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Detection of Foodborne Pathogen *Vibrio parahaemolyticus* in Milkfish and Nile Tilapia from Selected Wet Markets in Quezon City, Philippines

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Abstract

Vibrio parahaemolyticus is one of the foodborne pathogens causing public concern in both aquaculture and the medical field. In this study, presence of *V. parahaemolyticus* was detected in 84% (n = 84) of the total fish samples using thiosulfate-citrate-bile salts-sucrose (TCBS) agar, supplemented with 3% salt. Among these, 55.95% (n = 47) were from milkfish, while 44.05% (n = 37) were from tilapia, highlighting the halophilic nature of *V. parahaemolyticus*. Incidence of pathogenic *V. parahaemolyticus* with thermostable-direct hemolysin (*tdh*) gene was detected through polymerase chain reaction (PCR) (20.24% positivity rate) and loop-mediated isothermal amplification (LAMP) (21.43% positivity rate). In addition, all the confirmed pathogenic *V. parahaemolyticus* in both amplification methods were subjected to antimicrobial resistance assay, and were found to be resistant against sulfamethoxazole. Out of the 18 isolates, 28% (n = 5) were resistant to oxytetracycline; whereas 6% (n = 1) were resistant to florfenicol. Overall, 28% (n = 5) *V. parahaemolyticus tdh*+ isolates were found to be multi-drug resistant. The presence of pathogenic and multi-drug resistant *V. parahaemolyticus* in food sources is a major health and farming concern that could affect the sectors of food production, aquaculture, and public health. Thus, the results of this study could serve as the baseline for the development of microbiological criteria in aquaculture in the Philippines.

Keywords: Antibiotic resistance; *V. parahaemolyticus*; Loop-mediated Isothermal Amplification; Polymerase Chain Reaction; *tdh* gene

1. Introduction

Food quality and safety assessments are crucial in securing public health and ensuring that no contamination of the product will occur throughout the production line. These are also to reduce the chances of an outbreak that might cause injury and loss of life (Sadeghi *et al.,* 2021). Alongside *Salmonella* spp., *Clostridium botulinum* and *Listeria monocytogenes*, *Vibrio parahaemolyticus* ranks among the most common pathogens responsible for disease outbreaks linked to seafood. It has become one of the main causes of gastrointestinal illnesses, leading to outbreaks and sporadic cases of foodborne illnesses linked to the consumption of raw or undercooked contaminated seafood (Roy *et al.,* 2024).

Aside from being a public health concern, *V. parahaemolyticus* is also considered as a pathogen in the aquaculture industry as it causes bacterial infections in aquaculture products leading to high disease and death rates, as well as a lowered food production rate

(Sadeghi *et al.,* 2021). Though vaccination and improved husbandry practices are widely implemented to reduce use and make antibiotics the last option to combat diseases, many aquaculture-producing countries are still using antibiotics for therapeutic and prophylactic purposes (Lulijwa *et al.,* 2019). In this, the whole fish population is treated orally including the population of healthy fish to protect the fish from infection, thereby lowering the death rate and minimizing production losses (FAO, 2020). However, this practice enables the development of microbial resistance against the antibiotic applied because the infection is often not completely eradicated and cleared. The problem with antibiotic resistance is further amplified by human consumption of the affected fish, transferring the antibiotic resistance to humans leading to gastrointestinal diseases. Furthermore, it will be difficult for the healthcare industry to provide the appropriate treatment due to the resistance, which in turn increases costs and the number of days needed for recovery (Towers, 2017).

Several advances in terms of detection methods in food safety and quality assessment were developed throughout the years to ensure a safe food supply. Though traditional techniques still have adequate sensitivity and specificity for microbial detection, the results take longer to obtain depending on the rate of pathogen growth. Thus, several molecular techniques that are more sensitive, specific, and quick have emerged and are used nowadays (Teh *et al.,* 2020). One of these techniques, the Polymerase Chain Reaction (PCR) method, is a rapid screening method used in molecular biology that can detect the target nucleic acid sequences in environmental samples as well as to amplify weak input signals with high sensitivity and good reproducibility (Basu, 2021). Though the PCR method is widely used and performed due to its rapid and sensitive characteristics, it requires post-amplification gel electrophoresis which makes it laborious and expensive when dealing with large sample sizes. Thus, it has become necessary to find other rapid alternative for *V. parahaemolyticus* detection (Teh *et al.,* 2020).

