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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*V*ibrio 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*.

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 Iida 1993), which is primarily responsible for β-hemolytic 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; Nemoto et al. 2009; Yamazaki et al. 2010). However, studies on its application in detection of *V. parahaemolyticus* from fish samples have been limited. Moreover, there is a lack of baseline information on the occurrence of *V. parahaemolyticus* in the Philippines (Vinarao et al. 2017), leaving the potential risks unknown. Thus, in this study, LAMP assays for the detection of total and TDH-producing *V. parahaemolyticus* in fish samples from wet markets in Laguna, Philippines, were developed and compared with SYBR® Green real-time PCR assays. LAMP and real-time PCR reactions were optimized based on the *tlh* and *tdh* genes, which were regarded as markers of total and TDH-producing *V. parahaemolyticus*, respectively. The analytical specificities and sensitivities of the LAMP and real-time PCR assays in detecting *V. parahaemolyticus* from pure cultures were also evaluated. The assays were then used to detect *V. parahaemolyticus* in flesh, gills, and intestines of milkfish (*Chanos chanos*) and Nile tilapia (*Oreochromis niloticus*) samples, and the prevalence of total and TDH-producing *V. parahaemolyticus* from these samples were determined.

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, Microbiologics, 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™ (*tdh*−) 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 Biotek-M™ 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 (Bio-Rad, Hercules, CA, USA) with the following thermal cycling

Table 1: LAMP and PCR primers targeting *tlh* and *tdh* genes of *V. parahaemolyticus*.

Primer	Sequence (5'–3')	Melting Temperature (°C)	Amplicon Size	Reference
tlh-FIP	ATGTTTTTAAATGAAACGGAGCTCCGGCAA AAAACGA AGATGGT	76.1		
tlh-BIP	ACGTCGCAAAACGTTATCCGGCGAAGAACG TAATGTC TG	77.9		
tlh-F3	AGCTACTCGAAAGATGATCC	56.4	variable	Yamazaki et al. (2008)
tlh-B3	GGTTGTATGAGAAGCGATTG	56.4		
tlh-LF	ACCAGTAGCCGTCAATG	52.4		
tlh-LB	TTAGATTTGGCGAACGAGA	53.0		
tlh-forward	ACTCAACACAAGAAGAGATCGACAA	62.5	208 bp	Nordstrom et al. (2007)
tlh-reverse	GATGAGCGGTTGATGTCCAA	58.4		
tdh-FIP	GTACCTGACGTTGTGAATACTGATTGTCTCT GACTTTT GGACAAAC	78.0		
tdh-BIP	TGACATCCTACATGACTGTGAACACTTATA GCCAGAC ACCGC	78.9		
tdh-F3	AGATATTGTTTGTTGTTCGAGAT	55.7	variable	Yamazaki et al. (2008)
tdh-B3	AACACAGCAGAATGACCG	53.9		
tdh-LF	GTACGGTTTTCTTTTTACATTACG	58.4		
tdh-LB	AAGACTATACAATGGCAGCG	56.4		
tdh-forward	CTGTCCCTTTTCCTGCCCCCG	67.2	245 bp	Gutierrez West et al. (2013)
tdh-reverse	AGCCAGACACCGCTGCCATTG	65.3		

