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Antibiogram of *Aeromonas hydrophila* isolated from diseased Blood Red Parrot Cichlid fish *(Amphilophus Citrinellus x Heros Severus)* of aquarium shop

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DECLARATION

I hereby declare that the work embodied here is the result of my own research except for the except as cited in the references.

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Antibiogram of *Aeromonas hydrophila* isolated from diseased Blood Red Parrot Cichlid fish *(Amphilophus Citrinellus x Heros Severus)* of aquarium shop

ABSTRACT

In the current study, an antibiogram of *Aeromonas hydrophila* isolated from diseased Blood Red Parrot Cichlid fish of aquarium shop was carried out. Ornamental fish have been kept as a pet because of their attractiveness based on their body color, body shape, sizes, behaviors, and the way they are swimming such as Blood Red Parrot Cichlid fish with their unique characteristic. Blood Red Parrot Cichlid fish is one of the famous hybrid ornamental fish. They were valued for their calm personality and ability to live in a community tank with the same fish species or other peaceful fish communities. Ornamental fish market demand has been increased lately in Malaysia. However, *A. hydrophila* has been a threat to ornamental fish since it will cause bacterial disease on fish and lead to mortality. The bacteria had been infected the fish organs and ulcers of the skin. Although there are a few research about the isolation of *Aeromonas hydrophila* from infected ornamental fish, there is insufficient information on the most suitable antibiotic that applies to treat *A. hydrophila* infection on ornamental fish. The method that will be used in this study is the antibiotic sensitivity test. The present bacteria was subjected to this test by using the disk diffusion method with a total of 16 types of antibiotics was used in this study. The 16 types of antibiotics are amoxycillin 25µg, amplicillin 10µg, chloramphenicol 30µg, compound sulphonamides 300µg, doxycycline 30µg, erythromycin 15µg, flumequine 30µg, fosfomycin 50µg, kanamycin 30µg, nalidixic acid 30µg, novobiocin 30µg, oxolinic acid 2µg, oxytetracycline 30µg, sulphamethoxazole 25µg, spiramycin 100µg, and tetracycline 30µg. Based on the antibiotic sensitivity results, the most suitable antibiotic that can be applied on *A. hydrophila* disease-infected ornamental fish is erythromycin (E) and flumequine (UB) as both of these antibiotics showed the highest sensitivity in this study. In the interval, it could decide that these antibiotics should replace the existing antibiotic for the *A. hydrophila* disease treatment.

Keywords: antibiogram, *Aeromonas hydrophila*, ornamental fish, Blood Red Parrot Cichlid fish, antibiotic

Pengambilan antibiogram *Aeromonas Hydrophila* daripada ikan Parrot Merah Darah *(Amphilophus Citrinellus x Heros Severus)* yang berpenyakit di kedai akuarium

ABSTRAK

Dalam kajian semasa, pengambilan antibiogram *Aeromonas hydrophila* daripada ikan Parrot Merah Darah yang berpenyakit di kedai akuarium telah dijalankan. Ikan hiasan telah dipelihara sebagai haiwan peliharaan kerana daya tarikannya berdasarkan warna badan, bentuk badan, saiz, tingkah laku dan cara mereka berenang seperti ikan Parrot Merah Darah dengan ciri uniknya. Ikan Parrot Merah Darah ialah salah satu ikan hiasan hibrid yang terkenal. Mereka bernilai kerana personaliti mereka yang tenang dan keupayaan untuk hidup dalam tangki komuniti dengan spesies ikan yang sama atau komuniti ikan damai yang lain. Permintaan ikan hiasan dalam pasaran telah meningkat sejak kebelakangan ini di Malaysia. Walau bagaimanapun, *A. hydrophila* telah menjadi ancaman kepada ikan hiasan kerana ia akan menyebabkan penyakit bakteria pada ikan dan membawa kepada kematian. Bakteria telah menjangkiti organ ikan dan ulser pada kulit. Walaupun terdapat beberapa kajian tentang pengambilan *Aeromonas hydrophila* daripada ikan hiasan yang dijangkiti, terdapat maklumat yang kurang tentang antibiotik yang paling sesuai digunakan untuk merawat jangkitan *A. hydrophila* pada ikan hiasan. Kaedah yang akan digunakan dalam kajian ini ialah ujian sensitiviti antibiotik. Bakteria yang hadir telah dikenakan ujian ini dengan menggunakan kaedah reserapan cakera dengan sejumlah 16 jenis antibiotik yang digunakan dalam kajian ini. 16 jenis antibiotik itu adalah amoxycillin 25µg, amplicillin 10µg, chloramphenicol 30µg, compound sulphonamides 300µg, doxycycline 30µg, erythromycin 15µg, flumequine 30µg, fosfomycin 50µg, kanamycin 30µg, nalidixic acid 30µg, novobiocin 30µg, oxolinic acid 2µg, oxytetracycline 30µg, sulphamethoxazole 25µg, spiramycin 100µg, and tetracycline 30µg. Berdasarkan keputusan sensitiviti antibiotik, antibiotik yang paling sesuai yang boleh digunakan pada ikan hiasan yang dijangkiti penyakit *A. hydrophila* ialah erythromycin (E) dan flumequine (UB) kerana kedua-dua antibiotik ini menunjukkan sensitiviti yang paling tinggi dalam kajian ini. Dalam selang waktu itu, ia dapat memutuskan bahawa antibiotik ini dapat menggantikan antibiotik sedia ada untuk rawatan penyakit *A. hydrophila*.

Kata kunci: antibiogram, *Aeromonas hydrophila,* ikan hiasan, ikan Parrot Merah Darah, antibiotik

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LIST OF ABBREVIATIONS

FAO	Food and Agriculture Organization
MCO	Movement Control Order (MCO) by Malaysia Government
SOP	Standard Operating Procedure
US	United States
AAPMA	American Pet Products Association
MAS	Motile Aeromonas Septicemia
AIM	Aeromonas Isolation Medium
TSA	Tryptic Soya Agar/Tryptone Soy Agar
NaCl	Sodium Chloride
ECP	Extracellular Products
TSB	Tryptic Soy Broth
SDA	Saboraud Dextrose Agar
MAR	Multiple Antibiotic-Resistant
CB	Carbenicillin
FOX	Cefoxitin
CIP	Ciprofloxacin
AMG	Aminoglycoside
QNs	Quinolones
SXT	Trimethoprim/sulfamethoxazole
NEO	Neomycin
AMK	Amikacin
GEN	Gentamicin
NOR	Norfloxacin
TMP	Trimethoprim
AML	Amoxycillin

AMP	Amplicillin
C	Chloramphenicol
CL	Cloxacillin
DO	Doxycycline
E	Erythromycin
FLO	Florfenicol
UB	Flumequine
FOF	Fosfomycin
K	Kanamycin
LCM	Lincomycin
NA	Nalidixic Acid
NV	Novobiocin
OM	Oleandomycin
OA	Oxolonic Acid
OT	Oxytetracycline
S3	Compound Sulphonamides
SMX	Sulphamethoxazole
SP	Spiramycin
S	Streptomycin
TE	Tetracycline
CLSI	Clinical Laboratory and Standards Institute
USD	United States Dollars
RM	Ringgit Malaysia
S	Sensitive
I	Immediate sensitive
R	Resistance
B	Beta

