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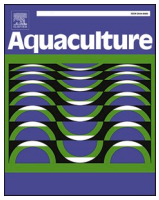

A rapid purification-free LAMP assay for detection of largemouth Bass Ranavirus using minimally invasive samples

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ABSTRACT

Largemouth Bass Ranavirus (LMBV) causes severe mortality in largemouth bass (*Micropterus salmoides*), significantly threatening aquaculture sustainability. Conventional PCR-based methods for LMBV detection are resource-intensive, requiring specialized equipment, skilled personnel, and purified DNA samples, thus limiting their practical applications. Here, we established a simplified, purification-free loop-mediated isothermal amplification (LAMP) assay integrated with a portable fluorescence detection device (WeD-1) for rapid and sensitive detection of LMBV. Using an in vitro infection model, we demonstrated that crude lysates from minimally invasive samples, particularly peritoneal fluid, could be directly used in LAMP assays without nucleic acid purification. The entire detection workflow—from sample collection to result interpretation—was completed within 40 minutes (min). This approach provides a sensitive, rapid, and cost-effective diagnostic method for early detection of LMBV and can potentially be extended for rapid detection of other aquatic pathogens.

1. Introuduction

Largemouth bass (*Micropterus salmoides*), originally native to North America, has emerged as a cornerstone species in Asian aquaculture, especially in China (Li et al., 2022; Yang et al., 2024a). However, rapid expansion of aquaculture combined with environmental degradation has resulted in frequent disease outbreaks, significantly threatening industry sustainability (Lafferty et al., 2015; Yang et al., 2024a). Among these threats, Largemouth Bass Ranavirus (LMBV) is the most severe, causing high mortality rates and posing major challenges for disease management globally (Yang et al., 2024b; Yang et al., 2024c). LMBV, a large double-stranded DNA virus of the genus Ranavirus within the Iridoviridae family, replicates in the host cytoplasm. Currently, no effective treatments exist for LMBV infections (Chen et al., 2024; Wang et al., 2023), highlighting the critical importance of early diagnosis and routine pathogen screening in aquaculture systems (Cao et al., 2024; Guo et al., 2022; Zhang et al., 2022; Zhu et al., 2020).

Polymerase chain reaction (PCR) remains the standard diagnostic method for LMBV due to its high sensitivity. Nevertheless, PCR is resource-intensive, requiring specialized laboratories, expensive instruments, trained personnel, and lengthy procedures, all contributing to high per-sample costs (Yang et al., 2024b). Although rapid detection techniques have been introduced, most still involve DNA purification, which requires additional equipment and technical expertise, limiting practical field deployment (Guang et al., 2024; Guo et al., 2022; Jiang et al., 2023; Zhang et al., 2022; Zhu et al., 2022). Therefore, there is an

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urgent need for portable, user-friendly diagnostic tools suitable for on-site testing by farmers and hatchery operators without specialized training or infrastructure.

Loop-mediated isothermal amplification (LAMP) is a promising alternative, providing rapid nucleic acid detection with high efficiency, specificity, cost-effectiveness, and straightforward visual results (Ahmadi et al., 2025; Saifuddin et al., 2024; Yang et al., 2024a). Previously, we developed the handheld WeD-1 isothermal amplification device (Pang et al., 2024), which, combined with a customized LAMP MasterMix and an enhanced one-pot detection reagent based on prokaryotic Argonaute (PfAgo), enables real-time fluorescence monitoring and duplex visual detection of aquatic pathogens.

Despite these advances, practical implementation at pond-side faces two key challenges. First, the current approach involves two separate steps—initial LAMP amplification followed by PfAgo enzymatic digestion—which increases complexity and cost. Second, existing protocols typically require homogenization of internal tissues such as liver or spleen, restricting ease of sampling. Notably, viral genomes can also be detected in more accessible host fluids such as mucus (Pohl et al., 2024; Willner, 2010), blood (Moustafa et al., 2017), pleural or peritoneal fluid (Coccolini et al., 2020), and lymph (Melo-Silva and Sigal, 2024). These minimally invasive samples can be easily collected using throat swabs or simple collection tubes without sacrificing the fish. However, most current detection protocols still rely on purified DNA, limiting their true field applicability.

To address these limitations, we established an in vitro LMBV infection model and evaluated the feasibility of directly testing crude lysates obtained from mucus, blood, and peritoneal fluid collected via pharyngeal swabs. Using the portable WeD-1 device, we systematically assessed the effectiveness of various minimally invasive samples, developing a practical, cost-effective, and rapid field-ready method suitable for on-site LMBV detection in largemouth bass aquaculture.

