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Rapid Detection of Aeromonas hydrophila in Tilapia Samples Using Loop-Mediated Isothermal Amplification (LAMP) Targeting the Aerolysin Gene

Arren Christian M. de Guia[1,2*], Mary Rose D. Uy-de Guia[4], Juvy J. Monserate[3], Joel R. Salazar[3], Ravelina R. Velasco[2] and Karl Marx A. Quiazon[2]

[1]*Livestock Biotechnology Center, Philippine Carabao Center, Science City of Muñoz, Nueva Ecija, 3120*
[2]*Freshwater Aquaculture Center – College of Fisheries, Central Luzon State University, Science City of Muñoz, Nueva Ecija, 3120*
[3]*Department of Chemistry, College of Science, Central Luzon State University, Science City of Muñoz, Nueva Ecija, 3120*
[4]*Production Systems and Nutrition Section, Philippine Carabao Center, Science City of Muñoz, Nueva Ecija, 3120*

Email for correspondence: deguia.arren@clsu2.edu.ph; arrenchristian@gmail.com
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Abstract

Aeromonas hydrophila is a notorious pathogen in tilapia aquaculture, often linked to fish kills due to its virulence gene, aerolysin. Economic losses globally underscore the urgency for effective disease diagnosis. While PCR is the gold standard, its complexity limits field use. Thus, this study aimed to develop a sensitive, specific, and field-friendly detection kit using Loop-Mediated Isothermal Amplification (LAMP). Bacterial isolates from 12 farms across Luzon were collected and tested. LAMP primers, validated through parallel PCR testing, showed 100% accuracy, sensitivity, and specificity. The optimized incubation conditions (63°C for 1 hour) facilitated rapid and reliable detection. Notably, LAMP's simplicity and shorter processing time offer advantages over PCR, obviating the need for sophisticated equipment and highly skilled personnel. The developed protocol, with its high diagnostic reliability, holds promise for future surveillance of aerA gene-carrying *A. hydrophila* strains in aquaculture settings.

Keywords: Aeromonas hydrophila, aerolysin gene, LAMP assay, PCR assay, Primer Explorer

Introduction

Aeromonads, particularly *Aeromonas hydrophila*, are a significant cause of disease outbreaks in aquaculture, especially in tilapia farming (Bebak et al., 2015; Harikrishnan and Balasundaram, 2005). This bacterium is responsible for multiple infections, including hemorrhagic septicemia, epizootic ulcerative syndrome, and red body disease (Abdelhamed et al., 2017; Igbinosa et al., 2012), resulting in substantial economic losses in the global aquaculture industry.

Traditionally, the polymerase chain reaction (PCR) has been the gold standard for detecting bacterial pathogens, including *A. hydrophila*. While PCR is highly sensitive and specific, it has limitations, particularly in field settings, due to its reliance on thermal cycling equipment and the need for skilled personnel (Zhao et al., 2014). In recent years, Loop-

Mediated Isothermal Amplification (LAMP) has emerged as a promising alternative. LAMP is a nucleic acid amplification method that operates at a constant temperature, typically between 60°C and 65°C, eliminating the need for expensive thermal cyclers (Notomi et al., 2000). The reaction is completed within 30 to 60 minutes, making it a rapid and convenient method for pathogen detection, particularly in resource-limited settings (Li et al., 2022).

Several studies have demonstrated the advantages of LAMP over traditional PCR-based methods. LAMP is simpler as it operates under isothermal conditions, allowing amplification to occur in basic, portable devices, such as a heat block (Mori & Notomi, 2009). It also completes faster than PCR, typically within 60 minutes, significantly reducing the waiting time for diagnostic results (Nagamine et al., 2002). Furthermore, LAMP's field applicability is enhanced by the visual detection of the amplified product, such as through turbidity or color change, eliminating the need for sophisticated detection equipment, like that used in real-time PCR (Tomita et al., 2008).