Due to this challenge, many laboratories have opted to use cheaper alternatives, and among the most common substitutes is the loop-mediated isothermal amplification (LAMP) method that was developed by Notomi *et al.* (2000). It is considered among the most cost-effective substitutes for the PCR method offering higher sensitivity and accuracy by utilizing 6 primers, compared to 2 primers of PCR via stem-loop DNA series. Furthermore, it does not require a thermocycler as the isothermal amplification is done at a constant temperature (60 - 65 °C) providing high amplification efficiency of 10^9 to 10^{10} in 15 - 60 minutes thus significantly shortening the amplification duration (Notomi *et al.,* 2000). With the combination of speed, simplicity, and high specificity comparable to PCR, it is used for disease diagnostics and verification for the detection of several medically important pathogens such as *V. parahaemolyticus* (Foo *et al.,* 2020; Goodarzi *et al.,* 2018; Anupama *et al.,* 2021).

In this regard, this study aims to use a convenient and efficient technique, LAMP, to detect *Vibrio parahaemolyticus* in fish collected from fish markets in Quezon City, Philippines and compared this with conventional method, PCR. Further, confirmed isolates were subjected to antimicrobial resistance assay. Integrating LAMP, a novel molecular detection method, with antimicrobial testing offers a faster, cost-effective, and comprehensive diagnostic tool, which could significantly enhance pathogen control and promote responsible antibiotic use in the aquaculture industry.

2. Methodology

2.1 Sample collection, bacterial strains, processing, and labeling

The sample collection, processing, enrichment, and isolation were based on Bacteriological Analytical Manual (BAM) Chapter 9: *Vibrio* with modifications from U.S. Food and Drug Administration (2019). The major wet markets mentioned experience high consumer demand and hold strategic locations, making them key suppliers to small distributors, retailers, and other markets across Metro Manila. As a result, they serve

as important distribution hubs for fresh fish, ensuring that the collected samples represent the quality and safety of fish consumed by a significant portion of the Metro Manila population. Furthermore, these markets are near the laboratory where the study was conducted, facilitating efficient sample transport and handling. All the samples were collected between the months of November 2022 to March 2023. A total of 10 individual fish consisting of 5 Nile tilapia and 5 milkfish were bought from each of the 5 wet markets, for a total of 50 fish samples.

The reference strains used were from KWIK-STIK™ ATCC® (Microbiologics, USA) lyophilized microorganism and from fish samples. *Vibrio parahaemolyticus* ATCC® BAA-239TM (*tdh*+) and *Vibrio* alginolyticus ATCC® 17749 (*tdh*-) served as positive and negative *Vibrio* controls, respectively. *Escherichia coli* O157:H7 served as non-*Vibrio* negative control. All *Vibrio* positive, negative control and fish isolates were cultured in Trypticase Soy Broth (Condalab, Spain) with 3% salt (TSBS), while non-*Vibrio* negative control were cultured in Trypticase Soy Broth (TSB). All cultures were incubated at 35 ± 2 °C for 24 - 48 hrs.

After sample collection from different wet markets, both the milkfish and tilapia samples were placed in sterile plastic container and stored in an ice chest, without direct contact with the ice, upon transit to the Biological Research and Services Laboratory of the Natural Sciences Research Institute, University of the Philippines Diliman, and were processed within 1 hour after collection. From each fish, 2 samples, one from the flesh and one from the intestine, were obtained for a total of 100 samples. Five grams of flesh and 5 g intestine from each fish were obtained aseptically. For the flesh samples, the scales and skin of the fish were removed, followed by surface sterilization using 70% ethanol.

Next, a thin layer of flesh, without skin, was obtained using a sterile scalpel. For the intestine samples, the fish was cut in between the pectoral fin and ventral fin aseptically. Then, the intestinal tract from the posterior to anterior portion of the fish was cut using sterile dissecting scissors. The weighed flesh and intestine samples per fish were aseptically cut into small pieces using sterile dissecting scissors prior to homogenization.

2.2 Enrichment and isolation of presumptive V. parahaemolyticus

The small pieces of flesh and intestine samples from each milkfish and tilapia samples were homogenized using a handheld blender for about 1-2 minutes to make a 1:10 dilution mixture using Alkaline Peptone Water (TM Media, India) as shown in Figure 1.

