Detection of *Vibrio parahaemolyticus* in fish samples from selected wet markets in Laguna, Philippines, using loop-mediated isothermal amplification (LAMP) and real-time polymerase chain reaction (qPCR)

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*Vibrio parahaemolyticus* is known to be the leading cause of seafood-borne bacterial gastroenteritis worldwide, with strains that are pathogenic to both fish and humans. A rapid but cost-effective method of detecting *V. parahaemolyticus* is therefore necessary as a concern for both food safety and aquaculture maintenance. In the present study, loop-mediated isothermal amplification (LAMP) assays were evaluated and applied in comparison with real-time polymerase chain reaction (qPCR) assays to detect *V. parahaemolyticus* in flesh, gills, and intestines of *Oreochromis niloticus* (n = 15) and *Chanos chanos* (n = 15) samples from three wet markets in Laguna, Philippines. Specifically, assays targeting the *tlh* and *tdh* genes, which serve as markers for total and pathogenic *V. parahaemolyticus*, respectively, were optimized and assessed in terms of analytical specificity and sensitivity. The LAMP assays were found to be 100% specific and capable of detecting as low as 18 pg of DNA per reaction, matching the analytical specificity and sensitivity of the real-time PCR assays. Among fish samples tested, 20% (6/30) were positive for *V. parahaemolyticus*; none of these possessed the *tdh* gene. Overall, this study demonstrated the potential of LAMP as a rapid and sensitive alternative for detection of *V. parahaemolyticus*.

**KEYWORDS**  
*Vibrio parahaemolyticus*, *Oreochromis niloticus*, *Chanos chanos*, food safety, molecular detection, qPCR, LAMP

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INTRODUCTION

*Vibrio parahaemolyticus* is a Gram-negative, rod-shaped bacterium known to be the leading cause of seafood-borne bacterial gastroenteritis worldwide, usually accompanying consumption of raw or undercooked seafood (Quilici et al. 2005). One major virulence factor produced by pathogenic strains of this bacterium is the thermostable direct hemolysin (TDH) (Honda and lida 1993), which is primarily responsible for β-hemolysis and enterotoxigenic properties often associated with strains isolated from clinical samples (Miyamoto et al. 1969; Nishibuchi et al. 1992; Shinoda 2011). TDH-producing strains of *V. parahaemolyticus*, however, are rarely isolated from seafood and environmental samples, presumably due to their presence in low concentrations in the environment (Shinoda 2011). Because of this difficulty, total *V. parahaemolyticus* has instead been widely used to assess microbiological quality of seafood (Yamazaki 2008). Thus, there is a need for a sensitive and definitive method for the detection of both total and TDH-producing *V. parahaemolyticus* in fish in order to understand the apparent and actual risks this bacterium may bring to fish consumers.

Conventional methods of detecting *V. parahaemolyticus* from fish samples, such as bacterial isolation with subsequent biochemical identification, are labor-intensive and time-consuming, usually taking at least three days from isolation to identification. Polymerase chain reaction (PCR) assays, on the other hand, are more rapid and sensitive (Bej et al. 1999) but these require post-amplification gel electrophoresis, which may also be arduous when dealing with voluminous samples. Real-time PCR addresses such issues with regular PCR (Klein 2002); however, it requires a thermal cycler with a fluorescence detector, which may be expensive especially for small-scale or field laboratories. It is therefore necessary to develop a likewise rapid alternative for *V. parahaemolyticus* detection.

One such method is the loop-mediated isothermal amplification (LAMP) assay, which is a novel DNA amplification technique developed by Notomi et al. (2000). LAMP is an auto-cycling strand displacement DNA synthesis carried out by a special type of DNA polymerase from *Bacillus stearothermophilus* called Bst DNA polymerase, which eliminates the need to have cycles of different temperatures for denaturation, annealing, and extension. This technique utilizes 4–6 primers which recognize 6–8 sequences in the target gene (Notomi et al. 2000; Nagamine et al. 2002), making it much more specific compared to PCR assays which only utilize two primers. The LAMP reaction can also be performed within an hour, and amplicons can easily be viewed using fluorescent or colorimetric dyes (Notomi et al. 2000; Caipang et al. 2012; Tomita et al. 2008), thus making LAMP a rapid, sensitive, and specific method all rolled into one.

