ANTIBIOGRAM OF *ESCHERICHIA COLI* ISOLATED FROM DISEASED FLOWER HORN FISH *(Cichlasoma sp.)* OF AQUARIUM SHOP

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A thesis submitted in fulfilment of requirement for degree of Bachelor of

Applied Science (Animal Husbandry Science) with Honours

FACULTY OF AGRO BASED INDUSTRY

UNIVERSITY MALAYSIA KELANTAN

2022

DECLARATION

I hereby declare that the work embodied in this report is the result of my own research except individual citations and summaries that I have explained their sources.

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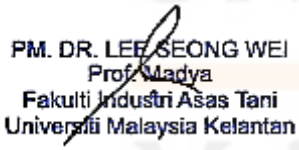

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ACKNOWLEDGEMENT

There are several persons I wish to deliver my thanks to upon finishing my final year project. First and foremost, I wish to deliver my gratefulness to my parents, Mr. Ong Lay Seong and Mrs. Koay Lee Eng for their trust on me, giving me permission to stay and complete this research during semester break. In spite of mental support, they also support me financially through providing me funds needed for basic needs.

Next, I wish to deliver my thanks to my supervisor, Assoc Prof. Madya Dr. Lee Seong Wei. He is ready and willing to help students to solve their problems during research conduct. Suggestions provided by him are helpful. He has assisted and provided me with excellent guidance, supervision, knowledge, and suggestion throughout my research journey and in the completion of this final year project thesis. Without his continual inspiration, it would have been impossible for me to complete my research.

Besides, appreciation is conveyed to Dean of Faculty of Agro Based Industry (FIAT) for the facilities provided for research completing.

I have been blessed with my amazing boyfriend, Ong Shen Han who always accompanied me in conducting the experiment during the semester holidays and for the constant support during my thesis writing. He also gives me some suggestion to solve my difficulty.

Lastly, I would like to express my appreciation to all of my lecturers and friends in the UMK Jeli campus who had, directly and indirectly, assisted me throughout the research. Thanks for all the support from them who gave me, whether spiritually or financially, support. This report would not have been accomplished without all of these people.

Antibiogram of *Escherichia coli* isolated from diseased Flower Horn fish (*Cichlasoma sp.)* of aquarium shop

ABSTRACT

The ornamental fish trade is part of a wide and global market for products and resources, offering new professionals with services and employment possibilities while also assisting underdeveloped nations in their economic growth. *Escherichia coli* has become worldwide disease and is considered as main bacteria in ornamental fish. In this study, antibiograms of *Escherichia coli* are isolated and classified from diseased Flower Horn *(Cichlasoma sp.)* fish in an aquarium shop. *Cichlasoma sp.* is the scientific term for Flower Horn, also known as Hua Luo Han or Flower Horn cichlid, where most popular of hobbies with millions of enthusiasts worldwide. This study aim is to characterize the antibiogram of *Escherichia coli* via Most Probable Number (MPN) analysis and identify antibiogram of *E. coli* which isolated from aquarium shop ornamental Flower Horn fish for better antibiotic in future use. *Escherichia coli* are isolated from Flower Horn by direct streaking from abdomen, eye, gill, kidney, and skin onto the agar. All isolates are identified through clinical examination, Most Probable Number (MPN) Test and Antibiogram Susceptibility Test. Furthermore, *E. Coli* are tested with Multiple Antibiotic Resistances (MAR) using 16 type of antibiotic discs which are Novobiocin (30 µg), Tetracycline (30 µg), Flumequine (30 µg), Sulphamethoxazole (25 µg), Amoxycillin (25 µg), Chloramphenicol (30 µg), Compound Sulphonamides (300 µg), Spiramycin (100 µg), Ampicillin (10 µg), Oxytetracycline (30 µg), Doxycycline (30 µg), Nalidixic Acid (30 µg), Erythromycin (15 µg), Kanamycin (30 µg) and Oxolinic Acid (2 µg). The results show that the most resistant antibiotic towards *E. coli* is Compound sulphonamides (93.33%) while sensitive antibiotics are Erythromycin and Oxolinic Acid (93.33%). Multiple Antibiotic Resistance (MAR) index value are 0.50. Their value are more than 0.2 and indicated as high-risk antibiotic expose. This research is beneficial for researchers to establish effective tank management and a water-recirculation system, allowing Flower Horn to have a consistent supply of pollution-free freshwater and increase in health level.

Keywords: aquarium shop, antibiogram, diseased ornamental fish, Flower Horn (*Cichlasoma sp*), *Escherichia coli*

Antibiogram Escherichia coli diasingkan daripada hiasan berpenyakit Ikan Flower Horn (*Cichlasoma sp*.) kedai akuarium

ABSTRAK

Perdagangan ikan hiasan adalah sebahagian daripada pasaran yang luas dan global untuk produk dan sumber, menawarkan profesional baharu dengan perkhidmatan dan peluang pekerjaan sambil turut membantu negara kurang membangun dalam pertumbuhan ekonomi mereka. *Escherichia coli* telah menjadi penyakit di seluruh dunia dan dianggap sebagai bakteria utama dalam ikan hiasan. Dalam kajian ini, antibiogram *Escherichia coli* diasingkan dan dikelaskan daripada ikan Flower Horn (*Cichlasoma sp.)*yang berpenyakit di kedai akuarium. *Cichlasoma sp*. ialah istilah saintifik untuk Flower Horn, juga dikenali sebagai Hua Luo Han atau Flower Horn cichlid, di mana hobi paling popular dengan berjuta-juta peminat di seluruh dunia. Kajian ini bertujuan untuk mencirikan antibiogram *Escherichia coli* melalui analisis Most Probable Number (MPN) dan mengenal pasti antibiogram *E. coli* yang diasingkan daripada ikan Flower Horn hiasan kedai akuarium untuk antibiotik yang lebih baik untuk kegunaan masa hadapan. *Escherichia coli* diasingkan daripada Flower Horn melalui coretan terus dari perut, mata, insang, buah pinggang, dan kulit ke dalam agar. Semua isolat dikenal pasti melalui pemeriksaan klinikal, Ujian Most Probable Number (MPN) dan Ujian Antibiogram Susceptibility. Tambahan pula, *E. Coli* diuji dengan Multiple Antibiotic Resistances (MAR) menggunakan 16 jenis cakera antibiotik adalah Novobiocin (30 µg), Tetracycline (30 µg), Flumequine (30 µg), Sulphamethoxazole (25 µg), Amoxycillin (25 µg), Chloramphenicol (30 µg), Compound Sulphonamides (300 µg), Spiramycin (100 µg), Ampicillin (10 µg), Oxytetracycline (30 µg), Doxycycline (30 µg), Nalidixic Acid (30 µg), Erythromycin (15 µg), Kanamycin (30 µg) and Oxolinic Acid (2 µg). Keputusan menunjukkan bahawa antibiotik yang paling tahan terhadap *E. coli* ialah Compound sulphonamides (93.33%) manakala antibiotik sensitif ialah Erythromycin dan Oxolinic Acid (93.33%). Nilai indeks Multiple Antibiotic Resistances (MAR) adalah 0.50. Keputusan tersebut sudah lebih daripada 0.2 dan ditunjukkan sebagai pendedahan antibiotik berisiko tinggi. Penyelidikan ini memberi manfaat kepada penyelidik untuk mewujudkan pengurusan tangki yang berkesan dan sistem peredaran semula air, membolehkan Flower Horn mempunyai bekalan air tawar bebas pencemaran yang konsisten dan peningkatan tahap kesihatan.