LIST OF SYMBOLS

h	Hour
min	Minutes
%	Percentage
ml	Millilitre
mm	Millimetre
°C	Degree Celcius
µg	Microgram
µm	Micrometre
µl	Microlitre

LIST OF EQUATIONS

3.2.3 MAR Index = Number of Antibiotics To Which The Isolate Showed Resistance / Number of Total Antibiotics Exposed To The Isolate

CHAPTER 1

INTRODUCTION

1.1 Research Background

Ornamental fish has been defined by the Law Insider dictionary as any species of aquatic animals that are reared or marketed for their beauty or exotic characteristics, rather than for consumptive or recreational use. It can be classified as one type of pet and is usually kept in an aquarium and fishpond. They come from many species. They have been kept because of their attractiveness based on their body color, body shape, sizes, behaviors, and the way they are swimming such as Blood Red Parrot Cichlid fish with their unique characteristic. Blood Red Parrot Cichlid fish is one of the famous hybrid ornamental fish with the scientific name *Amphilophus Citrinellus x Heros Severus*. They were valued for their calm personality and ability to live in a community tank with the same fish species or other peaceful fish communities. The ornamental fish market and trade got many interesting tricks and secrets to create a beautiful creature of ornamental fish. In ancient times, the Chinese, Japanese, Egyptians, and Romans would be the first to enjoy ornamental fish keeping (Swann, 1992). The ornamental fish trade is worth around USD 278 million worldwide (FAO, 2005), and the aquarium industry is worth more than USD 1 000 million (Cato & Brown, 2003). In Malaysia, Johor was one of the first fish dealers and has since risen to the top of the industry, which is worth RM 353 million (Ng C., 2016).

Ornamental fish care is fully dependent on their owner since they are kept in one place. Owners of pets including ornamental fish in England and Wales are required by the Animal Welfare Act 2006 should ensure that all animals they are responsible for stay healthy and happy. In general, the Act holds owners and keepers committed to guaranteeing that their animals' welfare needs are met, including the environment, diet, and safety from damage or other else (Animal Welfare Foundation, n.d.). Therefore, the water quality of Blood Red Parrot Cichlid fish that is kept is a crucial part to maintain their health and free from any diseases.

The most common infectious problem of ornamental fishes is bacterial diseases that cause by problems in water quality. The uncleaned or problematic water quality could be a site for bacteria to breed and bacterial disease will occur. The majority of bacterial infections occurred in ornamental fish are caused by Gram-negative organisms including the following pathogenic genera such as *Aeromonas*, *Citrobacter*, *Edwardsiella*, *Flavobacterium (Flexibacter)*, *Mycobacterium*, *Pseudomonas*, *and Vibrio*, and *Streptococcus*, a Gram-positive genus (Greg Lewbart M.S. et al., 2001). However, bacterial disease not only occurs due to poor water quality but also could occur due to external stress such as shipping, crowding, and inadequate nutrition.

Aeromonas hydrophila is a ubiquitous gram-negative, motile, rod-shaped that can commonly be isolated from freshwater ponds and also a normal inhabitant of the gastrointestinal tract of fish (Randy White, n.d.). It has been a threat to ornamental fish since it will cause bacterial disease on fish and lead to mortality. The bacteria will infect the fish organs and ulcers of the skin. There have been reports of *A. hydrophila* is stand as a primary pathogen in fish. Their isolation differs greatly in their pathogenicity with some strains being highly virulent and others non-virulent. Disease caused by *A. hydrophila* such as "Hem-morhagicSepticemia", "Motile Aeromonas Septicemia", "Ulcer Disease", or "Red-Sore Disease". The disease caused by these bacteria has given a big economic impact on the ornamental fish market.

In the present study, *A. hydrophila* was isolated from diseased Blood Red Parrot Cichlid fish of aquarium shop and an antibiogram test is carried out. The antibiogram test was carried out by using the disk diffusion method. The test was identified the most suitable antibiotics for treatment purposes on the *A. hydrophila* disease infected ornamental fish based on 16 types of antibiotics that was use in this study.

1.2 Problem Statement

Ornamental fish are mostly infected by bacterial disease due to poor water quality since ornamental fish need high care from the owner and producer. The bacterial disease that infected them has been caused mortality and morbidity among ornamental fish. It also would affect the ornamental fish market since it has been in high demand among Malaysians and other countries too. Therefore, this study was designed to isolate and identify *Aeromonas hydrophila* from the diseased Blood Red Parrot Cichlid in order to find the most suitable antibiotic to apply to the infected ornamental fish.

Recently, *A. hydrophila* has been found as the primary pathogen in fish and has mostly been found in them. *A. hydrophila* is the most organism related to the aquatic environment. *A. hydrophila* is typically recognized as an opportunistic pathogen or secondary invader (Austin and Austin, 1987). It can be said that these bacteria will easily infect the ornamental fish so that, it is important to carry this study to identify the find the most suitable antibiotic to apply on the infected ornamental fish. Hence, the morbidity caused by *A. hydrophila* could be reduced and eliminated.

1.3 Objectives

The objectives of the current study are :

1. To isolate and identify *Aeromonas hydrophila* from Blood Red Parrot Cichlid fish.
2. To characterize the antibiogram of isolated *Aeromonas hydrophila*.

1.4 Scope of Study

Microbiology

1.5 Significance of Study

In the present study, the diseased Blood Red Parrot Cichlid fish from the aquarium shop was identified and monitored. The antibiogram test of isolated *Aeromonas hydrophila* was characterized the most suitable antibiotic to be applied on the *A. hydrophila* disease-infected ornamental fish for the future treatment procedure. *A. hydrophila* has also been said as the primary pathogen in fish by a few research. Besides, there is not much research on the ornamental fish diseases on which bacteria is causing the disease so that, the treatment that is applied to the ornamental fish may not effectively cure the disease. In this study, isolation, and identification of bacteria from diseased Blood Red Parrot Cichlid fish was fully understood and carried out carefully so that, the most suitable antibiotic was decided based on the specific bacteria, *A. hydrophila.*

Figure 1.1: The diseased Blood Red Parrot Cichlid fish from the aquarium shop

1.6 Limitation of Study

The limitation in this study is not much research about the antibiogram of *Aeromonas hydrophila* isolated from diseased ornamental fishes to be referred and limited of specific information that is related to the present study. Even there is a few research on antibiogram of *A. hydrophila* but they are more focusing on aquaculture species that can be eaten to study the effect on humans or potential health hazards. Hence, can't compare the suitable antibiogram result that comes out in this study with other researchers. In addition, the type of antibiotics needs to be added so that, could compare the results well with the others latest study. The findings that I found out were not many antibiotics that same as the current study to be compared of and it was quite difficult to determine the end results.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction to Ornamental Fish

Ornamental fishes are defined as attractive, colorful, and unique types of fishes that are being kept as a pet in one place to enjoy their beauty and give a pleasant pleasure in the eyes of the beholder. They usually being kept in aquariums and fish ponds (Miller, S. M et al., 2009). They have been kept because of their attractiveness based on their body colour, body shape, sizes, behaviors, and the way they swim. Ornamental fishes also being called “live jewels” for their beauty and color. There are many types and species of ornamental fishes such as guppy, gourami, swordtail, betta, goldfish, koi, and more. Keeping ornamental fishes as pets have a few advantages such as no messy home and no disruptive noise. Nowadays, the ornamental fishes trade is rising around the world. There are 259 ornamental fish exporters currently in Malaysia (Department of Fisheries, 2016). The collection, breeding, and marketing of ornamental fishes is being a great business opportunity and could generate higher income since the number of collectors of ornamental fishes is increasing rapidly. Although the European Union is the world's largest market for ornamental fish, the United States (US) is the world's largest importer of ornamental fish (FAO, 1996-2005; Chapman, 2000). The worldwide value of ornamental fish and invertebrates shipped into various countries is estimated to be 278 USD (FAO, 1996-2005). According to pet industry analysts, the aquarium industry is worth over a billion USD, despite the fact that some people keep their ornamental fish in ponds (Cato and Brown, 2003; AAPMA, 2005). In Malaysia, Johor has been the early fish dealer and become the clear market leader in the industry that valued at RM353 million (Ng. C, 2016). Since then, the ornamental fishes market and trade in Malaysia have been growing steadily and soared up.