Despite these advantages, LAMP does have some limitations. The method is highly sensitive to contamination, and improper primer design can result in nonspecific amplification (Lucchi et al., 2016). Although LAMP does not require expensive equipment, the reagents and enzymes involved, such as Bst polymerase, may still be costly in certain settings (Parida et al., 2008).

This study aims to develop a LAMP assay for detecting *A. hydrophila* by targeting the aerolysin gene (aerA), a key virulence factor in fish infections. The aerA gene is associated with the cytotoxic effects of *A. hydrophila*, contributing to its pathogenicity in aquatic organisms (Bücker et al., 2011). The primary goal is to create a sensitive and specific LAMP protocol that can be used for rapid field detection in tilapia farms, thus enhancing disease surveillance and management in aquaculture settings.

Materials and Methods

Sample Collection and Sampling Frame:

The study was conducted across five major tilapia-producing provinces in Luzon, Philippines: Pampanga, Isabela, Batangas, Laguna, and Nueva Ecija. A total of 12 farms were selected based on their production capacity, history of Aeromonas-related disease outbreaks, and geographical distribution to represent a diverse range of farming practices and environmental conditions. Each farm served as a sampling unit in this study.

The sampling frame included all commercial tilapia farms within these provinces that met the following criteria:

1. **Farm Size and Production Capacity:** Farms with a minimum production capacity of 5 metric tons per year were selected to ensure that the sampling captured data from significant contributors to the local aquaculture industry.
2. **Disease History:** Farms that had previously reported incidents of Aeromonas hydrophila infections were prioritized to increase the likelihood of detecting the pathogen.
3. **Geographical Representation:** Farms were selected to cover the main tilapia-producing regions in Luzon, ensuring that the study encompassed various environmental conditions and management practices.

From each of the 12 selected farms, five fish samples were collected, yielding a total of 60 samples. These samples were subjected to bacterial isolation, resulting in 257 bacterial isolates. Each isolate was then subjected to DNA extraction and subsequent molecular analysis. The collection was carried out by trained personnel under the supervision of the project lead, ensuring that standardized procedures were followed to minimize contamination and maintain sample integrity.

The isolates were purified through repeated sub-culturing, and DNA was extracted using the boiling method as described by Dashti et al. (2009). All DNA samples were diluted to a concentration of 50 ng/µL to avoid false-positive results during the PCR experiments. A positive control isolate of *A. hydrophila* was provided by the Bureau of Fisheries and Aquatic Resources-National Fisheries Laboratory Division to validate the assays.

Primer Designing. Primers used in this study were designed in the *A. hydrophila aer*A gene. Briefly, multiple sequence alignment was carried out to determine the conserved region of the *aer*A gene. Next, the conserved region of the gene was uploaded to Primer Explorer

version 5 for the primer design (https://primerexplorer.jp/e/). The designed primer sequences were sent to Macrogen Inc. (South Korea) for synthesis.

Touchdown PCR Assay. PCR assay was initially performed to screen all the *aer*A gene-positive samples. The F3 and B3 primers designed by the Primer Explorer version 5 (https://primerexplorer.jp/e/) (Table 1) were used to determine samples possessing the target gene. The PCR master mix (Promega, USA) was composed of 9.0 µL ultrapure water, 6 µL PCR buffer, 2 µL $MgCl_2$, 0.3 µL dNTPs, 0.3 µL of each primer, 0.3 µL *Taq* polymerase, and 2 µL DNA template. The thermal profile for touchdown PCR is as follows: 1 cycle of 95°C for 5 min, 40 cycles (of 95°C for 30 secs, 65°C with 1.0°C heat decrement each cycle for 30 secs, and 72°C for 5 min) and finally 1 cycle of 72°C for 5 min. Touchdown PCR was performed in a thermal cycler (Applied BioSystem, USA).

