PUNGGAWA
GLOBAL RESEARCH:
JURNAL MULTIDISIPLIN

Punggawa Global Research: Jurnal Multidisiplin
Volume 1, Nomor 3, 2026
E-ISSN: 3164-0117
Open Access: punggawajournal.com/pgr

Development of *aerA* Gene-Based PCR Primers as DNA Biomarkers for the Molecular Detection of *Aeromonas hydrophila* Using an In Silico Approach

Septianto Wikan Nurhidayat[1], Naina Nur Prastiti[2]
[1,2] Fakultas Sains dan Teknologi, Universitas Aisyiyah Yogyakarta
Correspondence: septiantowikannurhidayat@unisayogya.ac.id

Article Info

Article history:
Received Aug 1th, 2026
Revised Aug 5th, 2026
Accepted Aug 8th, 2026

Keyword:
Aeromonas hydrophila; *aerA* gene; DNA biomarker; *In Silico* PCR; Primer3Plus.

ABSTRACT

Aeromonas hydrophila is one of the major bacterial pathogens affecting cultured fish, causing Motile Aeromonas Septicemia (MAS) and substantial economic losses in aquaculture. Early detection of this pathogen is essential for effective disease prevention and control. This study aimed to design a primer pair targeting the *aerA* virulence gene as a DNA biomarker for the molecular detection of *A. hydrophila* using an *in silico* approach. The *aerA* gene sequence (GenBank accession number EU254217.1) was retrieved from the NCBI database and used as the template for primer design using Primer3Plus. The designed primers were evaluated based on primer length, melting temperature (Tm), GC content, predicted amplicon size, and the potential formation of secondary structures. Primer specificity was subsequently assessed using *In Silico* PCR against several Aeromonas genomes. The selected primer pair consisted of the forward primer 5'-GAGAAGACGGCCATCAAGGT-3' and the reverse primer 5'-TGTGGTTCCAGTTCGGGC-3', producing a predicted amplicon of 529 bp. The primers exhibited melting temperatures of 59.7°C and 59.9°C with GC contents of 55.0% and 61.1%, respectively, indicating suitable characteristics for PCR amplification. In Silico PCR analysis demonstrated successful amplification exclusively in *A. hydrophila* genomes, while no amplification was detected in the non-target Aeromonas species evaluated. These findings suggest that the *aerA* gene is a promising DNA biomarker for the molecular detection of *A. hydrophila* . However, further in vitro validation is required to confirm the sensitivity and specificity of the designed primers using field isolates.

INTRODUCTION

The intensification of aquaculture systems has resulted in a higher incidence of bacterial infections, notably those caused by *Aeromonas hydrophila*. This pathogen is responsible for outbreaks of Motile Aeromonas Septicemia (MAS), which cause significant economic losses (Rasmussen-Ivey et al., 2016; Abdella et al., 2024). Additionally, *A. hydrophila* is of considerable relevance within the One Health framework due to its antimicrobial resistance and its capacity to infect humans via environmental and foodborne routes (Fernández-Bravo & Figueras, 2020). The pathogenicity of *A. hydrophila* is attributed to several virulence factors, with the *aerA* gene encoding aerolysin being highly conserved among pathogenic isolates. (Ahangarzadeh et al., 2022) This conservation makes the *aerA* gene a promising DNA biomarker target for molecular identification (Tomás, 2012; Rasmussen-Ivey et al., 2016; Majeed et al., 2023).

Conventional identification methods are limited by time constraints and insufficient accuracy. (Qu et al., 2021) As a result, molecular techniques based on Polymerase Chain Reaction (PCR) have emerged as the primary alternative due to their speed, sensitivity, and high specificity (Lin et al., 2022; Abdelsalam et al., 2023). Recent advances in genomic technologies, including whole-genome sequencing, have demonstrated that virulence genes serve as more specific diagnostic targets than conserved genes such as 16S rRNA (Abdella et al., 2023; Lee et al., 2023). Although multiple PCR methods have been developed for detecting *A. hydrophila*, ongoing updates to genomic databases and

the discovery of genetic variation among isolates require continuous evaluation and development of improved primers (Lin et al., 2022; Abdelsalam et al., 2023).