2.2 Introduction to Blood Red Parrot Cichlid Fish

The Blood Red Parrot Cichlid fish is a hybrid ornamental fish that possibly crosses with Midas cichlid (*Amphilophus Citrinellus*) and the Gold Severum cichlid (*Heros Severus*) as shown in Figure 2.1 and Figure 2.2. The breeders also can't confirm its true parent species among them. It was first created in Taiwan in the 1980s. They were valued for their calm personality and ability to live in a community tank with the same fish species or other peaceful fish communities. Its body has a bright orange color and makes them noticeable compared to other fishes around in the same aquarium. They also got another patch of color on their body that naturally produced such as red, yellow, or grey. They are usually sold as blood parrots or bloody parrots and should not be mistaken with freshwater parrot cichlids (*Hoplarchus Psittacus*) or saltwater parrotfish (*Callyodon Fasciatus*). However, Blood Red Parrot Cichlid fish were not commonly found in pet stores before the year 2000, despite the fact that they have been on the market for some time. It was because of the controversy that was created by the original breeders of this species. People were banned from this species in the market because they felt that this species can’t be created based on the ethics of crossbreeding. They are concerned about this species due to some bordering on deformities that cause difficulties for the fish. The fish's mouth is relatively tiny and irregularly formed, which may hinder its ability to feed. When it comes to feeding time, Blood Red Parrot Cichlids fish may struggle to compete with tankmates that are more aggressive and have larger mouths. They also have spinal and swim bladder abnormalities that limit their swimming ability. Many people believe that creating a fish with such malformations is unethical and even cruel, and some ornamental fish fans have gone so far as to boycott stores that sell this hybrid.

Figure 2.1: Midas cichlid (*Amphilophus Citrinellus*) (Source: Zoo in Ukraine, n.d.)

Figure 2.2: Gold Severum cichlid (*Heros Severus*) (Source: petesaquariums.com, n.d.)

Based on research carried out by Naser. A et al (2020), one species of cichlid, *Amphilophus citrinellus*, and its hybrid the blood parrot cichlid (*Amphilophus citrinellus* × *Vieja melanura*) was recorded in the rice field ecosystem. They were believed that the Blood Red Parrot Cichlids fish found were possibly escapes or abandoned fish from aquarium dumping. The reason they were removed from the aquarium shop was because of lacked bright orange colour, deformities, or undesirable traits such as body shape and colors that were not favored for the ornamental fish trading. Cichlids have developed and become well-established throughout Peninsular Malaysia.

2.3 Introduction to *Aeromonas hydrophila*

Based on research carried out by Dias. D et al (2012), *Aeromonas* species has been found out as common inhabitants of aquatic ecosystems. 288 bacteria were isolates and seven different species are belong to this genus were found through genetic sequestration. *Aeromonas spp.* are commonly found in ornamental fish and have a wide range of species. *Aeromonas* is a genus of *Gammaproteobacteria* that belongs to the *Aeromonadaceae* family. *Aeromonas spp.* is Gram-negative, non-spore-forming, motile bacilli or coccobacilli rods with rounded ends measuring 1-3,5 µm in diameter. They are facultatively anaerobic, oxidase, catalase, and indol-positive, able to reduce nitrate to nitrite, and are usually immune to the vibriostatic agent O/129 since they ferment glucose. *Aeromonas spp*. can be found in a wide range of ecosystems and also can survive in any kind of water. *Aeromonas hydrophila* has been identified as causative agents or pathogens of bacterial fish disease as they are also the most common bacteria that are being found on infected fish.

Aeromonas hydrophila causes disease in fish known as "Motile Aeromonas Septicemia" (MAS), "Hemorrhagic Septicemia," "Ulcer Disease," or "Red-Sore Disease" that are present within numerous organs of the fish, and ulcers of the fish's skin (White. M. R, n.d). *A. hydrophila* is always able to produce disease if they got the chance and the symptoms are varied. The common causes of *A. hydrophila* diseases are not only because of poor water quality but also related to stress conditions such as transportation, poor handling, and poor nutrition. *A. hydrophila* is a usual flora as well as a primary and secondary fish pathogen that influences ornamental fish (Austin and Austin, 1999). There were reported that the most bacteria isolated from aquarium shops in Malaysia is *A. hydrophila* which is 60% of them.

2.4 Isolation and Identification of Bacteria

According to research conducted by Mamnur Rashid et al. (2014), fish that showed Motile Aeromonas Septicemia (MAS) like reddish head and anal region, pale body color and external haemorrhages is the common infection that caused by *Aeromonas hydrophila* were taken as samples. Intestine, liver, kidney, and environmental samples such as sediment, feed, and water are also being taken. Then, they were inoculated onto the Aeromonas Isolation Medium (AIM) and Tryptone Soya Agar (TSA) plates. The AIM colonies were undergone specific characterization for *Aeromonas hydrophila*. All the grown colonies of *Aeromonas spp.* from the streaked and spread plates of AIM were subcultured onto TSA plates to obtain fresh culture. The isolates gave rise to yellowish opaque colonies on TSA agar. Then, they have undergone morphological characteristics such as shape, size, Gram character, flagellation, and motility test. Physiological characters were studied by observing the growth of each isolate at temperatures of 4°C, 37°C, and 40°C in different concentrations of NaCl as 0%, 1%, 2%, 3.5%, and 4% to confirm the identification of the *A. hydrophila* bacteria. The result that came out shows that, need to take proper management practices to avoid bacterial diseases such as *Aeromonas hydrophila.*

Citarasu. T. et al. (2011) is isolated *A. hydrophila* from various tissues and body fluids of infected Koi (*Cyprinus carpio koi)* and goldfish (*Carrassius auratus)* samples were taken based on morphological and biochemical confirmative tests and identified the virulence factors. *A. hydrophila* produces a variety of biologically active extracellular products (ECP), including enzymes and hemolysin compounds (Wretlind et al., 1973). The dominant colonies were isolated from the hemorrhages as well as the body fluids of the infected fish. Triplicate samples were aseptically blended with Tryptic Soy Broth (TSB) and incubated at 37℃ overnight for enrichment. The culture was plated on Tryptic Soy Agar (TSA). Then, the confirmations test was run by observing their morphological, physiological, and biochemical based on the characteristics described in Bergey's Manual of Systematic Bacteriology (Holt et al., 1994). The result has shown that isolated *Aeromonas spp.* is dominant over the other bacteria by 80%. The other bacteria are *Vibrio spp.* (12%), *Bacillus spp.* (5%) and *E. coli* (3%) respectively.