LAMP Assay. LAMP reaction master mix consisted of NFW, 2.5mM dNTPs, 100 mM betaine, 10 × LAMP buffer, 150mM MgSO4, 2.5 mM dNTPs, 10 mM of each primer, 8u/uL Bst polymerase (NEB, USA) and 2 uL DNA template. This study determined the optimum temperature and incubation duration to know the condition that will be used in every run. Three different temperatures of 63, 64, and 65°C were used to evaluate the optimum temperature using digital heat block dry bath (Benchmark, USA). The temperature that gave the most intense ladder-like bands on the agarose gel was selected. On the other hand, the incubation duration was also tested using 30, 60, and 90 minutes of incubation time. The shortest time to give ladder-like bands was used for further testing. The minimum detectable limit, as well as the specificity of the LAMP assay, were also determined. The positive control was serially diluted for the detection limit into eight different concentrations to determine the minimum detectable limit for LAMP assay. The result of the LAMP assay was compared to that of the PCR to evaluate the specificity. For the visual determination of the result, addition of intercalating dye SyBr green was done

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Table 1. List of primers designed and used for LAMP assay.

PRIMER NAME	SEQUENCE (5'-3')
F3	GTGGGGAGCAAACAGGATT
B3	CCAGGTAAGGTTCTTCGCG
FIP	CCGGAAGCCACGTCTCAAGGGTAGTCCACGCCGTAAACG
BIP	GGAGTACGGCCGCAAGGTTAACATGCTCCACCGCTTGTG

Statistical Analysis

All statistical analyses were performed using Medcalc diagnostic test evaluation calculator (https://www.medcalc.org/calc/diagnostic_test.php).

Accuracy, sensitivity, and specificity were computed based on the result of PCR using the formulae proposed by Baratloo *et al.* (2015).

$$Accuracy = \frac{TP + TN}{TP + TN + FP + FN}$$

$$Sensitivity = \frac{TP}{TP + FN}$$

$$Specificity = \frac{TN}{TN + FP}$$

Legend: TP = True Positive
TN = True Negative
FP = False Positive
FN = False Negative

$$\boldsymbol{PPV = \frac{sesitivity\ x\ prevalence}{sesitivity\ x\ prevalence + (1 - specificity)\ x\ (1 - prevalence)}}$$

$$\boldsymbol{NPV = \frac{specificity\ x\ (1 - prevalence)}{(1 - sensitivity)\ x\ prevalence + specificity\ x\ (1\ - prevalence)}}$$

Results and Discussion

Out of 257 isolates, only the positive control isolate showed a band for the *aer*A gene amplification (Figure 1). The *aer*A gene fragment produced 232 bp amplicon. The touchdown PCR assay showed no unspecific bands. The result of the assay was used as the basis for the specificity of the LAMP assay by comparing the result of both assays.

Figure 1. Agarose gel image showing amplified product of *aerA* gene at 232 bp. Lane M = 100 bp Marker, lane 1 = positive control, lanes 2-4 = isolate 1-3, lane 5 = negative control

Figure 1 illustrates the results of the touchdown PCR assay, with the positive control showing a clear band at 232 base pairs, corresponding to the *aerA* gene fragment. The absence of bands in Lanes 2-4 (sample isolates) is consistent with the fact that none of the tested field samples carried the *aerA* gene. The positive control, located in Lane 1, successfully amplified the target gene, while the tested samples showed no amplification, confirming the absence of the gene. This figure supports the specificity of the assay and validates the PCR results, ensuring that no unspecific amplification occurred in the tested samples

Figure 2 presents the optimization results of the LAMP assay. In **Figure 2A**, the LAMP products were incubated for 30, 60, and 90 minutes, with the most intense bands observed after 60 minutes, indicating that 60 minutes is the optimal incubation time for the assay. **Figure 2B** displays the results at different incubation temperatures (63°C, 64°C, and 65°C), with the clearest ladder-like bands visible at 63°C, establishing this as the optimal temperature for the amplification process. Finally, **Figure 2C** shows the minimum detectable limit of the LAMP assay, indicating that it could detect DNA concentrations as low as 3.58x10^-6 ng/µL, which is comparable to the sensitivity of the PCR assay. These results demonstrate that the LAMP assay is both time-efficient and highly sensitive, offering a valuable tool for field diagnostics where time and equipment are limited

Table 2. Summary of LAMP assay targeting *aer*A gene test result.