The effectiveness of PCR detection depends on the optimal design of primer pairs, which can now be achieved through computational tools (Untergasser et al., 2012). Integrating bioinformatics-based primer design with in silico PCR validation enables researchers to assess primer specificity against target genomes efficiently, without preliminary laboratory experiments (Bikandi et al., 2004; San Millán et al., 2013). Following this approach, the present study aims to design specific primer pairs targeting the *aerA* gene using Primer3Plus and to evaluate their performance through in silico PCR. This work provides a foundation for the development of rapid and accurate early diagnostic methods for *A. hydrophila* in the aquaculture industry.

RESEARCH METHODS

This bioinformatics (in silico) study aims to evaluate the potential of the *aerA* gene as a DNA biomarker for the rapid detection of *Aeromonas hydrophila* without involving laboratory (in vitro) testing. The in silico approach is widely used as an initial step in the development of molecular diagnostics to efficiently evaluate primer performance prior to experimental validation (Untergasser et al., 2012; Bikandi et al., 2004; San Millán et al., 2013). The first stage began with the retrieval of the *aerA* gene nucleotide sequence (accession number EU254217.1) in FASTA format from the verified NCBI GenBank database. The use of this public database serves as a template for primer design to ensure the reliability and reproducibility of the research.

The primer design process was conducted using Primer3Plus software, which can automatically optimize thermodynamic parameters in accordance with diagnostic PCR standards (Untergasser et al., 2012). The established parameter criteria included a primer length of 18-24 bp, a melting temperature (Tm) between 58-62°C with a maximum difference of ≤2°C, a GC content of 40-60%, and an amplicon size of 300-600 bp. In addition to these criteria, the primer pairs were evaluated for their physical and thermodynamic characteristics to ensure binding stability and the absence of secondary structure formations that hinder amplification, such as hairpins, self-dimers, and cross-dimers (Dieffenbach et al., 1993; Untergasser et al., 2012).

Primer specificity validation was tested using the UCSC In Silico PCR application by comparing the *A. hydrophila* genome against non-target species (*A. salmonicida* and *A. veronii*) to predict amplification success and reduce the risk of in vitro optimization failure (Bikandi et al., 2004; San Millán et al., 2013). Data were analyzed descriptively by assessing the successful formation of virtual DNA bands specific to the target genome. This final interpretation was used to confirm the feasibility of the *aerA* gene as a molecular diagnostic biomarker, which will later be compared with various previous studies regarding pathogen identification in aquaculture (Lin et al., 2022; Abdella et al., 2023; Abdelsalam et al., 2023).

RESULTS AND DISCUSSION

The development of molecular-based disease diagnostic methods for fish is crucial for the early detection of *Aeromonas hydrophila*, the primary bacterium causing outbreaks in freshwater fish. Virulence genes are considered more effective as PCR targets compared to conserved genes (such as 16S rRNA) because they are able to differentiate between pathogenic and non-pathogenic isolates (Tomás, 2012; Rasmussen-Ivey et al., 2016; Lin et al., 2022).