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
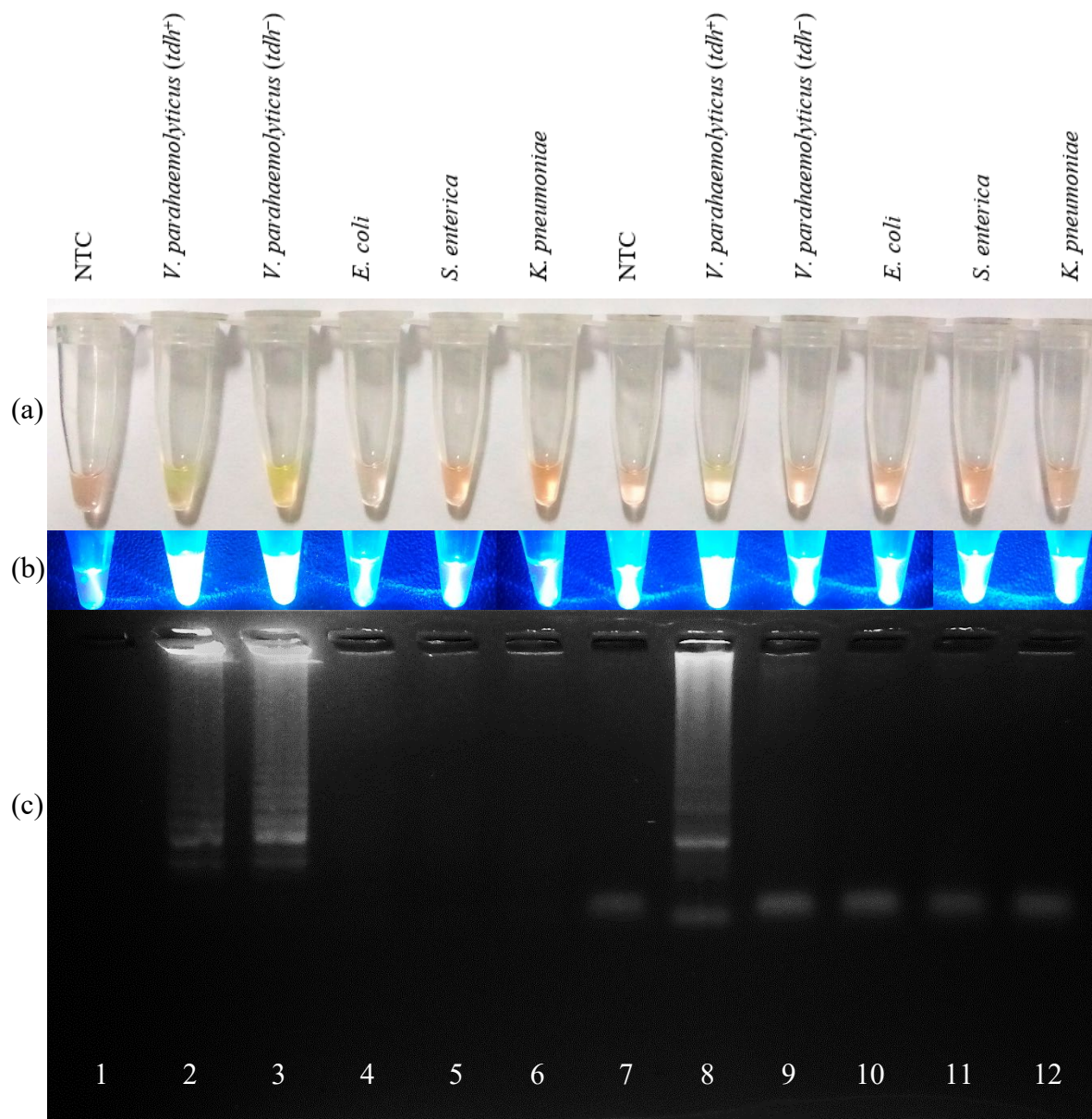
Figure 1: Representative amplicons of various strains subjected to *tlh*- and *tdh*-LAMP as seen by naked eye (A), under blue LED (B), and subjected to agarose gel electrophoresis (C). Samples 1–6 amplified using *tlh*-LAMP were NTC (lane 1), *V. parahaemolyticus* ATCC® BAA-239™ (lane 2), *V. parahaemolyticus* 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).