According to a study carried out by Kusdarwati. R. et al. (2017), one of the causes of the infected catfish's high mortality has been *A. hydrophila and Saprolegnia sp*. In a short period of 1-2 weeks, *A. hydrophila* can harm fish farming and often triggers disease outbreaks, with mortality rates as high as 80% until 100%, resulting in significant losses in catfish farming. The medium used to isolate *A. hydrophila* was Trypticase Soy Agar (TSA), while Saboraud Dextrose Agar was used to isolate *Saprolegnia sp*p. (SDA). Bacterial identification was conducted with the macroscopic test, Gram staining, and biochemistry test. The result showed that all 20 catfish samples would have clinical symptoms such as wounds on the skin, as well as damaged fins and tails. A. hydrophila bacterial infection can demonstrate itself within four different stages: acute septicaemia, sub-acute septicaemia, chronic septicaemia, and latent septicaemia.

2.5 Antibiotic Sensitivity Test

Based on research carried out by Dias. D. et al (2012) on antimicrobial resistance patterns of *Aeromonas spp*. isolated from ornamental fish, *Aeromonas spp.* strains were isolated from the water and skin of ornamental fish and tested their resistance to antibiotics. The most common bacterial disease of freshwater fish are infections caused by motile aeromonads and antibiotics that are commonly used are no longer effective. This research used 28 antimicrobials that are commonly used by the disk-diffusion technique. A total number of 299 isolates were obtained from aquaria of ornamental fish shops and *A. hydrophila* is the most common bacteria. The results show that there are differences in some of the antibiotics tested according to the species and a high incidence of resistance of *Aeromonas* isolates to β-lactams antibiotics, as 95% were resistant to amoxicillin (AML), 96% to carbenicillin (CB), and 94% to ampicillin (AMP). There was a crossed multi-resistance between aminoglycoside (AMG), quinolones (QNs), tetracycline (TE), chloramphenicol (C), erythromycin (E), and trimethoprim/sulfamethoxazole (SXT) so that, it has been escalated their potential health hazard and be potential reservoirs of antibiotic resistance genes.

Jongjareanjai. M. et al. (2009) is research on In Vitro antibiotic susceptibility of *Aeromonas hydrophila* isolated from disease ornamental fish and 30 bacterial were isolated. The antibiotics susceptibility testing was performed by disc diffusion method, using 24 types of antibiotic discs (OXOID, Oxoid Ltd, UK). *A. hydrophila* has been recognized as a primary and secondary fish pathogen in ornamental fish (Austin and Austin, 1999), with the majority being isolated in this study. The results showed that 100% of the bacteria tested were resistant to metronidazole, 92.31% were resistant to penicillin and amoxicillin, 88.89% were resistant to colistin, 84.62% were resistant to oxytetracycline (OT), 83.33% were resistant to ampicillin (AMP), 81.82% were resistant to tetracycline (TE), 80.95% were resistant to neomycin (NEO), and 80.0% were resistant to novobiocin (NV). Chloramphenicol (C) showed the highest efficacy against the bacterial strains tested (59.09% sensitive and 31.82% resistant). Other effective antibiotics were sulphamethoxazole-trimethoprim (SXT) (58.33% susceptibility and 41.67% resistance), amikacin (AMK) (50.00% sensitive and 50.00% resistant). Antibiotic resistance was found in all bacterial strains, which was induced by antibiotic consumption in aquatic animals. According to Norvick (1981), the use of antibiotics at subtherapeutic levels in animal husbandry has encouraged the emergence and maintenance of multiple antibiotic-resistant (MAR) pathogenic bacteria.

Kanchan. C. et al. (n.d.) has been conducted research on the antibiotic susceptibility test of three strains of *Aeromonas hydrophila* isolated from diseased catfish by using the disk diffusion method. 13 types of antibiotics was involved which is amoxycillin (AML), ampicillin (AMP), cefoxitin (FOX), chloramphenicol (C), ciprofloxacin (CIP), erythromycin (E), gentamicin (GEN), kanamycin (K), novobiocin (NV), oxytetracycline (OT), norfloxacin (NOR), sulphamethoxazole-trimethoprim (SXT), and trimethoprim (TMP). A few species of fish have been observed being infected by *Aeromonas hydrophila* so that, *A. hydrophila* is an important bacterial pathogen in fish species based on a few research. Bacteria were isolated from the kidneys and liver of diseased catfish into Tryptic Soy agar (TSA, Difco, USA) plates and incubated at 30 °C until pure cultures were obtained. According to the results was obtained, the three strains of *A. hydrophila* were all resistant to amoxicillin (AML), trimethoprim (TMP), and novobiocin (NV) but susceptible to cefoxitin (FOX) and gentamicin (GEN). It has been suggested that gentamicin was an effective drug for curing bacterial pathogens namely *A. hydrophila* in diseased catfish.

CHAPTER 3

MATERIALS AND METHOD

3.1 Materials

3.1.1 Chemicals and Reagents

Diseased Blood Red Parrot Cichlid fish, GSP media (Oxoid, Basingstoke, UK), Tryptic Soy agar (TSA, Difco, USA), Tryptone Soy Broth (TSB, Himedia, India), distilled water, antibiotic test discs: amoxycillin (AML) 25µg, amplicillin (AMP) 10µg, chloramphenicol (C) 30µg, compound sulphonamides (S3) 300µg, doxycycline (DO) 30µg, erythromycin (E) 15µg, flumequine (UB) 30µg, fosfomycin (FOS) 50µg, kanamycin (K) 30µg, nalidixic acid (NA) 30µg, novobiocin (NV) 30µg, oxolinic acid (OA) 2µg, oxytetracycline (OT) 30µg, sulphamethoxazole (RL) 25µg, spiramycin (SP) 100µg, and tetracycline (TE) 30µg (OXOID, Oxoid Ltd, UK), alcohol, clove oil.

3.1.2 Equipment and Apparatus

Inoculating loop, spatula, electronic weighing balance (Shimadzu, Japan), Petri dishes, L-shaped hockey sticks, bijou bottles (Samco, England), Bunsen burner, pipette (Microlit, India), laboratory film (Parafilm M, USA), test tube, Durham tube, cotton bud (Premier, Malaysia), vortex, BBL Crystal kit (BD, USA), forceps, zipper plastic bag, filter papers (Smith, UK), scalpel (Albion, UK), laminar flow (Esco, Malaysia), incubator (Jeiotech, Korea).

3.2 Methods

3.2.1 Sample Collection and Preparation

The diseased Blood Red Parrot Cichlid fish was collected from a local aquarium shop in Kota Bharu, Kelantan. The samples were collected in sterile polyethylene bags and transported to the laboratory in an icebox. The samples were processed for bacteria isolation within 2h after collection. The Blood Red Parrot Cichlid fish was euthanized humanely by using clove oil. The clove oil was mixed in lukewarm water and poured into the fish container. After euthanization, the fishes were dissected using a scalpel to swab its body and organ. All the processed had to be done as soon as possible.