Kata kunci: kedai akuarium, antibiogram, ikan hiasan berpenyakit, Flower Horn (*Cichlasoma sp*), *Escherichia coli*

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LIST OF ABBREVIATIONS

AST = Antibiogram susceptibility test

CFU = Colony Forming Unit

EAEC = enteroaggregative *E. coli*

EMB = Eosin Methylene Blue agar

EPEC = Enteropathogenic *E. coli*

ETEC = enterotoxigenic *E. coli*

FAO = Food and Agriculture Organization

GO = Graphene oxide

LB = Lactose Broth

MAR = Multiple Antibiotic Resistant

MPN = Most Probable Number

STEC = Shiga toxin-producing *E. coli*

TNTC = too numerous to count

TSB = Tryptic soy broth

LIST OF SYMBOLS

%	Percent
°C	Degree Celsius
µg	Microgram
Mm	Millimetre
dHG	Degrees of general hardness

LIST OF EQUATIONS

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CHAPTER 1

INTRODUCTION

1.1 Research Background

The ornamental fish trade is a vast and global market that moves large quantities of goods and resources, providing new professionals with services and job opportunities, and contributing to the economic development of developing countries. (Ladisa, Bruni, & Lovatelli, 2017) Ornamental fish keeping is a common hobby among hobbyists who are attracted by the colours, shapes, and swimming behaviours that various types of fish exhibit in an aquarium. In Malaysia, there are currently over 250 ornamental fish exporters, as the selection, breeding, and marketing of ornamental fish is a significant industry that generates employment and foreign exchange. However, there are questions about the industry's economic viability and environmental sustainability. In addition to harvesting fish from the wild, early aquarists learned to breed the fish and create varieties that were very different from their wild counterparts. (Ng, 2016)

The ornamental aquaculture industry's rapid growth over the last few decades has necessitated the use of antibiotics to prevent a diverse range of infectious diseases. Antibiotics are routinely used in aquaculture, according to the FAO, in addition to disease

prevention, as a growth promoter and to lower mortality rates. (Preena, Dharmaratnam, Raj, Kumar, Raja, & Swaminathan, 2019) Infections with *E. coli* is most often caused by consuming infected water or food. The infection's most common symptom is diarrhoea, which can lead to death in immunocompromised people, due to dehydration from prolonged illness affects the very young and the elderly. (Nontongana, Sibanda, Ngwenya, & Okoh, 2014) The aim of this research is to isolate bacteria from diseased Flower Horn ornamental fish and study the antibiogram of *Escherichia coli (E. coli*) in order to improve antibiotic treatment options in aquarium shops.

1.2 Problem Statement

Infections with *E. coli* are most often caused by consuming infected water or food. The infection's most common symptom is diarrhoea, which may lead to death in immunocompromised people, due to dehydration from prolonged illness. As a result, finding an appropriate antibiotic for managing *E. coli* infection is critical. Therefore, *E. coli* bacteria from ornamental fish must be isolate and identify, so fish poisoning caused by *E. coli* toward ornamental fish of aquarium shop can be avoided. (Nontongana, Sibanda, Ngwenya, & Okoh, 2014)

The need for successful therapy against diseased ornamental fish pathogens has increased as aquaculture has become more intensive. The quest for alternative antibiotics

and other disease control measures was necessitated by a lack of knowledge on effective antibiotics for aquaculture and a growing rate of resistance among bacterial pathogens to widely used antibiotics. Therefore, help in antibiogram to characterize the of *E. coli* and create suitable antibiotics to decrease the rate of ornamental fish of aquarium shop get effected to the disease. (Abraham, Sasmal, & Banerjee, 2004)

1.3 Objective

The objectives of the present study are:

1. To isolate and identify *Escherichia coli* bacteria from ornamental fish
2. To characterize the antibiogram of *Escherichia coli* isolated from diseased ornamental fish of aquarium shop.

1.4 Scope of Study

Microbiology and health field

1.5 Significance of Study

It is critical in this study to identify and track a suitable environment for ornamental fish, so *E. coli* pollution in aquarium shop ornamental fish will be controlled. The antibiogram of *E. coli* are characterised, and the results are useful and informative in determining most effective of antibiotic in treating *E. coli* infections. Furthermore, the powerful antibiotic may be able to be used in the future for medicinal purposes, as well as a recommendation for aquarium shops to produce safe ornamental fish. Antibiogram is also used in aquaculture as a prophylactic and treatment measure since there are no antibiotics specifically formulated for aquaculture. The ornamental fish which are carry out in this experiment is Flower Horn (*Cichlasoma sp.*).

1.6 Limitation of Study

The aim of this study is to determine the health status of ornamental fish in an aquarium store, as well as to perform an antimicrobial susceptibility test on *E. coli* isolated from diseased ornamental fish. For the other limitation in this project is the number of antibiotics, due to only 16 antibiotics are used for the research, it is not enough to acquire a total accurate result due to there are many more types of antibiotic are available throughout the world and to ensure the result of this experiment to be more accurate, more antibiotic are needed to be use to get a better and accurate result. Besides that, due to the pandemic, this cause lack of workers to work as usual and unable to maintain health status of ornamental fish. As for the recorded unique database on the relevant to this study will be harmed as a result of this restriction.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction to Ornamental Fish, Flower Horn (*Cichlasoma sp.*)

Ornamental fish is known as fishes which are kept in tanks, other aquarium systems and home aquariums where encompass a wide variety of species, of many shapes, sizes, and colours. Aquarium keeping is a hobby that is very popular with millions of enthusiasts worldwide. (Ornamental Fish | Roger Williams University, 2021) While most aquarium fish are freshwater, the popularity of marine ornamental fish has grown dramatically, thanks to children's movies featuring charismatic colourful fish and other creatures. The hobby has been made even easier by recent advancements in fish husbandry and aquarium equipment technology. Water quality is critical in aquaculture since the survival of the fish is entirely dependent on the caretaker, whether the water is freshwater or marine. (Livengood & Chapman, 2007)

Domain: Eukaryota Kingdom: Animalia

Phylum: Chordata Subphylum: Vertebrata

Class: Actinopterygii Order: Cichliformes

Family: Cichlidae Genus: *Cichlasoma*

Cichlasoma sp. is the scientific term for Flower Horn, also known as Hua Luo Han or Flower horn cichlid, and since the late 1990s, it has been widely distributed in temperate and tropical regions. Many introduced populations of *Cichlasoma sp.* have been established in many countries because of their very dynamic character. They've been taught to respond to humans in their environment, and some may even raise their nuchal hump out from the water to be petted! *Cichlasoma sp.* is currently found in tropical and temperate freshwater environments all over the world. The Flower Horn is an omnivorous creature that feeds on worms, plant particles, and grasshopper. (Jason, 2018)

