Antibiogram of *Vibrio parahaemolyticus* isolated from diseased Climbing Perch (*Anabas testudineus*) of aquarium shop

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DECLARATION BY STUDENT

I hereby declare that the work embodied in this report is the result of the original research and has not been submitted for a higher degree to any institutions

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I certify the report of this final year project entitled 'Antibiogram of *Vibrio parahaemolyticus* isolated from diseased *Anabas testudineus* of aquarium shop' by Nur Alyani binti Mohamad Amin, matric number F18B0133 has been examined and all the correction recommended by examiners have been done for the degree of Bachelor Of Applied Science (Animal Husbandry) with Honors, Faculty of Agro-Based Industry, Universiti Malaysia Kelantan.

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ABSTRACT

The ornamental fish industry has contributed to billions of dollars market value in the world. However, *Vibrio parahaemolyticus* is posed a threat to the industry where it might cause a severe economic loss but there is scarce information available related to the *V. parahaemolyticus* infection in ornamental fish. In the present study, *V. parahaemolyticus* was isolated and identified from a diseased *Anabas testudineus* from local aquarium shop. Thiosulphate-citrate-bile salts-sucrose (TCBS) agar was used to isolate *V. parahaemolyticus* and BBL Crystal kit was used for identification. A total of 30 bacteria colonies was isolated from abdomen, eye, gill, kidney and skin were tested to a total of 16 types of antimicrobial susceptibility test discs by using disk diffusion method. The antimicrobial susceptibility test tested in this study were amoxicillin (25 µg), ampicillin (10 µg) chloramphenicol (30 µg), compound sulphonamides (300 µg), doxycycline (30 µg), erythromycin (15 µg), flumequine (30 µg), fosfomycin (50 µg), kanamycin (30 µg), nalidixic acid (30 µg), novobiocin (30 µg), oxolinic acid (2 µg), oxytetracycline (30 µg), spiramycin (100 µg), sulphamethoxazole (25 µg) and tetracycline (30 µg). It was found that all isolates have higher resistance rate towards compound sulphonamides (96.6%), ampicillin (76.6%), sulphamethoxazole (70%) and amoxicillin (66.6%), but exhibit sensitivity to oxolonic acid (76.6%). The antimicrobial susceptibility test indicated that oxolonic acid is the one that effective in treat *V. parahaemolyticus* infections efficiently. The multiple antibiotic resistance (MAR) index for *V. parahaemolyticus* was 0.81. Thus, it indicates that the samples were highly exposed to the test antimicrobials.

Key words: Ornamental fish, *Vibrio parahaemolyticus*, antibiogram, MAR index

ABSTRAK

Industri ikan hiasan telah menyumbang kepada nilai pasaran jutaan ringgit di dunia. Walau bagaimanapun, *Vibrio parahaemolyticus* telah menimbulkan ancaman kepada industri di mana ia boleh menyebabkan kerugian ekonomi yang teruk tetapi terdapat sedikit maklumat yang berkaitan dengan jangkitan *V. parahaemolyticus* pada ikan hiasan. Dalam kajian ini, *V. parahaemolyticus* diasingkan dan dikenal pasti dari seekor *Anabas testudineus* yang berpenyakit dari kedai akuarium tempatan. Agar Thiosulphate-citrate-bile salts-sucrose (TCBS) digunakan untuk mengasingkan *V. parahaemolyticus* dan kit BBL Crystal digunakan untuk pengenalan bakteria. Sebanyak 30 koloni bakteria telah diasingkan dari perut, mata, insang, ginjal dan kulit telah diuji dengan sejumlah 16 jenis cakera ujian kerentanan antimikrob dengan menggunakan kaedah ujian resapan cakera. Ujian kerentanan antimikrob yang diuji dalam kajian ini adalah amoxicillin (25 µg), ampicillin (10 µg) chloramphenicol (30 µg), compound sulphonamides (300 µg), doxycycline (30 µg), erythromycin (15 µg), flumequine (30 µg), fosfomycin (50 µg), kanamycin (30 µg), nalidixic acid (30 µg), novobiocin (30 µg), oxolinic acid (2 µg), oxytetracycline (30 µg), spiramycin (100 µg), sulphamethoxazole (25 µg) and tetracycline (30 µg). Didapati bahawa semua isolat mempunyai kadar rintangan yang lebih tinggi terhadap compound sulphonamides (96.6%), ampicillin (76.6%), sulphamethoxazole (70%) dan amoxicillin (66.6%), tetapi menunjukkan sensitiviti terhadap oxolinic acid (76.6%). Ujian kerentanan antimikrob menunjukkan bahawa oxolinic acid adalah yang berkesan dalam merawat jangkitan *V. parahaemolyticus* dengan cekap. Indeks ketahanan pelbagai antibiotik (MAR) untuk *V. parahaemolyticus* adalah 0.81. Oleh itu, ini menunjukkan bahawa sampel sangat terdedah kepada antimikrob\ yang digunakan.

Kata kunci: Ikan hiasan, *Vibrio parahaemolyticus*, antibiogram, MAR indeks

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LIST OF ABBREVIATIONS

TCBS	Thiosulphate-Citrate-Bile Salts-Sucrose
MAR	Multiple antibiotic resistance
US	United States
H_2S	Hydrogen sulfide
SOP	Standard operating procedure
APW	Alkaline peptone water
TGA	Trptone glucose agar
TPC	Total plate count
TSA	Tryptone soy agar
rpm	Revolutions per minute
PV	Presumptive vibrios
PVP	Presumptive Vibrio parahaemolyticus
BHI	Brain heart infusion
SIM	Sulphide indole motility
TSIA	Triple sugar iron agar
MR-VP	Methyl Red voges-Proskauer
OF	Oxidative fermentative
LB	Lysogeny broth

LIST OF SYMBOLS

$	Dollar
%	Percentage

LIST OF UNITS

µ	Micro
g	Gram
˚C	Degree Celsius
ml	Milliliter

LIST OF EQUATIONS

$$MAR = \frac{Number\ of\ antibiotics\ to\ which\ the\ isolate\ showed\ resistance\ (a)}{Total\ number\ of\ antibiotics\ tested\ (b)} \quad 22$$

CHAPTER 1

INTRODUCTION

1.1 Research Background

The most popular hobbies worldwide is ornamental fish keeping. The growing popularity of aquarium fishes has resulted in a steady rise in aquarium fish trade internationally. The industry has a lot room of opportunities as the average annual value for global import from 1983 to 1992 was US$130 million with an average annual growth of 21% (Rodríguez, 2006). During 1992, the top three global exporters were Singapore, Hong Kong, and the United States (US) (Rodríguez, 2006). Many developing countries have recognized the economic value of ornamental fisheries in terms of employment generation and livelihoods. Freshwater, brackish water, and saltwater fish are the three main types of fish kept in captivity. The primary distinction between the three groups is the relative density of the water in which they dwell. The

cultivation of freshwater species of ornamental fish is the most favourable; for example, in Florida, there are 800 varieties. In comparison, only 25 species of marine fish are commercially cultured (Ladisa, 2017). However, infectious diseases have been identified as a significant impediment to the development of this industry, which bring about serious financial losses (Marudhupandi et al., 2017). Parasitic invasions, fungi, viruses and bacteria cause the vast majority of infectious diseases. Bacterial infections are mostly sustained by opportunistic pathogens and can be considered the most serious issue in the marine ornamental fish culture (Marudhupandi et al., 2017).