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

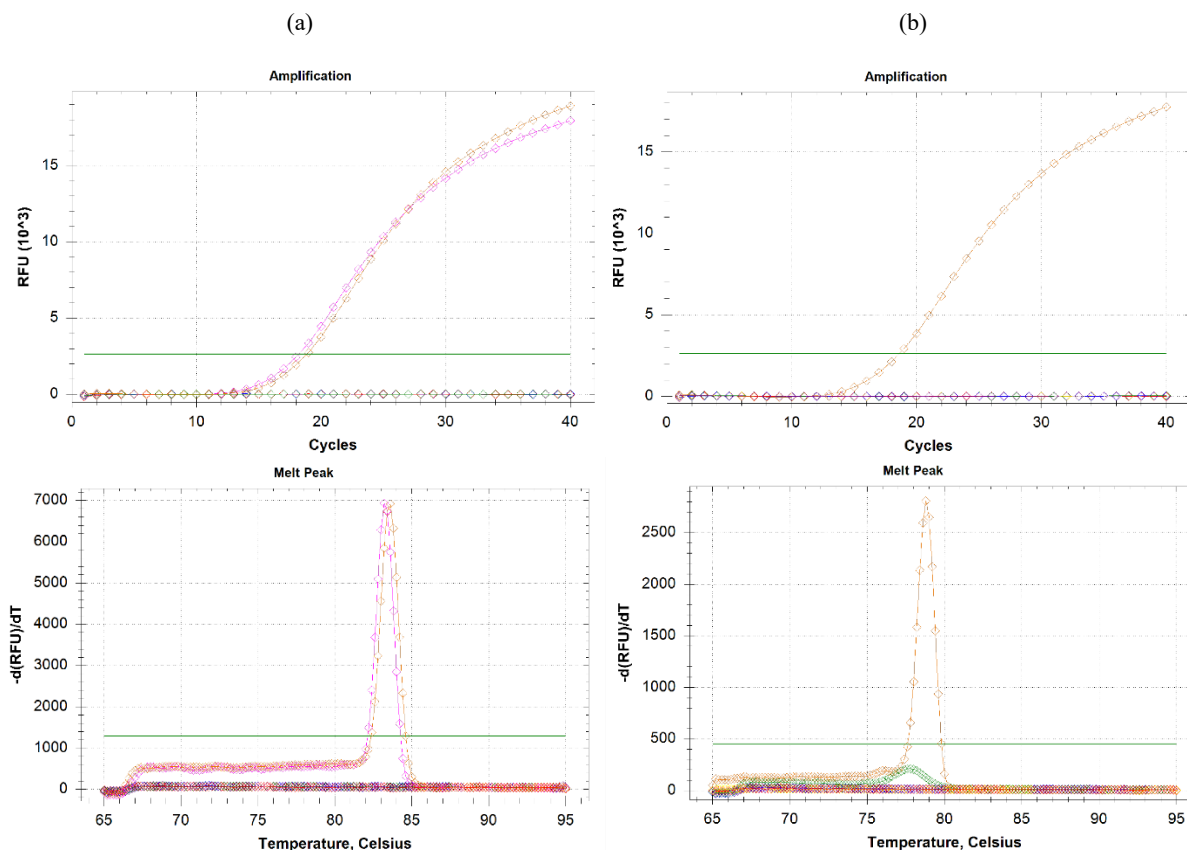| (a) | (b) |

Figure 2: Representative DNA amplification curves and melt peaks of various strains in real-time PCR targeting the *tlh* gene (A) and the *tdh* gene (B). Curves shown are those of NTC (red), *V. parahaemolyticus* ATCC® BAA-239™ (orange), *V. parahaemolyticus* ATCC® 17802™ (magenta), *E. coli* ATCC® 25922™ (green), *S. enterica* (blue), and *K. pneumoniae* (violet).

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*⁺ and *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.

Table 2: Summary of LAMP and real-time PCR specificities targeting the *tlh* and *tdh* genes of *V. parahaemolyticus*.

| Species | Strain | LAMP | | Real-time PCR | |
		tlh	*tdh*	*tlh*	*tdh*
Vibrio parahaemolyticus	ATCC® BAA-239™ (*tdh*⁺)	+	+	+	+
Vibrio parahaemolyticus	ATCC® 17802™ (*tdh*⁻)	+	−	+	−
Escherichia coli	ATCC® 25922™	−	−	−	−
Salmonella enterica	−	−	−	−	−
Klebsiella pneumoniae	−	−	−	−	−

Table 3: Sample source and presence of *tlh* and *tdh* genes of *V. parahaemolyticus* isolates confirmed by LAMP and real-time PCR targeting the *tlh* gene.

Isolate	Sampling Site	Fish Species	Fish Part	Inoculum Source	*tlh* gene	*tdh* gene
PT₅FA₂	Pila	*O. niloticus*	Flesh	Top 1 cm	+	−
BT₁FA₂	Biñan	*O. niloticus*	Flesh	Top 1 cm	+	−
BT₂FB₁	Biñan	*O. niloticus*	Flesh	Vortexed	+	−
BT₅GB₁	Biñan	*O. niloticus*	Gills	Vortexed	+	−
BB₄GA₁	Biñan	*C. chanos*	Gills	Top 1 cm	+	−
BB₄GA₂	Biñan	*C. chanos*	Gills	Top 1 cm	+	−
BB₄GA₃	Biñan	*C. chanos*	Gills	Top 1 cm	+	−
BB₅GA₂	Biñan	*C. chanos*	Gills	Top 1 cm	+	−

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; Soltany-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 *tlh* 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 *tlh* 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

discounted. This is exemplified by the occurrence of pathogenic *V. parahaemolyticus* which do not possess these well-known hemolysins, suggesting that other virulence factors may also contribute to the pathogenicity of *V. parahaemolyticus* (Jones et al. 2012; Lynch et al. 2005).

Previous studies on the prevalence of *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 *Vibrio* spp. In the present study, the detection of *V. parahaemolyticus* in fish reared in freshwater environments is alarming since these kinds of environments are supposedly unfavorable for *Vibrio* spp. survival. It is possible that these fish act as more favorable environmental sinks for strictly halophilic *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 *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 *Vibrio* spp. *V. parahaemolyticus* is simply one of the 11 known pathogenic species of *Vibrio* (Farmer et al. 2015). Thus, the absence of *V. parahaemolyticus* in some of the fish samples does not automatically discount the possibility of other pathogenic *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 *tlh* and *tdh* genes of *V. parahaemolyticus*. The assays were all found to be specific enough to differentiate *V. parahaemolyticus* from non-*Vibrio* strains, as well as *tdh*+ from *tdh*− strains of *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 *V. parahaemolyticus*; none of these possessed the *tdh* gene. The presence of *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 *V. parahaemolyticus* in fish samples from wet markets using LAMP as a rapid, sensitive, and definitive alternative to real-time PCR for detection of *V. parahaemolyticus*.

ACKNOWLEDGMENT

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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