Cichlasoma sp. is a solitary freshwater fish that due to the destructive and exploratory character of these fish, they tend to be relatively naked. The gestation period for Flower Horn occurs for 1 to 2 weeks. Males are generally larger than females, though there are few outliers. Flower Horn males have the nuchal hump, or the kok, on their foreheads. Males' hues are usually brighter and more vivid. Females have black spots on their dorsal fins in most breeds, whilst males have longer anal and dorsal fins. Females, especially when preparing to reproduce, have an orange belly. The male's mouth is larger and more prominent than the female's. Flower Horn's sex can be determined by the fact that mature females will deposit eggs every month even if the male is not there. The life

span of the Flower Horn is about 12 years, will reach sizes around 12 to 16 inches, and about 0.5kg in weight. (Flower Horn Fish Facts | *Cichlasoma sp.* | AZ Animals., 2021)

Due to their incredibly involved character, *Cichlasoma sp.* are the ultimate "pet" fish. They can also tolerate a broad of temperatures and salinities range of 25 to 30°C, although they are most frequently found in tropical seas that are warm and rather hard. The ideal pH range for the Flower Horn to live and breed is 6 to 8, with 9 to 20 dGH of water hardness ranging. (Jessie Sanders, 2021). Unless place in a quite huge tank with at least 200 gallons, these fish do not make suitable social fish. Flower Horns are extremely territorial and aggressive, to the point where any other fish in the aquarium is likely to be attacked. Despite their strange, exotic appearance, these fish are rather easy to care for as long as a large tank is provided for them, monitor and control the water conditions in the tank, and feed Flower Horn a high-quality diet that has all of the nutrients it requires to grow. (Alison Page, 2021)

2.2 Introduction to Escherichia Coli

Domain:	Bacteria	Phylum:	Proteobacteria
Class:	Gammaproteobacteria	Order:	Enterobacterales
Family:	Enterobacteriaceae	Genus:	*Escherichia*

A gram-negative bacterium *E. coli* is with a rod-shaped microscopic structure that can be found in the atmosphere, foods, and people's and animals' intestines. While the majority of *E. coli* strains are harmless, others can cause severe food poisoning, diarrhoea, urinary tract infections, respiratory disease, pneumonia, and other illnesses. The harmless strains are part of the natural gut microbiota and give benefit to their hosts by developing vitamin K2, which aids blood clotting and prevents pathogenic bacteria from colonising the intestine, forming a mutualistic relationship. Faecal matter is used to remove *E. coli* into the atmosphere. For three days, the bacterium grows rapidly in fresh faeces under aerobic conditions, but its numbers gradually decline after that. (What Is *E. coli*?., 2021)

E. coli contain a high growth rate and can replicate in less than 20 minutes, the commensal and *E. coli* pathogenic strains were categorised based on their virulence properties and pathogenicity mechanisms. There are different pathogenic strains in *E. coli* like enteroaggregative *E. coli* (EAEC), diffusely adherent *E. coli*, Enteropathogenic *E. coli* (EPEC), Shiga toxin-producing *E. coli* (STEC), Enteroinvasive *E. coli* and

enterotoxigenic *E. coli* (ETEC). However, abiotic factors such as surrounding temperature and nutrient availability, as well as biotic factors such as *E. coli*'s ability to use nutrients, can affect the growth and survival rate. Most common cause of *E. coli* infections is by consumption of infected water or food. The best way to avoid contracting this disease is to practise good hygiene by cleaning the tanks on a regular basis, keeping clean while giving feed or cleaning to prevent cross-contamination, and giving fresh, uncontaminated pellets or live feed. (Prevent Shiga Toxin-Producing *E. coli* Infection., 2018)

2.3 Isolation and Identification of Bacteria

Isolation is the process of separating a strain from a naturally mixed population of living microbes found in the environment, such as water or living beings, in order to distinguish the microbe of interest. In the 19th century the isolation techniques in the laboratory were first introduced in the fields of bacteriology and parasitology. Microbial isolation methods have improved dramatically over the last 50 years, both in terms of labour and in terms of the technologies involved, and the results are now faster and more accurate. Bacteriological studies begin with bacterial identification, isolation, and purification. Isolation is performed to get the pure bacterial cultures. Bacteria are most commonly isolated from the kidneys and spleens of fish, as well as the lymphoid organ, hepatopancreas, and muscles of shrimp. These organs serve as monitors and are known to host disease-causing bacteria during infection.

Firstly, to gain a pure bacterial culture is through bacterial identification. Pure culture is critical for studying a bacterial strain's anatomy, susceptibility to antimicrobial agents, physiology, and biochemical characteristics. Pour plate, solid media, or streak plate methods are the best ways to obtain pure cultures. If done correctly, the streak plate is the most realistic tool. In the streak plate phase, a loopful of inoculum is placed near the plate's periphery and spread or streaked on the upper portion of the plate with parallel overlapping strokes. Other parts of the plate are streaked with inoculum so that isolated colonies can be seen in the last streaked region. In fish diagnosis, identifying a bacterial pathogen is important. Treatment can begin only after the causative agent or bacterium has been identified. Bacterial organisms have physiological, biochemical differences, and morphological that can be used to code or label for future use. As a result, recognition is achieved through a variety of morphological, physiological, and biochemical measures. The results of these experiments are compared to taxa or identification schemes that are well-known.

Bacterial cultures should be held for future study indefinitely. Bacterial cultures can be preserved by storing them in the right medium. Subculturing or moving the organism to fresh solid medium with a minimal nutrient content to avoid bacterial overgrowth is the simplest process. Before being stored in the refrigerator, the bacteria are allowed to grow, or they are kept at room temperature in the dark after covered in paraffin oil. Another simple method is to deep-freeze the bacterial culture in a broth medium containing glycerol. Glycerol is added to keep bacterial cells from drying out. Bacterial cultures may also be frozen or lyophilized for storage. Sublimation under vacuum extracts water from the frozen bacterial suspension in this process. Before storing bacterial cultures, they should be properly labelled or coded. It is important to use an

indelible ink to mark the tube or vial used to store bacterial cultures. The reference number, as well as other pertinent details such as the source of the sample with the date of isolation, host animal, special properties, location, identity, name of the individual who isolated the organism, and the date of stock culture preparation should all be included on the label or code. (Ruangpan & Tendencia, 2004)

2.4 Most Probable Number (MPN) Analysis

Centerer on the theory of extinction dilution, the most probable number (MPN) is an estimating tool for bacteria in a sample with the method inoculating broth in 10-fold dilutions and produce viable numbers in a statistic format. The MPN test is used in this study to assess the presence of coliform bacteria in guppy. It's a statistical multiple assay that includes presumptive, proof, and completed tests. Coliform detection may be used as a water salinity indicator or a general indicator of sanitary conditions in a food-processing system. It's often used to estimate the number of bacterial cells in food and water.