After incubation for 24 hrs, the enriched samples were further diluted to 10^{-2}, 10^{-3}, and 10^{-4}. The samples were then aseptically streaked into thiosulfate-citrate-bile salts-sucrose (TCBS) (Himedia, India) agar plates followed by incubation at 35 ± 2 °C for 24 - 48 hrs. The resulting isolates that showed round green colonies with 2-3 mm diameter in TCBS plates were considered as presumptive *V. parahaemolyticus*. The presumptive colonies were inoculated to TSBS (Condalab, Spain) then incubated at 35 ± 2 °C for 24 - 48 hrs until growth and turbidity were observed.

Figure 1. Enrichment step of the fish samples in 45 ml APW prior to streak plating in TCBS (from BAM Chapter 9: *Vibrio* with modifications (U.S. Food and Drug Administration, 2019)

2.3 DNA extraction of presumptive V. parahaemolyticus isolates

The DNA of presumptive *V. parahaemolyticus* isolates in TSBS were extracted using GF-1 Bacterial DNA Extraction Kit (Vivantis Technologies, Malaysia) following the manufacturer's protocol. The eluted DNA was stored at 4°C and served as a DNA template for PCR and LAMP assay. Concentration of the presumptive *V. parahaemolyticus* gDNA was quantified using Qubit 4 Fluorometer (ThermoFisher Scientific, USA).

2.4 Molecular detection of tdh+ V. parahaemolyticus using PCR and LAMP method

2.4.1 PCR amplification

The following primers, *tdh* – FP: CTGTCCCTTTTCCTGCCCCCG and *tdh*-RP: AGCCAGACACCGCTGCCATTG, from the study of Yang et al. (2017), were used to detect *tdh* genes at 245 bp using PCR amplification in *V. parahaemolyticus* isolated from fish isolates.

Ready-to-use PCR Master Mix (Promega, Germany) 2X solution was used for efficient amplification of the DNA template by PCR. The solution was composed of 50 units/ml of *Taq* polymerase supplied in the reaction buffer (pH 8.5) of 400µM dATP, 400 µM dGTP, 400 µM dCTP, 400 µM dTTP and 3mM $MgCl_2$. A volume of 1 µL each of FP and RP along with 1 µL DNA template sample were added to 12.5 µL PCR Master mix. Nine and a half (9.5) µL of nuclease-free water was added to the sample to obtain 25 µL working volume of the reaction tube.

The amplification conditions from the study of Yang *et al.* (2017) were followed. The samples were exposed to 95 °C for 5 minutes (initialization) followed by 40 cycles of the amplification phase. This phase is composed of denaturation (95 °C for 1 min), annealing (62 °C for 1 min) and extension (72 °C for 1 mins). Afterwards, final extension of the amplification was done at 72 °C for 5 mins, followed by the holding stage at 4 °C.

Two µL of the PCR amplified product were added to 2 µL loading dye before being loaded into the 2 % agarose gel submerged in gel electrophoresis set-up with 1X Tris-Acetate EDTA (TAE; Vivantis, Malaysia) buffer, with the voltage set to 135 volts for 30 minutes.

The dye was stained with Diamond Nucleic Acid Stain (Promega, Germany) with a concentration of 1:10,000 for 10-15 minutes in a shaking dry bath at 20 - 25 °C. LED Transilluminator (Hercuvan Lab Systems, UK) was used to visualize a clear band of the specific PCR products at 245 bp with respect to the loaded molecular ladder.

2.4.2 LAMP amplification

The technical details and specifications of the LAMP assay methodology followed the optimized protocol of the project study "Development of Loop-Mediated Isothermal Amplification (LAMP)-Lateral Flow Assay (LFA) Based Kit to Detect *Vibrio* infections in fisheries", led by the Natural Sciences Research Institute, University of the Philippines Diliman funded by the Department of Agriculture – Biotechnology Program Office. Due to proprietary information of said project and instructions from the UP Technology Transfer and Business Development Office, exact specifications of the reaction cannot be provided.

Two µL of SYBR Green I (Lonza, USA) was added to each resulting LAMP tube before being observed under the UV transilluminator for fluorescence visualization. Further amplification confirmation of the target DNA sequences was done by visualizing 2 µL of the LAMP amplified product in 2 % agarose gel using gel electrophoresis with 1X TAE buffer. The gel was stained with Diamond Nucleic Acid Dye with a ratio of 1:10,000.

2.5 Antibiotic Susceptibility Test of pathogenic V. parahaemolyticus

Following the Kirby-Bauer Disk Diffusion Susceptibility Test Protocol from Hudzicki (2009), direct suspension of 4-5 colonies grown in tryptic soy agar (TSA)

(Condalab, Spain) with 3% NaCl (ACl Labscan, Thailand). The suspension was homogenized and adjusted based on the 0.5 McFarland standard (Himedia, India). Using sterile swab, the suspension was swabbed uniformly on the Mueller Hinton agar (MHA) (Himedia, India) with 3% NaCl plate.

