic tree showed a kinship relationship with the bacteria *Acinetobacter* sp. because is in the same cluster and the PBA-2 isolate shows a relationship with *Enterobacter* sp. bacteria, and both belong to the Enterobacteriaceae group of bacteria which are opportunistic pathogens. Based on the phylogenetic tree, both are clearly separated from the group of Gram-positive bacteria, *Staphylococcus aureus*.

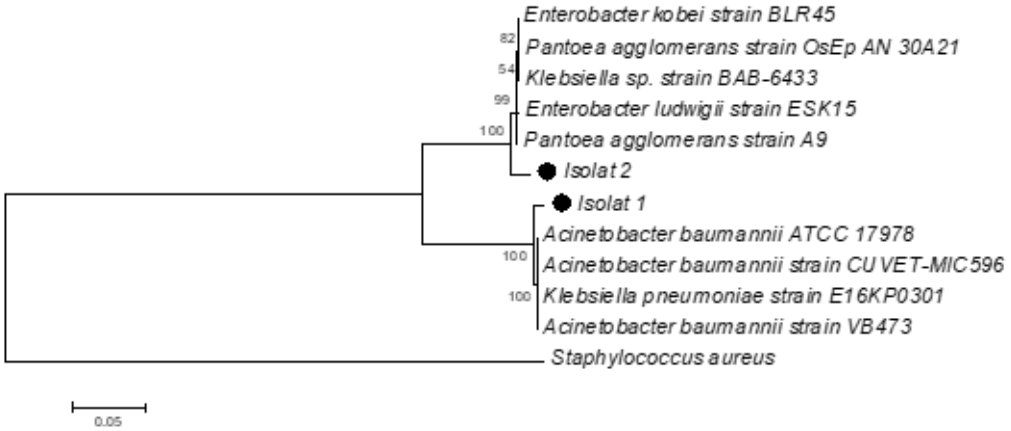


Figure 5 Phylogenetic tree of bacterial 16S rRNA gene from leatherback turtle eggshell, using neighbor joining tree method with 1000x bootstrap.

DISCUSSION

Purification aims to obtain the desired pure culture so that there are no contaminants from other microbes. Selection of purified bacterial colonies based on visible colony morphology differences. Purification of bacterial isolates was carried out by transferring bacteria using the streak method which was then grown on NA media (Edhar, 2017). The bacteria that grew in the media were then carried out with Gram staining to see the Gram properties of each bacterium.

After Gram staining, bacterial isolates from slanted NA were grown on blood agar medium and incubated at 37 °C for 24 hours. Blood agar is a standard growth medium to identify the type of bacteria and as a medium for antibiotic sensitivity tests of various pathogenic bacteria (Krihariyani, 2016). The addition of blood aims to fertilize the seeds and to grow bacteria that are difficult to grow in ordinary seeds. In addition, this media can also see the characteristics of bacteria and the ability of bacteria to destroy erythrocytes (Entjang, 2003). The technique commonly used on blood agar plates is streak plate. The hemolysis test aims to determine the ability of isolates to be potentially pathogenic or not in humans or animals (Sodiq et al., 2019). Hemolysin itself is an enzyme that can cause red blood cells to lyse and also increase cell premiabilities so that cells tend to be more susceptible to bacterial infections (Khairunnisa, 2018).

Although the hemolysis test is one of the tests to identify pathogenic bacteria, the absence of hemolysis in the blood cannot be used as a reference that there are no pathogenic bacteria. The purpose of this study was to isolate and identify pathogenic bacteria in turtle eggshells that succeeded and failed to hatch. However, based on isolation, bacterial isolates were not able to hemolyze blood but the molecular identification process was continued because bacteria that were unable to hemolyze blood could be normal florals that could become opportunistic pathogens.

Based on observations made on bacterial DNA isolation using PCR from leatherback turtle eggshells with 1% gel electrophoresis, Figure 4 shows the bacterial isolates PBA-1 and PBA-2 have the same location and size, namely ~1500 bp and with good DNA band thickness. Thick and clumping (not spreading) DNA bands indicate a high concentration and the total extracted DNA is intact, while the scattered DNA bands indicate that the bonds between DNA molecules are broken during the extraction process, so that the DNA genome is cut into pieces smaller part. The breaking of the bonds between the molecules can be caused by excessive physical movements that can occur in the pipetting process, when being turned over in an ependorf, centrifuged, or even because the temperature is too high and because of the activity of certain chemicals (Irmawati, 2003). DNA amplification results obtained have been successful.

The success of amplification of DNA fragments is influenced by several factors, namely DNA templates, primers, PCR buffers, $MgCl_2$, polymerase enzymes, temperature, time and number of cycles (Sambrook and Russell, 2001). The sequencing process is carried out by sending DNA PCR products to 1st BASE, Malaysia. Data analysis in this study used the Basic Local Alignment Search Tool (BLASTN) software application, which is a bioinformatics tool related to the use of biological sequence databases (Pratiwi et al., 2018).
The percentage of similarity that can be accepted is at least 95%, except for sequences with lower readings, 75% is applied. Good sequencing results are shown by graphs with high peaks and are separated from each other. In contrast to the poor sequencing results, this is indicated by the peaks being gentle and not separated from each other or there are double peaks on the chromatogram (Untul et al., 2015).

The phylogenetic tree analysis aims to determine the genetic relationship distance between one species and another species to be studied. The phylogenetic tree construction is made based on the alignment of DNA sequences using the neighbor join tree program with a boostrap value of 1000x in the application software MEGA 5.05.

