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

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ABSTRACT

Aquatic mammals in Indonesia are officially protected. However, there is a lack of research on these mammals, particularly in relation to potential disease-causing bacteria. A study was conducted in a conservation pond to address this gap, where swab samples were taken from the blowholes of aquatic mammals. The focus was on identifying bacteria that could potentially cause infectious diseases in these animals. The results revealed *V. parahaemolyticus* bacterial isolates, which showed a 98% similarity to the registered *V. parahaemolyticus* in NCBI. These bacterial isolates exhibited hemolysin properties and demonstrated resistance to trimethoprim, streptomycin, cephalothin, and penicillin antibiotics.

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

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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. 0All 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

accordance with the Guidelines for the Care and Use of Animals in Research, which the National Research and Innovation Agency issues under registration number 075/KE.03/SK/07/2023.

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).

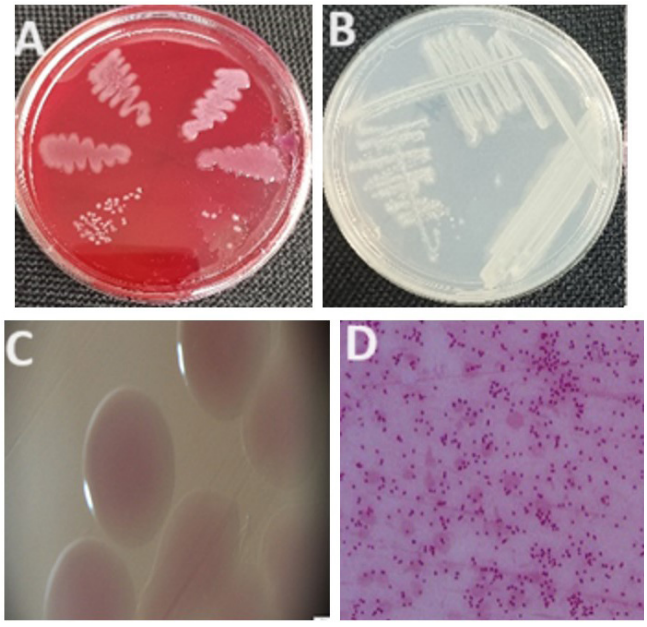

Figure 1. (A) Isolation of bacteria on GSP selective media, (B) pure isolates of target bacteria on TSA general media, (C) single colonies of target bacteria on TSA general media, (D) gram stain results

Table 1. Results of bacterial identification with the malditof method based on data stored in the library

Organism	Strain	Score
Vibrio parahaemolyticus	FBD 362018	2.24
Vibrio parahaemolyticus	FBD 361185	2.04
Vibrio parahaemolyticus	FBD 361360	1.90
Vibrio parahaemolyticus	FBD 364053	1.83
Vibrio parahaemolyticus	ZYAC 07190003A	1.73
Vibrio alginolyticus	FBD 363181	1.69
Vibrio parahaemolyticus	BJTR 07190002A	1.69
Vibrio harveyi	FBD 364067	1.66
Vibrio parahaemolyticus	FBD 362221	1.63
Vibrio parahaemolyticus	FBD 363775	1.61

To verify the identification results using the malditof method, the same isolate was then extracted and amplified using universal 16SrRNA 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).

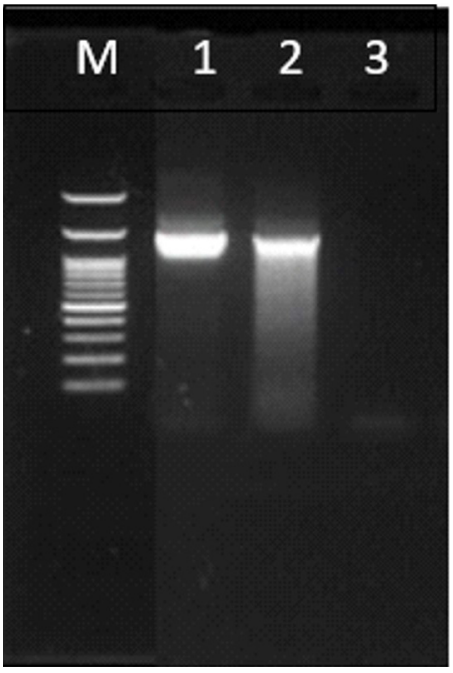

Figure 2. PCR result from the sample of *V. parahaemolyticus* isolated from the dolphin, M: Marker; 1: Sample, 2: Pos control, 3: Neg Control