Each swabbed plate was allowed to dry for 1-2 minutes before manually placing the antibiotic disk (Oxoid, England) using sterile forceps. The plates were then incubated at 35 ± 2 °C for 18 - 24 hrs. The zone of inhibition was measured using a digital caliper and the zone diameter value was interpreted according to CLSI recommendation breakpoint.

3. Results and Discussion

3.1 Enrichment and isolation of presumptive V. parahaemolyticus

Eighty-four percent (84%) of the streaked TCBS agar plates exhibited a positive green colony growth, which indicated the presence of *V. parahaemolyticus*. This high rate of *V. parahaemolyticus* presence in environmental samples highlighted the importance of the enrichment step for detection methods, recommended by both BAM and ISO 21872-1:2017 methods, to help in the recovery of injured isolates that were exposed to various environmental stresses during distribution of the fish from the farm to the wet market (Loo *et al.* 2022).

The high prevalence of presumptive *V. parahaemolyticus* in fish samples offered on the market presents a risk to consumers' health (Stratev *et al.,* 2023). Generally, fish are more perishable compared to meat and poultry due to their high unsaturated fat content and water content (Tavares *et al.,* 2021; Tan *et al.,* 2017). Because of this, fish need strictly controlled storage requirements in order to maintain its freshness and limit the growth of harmful microorganisms (Tan *et al.,* 2017). As observed in wet markets, milkfish and tilapia, which are all locally produced by fish farms in Dagupan, Batangas and Bulacan, are usually displayed open-air and side-by-side on a tray or directly to the stall surface with little or no ice left as shown in Figure 2. Additionally, the fish from the farm that are directly delivered to the store owners are transported by huge ice boxes where temperature control is minimal.

Prevalence of *V. parahaemolyticus* is dependent on the temperature and salinity of the environment (Padovan *et al.,* 2021). However, based on the results there was no significant difference between the number of isolates from fish samples taken from the monsoon (November and January) and dry (February and March) seasons. The optimal growing temperature of *V. parahaemolyticus* is between 20 - 35 °C, a range which covers the temperature changes in the Philippine climate (Florida Department of Health, 2022). Moreover, the discrepancy of water runoff in different regions which may contain high levels of nutrients and sediments to streams and rivers affecting eutrophication and plankton biomass is a consideration for diversity of the *Vibrio* community (Hartwick *et al.,* 2019). This phenomenon of increased eutrophication further distorts the correlation between the season and prevalence of *V. parahaemolyticus*.

A) Tilapia fish displayed open-air directly on the stall surface; B) Milkfish displayed open-air on a tray with little ice; C) Milkfish and Tilapia displayed open-air side-by-side directly on the stall surface

Figure 2. Fish samples from selected wet markets in Quezon City

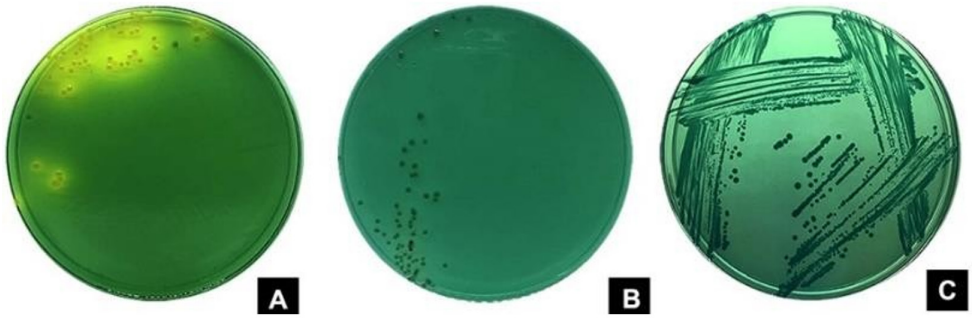
On the other hand, there is a significant difference in the number of isolates between the type of fish samples. As shown in Figure 3, higher abundance of green colonies of presumptive *V. parahaemolyticus* were observed in milkfish samples compared to Tilapia. Furthermore, out of 84 presumptive *V. parahaemolyticus* isolates, 47 isolates (55.95%) were isolated in milkfish samples, compared to the 37 isolates (44.05%) in tilapia samples.