The 16S ribosomal RNA (16S rRNA) gene is used as a molecular marker because it has several advantages that strengthen its use as an identification tool, namely (a) ubiquity with identical functions in all organisms, (b) can change according to evolutionary distance, so that it can be used as a good evolutionary chronometer, (c) has several regions with relatively conservative base sequences to construct a universal phylogenetic tree, Because it changes relatively slowly and reflects the chronology of Earth's evolution, (d) it has several varied regions that can be used to track diversity and place strains within a species (Pangastuti, 2006). In addition, among the sequence-based microbiome studies, the 16S rRNA genes have been the most predominantly

utilized molecular marker for bacterial classification (Srinivasan et al., 2015). The bacterial 16S rRNA gene is around 1500 bp long and contains both conserved and variable regions that advance at different rates. The slow evolution rates of the previous regions enable the design of universal primers that amplify genes across different taxa, while fast-evolving regions reflect differences between species and are useful for taxonomic classification (Clarridge, 2004).

*Acinetobacter*are hydrophilic organisms and preferentially colonize in aquatic environments. This organism is able to spread to almost all parts of the body either transiently or as normal flora. *Acinetobacter baumanii* is an opportunistic pathogen that can cause suppurative infections in organs or tissues. *Acinetobacter* species have low virulence but are capable of causing infection. The virulence factors in this species are difficult to explain because they allow them to escape from phagocytosis and produce exopolysaccharides to protect themselves from other and innate immune mechanisms (Nugroho et al., 2012).

Enterobacter kobei is a species of *Enterobacter cloacae* which is phenotypically most closely related to *E. cloacae* species. *Enterobacter* bacteria including bacteria that are pathogenic to reptiles. According to Borrow and Filtham (2003) these bacteria are a group of Enterobacteriaceae bacteria which are Gram negative, rod-shaped and capable of fermenting lactose and glucose. Bacteria that are opportunistic pathogens are normal flora that can live in a normal human body, one of which is in the human intestine. So, it is assumed that the presence of these bacteria is thought to be due to contamination by feces from the digestive tract. These bacteria are found in water, soil and air as well as the digestive tract of humans and animals. Bacteria of the *Enterobacter* genus are a group of coliform bacteria whose presence can be an indicator of contamination of animal and human feces (Nursanty et al., 2019).

The inability of bacteria to hemolyze blood because both bacteria are normal flora but these bacteria can be one of the causes of egg failure if these bacteria are predisposed to cause the bacteria to become pathogenic. Virulence factors can also contribute to causing infection. Virulence factors can be in the form of adhesion (fimbrial adhesion and afimbrial adhesion), hemagglutinin protein, capsule, lipopolysaccharide (endotoxin), serum resistance, and siderophores (Ragione and Woodward, 2002).

Bacteria are said to be pathogenic if they have the ability to transmit, attach to host cells and carry out multiplication, use nutrients from host cells, invade and cause damage to tissue cells (Pratiwi, 2017). Non-pathogenic bacteria are bacteria that are harmless and can act as normal flora. If a non-pathogenic bacterial organism moves from its place of origin it will be able to cause disease and is called an opportunistic pathogen. Bacteria that are opportunistic pathogens are normal flora that live in normal human and animal bodies (Padoli, 2016).

There are two main factors that greatly affect the success of eggs directly while in semi-natural nests, namely temperature and humidity (Pitriani et al., 2017). Environmental conditions in natural nests are the best conditions for hatching turtle eggs but considering the presence of predators, turtle eggs are relocated or moved to semi-artificial nests as a conservation effort (Samosir et al., 2018). Hatching failure is the impact of the results of the hatching process that is not optimal, it can be influenced by many factors. Both internal factors,

namely the development of the eggs themselves, as well as external factors, namely from the environment around the eggs (Khushartono et al., 2016).

The hatching period for leatherback turtle eggs is in the rainy season, which is in the period from December to January. Indonesia experiences high rainfall between 200-500 mm from January to April 2021 (BMKG, 2020). Rainfall can affect fluctuations in temperature and water content which then affects the success of hatching. The low water content in the nest will make water come out of the eggs while the high environmental water content can kill the developing fetus so that the turtle eggs fail to hatch (Pitriani et al., 2017). Failure in hatching eggs can also be affected by shocks in the early stages of egg development so that the division process is disrupted so that the embryo is not formed (Kushartono et al, 2016). The effect of shock on the early stages of egg development can occur when eggs are transferred from natural nests to be relocated to semi-natural nests. According to a personal communication with conservation authorities, egg transfer also occurs when the sand is damp, eggs exposed to moisture from the sand are moved from the bottom of the nest to the top of a semi-natural nest, in addition, eggs that fail to hatch can also be caused by eggs that are infertile and fail to fertilize (Umama et al., 2020).

Based on the results of the study, the five samples from leatherback turtle egg shells, both those that failed to hatch or successfully hatched were unable to hemolyze blood. The results of DNA extraction using 1% gel electrophoresis were seen from intact and there was no smear on the DNA. The amplified DNA obtained was successful with ~1500 bp band. The results of molecular analysis based on 16S RNA gene sequences using BLASTN and phylogenetic tree construction showed that PBA 1 isolate had similarities with *Acinetobacter baumannii* with a 98.64% similarity percent while PBA 2 isolates had a similarity with *Enterobacter kobei* with 97.82% similarity percent, both of these bacteria were a group of bacteria. It can be concluded that there were two pathogenic bacteria can potentially be opportunistic pathogens found in leatherback turtle eggshells that failed or succeeded in hatching, namely *A. baumannii* and *E. Kobei*.

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AUTHOR CONTRIBUTIONS

WES and AS: Conceptualized and designed the study. WES, NNA, and AS: Carried out the experiment. WES, NNA, AS, and MHF: Analysis of the data. WES, NNA, MH, HV, RSZ, NN, and AA: Drafted and revised the manuscript. All authors have read and approved the final manuscript.

CONFLICT OF INTEREST

There is no conflict of interest of this research.

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