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Multiplex PCR Targeting Multiple Genome Segments for Enhanced Detection of Tilapia Lake Virus (TiLV) in Tilapia

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ABSTRACT

Background: Tilapia Lake Virus (TiLV) is an emerging threat to global aquaculture and current PCR assays that target a single genome segment is vulnerable to false negatives due to genetic variability and viral reassortment. A robust diagnostic tool is needed for reliable detection and surveillance.

Methods: We developed a multiplex polymerase chain reaction (mPCR) assay targeting three conserved genome segments (2, 3 and 8) using TiLV Primer sets that were optimized through uniplex and multiplex reactions and assay conditions were standardized at 58°C. Sensitivity was assessed using serial dilutions of TiLV-positive cDNA, while specificity was evaluated against non-TiLV aquatic pathogens. Diagnostic performance was compared with semi-nested reverse transcription polymerase chain reaction (semi-nested RT-PCR) and validated using Receiver Operating Characteristic (ROC) analysis.

Result: The optimized mPCR assay consistently amplified all three segments in a single reaction, producing clear and specific bands without non-specific amplification. The detection limit was 100 (picogram) pg/µL of TiLV cDNA and no cross-reactivity was observed with non-TiLV pathogens. ROC analysis yielded an AUC value of 1.0, indicating perfect sensitivity and specificity. This multi-segment approach minimizes false negatives and offers a reliable tool for TiLV detection in tilapia aquaculture.

Key words: Aquaculture diagnostics, Multiplex PCR, Tilapia lake virus, Viral detection.

INTRODUCTION

Tilapia is the world's second most cultivated freshwater fish and has become an important source of dietary protein as well as a substantial economic resource in developing nations (FAO, 2022; Hounmanou *et al*., 2018). Global tilapia production, including other cichlids, reached 6.1 million metric tons in 2020, with China, Thailand, Ecuador, Egypt and Indonesia as major contributors (Harvey, 2016). Despite their relative resistance, farmed tilapia are susceptible to a variety of disease-causing organisms (Machimbirike *et al*., 2019; Surachetpong *et al*., 2020; Kaviarasu *et al*., 2022).

Diseases of viral origin are considered among the most significant threats in tilapia aquaculture and Tilapia Lake Virus (TiLV) has emerged as one of the most important pathogens in recent years (Eyngor *et al*., 2014; Ferguson *et al*., 2014). TiLV, also known as *Tilapia tilapinevirus*, is an enveloped virus with a 10-segment, negative-sense RNA genome encoding 14 predicted proteins belonging to the genus *Tilapine virus* within the family *Amnoonviridae* (Koonin *et al*., 2023) and is listed as an emerging finfish disease by the World Organisation for Animal Health (WOAH, 2022).

Detection of TiLV generally relies on molecular techniques that identify viral genetic material in clinical specimens. Several diagnostic assays have been developed, including RT-PCR (Eyngor *et al*., 2014), nested RT-PCR (Kembou *et al*., 2017), semi-nested RT-PCR (Dong *et al*., 2017), RT-qPCR (Tattiyapong *et al*., 2018; Waiyamitra *et al*., 2018; Taengphu *et al*., 2020; Taengphu *et al*., 2022), RT-LAMP (Phusantisampan *et al*., 2019; Yin *et al*., 2019; Kampeera *et al*., 2021) and nanopore-based PCR amplicon sequencing (Delamare-Debouteville *et al*., 2021). However, most of these assays target a single genome segment, which may reduce diagnostic reliability

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when a probable genetic variation occurs in the primer-binding region.

Growing evidence indicates that TiLV undergoes frequent genetic reassortment, which is a major driver of its genomic diversity (Chaput *et al*., 2020; Thawornwattana *et al*., 2021; Verma *et al*., 2022). As a result, assays that amplify only one segment may fail to detect variants carrying mutations in that locus (Kembou *et al*., 2017; Dong *et al*., 2017; Waiyamitra *et al*., 2018; Yin *et al*., 2019). This problem has become more evident with reports of TiLV-negative fish that nevertheless display typical syncytial hepatitis, one of the hallmark lesions of TiLV infection indicating that the viral variants may escape detection due to mutations or reassortment in the targeted genome region (Taengphu *et al*., 2020).