Salinity also plays a more dominant role than temperature in the prevalence of *Vibrio* species (Deeb *et al.,* 2018). *V. parahaemolyticus* is a halophilic bacterium capable of surviving in NaCl concentrations ranging from 0.5% to as high as 9%, with optimum growth at 3%, demonstrating that the strains rapidly adapt to environmental changes (Zhang *et al.* 2023). Seawater in the world's oceans typically has a salinity of around 3.5%, with sodium and chloride ions as predominant dissolved salts (Nayfeh and Nayfeh, 2023). The favorable salinity of the seawater enhances the growth of presumptive *V. parahaemolyticus* more in milkfish samples compared to the tilapia samples.

Moreover, a significant difference in the number of isolated presumptive *V. parahaemolyticus* between the chosen fish parts with the pathogen being found more frequently in the intestinal isolates (58.33%) over the flesh (41.67%). The highly complex gut microbiota of most fish is 90% composed of various bacterial species such as *Proteobacteria, Firmicutes*, and *Bacteroides.* These species serve diverse advantageous purposes in the host organism's body (Cui *et al.,* 2022). Despite the complex immune defenses, pathogens can still invade the ecosystem of the gut microbiota due to various disturbances, such as environmental stress and antibiotic use, which may further increase the pathogen's virulence (Xiong *et al.,* 2019). *Vibrio* spp., as one of the common inhabitants of aquatic ecosystem, is among the prevalent bacterial colonizers and pathogens, under phylum *Proteobacteria,* found in the gastrointestinal tract in freshwater and marine fish, particularly in juveniles (Manchanayake *et al.,* 2023). This phenomenon could explain the prevalence of *V. parahaemolyticus* in the intestine of both the milkfish and tilapia samples as they are part of the normal flora of marine environments as well as the intestines of many aquatic species.

3.2 Molecular detection of tdh + V. parahaemolyticus using PCR and LAMP method

Figure 4 shows the visualization of PCR and LAMP amplified products targeting the *tdh* gene in 2% agarose gel and under the blue light. As observed, only 17 (20.24%) and 18 (21.43%) isolates of *V. parahaemolyticus* were detected to have the pathogenic *tdh* gene using PCR and LAMP assay, respectively, out of 84 total presumptive *V. parahaemolyticus* isolated previously. Among all isolates, isolate 48 was detected through LAMP assay but not in the PCR assay. Despite the wide use of PCR assay in microbial pathogen detection, however, it was still hampered by limitations due to potential PCR inhibitors (Kim *et al.,* 2023)

