Original Article

Preliminary development of visual and real-time loop-mediated isothermal amplification assays for *Megalocytivirus* detection in Betta fish (*Betta splendens*)

Kitikarn Sakuna[1*] Supaporn Noochu[1] Kittichon U-Taynapun[2]

Nion Chirapongsathonkul[2] Saransiri Nuanmanee[3]

Abstract

This study developed rapid detection of *Megalocytivirus* using visual and real-time loop-mediated isothermal amplification (LAMP) assays. The primer was designed with the major capsid protein gene of *Megalocytivirus,* including red sea bream iridovirus (RSIV), infectious spleen and kidney necrosis virus (ISKNV), and turbot reddish body iridovirus (TRBIV). The visual and real-time LAMP assays were evaluated using DNA extracted from symptomatic betta fish that tested negative for *Megalocytivirus* by the broad-range PCR assay, as well as a chemically synthesized *Megalocytivirus* DNA template. Visual LAMP was found sensitive enough to detect up to 10 pg per reaction of the target DNA within 30 min. Real-time LAMP efficiently detected 10 ng per reaction of the target DNA in around 10 min. Compared with the broad-range PCR assay, the novel assays demonstrated 100% positive percent agreement when tested with the chemically synthesized *Megalocytivirus* DNA template and 100% negative percent agreement when evaluated using DNA extracted from symptomatic betta fish that tested negative for *Megalocytivirus*. The developed LAMP assays when combined with a fast DNA extraction method, could facilitate on-site *Megalocytivirus* screening.

Keywords: betta fish, LAMP assay, *Megalocytivirus*, portable *Megalocytivirus* screening, rapid detection, real-time LAMP assay

[1]*Faculty of Veterinary Science, Rajamangala University of Technology Srivijaya, Nakhon Si Thammarat 80240, Thailand*
[2]*Faculty of Agiculture, Rajamangala University of Technology Srivijaya, Nakhon Si Thammarat 80240, Thailand*
[3]*Songkhla Aquatic Animal Health Research and Development Center, Songkhla 90100, Thailand*
***Correspondence**: kitikarn.s@rmutsv.ac.th* (K. Sakuna)
Received November 10, 2025
Accepted January 13, 2025
https://doi.org/10.56808/2985-1130.3964

Introduction

Betta fish (*Betta splendens*) is one of the most commercially exported ornamental fish species in Thailand and other Southeast Asian countries (Sermwatanakul, 2019; Malawa *et al*., 2023). However, the strict infectious disease requirements imposed by importing countries remain a major obstacle to the expansion of the betta export market. *Megalocytivirus*, a genus within the Iridoviridae family, has become increasingly widespread through the international movement of freshwater ornamental fish such as swordtail (Xiphophorus helleri), molly (Poecilia sphenops), and three-spot gourami (Trichopodus trichopterus) (Go *et al*., 2006; Joon *et al*., 2008; Zainathan *et al*., 2017). This virus poses a substantial biosecurity threat due to its broad host range and pathogenicity, highlighting the risks associated with global ornamental fish trade.

The detection of *Megalocytivirus* in various ornamental fish species, including betta fish intercepted during border quarantine, has been documented (Nolan *et al*., 2015). Although most infected betta fish are asymptomatic, some may exhibit non-specific clinical signs such as hemorrhages, loss of body coloration, pale gills, skin darkening, lethargy, and inappetence, usually without significant mortality (Zainathan *et al*., 2017; Baoprasertkul and Kaenchan, 2019). The lack of specific clinical signs makes *Megalocytivirus* difficult for farmers to detect, resulting in asymptomatic, infected fish being exported during the carrier stage.

Current international efforts to prevent *Megalocytivirus* transmission rely heavily on early diagnostic screening during quarantine in importing countries. However, long-term sustainability for exporting countries depends on developing farming systems capable of producing *Megalocytivirus*-specific pathogen–free (SPF) fish. Establishing such SPF systems requires reliable diagnostic methods to screen and exclude infected fish at the farm level.

Several nucleic acid amplification methods have been developed for the detection of one or two *Megalocytivirus* genotypes, including red sea bream iridovirus (RSIV), infectious spleen and kidney necrosis virus (ISKNV), and turbot reddish body iridovirus (TRBIV). These include conventional PCR (Nolan *et al*., 2015), SYBR Green real-time PCR (Gias *et al*., 2011; Go *et al*., 2016), and TaqMan real-time PCR (Mohr *et al*., 2015).