The system, however, is only capable of counting living organisms. The number of *E. coli* in water samples is calculated as the inverse of the smallest portion of the sample in a geometric sequence of dilutions that yields a positive test result. This means that the sample dilutions are such that the last few dilutions yield negative results, which can be confusing when "skips" occur in the series of dilutions and when more than one

observation is obtained from a single sample. A plate count may be directly translated into a number that represents the most likely number of bacteria, which is a linear function of the plate count. However, with a multiple tube result, the most likely number of bacteria is a logarithmic feature of the result, necessitating additional calculations to translate the results to the MPN value. (Oblinger & Koburger, 1975)

The sample is applied to a lactose broth containing Durham's tube and divided into three parts: 10ml, 1ml, and 0.1ml in the presumptive test. (Ivy & Wiedmann,2014) After a certain period of incubation, turbidity and gas output can be observed. Lactose broth is a type of pre-enrichment medium that provides nutrient for the gram-negative bacteria growth due to the presence of lauryl sulphate. When lactose broth turns turbid, it indicates that bacteria colony is present and consuming the nutrient. As in the verified test, the positive tubes from the presumptive stage will be streaked on selective agar based on the bacteria that will be studied further and incubated for a set period of time. Following the completion of the test, the selective colonies will be inoculated with hockey stick and transferred back to lactose broth with Durham's tube. The positive tube with the gas bubble is streaked on Trypticase Soy agar (TSA) for identification after incubation.

2.5 Antibiogram Susceptibility Test (AST)

Antibiotic susceptibility testing, or AST, is a commonly used tool in clinical settings for assessing antibiotic resistance and deciding patient care plans. When fungi and bacteria are unable to thrive in the existence of single antimicrobial drugs or more, this is referred to as susceptibility. After an individual's infection caused by bacteria or fungi have been healed in a specimen culture, susceptibility testing is done. Testing is done to see whether a specific antibiotic is successful against bacteria and to see if the bacteria have established resistance to such antibiotics. The findings of this test can be used to aid in the selection of the most appropriate medication for infection treatment. Fungi and bacteria have the ability to develop antibiotic and antifungal drug resistance at any time. Antibiotics that were once successful in killing or ineffective in inhibiting their growth. Despite the fact that viruses are bacteria, antiviral drug resistance research is done less regularly and with different test methods. The focus of this article is on bacterial and fungal susceptibility research. (Antibiotic Susceptibility Testing | Lab Tests Online, 2021)

Agar dilution, broth dilution, and disc diffusion assays are some of the common AST methods. To recover the fungus or bacteria that is causing the infection, from contaminated site sample is cultured on specialised media. The type of culture conducted determines the process used to collect a sample from a person. Using the disc diffusion process, bacteria are spread on an agar plate and paper discs impregnated with antibiotics are placed on the plate. Bacterial growth is monitored after incubation. Zones of inhibition are areas around the antibiotic disc where no bacterial growth can be seen. These zones

indicate that an antibiotic has been effective in halting or destroying bacterial development. We may compare antibiotic efficacy and monitor antimicrobial resistance by measuring the diameter of these areas.

Pathogens are isolated during the culture process and segregated from all other microbes present. Biochemical, enzymatic, and molecular tests are used to identify each pathogen if it is present. It is possible to decide if susceptibility testing is needed once the pathogens have been established. Not every pathogen is tested for susceptibility as some pathogens lead to well-established standard treatments. Susceptibility testing is done on any fungi or bacteria that may be important to the patient's care but whose susceptibility to treatment is unknown. Antimicrobials' ability to inhibit pathogen growth is checked for each pathogen separately. This can be directly assessed by combining the pathogen and antibiotic in a growing setting, such as nutrient media in a test tube or an agar plate, and observing the antibiotic's effect on the bacteria's development. Resistance can also be calculated by detecting a gene that has been linked to antibiotic resistance in the past. (Antibiotic Susceptibility Testing (AST) – Synbiosis, 2021)

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemicals and Reagents

Cichlasoma sp., tank water, plastic zipper bag, Tryptic soy broth (TSB), Trypticase soy agar (TSA), Lactose broth (LB), antibiotics disc, BBL Crystal kit, alcohol, parafilm, aluminium foil, Eosin Methylene Blue (EMB) agar

3.1.2 Equipment and Apparatus

Scalper, ruler, board, maker pen, cotton bud, mask, gloves, petri dish, Scott bottle, measuring cylinder, beaker, Durham tube, inoculate wire loop, test tubes, bijou bottle, vortex machine, pipette, Lamina flow cabinet, autoclave machine, electronic balance, Bunsen burner, incubator, hockey stick, filter paper

3.2 Methods

3.2.1 Sample Preparation and Collection

600ml of the water sample and Flower Horn, *Cichlasoma sp.* is collected from the tank. Opened the Flower Horn samples with a scalper that has been cleaned and disinfected. Placed the Flower Horn on the board with care and scrape the flesh out with the blunt end of the scalper. Collect and seal the flesh inside a clean plastic zipper bag respectively, homogenised gently with hand and total weight is recorded. The sample must be use as soon as possible while store at room temperature.

3.2.2 Most Probable Number (MPN) Test

3.2.2.1 Presumptive Test

60 capped test tubes are divided into four sets which Lactose Broth (LB) and Durham ‘s tubes are insert upside down into 15 test tubes. Then, used a sterile pipette adds 10ml of lactose broth is fill into every test tube and 10ml, 1ml and 0.1ml of Flower Horn into the first set respectively. (R.A. Ivy, M. Wiedmann,2014) The tubes are incubated for 24 hours or more at 25°C in incubator. The present of air bubbles due to fermentation of bacteria in the tube was observed and it indicated positive result. The number of tubes gave positive result were compared to the standard chart and recorded. (NA, 2021)

3.2.2.2 Confirmation Test

The selected bacteria from presumptive test are spread on two plates of selective medium that is Eosin Methylene Blue (EMB) agar. The plates are incubated for 24 hours or more in incubator at 25°C.

3.2.2.3 Complete Test

Single colony of bacteria are inoculated into Lactose Broth (LB) containing Durham's tube and incubate overnight. Incubated the tubes at 25°C in incubator for 24 hours or more. Positive tube with the present of gas bubble indicated the present of bacteria are streak on the Trypticase soy agar (TSA) for further identification. The positive data are recorded and the refer to the MPN table for identifying the MPN index.

