Isolation and Identification of *Vibrio parahaemolyticus* Bacteria in Bottlenose Dolphins (*Tursiop truncates*) in Kendal Conservation Pond, Central Java

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**1. Introduction**

Bottlenose dolphins are one of the aquatic animals whose existence is protected by law in Indonesia through PP Number: 7 of 1999 concerning the preservation of plant and animal species and Decree of the Minister of Marine Affairs and Fisheries Number: 79/KEPMEN-KP/2018 concerning the National Action Plan for Marine Mammal Conservation. One of these protection laws regulates information about marine mammal diseases. Disease agents in marine mammals can be bacteria, protozoa, helminths, and viruses.

Diseases caused by bacteria are often found in animals living in the water column, both cultured and wild aquatic animals. Molecular biology detection can determine the presence of disease in the host. Still, the presence of isolates of bacterial pathogens is important for more detailed characterization and identification of disease pathogens.

Bacterial consortia in marine waters are very rich and diverse, in which there is the potential for bacteria as disease control or otherwise pathogenic. In fish farming, some bacteria are known to be able to be pathogenic to their hosts. One of the pathogenic bacteria that is often associated with disease is bacteria of the *Vibrio* type, which is a normal flora in seawater and, under certain conditions, causes disease attacks in both fish and shrimp aquaculture (Mohamad *et al.* 2019; De Souza Valente and Wan 2021).

Information on diseases caused by bacterial pathogens that attack dolphins as protected animals still needs to be improved (Field 2022). *Vibrio* in aquatic mammals of the species *Vibrio vulnificus* was found to cause septicemia and death in spotted seals (Li *et al.* 2018). In addition, as organisms of the same class, it is feared that pathogens in mammals are also dangerous to humans. There have been no reports of...
disease in dolphins caused by vibrio bacteria, as is the case with fish and shrimp. However, it does not rule out the possibility that vibrio bacteria can infect dolphins and cause diseases that are different from fish and shrimp.

*Vibrio parahaemolyticus* is also known to cause disease in humans that can be spread through seafood consumption (Beshiru and Igbinosa 2023). Although aquatic mammals are protected animals that should not be consumed, the presence of zoonotic bacteria is very important information to minimize the risk of disease spread through water.

Therefore, research is needed to determine the types of bacteria that can become pathogenic agents for aquatic mammals. Therefore, this study was conducted to obtain evidence of the existence of pathogenic bacteria in the body of healthy aquatic mammals that do not show symptoms of disease either due to the presence of *Vibrio* bacteria or other pathogens so that it can be used as an early warning of possible disease transmission from aquatic mammals to humans.

2. Materials and Methods

2.1. Sampling and Bacterial Isolation Procedure

A total of 5 marine mammals in a conservation institution located in Kendal Regency, Central Java, were used as research subjects. The sampling was done using a non-lethal sampling procedure. Samples were collected from the external organs, including the mouth, blowhole, anal and reproductive organs of the subject mammals by swabbing process (Coutinho et al. 2023). The swabbed samples were stored in sterile phosphate buffer saline (PBS) at a cool temperature during transport (Frosth and Lewerin 2019).

A total of 10 µl of PBS solution was cultured on Tryptic Soy Broth (TSB, Merck) supplemented with 0.85% Sodium Chloride (NaCl, Oxoid) and incubated at 30°C overnight. A loopfull suspension was cultured on Tryptic Soy Agar (TSA, Oxoid) medium that was added with 0.85% NaCl and incubated again overnight at 30°C. Afterward, one loopfull of the growing bacteria was re-cultured using the quadrant streak method on selective MacConkey agar media (Oxoid) and 0.85% NaCl. All re-cultured bacteria were incubated overnight at 30°C. The quadrant-plate procedure is designed to isolate pure cultures of bacteria, or colonies, from mixed populations by simple mechanical separation. Single colonies are comprised of millions of cells growing in a cluster on or within an agar plate (Sanders 2012). Different colonies were taken using a loop inoculation and re-cultured on the identical selective media; the re-culture process was repeated until homogeneous colonies were obtained in one Petri dish. The quadrant process was carried out up to 3 times to obtain homogeneous bacterial colonies (Al-blooshi et al. 2021). For the biochemical process for identification purposes, a set of observations was done following Benson (2002).