These concerns highlight the need for diagnostic strategies that simultaneously target multiple conserved genomic regions to improve robustness and reduce the likelihood of false negatives. Therefore, the present study aimed to develop a mPCR assay targeting TiLV segments 2, 3 and 8. By amplifying three conserved regions in a single reaction, this multi-segment approach provides a more reliable and comprehensive detection tool for TiLV in tilapia aquaculture.

MATERIALS AND METHODS

Sample collection

Samples used in this study were obtained from a previously published TiLV surveillance investigation conducted across 15 tilapia farms in Tamil Nadu, India (Ponsrinivasan *et al*., 2025). The original surveillance included both clinically affected and apparently healthy fish, providing a broad representation of farm-level infection status. For the present study conducted during 2023 and 2024, a subset of TiLV-positive (n = 100) and TiLV-negative (n = 100) samples previously confirmed by semi-nested RT-PCR targeting segment 3 (Dong *et al*., 2017) was randomly selected ensuring unbiased representation of the broader dataset. All laboratory analyses in the present study were performed at the State Referral Laboratory for Aquatic Animal Health (AAH), Madhavaram, Chennai.

RNA extraction and cDNA synthesis

Total RNA was extracted from the eye, liver, brain, gills, kid-ney and spleen tissues using RNAiso Plus reagent (Takara Bio) according to the manufacturer's protocol. RNA quality and concentration were assessed using a Nanodrop ND-1000 spectrophotometer (Thermo Fischer, USA). The RNA was then transcribed to cDNA using a first-strand cDNA synthe-sis kit (QIAGEN, Germany, Thermo Scientific) and stored at -80°C.

Primer designing

Primers were designed to target conserved regions of TiLV genome segments 2, 3 and 8. Representative sequences from multiple countries (Table 1) were retrieved from GenBank and aligned using Clustal Omega (https://www.ebi.ac.uk/jdispatcher/msa/clustalo) to identify conserved regions. Primers were then designed using Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) using the following criteria: GC content: 40-60%, Melting temperature (Tm): 52-62°C, Low self-complementarity; avoidance of hairpins and dimers, High specificity to TiLV with no predicted cross-reactivity. The designed primers were examined for multiplex compatibility and synthesized by Eurofins Technologies (Bangalore, India). Primer

Table 1: TiLV genome sequences used for primer design and multiple sequence alignment (MSA).

Accession number	Location	Year (Collection)
Segment 2		
MZ493942	India	2021
MZ297924	India	2022
PP600916	India	2022
PP600910	India	2023
MK392373	Ecuador	2019
MN193524	USA	2019
ON376573	Vietnam	2020
ON376583	Vietnam	2020
MT466448	Bangladesh	2019
MT466458	Bangladesh	2019
MN687766	Thailand	2019
MH319379	Thailand	2019
Segment 3		
MZ493941	India	2021
PQ523715	India	2022
PP600911	India	2023
OL539822	Indonesia	2018
MK392374	Ecuador	2012
MN193525	USA	2019
MT466459	Bangladesh	2019
MN939374	Bangladesh	2019
MN687767	Thailand	2019
OL469275	Thailand	2019
OM417602	China	2021
KX631923	Thailand	2021
Segment 8		
OM469317	India	2020
MZ475949	India	2021
PP623819	India	2022
PP600913	India	2023
ON376589	Vietnam	2019
MN193530	USA	2019
MK425017	Peru	2018
MK392379	Ecuador	2012
MT466444	Bangladesh	2018
MT466454	Bangladesh	2019
MN687772	Thailand	2019
KX631928	Thailand	2016

sequences and corresponding amplicon sizes are listed in Table 2.