3.2.3 Isolate and Identify *Escherichia Coli* from Samples

3.2.3.1 Isolation of *Escherichia Coli* by Using Spread Plate

100µl of mixture from 10^{-1} test tube is plated on Eosin Methylene Blue (EMB) agar by using spread plate method and triplicate are done for each dilution through use of hockey sticks. The petri dish sealed with parafilm and incubate at 25°C in incubator for 24 hours or more. (Ruangpan, & Tendencia, 2004)

3.2.3.2 Total Plate Count and Colony Forming Unit (CFU) Calculation of Bacteria Isolates

Standard colony counts are made for plate that has 30 to 200 colonies. Single and pure colony of bacteria is marked with market pen. If the number of colonies are more than 200 then recorded as too numerous to count (TNTC). All the bacteria grow will be recorded. To determine the CFU/ml in the original sample, the result must multiply by dilution factor in order.

$$\frac{\text{CFU}}{\text{ml}} = \frac{\text{CFU per plate} \times \text{dilution factor}}{\text{Volume of sample taken (ml)}}$$

3.2.3.3 Selection and Inoculation of Typical Single Colonies into Tryptone Soy Broth (TSB)

The 25 single colonies for each sample are selected by using sterile inoculate wire loop from the petri dishes with EMB agar. Next, each single colony are subculture into Tryptone Soy Broth (TSB) and vortex for few seconds and incubate at 27°C for 24 hrs in incubator. (Ruangpan & Tendencia, 2004)

3.2.3.4 Bacteria Identification Using BBL Crystal Kit

The inoculum fluid are poured into the target area base and make sure it fills the entire well. The lid will be placed back until it snacked into place. After incubated at 27°C for 24 hours in incubator, the colour changed on the BBL crystal kit was observed by referring to the colour reaction chart. BBL Crystal kit consists of panel lid, bases, and incubation tray. Each positive test result is assigned a value of 4, 2, or 1 to the row in which the test is located. The positive reaction is total up and the 10-digit number obtained are profile number for the test. The data are generated key in the coding by using software providing by the manufacturer. (NA, 2021)

3.2.4 Antibiogram Susceptibility Test

Antibiogram sensitivity test is applied to identify the sensitivity of selected bacteria to particular drugs of choice. A sterile cotton bud is soaked in each bijou bottles for few minutes. Then, remove excess liquid on cotton bud and swab onto petri dish with Trytone Soy Agar (TSA). Do triplicate of petri dish with TSA for each inoculum, where continue this process for all incubated bijou bottle. (Abd El Tawab, Ammar, Nasef, & Reda, 2015) Next, 16 antibiotics that select are novobiocin (30 µg), tetracycline (30 µg), flumequine (30 µg), sulphamethoxazole (25 µg), amoxycillin (25 µg), chloramphenicol (30 µg), compound sulphonamides (300 µg), spiramycin (100 µg), ampicillin (10 µg), oxytetracycline (30 µg), doxycycline (30 µg), nalidixic acid (30 µg), erythromycin (15 µg), kanamycin (30 µg) and oxolinic acid (2 µg) are place on the agar plates by disk diffusion method. The petri dish is sealed with the parafilm and incubating for 24 hours at 27°C in incubator. After the incubation, diameter of inhibition zone is measured in millimetre and recorded in a table. The inhibition zone is a clear region that indicated the absence of microbial growth effect of expose to antibiotic. The zone of inhibition will be classified into three groups which are resistance (R) (less than 15mm), intermediate sensitive (IS) (16- 17mm) and sensitive (S) (more than 18mm) according to Clinical and Laboratory Standards Institute standard guidelines. To identify the isolates are either from high or low antibiotic use region and this related with health risk assessment Multiple Antibiotic Resistance (MAR) index is used. The MAR index which is more than 0.2 showed that the guppy is exposed highly to antibiotics while MAR index less than 0.2 is less exposed to antibiotic. (Bawanti, Siregar, Kristianty, & Indriati, (2019)

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

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Clinical Examination of Infected Ornamental Fish

Ornamental fish infections are linked to a wide spectrum of bacteria. The presence of various clinical signs such as pale colour, slimy skin, detachment of scales and haemorrhagic skin, exophthalmia, sluggish movement, loss of appetite, swimming near the water surface, gasping air, and showing an increase in breathing frequency were observed in this study's clinical examination of randomly collected ornamental fishes. The fish rubbed their bodies against hard, immovable things as well. Fried fins and tail rot, scale loss, erosion, and rubbing sores were all visible on the skin. Gills seemed to be pallid. Other affected fish suffered from dullness and the presence of a turbid greyish-white film of mucus on fins and skin, and other fish from the presence of white spots over the body surface and fins, which may clump together to form white spot patches of various sizes, and the fish may appear as if they have been sprinkled with salt or sugar (Figure 1). (Aya Galal Saad El-Deen and Mohammed Rawway, 2014). Body deformity with external haemorrhages was discovered in naturally sick ornamental fish, which had bloody serous fluid in the abdominal cavity, a clogged and friable liver, and a clogged and enlarged kidney (Figure 2). These symptoms, on the other hand, might be due to various viral and/or non-infectious factors that are common in the aquatic environment.

Figure 1: Experimentally infected Flower Horn (*Cichlasoma sp.*) showing exophthalmia, detachment of scales and haemorrhagic skin

Figure 2: Experimentally infected Flower Horn (*Cichlasoma sp*.) showing congested kidney, bloody exudate in the intestine and peritoneum

4.2 Antibiogram Susceptibility Test

Antibiogram Susceptibility was conducted using 26 different of antibiotic discs. Twenty-six antibiotic discs were placed neatly onto Trytone Soy Agar (TSA) (Figure 3). Inhibition zones were measured and marked as Sensitive (S), Intermediate sensitive (IS) and Resistance (C) (Appendix A).

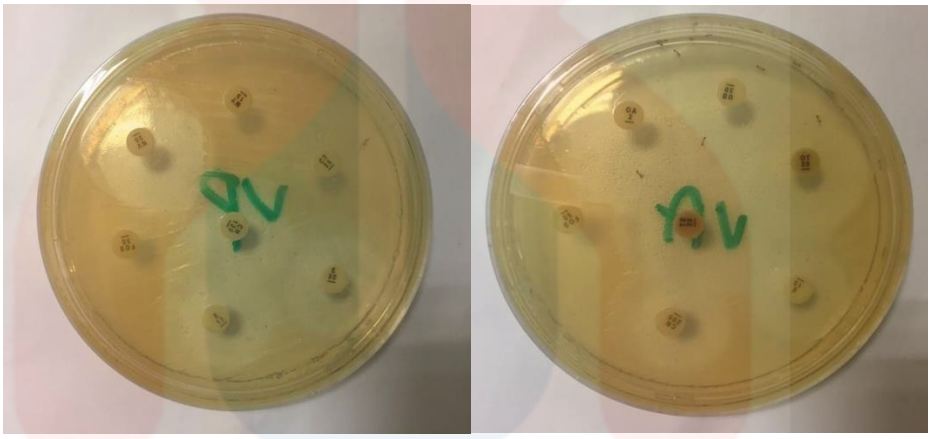

Figure 3: Different antibiotic discs used on Trytone Soy Agar (TSA)