1. Results of *aerA* Gene Primer Design

The *aerA* gene sequence of *A. hydrophila* (GenBank EU254217.1) was used as a template for primer design via Primer3Plus. As a result, one optimal primer pair was obtained with the following specifications: Forward Primer (5'-GAGAAGACGGCCATCAAGGT-3'), Reverse Primer (5'-TGTGGTTCCAGTTCGGGC-3'), Primer length 20 & 18 bp, Melting temperature (Tm) 59.7°C & 59.9°C, GC content 55.0% & 61.1%, PCR Product size-529 bp. Based on thermodynamic and structural parameters, a primer length ranging from 18–20 bp with a very small melting temperature difference (0.2°C) supports efficiency and binding synchronization during the annealing process. The GC content of the primers is also within the optimal range to maintain thermodynamic stability, supported by the absence of potential secondary structure formations such as hairpins, self-dimers, and cross-dimers that could inhibit the PCR reaction (Dieffenbach et al., 1993; Untergasser et al., 2012). Furthermore, the

predicted amplicon size of 529 bp is highly ideal for visualization using conventional PCR (Abdelsalam et al., 2023). This aligns with the selection of the *aerA* gene, which has been proven to have a high level of conservation in pathogenic isolates, making it a much more stable target compared to the 16S rRNA gene and other secondary virulence genes such as hlyA, act, alt, and ast (Tomás, 2012; Lin et al., 2022; Majeed et al., 2023; Abdella et al., 2023; Lee et al., 2023).

2. Results of In Silico PCR Analysis

Specificity validation was computationally performed using UCSC In Silico PCR by testing the primer pair against target and non-target genomes (Figures 1).