Vibrio parahaemolyticus is a Gram-negative, singularly or straight curved rod shape bacterium with high motility by means of a single polar flagellum. They are oxidase-positive facultative anaerobic bacterium where the source of carbon and energy are exclusively or only coming from D-glucose and they do form endospores or microcysts (Ramamurthy & Nair, 2014). This bacteria is naturally present in brackish and warm coastal waters. Sometimes the bacteria clinging to fish, selfish, zooplankton, sediments or can be discovered swimming freely (de Souza Santos et al., 2015). *V. parahaemolyticus* is a significant bacterial pathogen that causes high mortality in a variety of fish species, including marine ornamental fish (Marudhupandi et al., 2017). The most common cause of seafood-borne acute gastroenteritis is ingestion of raw or undercooked fish contaminated with *V. parahaemolyticus* (de Souza Santos et al., 2015). *V. parahaemolyticus* incubation time varies from 4 to 48 hours (Saha & LaRocque, 2013). It can also induce inflammation of open cuts introduced to open ocean. In the previous research, *V. parahaemolyticus* has been extracted from a rot tail of a clownfish *Amphiprion sebae* by using spread plate method on Zobell marine agar (Marudhupandi et al., 2017). Then, the biochemical

identification is done by presumptive tests such as Gram staining, fermentation of glucose sugar production, Decarboxylate of lysine, Decarboxylate omithine, Heamolysis, Arginine dihydrolase, H_2S production, Urease, Catalase, Cytochrome oxidase, Glycerol, Cellobiose protease, Tryptophan deaminase, Indole production, Voges-Proskeur, utilization of citrate and Nitrate reduction (Marudhupandi et al., 2017).

1.2 Problem Statements

In recent years, the rate of outbreak incidence of *Vibrio parahaemolyticus* is assumed to increase by years. Tail and fin rot are the most prevalent *V. parahaemolyticus* diseases present in many fish farms. The incident resulted in a higher mortality rate (Marudhupandi et al., 2017). Since the ornamental fish trade is a worth billions of dollars business, a thorough knowledge of this bacteria is needed to prevent a significant loss in the industry. Therefore, this study is designed to monitor the characteristics of the bacteria. Besides, a scientific studies on the identification of specific pathogens for tail rot disease in many fishes are yet to be discovered for the effective diagnosis and treatment. Therefore, it is important to identify effective antimicrobial in controlling *V. parahaemolyticus* infection.

1.3 Objectives

The objectives of the present study are:

1. To isolate and identify *Vibrio parahaemolyticus* from *Anabas testudineus*.
2. To characterize antibiogram of isolated *Vibrio parahaemolyticus* using antimicrobial susceptibility test.

1.4 Scope of study

Aquatic animal health

1.5 Significance of Study

One of the most crucial aspects of the ornamental fish trade is the health care of the fish. As the business keep growing, new information and discoveries about the

fish's health are needed to ensure the industry's long-term viability. This study's findings can lead to a better understanding of *V. parahaemolyticus* and their resistance to antibiogram pattern. Therefore, it is important to characterize and to identify effective antimicrobial of *V. parahaemolyticus* from the ornamental fish. In the future, the results collected can be used in managing ornamental fish health.

1.6 Limitation of Study

The limitation in this study is a restriction on using the laboratory. Due to covid-19 pandemic, the students must strictly adhere to the laboratory's standard operating procedure (SOP), which allows only a limited number of individuals to be present at one time. Furthermore, the number of antimicrobials used in this study is limited. There were only 16 antimicrobials provided. A wide range of antimicrobials can help to produce more accurate results.

CHAPTER 2

LITERATURE REVIEW

2.1 Ornamental fish

The aquarium fish market is a huge and international industry that moves significant volumes of products and revenue while providing services and employment openings to young people and promote the economic growth of developing nations (Ladisa, 2017). Based on article written by Rodríguez (2006), the ornamental fish trade involves more than 100 countries, and approximately one billion fish are transported between trading blocks each year. The tropical countries, Asia, South America, Africa, and the Caribbean are known to provide the majority of the world's supply of ornamental tropical fish to the rest of the world. The European Union as a whole is the nation's biggest industry for ornamental fish, however the United States (US) is the world's leading importer (Livengood & Chapman, 2007;

Whittington & Chong, 2007). According to Ladisa (2017), the overall ornamental fish business is worth around USD 15 billion, and around 2000 to 2011 the international ornamental fish exports increased from USD 181 million to USD 372 million. Two studies have shown that the vast majority of ornamental fishes in the aquarium trade are of freshwater descent and farm-raised (Livengood & Chapman, 2007; Ladisa, 2017). Singapore, China, Hong Kong, Japan, Thailand and Malaysia have all traditionally specialized in the propagation and production of freshwater ornamental fishes. The small-scale design of an ornamental fish farming scheme, whether outdoors or indoors, minimizes environmental effects and helps to maximize the use of space, labour, resources, and operating costs (Livengood & Chapman, 2007). Despite the industry's economic significance, ornamental fish diseases and illnesses have gained little recognition over the years. Diseases of ornamental fish appear infrequently in books mainly concerned with food fish. As the value of the aquarium fish industry soared and surpassed that of dogs and cats in the Britain during the late 1970s, then the veterinary physicians became mindful of the need for knowledge on ornamental fish diseases (Rodríguez, 2006). As stated in research before, the infectious diseases is deemed to be the greatest obstacle for ornamental fish industry (Marudhupandi et al., 2017). The most frequent infectious threat to ornamental fishes is bacterial disease. Pathogenic bacteria such as *Pseudomonas, Vibrio, Aeromonas, Citrobacter, Edwardsiella*, and *Mycobacterium* cause bacterial infections alongside predominantly gram-negative organisms. Bacteria species may be the main sources of illness, or they may be additional agents, exploiting an opening in the fish's integument or a compromised immune system. Almost all of bacterial fish organisms are indigenous to the aquatic ecosystem, either seawater or freshwater. An external

environment that creates stress such as transportation, low water quality, overcrowding and undernutrition may potentially expose an ornamental fish to bacterial disease (Lewbart, 2001).