PCR amplification and optimization

PCR amplification was first optimized in uniplex format to evaluate the performance of each primer pair targeting TiLV genome segments 2, 3 and 8. Each 25 µL uniplex reaction contained 12.5 µL of 2× Red PCR Master Mix (Amplicon, Denmark), 1 µL each of forward and reverse primers (10 µM; final concentration 0.4 µM), 1 µL of cDNA template and nuclease-free water to a final volume of 25 µL. Thermal cycling conditions consisted of an initial denaturation at 95°C for 5 min, followed by 35 cycles of 95°C for 30 s, annealing at a 52-60°C gradient for 30 s and extension at 72°C for 45 s, with a final extension at 72°C for 5 min. Gradient optimization showed that 58°C was the most suitable annealing temperature across all three segments. This temperature produced strong, clear and distinct amplicons for segments 2, 3 and 8, eliminated non-specific bands and ensured balanced amplification efficiency among primer sets.

For mPCR, all three primer pairs were combined in a single 25 µL reaction using the same reagent composition as the uniplex assays, with each primer maintained at a final concentration of 0.4 µM. Cycling conditions were identical to the optimized uniplex protocol, using the standardized 58°C annealing temperature. PCR amplicons were resolved on 1.5% agarose gels prepared in 1× TAE buffer, stained with 0.5 µg/mL ethidium bromide and visualized using a Bio-Rad gel documentation system (Bio-Rad, Germany). Both uniplex and multiplex assays showed consistent and reproducible amplification profiles across three independent runs.

Analytical sensitivity and specificity

Analytical sensitivity was evaluated using 10-fold serial dilutions of TiLV-positive cDNA, starting from 100 ng per reaction. All assays were carried out in three independent runs in triplicates to ensure consistency and the detection limit was defined as the lowest dilution at which all three TiLV target segments produced visible amplicons. Gel densitometry was performed using ImageJ 1.x to objectively quantify band intensities (Schneider *et al*., 2012). Each PCR lane was selected with the rectangular ROI tool and lane profiles were generated using Analyse → Gels → Select First Lane/Select Next Lane → Plot Lanes. Peak detection was carried out on the resulting intensity profiles. A band was considered as detectable when a corresponding peak was present above baseline noise. Peak area (integrated optical density) and relative intensity were used to compare detection across template dilutions. This analysis allowed objective determination of the lowest template concentration at which at least one amplicon peak remained detectable.

Assay specificity was assessed using DNA/cDNA from non-TiLV aquatic viral and bacterial pathogens, such as Tilapia parvovirus, Lymphocystis disease virus, Carp edema virus, *Aeromonas veronii*, *Streptococcus agalactiae*, *Enterococcus* sp. and *Pseudomonas* sp. Specificity testing was also conducted in three independent runs in triplicates and no amplification was observed for any non-TiLV organisms.

Diagnostic accuracy assessment

Diagnostic performance of the mPCR assay was compared with the reference semi-nested RT-PCR method (Dong *et al*., 2017) using 200 field samples. Samples were classified as true positives, true negatives, false positives, or false negatives. Receiver Operating Characteristic (ROC) analysis was carried out in R version 4.3.0 using the pROC package (R Core Team, 2021). Band presence/absence (binary scoring) was used for diagnostic classification of the positive and negative samples. The area under the curve (AUC) was calculated to assess overall diagnostic accuracy.

RESULTS AND DISCUSSION

Sample validation

Tilapia Lake Virus (TiLV) has emerged as a major transboundary pathogen in tilapia aquaculture, with reports from several countries including Colombia, Chinese Taipei, Egypt, Thailand, Malaysia (Jansen *et al*., 2019). In India, TiLV has been recently confirmed in farmed tilapia, (Rao *et al*., 2024; Rebecca *et al*., 2025) underlining the urgent need for sensitive and reliable diagnostic tools to support surveillance and early intervention strategies. To address this issue, we conducted an on-field surveillance as part of our research work and collected samples with visual clinical signs of exophthalmia, skin hemorrhages, fin erosion and abdominal distension (Fig 1). Samples selected from the previous surveillance study (Ponsrinivasan *et al*., 2025)

Table 2: Primer sets developed for the mPCR assay targeting tilapia lake virus (TiLV) segments 2, 3 and 8.