Province	True Positive (TP)	False Positive (FP)	True Negative (TN)	False Negative (FN)	Total Samples
Pampanga	0	0	48	0	48
Laguna	0	0	57	0	57
Batangas	0	0	47	0	47
Nueva Ecija	0	0	37	0	37
Isabela	0	0	67	0	68
Total	0	0	257	0	257

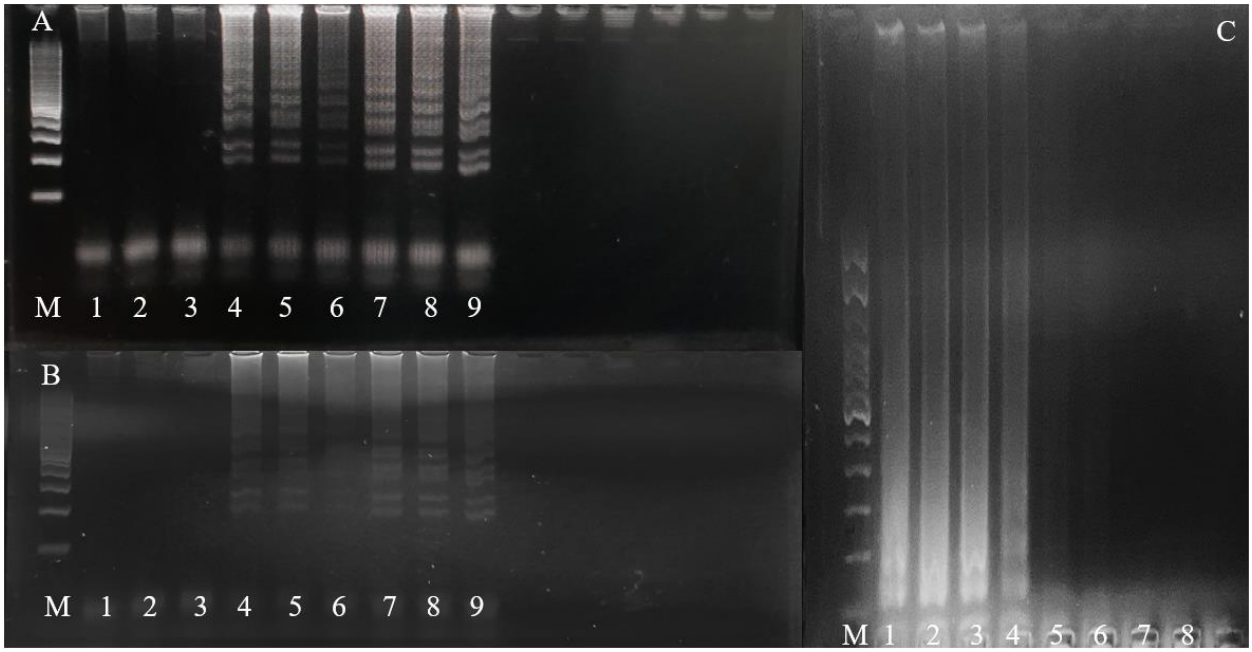

Figure 2. Agarose gel image showing the optimized incubation duration (A) (lane M = 100 bp marker, lanes 1-3 = 30 min., lanes 4-6 = 60 mins. and lanes 7-9 = 90 mins), incubation temperature (B) (lane M = 100 bp marker, lanes 1-3 = 65°C, lanes 4-6 = 64°C and lanes 7-9 = 63°C) and minimum detectable limit (C) (lane M = 100 bp marker, lanes 1-6 = 3.58 x 10^{-1} to 3.58 x 10^{-6} respectively)

