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Antimicrobial Resistance Profiles in *Vibrio* spp. and *Aeromonas* spp. from Pangasius Fish: Detection and Identification from the Three Markets at Siem Reap Province, Cambodia

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ABSTRACT

Pangasius fish carry various bacteria, notably *Vibrio* spp. and *Aeromonas* spp., which concern human health risks. They affect fish production and also produce harmful toxins for humans. The more antibiotics used in production, the greater the risk of antibiotic-resistant bacteria dominating competition at the antimicrobial resistance level. The study used 30 samples collected from three markets in Siem Reap, which were tightly packed in cooler bags during transport. The experiments were performed at the Microbiology Laboratory at RUA. These two bacteria can be detected using selective mediums known as TCBS Agar and MacConkey Agar. The purified colonies were then identified and characterized by gram stain, biochemical tests including catalase, oxidase, TSI, and motility test, and then confirmed strains using API 20E Version 5.0 system. The antibiotic susceptibility test was conducted using the disk diffusion method with 7 different drugs. The result showed that the total presence of *Vibrio parahaemolyticus* (n=12) was about 40% and *Aeromonas hydrophila* (n=14) was about 46.67%. Consequently, both bacteria had strong resistance to Ampicillin and Colistin Sulphate but were highly sensitive to Florfenicol, Sulfamethoxazole/Trimethoprim, and Oxytetracycline. In conclusion, the significant presence of these bacteria in pangasius fish poses health risks. The use of antibiotics often leads to bacterial immunity and antimicrobial resistance in which both bacteria show resistance to Ampicillin and Colistin Sulphate. This emphasizes the need for a cautious approach to antibiotic use to safeguard human health and maintain efficacy in pangasius fish.

INTRODUCTION

Antimicrobial resistance (AMR) has emerged as a major threat to human health. Global mortality and the economic burden caused by AMR are anticipated to continue increasing if it is left unchecked. In Cambodia, Antibiotics have been applied in aquaculture with the feed or used to cure infections. *Vibrio* spp. and *Aeromonas* spp. are common microorganisms found in pangasius fish and other marine life which are known as a major cause of infection including an effect toward food poisonous as safety concern (Reed *et al.*, 2019). Pangasius fish is popular for cooking and processing into various side dishes such as Prahok and stew. Many types of bacteria are found in fish including the most common and notable bacteria known as *Vibrio* spp. and *Aeromonas* spp. Both bacteria can cause diseases in humans, such as liver disease, gastritis, diarrhea, typhoid fever, and in some severe cases can be fatal. Meanwhile, *Aeromonas* spp. is an infectious bacterium which affects fish production as well as producing toxin substances in food that can be harmful to humans. At the same time, antibiotics are used to control and treat diseases or infections caused by bacteria. The greater the amount of antibiotics used, the higher the risk that the number of antibiotic-resistant bacteria will dominate the competition for survival at the level of antimicrobial resistance (Rasul & Majumdar, 2017).

Vibrio spp. is a gram-negative that has lipopolysaccharide as a cell wall. More than 148 species have been recognized (Wright *et al.*, 1996). *Vibrio* spp. causes gastroenteritis, nausea, and fever may be fatal. Many sources of disease are related to this type of bacteria, including the presence of bacteria or toxins from bacteria present in many organs of the fish and ulcers of the skin of the fish (FAO, 2021). Three main *Vibrio* spp. species; *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus* are global concern due to its infectious disease and contamination (Farmer *et al.*, 2015). Many hypotheses have been raised about cases of outbreaks, most of which are in industrialized and developing countries, as high population growth and high demand for seafood increase in food production in the industry that contributing to safety concerns.