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------------------------------------------------------------------------------
Score              Expect       Identities      Gaps            Strand
1367 bits(740)     0.0          766/779(98%)    1/779(0%)       Plus/Plus
------------------------------------------------------------------------------
Query  10   CTTC-GGNGACGATAACGGCGTCGAGCGGCGGACGGGTGAGTAATGCCTAGGAAATTGCC  68
            ||||  || ||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  36   CTTCGGGGGACGATAACGGCGTCGAGCGGCGGACGGGTGAGTAATGCCTAGGAAATTGCC  95

Query  69   CTGATGTGGGGGATAACCATTGGAAACGATGGCTAATACCGCATGATGCCTACGGGCCAA  128
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  96   CTGATGTGGGGGATAACCATTGGAAACGATGGCTAATACCGCATGATGCCTACGGGCCAA  155

Query  129  AGAGGGGGACCTTCGGGCCTCTCGCGTCAGGATATGCCTAGGTGGGATTAGCTAGTTGGT  188
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  156  AGAGGGGGACCTTCGGGCCTCTCGCGTCAGGATATGCCTAGGTGGGATTAGCTAGTTGGT  215

Query  189  GAGGTAAGGGCTCACCAAGGCGACGATCCCTAGCTGGTCTGAGAGGATGATCAGCCACAC  248
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  216  GAGGTAAGGGCTCACCAAGGCGACGATCCCTAGCTGGTCTGAGAGGATGATCAGCCACAC  275

Query  249  TGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATG  308
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  276  TGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATG  335

Query  309  GGCGCAAGCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCAC  368
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  336  GGCGCAAGCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCAC  395

Query  369  TTTCAGTCGTGAGGAAGGTGGTGTAGTTAATAGCTGCATTATTTGACGTTAGCGACAGAA  428
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Sbjct  396  TTTCAGTCGTGAGGAAGGTGGTGTAGTTAATAGCTGCATTATTTGACGTTAGCGACAGAA  455

Query  429  GAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCGAGCGTTAATC  488
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  456  GAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCGAGCGTTAATC  515

Query  489  GGAATTACTGGGCGTAAAGCGCATGCAGGTGGTTTGTTAAGTCAGATGTGAAAGCCCGGG  548
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  516  GGAATTACTGGGCGTAAAGCGCATGCAGGTGGTTTGTTAAGTCAGATGTGAAAGCCCGGG  575

Query  549  GCTCAACCTCGGAATTGCATTTGAAACTGGCAGACTAGAGTACTGTAGAGGGGGGTAGAA  608
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  576  GCTCAACCTCGGAATTGCATTTGAAACTGGCAGACTAGAGTACTGTAGAGGGGGGTAGAA  635

Query  609  TTTCAGGTGTAGCGGTGAAATGCGTAGAGATCTGAAGGAATACCGGTGGCGAAGGCGGCC  668
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Sbjct  636  TTTCAGGTGTAGCGGTGAAATGCGTAGAGATCTGAAGGAATACCGGTGGCGAAGGCGGCC  695

Query  669  CCCTGGACAGATACTGACACTCAGATGCGAATGCGTGGGGAGCAAACAGGATGAGATACC  728
            |||||||||||||||||||||||||||||| |||||||||||||||||||| |||||||
Sbjct  696  CCCTGGACAGATACTGACACTCAGATGCGAAAGCGTGGGGAGCAAACAGGATTAGATACC  755

Query  729  CTGGTAATCCACGCCGCAAACGAAGTCTACTGGGATGTGGTGGCTTGGTGCCGTGCTTT  787
            |||||| ||||||||| |||||| ||||||| ||| || ||||| | | ||||||||||
Sbjct  756  CTGGTAGTCCACGCCGTAAACGATGTCTACTTGGAGGTTGTGGCCTTGAGCCGTGCTTT  814
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Figure 3. Aligment of *V. parahemolyticus* sequenced sample with *V. parahaemolyticus* species from NCBI (EU155529.1)