2.2 *Vibrio parahaemolyticus*

According to research studies, Tsunesaburo Fujino discovered *Vibrio parahaemolyticus* from a foodborne case of gastroenteritis caused by the ingestion of semidried sardines in Osaka, Japan in 1950 (de Souza Santos et al., 2015; Ramamurthy & Nair, 2014; Todd, 2014). The incidents happened during fall season where it caused 272 sickened individuals and 20 fatalities. As stated in de Souza Santos et al. (2015) study, the halophilic characteristics of *V. parahaemolyticus* were discovered five years after the bacterium was identified, and the start of cultivation media containing sodium chloride being used in bacterial isolation. This discovery fits with reports that *V. parahaemolyticus* is native to seawater and estuarine ecosystems. The bacterium does well in warm water and can be present during the year in places where the temperatures of the water do not fall below 15˚C. *V. parahaemolyticus* infections have risen worldwide since the 1950s, with the most common cause was consumption of fresh, poorly prepared or cooked, recontaminated fish and shellfish (Todd, 2014). Based on studies, only till 1996 the *V. parahaemolytic*us infections were intermittent and prompted by numerous and varied

serotypes, which each was limited to a particular geographical region (de Souza Santos et al., 2015). Nevertheless, a significant shift in the epidemiology of *V. parahaemolyticus* took place in September 1996, when an incidence of diarrhea in Kolkata, India was due to the emergence of a novel clone of O3:K6 (de Souza Santos et al., 2015; Todd, 2014). The ensuing escalate quickly to several nations resulting in many diarrheal outbreaks indicated the very first of *V. parahaemolyticus* infection pandemic (de Souza Santos et al., 2015; Ramamurthy & Nair, 2014). Multiple occurrence reports and infections of this bacteria in shellfish and crustaceans have been widely documented. However, there is little data on the quantitative identification of *V. parahaemolyticus* in aquarium fish species. In recent years, there have been increase of study of *V. parahaemolyticus* in aquarium fish as bacterial infection is one of a dangerous threat to this industry.

2.3 Isolation and Identification of *V. parahaemolyticus*

The isolation and identification of *V. parahaemolyticus* from clinical specimens are well established. An enrichment step can be used before culture the *V. parahaemolyticus* on agar as the density of the bacteria in the samples may not be very high. The alkaline peptone water (APW) with pH of 8.0-8.7 supplemented with 1-3% NaCl is the best option for initial enrichment (Ramamurthy & Nair, 2014). Then, the selective plating method is the most preferable for *Vibrio* species isolation.

This can be found in research by Chakraborty et al. (2008) where it stated that the alkaline peptone water (APW) broth (225 ml) is used for homogenizing the samples (25g) in a sterile polythene stomacher bag at 230 rpm for one minute, and enriched it for 18-24 hour. The research that have been conducted is about *V. parahaemolyticus* from seafoods along the southwest coast of India. They used seven species of finfish, eight species of shellfishes and two species of cephalopod. Then, 0.5 ml of APW broth was a sceptically pipetted into thiosulphate-citrate-bile salt-sucrose agar (TCBS) and tryptone glucose agar (TGA) plates where the plates were incubated at 37˚C for 24-30 h and 37˚C for 24-48 h, respectively. The TGA plates results indicates number of colonies ranging from 30 to 300 which are reported as total plate count (TPC) while TCBS plate resulting in 3 to 4 standard colonies with green or bluish green colouration and a dark blue or green center measuring between 3 to 5 mm. Both TCBS colonies were inoculated into sterile sucrose broth supplemented with NaCl (3% w/v) and for further details, tryptone soy agar slants (TSA) supplemented with NaCl (3%w/v) was used by sucrose non-fermenting colonies through streaking method at room temperature. To better identify the bacteria, arginine dehydrolase, lysine and ornithine decarboxylase, O/129 susceptibility, gram staining, catalase, cytochrome oxidase, halophilism tests, triple sugar iron and lysine iron agar were performed. By using Hugh-Leifson broth, the oxidation-fermentation glucose tests (OF glucose test) were also done. Unless otherwise stated, all media were supplemented with NaCl (3% w/v). According to the findings, all colonies forming on TCBS are consider as presumptive vibrios (PV), and colonies that present as green colonies with dark blue or green centers measuring between 3-5 mm as *V. parahaemolyticus* are deemed as presumptive *V. parahaemolyticus* (PVP). Another study from Khouadja et al. (2013)

also start their isolation method by enrichment method for their samples. The research focuses on the description and pathogenicity of *V. parahaemolyticus* strains collected from sea bass (*Dicentrarchus labrax*) from marine cage farm and inshore fish farm in Tunisia. Ten juvenile and ten adults of *D. labrax* were acquired from each fish farm, with samples taken from the skin, kidney, liver and spleen. The samples then were cultivated on alkaline peptone water (1% NaCl, pH 8.6) and incubated for 18 to 24 h at 37˚C. The enrichment culture was then streaked onto thiosulphate-citrate-bile salt-sucrose (TCBS) agar for selective isolation of *Vibrio* strains and incubated for 18 to 24 h at 37˚C. The results from TCBS plate were green colonies. The green colonies were chosen at random and subcultured on tryptic soy agar (TSA) containing 1% NaCl. The isolated bacteria were frozen at -80˚C in a glycerol solution 20% (v/v) for further analysis. Indole production, O-F test, motility (mannitol-motility agar), susceptibility to the vibriostatic compound O/129, gram staining, cell morphology, the oxidase and catalase were all used for the identification of *Vibrio* genus. All microtubes were supplemented with 5 ml of 2.5% NaCl solution and incubated for 24 h at 30˚C. The identification was obtained using the APILAB PLUS software and was deemed appropriate because the likelihood was equal to or greater than 80%. Further tests such as arginine dehydrolase test and halophilism tests were performed.

However, there are some studies that do not used enrichment step before the isolation and identification of the bacteria. According to Saad El-deen and Elkamel (2015) *vibriosis* is one of dangerous bacteria diseases in ornamental fishes. In Egypt, there has been a lack of research on the microbial species correlated with ornamental fishes or the aquarium water in which they are imported and stored. Therefore, this study conducted to investigate the infections with *vibriosis* among some species of

ornamental fishes. A total number of 100 ornamental fishes that showing signs of septicaemia were collected from ornamental fish shops as the samples. The 100 ornamental fishes including 73 Moribund fantail (*Carassius auratus auratus*), 17 black molly (*Poecillia latipinna*) and 10 koi carp (*cyprinus carpio*) with body weight between 10 to 25 gram. The brain heart infusion agar (BHI) supplement with 3, 6, or 8% NaCl and TCBS agar were used for isolation directly from the liver and kidney of fantail fish. Then the plates were incubated for 48 h at 28˚C. The colonies were selected for further study based on morphological characteristics, purified by sub-culturing, and stored on BHI slants at 4˚C. Then, the characterization of the bacteria were determined by oxidase, catalase, simmon citrate, O-F test, sulphide indole motility test (SIM), methyl red, voges proskauer and spring test. The salt tolerance (NaCl 3%, 6% and 8%) and oxidation-fermentation tests (lactose, sucrose, mannose) also were performed. This study found 7 species of *Vibrio* from the biochemical characterization as: *V. vulnificus* (23, 38.98%), *V. parahaemolyticus* (17, 28.81%), *V. harveyi* (7, 11.86%), *V. ordalii* (4, 6.78%), *V. alginolyticus* (3, 5.08%), *V. mimicus* (3, 5.08%), and *V. fisheri* (2, 3.39%).