Aeromonas spp. is a rod gram negative bacteria categorized as Enterobacteriaceae. *Aeromonas hydrophila* causes a disease that can be transmitted from animals to humans. However, the danger arises when eating fish. People who do not have an immune system, such as children, the elderly, or people with other health problems, are at the highest risk of being infected (Swann, 2007). Recent studies have identified the presence of two Shiga toxins (stx1 and stx2) in *Aeromonas* cells from patients with intestinal infections and diarrhea (Alperi and Figueras, 2010). Shiga toxin produced in bacteria can cause diarrhea and in rare cases can cause colitis and kidney damage. *Aeromonas* spp. identified and detected most presence in contaminated water, soil, fish, food, domestic animals,

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vertebrates, and birds (Lamy *et al*., 2009).

A historical event on September 3, 1928, led to the discovery of the antibiotics called penicillin by Alexander Fleming (Fleming, 1929). During the 1930s, the first widely used antiviral drug, Prontosil, was synthesized from the organic chemical sulfonamide by German chemist Gerhard Domagk (Sneader, 2001). In 1940, the Oxford team, led by Howard Florey and Ernst Chain, published a description of the purification of sufficient penicillin for clinical trials. This post-publication boosted production momentum and antibiotics were widely distributed in 1945 (Chain *et al*., 2005). In 1947, Waksman considered antibiotics to be produced by microbes that retain and kill microbes (Waksman, 1947).

Antimicrobial Resistance (AMR) may be an effective transformation of bacteria through genes that require the rapid development of multidisciplinary drugs to prevent risks to human health (McMillan *et al*., 2019). Antimicrobial resistance increases when bacteria or fungi have the ability to destroy drug components, as bacteria increase, making the drug incapable of destroying bacterial structures and killing germs. Bacterial infections that are resistant to antimicrobials are difficult to deal with and sometimes uncontrollable (WHO, 2014).

The main objectives of the study are (1) to detect and identify the presence of *Vibrio* spp. and *Aeromonas* spp. in pangasius fish from three targeted markets in Siem Reap. (2) to analyze susceptibility to seven antibiotics and determine the resistance profiles. The study lasted for three months and was conducted at the Microbiology Laboratory of the Faculty of Agro-Industry of the Royal University of Agriculture from December 2022 until February 2023.

LITERATURE REVIEW

Pathogens Threatening Pangasius Fish Survival

Pangasius fish are known to survive in polluted environments that undergo seasonal changes, as well as in high-density populations. However, these fish are prone to infection problems (Nam, 2009). *Aeromonas* spp., particularly *Aeromonas hydrophila*, have been identified as major pathogens for fish transmission (Ferguson *et al*., 2001). Other pathogens such as *Vibrio* spp., *Mycobacterium* spp., *Listonella anguillarum, Vibrio salmonicida*, and *Photobacterium damsela* (Lafferty *et al*., 2015) can also significantly affect the health and survival of fish.

The Case Study of Foodborne Illness in Cambodia

Attention to tightening food safety in Cambodia is still limited. Foodborne illness caused by microorganisms is a matter of concern because the health of many people who eat it is affected, so food quality factors need to be taken into account. Precautions for food safety reduce the risk of contamination and protect consumers from food-related diseases and injuries (Shahen, 2024). Pangasius is a type of fish that is popularly eaten and processed into a variety of food products. The popularity of eating this type of fish requires careful caution regarding to the safety of harmful microorganisms present in the body of the fish. Due to an incident that occurred on April 10, 2012, the administration of Kampong Speu Provincial Hall informed the Department of Infectious Diseases Management that 49 cases of diarrhea and severe vomiting were found two days after most patients attended the wedding in Tbong Boeng village on April 8. The next conclusion is that these cases are food poisoning caused by *Vibrio* spp. which *V. parahaemolyticus* species were present in wedding dishes (Vandy *et al*., 2012).

Environmental Resilience and Health Risks of *Vibrio* spp.