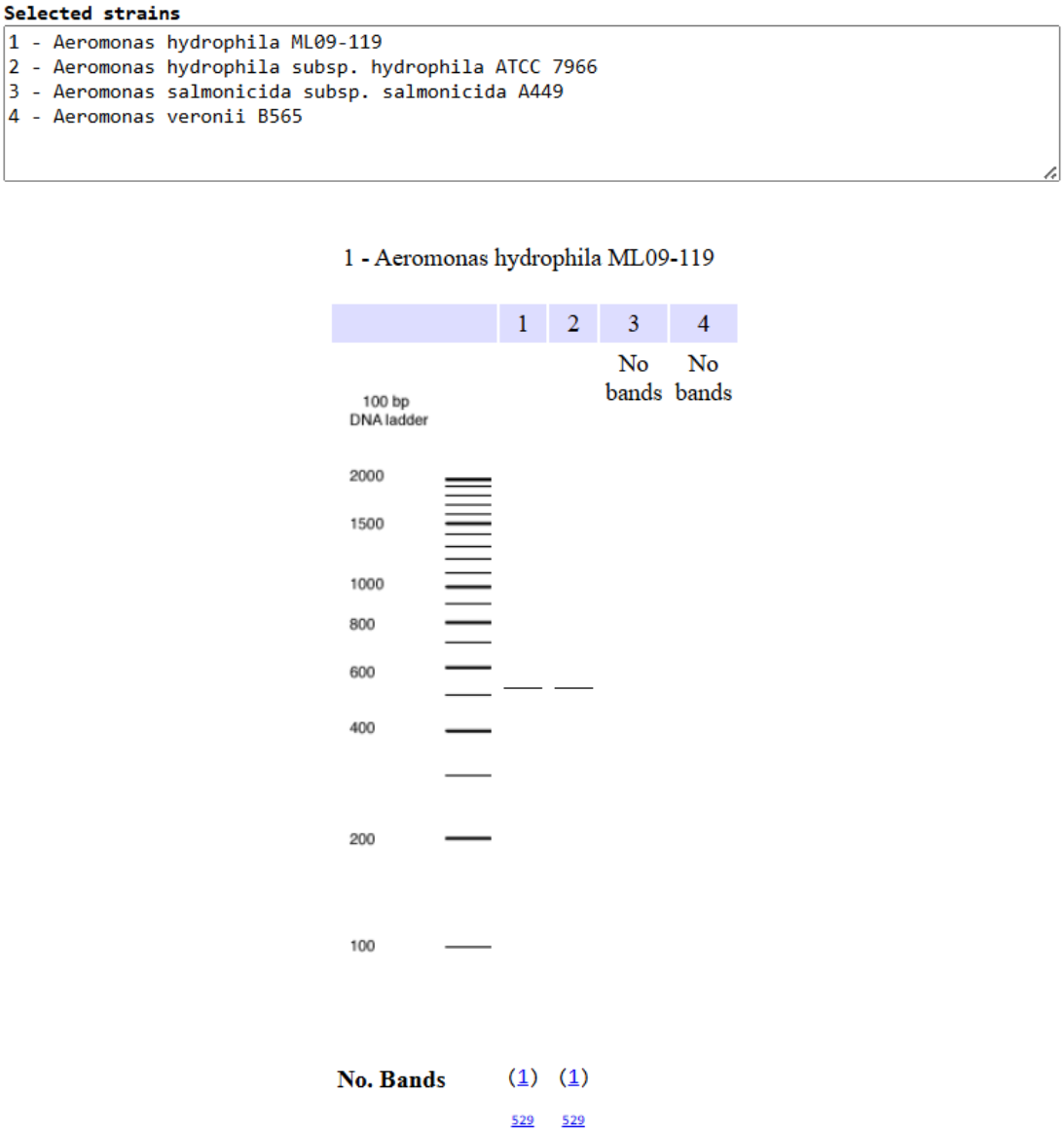

Figure 1. In silico PCR assay using Pair 2 primer and 4 Aeromonas genomic DNA resulted two single 529-bp DNA bands belonging to all *A. hydrophila*

The simulation results demonstrated a high level of specificity, where the primer pair successfully generated a 529 bp virtual DNA band exclusively on the *A. hydrophila* genome, without triggering cross-amplification in the comparative species, thereby minimizing the risk of false-positive results (Bikandi et al., 2004; San Millán et al., 2013). This success is supported by genomic evidence showing that the *aerA* gene is the most consistent virulence determinant in *A. hydrophila* compared to other genes whose distributions frequently vary among species, making it an ideal DNA biomarker for the

accurate diagnosis of Motile Aeromonas Septicemia (MAS) (Rasmussen-Ivey et al., 2016; Abdella et al., 2023; Lee et al., 2023; Majeed et al., 2023). Although this in silico approach offers significant potential for early detection that can reduce mortality rates and avoid the excessive use of antibiotics in the aquaculture sector (Abdelsalam et al., 2023), this testing still requires an in vitro laboratory-based validation stage using actual field isolates.

3. Discussion

This in silico study reinforces the position of the *aerA* gene as a highly potential DNA biomarker with a strong biological basis of pathogenicity in *Aeromonas hydrophila*. This gene specifically encodes aerolysin, a β-pore-forming toxin that serves as the primary determinant causing host cell damage, erythrocyte hemolysis, and facilitating bacterial invasion into fish tissues. The utilization of this gene is directly related to the bacterial pathogenesis mechanism that triggers the clinical symptoms of Motile Aeromonas Septicemia (MAS) (Tomás, 2012; Rasmussen-Ivey et al., 2016). Fundamentally, aerolysin is widely recognized as one of the most lethal and highly conserved virulence factors among pathogenic Aeromonas strains (Janda & Abbott, 2010; Abdella et al., 2023), thus making it a highly logical and stable molecular target for detecting the presence of potentially harmful isolates in aquaculture systems.

The advantage of the *aerA* gene as a single biomarker provides a much sharper specificity resolution in differentiating pathogenic isolates compared to standard operational genes such as 16S rRNA, which is highly conserved but often fails to distinguish closely related Aeromonas species phylogenetically. On the other hand, although secondary virulence genes such as *hlyA* (hemolysin) and act (enterotoxic cytotoxin) also contribute to pathogenesis, their distribution and expression levels frequently vary inconsistently due to the high genomic plasticity in Aeromonas (Stratev & Odeyemi, 2016; Majeed et al., 2023). This natural genetic variation makes the use of *aerA* a more representative and accurate choice, as the gene is consistently found in *A. hydrophila* isolates without triggering cross-amplification with non-target species such as A. salmonicida and A. veronii (Lee et al., 2023).