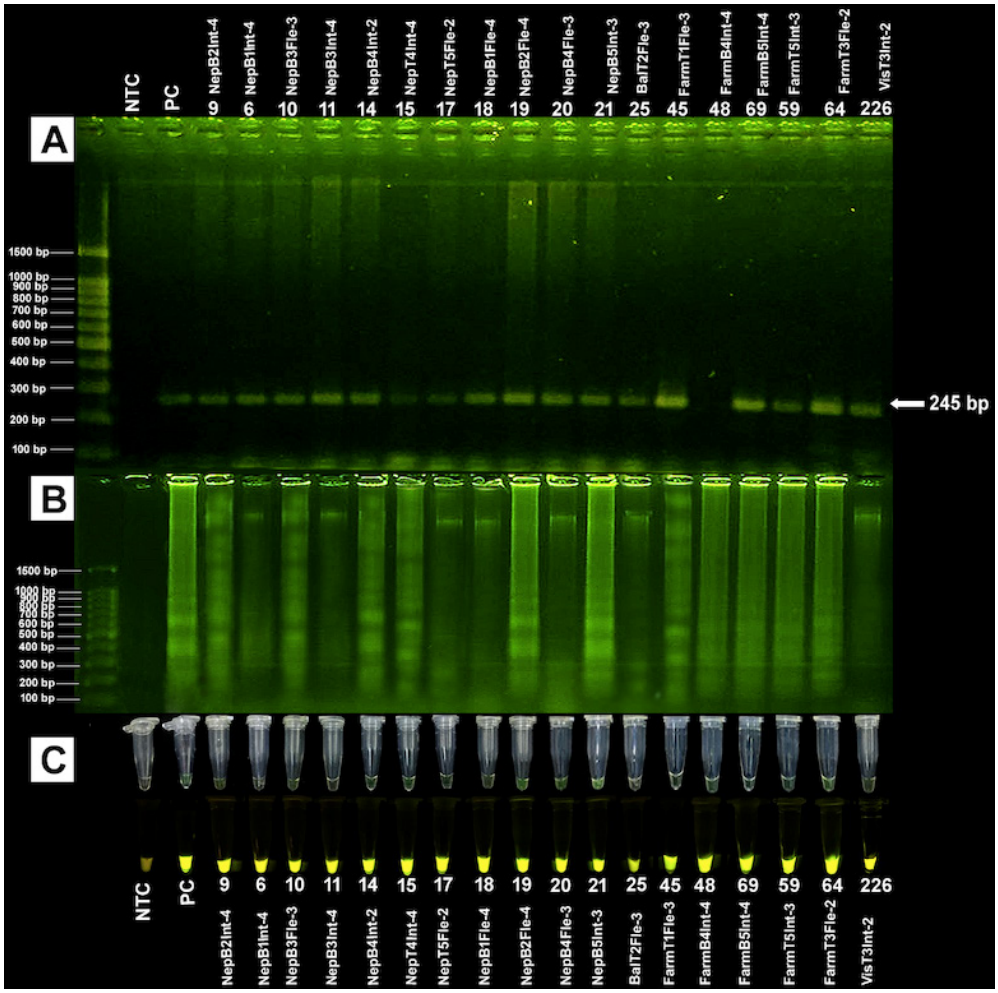


A) NepT4Int-4 isolated from Tilapia sample; B) NepB2Int- 4 isolate from Milkfish sample; C) Pure colony of NepB2Int-4 isolate

Figure 3. Presumptive colonies of *V. parahaemolyticus* in TCBS agar after 24 - 48 hrs incubation at 35 ± 2 °C

The major contributor to the PCR inhibition in the experiment was the presence of NaCl salts. Since the target microorganism is halophilic, all media used to obtain optimum growth were supplemented with 3% salt. High concentration of salt affects the function of the Mg^{2+} in PCR assays (Lee *et al.,* 2021). Agents that may reduce Mg^{2+} availability or competes with its binding to the Taq polymerase inhibits PCR amplification (Kuffel *et al.,* 2020). Furthermore, the *Taq polymerase* that is commonly used in PCR assays may not effectively amplify the target gene when DNA is contaminated with NaCl concentrations exceeding 40 mM (Lee *et al.,* 2021).

The stated limitations of PCR were not a problem in LAMP assay. Furthermore, studies revealed that LAMP assays were 10-fold more sensitive compared to conventional PCR (Sadeghi *et al.,* 2021; Anupama *et al.,* 2021; Obande and Singh, 2020). Although the *Bst* polymerase lacks exonuclease activity, its characteristic of being a highly strand-displacing polymerase, plus taking advantage of having 4 - 6 primers that binds to 6 - 8 separate specific regions in the target sequence, contributes to its sensitive and specific exponential amplification with process time usually less than or equal to 1 hour, yielding up to 10^9 copies (Milligan *et al.,* 2018). Using the designed primers, the



A) PCR products in 2% Agarose Gel at 135 V for 30 mins; B) LAMP products in 2% Agarose Gel at 135 V for 30 mins; C) LAMP tubes under blue light
PC Positive Control; NTC Negative Control

Figure 4. LAMP and PCR products targeting *tdh* gene in fish samples

LAMP assay was conducted at 61.6 ℃ for 1 hour. However, the PCR assay took 2 hrs and 37 minutes to finish the amplification process. This showed that LAMP assay was more time efficient compared to the PCR assay in terms of the amplification process.

Several parameters can be used to assess the efficiency of the LAMP and PCR methods. In general, both the PCR and LAMP assay were able to detect the *tdh* gene confirming the pathogenic strain of *V. parahaemolyticus* collected from fish samples. However, the low concentration of the genomic DNA in the samples and the possibility of high concentration of PCR inhibitors could have led to a higher chance of detection through LAMP than the PCR assay. Furthermore, LAMP assay results can be obtained three times faster relative to the overall PCR assay process inclusive of post-amplification steps. Overall, LAMP assays proved to be more efficient in determining the presence of *V. parahaemolyticus* in fish samples compared to the PCR method in terms of tolerance to inhibitors and time needed for the amplification and post-amplification step.

