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Population Dynamics and Molecular Identification of *Vibrio* spp. on Rearing Water of Olive Ridley Turtles (*Lepidochelys olivacea*) at Turtle Conservation and Education Center (TCEC) Serangan Bali

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ABSTRACT

Bacteria from the Genus *Vibrio* are among those that can contaminate turtle rearing water. These bacteria are categorized as opportunistic pathogens affecting aquatic organisms. Olive ridley turtles rescued from the wild are susceptible to infection by pathogenic bacteria due to wounds on their body surface. This study aimed to assess the population dynamics and molecular identification of *Vibrio spp.* in the rearing water of olive ridley sea turtles (*Lepidochelys olivacea*) at TCEC Serangan Bali. Rearing water samples were collected using a composite sampling method from three different rearing water. Bacterial enumeration, including total bacterial count (TBC), total Vibrio count (TVC), total yellow colony (TYC), and total green colony (TGC), was conducted in the olive ridley sea turtle rearing water on days 0, 15, and 30. Serial dilution and the plate count method were used to determine the number of colonies that developed. Bacterial isolates of *Vibrio spp.* that successfully grew were confirmed by molecular identification using PCR. The TBC in the rearing water fluctuated during the study period. The highest TBC value was 63.02 log CFU/mL on day 15 in rearing water 2, and the lowest TBC value was 4.30 log CFU/mL on day 30 in rearing water 3. The highest TVC value was 43.27 log CFU/mL on day 15 in rearing water 2, and the lowest was 9.37 log CFU/mL on day 0 in rearing water 2. The highest TGC value was 7.64 log CFU/mL in rearing water 1 on day 0, and the lowest was 4.77 log CFU/mL in rearing water 3 on day 30. The highest TYC result was 38.08 log CFU/mL in rearing water 2 on day 15, and the lowest was 4.39 log CFU/mL in rearing water 1 on day 0. Electrophoresis showed that the 16S rRNA gene in *Vibrio* bacterial DNA was successfully amplified and indicated by a band of approximately 1416 bp. One bacterial isolate, *Vibrio* parahaemolyticus, was identified from this study.

Keywords: *Olive ridley turtles, Vibrio parahaemolyticus, zoonosis.*

ABSTRAK

Salah satu bakteri yang dapat mencemari air kolam penyu yaitu bakteri dari Genus *Vibrio* yang termasuk dalam patogen oportunistik biota perairan. Penyu lekang yang merupakan hasil penyelamatan di alam liar memiliki kerentanan terinfeksi bakteri patogen karena munculnya perlukaan pada permukaan tubuhnya. Tujuan dari penelitian ini adalah untuk mengetahui dinamika populasi dan identifikasi molekuler *Vibrio* spp pada air pemeliharaan Penyu Lekang (*Lepidochelys olivacea*) di TCEC Serangan Bali. Pengambilan sampel air dilakukan secara komposit pada tiga kolam yang berbeda. Pengukuran kelimpahan bakteri meliputi total bacterial count (TBC), Total *Vibrio* Count (TVC), Total yellow colony (TYC), dan total green colony (TGC) pada media pemeliharaan penyu lekang yang dilakukan tiga kali (hari ke-0, 15, dan 30) dengan pengenceran berseri metode cawan sebar untuk dihitung koloni yang tumbuh. Isolat bakteri *Vibrio spp.* yang berhasil tumbuh dikonfirmasi jenisnya secara molekuler dengan metode PCR. Hasil penelitian menunjukkan nilai TBC pada air kolam pemeliharaan penyu lekang selama penelitian berfluktuasi. TBC tertinggi yaitu 63,02 log CFU/ml pada hari ke-15 di kolam 2 dan TBC terendah yaitu 4,30 log CFU/mL pada hari ke-30 di kolam 3. Nilai TVC tertinggi yaitu 43,27 log CFU/mL pada hari ke-15 di kolam 2 dan terendah yaitu 9,37 log CFU/ mL pada hari ke-0 di kolam 2. Hasil TGC tertinggi yaitu 7,64 log CFU/mL pada kolam 1 hari ke-0 dan terendah yaitu 4,77 log CFU/ mL pada kolam 3 hari ke-30. Hasil TYC tertinggi yaitu 38,08 log CFU/mL pada kolam 2 hari ke-15 dan terendah yaitu 4,39 log CFU/ mL pada kolam 1 hari ke-0. Hasil elektroforesis menunjukkan bahwa gen 16S rRNA pada DNA bakteri *Vibrio* berhasil diamplifikasi dan ditunjukkan dengan pita yang berukuran kurang lebih 1416 bp. Teridentifikasi satu isolat bakteri *Vibrio parahaemolyticus* dari hasil penelitian ini.