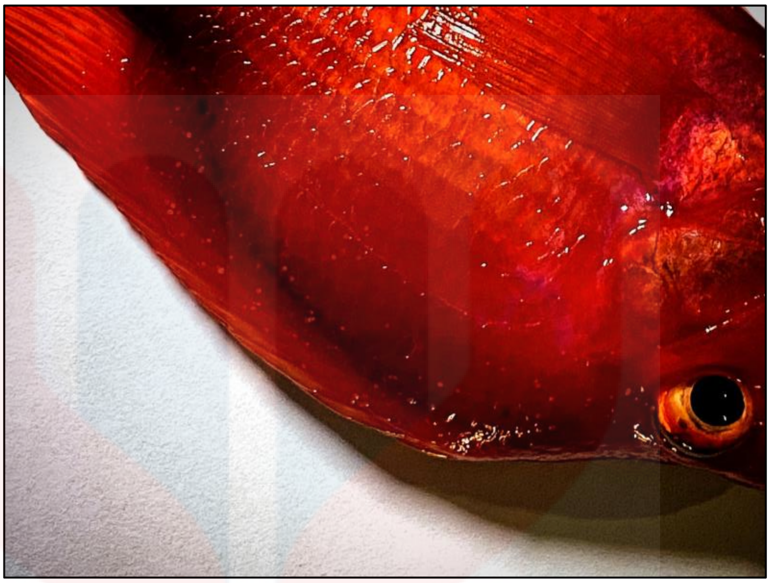

Figure 3.1: The diseased Blood Red Parrot Cichlid fish with white spot

Figure 3.2: The dissected Blood Red Parrot Cichlid fish

3.2.2 Isolate and identify *Aeromonas hydrophila* from samples

1. Isolation of *Aeromonas hydrophila* by using spread plate

The dissected fish sample was swabbed using sterile cotton bud on its abdomen (A), eyes (E), gill (G), kidney (K), and skin (S). There is a total of 30 samples that were obtained from swabbed its body. The swab cotton buds were streaked immediately on GSP media agar (Oxoid, Basingstoke, UK) by using the spread plate method. This media was used to isolate typical colonies, such as yellow on GSP agar, and purify the strains (Dias. C, 2012). The plate was sealed and incubated at 30℃ for 24h to obtain the pure culture.

Figure 3.3: Isolation of present bacteria by swabbed the fish skin (S)

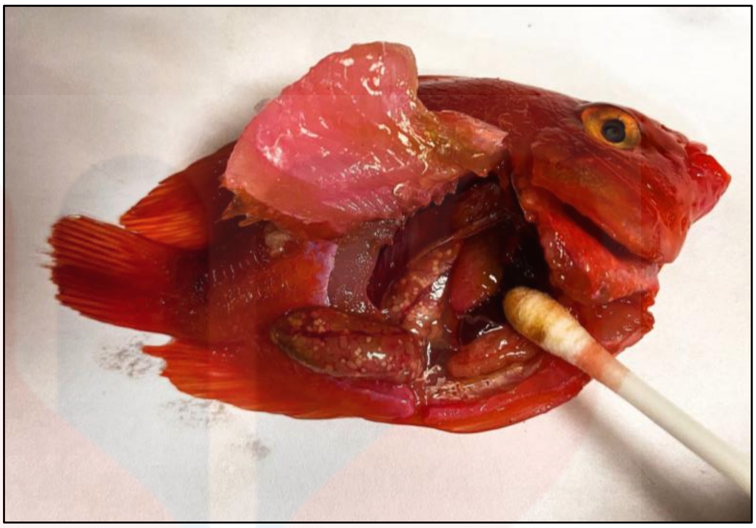

Figure 3.4: Isolation of present bacteria by swabbing the fish organs

2. Selection and inoculation of typical single colonies into Tryptone Soy Broth (TSB)

The single colonies were selected for each sample by using a sterile wire loop from the plates with GSP agar. Next, every single colony was inoculated into Tryptone Soy Broth (TSB) (Himedia, India) and vortex for a few seconds. Then, the sample was incubated at 27°C for 24h in the incubator (Jeiotech, Korea). The isolated Aeromonas hydrophila was identified using extensive morphological, physiological, and biochemical analysis.

3. Bacteria identification using BBL Crystal kit

The inoculum fluid TSB was poured into the target area base by filling the entire well. The lid was placed back until it snapped into place. After incubated at 27°C for 24h in the incubator (Jeiotech, Korea), the color change on the kit was observed by referring to the color reaction chart. The data were generated in the coding by using software provided by the manufacturer.

Figure 3.5: BBL Crystal kit for bacteria identification

3.2.3 Antibiotic Sensitivity Test

A. hydrophila which is isolated from diseased Blood Red Parrot Cichlid fish was undergo an antibiotic sensitivity test by the disc diffusion method. Total 16 types of antibiotics were used which is amoxycillin (AML) 25µg, amplicillin (AMP) 10µg, chloramphenicol (C) 30µg, compound sulphonamides (S3) 300µg, doxycycline (DO) 30µg, erythromycin (E) 15µg, flumequine (UB) 30µg, fosfomycin (FOS) 50µg, kanamycin (K) 30µg, nalidixic acid (NA) 30µg, novobiocin (NV) 30µg, oxolinic acid (OA) 2µg, oxytetracycline (OT) 30µg, sulphamethoxazole (RL) 25µg, spiramycin (SP) 100µg, and tetracycline (TE) 30µg (OXOID, Oxoid Ltd, UK).

A sterile cotton bud was soaked in each bijou bottle for a few min. Then, excess liquid on cotton buds was removed and swabbed onto plates with Tryptic Soy agar (TSA, Difco, USA). Triplicate of plates was done with TSA for each inoculum. The process was continued for all incubated bijou bottles. Sterile punched filter papers were soaked in selected 16 types of antibiotics for a few seconds before being placed on the TSA. Six antibiotics discs for each TSA media were placed by using forceps. The plates were sealed using parafilm and incubated at 27°C for 24h.

After the incubation, the diameter of the inhibition zone was measured in millimeters (mm) using Vernier calliper and recorded in a table. The inhibition zone is a clear region that indicated the absence of microbial growth effect of exposure to the antibiotic. The zone of inhibition was classified into three groups which are resistance (less than 15mm), immediate sensitive (16-18mm), and sensitive (more than 19mm) according to Clinical Laboratory and Standards Institute (CLSI, 2017).

Multiple antibiotic resistance (MAR) index is used to identify the isolates are either from high or low antibiotic use regions which related with health risk assessment. The MAR index which is more than 0.2 showed that the ornamental fish is highly exposed to antibiotics that are used from contamination sources while the MAR index less than 0.2 is less exposed to antibiotics in which the fish rarely used antibiotics. MAR index is calculated by using the following formula (Lee and Wee, 2012):

$$\text{MAR Index} = \frac{\text{Number of antibiotics to which the isolate showed resistance}}{\text{Number of total antibiotics exposed to the isolate}} \qquad (3.1)$$

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Results of Antibiogram Test

In the present study, 30 samples of Blood Red Parrot Cichlid fish that was swabbed from different places of body and organs which is abdomen (A), eyes (E), gill (G), kidney (K), and skin (S) was subjected to antibiogram test. The antibiogram test had been used the disk diffusion method and 16 types of antibiotics namely, amoxycillin (AML) 25µg, amplicillin (AMP) 10µg, chloramphenicol (C) 30µg, compound sulphonamides (S3) 300µg, doxycycline (DO) 30µg, erythromycin (E) 15µg, flumequine (UB) 30µg, fosfomycin (FOS) 50µg, kanamycin (K) 30µg, nalidixic acid (NA) 30µg, novobiocin (NV) 30µg, oxolinic acid (OA) 2µg, oxytetracycline (OT) 30µg, sulphamethoxazole (RL) 25µg, spiramycin (SP) 100µg, and tetracycline (TE) 30µg. The samples isolated had been evaluated by three groups which are resistance (less than 15mm), immediate sensitive (16-18mm), and sensitive (more than 19mm) according to Clinical Laboratory and Standards Institute (CLSI, 2017) based on their zone of inhibition as shown in Table 4.1.