The use of an integrated bioinformatics approach, which combines the convenience of Primer3Plus design and In Silico PCR simulation, has proven capable of significantly increasing the efficiency of diagnostic research. This computational process facilitates the strict evaluation of thermodynamic parameters—such as GC content, melting temperature, and the avoidance of secondary structures (hairpins, dimers)—while simultaneously validating primer specificity against thousands of public genomic data sets (Untergasser et al., 2012). This multi-layered diagnostic strategy drastically cuts down time, operational costs, and the risk of optimization failure before the research moves to the wet laboratory stage, providing a very solid theoretical foundation to prevent false-positive results during the experimental phase (Bikandi et al., 2004; San Millán et al., 2013).

For future field application projections, this *aerA* primer pair design holds strategic value in aquaculture biosecurity management, particularly for detecting infections prior to massive outbreaks. Accurate early detection capabilities have the potential to drastically reduce fish mortality rates and prevent the empirical use of antibiotics, which often triggers antimicrobial resistance (AMR). This primer pair is not only ideal for application in conventional PCR but is also highly adaptable for further development into faster and more portable advanced diagnostic technologies such as qPCR, LAMP, and RAA-CRISPR/Cas12a (Lin et al., 2022; Abdelsalam et al., 2023). As an absolute follow-up step, in vitro laboratory validation using clinical and environmental isolates from aquaculture sites remains essential to demonstrate the sensitivity and performance of the primers in overcoming the challenges of PCR inhibitors in real samples.

CONCLUSION

This study successfully designed an *aerA* gene-based primer pair as a DNA biomarker for the molecular detection of *Aeromonas hydrophila* using an in silico approach. The obtained primer pair possesses characteristics that meet the criteria for optimal PCR primers, including primer length, melting temperature, GC content, and amplicon size, while demonstrating no potential for secondary structure formations that could interfere with the amplification process. The In Silico PCR analysis results demonstrated that the primers could specifically amplify the target *aerA* gene in the *A. hydrophila* genome without generating amplification in the tested comparative species. These findings indicate that the *aerA* gene has the potential to be utilized as a DNA biomarker in the development of rapid, specific, and accurate molecular detection methods for *A. hydrophila* . Nevertheless, in vitro

validation using clinical and field isolates remains necessary to ensure the sensitivity, specificity, and reproducibility of the primers prior to their application as a diagnostic tool in fish health and aquaculture.

REFERENCES

Abdella, B., Abozahra, N. A., Shokrak, N. M., Mohamed, R. A., & El-Helow, E. R. (2023). Whole spectrum of Aeromonas hydrophila virulence determinants and the identification of novel SNPs using comparative pathogenomics. *Scientific Reports*, *13*(1), 1–11. https://doi.org/10.1038/s41598-023-34887-1

Abdella, B., Shokrak, N. M., Abozahra, N. A., Elshamy, Y. M., Kadira, H. I., & Mohamed, R. A. (2024). Aquaculture and Aeromonas hydrophila: a complex interplay of environmental factors and virulence. *Aquaculture International*, *32*(6), 7671–7681. https://doi.org/10.1007/s10499-024-01535-y

Abdelsalam, M., Elgendy, M. Y., Elfadadny, M. R., Ali, S. S., Sherif, A. H., & Abolghait, S. K. (2023). A review of molecular diagnoses of bacterial fish diseases. *Aquaculture International*, *31*(1), 417–434. https://doi.org/10.1007/s10499-022-00983-8

Ahangarzadeh, M., Najafabadi, M. G., Peyghan, R., Houshmand, H., Rohani, M. S., & Soltani, M. (2022). Detection and distribution of virulence genes in Aeromonas hydrophila isolates causing infection in cultured carps. *Veterinary Research Forum*, *13*(1), 55–60. https://doi.org/10.30466/vrf.2020.115998.2761