Vibrio spp. is a gram-negative bacteria that contain lipopolysaccharide outside the cell membrane. It belongs to the *Vibrionaceae* family which can survive in water that is resistant to salinity, including hot water, clear water, sewage and seawater. *Vibrio* is a multi-genetic group with genetic evolution and rod shape (Thompson *et al*., 2005). It is important to note that three of *Vibrio* spp. are most commonly found in seawater and freshwater known as *V. cholerae, V. parahaemolyticus,* and *V. vulnificus*. Risks caused by *Vibrio* spp. to the environment, animals, and human health are believed to be spread by factors of climate change, especially global warming, which also affects seawater (Vezzulli *et al*., 2013). Rising temperatures may directly or indirectly contribute to mutations in the genes of viruses and the recent geographical adaptation of *Vibrio* spp (Greenfield *et al*., 2017). Significant factors of cholera transmission caused by *Vibrio* spp. occur through water and food. Conditions that favor the occurrence of cholera are due to poor quality sanitation of water sources (EFSA, 2021).

Aeromonas spp. as a Threat to Fish Health and Public Health

Aeromonas spp. is a gram negative, rod-shaped, 1 to 3.5 µm long, belonging to the family Enterobacteriaceae (Martin *et al*., 2005). On the other hand, the presence of *Aeromonas* spp. is also a problem that contributes to foodborne illness. Experiments have shown that fish in poor environments due to improper water quality, such as high nitrate levels, low dissolved oxygen levels or high carbon dioxide levels, are vulnerable to *Aeromonas hydrophila* infections. The presence of this bacterium not only causes disease but also affects fish production, causing damage and economic loss (Amber, 2022). The risk of transmitting *A. hydrophila* to humans is through the consumption of infected fish, which can cause gastrointestinal diseases, pneumonia, diarrhea and meningitis, so attention should be paid to the presence of this bacterium crucially for public health (Kirov, 1993).

Antimicrobial Resistance in Cambodia as a Part of Growing Global Concern

Meanwhile, in response to the antimicrobial resistance problem, which is a major global issue, as well as part of the vision of a multi-sectoral action plan on antimicrobial resistance in Cambodia, the use of non-compliance

with technical principles and standards recommended by livestock experts has led to bacterial resistance to antimicrobials and natural immunity. Antimicrobial residues such as tetracycline, ciprofloxacin, enrofloxacin and amoxicillin are found in meat and eggs sold for human consumption (Islam *et al*., 2016). Antimicrobial resistance occurs when a type of microbe becomes resistant to antibiotics due to overuse. These immunocompromised microbes can infect humans or animals and are more difficult to treat than diseases caused by non-immune bacteria (O'Neill, 2015). Antibiotics have been used in human treatment, animal production and in preventing disease. It has been mixed with feed or water for animals or if the animal has a serious disease, the injection with antibiotics has been applied (Rahman *et al*., 2021). Hence, food systems are delicate, and current farming methods have a big impact on food security. (Chapagai *et al*., 2023).

Treatment Approaches for *Vibrio* spp. Infections

Most infections that caused by *Vibrio* spp. does not require much clinical treatment, but is often treated with antibiotics in case of severe *Vibrio*sis (Loo *et al*., 2020). Currently, in some cholera treatments, the antibiotic known as Azithromycin, Doxycycline (Tetracycline) or Ciprofloxacin (Aquinolone) are used (Das *et al*., 2020). Doxycycline and third-generation cephalosporins are currently recommended for *V. vulnificus* as a primary treatment, while Doxycycline or Quinolone are for *V. parahaemolyticus*. *V. vulnificus* infections often have serious consequences, even when prescribed antibiotic therapy is applied at an early stage (Hendren *et al*., 2017). Increased immunity to *Vibrio* spp. is due to the association or transfer of genes between immune and non-immune strains, including the multifaceted environmental factors that people use (Pérez *et al*., 2021).

Antibiotic Resistance in *Aeromonas* spp.

Ciprofloxacin was effective against *A. hydrophila* as well as effective against bacteria *A. caviae* and *A. veronii bv sobria* (Ko *et al*., 2003). Susceptibility of *Aeromonas* spp. that resistance to the antibiotics such as Cefotaxime, Ciprofloxacin, Trimethoprim, Chloramphenicol, Tetracycline and Cotrimoxazole has also been reported in a separate study (Vila *et al*., 2003). The presence of immunity in the food and water in which *Aeromonas* spp. bacteria are present has been observed to increase their ability to survive and grow even under antimicrobial conditions. This suggests that the immunity plays a crucial role in the persistence of these bacteria in different environments (Alcaid *et al*., 2010). The widespread use of antimicrobials for the prevention and treatment of humans and fish actually contributes to the increasing number of *A. hydrophila* species that can develop its immunity.