Numerous LAMP assays optimized for the detection of *V. parahaemolyticus* in seafood samples have been developed (Yamazaki et al. 2008; Chen and Ge 2010; Nemoto et al. 2011; Niiho et al. 2002; Nagamine et al. 2002), making it much more specific compared to PCR assays which only utilize two primers. The LAMP reaction can also be performed within an hour, and amplicons can easily be viewed using fluorescent or colorimetric dyes (Notomi et al. 2000; Caipang et al. 2012; Tomita et al. 2008), thus making LAMP a rapid, sensitive, and specific method all rolled into one.

**MATERIALS AND METHODS**

**Bacterial Strains and Culture**

Strains used in this study were obtained from the Philippine National Collection of Microorganisms (PNCM) in BIOTECH, University of the Philippines Los Baños, Microbiologies, Inc., the Natural Sciences Research Institute in University of the Philippines Diliman, and from fish samples. *Vibrio parahaemolyticus* ATCC® BAA-239™ (*tdh*) and ATCC® 17802™ (*tlh*) were used as positive controls for LAMP and real-time PCR. Non-*Vibrio* strains used in this study were *Escherichia coli* ATCC® 25922™, *Salmonella enterica*, and *Klebsiella pneumoniae*. *V. parahaemolyticus* from fish samples were isolated using thiosulfate citrate bile salts sucrose (TCBS). All *V. parahaemolyticus* positive controls and isolates were maintained in tryptic soy agar supplemented with 3% NaCl, while other non-*Vibrio* strains were maintained in TSA without NaCl. Tryptic soy broth (TSB) with 0% or 3% NaCl were used to culture strains prior to molecular analyses. All cultures were incubated at 35°C for 18–24 h.

**DNA Extraction**

DNA was extracted from bacteria using a simple boiling method. Briefly, a broth culture was centrifuged at 6 000 × g for 5 min, and the resulting pellet was resuspended and vortexed in 200 μL of DEPC-treated water (Invitrogen, Carlsbad, CA). The bacterial suspension was then heated to 100°C for 10 min using a dry heating block, after which it was centrifuged at 6 000 × g for 5 min. The supernatant was collected and used as DNA template for the LAMP and PCR reactions.

**LAMP and Real-time PCR**

LAMP and PCR primers used in this study (Table 1) were based on primers previously designed from different studies (Yamazaki et al. 2008; Yamazaki et al. 2010; Nordstrom et al. 2007; Gutierrez et al. 2013). All primers were custom synthesized by Macrogen Inc., Korea.

The 25 μL LAMP reaction mixture for both *tlh* and *tdh* genes consisted of the following: 1X DNA polymerase buffer (Lucigen, Middleton, WI, USA), 6 mM of MgSO4, 0.8 M of betaine, 1.4 mM of deoxynucleotide triphosphate (dNTP) mix, 16 U of Bst DNA polymerase (Lucigen, Middleton, WI, USA), 0.48 μM of each inner primer (FIP and BIP), 0.2 μM of each outer primer (F3 and B3), 0.24 μM of each loop primer (LF and LB), and 3 μL of DNA template. LAMP reactions were carried out in a Biomek-M3™ LAMP heater (Manila HealthTek, Marikina, Philippines) at 63°C for 40 min and terminated at 80°C for 2 min. LAMP products were visualized straight from the tubes by adding 2 μL (1:20) of SYBR® Green I (Manila HealthTek, Marikina, Philippines) and observing color change from orange to green under naked eye and under blue LED using an Easyview™ LED transilluminator (Manila HealthTek, Marikina, Philippines).