Rukmana et al. (2019) conducted a study to classify the bacterial species that could attack marine ornamental fish in Denpasar, Bali's Fish Quarantine, Quality Control, and Fishery Products Safety Class I. The infected fish included *Dascyllus trimaculatus, Dascyllus arwanus, Dascyllus melanurus, Dascyllus carneus, Pseudochromis, Abudefduf sp., Pomacentrus* and *Chromis viridis*. The bacterial isolation is isolated from gill organs to 3% of Triptic Soy Agar (TSA) and TCBS agar by streaking method for 24 h at 28˚C. TCBS agar is used to identify *Vibrio* bacteria because it is a selective medium. They eventually grew a single colony from TSA

agar (3%) to TCBS agar for bacterial purification. Because of the bacteria's ability to digest sucrose and reduce pH, yellow colonies suggested *Vibrio cholera* and *Vibrio alginolyticus,* while green colonies indicated *Vibrio* that are unable to ferment sucrose. *V. parahaemolyticus* is the bacteria that will give green center colonies. Then, three presumptive tests were performed, including a Gram test supplemented with KOH solution (3%), a catalase test with H2O2 (3%) inclusion, and an oxidase test with oxidase test strips to determine the nature of microbes. The biochemical test included oxidative fermentative testing (OF), nitrate testing, lysine testing, ornithine testing, sugar testing, Methyl Red voges-Proskauer testing (MR-VP), Sulfide Indole Motility (SIM) testing, citrate testing, gelatine testing, urea testing and acid fermentation in Triple Sugar Iron Agar (TSIA) media. Bergey's Manual of Determinative Bacteriology was referred for the outcome of the biochemical test. *Vibrio furnissii, V. alginolyticus*, and *V. parahaemolyticus* were identified as the bacterial that infected the marine ornamental fish based on the results of the biochemical tests. This indicated that biosecurity was not really implemented in the cultivation method.

2.4 Antimicrobial Susceptibility Test

Antimicrobial are effective weapons in clinical medicine, but they selectively select for resistance in pathogens and therefore can become inefficient. A

comprehensive analysis of drug-resistant processes in pathogens has been conducted and still ongoing in the latest years (Carlson, 2017). Antimicrobial susceptibility in the aquaculture industry is one field of study that is currently being investigated. In the research of Yu et al. (2016), an antimicrobial susceptibility test on *Vibrio parahaemolyticus* isolated from supermarket shellfish in Shanghai is done. The aim of this research was to look into the prevalence and drug tolerance of *V. parahaemolyticus*. They used 140 shellfish as the samples including 24 scallop (*Chlamys nobilis*), 35 razor clam (*Sinonovacula constricta*), 38 oyster (*Crassostrea ariakensis*) and 43 clam (*Meretrix meretrix*). According to the Clinical and Laboratory Standards Institute, the samples were tested using disc-diffusion method in Mueller-hinton Agar augmented with NaCl (0.85% w/v) using antimicrobial discs. There were 18 antimicrobial discs used such as ciprofloxacin, norfloxacin, nalidixic acid, sulphamethoxazole/trimethoprim, chloramphenicol, piperacillin tazobactam, cephazolin, cephalothin, ampicillin, piperacillin, amoxicillin/calvulanic acid, imipenem, aztreonam, gentamycin, amikacin, tetracycline, cefotaxime and ceftazidime. The isolates were incubated overnight in lysogeny broth (LB) liquid medium containing NaCl (3% w/v), and modified to 0.5 McFarland using sterile physiological saline before being swabbed onto Mueller-Hinton medium and incubated for 18 h at 37˚C. The results from this study showed that 87.5% of the 96 isolates analyzed were resistant to ampicillin, with a small number being resistant cephazolin (31.3%), cephalothin (6.3%), amoxicillin/calvulanic acid (6.3%), piperacillin (6.3%), and amiakcin (3.1%). While no drug resistance was found in azteonam isolates, a large number of them (40.6%) showed intermediate resistance. In ceftazidime, gentamycin, nalidixic acid, norfloxacin,

sulphamethoxazole/trimethoprim, imipenem and chloramphenicol, all isolates showed sensitive result. Using the MAR index, 34% of *V. parahaemolyticus* isolates display multiple antimicrobial resistance to at least two antimicrobials.

Based on another research from Yaashika et al. (2016), an antibiotic study have been conducted on prawn (*Penaeus monodon*) to determine the *Vibrio* species' resistance and sensitivity. Before starting the antibiotic susceptibility test, the test culture was placed in a sterilized broth and incubated for a few hours at 35˚C until it became mildly turbid. The bacteria were then evenly inoculated on the top layer of sterile Muller Hinton Agar plates with a sterile cotton swab before put the antibiotic discs. For 24 hours, the plates were incubated at 37°C. This study only used four antibiotics such as penicillin G, clindamycin, piperacilin and co-trimoxazole. From the result obtained, the *Vibrio parahaemolyticus* show resistant towards penicillin G, clindamycin and piperacilin while very sensitive to co-trimoxazole. The rapid evolution of antibiotic resistance in bacteria, as well as the advent of drug-resistant in diseases caused by microorganisms in aquaculture industry, poses significant environmental, economic, and management issues, as well as human health risks.

There is also a report in Malaysia that looks at the prevalence and antimicrobial resistance of *Vibrio parahaemolyticyus* extracted from short mackerels (*Rastrelliger brachysoma*) (Tan et al., 2017). A total of 67 bacteria colony from each sampling is used in antimicrobial susceptibility test. The discs diffusion technique was used in this study. Before inoculating the bacteria for antimicrobial susceptibility test, the samples were grown in Mueller-Hinton broth (5 ml) augmented with NaCl (3% w/v) and incubated for 24 h, 120 rpm at 37˚C. Using a sterile cotton swab, the bacteria will

be swabbed over the top layer of Mueller-Hinton agar augmented with NaCl (3% w/v) and allowed to dry for 3-5 minutes. The antimicrobial susceptibility discs were then placed on top of the agar and incubated for 24 hours at 37˚C. There were 17 antimicrobial susceptibility test discs used in this research, including tetracycline, streptomycin, penicillin, meropenem, ampicillin, ampicillin sulbactam, amikacin, amoxicillin/clavulanic acid, ceftazidime, cefotaxime, cephalothin, levofloxacin, imipenem, gentamicin, doxycycline, ciprofloxacin and chloramphenicol. The results found that the majority of the isolates studied were susceptible to antimicrobial such as ampicillin sulbactam (100%), meropenem (100%), ceftazidime (98.5%), and imipenem (98.5%). Penicillin G (92.5%) and ampicillin (82.1%) showed a high degree of resistance. According to the MAR index value, 89.6% of the isolates were resistant to two or more antimicrobials, with two isolates having the highest MAR index value of 0.41 and resistance to seven different antimicrobials.

CHAPTER 3

METHODOLOGY

3.1 Materials

3.1.1 Chemicals and Reagents

Thiosulphate-Citrate-Bile Salts-Sucrose (TCBS), alkaline peptone water (APW) with 1% NaCl, Trypticase soy agar (TSA), antibiotic discs, BBL diluent, nutrient broth, Mueller-Hinton Agar, amoxicillin (25 µg), ampicillin (10 µg) chloramphenicol (30 µg), compound sulphonamides (300 µg), doxycycline (30 µg), erythromycin (15 µg), flumequine (30 µg), fosfomycin (50 µg), kanamycin (30 µg), nalidixic acid (30 µg), novobiocin

(30 µg), oxolinic acid (2 µg), oxytetracycline (30 µg), spiramycin (100 µg), sulphamethoxazole (25 µg), tetracycline (30 µg)

3.1.2 Raw material

Climbing perch (*Anabas testudineus*)

3.1.3 Equipment

Inoculating loop, petri dish, Bunsen burner, incubator, laminar flow cabinet, forceps, BBL Crystal Kit, cotton swab, laboratory film, cling film, test tube, marker pen, mask, gloves, conical flask and beaker

3.2 Methods

3.2.1 Sample collection and bacterial isolation

An *Anabas testudineus* was bought from aquarium store. The samples were collected from each abdomen, eye, gill, kidney and skin of the diseased *Anabas testudineus*. Next, all the samples were cultured on alkaline peptone water (APW) with NaCl (1%), pH 8.6 and incubated for 18 to 24 hours at 37˚C.