Loop-mediated isothermal amplification (LAMP) has become an attractive diagnostic tool in aquaculture (Sakuna *et al*., 2018; Fei *et al*., 2023) because it amplifies nucleic acids at a constant temperature using a strand-displacing polymerase and four to six primers targeting multiple regions of the gene. Amplification is typically completed within 30 to 60 minutes. Advantages of LAMP include the lack of requirement for a thermocycler, rapid turnaround time, high amplification efficiency and sensitivity, and suitability for simple visual detection using colorimetric dyes. However, LAMP also has limitations: primer design is more complex, it is more prone to non-specific amplification if primers are suboptimal, and quantification is less precise than real-time PCR. In addition, the interpretation of visual assay results can vary depending on the detection format employed. LAMP has been successfully applied for the rapid detection of ISKNV and TRBIV (Zhang *et al*., 2009; Subramaniam *et al*., 2014).

Although infection with RSIV has not been reported in betta fish, ISKNV has been documented in betta fish in Thailand (Baoprasertkul and Kaenchan, 2019). The global ornamental fish trade may facilitate the spread of *Megalocytivirus* and enable disease emergence in new host species across distant regions. Do *et al*. (2004) reported that epizootic rock bream iridovirus (RBIV) is closely related to RSIV, grouper sleepy disease iridovirus (GSDIV), dwarf gourami iridovirus (DGIV), and ISKNV, underscoring the potential for cross-species transmission. Therefore, developing diagnostic methods capable of detecting all three *Megalocytivirus* genotypes is essential.

To date, the conventional PCR assay described by Kurita and Nakajima (2012) is the only method reported to detect all three *Megalocytivirus* genotypes. Compared with conventional PCR, LAMP-based diagnostics offer better practicality for use at farms and during border quarantine. In this study, preliminary development of real-time and visual LAMP assays targeting the major capsid protein (MCP) gene were established to enable detection of all three *Megalocytivirus* genotypes.

Materials and Methods

Primer design for LAMP assays: The LAMP primers targeting the MCP genes of RSIV, ISKNV, and TRBIV were designed using the NEB LAMP Primer Design Tool (New England Biolabs; https://lamp.neb.com /#!/). The primer sequences are listed in Table 1.

Preparation of virus and viral DNA templates: When pathogen-positive samples are unavailable, synthetic DNA or RNA standards have been shown to be effective substitutes (Martínez-Martínez *et al*., 2011; Lima *et al*., 2017; Morfin *et al*., 2023). For this study, a *Megalocytivirus* DNA template was chemically synthesized by GenScript Biotech Co., Ltd. (Nanjing, China) based on conserved regions of the MCP gene from RSIV, ISKNV, and TRBIV sequences retrieved from the GenBank database of the National Center for Biotechnology Information (NCBI), including RSIV (GenBank accession no. AB080362.1), ISKNV (GenBank accession no. AAL98730.1), and TRBIV (GenBank accession no. AY590687.2). Conserved regions were identified using Sequencher version 5.0 (Gene Codes Corporation, Ann Arbor, MI, USA). The synthesized fragment contained the MCP target sequence used in the LAMP assays.

Table 1 Loop-mediated isothermal amplification (LAMP) primers designed from the MCP gene of *Megalocytivirus*.

Primer	Sequence (5′-3′)
FIP	TGCGGTGGGTGACGTTCTTTACCTTGGTGCATCTGGACCTC
BIP	ACGTGCAAAGCAATTACACCGCGCAAAGGCAGATTCACCTTG
F3	GTCACACCCGCAGACAAT
B3	ACCTCGGACAGGGGATTG
LF	TCACGGGGTGACTGAACCT
LB	GGCCAGTCCCGTGTATGTCA