For real-time PCR reactions, 20 μL reaction mixtures consisted of the following: 10 μL of PowerUp™ SYBR® Green Master Mix (Applied Biosystems, Austin, TX, USA), 0.5 μM of each primer, and 3 μL of DNA template. Reactions were carried out in a CFX96 Touch™ Real-Time PCR Detection System (BioRad, Hercules, CA, USA) with the following thermal cycling...
Table 1: LAMP and PCR primers targeting *tlh* and *tdh* genes of *V. parahaemolyticus*.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’–3’)</th>
<th>Melting Temperature (°C)</th>
<th>Amplicon Size</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>tlh</em>-FIP</td>
<td>ATGTTTTTAATGAAAGCCGAGCTCCGGCAA</td>
<td>76.1</td>
<td>variable</td>
<td>Yamazaki et al. (2008)</td>
</tr>
<tr>
<td><em>tlh</em>-BIP</td>
<td>AAAACGA AGATGGT</td>
<td>77.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>ACGTCGAAAACGTATTCCGGCGAAGACG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>tlh</em>-F3</td>
<td>AGCTACTCGAAAAGATGATCC</td>
<td>56.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>tlh</em>-B3</td>
<td>GGTGTATAGAAGCGATTG</td>
<td>56.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>tlh</em>-LF</td>
<td>ACCAGTAGCCGTCATG</td>
<td>52.4</td>
<td></td>
<td></td>
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<tr>
<td><em>tlh</em>-LB</td>
<td>TAGATTTGGCGAAGACGAGA</td>
<td>53.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>tlh</em>-forward</td>
<td>ACTCAAACAAAGAAGATCGACAA</td>
<td>62.5</td>
<td>208 bp</td>
<td>Nordstrom et al. (2007)</td>
</tr>
<tr>
<td><em>tlh</em>-reverse</td>
<td>GATGAGCGGTGTAGTGGCAAA</td>
<td>58.4</td>
<td></td>
<td></td>
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<tr>
<td><em>tdh</em>-FIP</td>
<td>GTACCTGAGCTTTGTGAAACTGATTTGCTCT</td>
<td>78.0</td>
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<tr>
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<tr>
<td><em>tdh</em>-F3</td>
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<td>55.7</td>
<td>variable</td>
<td>Yamazaki et al. (2008)</td>
</tr>
<tr>
<td><em>tdh</em>-B3</td>
<td>AACACACGAGAATGACCG</td>
<td>53.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>tdh</em>-LF</td>
<td>GTACCGTTTTCTTTTACATTACG</td>
<td>58.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>tdh</em>-LB</td>
<td>AAGACTATAACATGGCCAGCG</td>
<td>56.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>tdh</em>-forward</td>
<td>CTGTTCCCTTTCTCGCCCCCG</td>
<td>67.2</td>
<td>245 bp</td>
<td>Gutierrez</td>
</tr>
<tr>
<td><em>tdh</em>-reverse</td>
<td>AGCCAGACACCGCTGCCATTG</td>
<td>65.3</td>
<td></td>
<td>West et al. (2013)</td>
</tr>
</tbody>
</table>

protocol: one cycle of 50°C for 2 min, one cycle of 95°C for 2 min, and 40 cycles consisting of 95°C for 3 s and 58°C for 30 s. Melt curve analyses were performed at 65°C to 95°C in 0.2°C increments for 10 s per increment. Data acquisition was done using Bio-Rad CFX Manager version 1.6 after each cycle and melt curve increment.

Specificity of the LAMP and Real-time PCR Assays
The analytical specificity of the assays in terms of inclusivity (diagnostic sensitivity) and exclusivity (diagnostic specificity) were evaluated by subjecting DNA samples from the previously mentioned bacterial strains to the optimized reaction mixture and temperatures of LAMP and real-time PCR. DNA extracted from each strain was subjected to LAMP and real-time PCR in duplicate and was regarded as positive only when the two replicates tested positive. Additionally, LAMP products were subjected to agarose gel electrophoresis using 2% agarose stained with 1% GelGreen™ (Biotium, Fremont, CA, USA) to confirm positive or negative results as indicated by continuous ladder-like gel profiles. Representative real-time PCR products for each gene, on the other hand, were sent to Macrogen, Inc., Korea, for sequencing. Species and gene identities of the
representative PCR amplicons were confirmed using Basic Local Alignment Search Tool (BLAST) (NCBI). Percent inclusivity was obtained by dividing the number of true positive results by the sum of the false negative and true positive results, while percent exclusivity was obtained by dividing the number of true negative results by the sum of the false positive and true negative results.