Bikandi, J., Millán, R. S., Rementeria, A., & Garaizar, J. (2004). In silico analysis of complete bacterial genomes: PCR, AFLP-PCR and endonuclease restriction. *Bioinformatics*, *20*(5), 798–799. https://doi.org/10.1093/bioinformatics/btg491

Dieffenbach, C. W., Lowe, T. M. J., & Dveksler, G. S. (1993). General concepts for PCR primer design. *Genome Research*, *3*(3). https://doi.org/10.1101/gr.3.3.S30

Fernández-Bravo, A., & Figueras, M. J. (2020). An update on the genus Aeromonas: Taxonomy, epidemiology, and pathogenicity. In *Microorganisms* (Vol. 8, Issue 1). https://doi.org/10.3390/microorganisms8010129

Janda, J. M., & Abbott, S. L. (2010). The genus Aeromonas: Taxonomy, pathogenicity, and infection. *Clinical Microbiology Reviews*, *23*(1), 35–73. https://doi.org/10.1128/CMR.00039-09

Lee, H. J., Storesund, J. E., Lunestad, B. T., Hoel, S., Lerfall, J., & Jakobsen, A. N. (2023). Whole genome sequence analysis of Aeromonas spp. isolated from ready-to-eat seafood: antimicrobial resistance and virulence factors. *Frontiers in Microbiology*, *14*(June). https://doi.org/10.3389/fmicb.2023.1175304

Lin, Z., Lu, J., Wu, S., Lin, X., Zheng, L., Lou, Y., & Xiao, X. (2022). A novel detection method for the pathogenic Aeromonas hydrophila expressing aerA gene and/or hlyA gene based on dualplex RAA and CRISPR/Cas12a. *Frontiers in Microbiology*, *13*(October), 1–10. https://doi.org/10.3389/fmicb.2022.973996

Majeed, S., De Silva, L. A. D. S., Kumarage, P. M., & Heo, G. J. (2023). Occurrence of potential virulence determinants in Aeromonas spp. isolated from different aquatic environments. *Journal of Applied Microbiology*, *134*(3), 1–12. https://doi.org/10.1093/jambio/lxad031

Qu, Y., Wang, Q., Li, Y., Wang, Y., Yin, J., Ren, Y., Liu, C., Liu, X., Wang, Y., & Zeng, W. (2021). Development of a real-time recombinase polymerase amplification assay for rapid detection of Aeromonas hydrophila. *Journal of Fish Diseases*, *44*(4), 469–477. https://doi.org/10.1111/jfd.13291

Rasmussen-Ivey, C. R., Figueras, M. J., McGarey, D., & Liles, M. R. (2016). Virulence factors of Aeromonas hydrophila: In the wake of reclassification. *Frontiers in Microbiology*, *7*(AUG), 1–

10. https://doi.org/10.3389/fmicb.2016.01337

San Millán, R. M., Martínez-Ballesteros, I., Rementeria, A., Garaizar, J., & Bikandi, J. (2013). Online exercise for the design and simulation of PCR and PCR-RFLP experiments. *BMC Research Notes*, *6*(1), 2–5. https://doi.org/10.1186/1756-0500-6-513

Stratev, D., & Odeyemi, O. A. (2016). Antimicrobial resistance of Aeromonas hydrophila isolated from different food sources: A mini-review. *Journal of Infection and Public Health*, *9*(5), 535–544. https://doi.org/10.1016/j.jiph.2015.10.006

Tomás, J. M. (2012). The Main Aeromonas Pathogenic Factors . *ISRN Microbiology*, *2012*, 1–22. https://doi.org/10.5402/2012/256261

Untergasser, A., Cutcutache, I., Koressaar, T., Ye, J., Faircloth, B. C., Remm, M., & Rozen, S. G. (2012). Primer3-new capabilities and interfaces. *Nucleic Acids Research*, *40*(15), 1–12. https://doi.org/10.1093/nar/gks596