One of the most common causes of illness problems in ornamental fish is *E. Coli*. Antimicrobial susceptibility tests were used to describe *E. Coli* isolates from sick ornamental fish. In this investigation, 30 *E. Coli* isolates were obtained from sick ornamental fishes from various regions of the animal. The E. Coli bacteria may be discovered in the fish's skin, eye, kidney, gills, and abdomen. Stress, changes in environmental circumstances, overcrowding, handling, transportation, poor water quality, temperature variations, low dissolved oxygen, high CO2 levels, high nitrite levels, and high ammonia levels are all common causes of disease outbreaks. (PMC, E., 2022). The

following are the most prevalent predisposing factors for *E. Coli* infections. Furthermore, *E. Coli* pathogenicity appears to be linked to host stress; high-virulence *E. Coli* may infect healthy fish; however, stress from intensive fish farming also contributes to and drives outbreaks (Austin and Austin 2007). Sixteen antibiotics that select are novobiocin (30 µg), tetracycline (30 µg), flumequine (30 µg), sulphamethoxazole (25 µg), amoxycillin (25 µg), chloramphenicol (30 µg), compound sulphonamides (300 µg), spiramycin (100 µg), ampicillin (10 µg), oxytetracycline (30 µg), doxycycline (30 µg), nalidixic acid (30 µg), erythromycin (15 µg), kanamycin (30 µg) and oxolinic acid (2 µg).

Table 1: Table of antimicrobial susceptibility test of Escherichia Coli on Cichlasoma sp.

Type of antimicrobial agent	Concentration (µg/disk)	Bacteria Isolated (*E. coli)*		
		Sensitive (%)	Intermediate sensitive (%)	Resistance (%)
Amoxycillin (AML)	25	3.33	16.67	80.00
Ampicillin (AMP)	10	0.00	50.00	50.00
Chloramphenicol (C)	30	56.67	30.00	13.33
Compound sulphonamides (S3)	300	0.00	6.67	93.33
Doxycycline (DO)	30	63.33	36.67	0.00
Erythromycin (E)	15	93.33	6.67	0.00
Flumequine (UB)	30	86.67	13.33	0.00
Fosfomycin (FOS)	50	70.00	20.00	10.00
Kanamycin (K)	30	76.67	23.33	0.00
Nalidixic Acid (NA)	30	80.00	20.00	0.00

Novobiocin (NV)	30	6.67	63.33	30.00
Oxolinic Acid (OA)	2	93.33	6.67	0.00
Oxytetracycline (OT)	30	53.33	43.33	3.33
Sulphamethoxazole (RL)	25	0.00	43.33	56.67
Spiramycin (SP)	100	76.67	23.33	0.00
Tetracycline (TE)	30	33.33	66.67	0.00

Antimicrobial susceptibility was determined for the 30 samples from *Cichlasoma sp*. The isolated bacteria exhibited variable resistance rates to 16 antimicrobial agents (Table 1). Interestingly, the highest resistance was observed for Compound sulphonamides with 93.33%, followed by Amoxycillin with 80.00%. In contrary, some isolates of *E. coli* showed the highest intermediate sensitive percentage was Tetracycline with 66.67%, the less sensitive was Ampicillin, Compound sulphonamides, and Sulphamethoxazole with 0%. Finally, this study adds to our knowledge of the principal aerobic bacterial species found in Malaysian ornamental fish. *E. coli* is a serious illness concern in ornamental fish and choosing the most efficient antimicrobials to combat it is crucial (AYA GALAL SAAD EL-DEEN and MOHAMMED RAWWAY, 2014).

4.3 Discussions

Escherichia coli is a common pathogen that causes severe mortality in Flower Horn populations throughout Asia. It's a widespread issue in aquarium stores that can harm both sellers and buyers. In Malaysia, shopkeepers preferred Nalidixic acid and Oxolinic acid for treating *Escherichia Coli* in seafood (Yanong, R. P., 2003). Antibiotics were commonly employed in pellet feed and taken orally. Microbial genetic ecology influences antibiotics used in aquariums through mutation, selection, and flow of genetic information, also known as horizontal gene transfer between microbes (Matyar et al., 2008). Overuse of antibiotics in aquariums has resulted in the proliferation of drug-resistant bacteria, which have no effect on humans if they are infected by bacteria after intake (Mitchell, 2015).

Several investigations of antibiotic resistance in *E. Coli* were conducted in Malaysia. In this investigation, the highest resistance was observed for Compound sulphonamides with 93.33%, followed by Amoxycillin with 80.00%. Because Compound sulphonamides are mostly synthetic antibacterial compounds with a broad spectrum of action against a wide range of bacterial species, this is the case. (Chaves Hernández, A., 2014). This study found high resistance of *E. coli* to the antimicrobial drugs tested, similar to what was shown by who found very high resistance of *E. coli* isolates to Amoxycillin. The resistance to Amoxycillin found in this study was like that seen in South Africa, Israel (62 to 84%), and Hong Kong, Philippines (64 to 82%). (Desenclos, J. C., & et, 1988). Then, the next resistant antibiotic is Sulphamethoxazole and Ampicillin with 56.67% and 50.00% respectively. According to Liu, Y. et al (2012), Sulphamethoxazole is widely

used in the treatment of infectious diseases caused by bacterial pathogens in aquaculture. In their work on antibiotic resistance detection in *E. coli* strains obtained from two different aquaculture systems in South China, Zhang et al (2013) found high Ampicillin resistance. Ampicillin resistance was also found to be high by Hatha et al. (2005). In another investigation, isolates from Malaysian mangrove soil were found to be 100.00% resistant to Ampicillin. In Australia, according to Akinbowale et al. (2006), 54.80% of aquaculture bacteria are resistant to Ampicillin.

According to Bacolla et al (2006), a DNA gyrase inhibitor, which also acts as an enzyme that introduces negative supercoil tension in *E. coli*, totally prevented cell death, resulting in poor resistance of only 30.00 percent. Since 1983, the use of chloramphenicol in aquaculture has been prohibited in a number of nations, including Malaysia, Korea, and Japan. This is due to Chloramphenicol's harmful effect in humans, even at extremely low doses, which can result in various side effects such as serious or fatal blood issues. Anaemia and "grey syndrome," a cyanosis and cardiovascular collapse condition that occurs most commonly in new born, are examples of blood disorders. Workers handling antibiotic-containing items are at danger as a result of this. Antibiotics have been banned, which has helped to reduce the amount of antibiotic-resistant microbes in the environment. With 13.33% resistance to Chloramphenicol, this study found a fairly low percentage of resistance (Kathleen et al, 2016). Despite the fact that lack of Fosfomycin susceptibility was uncommon in the current investigation (10.00%), Fosfomycin susceptibility was not identified for all isolates and cannot be ruled out as a reason of diminished clinical and bacteriologic response to treatment. When compared to other antibiotics, Fosfomycin is well tolerated, can be used in special fish populations such as liver impairment, pregnancy, and renal dysfunction, and has a low frequency of hypersensitivity, allergy, and adverse responses (Derington et al, 2020). Antibiotic resistance is as follows, 3.33%

Oxytetracycline is not entirely absorbed or digested in the organisms to whom it is given, and around 30% to 90% of the utilised quantity is excreted into the environment by urine and faeces. Oxytetracycline is a tetracycline antibiotic having broad range action against gramme positive and gramme negative microorganisms. Interfering with bacterial protein production is how the product works. (Oxytetracycline, 2022).