After that, a loopful of enrichment culture were transfer onto thiosulphate-citrate-bile salt-sucrose (TCBS) agar by streaking method. The selective agar is used for isolation of Vibrio strains. Then, the plates were incubated for 18 to 24 h at 37˚C. There will be standard colonies having green or bluish green colour as *Vibrio parahaemolyticus* cannot degraded the sucrose in the agar.

3.2.2 Selection and inoculation of typical single colonies into trypticase soy agar (TSA)

One single green colony from each sample were selected randomly from TCBS agar by using sterile inoculating loop. Then, the bacteria were subcultured onto trypticase soy agar (TSA) using streaking method. The plates were incubated for 18 to 24 h at 37˚C.

3.2.3 Bacteria identification using BBL Crystal Kit

A loopful of large isolated colony from TSA medium were transfer into BBL diluent. The suspension was mixed well by shaking the bottle. Then, the inoculum fluid was poured into the target area of the base. The inoculum liquid was gently roll along the tracks until all the wells are filled. After that, the lid was align with the base and it was pushed down until it snap into place with two clicks. The BBL panel was wrapped with cling film to prevent drying out. After incubated for 37˚C for 18 to 24 h, the colour changed on the kit was observed by referring to the colour

reaction chart. The ten digit profile number from the BBL Crystal Kit was enter into BBL Crystal database to identify the bacteria.

3.2.4 Antimicrobial susceptibility test

The isolates was placed in a sterilized nutrient broth. The broth was incubated at 35˚C for a few hours until it becomes mildly cloudy. Using a sterile cotton swab, the standardized bacterial test suspension was inoculated uniformly on the top layer of sterile Mueller-Hinton Agar plates and allowed to dry for 3-5 minutes. The antimicrobial discs were arranged on the inoculated agar plate using a disc dispenser and incubated for 24 hours at 37˚C. A total of 16 antimicrobial susceptibility test discs were used including nalidixic acid (30 µg), oxolinic acid (2 µg), compound sulphonamides (300 µg), doxycycline (30 µg), tetracycline (30 µg), novobiocin (30 µg), chloramphenicol (30 µg), kanamysin (30 µg), sulphamethoxazole (25 µg), flumequine (30 µg), erythromycin (15 µg), ampicillin (10 µg), spiramycin (100 µg), oxytetracycline (30 µg), amoxycillin (25 µg), fosfomycin (50 µg). The antimicrobial activity was determined by measuring the diameter of the inhibition area to the nearest whole millimeter. The inhibition zone results are categorized as sensitive

(more than 18mm), intermediate (16-17mm) and resistance (less than 15mm).

3.2.5 Multiple Antibiotic Resistance

In order to determine the multiple antibiotic resistance (MAR) index value, the formula a/b was used, where "a" represents the number of antibiotics to which the particular isolate was resistant and "b" represents the total number of antibiotics tested for the isolate (Tan et al., 2017).

MAR index:

$$MAR = \frac{Number\ of\ antibiotics\ to\ which\ the\ isolate\ showed\ resistance\ (a)}{Total\ number\ of\ antibiotics\ tested\ (b)}$$

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Results

In the present study, a diseased *Anabas testudineus* was analyzed for isolation of *Vibrio parahaemolyticus* from local aquarium shop. First, the bacteria were isolated using streaking method on thiosulphate-citrate-bile salt-sucrose (TCBS) agar. The selective agar was used for isolation of Vibrio strains. The agar findings revealed green colonies. The green colonies on the plates confirmed the presence of *Vibrio parahemolyticus.* The *V. parahaemolyticus* was successfully isolated from abdomen, eye, gill, kidney and skin. Next, a total of 16 commonly used antimicrobials were chosen for antimicrobial susceptibility testing, and the results were summarized in Table 1. Based on the antimicrobial susceptibility tests, the resistance rate of the 30 bacteria colonies of *V. parahaemolyticus* isolates in this study was 96.6% to compound sulphonamides, 76.6%

to ampicillin, 70% to sulphamethoxazole and 66.6% to amoxicillin. This study also documented 76.6% and 70% of bacteria isolates were sensitive to oxolinic acid and doxycycline, respectively. Then, the percentage of sensitivity of present bacterial isolates to flumequine, erythromycin and fosfomycin is same (46.6%). Then, the intermediate resistance was exhibited against kanamysin (80%), chloramphenicol (70%), oxytetracycline (60%), novobiocin (56.6%), spiramycin (53.3%), tetracycline (50%) and nalidixic acid (40%). The multiple antibiotic resistance (MAR) index of the *V. parahaemolyticus* isolates is 0.81.

Table 1: Antimicrobial susceptibility test of *Vibrio parahaemolyticus* on *Anabas testudineus*

Types of antimicrobial agent	Concentration (µg/disk)	Bacteria isolated (*Vibrio parahaemolyticus*)		
		Resistance (%)	Intermediate resistance (%)	Sensitive (%)
Amoxicillin (AML)	25	66.6	33.3	0
Ampicillin (AMP)	10	76.6	23.3	0
Chloramphenicol (C)	30	0	70	30
Compound Sulphonamides (S3)	300	96.6	3.3	0
Doxycycline (DO)	30	6.6	23.3	70
Erythromycin (E)	15	16.6	36.6	46.6
Flumequine (UB)	30	13.3	40	46.6
Fosfomycin (FOS)	50	13.3	40	46.6
Kanamycin (K)	30	3.3	80	16.6
Nalidixic Acid (NA)	30	20	40	40
Novobiocin (NV)	30	26.6	56.6	16.6
Oxolinic Acid (OA)	2	0	23.3	76.6
Oxytetracycline (OT)	30	6.6	60	33.3
Spiramycin (SP)	100	0	53.3	46.6
Sulphamethoxazole (RL)	25	70	26.6	3.3
Tetracycline (TE)	30	6.6	50	43.3

4.2 Discussion

Based on the results obtained, the resistance of *Vibrio parahaemolyticus* to compound sulphonamides is the highest 96.6%. The report of this antibiotics is limited and only a studies from Banerjee et al. (2012) showed the antimicrobial susceptibility test on *Vibrio parahaemolyticus*. As such, in contrast with the present study the *V. parahaemolyticus* is 100% sensitive to compound sulphonamides (Banerjee et al., 2012). Then, the second most resistant antibiotic is ampicillin (76.6%). These findings were consistent with prior research by Chakraborty et al. (2008) and Ghenem & Elhadi (2018), in which found 80.9% and 88% of *V. parahaemolyticus* isolates were resistant to ampicillin respectively. This is to be expected given that ampicillin has been actively used since 1960, and ampicillin resistance is also often reported (Shaw et al., 2014; Silvester et al., 2015; He et al., 2016; Ghenem & Elhadi, 2017). Interestingly, another two studies discovered that ampicillin resistance to *V. parahaemolyticus* was approximately 100% (Devi et al., 2009; Sudha et al., 2014). Besides, it is believe that *V. parahaemolyticus* resistance to ampicillin and other penicillins due to its chromosomally encoded β-lactamase (Devi et al. 2009). In addition, over 60% of *V. parahaemolyticus* isolates were resistant to amoxicillin, which agrees with the results published by Tunung et al. (2012) (80.43%). It is consistent with the statement above, as amoxicillin is under penicillin family. For sulphamethoxazole antibiotic, the present study reported a 70% resistance rate. These result are comparable with the finding by Tunung et al. (2012) (73.91%). Therefore, ampicillin, amoxicillin and sulphamethoxazole should be phased-out for treating *V. parahaemolyticus* infections.