Kata kunci: Penyu lekang, *Vibrio parahaemolyticus*, zoonosis.

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INTRODUCTION

Sea turtles are crucial for maintaining the balance of marine ecosystems, contributing to productive coral reef habitats and facilitating the transport of nutrients from the sea to the coastline (Cazabon-Mannette et al., 2017). However, the global population of sea turtles has declined due to various factors, including human activity. The number of sea turtles is now declining across their entire natural range. Six out of seven species are classified as endangered or critically endangered according to the IUCN Red List (International Union for Conservation of Nature) (Beber et al., 2024). Various direct and indirect effects have contributed to the decline in sea turtle populations, including bycatch, large-scale coastal development, illegal hunting of nesting female turtles, illegal egg collection, and climate change (Biddiscombe et al., 2020; Pelupessy et al., 2021; Pheasey et al., 2021).

Wild sea turtles frequently face death or injury from entanglement in human traps or from diseases (Radulski et al., 2023). Infectious diseases and parasites significantly affect the health of sea turtles, potentially leading to population declines both in the wild and captive breeding programs (Warwick et al., 2013; Mashkour et al., 2020; Milotic et al., 2020). Bacteria in aquatic environments may act as primary or secondary pathogens (Oberbeckmann et al., 2018; Sooriyakumar et al., 2022; Zhong et al., 2023). Many of these bacteria have the potential to infect various animals, including humans. Consequently, both direct and indirect human interactions with sea turtles and their ecosystems may result in One Health issues (Mashkour et al., 2020).

The Turtle Conservation and Education Center (TCEC), located on Serangan Island, Denpasar, is one of the breeding centers in Bali (Pelupessy et al., 2021). *Ex-situ* turtle conservation is an effort aimed at preventing the loss of turtle ecosystems and commercial exploitation of turtles for their eggs, meat, or shells. *Ex-situ* conservation can also be used to educate the general public on the importance of sea turtle conservation, aiming to protect their habitats in Indonesia and avert extinction (Barik et al., 2023; Robinson et al., 2023; Virgili et al., 2024).

The olive ridley sea turtle (*Lepidochelys olivacea*) is one of the sea turtle species kept by TCEC. A common problem faced by olive ridley sea turtles is that they are found injured on the coast or entangled in fishing nets. These injuries pose a risk to the health of the turtles in captivity.

The quality of rearing water in the pool is a significant factor influencing the survival of sea turtles in captivity. Regular management and monitoring of rearing water quality are essential to prevent excessive bacterial growth and ensure the health of the turtles. Contaminated rearing water poses a significant threat to the health and survival of the resident sea turtles. Similarly, opportunistic pathogenic bacteria such as *Vibrio spp.*, commonly found in marine life rearing water, pose a significant concern.

Isolates of *Escherichia coli* and *Vibrio parahaemolyticus* were characterized and tested for antibiotic sensitivity from the oral cavity and cloaca of the olive ridley sea turtle in the Gulf of California. The findings indicated 100% bacterial resistance to antibiotics and the presence of multidrug-resistant (MDR) and extensively drug-resistant (XDR) strains (Jorge et al., 2023). A subsequent study reported the presence of 523 gram-negative *Enterobacteriaceae* isolates from seven sea turtle species, indicating the presence of *V. fluvialis* and *Burkholderia cepacia* with zoonotic properties (Resendiz and Fernandez-Sanz 2021). Currently, reports on the monitoring of rearing water quality in captive breeding facilities for the olive ridley sea turtle are scarce.

This study focused on the isolation and molecular identification of *Vibrio spp.* isolates from olive ridley sea turtle rearing water at the TCEC in Serangan, Bali. Monitoring the abundance of *Vibrio spp.* is essential for mitigating diseases caused by secondary infections from isolates and for implementing health management strategies for olive ridley sea turtles in rearing water.

MATERIALS AND METHOD

Preparation of Thiosulfate Citrate Bile Salt Sucrose (TCBS) Medium

A total of 88 grams of TCBS was added to the Erlenmeyer flask. Subsequently, 1000 mL of distilled water was added and heated. The 500-ppm concentration medium solution was homogenized at 90°C using a hot plate and stirrer bar for 5 minutes until homogenous. The TCBS medium solution was sterilized in an autoclave at 121°C for 15 minutes.