2. Materials and methods

2.1. Ethics

All procedures were conducted in accordance with the guidelines of the Committee on Laboratory Animal Care and Use of Shanghai Ocean University under protocol SHOU-DW-2023-076.

2.2. Fish, cell line and virus

Healthy largemouth bass (*Micropterus salmoides*), weighing 9.16 ± 2.37 g and measuring 9.00 ± 0.69 cm in length, were purchased from Zhejiang Huzhou Nanxun Linghu Chenhao Aquatic Products Operation Department (Zhejiang, China). Fish were acclimated for one week in the laboratory prior to experimentation (Li and Huang, 2024). The LMBV strain (10^6 $TCID_{50}$/0.1 mL) and the fathead minnow (FHM) cell line were obtained from the National Aquatic Animal Pathogen Bank of Shanghai Ocean University. Virus preparation followed with previous described method (Dai et al., 2025). Briefly, FHM cells were cultured in Medium 199 (Gibco, USA, 31100–035) supplemented with 10 % fetal bovine serum (Gibco, USA, A5669701) and maintained at 27 °C in a 5 % CO_2 incubator. Once the cells reached approximately 90 % confluence, they were inoculated with LMBV. When 90 % of the cells exhibited cytopathic effects (CPE), the virus was harvested and stored at −80 °*C. prior* to the viral infection experiment, the viral stock was thawed at room temperature just until fully defrosted, then re-frozen at −80 °C. After three freeze–thaw cycles, the virus was stored at −80 °C for long-term preservation.

2.3. LMBV infection model and sample collection

A total of 50 largemouth bass were randomly allocated into two tanks (100 L each) maintained at 25 ± 2 °C. The LMBV suspension used for infecting largemouth bass was prepared from the viral stock solution described in Step 2.2. Prior to infection, the LMBV titer was determined and then diluted with M199 medium to a final concentration of 10^6 $TCID_{50}$/0.1 mL. Ten fish served as the uninfected control group, while 40 fish were intraperitoneally injected with 200 μL of the LMBV suspension. Gill mucus, head mucus, dorsal mucus, anal mucus, peritoneal fluid, and blood samples were collected at 24, 48, 72, and 96 h post-infection (hpi), as shown in Fig. 3B. Mucus samples were collected using DNA collection tubes with swabs, and blood and peritoneal fluid were collected via syringe to prevent contamination. Those Crude DNA extracts were either immediately used for analysis or stored at −20 °C. Tail fin tissue samples were collected using sterilized scissors and stored at −80 °C.

2.4. Total DNA extraction

Genomic DNA was extracted from tail fin tissues using the Blood/Cell/Organ Tissue Genomic DNA Extraction Kit (TIANGEN, China, Cat. No. DP304–3). DNA from crude extracts of gill mucus, head mucus, dorsal mucus, anal mucus, peritoneal fluid, and blood was further purified with the DNA Viral Genome Extraction Kit (Solarbio, China, Cat. No. D2400) for subsequent absolute quantitative PCR analysis.

2.5. Construction of recombinant plasmid pMD™19-T-MCP

The full-length Major capsid protein (MCP) gene of LMBV (1410 bp) was amplified using 2× Taq Master Mix (Vazyme, China, Cat. No. P112). The PCR reaction mixture (20 μL) consisted of 10 μL 2× Taq Mix, 1 μL forward primer (pMCPF, 10 μM), 1 μL reverse primer (pMCPR, 10 μM), 300 ng total DNA, and nuclease-free water. The PCR primer sequences are shown in Table 1. PCR conditions were as follows: initial denaturation at 95 °C for 5 min; 10 cycles of 95 °C for 30 s, 50 °C for 30 s, 72 °C for 90 s; followed by 25 cycles of 95 °C for 30 s, 60 °C for 30 s, 72 °C for 90 s; and a final extension at 72 °C for 10 min. Amplified products were visualized by 1 % agarose gel electrophoresis, purified using the QIAquick Gel Extraction Kit (QIAGEN, Germany, Cat. No. 28106) and ligated into the pMD™19-T vector (Takara, Japan, Cat. No. 6013). The recombinant plasmid was transformed into *E. coli* DH5α cells. Plasmid DNA was extracted using the EndoFree Plasmid ezFlow Miniprep Kit (Biomiga, USA, Cat. No. BW-PD1220) and quantified using a NanoDrop One spectrophotometer (Thermo Fisher Scientific).