Primer name	5′-3′ sequences	Product size
SEG2F	GCACATGTATGACGGGTTCC	724
SEG2R	CGTGCCTGGACTACATGAGA	
SEG3F	TGCCACATACGTTTGCTCTG	553
SEG3R	CTTTCCAGAGTCGCGCATGA	
SEG8F	CTCAACGGCATGACAGTGAC	331
SEG8R	CGACACAGTTAATCCCAGGG	



Fig 1: TiLV infected fish showing skin hemorrhages, exophthalmia, scale loss and fin erosion.

were reconfirmed using semi-nested RT-PCR targeting segment 3, yielding the expected 415 bp and 250 bp amplicons (Fig 2). This confirmation ensured that only verified positive and negative samples were used for subsequent assay validation.

PCR optimization

Uniplex PCR assays were performed to evaluate the performance of primers targeting segments 2, 3 and 8 across annealing temperatures from 52°C to 60°C. Each primer pairs produced clear amplicons of 724 bp, 553 bp and 331 bp, respectively (Fig 3). The amplification profiles were reproducible across three independent runs, with each primer pair yielding amplicons of identical size with no nonspecific bands. Gradient analysis showed that 58°C yielded the strongest, most distinct bands for all three segments and this temperature was selected for multiplex optimization. Using the optimized annealing temperature of 58°C, the mPCR successfully amplified all three segments simultaneously, generating distinct bands of the expected sizes without cross-reactivity or primer interference (Fig 4). The ability to amplify three conserved genomic regions in a single reaction demonstrates the robust primer compatibility and enhances diagnostic confidence by reducing the risk of false negatives.

The choice of these segments was guided by their diagnostic and evolutionary relevance, as segment 2 is evolutionarily stable and less prone to reassortment, making it a reliable diagnostic marker (Chaput *et al*., 2020). Segment 3 has been widely used in TiLV detection and phylogenetic studies (Verma *et al*., 2022; Tattiyapong *et al*., 2018; Waiyamitra *et al*., 2018; Taengphu *et al*., 2022), while segment 8 is highly conserved with minimal reassortment (Thawornwattana *et al*., 2021). Notably, studies on TiLV genome dynamics indicate that reassortment is a dominant evolutionary force, highlighting the importance of multi-target assays to capture genetic diversity and ensure reliable detection (Verma *et al*., 2022; Tran *et al*., 2022). By targeting a combination of segments with different evolutionary stability, the assay enhances detection accuracy across genetically diverse isolates and reduces the risk of false negatives caused by mutations or segment reassortment.

Sensitivity and specificity

The analytical sensitivity of the optimized mPCR was evaluated using serial ten-fold dilutions of TiLV template cDNA ranging from 100 (nanogram) ng to 10 pg. It revealed that the mPCR could detect TiLV concentrations as low as 100pg/µL (Fig 5). Sensitivity testing was repeated across three independent runs and the detection limit remained consistent, confirming assay reliability. To provide an objective measure of band detectability, densitometric peak analysis was performed in ImageJ. Lane profiling produced three clear peaks corresponding to the multiplex amplicons in the 100 ng, 10 ng and 1 ng lanes. At 100 pg, a two small peak remained detectable, while the remaining target fell below the peak detection threshold. No measurable peaks were detected in the 10 pg reaction, consistent with the absence of visible bands on the gel (Fig 6). Together, these objective measurements establish an analytical sensitivity of 100 pg for the mPCR assay, with no detectable amplification observed at 10 pg. Although primer competition contributed to reduced band intensity at low template concentrations, detection across all targets indicates the assay's suitability for surveillance and early-stage infection detection.