Preparation of Tryptone Soya Agar (TSA) Medium

Forty grams of TSA were placed in an Erlenmeyer flask and added with 1000 mL of distilled water. The 500-ppm concentration medium solution was homogenized at 90°C using a hot plate and stirrer bar for 5 minutes until homogenous. The TSA medium solution was sterilized using an autoclave at 121°C for 15 minutes.

Isolation

Rearing water from the pool was pipetted using a micropipette aseptically to a volume of 1 mL and transferred to a 10-1 reaction tube containing 9 mL of sterile distilled water. Homogenization was then performed using a vortex. From the 10-1 reaction tube, 1 mL of the solution was transferred to a 10-2 reaction tube containing 9 mL of sterile distilled water. The solution was then homogenized until thoroughly mixed. Next, 1 mL was taken from the 10-2 test tube and transferred into a 10-3 test tube containing 9 mL of sterile distilled water and homogenized. Then, 1 mL was taken from tube 10-3 and added to test tube 10-4, which already contained 9 mL of sterile distilled water, and homogenized. Next, 1 mL was taken from tube 10-4 and added to test tube 10-5, which already contained 9 mL of sterile distilled water, and homogenized. About 1 mL of sample from tube 10-1 was put in a Petri dish. TCBS and TSA media that have been cooled were added and left until the media froze. This procedure was also repeated for tubes 10-2 to 10-5. The culture in Petri dishes was incubated upside down for 24 hours at a temperature of 30°C for further purification and identification.

Purification Streak for Singles

The inoculating needle was heated and cooled over a Bunsen burner. Then, one bacterial inoculum was taken and streaked onto the surface of TCBS and TSA agar media. Streaking began at one end of the Petri dish. The inoculation needle was heated and cooled before streaking for the next quadrant. Incubation was carried out at 30°C for 48 hours, and growth was observed (Lay and Jutono, 2016).

Gram Staining

Each bacterial smear was fixed with heat. The slide was placed in a staining tray, and Crystal Violet stain was dropped or poured onto the specimen. The staining procedure was completed by allowing it to settle for one minute. Then, the specimen was carefully washed with running water. Lugol's solution was added and allowed to stand for one minute before being rinsed with running water. 96% alcohol was poured into the slide until the purple color disappeared. It was then rinsed with running water. Safranin was added and allowed to stand for two minutes before being rinsed with running water and dried with tissue. The staining was complete and observed under a microscope.

Electrophoresis of PCR Products (Polymerase Chain Reaction)

Conventional PCR worked by amplifying specific DNA fragments in several cycles. The stages of the process are as follows:

1. The denaturation reaction aimed to create single-stranded DNA by breaking the hydrogen bonds in DNA. This reaction took place at high temperatures, between 95°C and 97°C (Mulado, 2010).
2. The annealing reaction in this stage began when the primer moved to a specific area complementary to the primer sequence—hydrogen bonds formed between the primer and the complementary sequence on the template (Gaffar, 2007).
3. Elongation occurred at 72°C, which was the ideal temperature for Taq polymerase. By adding dNTP, the previously attached primer was extended on its 3' end (Gaffar, 2007).

PCR amplification was performed using the 16S rRNA gene (Kai et al., 2019). Using a sterilized toothpick, a single bacterial colony was removed from a Petri dish and placed at the bottom of a tube. Then, 17.5 µL of PCR mix, 1.4 µL of forward primer, 1.4 µL of reverse primer, and 13.7 µL of nucleic acid-free water were added. PCR amplification was performed using a thermal cycler, with initial denaturation at 94°C for 5 minutes, followed by 30 cycles of denaturation at 94°C for 1 minute, annealing at 58°C for 1 minute, extension at 72°C for 2 minutes, and final extension at 72°C for 10 minutes.

DNA quality testing was performed using electrophoresis. The first step was to prepare an agarose gel. TAE buffer 1x was diluted by mixing 50 mL of TAE buffer 10x with 450 mL of distilled water. A 1% agarose gel was prepared by dissolving 0.3 g of agarose in 30 mL of TAE buffer 1x. The agarose solution was homogenized and poured into an agarose gel mold, then allowed to set. Once the gel had hardened, 5 µL of the DNA sample was added to the wells. Electrophoresis was performed at 100 V for 30 minutes. The electrophoresis results were documented using a Gel Doc.