2.6. Absolute quantitative PCR

The recombinant pMD™19-T-MCP plasmid was serially diluted (10-fold) to generate standards ranging from 10^5 to 10^{12} copies/mL. qPCR assays were performed using SsoFast EvaGreen Supermix (Bio-Rad, USA, Cat. No. 1725201) on a CFX Real-Time PCR System (Bio-Rad, USA, Model CT043335). Each 20 μL reaction included 10 μL EvaGreen Supermix, 1 μL each of forward (MCP-F1, 10 μM), and reverse primers (MCP-R1, 10 μM), 5 μL DNA template, and nuclease-free water. The PCR primer sequences are shown in Table 1. Cycling conditions were initial denaturation at 95 °C for 2 min, followed by 40 cycles of 95 °C for 30 s and 60 °C for 30 s. Standard curves were generated by plotting Ct values against $\log_{10}$ (copy number), and assays meeting quality criteria (E: 0.90–1.10; $R^2 > 0.98$) were considered valid. Viral load in samples was

Table 1
Primer sequences.

Target	Primer	Sequence	Length (bp)	Product (bp)
MCP gene	pMCPF	CGCGGATCCATGTCTTCTGTTACG	24	1410
	pMCPR	CGCGAATTCTTACAGGATGGGGAA	24	
MCP gene	MCP-F1	CTGTTGTTGGAGCGGGTAA	19	192
	MCP-R1	GGGCGTAAGAGTAGAGGTGG	20	

determined by interpolation.

2.7. LAMP assays

The LAMP MasterMix used for isothermal amplification, preloaded with LMBV-specific primer sets, was prepared following the protocol outlined in our previously published study (Pang et al., 2024). For LMBV LAMP assays, crude DNA extracts (from step 2.3) were centrifuged at 12,000 rpm for 5 min. Supernatants (25 μL) were transferred into homemade LAMP MasterMix tubes, gently mixed, and briefly centrifuged. Reaction tubes were placed into the portable WeD-1 isothermal amplification device. LAMP reactions proceeded at 65 °C for 30 min, with real-time fluorescence monitored and images captured at 1-min intervals using the device's mobile application.

2.8. Statistical analysis

Data are presented as mean ± standard deviation (SD) from at least three independent experiments. Statistical analyses were performed using IBM SPSS Statistics v25. Differences among multiple groups were assessed by one-way ANOVA followed by Tukey's post hoc test. The P value <0.05 was regarded statistically significant. Fluorescence intensity assays were performed using ImageJ. Graphs were generated using GraphPad Prism v8.

3. Result

3.1. Establishment of an in vitro LMBV infection model in largemouth bass

An in vitro infection model for LMBV was developed in largemouth bass, following the experimental workflow illustrated in Fig. 1A. Fish (weight: 9.16 ± 2.37 g; length: 9.00 ± 0.69 cm) were intraperitoneally injected with 200 μL of LMBV suspension (10^6 $TCID_{50}$/mL). Tail fin samples were collected at 24, 48, 72, 96, and 120 h post-infection (hpi) to quantify viral loads using absolute qPCR. Results revealed a significant, time-dependent increase in viral load within tail fin tissues (Tukey's test, $P < 0.05$). At 96 hpi, infected fish exhibited characteristic pathological features, including distinct white lesions on the liver, which corresponded to peak viral load and matched previously reported clinical observations (Xu et al., 2023; Zilberg et al., 2000). These results confirm the successful establishment of the LMBV infection model.

3.2. Peritoneal fluid showed the highest viral load among collected samples

To identify suitable samples for LAMP-based detection, we collected mucus from the gills, head, dorsal region, and anus of largemouth bass using pharyngeal swabs. Additionally, approximately 20 μL each of blood and peritoneal fluid were aspirated using syringes and placed into nucleic acid collection tubes (Fig. 2B). Viral genomic DNA from these crude extracts was purified using a viral DNA extraction kit and quantified by absolute qPCR according to the workflow shown in Fig. 2A. Quantification results revealed that peritoneal fluid consistently exhibited the highest viral load at each tested time point (Fig. 2C), displaying a statistically significant increase over time (Fig. 2I, $P < 0.05$). Gill mucus, dorsal mucus, anal mucus, and blood also showed significant increases in viral loads across these time points ($P < 0.05$). However, head mucus samples did not show statistically significant variation in viral load over time (Fig. 2D-H).