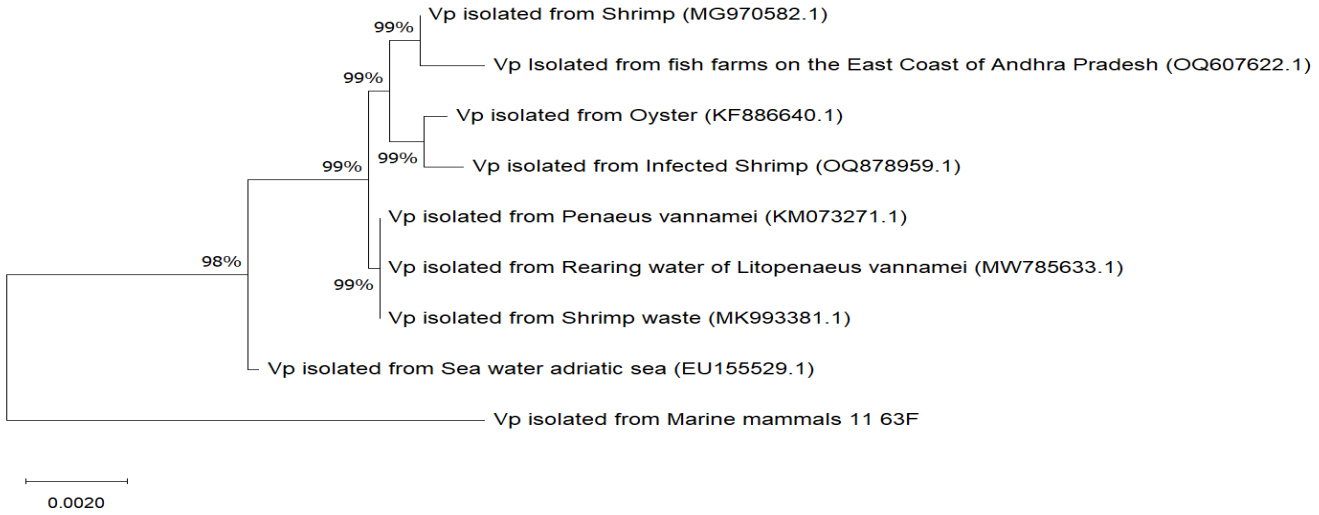

Figure 4. Phylogenetic tree of *V. parahaemolyticus* bacteria isolated from various source

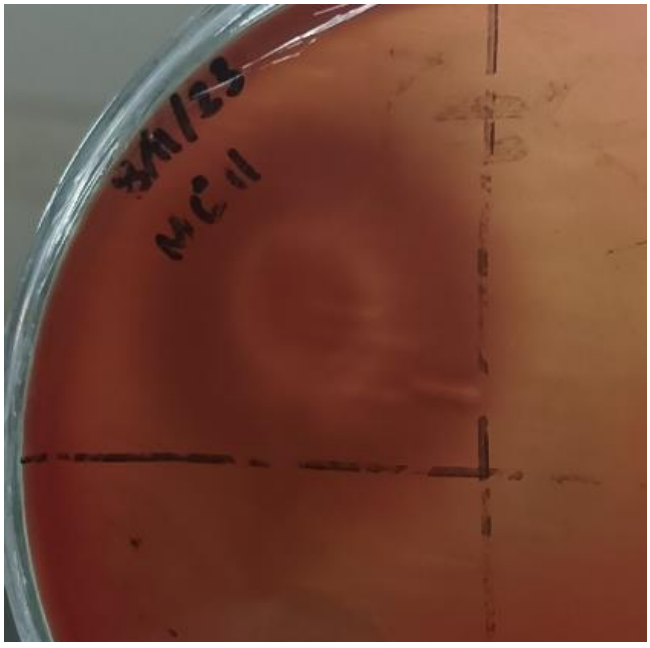

Figure 5. Kanagawa phenomenon test result of *V. parahaemolyticus* isolated from bottlenose dolphin