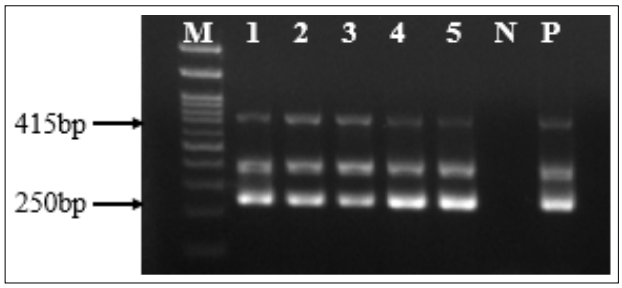


Fig 2: Semi-nested RT-PCR technique targeting the segment 3 of different isolates for detection of TiLV.

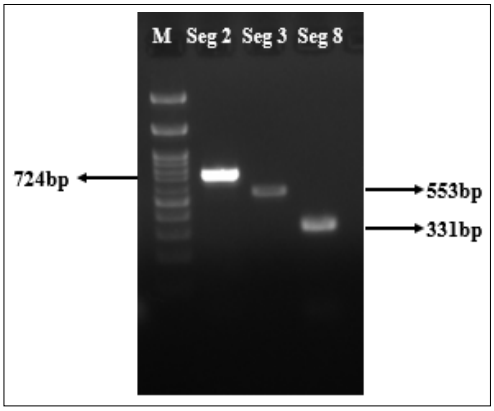


Fig 3: Uniplex PCR showing distinctive bands at respective bases-Segment 2 (724 bp), Segment 3-(553 bp), Segment 8-(331 bp).

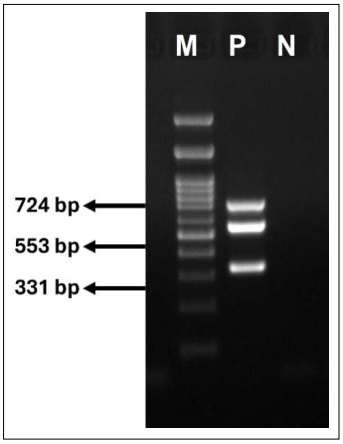


Fig 4: Optimized mPCR assay amplifying all three target segments within a single reaction.

Most published TiLV assays report detection limits in viral copy number, whereas our study reports sensitivity in template mass (100 pg/µL of cDNA). Because these units are not directly comparable without copy-number calibration, direct numerical comparison is not possible. Nested and semi-nested RT-PCR assays have demonstrated low-copy detection limits, including ~7 copies per reaction (Dong *et al*., 2017; Kembou *et al*., 2017). qRT-PCR assays remain the most sensitive, detecting as few as 2-62 copies per reaction (Tattiyapong *et al*., 2018; Megarani *et al*., 2022; Chengula *et al*., 2022), while iron-flocculation-coupled qPCR detects ~10 copies/µL in environmental samples (Taengphu *et al*., 2022). In contrast, the mPCR described here emphasizes diagnostic robustness through multi-segment detection rather than maximal analytical sensitivity, reducing the likelihood of false negatives due to segment-specific mutations or reassortment.

DNA from non-TiLV aquatic pathogens including Tilapia parvovirus, Lymphocystis disease virus, Carp edema virus, *Aeromonas veronii*, *Streptococcus agalactiae*, *Enterococcus* sp. and *Pseudomonas* sp. did not yield any amplification (Fig 7). Specificity testing was conducted in three independent runs for each pathogen and all runs produced identical results. These findings confirm that the assay specifically targets TiLV without cross-amplification of co-infecting bacterial or viral pathogens that commonly affect tilapia in aquaculture systems.