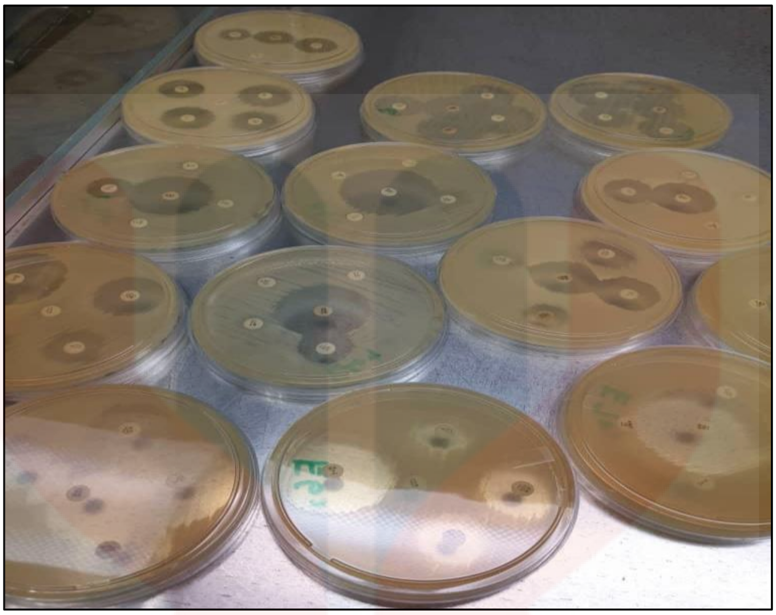

Figure 4.1: Zone of inhibition of each antibiotic type after undergoing the disk diffusion method

Based on the Table 4.1, show that the results of the antibiogram test of *Aeromonas hydrophila* isolates from Blood Red Parrot Cichlid fish sample was resistant to amoxycillin (AML), amplicillin (AMP), compound sulphonamides (S3), novobiocin (NV), and sulphamethoxazole (RL) with 83.3%, 80.0%, 96.7%, 63.3%, and 83.3% respectively. Then, erythromycin (E), flumequine (UB), fosfomycin (FOS), nalidixic acid (NA), oxytetracycline (OT), and spiramycin (SP) was reported sensitive with the *A. hydrophila* by 73.3%, 73.3%, 66.7%, 66.7%, 70.0%, and 70.0% respectively and the greatest number of antibiotics in cases. The others type of antibiotics was shown an immediate sensitive result with *A. hydrophila* which is chloramphenicol (C), 53.3%, doxycycline (DO), 60%, kanamycin (K), 83.3%, oxolonic acid (OA), 53.3%, and tetracycline (TE), 80.0% respectively. The sensitive cases have shown the highest cases in this study compared to the immediate sensitive and resistant cases of 16 types of antibiotics.

Table 4.1: The percentages of antibiogram test of *Aeromonas hydrophila* isolates from Blood Red Parrot Cichlid fish sample

Antibiotics (μg/disk)		**Sample (*Aeromonas hydrophila*)**		
		Sensitive, S (%)	**Immediate Sensitive, I (%)**	**Resistance, R (%)**
Amoxycillin	AML 25	0	16.7	83.3
Amplicillin	AMP 10	0	20.0	80.0
Chloramphenicol	C 30	20.0	53.3	26.7
Compound Sulphonamides	S3 300	0	3.3	96.7
Doxycycline	DO 30	33.3	60.0	6.7
Erythromycin	E 15	73.3	23.3	3.3
Flumequine	UB 30	73.3	23.3	3.3
Fosfomycin	FOS 50	66.7	23.3	10.0
Kanamycin	K 30	13.3	83.3	3.3
Nalidixic Acid	NA 30	66.7	33.3	0
Novobiocin	NV 30	10.0	26.7	63.3
Oxolonic Acid	OA 2	46.7	53.3	0
Oxytetracycline	OT 30	70.0	26.7	3.3
Sulphamethoxazole	RL 25	0	16.7	83.3
Spiramycin	SP 100	70.0	30.0	0
Tetracycline	TE 30	20.0	80.0	0

Hence, the data in the table was translated into a bar graph chart for a clear view of the present study results as shown in Figure 4.2. Based on the chart, the percentages results of antibiogram test of *A. hydrophila* isolates from Blood Red Parrot Cichlid fish sample with the highest percentage was the sensitive case which is 37.5% while resistance and immediate sensitive illustrated 31.25% of percentage for both cases. However, the highest sensitive type of antibiotics towards *A. hydrophila* is erythromycin (E) and flumequine (UB) by 73.3% for both types of antibiotics compared to the others. It had been shown that erythromycin (E) and flumequine (UB) were the most suitable antibiotics for treatment purposes on the *A. hydrophila* disease infected ornamental fish based on this present study. Besides that, the most resistant antibiotics towards *A. hydrophila* are compound sulphonamides (S3) with 96.7% and almost fully resistant. It is shown that compound sulphonamides (S3) are not suitable and designated to be an antibiotic for *A. hydrophila* diseases treatment. However, the MAR index that had been calculated was shown quite a high result which is 0.31 based on the antibiotics that showed resistance. If the value of the MAR index is exceeded than 0.2, it means that the high-risk source of contamination in where the antibiotics are frequently been used. Based on the current study, the aquarium shop where fish sample was collected have been used antibiotics frequently to treated their diseased ornamental fish.