**Sensitivity of the LAMP and Real-time PCR Assays**

The detection limits of the assays were determined in terms of DNA concentration (ng/µL) using DNA extracted from a pure culture of *V. parahaemolyticus ATCC® BAA-239™*. Concentration of stock DNA obtained using the aforementioned DNA extraction protocol was initially measured using a NanoDrop™ 2000 (Thermo Scientific, Wilmington, DE, USA), after which it was serially diluted tenfold using DEPC-treated water (Invitrogen, Carlsbad, CA, USA) until the assays could no longer detect DNA. Detection limits of the assays from pure culture were set as the concentration of the last dilution with two replicates showing positive LAMP and real-time PCR results.

**Application of LAMP and Real-time PCR Assays for Detection of *V. parahaemolyticus* in Fish Samples**

Fish samples were collected by convenience sampling from a total of three wet markets in the cities of Biñan, Pila, and Lawa in Laguna, Philippines, within the month of July 2018. A total of 10 fish, consisting of 5 Nile tilapia (*Oreochromis niloticus*) and 5 milkfish (*Chanos chanos*), were bought from each of the three wet markets. From each fish, three subsamples (flesh, gills, and intestine) were obtained for a total of 90 subsamples. Fish samples were bought and placed in sampling bags which were stored in an ice chest in transit from the sampling site to the laboratory, avoiding direct contact between the fish samples and the ice. All samples were obtained and processed immediately.

Up to 5 g of flesh, gills, and intestines from each fish were extracted aseptically and weighed in sterile containers. For flesh

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**Figure 1:** Representative amplicons of various strains subjected to *tlh* and *tdh*-LAMP as seen by naked eye (A), under blue LED (B), and subjected to agarose gel electrophoresis (C). Samples 1–6 amplified using *tlh*-LAMP were NTC (lane 1), *V. parahaemolyticus ATCC® BAA-239™* (lane 2), *V. parahaemolyticus ATCC® 17802™* (lane 3), *E. coli ATCC® 25922™* (lane 4), *S. enterica* (lane 5), and *K. pneumoniae* (lane 6). Samples 7–12 amplified using *tdh*-LAMP were NTC (lane 7), *V. parahaemolyticus ATCC® BAA-239™* (lane 8), *V. parahaemolyticus ATCC® 17802™* (lane 9), *E. coli ATCC® 25922™* (lane 10), *S. enterica* (lane 11), and *K. pneumoniae* (lane 12).
samples, fish scales were first removed, after which the skin was surface sterilized using 70% ethanol. A thin layer of flesh from the surface of the fish, including the skin, was obtained using a sterile scalpel. For gill samples, the operculum and surrounding skin was surface sterilized using 70% ethanol, after which the entire gill arches were extracted. For intestine samples, the ventral surface of the fish was sterilized using 70% ethanol prior to dissection. Using a sterile scalpel, the fish was cut along the median ventral line from the pectoral girdle to the anus. The intestines were then extracted starting from the posterior-most portion of the intestinal tract towards the anterior portion until the sample reached the desired weight.

Flesh, gill, and intestine samples were cut into small pieces aseptically and were each transferred to 50-mL centrifuge tubes containing alkaline peptone water (APW). The amount of enrichment broth was adjusted depending on the weight of the subsample such that the mixture was composed of 1:9 subsample/broth ratio (g/mL). The contents of the tubes were mixed thoroughly using a vortex for 10 s, after which the tubes were incubated at 35°C for 18 h. For each sample, a loopful of the enrichment medium obtained from the top 1 cm of the tube was streaked onto a TCBS plate using 3-way quadrant streak method. Each tube was then vortexed, after which a loopful of enrichment medium obtained from the mixed suspension was streaked onto another TCBS plate in the same manner.