MATERIALS AND METHODS

Study Area and Sampling Size

The study was conducted to isolate *Vibrio* spp. and *Aeromonas* spp. from three markets in Siem Reap province, in each market, 10 samples were randomly caught and done by the sellers who usually used to collect or sell to the buyers as usual. Each stall caught a total of 30 samples which were tightly packed in cooler bags during transport. The sample bag is tightly closed and does not sink or seep into the sample during transport to the laboratory at Royal University of Agriculture.

Sampling Methodology

10 grams of each fresh pangasius fish were cut into a sterile bag. Each 90 ml of Buffered peptone water was prepared to mix with the sample in a sterile bag. The sample bags were placed to be separated by the Stomacher machine at 1200 rpm for 2 minutes, equivalent to the first dispersal (10^{-1}). Then, 1 ml was taken from the sealed sample bag and separated with each prepared tip containing 9 ml of saline solution to be diluted. The samples were ready to be detected and identified for *Vibrio* spp. and *Aeromonas* spp.

Detection of *Vibrio* spp. and *Aeromonas* spp. Methodology

After the sample was prepared and diluted, each diluted tip was filtered by 0.1 ml spreading on TCBS Agar for detecting *Vibrio* spp. and the same for detecting *Aeromonas* spp. but on MacConkey Agar prepared in Petri dishes. After placing the bacteria in the incubator for 24 hours, if *Vibrio* spp., the colonies that grew on the surface of the seedlings would be yellow or green. If *Aeromonas* spp., the colonies would be gray. The suspected colonies were then purified on the nutrient agar for 24h incubation before the chemical test to confirm the strains. After the purification, the confirmed colonies were detected and ready to be identified.

Identification of *Vibrio* spp. and *Aeromonas* spp. Methodology

The identification process consisted of various steps. Starting from the pure culture colonies were tested for morphology and gram type of bacteria. *Vibrio* spp. is identically a gram-negative, in the form of curved rod-shaped bacteria rods, that can be clustered or solitary and move by flagellin and hairs. *Aeromonas* spp. is also identically a negative gram type that has a single round shape or a pair. After the gram staining, a catalase test was performed to test if the bacteria had the presence of the enzyme Catalase. This test used hydrogen peroxide (H_2O_2) because the enzyme Catalase has the ability to break down hydrogen peroxide into oxygen and water. The test performed by the colony must not last more than 24 hours, as enzyme activity may decrease. Both *Vibrio* spp. and *Aeromonas* spp. are identically catalase-positive. The catalase-positive colonies were then continued to oxidase test. The test was performed to confirm if the bacteria had the ability to produce the enzyme using Oxidase strips (OXIOD). Both *Vibrio* spp. and *Aeromonas* spp. are identically oxidase-positive. If the color on the strip changes to purple, it can be confirmed that it is an oxidase-

positive. Next, TSI test was performed to demonstrate the ability of bacteria to use sugars to ferment and produce hydrogen sulfide which both bacteria identically have. Using a sterile needle, the pure colony was scraped into the slant surface, poured into the center of the bottom of the test tube of TSI agar, and placed it in the incubator at 35 °C for 24 hours to read the results. A motility test was also performed simultaneously to test the ability of the bacteria to move on their own through a tail called flagella or by moving through a fiber called a fibril. This step is one of the necessary identification processes because both bacteria detected identically can move. A Sterile needle was used to extract the pure colony and inject it into the center of the TSA agar without reaching the bottom of the tube then incubated in an incubator at 35 °C for 24 hours. If the stain is still visible, it means that the bacteria are not moving (Non-Motile) and if there is movement, the bacteria will grow all over the surface of the tube. Lastly, to accurately confirm the identification of the bacteria strains, this experiment in the study included the confirmation process using the API 20E System Version 5.0. First, the refined colonies were mixed into a tube containing 7 ml of API AUX Medium using a loop. The lid of the API 20E Test kit was opened to drip distilled water through the small holes in the cover to maintain the level of evaporation during insertion into the incubator. Then the solution of 1 ml was pumped into each of the 20 holes of the chemical. Carefully, the lid of the API 20E Test kit closed and placed in the incubator at 35 °C for 24 hours. Then the results were recorded, interpreted, and analyzed the identification in the API System to confirm which strain of the bacteria in what species.