Until now, no data signifying the presence of *aer*A gene-possessing *A. hydrophila* has been reported in the Philippines. Several studies focusing on the prevalence of *A. hydrophila* were conducted in the country. Unfortunately, the determination of the presence of *aer*A gene was not part of that reported case. In an occurrence in Egypt, they found that two (9.09%) of the *Aeromonas* isolates tested positive for the hemolysin A (hylA) gene, four (18.18%) of the *Aeromonas* isolates tested positive for the cytotoxic enterotoxin gene, and 16 of the *Aeromonas* isolates (72.72%) tested positive for the aerolysin (*aer*A) gene. However, the cytotoxic heat-stable enterotoxin gene was absent from all other isolates (ast). The *A. hydrophila* isolates were tested for pathogenicity, which revealed a direct relationship between genotype and mortality rate. Mortality rates for isolates with two virulence genes (aer+ and act+ and aer+ andhylA+, respectively) were 63.3 and 73.3% higher than those for isolates with just one virulence gene were 40, 53.3, and 56.6% higher (El-Bahar *et al*., 2019).

Because of its high virulence, aerolysin makes *A. hydrophila* more pathogenic. According to an *in vitro* study, aerolysin lyses tight junction protein when used as a gastrointestinal mucosal barrier, which may lead to epithelial integrity issues (Bucker *et al*., 2011). However, studies on the molecular mechanisms behind aerolysin-induced cell lysis are still scarce. The only known example of aerolysin-related pathogenesis evidence is the possibility of *A. hydrophila* breaching the gastrointestinal barrier and systemically infecting other organs (Kong *et al*., 2017).

Because the LAMP assay approach depends on the presence of the target gene, it is more accurate than culturing and Gram staining procedures. According to previous studies, contamination is possible because culture techniques depend on the bacteria's morphology, shape, and enzymes (Pongsachareonnont *et al*., 2017). As an alternative to culture or Gram stain diagnosis, the detection of aerolysin toxin, a virulence agent employed by bacteria to enter the cell and cause a secondary pore ulcer, is advised (Geny and Popoff, 2006).

Over the past 10 years, the development of strand displacement amplification and nucleic acid sequence-based amplification assays (Down *et al*., 1996; Little *et al*., 1996; Pfyffer *et al*., 1999) has addressed the need for accurate and speedy detection of *A. hydrophila* infections. The main barriers to the widespread adoption of these technologies in relatively small-scale clinical laboratories, such as private clinics, are that they require precise

instruments for the amplification or complex methods for detecting the amplified products. The LAMP-based assay created in this work has the benefits of a quick reaction, straightforward operation, and straightforward detection (Down *et al*., 1996).

The sensitivity and specificity of the LAMP assay are comparable with that of the PCR assay. On the other hand, the bright side of using LAMP is that it does not require a thermal cycler.

Conclusion

In conclusion, the development of a Loop-Mediated Isothermal Amplification (LAMP) assay targeting the aerolysin gene (aerA) of *Aeromonas hydrophila* represents a significant advancement in the detection of this pathogen, particularly in aquaculture settings. This study demonstrated the efficacy of the LAMP assay in rapidly and accurately identifying *A. hydrophila* strains possessing the aerA gene, with a sensitivity and specificity of 100%. The optimized protocol allows for detection within a shorter timeframe compared to traditional PCR methods, making it highly suitable for field applications where sophisticated equipment may be lacking. Importantly, the LAMP assay offers a cost-effective and user-friendly alternative to PCR, addressing the challenges associated with traditional diagnostic methods in resource-limited settings.

Furthermore, the study sheds light on the prevalence of *A. hydrophila* carrying the aerA gene in tilapia farms across Luzon, Philippines, highlighting the importance of ongoing surveillance efforts in aquaculture. By providing a reliable tool for the rapid detection of this virulent pathogen, the developed LAMP assay offers valuable support for disease management strategies and contributes to safeguarding the health and productivity of farmed fish populations.

Overall, the findings of this study underscore the potential of LAMP-based assays as a practical and efficient tool for disease diagnosis in aquaculture, paving the way for improved disease control and management practices in the industry. Further research and application of LAMP technology hold promise for enhancing the resilience of aquaculture systems against microbial pathogens, ultimately benefiting both fish producers and consumers alike
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