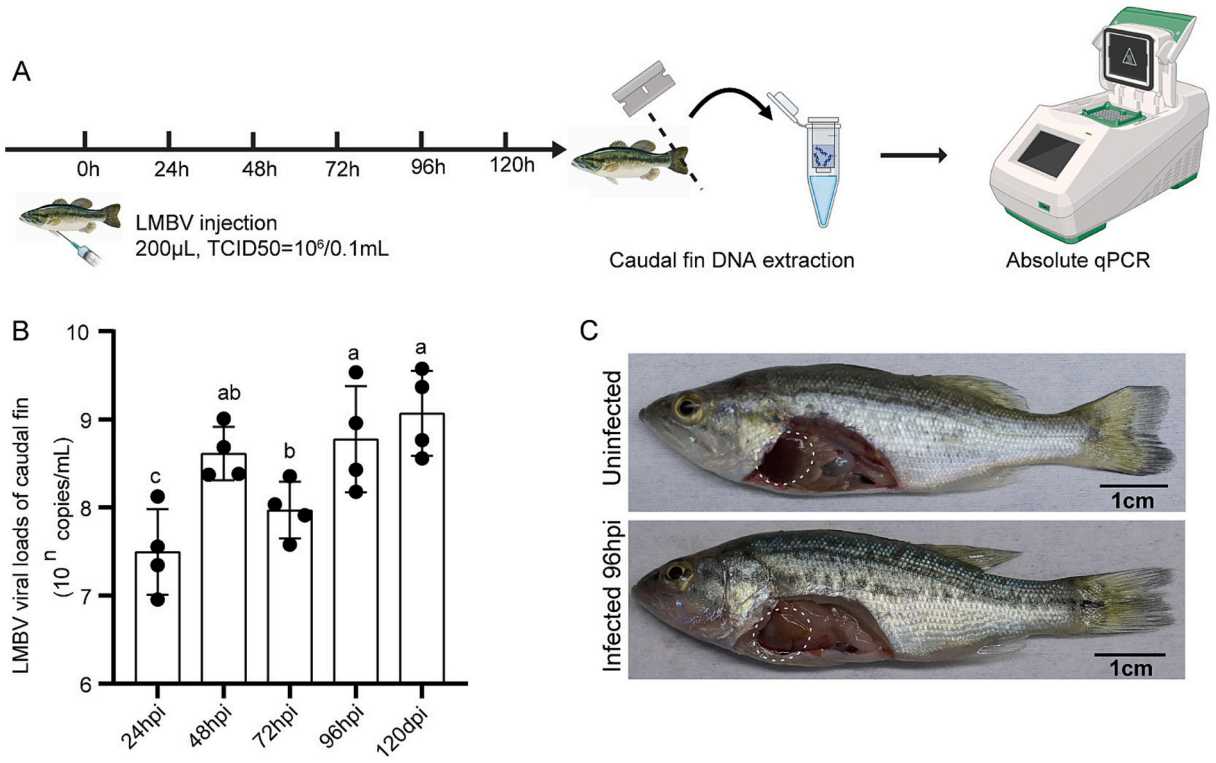

Fig. 1. Establishment of an in vitro LMBV infection model in largemouth bass.
(A) Schematic workflow for the establishment and phenotypic characterization of the LMBV infection model. Largemouth bass were injected with 200 μL of LMBV (10^6 $TCID_{50}$/0.1 mL), and tail fin tissues were collected at 24, 48, 72, 96, and 120 h post-infection (hpi) for quantification of viral load by absolute qPCR.
(B) Viral loads in tail fin tissues at various time points post-infection. Different letters (a–d) indicate significant differences (Tukey's test, $P < 0.05$).
(C) Anatomical image of largemouth bass at 96 hpi. White lesions in the liver region are indicated by white circles. Scale bar = 1 cm.