Specificity determination of the visual and real-time LAMP assays: For the specificity evaluation, eighteen DNA samples were randomly selected from 150 symptomatic betta fish that had previously tested negative for *Megalocytivirus* by the broad PCR assay (Kurita and Nakajima, 2012). In the absence of positive *Megalocytivirus* field samples, assay specificity was further assessed using a chemically synthetic *Megalocytivirus* DNA construct as a positive control. Three betta types were examined (Fig. 1C) wild betta (W), shortfin betta (SF), and longfin betta (LF). For each type, three males and three females were included in the analysis, resulting in a total of 18 fish (n=18). The sample size was intentionally limited to avoid an overrepresentation of true-negative samples, which could artificially influence preliminary assay performance (Riley *et al*., 2020). Each 25 µL LAMP reaction mixture contained 1.6 µM each of the forward inner primer (FIP) and backward inner primer (BIP), 0.2 µM each of the forward outer primer (F3) and backward outer primer (B3), and 0.4 µM of the backward loop primer (LB), along with 10 ng of DNA from symptomatic betta fish or 10 pg of the synthetic *Megalocytivirus* DNA template. The visual LAMP assay was carried out at 59°C for 30 min using the WarmStart Colorimetric LAMP 2X Master Mix (New England Biolabs, Ipswich, MA, USA), with reactions incubated in a Mini Heating Dry Bath Incubator (Major Science, New Taipei City, Taiwan). The real-time LAMP assays were performed at 59°C for 60 min (according to the manufacturer's instructions), with fluorescence acquisition every 60 s. Additional steps for melt-curve analysis were carried out under the following conditions: 95°C for 1 min, 40°C for 1 min, 70°C for 1 min, and a temperature increase of 0.5°C per 1 s up to 95°C (Caruso *et al*., 2024). All real-time LAMP reactions were performed using the WarmStart Fluorescent LAMP/RT-LAMP Kit (New England Biolabs, Ipswich, MA, USA) on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA). The optimal cutoff value for the positive detection of *Megalocytivirus* using the real-time LAMP quantification cycle (Cq) was determined through receiver operating characteristic (ROC) curve analysis based on 100 fg of synthetic *Megalocytivirus* DNA template and a no-template control. The ROC model yielded an area under the curve (AUC) of 0.900. Based on Youden's J statistic, the optimal cutoff value was 30.94, corresponding to a sensitivity of 0.80 and a specificity of 1.00.

Sensitivity determination of the visual and real-time LAMP assays: The analytical sensitivities of the newly developed real-time and visual LAMP assays were evaluated using a ten-fold serial dilution of DNA template, prepared from an initial concentration of 10 ng down to 10 fg per reaction. Serial dilutions were generated by sequentially diluting the DNA template in nuclease-free water, with thorough mixing at each step, and aliquots of each dilution were used as templates for LAMP amplification. All assays were performed in triplicate at each concentration. For the visual LAMP assay, reaction mixtures were incubated at 59°C for 30 min using the WarmStart Colorimetric LAMP 2X Master Mix (New England Biolabs, Ipswich, MA, USA), with incubation carried out in a Mini Heating Dry Bath Incubator (Major Science, New Taipei City, Taiwan). For the real-time LAMP assay, reactions were incubated at 59°C for 60 min using the WarmStart Fluorescent LAMP/RT-LAMP Kit (New England Biolabs, Ipswich, MA, USA) on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA), followed by a melt curve analysis as described previously.

Comparison of LAMP assays with broad Megalocytivirus PCR: A broad *Megalocytivirus* PCR assay for detecting RSIV, ISKNV, and TRBIV was conducted as previously described by Kurita and Nakajima (2012). To assess diagnostic sensitivity, a ten-fold dilution series ranging from 10 ng to 10 fg per reaction was prepared, whereas to assess diagnostic specificity, 10 pg of the synthetic *Megalocytivirus* DNA template and 10 ng of DNA from symptomatic betta fish representing all three betta types that had tested negative for *Megalocytivirus* by the broad PCR assay were used. The assays were performed independently in triplicate. All PCR reactions were carried out using OneTaq® 2X Master Mix (New England Biolabs, Ipswich, MA, USA) on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA) under the following conditions: 94°C for 30 s, followed by 30 cycles of denaturation at 94°C for 30 s and annealing/extension at 60°C for 1 min. The expected 777 bp PCR product was resolved on a 1.8% agarose gel stained with GelRed™ (Biotium, Fremont, CA, USA) and visualized using a Syngene InGenius LHR Gel Documentation System (Syngene International Ltd., Bangalore, India). Diagnostic performance was assessed by calculating true positives (TP), false positives (FP), false negatives (FN), and true negatives (TN). Sensitivity was calculated as [TP / (TP + FN)] × 100, specificity as [TN / (TN + FP)] × 100, and accuracy as [(TP + TN) / (TP + TN + FN + FP)] × 100 (Abramson and Abramson, 2009).