In this study, a low percentage of resistance towards the antibiotics was observed with the following antibiotics; novobiocin (26.6%), nalidixic acid (20%), erythromycin (16.6%), flumequine and fosfomycin (13.3%) and oxytetracycline, doxycycline and tetracycline (6.6%). In accordance, Drais et al. (2016) have reported novobiocin resistance with the percentage of 100%. Based on the results of this study, novobiocin should be considered for treatment of *Vibrio parahaemolyticus*. Previous studies have shown that nalidixic acid and tetracycline were sensitive against *V. parahaemolyticus* (Devi et al., 2009; Sudha et al., 2014; Tang et al., 2014; Shaw et al., 2014; Ghenem & Elhadi, 2017), but this contradicted our findings, which revealed resistance to nalidixic acid and tetracycline. In accordance, a recent research by Zaher et al. (2021) reported high resistance of *V. parahaemolyticus* to nalidixic acid (82.7%) and tetracycline (51.7%). It is quite concerning since tetracycline is one of the first-line antibiotics that was highly efficient against *Vibrio* spp (Sudha et al., 2014). Tetracycline's extensive usage due to its low toxicity and broad-spectrum antibiotic action against a wide range of Gram-positive and Gram-negative bacteria, as well as its successful prevention and treatment against *Vibrio*, might have resulted in significant resistance (Banerjee et al., 2012). Since nalidixic acid belongs to the quinolone class, resistance to it may be linked to chromosomal mutations or mutations producing lower drug accumulation, as well as quinolone resistance genes associated with plasmids (Fàbrega et al., 2009). Given the rise in resistance to tetracycline and nalidixic acid, the use of those antibiotics should be reduced for a period in order to decrease antibiotic resistance.

Similar to this study, Kang et al. (2017) and Amalina et al. (2019) have reported erythromycin resistance with the percentage of 11.4% and 18%, respectively which agrees with the present results. Due to their intrinsic resistance, gram-negative bacteria

are likely to develop resistance to erythromycin. In the current study, flumequine and fosfomycin had the same percentage of resistance rate (6.6%). The resistance of *V. parahaemolyticus* to flumequine has been previously reported by Khouadja et al. (2013) (22.2%). The comparison of flumequine resistance rates revealed that the first-generation fluoroquinolone antibiotic is still effective against *V. parahaemolyticus*, due to the fact that resistance rate is decreasing in the present day. In the meantime, in contrast with current findings, fosfomycin has been shown in prior studies to be 100% sensitive (Zago et al., 2020). Due to a major lack of understanding among veterinary experts, fosfomycin is frequently regarded as a second-line antibiotic (Pérez et al., 2014). However, the findings above indicate that these antibiotics have a high potential in the aquaculture business. Oxytetracycline is a tetracycline-based antibiotic with a broad range that is extensively used in aquaculture (Mog et al., 2020). According to the results of antimicrobial susceptibility testing for oxytetracycline, the resistance rate for *V. parahemolyticus* is 6.6 %. As such, the resistance of *V. parahemolyticus* to oxytetracycline has previously been reported by Devi et al. (2009) and Lee et al. (2018), which is 7.1% and 18%, respectively. This might be due to the isolates' intrinsic resistance to tetracycline, such as modifications to the membrane lipid barrier, resistance-nodulation-cell division (RND) efflux protein, and major facilitator superfamily (MFS) that helps the bacteria survive (Dutta et al., 2021). Doxycycline is a member of the same antibiotic family as oxytetracycline. Thus, it is plausible that the isolates' resistance stems from the same cause as oxytetracycline. However, doxycycline has the second highest sensitivity rate (70%), which is consistent with recent studies by Shaw et al. (2014) and Tan et al. (2020), which were 100% and 98.33%, respectively.

According to this study, kanamycin and chloramphenicol had a higher rate of intermediate resistance against *V. parahaemolyticus* isolates by 80% and 70%, respectively. Kanamycin findings is equivalent to the data of He et al. (2016), Xu et al. (2016), and Ghenem & Elhadi (2017), who found intermediate resistance to be 70%, 74.5%, and 71%, respectively. Earlier studies on chloramphenicol show a high sensitivity rate to *V. parahaemolyticus* (He et al., 2016; Xu et al., 2016; Lee et al., 2018; Ghenem & Elhadi, 2017; Tan et al., 2020), but the most recent study findings by Ashrafudoulla et al. (2021) agrees with this current study. Thus, kanamycin and chloramphenicol are not recommended in treatment regimens for *V. parahaemolyticus* infections. Although *V. parahaemolyticus* isolates from this study displayed high levels of resistance to compound sulphonamides, ampicillin and amoxicillin, as well as low resistance rate to novobiocin, nalidixic acid, erythromycin, flumequine, fosfomycin, oxytetracycline, doxycycline and tetracycline, in addition intermediate resistance rate to kanamycin and chloramphenicol, the antimicrobial susceptibility test revealed that most of the *V. parahaemolyticus* isolates was susceptible to oxolonic acid (76.6%). It is consistent with the findings from Ottaviani et al. (2001), Akinbowale et al. (2006), and Liu et al. (2008), who found that the sensitivity percentage of the isolates is 97.18%, 100%, and 100%, respectively. Therefore, these antibiotics might be utilized to treat *V. parahaemolyticus* infections efficiently.

In this study, the MAR index of *V. parahaemolyticus* is 0.81. A MAR index greater than 0.2 is a variable used to detect the spread of bacterial resistance in a certain population. A MAR index greater than 0.2 indicates that such bacteria strains came from high-risk sources such as a habitat where various antibiotics are used and antibiotic misuse in aquatic and environmental systems (Tang, et al. 2014; Drais, et al. 2016;

Ghenem & Elhadi, 2017 Tan, et al. 2020; Zaher, et al. 2021). Whereas, isolates having MAR indices less than 0.2 are less likely to be exposed to antibiotics. Thus, from this study, it showed that the samples were highly exposed to the tested antibiotics. This is not unexpected given that *V. parahaemolyticus* has demonstrated resistance to 13 antibiotics out of 16 tested. But, the MAR indices differ throughout research, with Ahmad et al. (2018) showing MAR indices ranging from 0.58 to 1, while Yu et al. (2016) reporting MAR indices ranging from 0.11 to 0.22 for *V. parahaemolyticus* isolates. Furthermore, the most recent study found a range of MAR indices of *V. parahaemolyticus* isolates, ranging from 0.04 to 0.71 for Tan et al. (2020) and 0.587 for Zaher et al. (2021). It is noted that the MAR index may represent the level of antimicrobial contamination; however, due to the number of antibiotics and types of antibiotics employed in the tests, it may not be suitable for comparison.