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). *Vibrio 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-

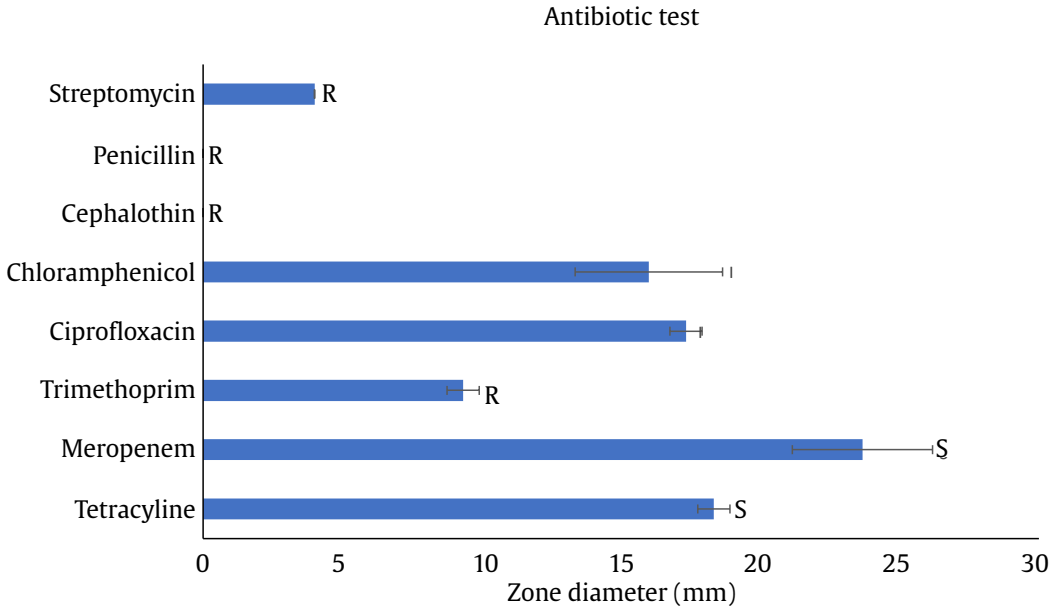

Figure 6. Kirby-bauer anti-susceptibility test of *V. parahaemolyticus* using eight different antibiotics

Urtaza *et al.* 2010). *V. parahamolyticus* 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. parahaemolytycus* 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.q. 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 haemolysing 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. parahaemolytic* bacteria, which are zoonotic and potentially dangerous to humans.

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References

Abdelaziz, M., Ibrahem, M.D., Ibrahim, M.A., Abu-Elala, N.M., Abdel-moneam, D.A., 2017. Monitoring of different *vibrio* species affecting marine fishes in Lake Qarun and Gulf of Suez: Phenotypic and molecular characterization. *The Egyptian Journal of Aquatic Research.* 43, 141–146. https://doi.org/10.1016/j.ejar.2017.06.002

Al-blooshi, S.Y., Latif, M.A.A., Sabaneh, N.K., Mgaogao, M., Hossain, A., 2021. Development of a novel selective medium for culture of Gram-negative bacteria. *BMC Res Notes.* 14, 211. https://doi.org/10.1186/s13104-021-05628-2

Aurilio, C., Sansone, P., Barbarisi, M., Pota, V., Giaccari, L.G., Coppolino, F., Barbarisi, A., Passavanti, M.B., Pace, M.C., 2022. Mechanisms of action of carbapenem resistance. *Antibiotics (Basel).* 11, 421. https://doi.org/10.3390/antibiotics11030421

Ayandele, A., Oladipo, E., Oyebisi, O., Kaka, M., 2020. Prevalence of multi-antibiotic resistant *Escherichia coli* and *Klebsiella* species obtained from a Tertiary Medical Institution in Oyo State, Nigeria. *Qatar Med J.* 2020, 9. https://doi.org/10.5339/qmj.2020.9

Benson, H.J., 2002. Microbiological Applications: A Laboratory Manual in General Microbiology, Complete Version, eighth ed. McGraw-Hill, Boston Burr Ridge Dubuque.