Doxycycline, Erythromycin, Flumequine, Kanamycin, Nalidixic Acid, Oxolinic Acid, Spramycin, and Tetracycline are among the antibiotics with zero percent resistance. Because Doxycycline is a safe, affordable, and nearly universally available antibiotic, further big in vitro and in vivo investigations are needed to better understand its potential as a novel supplementary treatment for multidrug-resistant *E. coli* infections. Although Doxycycline is a low-cost antibiotic, it has a broad spectrum of action against a variety of pathogens, including Gram-negative bacteria, and is still used to treat a variety of infectious disorders. Doxycycline is effective against multidrug-resistant organism infections, even in this period of growing occurrence of multidrug-resistant organism infections (Lai et al, 2016). In developing *Escherichia coli* cells, Erythromycin prevents the assembly of the big ribosomal subunit. The genetic origins of the strains, resistance features at the genus and species levels, kinds of mutations giving resistance, and fitness costs which defined as lower competitiveness in the absence of antibiotics that can all contribute to these disparities (Ge et al, 2017). Kanamycin is used to treat bacterial infections caused by one or more pathogens in the short term. Kanamycin, an aminoglycoside antibiotic, can stop *E. coli* from making peptides by stopping the translocation mechanism (Liu et al, 2020). Antibiotic resistance occurs when bacteria cease to respond to antibiotics intended to kill them, implying that the bacteria are not destroyed and continue to multiply. Tetracycline was outlawed as a growth enhancer for cattle in Sweden in the 1980s, and its usage in veterinary medicine has decreased as well.

Tetracycline, on the other hand, is still often used in human clinical practise (Karami et al, 2006).

The highest intermediate sensitive was observed for Tetracycline with 66.67%, followed by Novobiocin with 63.33% and Ampicillin with 50.00%. The fact that Tetracycline intermediate resistance in *E. coli* has not decreased in the last 20 years could indicate that the widespread use of Tetracycline in human medicine compensates for the reduced use of Tetracycline in farming animals in the selection of Tetracycline-resistant strains among commensal *E. coli* (Karami et al, 2006). Novobiocin is an antibiotic that prevents DNA from being unwrapped and rewrapped during DNA replication during the bacterial cell cycle. The current findings were like those of Najiah et al. (2009), who concluded that the antibiotics used were no longer effective in controlling bacterial growth. These findings are in line with others from throughout the world, which reveal that Ampicillin is the least effective antibiotic against *E. coli*, with resistance rates ranging from 50 to 75 percent. The frequent and improper use of Ampicillin in empirical therapy may be to blame for the high levels of *E. coli* resistance to this antibiotic. As a result, Ampicillin is no longer indicated as a first-line therapy for urinary tract infections (Yılmaz et al, 2016). The next high intermediate sensitive is Oxytetracycline and Sulphamethoxazole with same 43.33% index value. Oxytetracycline, a member of the tetracycline antibiotic family, is a broad-spectrum antibiotic that is effective against a wide range of bacteria. It is used to treat infections caused by Gram positive and Gram-negative bacteria such as *Mycoplasma pneumoniae, Pasteurella pestis, Escherichia coli, Haemophilus influenzae*, and *Diplococcus pneumoniae*. Sulphamethoxazole, on the other hand, is not frequently used because to a palatability issue that has a negative impact on fish kidneys (Yang et al, 2020).

E. coli were intermediate sensitive to Doxycycline with 36.67% and Chloramphenicol with 30.00%, while low level of intermediate sensitive was for Kanamycin and Spramycin with both 23.33%, and Fosfomycin and Nalidixic Acid with both 20.00%. This implies that a medium dose of antibiotic is required to inhibit growth, and they may encounter drug side effects as a result (Sensitivity Analysis, 2022).

There are few antibiotic agents which lower than 20 percent in intermediate sensitive, like Amoxycillin with 16.67%, Flumequine with 13.33%, and Compound sulphonamides, Erythromycin and Oxolinic Acid with 6.67%.

Interestingly, Erythromycin and Oxolinic Acid were shown to be the most susceptible medications. Because Erythromycin is most effective against gram-positive bacteria, while most bacteria that cause sickness in fish are gram-negative, it should only be administered when culture and sensitivity test results show that it is efficacious. Broad-spectrum antibiotics, such as Oxolinic acid, function against a large range of microorganisms (Yanong, R. P., 2003). Furthermore, Flumequine and Nalidixic Acid, both of which have a high susceptibility rate of 86.67% and 80.00%, were previously regarded as model antimicrobial agents due to their broad spectrum of action, favourable pharmacokinetics, and low incidence of toxicity (Daly, M., & Silverstein, D., 2009). Moreover, Kanamycin and Spiramycin, both have 76.67% sensitive. Both medicines are used to treat severe bacterial infections and operate by killing or stopping germs from multiplying (IBM Micromedex, 2022). Fosfomycin and Doxycycline were shown to be the susceptible medications, with a score of 70.00% and 63.33%. Interest in the use of Fosfomycin against multidrug-resistant (MDR) infections in additional indications has recently developed, motivating the development of a parenteral formulation for use in the

United States (Silver, L., 2017). Doxycycline is a broad-spectrum semi-synthetic antibiotic that has been used to treat germs in both animals and humans. Antibiotics have made a significant contribution to aquaculture output, even though their abuse can cause public health issues (Lee, K., 2020).

Furthermore, antimicrobial susceptibility tests for Chloramphenicol with 56.67% sensitive and Oxytetracycline with 53.33% sensitive, were rarely used in aquarium shops. Broad-spectrum antibiotics, such as oxytetracycline, are effective against a large range of microorganisms. Nonetheless, the unlawful use of restricted antimicrobial drugs in aquaculture, such as chloramphenicol, has become a serious problem in terms of consumer safety and the development of drug-resistant bacterium strains (Ng, et al., 2014). Tetracycline, Novobiocin and Amoxycillin were very rare used as the percentage is 33.33%, 6.67% and 3.33% respectively. Compound sulphonamides and Sulphamethoxazole were shown to be ineffective in preventing the growth of the isolates based on the susceptibility test results. Even though antibiotics may not be effective in treating some of the illnesses produced by this organism, this situation indicates that Streptomycin and Ampicillin should not be used to treat infections caused by this organism.