Results

Specificity of the visual and real-time LAMP assays: The specificities of the real-time and visual LAMP assays were shown in Fig. 1 and Supplementary Table S1. The reaction systems with the genomic DNA of

Megalocytivirus had exponential amplification, and the color of the reaction systems turned from pink to orange or yellow (the solution becomes acidic from released protons), while the other reaction systems had no exponential amplification or color change. Therefore, both the real-time and visual LAMP assays demonstrated specificity for *Megalocytivirus*, with amplification observed only for *Megalocytivirus* DNA. However, this specificity assessment was based on a limited number of samples and relied in part on synthetic DNA, which may have contributed to the high observed specificity.

Sensitivity of the visual and real-time LAMP assays: The sensitivities of the real-time and visual LAMP assays using a series of ten-fold dilutions of *Megalocytivirus* DNA template at 59°C were shown in Fig. 2 and Supplementary Table S2. The detection limit of the real-time LAMP was found to be 1 pg of *Megalocytivirus* DNA template. While the limit of the visual LAMP was found to be 10 pg, which was tenfold lower than the real-time LAMP assay. In Fig. 2C, three horizontal threshold lines (green: baseline; pink: peak-calling; blue: noise-filter) represent software-defined reference levels used to classify melt peaks as true amplification products. All melt peaks in this study exceeded these thresholds, confirming the high specificity of the LAMP assay.

Comparison of the LAMP assays with a broad Megalocytivirus PCR: The detection limit of a broad *Megalocytivirus* PCR assay was found to be 1 pg, similar to the limitation of the real-time LAMP (Fig. S1), but with the visual LAMP assay being a little less sensitive. In a comparison of LAMP assays with a broad *Megalocytivirus* PCR, the specificities were the same. Both the real-time and visual LAMP assays gave negative results when the PCR assay gave negative results. The statistical diagnostic performance was shown in Table 2. The novel real-time LAMP assay had 100% accuracy compared with the PCR assay when the visual LAMP assay had 92.9% accuracy.