Beshiru, A., Igbinosa, E.O., 2023. Surveillance of *Vibrio parahaemolyticus* pathogens recovered from ready-to-eat foods. *Sci Rep.* 13, 4186. https://doi.org/10.1038/s41598-023-31359-4

Bonnet, M., Lagier, J.C., Raoult, D., Khelaifia, S., 2019. Bacterial culture through selective and non-selective conditions: the evolution of culture media in clinical microbiology. *New Microbes New Infect.* 34, 100622. https://doi.org/10.1016/j.nmni.2019.100622

Brennan-Krohn, T., Pica, N., Sandora, T.J., McAdam, A., 2016. The brief case: safe to go back in the water? *Vibrio parahaemolyticus* wound infection associated with brackish water. *J Clin Microbiol.* 54, 1414–1415. https://doi.org/10.1128/JCM.02660-15

Brooks, G.F., Carrol, K.C., Butel, J.S., Mprse, S.A., Mietzner, T.A., 2013. *Medical Microbiology*, twenty-sixth ed. McGraw-Hill Companies Inc, New York.

Buck, J.D., Wells, R.S., Rhinehart, H.L., Hansen, L.J., 2006. Aerobic microorganisms associated with free-ranging bottlenose dolphins in coastal gulf of Mexico and Atlantic Ocean Waters. *Journal of Wildlife Diseases.* 42, 536–544. https://doi.org/10.7589/0090-3558-42.3.536

Correia Costa, I., Amorim de Oliveira, M., Wosnick, N., Ann Hauser-Davis, R., Siciliano, S., Nunes, J.L.S., 2022. Elasmobranch-associated microbiota: a scientometric literature review. *PeerJ.* 10, e14255. https://doi.org/10.7717/peerj.14255

Coutinho, C.D., Ford, C.E., Trafford, J.D., Duarte, A., Rebelo, R., Rosa, G.M., 2023. Non-lethal detection of ranavirus in fish. *Viruses.* 15, 471. https://doi.org/10.3390/v15020471

Davis, R., Brown, P.D., 2016. Multiple antibiotic resistance index, fitness and virulence potential in respiratory *Pseudomonas aeruginosa* from Jamaica. *Journal of Medical Microbiology.* 65, 261–271. https://doi.org/10.1099/jmm.0.000229

De Souza Valente, C., Wan, A.H.L., 2021. *Vibrio* and major commercially important vibriosis diseases in decapod crustaceans. *Journal of Invertebrate Pathology.* 181, 107527. https://doi.org/10.1016/j.jip.2020.107527

Di Renzo, L., Di Francesco, G., Profico, C., Di Francesco, C.E., Ferri, N., Averaimo, D., Di Guardo, G., 2017. *Vibrio parahaemolyticus* - and *V. alginolyticus* -associated meningo-encephalitis in a bottlenose dolphin (*Tursiops truncatus*) from the Adriatic coast of Italy. *Research in Veterinary Science.* 115, 363–365. https://doi.org/10.1016/j.rvsc.2017.06.023

Feng, H., Liu, X., Wang, S., Fleming, J., Wang, D.C., Liu, W., 2017. The mechanism of NDM-1-catalyzed carbapenem hydrolysis is distinct from that of penicillin or cephalosporin hydrolysis. *Nat Commun.* 8, 2242. https://doi.org/10.1038/s41467-017-02339-w

Field, CL, 2022. Bacterial Diseases of Marine Mammals-Exotic and Laboratory Animals-MSD Veterinary Manual. pdf. Available at: https://www.msdvetmanual.com/exotic-and-laboratory-animals/marine-mammals/bacterial-diseases-of-marine-mammals#top. [Date accessed: 27 February 2024]

Frosth, S., Lewerin, S.S., 2019. Survival of *Streptococcus equi* subsp. equi in normal saline versus phosphate-buffered saline and at two different temperatures. *Journal of Equine Veterinary Science.* 83, 102814. https://doi.org/10.1016/j.jevs.2019.102814