Data Analysis

TBC, TGC, and TYC data were tabulated using Microsoft Excel. CFU/mL colony data were transformed into log values and shown in tables and graphs. Results of molecular, macroscopic, and microscopic identification were descriptively displayed as pictures. Sequencing data were written in text format and analyzed using alignment software with Molecular Evolutionary Genetic Analysis Software version 10 or MEGA X (Tamura et al., 2007).

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RESULTS

Total Bacterial Count

Bacterial abundance parameters were calculated to distinguish bacterial abundance in each pool on different days. Table 1 presents the total bacterial count (TBC) data in the rearing water of the olive ridley sea turtle rearing water on days 0, 15, and 30.

Table 1. Prevalence of TBC

Day	Rearing water 1	Rearing water 2	Rearing water 3
	TBC (Log CFU/mL)		
Day - 0	5.24	4.31	5.03
Day - 15	6.88	63.02	18.71
Day - 30	6.97	4.94	4.30

Note:
TBC: Total Bacterial Count

On days 0, 15, and 30, the TBC in rearing water 1 rose to 5.24, 6.88, and 6.97 log CFU/mL. On days 0, 15, and 30, the TBC in rearing water 2 increased to 4.31, 63.02, and 4.94 log CFU/mL, respectively. On days 0 and 15, the TBC in rearing water 3 rose, and on day 30, they fell, registering 5.03, 18.71, and 4.30 log CFU/mL, respectively. TPC on TSA media was used to calculate the TBC present in the water samples from the olive ridley sea turtle pool.

The TBC in the olive ridley sea turtle breeding pool water fluctuated during the study. The highest TBC was 63.02 log CFU/mL, and the lowest total bacteria was 4.30 log CFU/mL.

Total Vibrio Count (TVC), Total Yellow Colony (TYC), and Total Green Colony (TGC)

The TVC of *Vibrio spp.* bacteria was determined by counting all bacterial colonies that grew on TCBS medium, including both yellow and green colonies. The TVC in rearing water 1 decreased from 10.94 CFU/mL on day 0 to 10.05 log CFU/mL on day 30. The TVC in rearing water 2 decreased on day 0 (9.37 CFU. mL), increased on day 15 (43.27 CFU/mL), and then decreased once more on day 30 (10.74 log CFU/mL). The TVC in rearing water 3 on day 0 decreased (11.12 CFU/mL), then increased on day 15 (13.01 CFU/mL) and decreased again on day 30 (11.16 log CFU/mL).

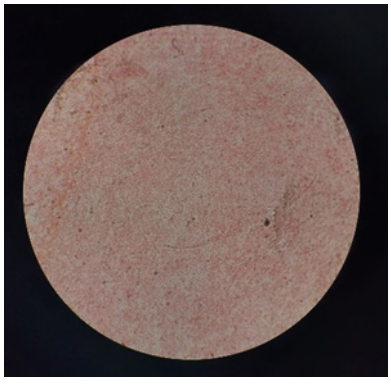
Total Yellow *Vibrio* (TYV) in rearing water 1 on day 0 decreased (4.39 CFU/mL), then increased on day 15 (5.16 log CFU/mL) and day 30 (5.01 log CFU/mL). TYV in rearing water 2 decreased on day 0 (4.41 log CFU/mL), then increased on day 15 (38.08 log CFU/mL) and decreased again on day 30 (5.37 log CFU/mL). TYV in rearing water 3 decreased on day 0 (4.61 log CFU/mL), increased on day 15 (7.64 log CFU/mL), and decreased again on day 30 (4.41 log CFU/mL).

Total Green *Vibrio* (TGV) in rearing water 1 on day 0 increased (6.55 log CFU/mL), then decreased on day 15 (5.37 log CFU/mL) and 30 (5.04 log CFU/mL). TGV in rearing water 2 decreased on day 0 (4.93 log CFU/mL) and increased on day 15 (5.19 log CFU/mL) and 30 (5.37 log CFU/mL). TGV in rearing water 3 increased on day 0 (6.51 log CFU/mL) and decreased again on days 15 (5.37 log CFU/mL) and 30 (4.77 log CFU/mL). The TVC of water samples from olive ridley sea turtle rearing water was measured using TPC on TCBS media. TVC in the olive ridley sea turtle breeding pool water fluctuated during the study. The highest TVC was 43.27 log CFU/mL, and the lowest was 9.37 log CFU/mL.