2.2. Identification Procedure

Single colonies taken from the third quadrant were then re-cultured on a common medium, Tryptic Soy Agar (TSA, Oxoid), for later identification using the maldi-tof method, which was carried out by following the standard protocol for pre-treatment sample microbe reagents (Zybio). For confirmation of identification, two full loops of bacteria from TSA media were extracted using reagents from Promega following the available protocol with minor modifications. The resulting DNA extract was then amplified using the 16SrRNA gene target sequence from Marchesi et al. (1998) and sequenced.

2.3. Antibiotic and Lysis Test on Blood Agar

The antibiotic test was performed by the Kirby-Bauer Disk Diffusion Assay method using eight antibiotics: meropenem, trimethoprim, chloramphenicol, ciprofloxacin, tetracycline, streptomycin, cephalothin, and penicillin. The reaction of isolates to antibiotics is described as Susceptible (S), Intermediate (I), or Resistant (R) according to CSLI from document M45 3rd Edition. The Multiple Antibiotic Resistance (MAR) Index value was calculated based on the formula: number of antibiotic-resistant isolates/total number of antibiotics used, with a value of 0.2 as the high-risk limit of the contamination source (Davis and Brown 2016; Ayandele et al. 2020). The Kanagawa phenomenon test was used to analyze the ability of bacteria to lyse blood on the media.

2.4. Ethical Clearance

This study was evaluated and approved by the Animal Ethics Committee of the Health Research Ethics Committee - National Research and Innovation Agency. Animal studies were performed in strict
To verify the identification results using the malditof method, the same isolate was then extracted and amplified using universal 16S rRNA primers. The results obtained were positive bands at a base size of 1,360 bp (Figure 2). The sequencing results were then matched with the base sequence contained in the National Centre for Biotechnology Information (NCBI) page. The results showed that there was a similarity of 98% with the species *V. parahaemolyticus* (EU155529.1). The nucleotide similarity of the two base sequences can be seen in Figure 3.

**V. parahaemolyticus** isolates obtained from dolphins have the highest similarity with *V. parahaemolyticus* isolated from Adriatic seawater (EU155529.1). Data at NCBI showed several isolates isolated from shellfish, seawater, aquaculture water, shrimp, and fish, which showed similarities of 98% when compared to *V. parahemolyticus* isolated from mammals, so one cluster was formed. However, isolates from dolphins have the closest distance to isolates from seawater (Figure 4). These results suggest that it is likely that the bacteria isolated from dolphins are the same species as bacteria that cause disease in humans spread through seafood. Moreover, the isolated *V. parahaemolyticus* also presented a hemolysis activity when it was tested using the Kanagawa phenomenon test (Figure 5).

**3. Results**

From the observation, the colony shape of isolate *V. parahaemolyticus* is round, with filiform growth type on inclined agar, convex elevation, and an entire margin type. Gram staining results showed that *V. parahaemolyticus* isolates were Gram-negative and short rod-shaped bacterial cells (Figure 1). The result of the isolate identification obtained from the isolation process is 2.24, which states that the isolate is *V. parahaemolyticus* species (Table 1).

![Image of bacterial colonies](image1.png)

**Figure 1.** (A) Isolation of bacteria on GSP selective media, (B) pure isolates of target bacteria on TSA general media, (C) single colonies of target bacteria on TSA general media, (D) gram stain results

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<thead>
<tr>
<th>Organism</th>
<th>Strain</th>
<th>Score</th>
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<tr>
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**Table 1.** Results of bacterial identification with the malditof method based on data stored in the library

![Image of PCR result](image2.png)

**Figure 2.** PCR result from the sample of *V. parahaemolyticus* isolated from the dolphin, M: Marker; 1: Sample, 2: Pos control, 3: Neg Control
Figure 3. Alignment of *V. parahemolyticus* sequenced sample with *V. parahaemolyticus* species from NCBI (EU155529.1)
Antibiotic tests showed resistant conditions in 4 types of antibiotics, namely trimethoprim, streptomycin, cephalothin, and penicillin. Two types of antibiotics showed intermediate conditions, namely chloramphenicol and ciprofloxacin. Meanwhile, tetracycline and meropenem showed susceptible results (Figure 6). The result of the MAR index calculation = 0.5, which exceeds the high-risk limit value.