In relation to the detection of pathogenic *V. parahaemolyticus* in fish samples using LAMP and PCR assay, most of the *tdh*+ isolates were isolated from the intestine (n = 10), which could be explained by the adaptations of *V. parahaemolyticus* in the antagonistic intestinal environment. Generally, the gastrointestinal tract is composed of acids, bile salts and enzymes which help in the breakdown of fats, absorption of vitamins and aids in digestion (Pazhani *et al.,* 2021). The antagonistic environment of normal gastrointestinal tract microflora inhibits pathogenic bacteria leading to inactivation of the bacterial toxins (Pazhani *et al,* 2021). Exposure to stress-inducing agents and conditions further triggers the stress response mechanism of pathogenic species which helps in environment adaptability and bacterial survival (Akolkar & Matson, 2023).

Bile salts present in the intestine of the fish also act as a bactericidal agent that disrupts bacterial membranes, denatures proteins, and causes oxidative damage to DNA (Urdaneta & Casadesús, 2017). Because of this, bile salts can be the potent host derived inducer of *tdh* gene under osmotic conditions as well as serve as a signal for the intestinal environment (Letchumanan *et al.,* 2017; Urdaneta & Casadesús, 2017).

3.3 Antibiotic Susceptibility Test of pathogenic V. parahaemolyticus

All of the 18 *tdh*+ *V. parahaemolyticus* isolates that showed amplification from LAMP and PCR have undergone the antibiotic susceptibility test. Each positive isolate was exposed to 3 types of antibiotics discs containing florfenicol 30 µg, oxytetracycline 30 µg and sulfamethoxazole 25 µg.

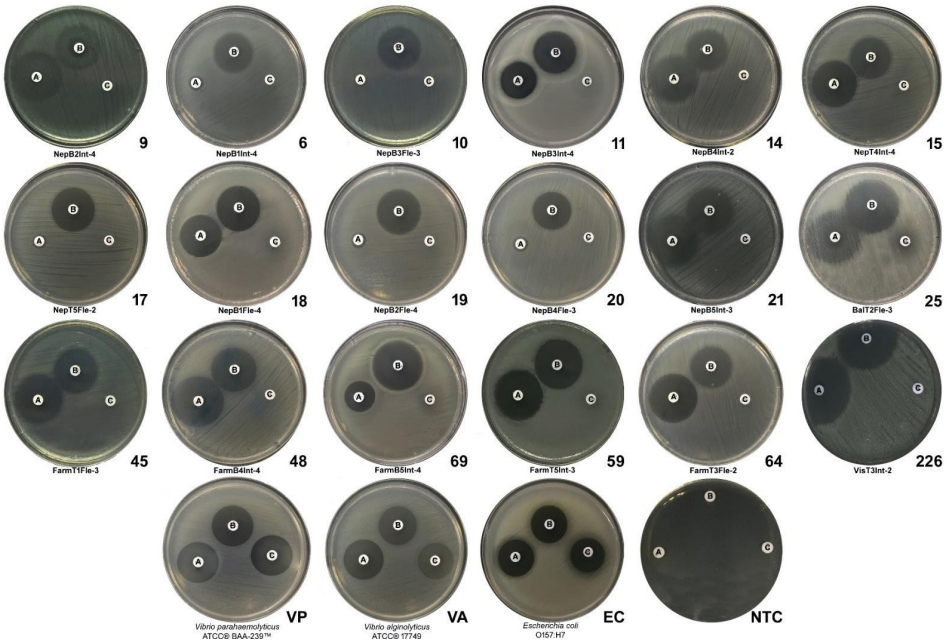
Florfenicol, under class of amphenicols, is a bacteriostatic agent that inhibits the peptidyl transferase activity of the ribosome by selectively binding to ribosomal 50S and 70S subunits suppressing transfer of amino acids to growing peptide chains and subsequent protein formation (Somogyi *et al.* 2023). Oxytetracycline, a broad-spectrum bacteriostatic agent under tetracycline class, primarily targets the 30S ribosomal subunit by blocking the attachment of aminoacyl tRNA to the A acceptor site, which halts further protein synthesis and inhibits ribosomal translation (Shutter and Akhondi, 2023). Sulfamethoxazole, a sulfonamide, inhibits bacterial dihydrofolic acid synthesis by acting as competitive inhibitor of para-aminobenzoic acid (PABA) which disrupts tetrahydrofolate production, hindering bacterial purine and DNA synthesis and resulting in a bacteriostatic effect (National Center for Biotechnology Information 2024).

As shown in Table 1, all of the isolates were resistant towards sulfamethoxazole and 28% (n = 5) to oxytetracycline. Furthermore, 89% (n = 16) showed susceptibility towards florfenicol and 61% (n = 11) towards oxytetracycline. Only 6% (n = 1) and 11% (n = 2) were under the intermediate category towards florfenicol and oxytetracycline, respectively.