From each TCBS plate, a maximum of four 2–3 mm green to blue colonies were picked and isolated using TSA with 3% NaCl. Isolates were then tested for growth in NB with 0% NaCl. After 24 h of incubation at 35°C, isolates with no growth were considered strict halophiles and regarded as putative _V. parahaemolyticus_ isolates. DNA from putative _V. parahaemolyticus_ isolates were then extracted and subjected to the optimized LAMP and real-time PCR assays.

**Statistical Analysis**

Statistical analyses were done using GraphPad Prism version 8.1 (GraphPad Software, San Diego, CA, USA). Chi-square test or Fisher’s exact test was used to determine if there are substantial associations between presence of _V. parahaemolyticus_ and fish species, source of inoculum from enrichment (top 1 cm or vortexed suspension), sampling site, or fish part. Results were considered statistically significant at _p_-value < 0.05.

**RESULTS AND DISCUSSION**

**Specificity of the LAMP and Real-time PCR Assays**

LAMP and real-time PCR assays targeting the _tlh_ gene were both able to detect _V. parahaemolyticus_ with 100% (2/2) inclusivity and 100% (3/3) exclusivity (Figs. 1 and 2). Both assays targeting the _tdh_ gene were also able to differentiate _tdh^+ _ from _tdh^- strains of _V. parahaemolyticus_ with 100% (1/1) inclusivity and 100% (4/4) exclusivity (Figs. 1 and 2). Real-time PCR amplicons from _tlh_ and _tdh_ amplifications were confirmed to be the respective genes from _V. parahaemolyticus_ via BLAST. Specificities of the assays are summarized in Table 2. As verified by our specificity tests, all 4 primer sets (Yamazaki et al. 2008; Yamazaki et al. 2010; Nordstrom et al. 2007; Gutierrez et al. 2013) were highly specific and were able to differentiate _V. parahaemolyticus_ from non-_Vibrio_ species, as well as _tdh^+ _ from _tdh^- strains of _V. parahaemolyticus_. In addition, amplification conditions were the same for the two genes of each method. This suggests that the primers, despite having different melting temperatures, affect the optimization of the assays to a lesser extent as compared to other variables such as reaction mixture components and temperature. Our integrated optimization of the primer sets in this study can be useful in future studies targeting _tlh_ and _tdh_ genes of _V. parahaemolyticus_ simultaneously.
Sensitivity of the LAMP and Real-time PCR Assays

Nucleic acid concentration of the stock DNA extract was determined to be 598.7 ng/µL. After tenfold serial dilution with subsequent LAMP and real-time PCR runs, the 10⁻⁵ dilution of the stock DNA was found to be the last dilution that consistently tested positive for both LAMP and real-time PCR targeting both genes. Hence, the detection limit for both assays targeting each of the two genes is 0.006 ng/µL, which corresponds to 18 pg per reaction. While to our knowledge there are no studies yet comparing the sensitivity of LAMP and SYBR® Green real-time PCR, previous reports comparing LAMP and TaqMan real-time PCR assays consistently showed that TaqMan real-time PCR assays have at least tenfold greater sensitivity compared to LAMP assays (Harper et al. 2010; Lin et al. 2012). On the other hand, when compared to SYBR® Green-based real-time PCR assays, TaqMan-based real-time PCR assays have also shown greater sensitivity in many studies (Cao and Shockey 2012; Kumar et al. 2012; Soltan- Rezaee-Rad et al. 2015). Therefore, LAMP and SYBR Green real-time PCR assays are expected to have similar sensitivities, as demonstrated in the present study.