He, J., Qiao, W., An, Q., Yang, T., Luo, Y., 2020. Dihydrofolate reductase inhibitors for use as antimicrobial agents. *European Journal of Medicinal Chemistry.* 195, 112268. https://doi.org/10.1016/j.ejmech.2020.112268

Ina-Salwany, M.., Al-saari, N., Mohamad, A., Mursidi, F., Mohd-Aris, A., Amal, M.N.A., Kasai, H., Mino, S., Sawabe, T., Zamri-Saad, M., 2019. Vibriosis in fish: a review on disease development and prevention. *J Aqua Anim Hlth.* 31, 3–22. https://doi.org/10.1002/aah.10045

Jeamsripong, S., Khant, W., Chuanchuen, R., 2020. Distribution of phenotypic and genotypic antimicrobial resistance and virulence genes in *Vibrio parahaemolyticus* isolated from cultivated oysters and estuarine water. *FEMS Microbiol Ecol.* 96, fiaa081. https://doi.org/10.1093/femsec/fiaa081

Jeamsripong, S., Thaotumpitak, V., Anuntawirun, S., Roongrojmongkhon, N., Atwill, E.R., Hinthong, W., 2022. Molecular epidemiology of antimicrobial resistance and virulence profiles of *Escherichia coli, Salmonella* spp., and *Vibrio* spp. isolated from coastal seawater for aquaculture. *Antibiotics.* 11, 1688. https://doi.org/10.3390/antibiotics11121688

Kapoor, G., Saigal, S., Elongavan, A., 2017. Action and resistance mechanisms of antibiotics: a guide for clinicians. *J Anaesthesiol Clin Pharmacol.* 33, 300–305. https://doi.org/10.4103/joacp.JOACP_349_15: 10.4103/joacp.JOACP_349_15

Kumarage, P.M., De Silva, L.A.D.S., Heo, G.J., 2022. Aquatic environments: a potential source of antimicrobial-resistant *Vibrio* spp. *Journal of Applied Microbiology* 133, 2267–2279. https://doi.org/10.1111/jam.15702

Letchumanan, V., Yin, W.F., Lee, L.H., Chan, K.G., 2015. Prevalence and antimicrobial susceptibility of *Vibrio parahaemolyticus* isolated from retail shrimps in Malaysia. *Front Microbiol.* 6, 33. https://doi.org/10.3389/fmicb.2015.00033

Li, M., Zhao, L., Ma, J., Zhao, N., Luo, J., Wang, C., Chen, L., Ma, G., Wang, Y., He, H., 2018. *Vibrio vulnificus* in aquariums is a novel threat to marine mammals and public health. *Transbound Emerg Dis.* 65, 1863–1871. https://doi.org/10.1111/tbed.12967

Lyu, Q., Bai, K., Kan, Y., Jiang, N., Thapa, S.P., Coaker, G., Li, J., Luo, L., 2019. Variation in streptomycin resistance mechanisms in *Clavibacter michiganensis*. *Phytopathology®*. 109, 1849–1858. https://doi.org/10.1094/PHYTO-05-19-0152-R

Manjano-Mendoza, A., Bravo-Fariñas, L., Fernández-Abreu, A., Martínez-Motas, I., Núñez, F., Mederos-Cuervo, L.M., Ramirez-Alvarez, M., Castro-Escarpulli, G., 2009. Caracterización fenotípica de bacilos gramnegativos anaerobios facultativos oxidasa positiva, aislados de pacientes con enfermedad diarreica aguda en Cuba. *Revista Biomédica.* 20, 25-32.

Martínez-Urtaza, J., Bowers, J.C., Trinanes, J., DePaola, A., 2010. Climate anomalies and the increasing risk of *Vibrio parahaemolyticus* and *Vibrio vulnificus* illnesses. *Food Research International*. 43:1780-1790. DOI:10.1016/j.foodres.2010.04.001

Marchesi, J.R., Sato, T., Weightman, A.J., Martin, T.A., Fry, J.C., Hiom, S.J., Wade, W.G., 1998. Design and evaluation of useful bacterium-specific PCR primers that amplify genes coding for bacterial 16S rRNA. *Appl Environ Microbiol.* 64, 795–799.