Disk Diffusion Method for Antibiogram

First, the purified colonies were transferred to a tube containing 4 ml of saline solution (0.9% NaCl) and mixed thoroughly. 0.1 ml of solution was transferred onto the Mueller Hinton Agar (MHA) using a spreader to cover the surface of the MHA. Sterile braces were carefully used to apply antibiotics to the measured surface of the agar. Then placed in an incubator at 35 °C for 24 hours. After the incubation, the immunity of the antibiotic was measured using diameter.

Table 1: Susceptibility to antibiotic resistance

No.	Antibiotics	Abbreviation	Amount	Critical concentration (mg/l)		Critical Diameters (mm)	
				S	R	S	R
1	Ampicillin	AMP	10µg	≤4	>16	≥19	<14
2	Florfenicol	FFC	30µg	≤8	>16	≥23	<19
3	Oxytetracycline	OT	30µg	≤4	>8	≥13	<10
4	Erythromycin	E	15µg	≤1	>4	≥22	<17
5	Colistin Sulphate	CL	10µg	≤2	>4	≥16	<10
6	Ciprofloxacin	CIP	5µg	≤1	>1	≥22	<22
7	Sulfamethoxazole/ Trimethoprim	SXT	1.25/23.75µg	≤2/38	>8/152	≥16	<10

Note: S: Sensitive, R: Resistant, I: Intermediate
Adapted from: CLSI Guideline (2006, 2016, & 2021)

RESULTS AND DISCUSSION

Detection and Identification of *Vibrio parahaemolyticus*

According to the result, out of the 30 samples collected from three markets in Siem Reap province, 12 were found to be positive for *Vibrio* spp, which represents 40% of the total samples tested. The API 20E System Version 5.0 tests have conclusively identified the presence of the *Vibrio parahaemolyticus* strain in all of the 12 samples. Furthermore, *Vibrio parahaemolyticus* was detected in 5 samples from the Domdek market, accounting for 41.7% of the samples. The Samaki market had 4 samples, making up 33.3%, while 3 samples from the Ler Thom Tmey market, making up 25%, were also identified as having the same strain.