ROC curve

Diagnostic performance was evaluated by comparing the developed mPCR with the semi-nested RT PCR assay of Dong *et al*. (2017) using 200 field samples (TiLV-positive and TiLV-negative). ROC curve analysis revealed an AUC value of 1.0, indicating perfect concordance with the reference method and demonstrating 100% diagnostic sensitivity (Fig 8). A reaction was scored positive only if a band appeared at the expected size and exceeded background noise under uniform exposure conditions. While this high AUC demonstrates excellent diagnostic discrimination, we acknowledge that perfect performance likely reflects the well-characterized nature of the validation set. Additional testing on a broader panel of field samples and diverse TiLV genotypes would further substantiate assay robustness.

Application and limitations

The developed mPCR provides a balanced compromise between sensitivity, specificity and operational accessibility. While qRT-PCR remains the most analytically sensitive method but requires real-time platforms, fluorescent chemistries and skilled personnel limiting its use in many routine diagnostic settings. LAMP assays are rapid and equipment-light but vulnerable to nonspecific amplification and aerosol contamination if strict workflow controls are not followed. This risk is particularly pronounced in non-segregated workspaces or high-template environments where aerosolised product can seed unintended amplification and lead to false positives. Although the mPCR requires a thermocycler, electrophoresis system and gel documentation unit, it is more accessible than qRT-PCR and more robust than single-segment PCR assays, particularly in the context of TiLV genome reassortment. Therefore, the assay is most

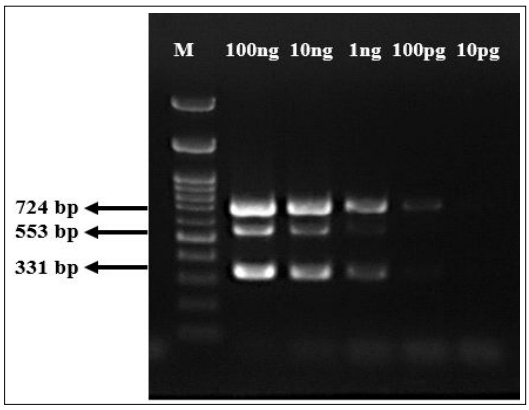
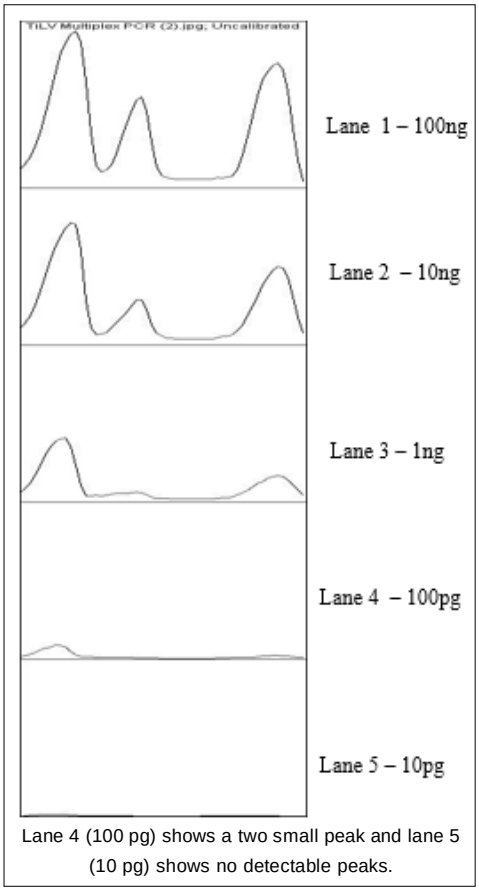


Fig 5: Analytical sensitivity of the TiLV mPCR assay showing reliable amplification of TiLV genome segments 2 (724 bp), 3 (553 bp) and 8 (331 bp) down to 100 pg.



Lane 4 (100 pg) shows a two small peak and lane 5 (10 pg) shows no detectable peaks.

Fig 6: Image J lane intensity plots of the sensitivity analysis gel showing three peaks are visible in lanes containing 100 ng, 10 ng and 1 ng.