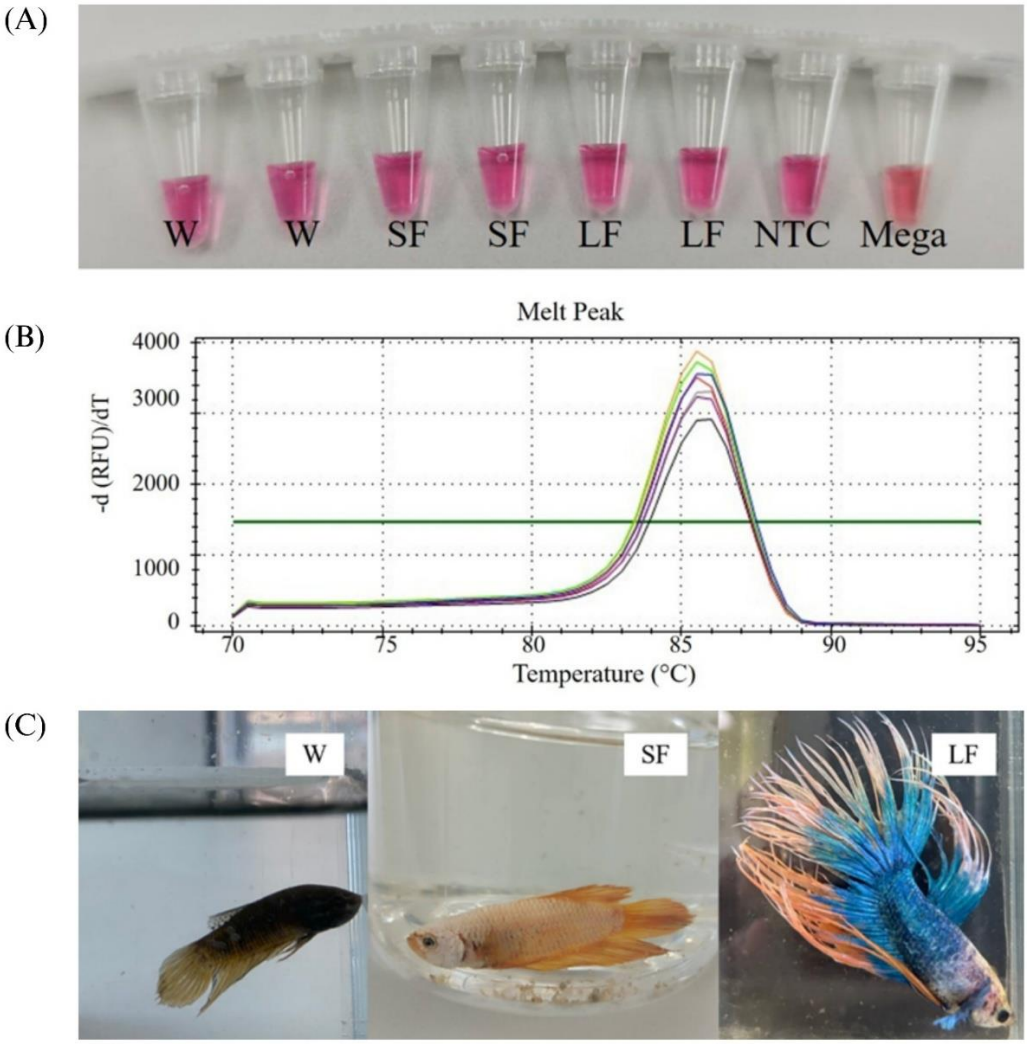

Figure 1 Specificity determination (A) Visual detection by colorimetric LAMP at 30 min. Positive reaction is indicated by turning to orange or yellow from pink. (B) Melt peak of amplified the genomic DNA of *Megalocytivirus* (10 pg). The horizontal green lines represent the baseline. (C) Representative images of symptomatic individuals from three betta fish types included in the study: wild betta (W), shortfin betta (SF), and longfin betta (LF), illustrating the nonspecific clinical signs such as loss of body coloration, skin darkening, and inappetence.
NTC: no-template control; Mega: 10 pg of *Megalocytivirus* DNA template.