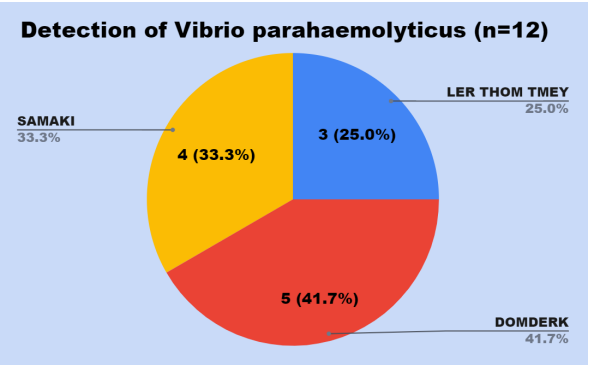

Figure 1: Detection of *Vibrio parahaemolyticus* from the three markets

Detection and Identification of *Aeromonas hydrophila*

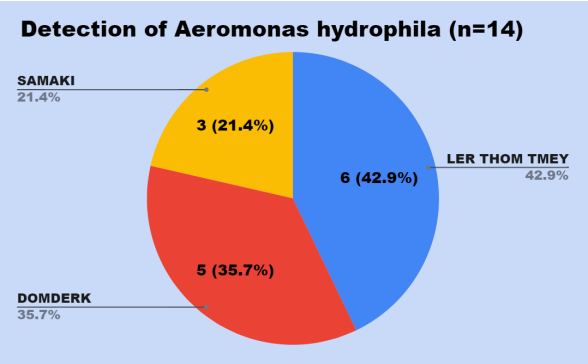

Figure 2: Detection of *Aeromonas hydrophila* from the three markets

According to the result, out of the 30 samples collected from three markets in Siem Reap province, 14 were found to be positive for *Aeromonas* spp., which represents 46.67% of the total samples tested. The API 20E System Version 5.0 tests have conclusively identified the presence of the *Aeromonas hydrophila* strain in all of the 14 samples. Furthermore, *Aeromonas hydrophila* was detected in 6 samples from the Ler Thom Tmey market, accounting for 42.9% of the samples. The the Domdek market had 5 samples, making up 35.7%, while 3 samples from the Samaki market, making up 21.5%, were also identified as having the same strain.

Antibiotic Resistance and Susceptibility Profiles of *Vibrio parahaemolyticus*

The results showed that *Vibrio parahaemolyticus* was resistant to Ampicillin at 83%, Florfenicol at 8.33%, erythromycin at 58%, and oxytetracycline at 50%, Colistin sulphate was 100%, ciprofloxacin was 25% and sulfamethoxazole/ Trimethoprim was 0%. Hence, *Vibrio parahaemolyticus* was 0% sensitive to Ampicillin, 83,33% to Florfenicol, 42% to Erythromycin, 8% to Oxytetracycline, 0% to colistin sulphate, 75% to ciprofloxacin and sulfamethoxazole/ Trimethoprim was 83%.