4. Discussion

Vibrio species are pathogenic bacteria in marine and brackish water that belong to the Gram-negative group. Vibrio is a normal flora in marine and brackish waters. One Vibrio species that can be isolated from all types of marine aquatic animals is V. parahaemolyticus. This species can be found in all fish, whether farmed or wild liar (Abdelaziz et al. 2017; Mohamad et al. 2019), crustaceans (De Souza Valente and Wan 2021), and elasmobranchs (Correia Costa et al. 2022).

In aquatic animals, Vibrio can generally result in Vibriosis disease characterized by clinical symptoms. Affected fish exhibit signs of fatigue, along with necrosis of the skin and appendages, resulting in deformities, stunted growth, liquefaction of internal organs, loss of vision, muscle opacity, and increased mortality rates (Ina-Salwany et al. 2019). The bacterium V. parahaemolyticus has been reported to be associated with meningoencephalitis in dolphins but has also been found in free-ranging healthy dolphins (Buck et al. 2006; Di Renzo et al. 2017). V. parahaemolyticus is a halophilic bacterium in marine ecosystems and several marine species (Liu 2011). Biofilm formation, antimicrobial resistance, salinity, temperature, pH, and aquatic biota influence the reproduction, survival, and adaptation to the environment as well as the distribution of V. parahaemolyticus in the aquatic environment, thereby making its eradication and control more difficult (Manjano-Mendoza et al. 2009, Martínez-
Urtaza et al. 2010). *V. parahaemolyticus* is a zoonotic bacterium capable of infecting humans and causing gastroenteritis. To date, *V. parahaemolyticus* bacteria have been reported to spread through food consumption (Raszl et al. 2016). According to Brooks et al. (2013), *V. parahaemolyticus* in humans causes acute gastroenteritis after consuming contaminated seafood with clinical signs such as nausea, vomiting, abdominal cramps, fever, and watery diarrhea after 12-24 hours. Wang et al. (2015) added pathological changes such as erosions of the jejunum and ileum, inflammation, and damage to several organs (liver, spleen, and lung). Therefore, the presence of *V. parahaemolyticus* in dolphins can transmit it to humans, considering that dolphins have the opportunity to contact humans. Opportunities for dolphins to contact with humans can be direct and indirect contact due to the dolphins being cared for in conservation institutions and on commercial exhibitions such as swimming with dolphins for recreational or therapeutic purposes, and because of professions such as veterinarian, marine biologist, paramedics who caring dolphins, rescue operations, and rehabilitation of stranded dolphins. The main virulence factors of *V. parahaemolyticus* that attack humans are direct thermostable hemolysin (tdh) and related hemolysin (trh) (Wang et al. 2015).

The isolation procedure used selective media with specific nutrients that support the growth of target bacteria and eliminate non-target bacteria. The primary purpose of selective media is to isolate specific strains of bacteria from a bacterial consortium (Bonnet et al. 2019). McConkey media is media that only cultivates negative bacteria, including vibrio species (Brennan-Krohn et al. 2016). The maldi-tof method (matrix-assisted laser desorption ionization-time of flight) is a new protein-based method for targeting bacteria, with the results in the form of a protein spectrum of the target bacteria. It compares with the protein spectrum of the dataset in the program. The reference value for species is >2.0. The protein spectrum match is then described in a table containing the probability of the detected species. Malditof is a reliable method of identifying unknown bacteria. The accuracy of the Malditof method is close to the accuracy of the DNA sequencing-based detection method, with higher accuracy than the detection method using Vitek 2 (Rudolph et al. 2019).

Wang et al. (2015) stated that the antibiotics that usually use for the therapy of *V. parahaemolyticus* infection are doxycycline, ciprofloxacin, or erythromycin. This research uses eight antibiotics with categories used in humans (meropenem, trimethoprim, chloramphenicol, ciprofloxacin, streptomycin, cephalothin, and penicillin), and only one antibiotic, namely tetracycline, usually used by aquaculture. The mechanism of antibiotics can be divided into two groups; the group with the mechanism of inhibiting cell wall synthesis is
carried out by antibiotic penicillin, meropenem, and cephalothin. In comparison, the second group inhibits the nucleic acid synthesis of bacteria, which is the mechanism of the antibiotic streptomycin, erythromycin, ciprofloxacin, trimethoprim, chloramphenicol, and tetracycline (Kapoor et al. 2017).