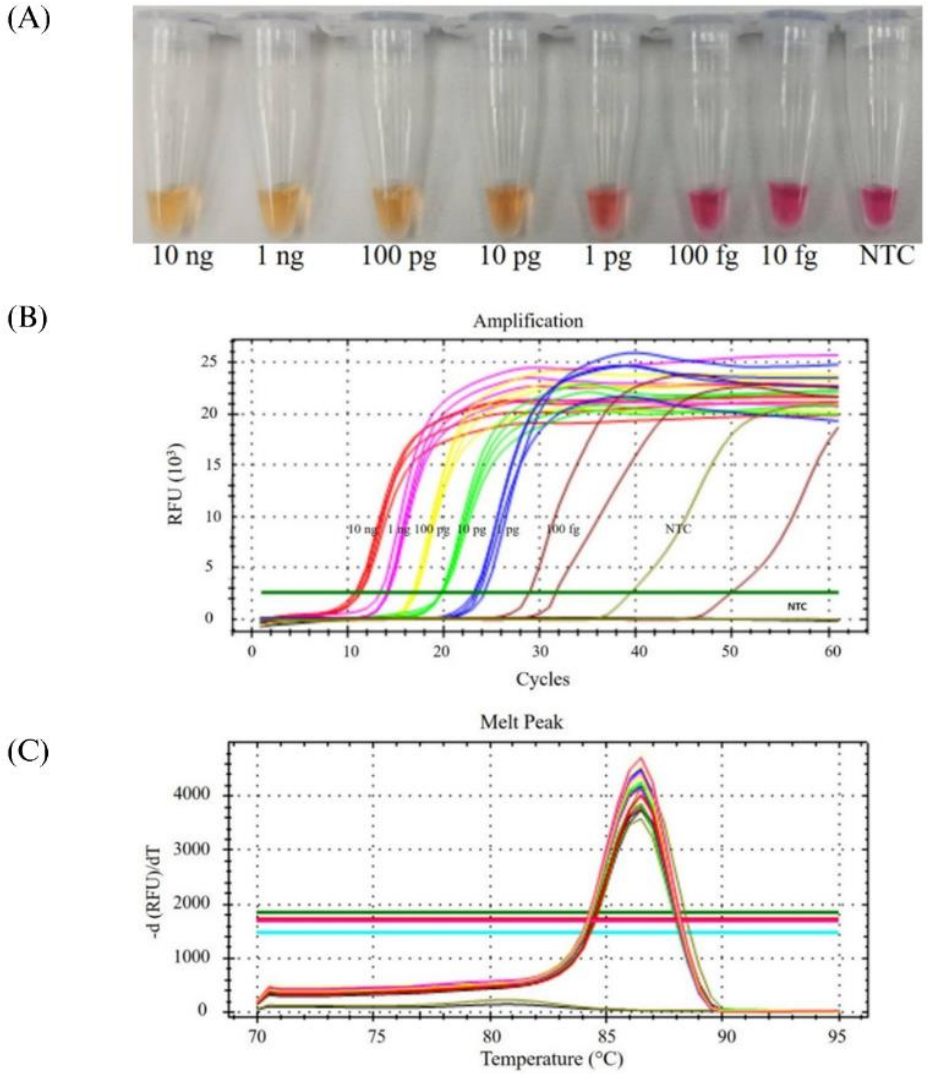

Figure 2 Sensitivity determination (A) Visual LAMP at 30 min. Positive reaction is indicated by turning to orange or yellow from pink. (B) Amplification results of the genomic DNA of *Megalocytivirus*, red color: 10 ng; pink color: 1 ng; yellow color: 100 pg; green color: 10 pg; blue color: 1 pg; brown color: 100 fg; black color: 10 fg; olive green color: no-template control. (C) Melt peak of amplified the genomic DNA of *Megalocytivirus*. Three horizontal threshold lines are included: green representing the baseline, pink representing the peak-calling threshold, and blue representing the noise-filter threshold.

Table 2 Diagnostic performance of visual and real-time LAMP assays using synthetic *Megalocytivirus* DNA and *Megalocytivirus*-negative betta fish samples.

		PCR assay	
		Positive	**Negative**
Visual LAMP assay	Positive	16(TP)	0 (FP)
	Negative	3 (FN)	23 (TN)
	Sensitivity	84.2%	
	Specificity	100%	
	Accuracy	92.9%	
Real-time LAMP assay	Positive	19 (TP)	0 (FP)
	Negative	0 (FN)	23 (TN)
	Sensitivity	100%	
	Specificity	100%	
	Accuracy	100%	

*The assays were performed independently three times; TP, true positive; FP, false positive; FN, false negative; TN, true negative; sensitivity = [TP/(TP + FN)]*100; specificity = [TP/(TN + FP)]*100; and accuracy = [(TP + TN)/(TP + TN + FN + FP)]*100.

Discussion

Megalocytivirus, members of the family Iridoviridae, are important pathogens responsible for high mortality in both ornamental and aquaculture fish species. Because early-stage infections often present with mild or no clinical signs, rapid and reliable diagnostic tools are essential to prevent farm-level outbreaks. Compared with conventional PCR, LAMP assays are highly practical for aquaculture settings due to their isothermal amplification, short turnaround time, and minimal equipment requirements. Visual LAMP further enables straightforward result interpretation without specialized instruments, making it particularly suitable for on-site screening and early disease control in fish farms.

In this study, visual and real-time LAMP assays targeting the MCP gene were developed and evaluated for the detection of all three recognized *Megalocytivirus* genotypes: RSIV, ISKNV, and TRBIV. Conserved regions of the MCP gene were identified using NCBI database sequences and subsequently synthesized for template preparation.

The diagnostic specificity of both assays when applied to clinical samples was comparable to that of the broad *Megalocytivirus* PCR assay described by Kurita and Nakajima (2012). Both the visual and real-time LAMP assays demonstrated high analytical specificity, distinguishing *Megalocytivirus* from other non-target pathogens and host genomic DNA, even when tested on clinically diseased fish exhibiting nonspecific signs. Nonspecific amplifications or contamination-derived false positives were not observed, even when the reaction was performed at a relatively low temperature (59°C). This contrasts with previous studies reporting non-specific amplification at 63°C, which required increasing the temperature to 65°C to eliminate non-specific or contamination-derived false positives (Liu *et al*., 2017). Validation using clinical samples is essential. We therefore attempted to obtain positive samples from other ornamental fish and tested 60 individuals, including 30 guppies (Poecilia reticulata) and 30 sailfin mollies (Poecilia latipinna). All samples were negative by broad-range *Megalocytivirus* PCR. Consequently, assay evaluation was performed using a synthetic *Megalocytivirus* DNA construct as a surrogate positive control, which we acknowledge as a limitation of this study.

The detection limits of the visual and real-time LAMP assays were 10 pg (10^7 copies of viral DNA) and 1 pg (10^6 copies), respectively, when evaluated against the broad-range *Megalocytivirus* PCR assay. Copy numbers were calculated based on the molecular weight of the chemically synthesized 791 bp double-stranded DNA fragment, assuming an average mass of 660 Da per base pair. Using this approach, 1 ng of the fragment corresponds to approximately 1.15×10^9 copies, with proportional values of approximately 1.15×10^7 copies for 10 pg and 1.15×10^6 copies for 1 pg, thereby confirming the accuracy of the reported detection limits. This demonstrates that the real-time LAMP assay exhibits a ten-fold higher analytical sensitivity than the visual LAMP assay, likely due to its fluorescence-based detection system, which allows earlier and more reliable discrimination of low-level amplification signals. In contrast, the visual LAMP assay relies on colorimetric change, which typically requires higher template concentrations to produce a detectable signal. Despite this difference in sensitivity, both assays maintained comparable analytical specificity. The real-time LAMP assay is therefore more suitable for applications requiring detection of lower viral loads, whereas the visual LAMP assay provides a simpler, equipment-free alternative that remains highly reliable for field-level screening.