CHAPTER 5

CONCLUSION

5.1 Conclusion

In conclusion, this is a study that isolate and identify *Vibrio parahaemolyticus* from *Anabas testudineus* and the antibiogram of isolated *V. parahaemolyticus*. This study revealed a useful information on the resistance pattern of *V. parahaemolyticus* towards several antimicrobials. Except for oxolonic acid (2 µg), no other antimicrobials were found effective against all the isolates. The multiple antibiotic resistance (MAR) index result show that the *V. parahaemolyticus* highly exposed to the antimicrobials and may pose danger in the future. Thus, bacterial population in the ornamental fish sector must be approached with precaution in order to prevent the formation of antimicrobial resistance strains. In addition, regular monitoring and surveillance programs should be continuous in order to prevent the potential spread of outbreak.

REFERENCES

Amalina, N.Z., Santha, S., Zulperi, D. et al. Prevalence, antimicrobial susceptibility and plasmid profiling of Vibrio spp. isolated from cultured groupers in Peninsular Malaysia. BMC Microbiol 19, 251 (2019).

Akinbowale, O. L., Peng, H. and Barton, M. D. (2006). Antimicrobial resistance in bacteria isolated from aquaculture sources in Australia

Ashrafudoulla, M., Na, K. W., Hossain, M. I., Mizan, M. F. R., Nahar, S., Toushik, S. H., Roy, P. K., Park, S. H. and Ha, S. D. (2021). Molecular and pathogenic characterization of Vibrio parahaemolyticus isolated from seafood. Marine Pollution Bulletin 172 (2021) 112927

Banerjee, S., Ooi, M. C., Shariff, M., & Khatoon, H. (2012). Antibiotic resistant Salmonella and Vibrio associated with farmed Litopenaeus vannamei. TheScientificWorldJournal, 2012, 130136. https://doi.org/10.1100/2012/130136

Carlson, J. M., Leonard A. B., R Hyde, E., Petrosino, J. F. and P Primm, T. (2017). Microbiome disruption and recovery in the fish Gambusia affinis following exposure to broad-spectrum antibiotic. Infect Drug Resist.

Chakraborty, R. D., Surendran, P. K. and Joseph, T. C. (2008). Isolation and characterization of Vibrio parahaemolyticus from seafoods along the southwest coast of India.

Devi, R., Surendran, P. K. & Chakraborty, K. (2009) Antibiotic resistance and plasmid profiling of Vibrio parahaemolyticus isolated from shrimp farms along the southwest coast of India. World J. Microbiol. Biotechnol. 25 (11), 2005–2012.

de Souza Santos, M., Salomon, D., Li, P., Krachler, A-M., Orth, K. (2015). The Comprehensive Sourcebook of Bacterial Protein Toxins || Vibrio parahaemolyticus virulence determinants. (2015), 230–260.

Drais, A. A., Usup, G. and Ahmad, A. (2016). Antibiotic Resistance of Vibrio Parahaemolyticus Isolated from Coastal Seawater and Sediment in Malaysia.

Dutta, D., Kaushik, A., Kumar, D. and Bag, S. (2021). Foodborne Pathogenic Vibrios: Antimicrobial Resistance. Front. Microbiol. 12:638331.

Ghenem, L., Elhadi, N. (2017). Isolation, molecular characterization, and antibiotic resistance patterns of Vibrio parahaemolyticus isolated from coastal water in the Eastern Province of Saudi Arabia. Journal of Water and Health.

He, Y., Jin, L., Sun, F., Hu, Q. & Chen, L. (2016). Antibiotic and heavy-metal resistance of Vibrio parahaemolyticus isolated from fresh shrimps in Shanghai fish markets, China. Environ. Sci. Pollut. Res. 23 (15), 15033–15040.

Kang, C. H., Shin, Y. J., Jang, S. C., Yu, H. S., Kim, S. K., An, S., Park, K., So, J S. (2017). Characterization of Vibrio parahaemolyticus isolated from oysters in

Korea: Resistance to various antibiotics and prevalence of virulence genes. Marine Pollution Bulletin

Khouadja, S., Lamari, F. and Bakhrouf, A. (2013). Characterization of Vibrio parahaemolyticus isolated from farmed sea bass (Dicentrarchus labrax) during disease outbreaks. International Aquatic Research (2013), 5:13.

Khouadja, S., Snoussi, M., Saidi, N. and Bakhrouf, A. (2013). Phenotypic characterization and RAPD fingerprinting of Vibrio parahaemolyticus and Vibrio alginolyticus isolated during Tunisian fish farm outbreaks. Folia Microbiol (2013) 58:17–26

Ladisa, C. (2017). Overview of Ornamental Species Aquaculture. FAO Aquaculture Newsletter.

Lewbart, G.A. (2001). Bacteria and ornamental fish. , 10(1), 0–56.

Lee, L-H., Ab Mutalib, N-S., Law, JW-F., Won,g S. H. and Letchumanan, V. (2018). Discovery on Antibiotic Resistance Patterns of Vibrio parahaemolyticus in Selangor Reveals Carbapenemase Producing Vibrio parahaemolyticus in Marine and Freshwater Fish. Front. Microbiol. 9:2513.

Livengood, E. J. & Chapman, F. A. (2007). The Ornamental Fish Trade: An Introduction with Perspectives for Responsible Aquarium Fish Ownership. EDIS 2007 (16).

Liu, P.-C., Chen, Y.-C., Huang, C.-Y. and Lee, K.-K. (2008). Virulence of Vibrio parahaemolyticus isolated from cultured small abalone, Haliotis diversicolor supertexta, with withering syndrome.

Marudhupandi, T., Kumar, T. T. A., Prakash, S., Balamurugan, J., Dhayanithi, N. B. (2017). Vibrio parahaemolyticus a causative bacterium for tail rot disease in ornamental fish, Amphiprion sebae. Aquaculture Reports, 8(2017), 39–44.

Miller, S. M. and Mitchell, M. A. (2009). Manual of Exotic Pet Practice.

Mog, M., Ngasotter, S., Tesia, S., Waikhom, D., Panda, S., Sharma, S. and Varshney, S. (2020). Problems of antibiotic resistance associated with oxytetracycline use in aquaculture

Qianqian Yu, Mengya Niu, Mengquan Yu, Yanhong Liu, Dapeng Wang and Xianming Shi. (2015). Prevalence and antimicrobial susceptibility of Vibrio parahaemolyticus isolated from retail shellfish in Shanghai. Food Control, (2015), S0956713515301407–.

Ottaviani, D., Bacchiocchi, I., Masini L., Leoni, F., Carraturo, A., Giammarioli, M. and Sbaraglia, G. (2001). Antimicrobial susceptibility of potentially pathogenic halophilic vibrios isolated from seafood. International Journal of Antimicrobial Agents 18 (2001) 135–140

Pérez, D. S., Tapia, M. O., & Soraci, A. L. (2014). Fosfomycin: Uses and potentialities in veterinary medicine. Open veterinary journal, 4(1), 26–43.