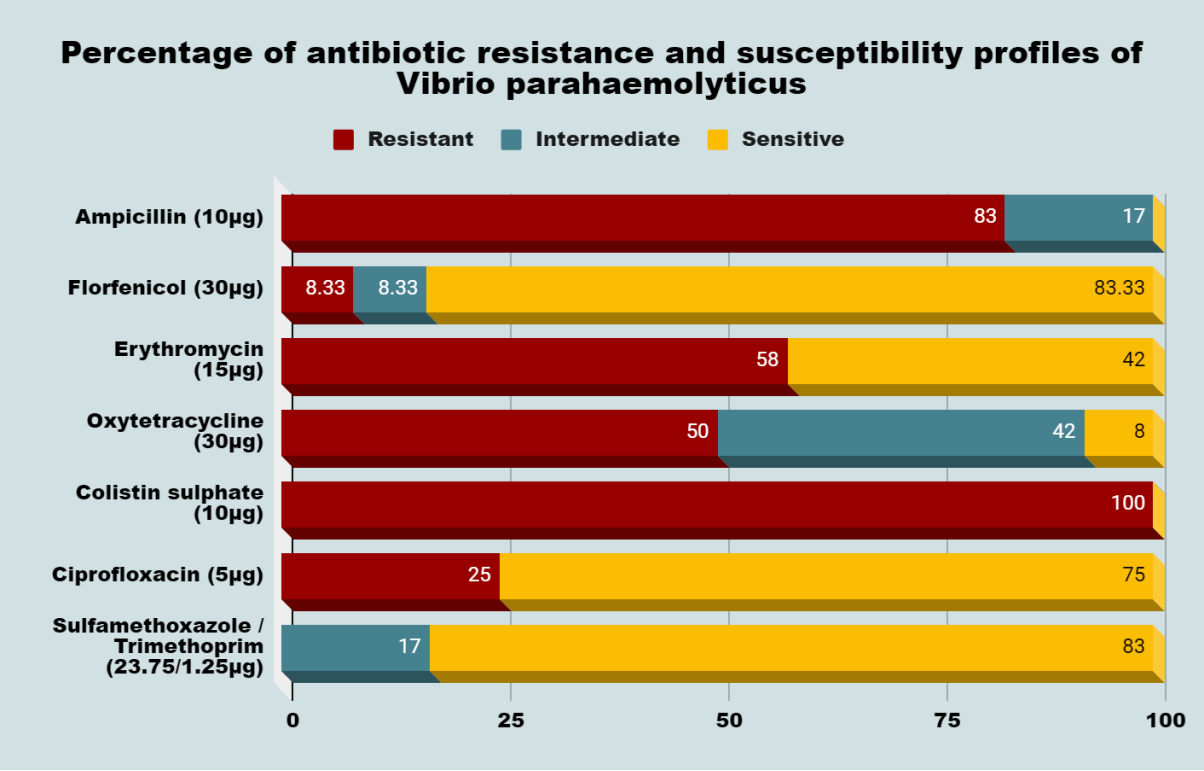

Figure 3: Antibiotic resistance and susceptibility profiles of *Vibrio* parahaemolyticus as percentage

Antibiotic Resistance and Susceptibility Profiles of *Aeromonas hydrophila*

The results showed that *Aeromonas hydrophila* was resistant to Ampicillin at 100%, Florfenicol at 14%, erythromycin at 21.5%, and oxytetracycline at 14%, Colistin sulphate was 57%, ciprofloxacin was 0% and sulfamethoxazole/ Trimethoprim was 29%. Hence, *Aeromonas hydrophila* was 0% sensitive to Ampicillin, 64% to Florfenicol, 57% to Erythromycin, 64% to Oxytetracycline, 0% to colistin sulphate, 100% to ciprofloxacin and sulfamethoxazole/ Trimethoprim was 71%. Ampicillin, as the first widely used drug, has been subject to extensive usage, leading to a considerable reduction in its efficacy. Consequently, the drug has become increasingly susceptible to bacterial resistance, thereby limiting its clinical utility in combating bacterial infections (Sudha *et al.*, 2014).

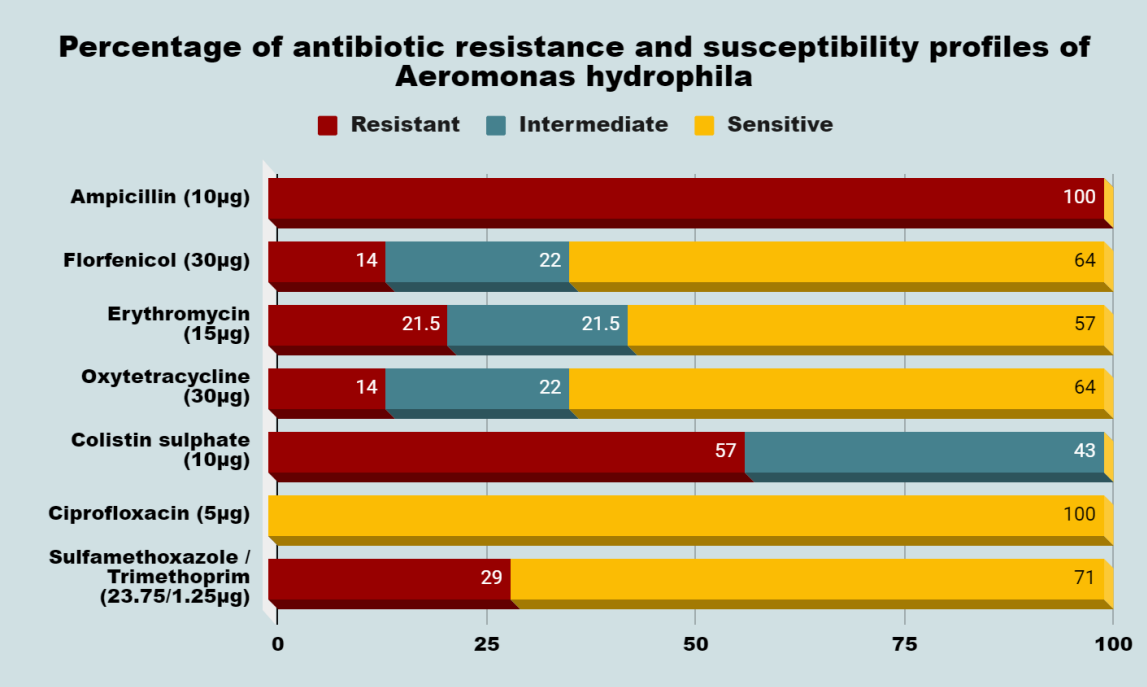

Figure 4: Antibiotic resistance and susceptibility profiles of *Aeromonas hydrophila* as percentage