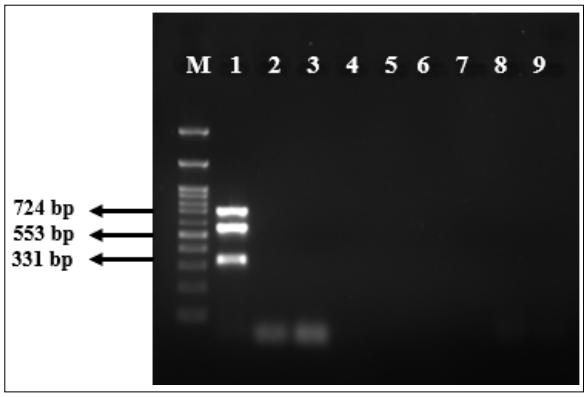


Fig 7: Specificity analysis of the optimized Lane 1-TiLV, Lane 2-Tilapia parvo virus, Lane 3-Carp edema virus, Lane 4 Lymphocystis disease virus, Lane 5-*Aeromonas veronii*, Lane 6-*Streptococcus agalactiae*, Lane 7-*Enterococcus* sp., Lane 8-*Pseudomonas* sp., Lane 9-Negative control.

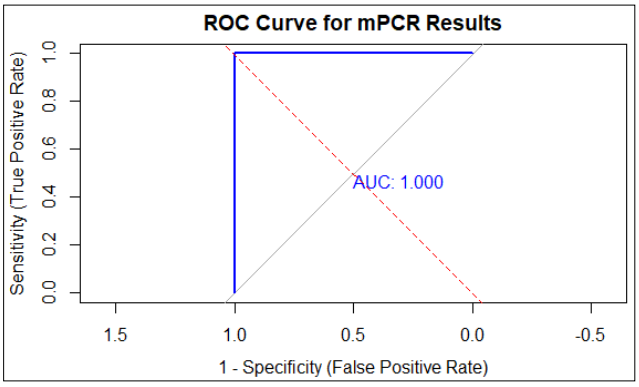


Fig 8: Receiver operating characteristic (ROC) curve for the mPCR assay.

suitable for regional diagnostic facilities rather than on-farm use and its practical role is within surveillance networks where farms can submit samples to nearby laboratories for routine screening.

Targeting three conserved segments may reduce the risk of false negatives, yet it is also important to know that highly divergent TiLV variants could still potentially affect primer binding. Broader validation and periodical update to primer sets using isolates from different regions and genetic backgrounds will be important for confirming its robustness. The assay was validated using tissue-derived cDNA from clinically affected fish, particularly from ponds experiencing clinical signs and mortality, where viral loads are expected to be sufficient for reliable detection; its application low-viral-load samples or alternative matrices warrants future evaluation.

CONCLUSION

The optimized mPCR assay targeting TiLV segments 2, 3 and 8 represents a reliable, sensitive and specific diagnostic platform. By addressing genomic variability and minimizing false negatives, it enhances detection accuracy and provides a robust tool for effective TiLV surveillance and disease management in tilapia aquaculture. Given the ongoing global threat posed by TiLV, the adoption of this multi-segment diagnostic strategy has the potential to significantly strengthen disease management frameworks, safeguard production and support the sustainability of tilapia aquaculture.

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Disclaimers

The views and conclusions expressed in this article are solely those of the authors and do not necessarily represent the views of their affiliated institutions. The authors are responsible for the accuracy and completeness of the information provided, but do not accept any liability for any direct or indirect losses resulting from the use of this content.

Informed consent

All animal procedures for experiments were approved by the Committee of Experimental Animal Care and Handling Techniques were approved by the University of Animal Care Committee.

Conflict of interest

The authors declare that there are no conflicts of interest regarding the publication of this article. No funding or sponsorship influenced the design of the study, data collection, analysis, decision to publish, or preparation of the manuscript.

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