Macroscopic and Microscopic Observation of Vibrio spp. isolates

Table 2 presents the results of *Vibrio* bacteria isolation from olive ridley sea turtle rearing water, both macroscopically and microscopically. In rearing water 1, *Vibrio spp.* colonies were successfully purified.

Table 2. Macroscopic and Microscopic Results

Macroscopic	Microscopic
	

Macroscopic observation revealed yellow and green colonies with a circular morphology, thin margins, and a smooth, mucoid colony surface. Microscopic observation revealed *Vibrio spp.* with curved rod-shaped (bacilli) morphology that stained well with the applied dye.

In rearing water 2, the *Vibrio spp.* colony that was successfully purified, macroscopically, the yellow and green colonies exhibited round and oval shapes with thin margins and a smooth, mucoid surface characteristic of *Vibrio spp.* Microscopically, *Vibrio spp.* appeared as curved rod-shaped (bacilli) bacteria that stained well with the applied dye.

In rearing water 3, the purified colonies of *Vibrio spp.* were macroscopically observed as yellow and green colonies with an oval shape, thin margins, and a smooth, mucoid surface. Microscopically, *Vibrio spp.* appeared as curved rod-shaped (bacilli) bacteria that stained well with the applied dye.

Results of PCR Amplification

The *Vibrio* species successfully identified from the water sample of the olive ridley sea turtle breeding pool were identified molecularly. The first step in molecular identification was the amplification of the 16S rRNA gene in *Vibrio* bacterial DNA. Electrophoresis was used to visualize the amplification findings, which showed green bands. These results indicate that the 16S rRNA gene in *Vibrio* bacterial DNA was successfully amplified, as shown in bands measuring approximately 1416 bp.

The green colonies in rearing water 1 and 3 had a 94% and 95% similarity percentage to the bacteria *Vibrio parahaemolyticus*, respectively, according to sequencing results compared with reference material in GenBank using the BLAST (Basic Local Alignment Search Tool) tool. This suggests that the isolate and the bacterium *Vibrio parahaemolyticus* have a similar basis, proving that the isolate is the native species of *Vibrio parahaemolyticus*. The yellow colony from rearing water 1, the green colony, and the yellow colony from rearing water 2 had a similarity percentage of 86% and 91% to the bacterium *Staphylococcus nepalensis*. The yellow colony in sample rearing water 3 had a similarity percentage of 84% to the bacterium *Staphylococcus lentus*. Six samples (YC1, GC1, YC2, GC2, YC3, and GC3) tested positive for *Vibrio spp.* bacteria. Samples YC1 and GC1 were from rearing water 1, samples YC2 and GC2 were from rearing water 2, and samples YC3 and GC3 were from rearing water 3. This can be seen from the electrophoresis results, which show six sample bands aligned with the positive marker (1416 bp).

Construction of a Phylogenetic Tree

BLAST similarity identification results indicated that *Vibrio parahaemolyticus* was the bacteria in the green colonies of rearing water 1 and 3. *Staphylococcus nepalensis* was the bacterium found in the yellow colony of rearing water 1, the green colony of rearing water 2, and the yellow colony of rearing water 2. The yellow colony of bacteria in rearing water 3 was *Staphylococcus lentus*. *Salmonella enterica*, *Bactrocera aeroginosa*, and *Aeromonas hydrophila* were included as outgroups in the phylogenetic tree.

According to the phylogenetic tree, *Vibrio parahaemolyticus* was the green colony in the bacterial samples from rearing water 1 and 3. The sea and estuaries are home to the curved or comma-shaped Gram-negative bacterium *Vibrio parahaemolyticus*. *Staphylococcus nepalensis* was the bacterium found in the rearing water 1 yellow colony, the rearing water 2 green colony, and the rearing water 2 yellow colony. *Staphylococcus lentus* was found in the rearing water 3 yellow colony.

These bacteria are facultative anaerobes, capable of surviving in both the presence and absence of oxygen. The ideal temperature for growth is 30°C. The culture medium utilized is a standard medium, specifically TSA.