Ramamurthy, T. and Nair, GB. (2014). Encyclopedia of Food Safety || Bacteria: Vibrio parahaemolyticus. (2014), 555–563.

Rodríguez, R. d. R., (2006). Ornamental Fish: Economic, Ecologic and Health Implications of The Trade. Vol 16(1)

Rukmana N. R., Mahasri, G., Hidayah, S. N., Ulkhaq, M. F. and Kenconojati, H. (2019). Bacterial Identification from Marine Ornamental Fish in Fish Quarantine, Quality Control and Fishery Products Safety Class I Denpasar, Bali.

Saad El-deen, A. G. and Elkamel, A. A. (2015). Clinical and Experimental Study on Vibriosis in Ornamental Fish.

Saha, D. and LaRocque, R. C. (2013). Hunter's Tropical Medicine and Emerging Infectious Disease || Cholera and Other Vibrios. (2013), 448–453.

Silvester, R., Alexander, D. & Ammanamveetil, M. H. A. (2015). Prevalence, antibiotic resistance, virulence and plasmid profiles of Vibrio parahaemolyticus from a tropical estuary and adjoining traditional prawn farm along the southwest coast of India. Ann. Microbiol. 65 (4), 2141–2149.

Shaw, K. S., Rosenberg Goldstein, R. E., He, X., Jacobs, J. M., Crump, B. C. & Sapkota, A. R. (2014). Antimicrobial susceptibility of Vibrio vulnificus and Vibrio parahaemolyticus recovered from recreational and commercial areas of Chesapeake Bay and Maryland Coastal Bays. PLoS One 9 (2), e89616.

Sudha, S., Mridula, C., Silvester, R. and Hatha, A. A. M. (2014). Prevalence and Antibiotic Resistance of Pathogenic Vibrios in Shellfishes from Cochin Market. Indian J. Geo-Mar. Sci. 43 (5), 815–824.

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. and Radu, S. (2017). Prevalence and Antimicrobial Susceptibility of Vibrio parahaemolyticus Isolated from Short Mackerels (Rastrelliger brachysoma) in Malaysia. Front. Microbiol. 8: 1087.

Tan, C. W., Rukayadi, Y., Hasan, H., Thung, T. T., Lee, E., Rollon, W. D., Hara, H., Kayal, A. Y., Nishibuchi, M. and Radu, S. (2020). Prevalence and antibiotic resistance patterns of Vibrio parahaemolyticus isolated from different types of seafood in Selangor, Malaysia. Saudi Journal of Biological Sciences

Tang, J. Y. H., Wan-Rosli, W. F., Abdul-Razak, N. H., Yeo, C. C., Abu Bakar, C. A. and Son, R. (2014). Incidence and antibiogram of Vibrio parahaemolyticus in processed and frozen bivalve mollusks in Kuala Terengganu, Malaysia. International Food Research Journal 21(3): 1349-1353 (2014)

Todd. (2014). Foodborne Diseases: Overview of Biological Hazards and foodborne Diseases, (2014), 221-242.

Tunung, R., Jeyaletchumi, P., Noorlis, A., Tang, Y. H., Sandra, A., Ghazali, F. M., Noranizan, M. A., Lesley, M. B., Haresh, K. K., Nakaguchi, Y., Nishibuchi, M. and Son, R. (2012). Biosafety of Vibrio parahaemolyticus from vegetables based

on antimicrobial sensitivity and RAPD profiling. International Food Research Journal 19(2): 467-474

Whittington, R. J. & Chong, R. (2007). Global trade in ornamental fish from an Australian perspective: The case for revised import risk analysis and management strategies.81(1-3), 92–116.

Xu, X., Cheng, J., Wu, Q., Zhang, J. and Xie, T. (2016). Prevalence, characterization, and antibiotic susceptibility of Vibrio parahaemolyticus isolated from retail aquatic products in North China. BMC Microbiology (2016) 16:32

Yaashika, P. R., Saravanan, A. and Kumar, P. S. (2016). Isolation and identification of Vibrio cholerae and Vibrio parahaemolyticus from prawn (Penaeus monodon) seafood: Preservation strategies. Microbial Pathogenesis, 99(2016), 5–13

You, K. G., Bong, C. W. and Lee, C. W. (2016). Antibiotic resistance and plasmid profiling of Vibrio spp. in tropical waters of Peninsular Malaysia. Environ Monit Assess. (2016), 188: 171

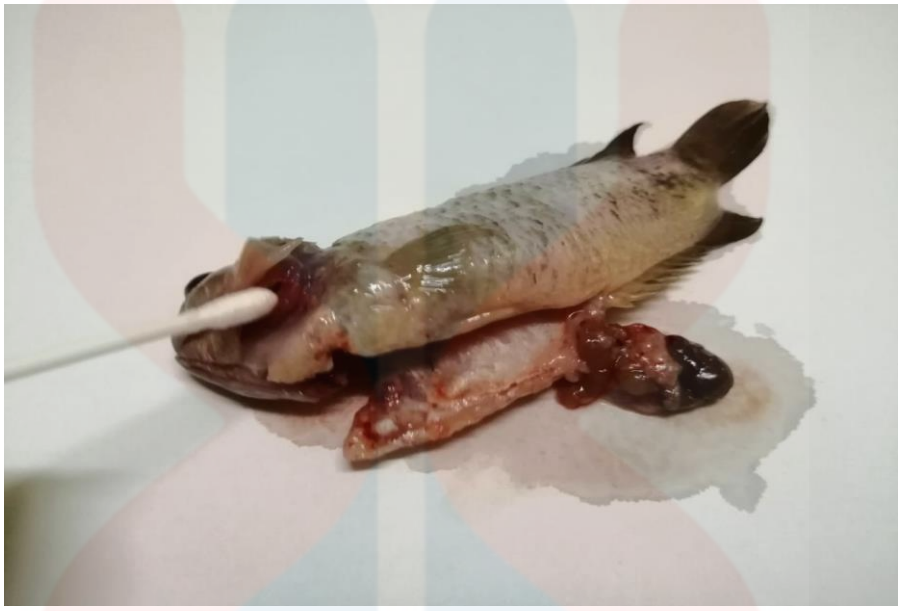
Yu, Q., Niu, M., Yu, M., Liu, Y., Wang, D., Shi, X. (2016). Prevalence and antimicrobial susceptibility of Vibrio parahaemolyticus isolated from retail shellfish in Shanghai. Food Control. 60, 263–268.

Zaher, H.A., Nofal, M.I., Hendam, B.M., Elshaer, M.M., Alothaim, A.S. and Eraqi, M.M. (2021). Prevalence and Antibiogram of Vibrio parahaemolyticus and Aeromonas hydrophila in the Flesh of Nile Tilapia, with Special Reference to Their Virulence Genes Detected Using Multiplex PCR Technique. Antibiotics 2021, 10, 654.

Zago, V., Veschetti, L., Patuzzo, C., Malerba, G., Lleo, M. M. (2020). Resistome, Mobilome and Virulome Analysis of Shewanella algae and Vibrio spp. Strains Isolated in Italian Aquaculture Centers. Microorganisms, 8(4), 572

APPENDIX A

Tittle: The Isolation of The Bacteria from Gill



Tittle: A Plate of Novobiocin Antimicrobial Activity Test on *Vibrio Parahaemolyticus* Isolate