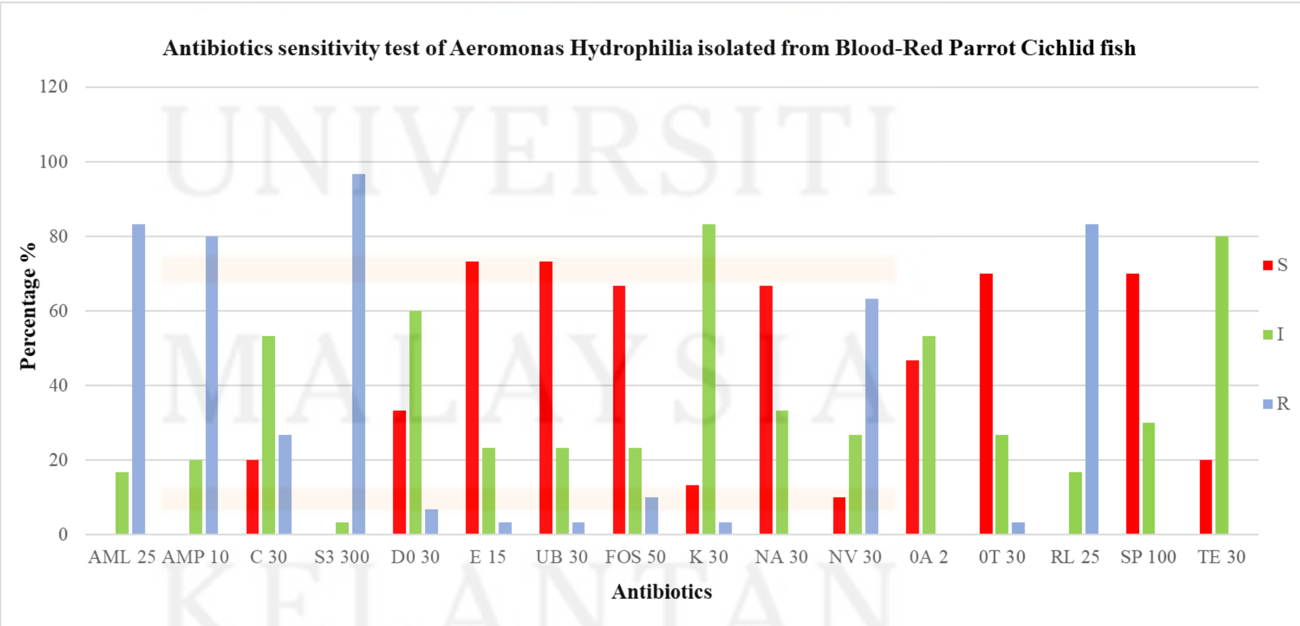

Figure 4.2: The bar graph chart of antibiogram test of *Aeromonas Hydrophilia* isolated from the Blood Red Parrot Cichlid fish

4.2 Discussions of Antibiogram Test

Aeromonas hydrophila was stood as a primary pathogen in fish and had been a threat to ornamental fish since it will cause bacterial disease on fish and lead to mortality based on a few studies. The bacteria will infect the fish organs and ulcers of the skin. Based on previous studies, *A. hydrophila* was successfully isolated from different types of animal species but most of them are from aquaculture species which is seabass in Thailand (Ruangpan, 1988), seabass in the Aegean Sea (Doukas et al., 1998), wild freshwater fish in Croatia (Topic et al., 2000), and wild *Piaractus Mesopotamicus* and *Oreochromis Niloticus* in Brazil (Belem-Costa and Cyrino, 2006]). It was discovered in Malaysia in numerous varieties of infected ornamental fish. (Najiah et al., 2008), American bullfrog and giant freshwater prawn (Lee et al., 2009). Therefore, it gave a clear message that *A. hydrophila* was a common bacteria found in bacteria-infected fish**.** *A. hydrophila* also could be found in other animals, water, and food. Hence, this present study was carried out to decide where it should replace the existing antibiotic for the *A. hydrophila* disease treatment by isolated it from diseased Blood Red Parrot Cichlid fish. The highest sensitive type of antibiotics towards *A. hydrophila* in this current study is erythromycin (E) and flumequine (UB) by 73.3% for both types of antibiotics compared to the others.

According to the research conducted by Wei. L.S. et al. (2015), *A. hydrophila* that were successfully isolated from diseased Red Hybrid Tilapia, *Oreochromis* spp., founded sensitive to nalidixic acid (NA), flumequine (UB), and oxytetracycline (OT). They were found effective in controlling all the bacterial isolates in this study. Except for oxytetracycline (OT), which is prohibited by the Malaysian government for use in aquaculture, they believe that nalidixic acid (NA) and flumequine (UB) can be utilized as treatments in commercial tilapia farms. Compared to the current study, flumequine (UB) also was discovered as the most sensitive antibiotic for *A. hydrophila* infected ornamental fish. Nevertheless, the percentage of sensitive cases by flumequine (UB) in the current study is not as much as Wei et al. (2015) research which is 100.0%. Another study by Lee S.W. et al. (2015) *Aeromonas* spp. isolated from Asian seabass (*Lates calcarifer*) hatchery also presented that nitrofurantoin (F), furazolidone (FR), tetracycline (TE), doxycycline (DO), flumequine (UB), oxolinic acid (OA), and florfenicol (FLO) were discovered to be highly sensitive towards the present bacteria. This would be strong evidence and agreement with the previous study by Wei. L.S. et al. (2015) that flumequine (UB) could be used as antibacterial agents for bacterial diseased ornamental fish.

However, in the other studies, most of them reported that *A. hydrophila* highly showed resistance towards erythromycin (E) antibiotics which is between 90.0% to 100.0%. It was the opposite with the present study that reported erythromycin (E) as highly sensitive to *A. hydrophila* that isolated from diseased Blood Red Parrot Cichlid fish with 73.3%. For example, in the study of W. S. Darwish et al. (2018), the samples isolated from different types of frozen fish in the Egypt market were 100% resistant to streptomycin (S), cloxacillin (CL), and erythromycin (E). This might be related to pathogen contamination of the aquatic environment as well as poor fish handling. Such germs may have a few public health consequences, particularly if infected seafood is ingested. As a result, sanitary measures should be implemented to limit microbial contamination in the aquatic environment or fish markets. Other’s example is research carried out by Omojowo F. S. and Omojasola F. P. (2013) that stated *A. hydrophila* was 100.0% resistant to tetracycline (TE), amoxycillin (AML), and erythromycin (E) that was isolated from cow dung used as fertilizer in Nigerian fishponds. According to the findings of Sayah et al. (2004), it is consistent with the research of Omojowo F. S. and Omojasola

F. P. (2013) that livestock is a reservoir of resistant bacteria for environmental pollution. Cow dung manure is a potential carrier of bacterial pathogens that possibly transmit zoonotic diseases to humans across contact with the manure. When this untreated manure is used to fertilize fishponds, it may lead to an increase in infectious diseases in the fish and act as a potential source of food-borne diseases for fish consumers.

According to the study by Sarker. B. et al. (2020), all *A. hydrophila* strains isolated from broiler chickens in Mymensingh Sadar, Bangladesh was high for ampicillin resistance, and a few isolates bacteria also highly tested resistance for co-trimoxazole (SMX) 84%, tetracycline (TE) 34%, and erythromycin (E) 34%. Other researchers Samal et al., (2014), Nagar et al., (2011), Vaseeharan et al., (2005), and Radu et al., (2003) have documented increased resistance to β-lactamases such as ampicillin (AMP). The development of inducible chromosomal β-lactamases that chromosomally encoded β-lactams is responsible for high resistance (Janda and Abbott, 2010). The present study has also shown that *A. hydrophila* isolated from diseased ornamental fish was high resistance to amoxycillin (AML), amplicillin (AMP), compound sulphonamides (S3), sulphamethoxazole (RL), and spiramycin (SP) in agreement with the previous study by Sarker B. et al. (2020), Samal et al., (2014), Nagar et al., (2011), Vaseeharan et al., (2005), and Radu et al., (2003).

Based on all the findings, resistance and sensitive cases of antibiotics differ according to the geographical origin of the imported fish and the variety of bacteria. The results for each study would not be exactly the same but some of them are just almost the same as the present study. The resistance and sensitive cases of antibiotics would be evolved from time to time as the bacteria would be developed a resistance towards the common antibiotics that are used to treat them. It was not a failure for this current study for not having the same result as other studies as the objective was to find the most suitable antibiotic to apply to the *A. hydrophila* infected ornamental fish and decide where it should replace the existing antibiotic for the *A. hydrophila* disease treatment.