Although the detection limits obtained in this study were higher than those previously reported for MCP-based real-time LAMP for ISKNV by Subramaniam *et al*. (2014) (10^4 copies), the assays developed here offer broader diagnostic capability because they can detect both ISKNV and TRBIV. Furthermore, the detection threshold of 10^7 copies aligns with the viral load commonly observed in fish following intraperitoneal challenge with cell culture–derived ISKNV (Becker *et al*., 2025). However, the limitation of sample size of the present study does not permit robust estimation of diagnostic sensitivity or specificity, and further large-scale field studies will be required for full clinical validation.

A realistic on-farm or import-country quarantine program would involve non-lethal sampling followed by testing with either the visual or real-time LAMP assay. Only betta fish that consistently test negative would proceed for broodstock selection or be cleared through quarantine. When paired with rapid on-site extraction methods, such as magnetic bead–based extraction (Farani *et al*., 2025), these LAMP assays have the potential to significantly improve *Megalocytivirus* screening efficiency across the ornamental fish industry.

In summary, visual and real-time LAMP assays targeting the MCP gene were successfully developed for the detection of all three recognized *Megalocytivirus* genotypes. Both assays demonstrated high analytical specificity and reliable discrimination of *Megalocytivirus* from non-target pathogens and host DNA, while the real-time LAMP assay showed greater analytical sensitivity than the visual format. Although validation using naturally infected clinical samples was limited by sample availability and required the use of synthetic DNA, the assays remain applicable to routine field screening. Future studies incorporating a larger number of naturally infected clinical samples are warranted to evaluate diagnostic sensitivity, specificity, and field performance under practical field conditions. These LAMP-based methods provide practical diagnostic tools for early detection and surveillance of *Megalocytivirus* in betta fish, supporting improved disease management in aquaculture and quarantine settings.

Acknowledgements

This work was supported by the Fundamental Fund" (FF) 2024 (Projects 192777) and Rajamangala University of Technology Srivijaya, Thailand.

References

Abramson JH and Abramson ZH 2009. Making Sense of Data: A Self-Instruction Manual on the Interpretation of Epidemiological Data. 3rd ed. New York: Oxford Academic.

Baoprasertkul P and Kaenchan N 2019. Distribution and detection of *Megalocytivirus* in ornamental fish in Thailand. J Fish Environ. 43: 11–24.

Becker JA, Hick PM, Megarani D, Siler H, Pierezan F, Gray SN and Subramaniam K 2025. Risk of spread of *Megalocytivirus* pagrus1 (Infectious spleen and kidney necrosis virus) from frozen fillets. J Fish Dis. 21: e70086.

Caruso AG, Ragona A, Agrò G, Bertacca S, Yahyaoui E, Galipienso L and Davino S 2024. Rapid detection of tomato spotted wilt virus by real-time RT-LAMP and in-field application. J Plant Pathol. 106: 697–712.

Chen F, Sun J, Han Z, Yang X, Xian J-a, Lv A and Shi H 2019. Isolation, identification and characteristics of Aeromonas veronii from diseased crucian carp (Carassius auratus gibelio). Front Microbiol. 10: 2742.

Do JW, Moon CH, Kim HJ, Ko MS, Kim SB, Son JH and Park JW 2004. Complete genomic DNA sequence of rock bream iridovirus. Virology 325: 351–363.

Farani PS, Lopez J, Faier-Pereira A, Hasslocher-Moreno AM, Almeida IC and Moreira OC 2025. Utility of magnetic bead-based automated DNA extraction to improve Chagas disease molecular diagnosis. Int J Mol Sci. 26: 937.

Fei Y, Wu Z, Shen K, Xue H, Wang S, Yao L and Wang L 2023. A contamination-free and visual method using LAMP and molecular beacon for sensitive and specific detection of rainbow trout (Oncorhynchus mykiss). Food Control. 154: 110014.