In this study, LAMP and SYBR Green real-time PCR were able to detect and amplify the same initial amount of template but with varying amplification times, all without the need for post-amplification electrophoresis. The total LAMP reaction time was sufficient to allow detection of amplicons within 45 min, whereas total real-time PCR reaction time needed at least 2 h for complete detection. LAMP is therefore more than twice as fast as real-time PCR to perform. Another point of comparison for the two assays is the cost of running each assay. The LAMP and SYBR Green real-time PCR reagents used in the present study had roughly the same price. However, the machines used for the two assays had a huge price difference. The real-time PCR system costed over ten times more than the LAMP heater used in the study. Hence, in this case, LAMP may be the cheaper option when used merely as a simple detection method compared to real-time PCR. Although one can argue that real-time PCR has quantitative capabilities, real-time LAMP reactions have reportedly been able to generate reliable quantification curves as well (Yamazaki et al. 2008; Chen and Ge 2010; Mori et al. 2004), demonstrating future potential for LAMP to be an alternative nucleic acid quantitation method.

Application of LAMP and Real-time PCR Assays for Detection of *V. parahaemolyticus* in Fish Samples

From all 90 fish subsamples, a total of 19 putative *V. parahaemolyticus* isolates were obtained, with multiple isolates coming from the same subsample. After subjecting pure cultures of these 19 isolates to real-time PCR targeting the *tdh* gene, 8 of them were found to be *V. parahaemolyticus* (Table 3). These isolates were also confirmed to be *V. parahaemolyticus* using LAMP assay targeting the *tdh* gene. These 8 isolates came from 20% (6/30) of all fish samples, 50% (3/6) of which were contaminated via flesh and 50% (3/6) via gills (Table 3). The absence of contamination via intestines is likely due to the rich gut flora of fish (Sivasubramanian et al. 2012), which may have competitively excluded *V. parahaemolyticus*, both *in vivo* and in the enrichment culture. The presence of *V. parahaemolyticus* was found to be significantly associated with sampling site (*p* = 0.0265) but not with fish species, fish part, and inoculum source. This suggests that the *V. parahaemolyticus* contamination originated somewhere along the production line from the rearing sites to the markets, and that the chances of contamination are unlikely to be influenced by differences in fish species, fish part, or inoculum source from enrichment. However, it must be noted that there were isolates obtained exclusively from the top 1 cm of the enrichment culture and exclusively from the vortexed enrichment culture. This suggests that obtaining the inoculum solely from the top 1 cm of the enrichment culture, as recommended by the U.S. Food and Drug Administration’s Bacteriological Analytical Manual (Kaysner and Depaola 2004), might not be able to capture all of the *V. parahaemolyticus* in the culture, making the test less sensitive. It is therefore recommended to also include vortexed enrichment cultures during isolation attempts in order to improve diagnostic sensitivity.

After subjecting the confirmed *V. parahaemolyticus* isolates to the LAMP and real-time PCR assays targeting the *tdh* gene, none were found to be positive for the gene (Table 3). This is in line with previous studies which had found that major virulence determinants in *V. parahaemolyticus*, such as the *tdh* and *trh* (TDH-related hemolysin) genes, are not commonly found in environmental samples (Shinoda 2011) and are thus unlikely to end up in fish reared in these environments. Although the *tdh* gene was not detected in any of the isolates, the possible presence of other virulence genes aside from *tdh* must not be

<table>
<thead>
<tr>
<th>Table 2: Summary of LAMP and real-time PCR specificities targeting the <em>tdh</em> and <em>tdh</em> genes of <em>V. parahaemolyticus</em>.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Species</td>
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<tr>
<td></td>
</tr>
<tr>
<td><strong>Vibrio parahaemolyticus</strong></td>
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<tr>
<td><strong>Vibrio parahaemolyticus</strong></td>
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<td><em>Escherichia coli</em></td>
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<td><em>Salmonella enterica</em></td>
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<td><em>Klebsiella pneumoniae</em></td>
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<tr>
<th>Table 3: Sample source and presence of <em>tdh</em> and <em>tdh</em> genes of <em>V. parahaemolyticus</em> isolates confirmed by LAMP and real-time PCR targeting the <em>tdh</em> gene.</th>
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<tr>
<td>Isolate</td>
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<td>PT:FA:</td>
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<tr>
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<td>BB:GA:3</td>
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<td>BB:GA:2</td>
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discounted. This is exemplified by the occurrence of pathogenic \emph{V. parahaemolyticus} which do not possess these well-known hemolysins, suggesting that other virulence factors may also contribute to the pathogenicity of \emph{V. parahaemolyticus} (Jones et al. 2012; Lynch et al. 2005).