DISCUSSION

Total Bacterial Count

The highest TBC was 63.02 log CFU/mL, and the lowest was 4.30 log CFU/mL (Table 3). These values are higher than those reported by Mangampa (2015), who found TBC in shrimp pool sediments ranging from 5 to 6 log CFU/g. Sabu et al. (2020) reported that TBC in vaname shrimp pool sediments could reach 10 log CFU/g. Fluctuations in TBC result from organic matter and bacterial competition, leading to an increase in bacterial populations. A decrease in TBC occurs when the available nutrients and energy in the pool are sufficiently to meet the nutritional needs of the existing TBC bacteria. Conversely, if nutrient levels are inadequate, a decline in the bacterial population is observed (Dahlan et al., 2017).

Total Vibrio Count (TVC), Total Yellow Colony (TYC) and Total Green Colony (TGC)

Several intrinsic and extrinsic variables, including work methods or application processes, as well as implicit influences, influence the growth of *Vibrio* bacteria at a specific location. Inherent characteristics encompass pH, water activity, oxidation-reduction

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capacity, nutrient content, materials containing bacteria, and the structural composition of food components at a specific location. Extrinsic elements influencing the development of *Vibrio* bacteria include storage conditions or temperature, humidity, gas pressure (O2), light, and UV light intensity in the area (Montieri et al., 2010).

The variation in TVC is believed to result from organic matter and bacterial competition, leading to an increase in the TVC population. The decline in TVC arises from the limited nutrients and energy present in the pool sediment, which can only satisfy the nutritional requirements of the current TVC bacteria. Consequently, if the nutrient levels are insufficient, a population decline will occur (Dahlan et al., 2017).

Table 3. Prevalence of TVC, TYC and TGC

Day	Rearing water 1	Rearing water 2	Rearing water 3
	TVC (Log CFU/mL)		
Day - 0	10.94	9.37	11.12
Day - 15	10.53	43.27	13.01
Day - 30	10.05	10.74	11.16
	TYC (Log CFU/mL)		
Day - 0	4.39	4.41	4.61
Day - 15	5.16	38.08	7.64
Day - 30	5.01	5.37	6.39
	TGC (Log CFU/mL)		
Day - 0	6.55	4.96	6.51
Day - 15	5.37	5.19	5.37
Day - 30	5.04	5.37	4.77

Note:
TVC: Total Vibrio Count
TYC: Total Yellow Colony
TGC: Total Green Colony

Macroscopic and microscopic observation of Vibrio spp. isolates

This study's bacterial isolation revealed two types of *Vibrio spp.* colonies: green and yellow (Figure 1). Green colonies represent *Vibrio* species that are unable to ferment sucrose, whereas yellow colonies can ferment sucrose and subsequently lower the pH in TCBS medium (Ihsan & Retnaningrum, 2017). Rahmanto et al. (2014) identified the type of *Vibrio* by analyzing the color of the colonies formed. Green *Vibrio* colonies cultivated on TCBS medium exhibited 86% similarity with *Vibrio* fishery and *Vibrio mimicus*, whereas yellow colonies demonstrated 90–95% similarity with *Vibrio alginolyticus* and *Vibrio harveyi*.

Vibrio spp. bacteria exhibit robust growth on TCBS media, producing yellow and green colonies. *Vibrio spp.* can proliferate on TCBS medium, as shown by Oliver and Kepper (2001), who identified TCBS as a selective and differential medium for isolating *Vibrio spp.* bacteria, including *Vibrio cholerae*, *Vibrio parahaemolyticus*, and *Vibrio vulnificus*. This medium contains bile salts that inhibit non-target bacterial growth, sodium chloride suitable for halophilic organisms, sodium trisulfate as a sulfur source, and iron citrate for detecting H_2S formation. *Vibrio spp.* are Gram-negative bacteria with a single-celled, small, curved or straight body measuring 1.4-5.0 µm in length (Bintari et al., 2016). The bacteria do not possess capsules or spores, and their cell walls change with age or culture (Nur, 2019). Gram staining was employed to examine the morphology of *Vibrio spp.* bacteria. *Vibrio* colonies were observed under a microscope, growing diffusely on the surface of a curved glass slide, exhibiting a comma-shaped morphology, and forming distinct colonies. The color observed under the microscope is red, indicating that *Vibrio* is a Gram-negative bacterium. According to Campbell et al. (2011), Gram-negative bacteria contain