Table 1. Antibiotic Susceptibility pattern of *tdh+ V. parahaemolyticus* obtained from fish isolates

Antibiotic	Interpretative Criteria		
	Susceptible (S)	Intermediate (I)	Resistant (R)
Florfenicol*	16 (89%)	1 (6%)	1 (6%)
Oxytetracycline**	11 (61%)	2 (11%)	5 (28%)
Sulfamethoxazole***	0	0	18 (100%)

Zone of diameter (mm) per Interpretive Criteria:
*S: >18 mm; I: 13 - 17 mm; R: < 12 mm
** S: >15 mm; I: 12 - 14 mm; R: < 11 mm
*** S: >16 mm; I: 11 - 15 mm; R: < 10 mm



A) Oxytetracycline (30 µg); B) Florfenicol (30 µg); C) Sulfamethoxazole (25 µg)
Positive controls (VP, VA, and EC), Negative control (NTC)

Figure 5. Disk Diffusion Assay of *tdh+ V. parahaemolyticus* in Mueller Hinton Agar with 3% salt after 18 - 24 hrs incubation at 35 ± 2°C.

Generally, *V. parahaemolyticus* is susceptible to all antibiotics targeting Gram negative bacteria, both in medical and veterinary uses (Costa *et al.,* 2021). However, as shown in Figure 5, all the *tdh+* isolates were resistant to sulfamethoxazole. On the other hand, there were 5 multi-drug resistant *V. parahaemolyticus tdh+* isolates, specifically isolate no. 6, 10, 19 and 20, that were resistant towards oxytetracycline and sulfamethoxazole, while isolate no. 9 is resistant to florfenicol, oxytetracycline and sulfamethoxazole. These can be attributed to the misuse of antibiotics in the aquaculture industry, or lateral gene transfer, or natural selection of microorganism due to prophylactic practice.

Ultimately, the presence of multi-drug resistant and virulent bacteria in food source is a major concern in fish farming and human health as this could increase the chance of disease-causing pathogenic bacteria in humans and spreading the antimicrobial resistance from the aquaculture to the consumer (Watts *et al.,* 2017).

In the Philippines, there are national food safety guidelines (Food Safety Act of 2013 or Republic Act No. 10611) that mandate food safety and security, with the Department of Agriculture – Bureau of Agriculture and Fisheries Standards (DA-BAFS) managing the creation and adoption of Philippine National Standards. In this instance, related standard

is the microbiological hazard limits which include *Vibrio* in fish and fishery products. With the limited studies and techniques in food safety regulatory agencies, this study may be used as baseline in developing assays to detect *Vibrio*, as well as reference in sustainable fisheries, by fish farmers, and the general aquaculture industry.

4. Conclusion

The study showed that there are high levels of *V. parahaemolyticus* in fish samples bought from selected wet markets in QC. The results showed that there is a higher presence of *V. parahaemolyticus* in milkfish compared to tilapia, emphasizing the halophilic nature of *V. parahaemolyticus*. The LAMP and PCR methods were both able to detect *tdh*+ *V. parahaemolyticus* from both fish samples. However, there were samples that were detected by the LAMP assay alone and not by the PCR assay, which indicated the higher sensitivity and time efficiency of the former over the latter.

The presence of pathogenic *V. parahaemolyticus* was found to be significantly associated with the fish part tested, as there were more *tdh*+ *V. parahaemolyticus* in the intestine compared to flesh. This suggest that the antagonistic environment created by the normal gastrointestinal tract microflora triggers a stress response mechanism in pathogenic species, aiding their adaptability and survival in this environment. Interestingly, no relationship was found between the level of *tdh*+ *V. parahaemolyticus* and the season with which the samples are collected. This could indicate that bacterial load is unaffected by seasonal changes implying that other external environmental factors (e.g. salinity, plankton biomass, water run-off) might play a more significant role in influencing its population within the fish. All of the 18 *tdh*+ *V. parahaemolyticus* isolated and tested were found to be resistant to sulfamethoxazole. Five of them showed multi-drug resistance to sulfamethoxazole, florfenicol, and oxytetracycline, which were all considered as common antibiotics used in aquaculture industry.

Overall, the presence of virulent antibiotic resistant *V. parahaemolyticus* in food sources that could be transferred to consumers may indicate a major problem affecting the sectors of food production, aquaculture, and public health.

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