Previous studies on the prevalence of \emph{V. parahaemolyticus} in fish samples from markets have found its presence ranging from 16.8\% and 56.7\% in South Korea (Lee et al. 2019; Kim et al. 2017) to 45.1\% and 66.7\% in India (Sudha et al. 2012; Pal and Das 2010). However, many of the samples included in these previous studies were sourced from marine environments, which are expected to naturally harbor halophilic \emph{Vibrio} spp. In the present study, the detection of \emph{V. parahaemolyticus} in fish reared in freshwater environments is alarming since these kinds of environments are supposedly unfavorable for \emph{Vibrio} spp. survival. It is possible that these fish act as more favorable environmental sinks for strictly halophilic \emph{V. parahaemolyticus} in the harsh freshwater environment (Nair et al. 2007; Sarkar et al. 1985). Cross-contamination of fish samples may have also possibly occurred due to common handling of these fish and other marine products in the markets. Further studies are needed to determine if these fish were naturally contaminated by their rearing environment or if they were contaminated due to handling. Nevertheless, to our knowledge, this study is the first to investigate the prevalence of \emph{V. parahaemolyticus} in fish samples from wet markets in the Philippines.

It is also worth noting that the TCBS plates used in isolation attempts contained a lot of other colonies which may have a good chance of being \emph{Vibrio} spp. \emph{V. parahaemolyticus} is simply one of the 11 known pathogenic species of \emph{Vibrio} (Farmer et al. 2015). Thus, the absence of \emph{V. parahaemolyticus} in some of the fish samples does not automatically discount the possibility of other pathogenic \emph{Vibrio} spp. in the samples. Early detection and surveillance of such pathogens is important in effective disease prevention. As such, development of alternatively rapid and definitive methods such as LAMP assays will be beneficial to the aquaculture industry as well as to food safety and public health overall.

**CONCLUSION**

The present study successfully optimized LAMP and real-time PCR assays targeting \emph{tdh} and \emph{tdh}+ genes of \emph{V. parahaemolyticus}. The assays were all found to be specific enough to differentiate \emph{V. parahaemolyticus} from non-\emph{Vibrio} strains, as well as \emph{tdh}+ from \emph{tdh}− strains of \emph{V. parahaemolyticus}. The sensitivity of LAMP was also found to be on par with real-time PCR at only a fraction of the reaction time and cost of equipment. Using these assays, 20\% (6/30) of fish samples from wet markets were found to be positive for \emph{V. parahaemolyticus}; none of these possessed the \emph{tdh} gene. The presence of \emph{V. parahaemolyticus} was found to be significantly associated with sampling site but not with fish species, fish part, nor inoculum source. This study is the first in the Philippines to assess the prevalence of \emph{V. parahaemolyticus} in fish samples from wet markets using LAMP as a rapid, sensitive, and definitive alternative to real-time PCR for detection of \emph{V. parahaemolyticus}.

**ACKNOWLEDGMENT**

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**CONFLICT OF INTEREST**

The authors declare no conflict of interest.

**CONTRIBUTIONS OF INDIVIDUAL AUTHORS**

Teh TRD and Vital PG conceived and conceptualized the study, analyzed and interpreted the data, and prepared the draft of and finalized the manuscript. Teh TRD and Santos JAP performed the experiments. Teh TRD and Cayetano MG collected samples and edited the manuscript.

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