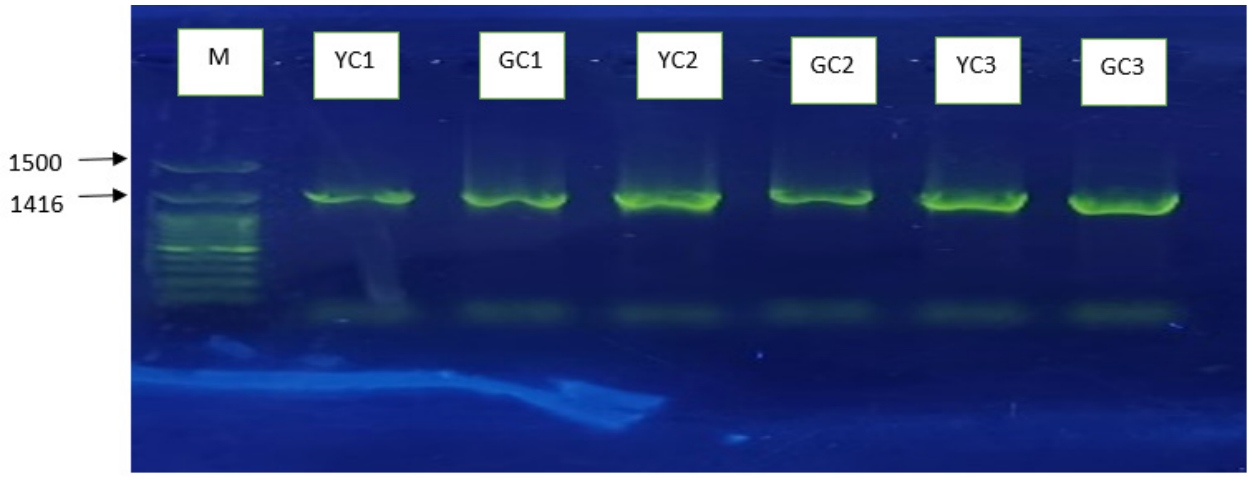

Figure 1. Results of electrophoresis

little peptidoglycan and have a complex cell wall structure.

Results of PCR Amplification

The 16S rRNA gene constitutes the small subunit of the ribosome and is utilized as a molecular marker for bacterial identification, with a total length of approximately 1500 bp. Johnson et al. (2019) employed the 16S rRNA gene to examine the microbiome at both the species and strain levels. This molecular marker is utilized for the identification of pathogenic bacteria and is a key parameter in the determination of new bacterial species (Srinivasan et al., 2015; Vetrovsky and Baldrian, 2013). The 16S rRNA gene measures approximately 1,500 to 1,550 bp in length and is characterized by a high content of guanine and cytosine (G+C) nitrogen bases. This gene contains a conserved region typically measuring 500-540 bp, as reported by Brown (1992).

Numerous studies in marine and fisheries science have utilized the 16S rRNA gene. Research in this field primarily seeks to identify bacteria that cause diseases and produce metabolites suitable for reuse across various domains. According to the study by Ramadhan et al. (2016), the identified bacteria are symbiotic with several macroalgae and can produce cellulase enzymes. These enzymes occur frequently in nature and are applicable in processes such as bioethanol production.

Construction of a Phylogenetic Tree

The similarity values presented in Table 4 indicate that a higher similarity value between samples Corre spools to a greater nucleotide similarity, suggesting a closer relationship (Sujaya et al., 2016). Figure 2 displays the results of identification derived from BLAST similarity. The green colonies from sample rearing water 1 and rearing water 3 exhibit similarities to the bacterium *Vibrio parahaemolyticus*. Bacterial samples of yellow colonies from rearing water 1, green colonies from rearing water 2, and yellow colonies from rearing water 2 exhibit similarities to the bacterium *Staphylococcus nepalensis*. In contrast, the yellow colony from rearing water 3 shows similarity to the bacterium *Staphylococcus lentus*.

The initial bioinformatics analysis for identifying bacterial species at the molecular level involved comparing the similarity of 16S rRNA genes from the sample to reference sequences in the NCBI GenBank database (National Center for Biotechnology Information). This also served as a foundation for determining bacterial species identification as either novel or identical species. The NCBI website offers storage and access to nucleotide and amino acid sequences contributed by various scientists, along with additional bioinformatics features. Bioinformatics through NCBI provides information related to genetic diversity in living organisms (Alifiansyah et al., 2024). The accuracy of similarity values can be confirmed in phylogenetic tree reconstruction. The application of genetic distances in a sample is essential for validating the results of similarity analyses and for supporting the construction of phylogenetic trees (Yan et al., 2019).