E. Coli may have contaminated the fish through feeding, the atmosphere of aquarium stores, or poor cleanliness, which might explain these results. Infection contamination might be caused by imported fish or improper raw material feeding, according to Sarter et al. (2007). The high frequency of drug resistance is a severe problem that necessitates the implementation of an antibiotic surveillance programme. To distinguish germs from various origins, researchers performed Multiple Antibiotic Resistance (MAR) analyses (Osundiya et al., 2013). In this investigation, high MAR indices were found, the Multiple Antibiotic Resistance (MAR) index value for all

bacterial *E. coli* isolates is 0.50 which are greater than 0.2 are indicators of high-risk sources, which might pose a risk to human health (Gwendelynne et al., 2005). Different forms of antibiotics supplied in aquarium shops may cause differences in resistance and susceptibility outcomes to the same medications. This might also be due to the diverse fish habitat or illness history. According to Su et al. (2017), water sources are the most significant channel for spreading antibiotic resistance genes. The discrepancies in various antibiotic resistance findings might be due to a variety of causes. According to Abuseliana et al. (2010), variables such as environmental variability, serotype variation, and frequent and non-guided treatment became the explanations for the differences in antibiotic resistance and sensitivity. Antibiotic use in medicine and animal husbandry has resulted in phenotypic alterations, often due to chromosomal mutations, according to WHO (2014), and antibiotic resistance in *E. coli* been detected internationally in isolates from human sources, animal and environmental. This finding points to the dangers of handling tainted water in an aquarium store.

CHAPTER 5

CONCLUSION AND RECOMENDATION

E. Coli's abilities as a bacterium must be further investigated in order to confirm their function in sickness and avert a potentially disastrous mass breakout. Bacteria were recovered from ornamental fish, *Cichlasoma sp*., based on isolation and identification qualities, as well as molecular testing. A total of 30 isolates from various areas of fish were successfully cultivated and identified as *E. Coli*, a pathogen that causes illness in ornamental fish, particularly in pet shops. *E. Coli* can be reliably identified and further characterised using the biochemical and molecular approaches utilised in this investigation. Furthermore, it aided study into *E. Coli* as a bacterium capable of causing sickness. The current investigation discovered that *E. Coli* infection may be linked to ornamental fish in pet stores. Erythromycin and Oxolinic Acid was the most effective antibiotics against *E. Coli* bacteria. Compound sulphonamides became the most resistances antibiotic in this study. In contrary, some isolates of *E. coli* showed the highest intermediate sensitive percentage was Tetracycline, the less sensitive was Ampicillin, Compound sulphonamides, and Sulphamethoxazole with. The MAR index value is 0.50 which are greater than 0.2 are indicators of high-risk sources. As a result, pet stores should avoid employing antibiotics to prevent the spread of antibiotic resistance in the aquaculture industry. Overall, the results demonstrated the need of proper aquaculture procedures in pet stores, as well as being selective and aware before utilising antibiotics. There have been few reports of *E. Coli* related economic losses in Malaysia, however

researchers have documented the development of *E. Coli* infection in ornamental fish in Malaysia.

Hopefully, in the future, researchers will be able to establish effective tank management and a water-recirculation system, allowing Flower Horn to have a consistent supply of pollution-free freshwater. Furthermore, instead of employing commercial antibiotics, novel antimicrobial compounds derived from natural resources should be developed to combat bacterial contamination of the Flower Horn. Whereas antimicrobial agents derived from natural resources are believed to be more environmentally friendly and safer to consume by humans and animals.

Furthermore, proper and appropriate sanitation facilities should be built to ensure that bacteria contaminated in Flower Horn is being breed or sold before being sold to clients without compromising the breed's quality or health. For example, a new E-Sensor technology should be developed to offer more rapid notice of Flower Horn health. It is critical that researchers, the Ministry of Agriculture, and the Argo-Based Industry work together to promote the use of the E-Sensor among Flower Horn breeders and pet store owners, as well as to educate them on the significance of health care in the decontamination of the Flower Horn. On the other hand, it is critical for researchers and the Ministry of Health to monitor the safety level of Flower Horn, as well as to educate communities, particularly consumers, about the need of being aware of the germs associated with the Flower Horn.

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APPENDIX A

Total of antibiotic Sensitive (S), Intermediate sensitive (IS) and Resistance (C) of the present isolate for Escherichia Coli

Sample	AML	AMP	C	S3	DO	E	UB	FOS	K	NA	NV	OA	OT	RL	SP	TE
A1	R	IS	S	R	S	S	S	IS	S	IS	IS	S	IS	IS	S	IS
A2	R	R	IS	R	IS	S	IS	IS	S	IS	R	S	IS	R	IS	IS
A3	R	R	S	R	S	S	S	S	S	S	IS	S	S	R	S	S
A4	R	IS	R	R	S	S	S	S	S	S	IS	S	S	IS	S	IS
A5	R	IS	S	R	S	S	S	S	S	S	R	S	S	IS	S	IS
A6	R	IS	S	R	S	S	S	S	S	S	IS	S	S	IS	S	IS
A7	R	IS	IS	R	S	S	S	S	S	S	IS	S	S	R	S	S
E1	R	R	S	R	IS	S	S	IS	S	S	IS	S	S	IS	IS	IS
E2	R	IS	IS	R	S	IS	S	S	S	S	IS	S	IS	R	S	S
E3	IS	IS	S	IS	IS	S	IS	IS	S	IS	IS	S	S	IS	S	IS
E4	R	R	S	R	S	S	S	S	IS	S	R	IS	IS	R	IS	S
E5	R	R	IS	R	S	S	S	S	S	S	R	S	R	IS	S	IS
E6	R	IS	S	IS	S	S	S	S	S	S	IS	S	IS	R	S	S
E7	R	R	R	R	S	S	S	S	S	S	R	S	S	R	S	S
G1	IS	R	IS	R	S	S	S	S	S	S	IS	S	S	R	S	S
G2	R	R	S	R	IS	IS	S	S	S	S	S	S	IS	IS	IS	IS
G3	R	IS	S	R	S	S	S	S	S	S	IS	S	S	R	S	IS
G4	R	R	IS	R	IS	S	IS	IS	S	S	R	S	S	R	S	IS
G5	IS	R	S	R	S	S	S	S	S	S	IS	S	S	IS	S	IS

G6	R	IS	S	R	S	S	IS	IS	S	S	IS	S	S	R	S	IS
K1	R	R	IS	R	IS	S	S	S	IS	S	IS	S	S	IS	S	IS
K2	R	R	S	R	S	S	S	S	S	S	S	S	IS	R	S	S
K3	S	IS	S	R	S	S	S	S	S	S	R	S	S	IS	IS	IS
K4	R	IS	S	R	IS	S	S	S	S	IS	IS	S	S	R	S	S
K5	IS	R	S	R	S	S	S	S	S	S	IS	S	IS	IS	S	IS
S1	R	IS	S	R	S	S	S	S	S	S	IS	S	S	R	S	IS
S2	R	IS	S	R	IS	S	S	S	S	S	R	S	S	R	S	IS
S3	R	R	S	R	S	S	S	S	S	S	IS	S	IS	R	S	IS
S4	R	R	S	R	S	S	S	S	S	S	R	S	S	IS	S	IS
S5	IS	IS	S	R	IS	S	S	S	S	S	IS	S	IS	R	S	IS

A Abdomen

E Eye

G Gill

K Kidney

S Skin

FYP 2

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