Gias E, Johnston C, Keeling S, Spence RP and Mcdonald WL 2011. Development of real-time PCR assays for detection of *megalocytivirus*es in imported ornamental fish. J Fish Dis. 34: 609–618.

Go J, Lancaster M, Deece K, Dhungyel O and Whittington R 2006. The molecular epidemiology of iridovirus in Murray cod (Maccullochella peelii peelii) and dwarf gourami (Colisa lalia) from distant biogeographical regions suggests a link between trade in ornamental fish and emerging iridoviral diseases. Mol Cell Probes. 20: 212–222.

Go J, Waltzek TB, Subramaniam K, Yun SC, Groff JM, Anderson IG, Chong R, Shirley I, Schuh JC, Handlinger JH, Tweedie A and Whittington RJ 2016. Detection of infectious spleen and kidney necrosis virus (ISKNV) and turbot reddish body iridovirus (TRBIV) from archival ornamental fish samples. Dis Aquat Organ. 122: 105–123.

Joon BJ, Ho YK, Lyu JJ, Ji HL, Nam GP, Joong KK and Hyun DJ 2008. Outbreaks and risks of infectious spleen and kidney necrosis virus disease in freshwater ornamental fishes. Dis Aquat Organ. 78: 209–215.

Kurita J and Nakajima K 2012. *Megalocytivirus*es. Viruses 4: 521–538.

Lima LR, Silva AP, Schmidt-Chanasit J and Paula VS 2017. Diagnosis of human herpes virus 1 and 2 (HHV-1 and HHV-2): use of a synthetic standard curve for absolute quantification by real-time polymerase chain reaction. Mem Inst Oswaldo Cruz. 112: 220–223.

Liu W, Huang S, Liu N, Dong D, Yang Z, Tang Y and Huang L 2017. Establishment of an accurate and fast detection method using molecular beacons in loop-mediated isothermal amplification assay. Sci Rep. 7: 40125.

Malawa S, Nuntapong N, Waeowannajit S and Thongprajukaew K 2023. Fermented water of Thai herbs effectively enhanced amputated fin regeneration in Siamese fighting fish (Betta splendens). Aquaculture 577: 739980.

Martínez-Martínez M, Diez-Valcarce M, Hernández M and Rodríguez-Lázaro D 2011. Design and application of nucleic acid standards for quantitative detection of enteric viruses by real-time PCR. Food Environ Virol. 3: 92–98.

Mohr PG, Moody NJ, Williams LM, Hoad J, Cummins DM, Davies KR and Crane MS 2015. Molecular confirmation of infectious spleen and kidney necrosis virus (ISKNV) in farmed and imported ornamental fish in Australia. Dis Aquat Organ. 116: 103–110.

Morfin N, Macías-Macías JO and Guzman-Novoa E 2023. Viral quantification in bee samples using synthetic DNA sequences with real-time PCR (qPCR). In: Virus-Host Interactions: Methods and Protocols. M Aquino de Muro (ed.). New York: Springer. 57–66 pp.

Nolan D, Stephens F, Crockford M, Jones JB and Snow M 2015. Detection and characterization of viruses of the genus *Megalocytivirus* in ornamental fish imported into an Australian border quarantine premises: an emerging risk to national biosecurity. J Fish Dis. 38: 187–195.

Sakuna K, Elliman J and Owens L 2018. Comparison of molecular detection PCR methods for chequa iflavirus in freshwater crayfish, Cherax quadricarinatus. J Virol Methods. 251: 139–144.

Sermwatanakul A 2019. Capacitating the local farmers to enhance global marketing of Thailand's national aquatic animal, the Siamese fighting fish. Fish People. 17: 42–48.

Subramaniam K, Shariff M, Omar AR, Hair-Bejo M and Ong BL 2014. Use of acridine orange to visually improve the loop-mediated isothermal amplification for detection of infectious spleen and kidney necrosis virus. Fish Pathol. 49: 173–180.

Zainathan S, Johan C, Subramaniam N, Ahmad A, Halim N, Norizan N and Ariff N 2017. Detection and molecular characterization of *Megalocytivirus* strain ISKNV in freshwater ornamental fish from Southern Malaysia. AACL Bioflux. 10: 1098–1109.

Zhang Q, Shi C, Huang J, Jia K, Chen X and Liu H 2009. Rapid diagnosis of turbot reddish body iridovirus in turbot using the loop-mediated isothermal amplification method. J Virol Methods. 158: 18–23.