The rate of mutation in evolution can influence genetic distance (Aprilyanto and Sembiring 2016). *Vibrio parahaemolyticus* is a bacterium that naturally occurs in brackish and coastal waters environments. It is one of the pathogenic species of *Vibrio spp.* affecting both shrimp and humans (Ceccarelli, Hasan, Hug, & Colwell, 2013; DePaola Kaysner, Bowers, & Cook, 2000). This bacterium is naturally present in aquatic environments and is frequently found in shrimp farming environments (Abu & Egenonu, 2008; Chitov, Wongdao, Thatum, Puprae, & Sisuwan, 2009; Newton, Kendall, Vugia, Henao, & Maho, 2012). The

Table 4. Data on bacterial species successfully isolated

No	Isolate Name	GenBank Isloate	Acc No.	Similarity
1	Yellow Colony in Pool 1	*Staphylococcus sp*. strain MBTDCMFRIWC29	KU554486.1	86%
2	Green Colony in Pool 1	*Vibrio parahaemolyticus* strain V7 16S ribosomal RNA gene, complete sequence	EU636231.1	95%
3	Yellow Colony Pool 2	Uncultured bacterium clone C3_9 16S ribosomal RNA gene, partial sequence	KP142336.1	91%
4	Green Colony in Pool 2	Uncultured bacterium clone C3_9 16S ribosomal RNA gene, partial sequence	KP142336.1	91%
5	Yellow Colony in Pool 3	*Staphylococcus sp*. b10 partial 16S rRNA gene, isolate b10	LK985359.1	84%
6	Green Colony in Pool 3	*Vibrio parahaemolyticus* strain NSTH08 16S ribosomal RNA gene, partial sequence	KF886619.1	94%

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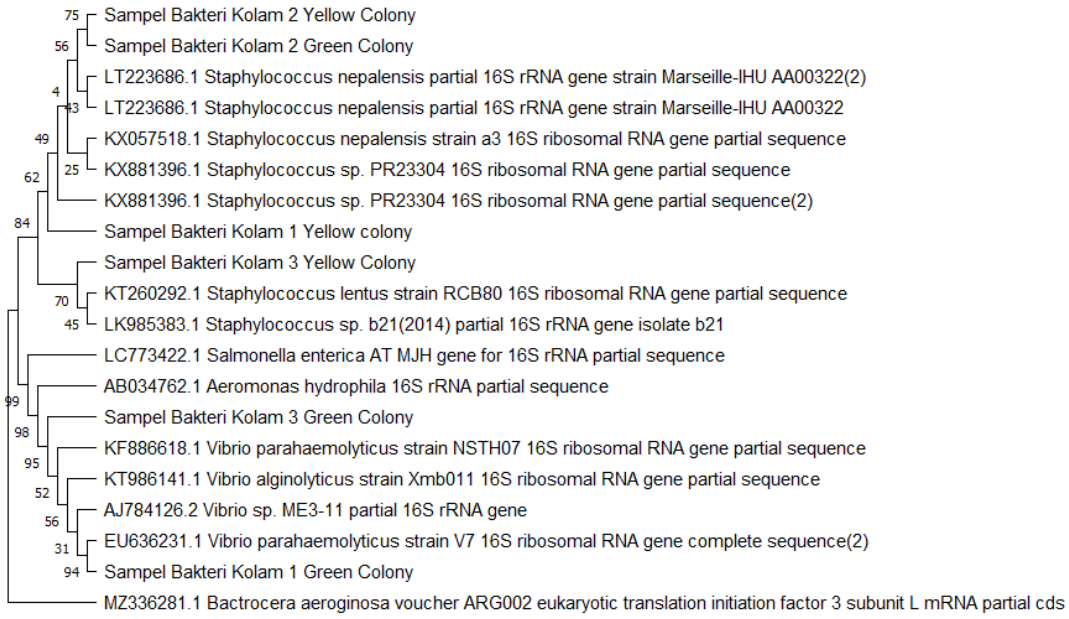

Figure 2. Phylogenetic tree constructed using MEGA11 software

natural habitat of *Staphylococcus* bacteria includes humans and animals. They are found on the skin, nose, and large intestine, particularly inhabiting external mucous membranes. However, these bacteria are often found in the environment (unprocessed water, soil, and contaminated objects) (Becker et al., 2014).

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