Multidrug resistance profiles of *Vibrio parahaemolyticus* Based on their susceptibility number by group of antimicrobials, *Vibrio parahaemolyticus* with multidrug resistance profiles that are resistant to more than two antimicrobials were grouped into 6 profiles.

Table 1: Multidrug resistance profiles of *Vibrio parahaemolyticus*

Profile of Multidrug Resistance	Sample Code	Group of antimicrobials	Number of Drugs
Profile 1	SR-DD-R14 and SR-SK-R26	AMP, E, OT, CL, CIP, SXT	6
Profile 2	SR-DD-R15	AMP, E, OT, CL, CIP	5
Profile 3	SR-DD-R16	AMP, E, OT, CL	4
Profile 4	SR-SK-R28	AMP, FFC, E, CL	4
Profile 5	SR-LT-R6	AMP, OT, CL	3
Profile 6	SR-LT-R10	E, OT, CL	3

Note: Confirmed strains resistant to more than two antimicrobials are considered multidrug resistance profiles.
Ampicillin (AMP), Florfenicol (FFC), Erythromycin (E), Oxytetracycline (OT), Colistin Sulphate (CL), Ciprofloxacin (CIP) and Sulfamethoxazole/Trimethoprim (SXT)

Multidrug resistance profiles of *Aeromonas hydrophila* Based on their susceptibility number by group of antimicrobials, *Aeromonas hydrophila* with multidrug resistance profiles that are resistant to more than two antimicrobials were grouped into 4 profiles.

Table 2: Multidrug resistance profiles of *Aeromonas hydrophila*

Profile of Multidrug Resistance	Sample Code	Group of antimicrobials	Number of Drugs
Profile 1	SR-LT-R2	AMP, FFC, E, SXT	4
Profile 2	SR-LT-R8	AMP, OT, CL, SXT	4
Profile 3	SR-LT-R1	AMP, FFC, E	3
Profile 4	SR-LT-R4	AMP, E, CL	3
Profile 5	SR-LT-R7	AMP, OT, SXT	3

Note: Confirmed strains resistant to more than two antimicrobials are considered multidrug resistance profiles.
Ampicillin (AMP), Florfenicol (FFC), Erythromycin (E), Oxytetracycline (OT), Colistin Sulphate (CL), Ciprofloxacin (CIP) and Sulfamethoxazole/Trimethoprim (SXT)

CONCLUSION

In conclusion, the results of the above experimental study can be concluded that the total presence of *Vibrio parahaemolyticus* bacteria (n=12) was about 40% and *Aeromonas hydrophila* (n=14) was about 46.67% of the total sample taken from pangasius fish in the three markets at Siem Reap province. This shows that *Aeromonas hydrophila* was 6.66% more than *Vibrio parahaemolyticus*. The presence of both bacteria is often a cause for concern for fish infections and food poisoning or disease in people who eat fish containing these toxins and bacteria. Antibiotics with the highest resistance to these two bacteria are Ampicillin and Colistin Sulphate, and the two most sensitive to Florfenicol, Sulfamethoxazole/ Trimethoprim and Oxytetracycline. The high doses of Ampicillin and Colistin Sulphate have been shown to induce bacterial immunity, both of which are not feared, and these drugs are not effective in killing or treating fish that contain both bacteria. Based on the result, the study suggests that farmers should follow the antibiotics guidelines recommended by veterinarians to avoid the misuse and overuse of Antibiotics which could lead to AMR. Moreover, next academic researchers should identify the gap between AMU and AMR including the alternative options. Significantly, Policy researchers and lawmakers should strengthen the effectiveness of AMU policy following the One Health guideline by WHO.

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