Meropenem, penicillin, and cephalothin are antibiotics of the same class, but meropenem showed different results compared to the other two antibiotics. Previous research also mentioned that V. parahaemolyticus isolated from mackerel showed resistance to penicillin but was sensitive to carbapenem (Tan et al. 2017). This is possible because of the difference in the structure of carbapenem from the other two groups (penicillin and cephalothin). In carbapenem, the sulfur-containing ring is replaced by a carbon atom, so it is thought that carbapenem has better antibiotic ability than penicillin and cephalothin (Meletis 2016; Feng et al. 2017; Aurilio et al. 2022).

Chloramphenicol is an antibiotic that has a mechanism that prevents protein synthesis by binding to the 50S subunit. The results of chloramphenicol are in accordance with previous research, which states that there is a high percentage of resistance in V. parahaemolyticus tested with chloramphenicol has a low percentage of resistance (Tan et al. 2017). Chloramphenicol has so far proven susceptible to V. parahaemolyticus and is even able to downregulate several virulence factors of V. parahaemolyticus (Sood 2016; Zhang et al. 2023).

Tetracycline and streptomycin have the same mechanism that inhibits protein synthesis by binding to the 30S subunit. However, the test results showed differences, where V. parahaemolyticus showed that it was susceptible to tetracycline and showed resistant results to streptomycin. The varied results of research on tetracycline susceptibility to V. parahaemolyticus bacteria indicate that the use of this antibiotic must be tightened so that cases of resistance can be reduced (Letchumanan et al. 2015; Kumarage et al. 2022). At the same time, streptomycin resistance cases were found in V. parahaemolyticus bacteria isolated from oysters and estuary water in previous studies (Jeamsripong et al. 2020). Resistance to the antibiotic streptomycin is known to be caused by the mechanism of gene mutation, enzyme activation, or efflux from bacteria (Lyu et al. 2019).

The results of the antibiotic susceptibility test on V. parahaemolyticus showed that there were cases of resistance to trimethoprim antibiotics. This is in accordance with previous research on antibiotic tests of isolates taken from shellfish and seawater (Jeamsripong et al. 2022). Trimethoprim is an antibiotic that inhibits the production of folate origin for bacterial cell growth (He et al. 2020). Resistance to trimethoprim antibiotics can be through several mechanisms, including transposons, the presence of impermeability, the presence of resistance genes in the Dihydrofolate reductase (DHFR) enzyme, the presence of excess DHFR enzyme production and alternative metabolic pathways (Wróbel et al. 2020).

Ciprofloxacin antibiotic is an antibiotic that inhibits the formation of DNA gyrase in bacteria. V. parahaemolyticus tested with ciprofloxacin showed intermediate cases as in previous research which showed intermediate case results on ciprofloxacin as much as 25% of the V. parahaemolyticus they tested (Tan et al. 2017). Although the results shown are not cases of resistance, the effectiveness of the antibiotic ciprofloxacin begins to decline in V. parahaemolyticus. Resistance to the antibiotic ciprofloxacin is thought to be due to bacterial mutations in the gyrA and parC genes (Zhou et al. 2019). The seven classes of antimicrobials identified in this research, apart from tetracycline, are classified by the World Health Organization as antimicrobials that are important for human medicine. There are two classifications related to antimicrobials for human medicine, namely levels—highly important and critically important antimicrobials. This makes it even more convincing that V. parahaemolyticus acquired its resistance properties, most likely due to the transfer of resistance genes between bacterial species in seawater. This can have an impact on the spread of resistance genes in other bacteria that are able to reach the aquatic environment and humans through, e.g. seafood, which has an impact on public health.

Hemolysin is one of the virulence factors in pathogenic bacteria, including V. parahaemolyticus. The presence of hemolysin activity can be known using the Kanagawa phenomenon (KP) test and PCR by detecting the tdh and trh genes. KP test is a method that is assisted by agar media, which is added with 5% blood (Sun et al. 2022). In this study, the KP test was used, with positive results and the
appearance of a clear zone around the culture area (Figure 5).

This study concluded that Vibrio bacteria are found in marine mammals but have not been found as disease-causing agents. The Vibrio bacteria found were haemolyzing and resistant to certain antibiotics. This can be an early warning system that you must be careful about when handling dolphins due to the presence of V. parahaemolyticus bacteria, which are zoonotic and potentially dangerous to humans.

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References