Next, referred to the study by Ayandele. A.A. et al. (2020), if the value of MAR index was larger than 0.2 indicates the high-risk source of contamination in the location where antibiotics are commonly utilized. It means that the place that the fish sample was collected contained high contamination and was potentially dangerous with present bacteria as the antibiotics have frequently been used there. The current findings had a MAR index with a 0.31 value which is high and claimed as dangerous. It was shown that the aquarium shop had been used antibiotics oftentimes to treat these fish. Based on the research by Adinortey, C. A., et al. (2020), the handling and subsequent consuming fish that containing multidrug-resistant bacteria poses a public health risk since these bacteria can cause a variety of human illnesses that are difficult to cure. Another likely possibility is that antibiotic-resistant genes carried by resistant bacteria will be passed on to other bacteria in consumers' gastrointestinal tracts, potentially resulting in life-threatening infectious illnesses. The authors also stated that the MAR indexing value is considered an efficient and cost-effective strategy for monitoring bacteria sources. It could be a valuable indicator for determining the risk of life-threatening pollution.

Finally, the most essential thing to know regarding *Aeromonas hydrophila* infected fish is that it is a zoonotic illness, which means that it may be transmitted from animals to humans and vice versa. Healthy people who are exposed to these bacteria are unlikely to get the illness, but it will do when we had an open wound such as cutting oneself when butchering infected fish or impaling a sharp fin into your palm is a definite way to become infected. People who are immunodeficient or immunocompetent, such as the very young, the elderly, or those with underlying illness issues, are more vulnerable. To avoid human exposure to this illness, good personal hygiene and thorough sanitation techniques should always be employed. These measures include using gloves while handling afflicted fish, seeking medical assistance for any cut or laceration, no matter how minor it is, and bandaging open wounds. While *A. hydrophila* infection in humans is often a rare infection case, it is prudent to seek medical assistance at the first sign of sickness following exposure to or handling of fish infected with this disease.

CHAPTER 5

CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

In the conclusion, the study had been demonstrated the most suitable antibiotic to apply to the *Aeromonas hydrophila* infected ornamental fish which was erythromycin (E) and flumequine (UB) with the highest sensitive case by 73.3% for both types of antibiotics compared to the other types. Therefore, these antibiotics could be replaced with the existing antibiotics treatment for *A. hydrophila* infected ornamental fish as both types of antibiotics were commonly used on the other bacterial diseases and easily to find. Hence, there is sufficient evidence and agreement with previous studies to conclude that erythromycin (E) and flumequine (UB) was the most suitable antibiotic to apply to the *Aeromonas hydrophila* infected ornamental fish.

5.2 Recommendations

It is recommended for having a further study about the antibiogram of *Aeromonas hydrophila* isolated from other ornamental fish species so that, could get an accurate result in the future and help to overcome the *A. hydrophila* infected diseased of ornamental fish. Hence, it could be used to compare the results of this study with further study to get an idea of how we could improve the *A. hydrophila* infection treatment. Moreover, the available research to refer to regarding the antibiogram of *A. hydrophila* is limited even though many studies had been mentioned about the occurrence of *A. hydrophila* in many places, species, and conditions. It had been reported there are many cases of *A. hydrophila* found in aquaculture species, freshwater, and fish ponds but not much research is conducted about it. It is critical to pay attention to the *A. hydrophila* infected diseased cases since it can affect humans and other animals too. Furthermore, it is a must to educate the breeders and sellers about the bacteria infection disease on ornamental fish. It can give them knowledge and information on how to overcome and treat the infected fish rather than they throw the fish anywhere that possibly can be a disturbance for our local fish ecosystem. Other than that, the government and Ministry of Fisheries Malaysia must give out strict regulations for the use and dosage of antibiotics for disease treatments. The frequent use of antibiotics would be a high potential risk for humans and animals if they are consuming it as the fish was contaminated with antibiotics. All these recommendations are needed to give a better future.

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APPENDIX A

TABLE A: Antibiogram test of *Aeromonas hydrophila* isolated from diseased Blood Red Parrot

SAMPLES	ANTIBIOTICS															
	S3	**DO**	**TE**	**NV**	**C**	**K**	**RL**	**UB**	**E**	**AMP**	**SP**	**OT**	**AML**	**FOS**	**NA**	**OA**
A1	0	17	18	12	15	17	15	21	22	15	21	19	8	20	22	18
A2	0	17	17	13	17	17	13	17	20	14	17	17	11	17	17	20
A3	8	17	18	16	16	17	11	16	21	15	24	24	12	23	19	18
A4	0	19	17	13	15	17	15	21	18	9	21	19	8	21	22	21
A5	11	19	18	13	19	18	16	19	22	16	21	19	9	20	18	19
A6	0	17	17	16	16	17	17	21	20	16	20	18	8	17	22	18
A7	0	18	18	13	17	18	15	18	18	13	18	19	13	18	17	18
E1	12	17	19	17	15	19	12	21	22	15	17	18	13	17	17	17
E2	8	21	18	11	16	16	11	21	17	17	21	21	8	20	22	18
E3	0	17	19	17	19	19	16	17	18	16	18	19	16	17	17	18
E4	0	17	18	13	17	16	15	21	22	13	17	17	14	20	22	17
E5	13	19	17	12	16	17	12	17	20	14	20	15	8	12	17	18
E6	0	17	18	13	21	18	13	21	21	13	21	19	17	20	21	21
E7	12	19	19	14	15	19	15	26	17	0	26	18	0	26	22	26
G1	8	19	18	13	16	17	12	21	22	15	19	19	8	21	19	19
G2	0	17	19	19	19	18	8	19	16	8	17	19	8	21	19	19
G3	14	19	18	17	16	17	15	21	21	12	21	21	15	20	22	18
G4	16	16	16	13	16	15	0	16	22	15	21	19	8	16	16	23
G5	0	17	18	17	19	18	14	21	18	14	21	18	16	20	18	18
G6	13	17	17	17	15	17	15	17	19	14	19	17	13	17	22	18
K1	14	17	18	13	17	17	15	20	22	15	20	19	8	20	16	20
K2	0	19	19	19	16	19	14	21	21	15	21	18	14	20	21	21
K3	12	15	18	15	16	18	16	15	24	16	17	24	16	15	22	18
K4	10	17	22	13	16	18	15	21	22	13	21	19	8	20	17	22
K5	9	19	18	16	15	18	16	20	20	15	17	20	16	20	19	20
S1	0	13	17	13	16	17	15	21	22	17	21	21	8	13	22	18
S2	0	17	18	13	16	18	12	23	23	12	21	23	12	20	23	23
S3	10	19	17	23	15	17	8	21	22	15	23	21	8	23	19	23
S4	0	17	18	13	21	18	15	21	14	12	17	21	10	21	22	18
S5	0	17	17	12	15	18	15	21	22	15	21	21	8	20	20	18
R	29	2	0	19	8	1	25	1	1	24	0	1	25	3	0	0
I	1	18	24	8	16	25	5	7	7	6	9	8	5	7	10	16
S	0	10	6	3	6	4	0	22	22	0	21	21	0	20	20	14

APPENDIX B

Calculation of MAR index value

MAR index

$$= \frac{\text{Number of antibiotics to which the isolate showed resistance}}{\text{Number of total antibiotics exposed to the isolate}}$$

$$= \frac{5}{16}$$

= 0.31