Meletis, G., 2016. Carbapenem resistance: overview of the problem and future perspectives. *Ther Adv Infect Dis.* 3, 15–21. https://doi.org/10.1177/2049936115621709

Mohamad, N., Amal, M.N.A., Yasin, I.S.M., Zamri Saad, M., Nasruddin, N.S., Al-saari, N., Mino, S., Sawabe, T., 2019. Vibriosis in cultured marine fishes: a review. *Aquaculture.* 512, 734289. https://doi.org/10.1016/j.aquaculture.2019.734289

Raszl, S.M., Froelich, B.A., Vieira, C.R.W., Blackwood, A.D., Noble, R.T., 2016. *Vibrio parahaemolyticus* and *Vibrio vulnificus* in South America: water, seafood and human infections. *J Appl Microbiol.* 121, 1201–1222. https://doi.org/10.1111/jam.13246

Rudolph, W.W., Gunzer, F., Trauth, M., Bunk, B., Bigge, R., Schröttner, P., 2019. Comparison of VITEK 2, MALDI-TOF MS, 16S rRNA gene sequencing, and whole-genome sequencing for identification of *Roseomonas mucosa. Microbial Pathogenesis.* 134, 103576. https://doi.org/10.1016/j.micpath.2019.103576

Sanders, E.R., 2012. Aseptic laboratory techniques: plating methods. *JoVE.* 63, 3064. https://doi.org/10.3791/3064

Sood, S., 2016. Chloramphenicol–a potent armament against multi-drug resistant (MDR) gram negative bacilli? *J Clin Diagn Res* 10, 1–3. https://doi.org/10.7860/JCDR/2016/14989.7167

Sun, J., Li, X., Hu, Z., Xue, X., Zhang, M., Wu, Q., Zhang, W., Zhang, Y., Lu, R., 2022. Characterization of *Vibrio parahaemolyticus* isolated from stool specimens of diarrhea patients in Nantong, Jiangsu, China during 2018–2020. *PLoS ONE* 17, e0273700. https://doi.org/10.1371/journal.pone.0273700

Tan, C.W., Malcolm, T.T.H., Kuan, C.H., Thung, T.Y., Chang, W.S., Loo, Y.Y., Premarathne, J.M.K.J.K., Ramzi, O.B., Norshafawatie, M.F.S., Yusralimuna, N., Rukayadi, Y., Nakaguchi, Y., Nishibuchi, M., Radu, S., 2017. Prevalence and antimicrobial susceptibility of *Vibrio parahaemolyticus* isolated from short mackerels (*Rastrelliger brachysoma*) in Malaysia. *Front. Microbiol.* 8, 1087. https://doi.org/10.3389/fmicb.2017.01087

Wang, R., Zhong, Y., Gu, X., Yuan, J., Saeed, A.F., and Wang, S., 2015. The pathogenesis, detection, and prevention of *Vibrio parahaemolyticus*. *Front. Microbiol.* 6, 44.

Wróbel, A., Arciszewska, K., Maliszewski, D., Drozdowska, D., 2020. Trimethoprim and other nonclassical antifolates an excellent template for searching modifications of dihydrofolate reductase enzyme inhibitors. *J Antibiot.*73, 5–27. https://doi.org/10.1038/s41429-019-0240-6

Zhang, M., Cai, L., Luo, X., Li, X., Zhang, T., Wu, F., Zhang, Y., Lu, R., 2023. Effect of sublethal dose of chloramphenicol on biofilm formation and virulence in *Vibrio parahaemolyticus. Front. Microbiol.* 14, 1275441. https://doi.org/10.3389/fmicb.2023.1275441

Zhou, H., Liang, Y., Gao, L., Ren, J., Xue, F., Guo, D., Jiang, Y., Yang, Z., Lian, L., Dai, J., 2019. Identification and expression analyses of new genes associated with ciprofloxacin resistance in *Vibrio parahaemolyticus. Food Research International* 125, 108629. https://doi.org/10.1016/j.foodres.2019.108629