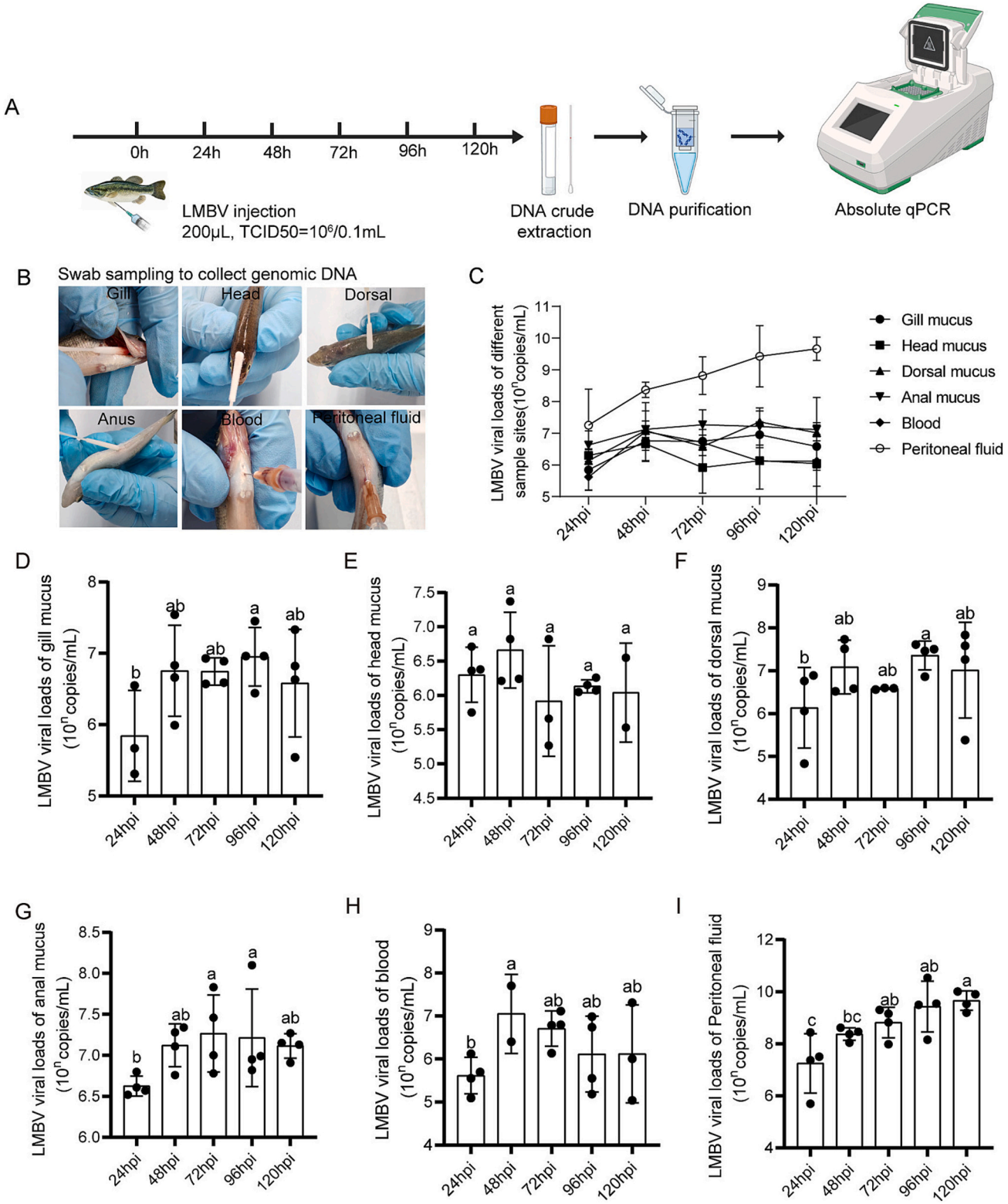

Fig. 2. Absolute quantification of LMBV loads in mucus, blood, and peritoneal fluid samples from largemouth bass.
(A) Experimental workflow for viral DNA quantification. Fish were injected with 200 μL LMBV (10^6 $TCID_{50}$/0.1 mL), followed by sample collection at multiple time points. Purified DNA extracts were analyzed by absolute qPCR.
(B) Representative images of sample collection from different anatomical sites.
(C) Comparative viral loads across different sample types and time points, highlighting consistently highest levels in peritoneal fluid.
(D-I) Individual graphs showing viral loads in gill mucus, head mucus, dorsal mucus, anal mucus, blood, and peritoneal fluid. Different letters (a–d) indicate significant differences (Tukey's test, $P < 0.05$).

3.3. Peritoneal fluid provided the highest detection efficiency using the LAMP assay and WeD-1 device

To evaluate the feasibility of using crude DNA extracts directly for LAMP detection, 25 μL of each crude DNA extract was added to the LAMP reaction mix. Following 30 min of amplification, peritoneal fluid and anal mucus yielded the highest positive detection rates (7/8 each), while gill mucus exhibited the lowest detection rate (1/8; Fig. 3B). During the experiment, we observed that cell debris in the crude DNA extracts inhibited the LAPM reaction. In the subsequent LAMP analysis, we centrifuged the crude extract at 12000 rpm for 5 min and used the supernatant for the LAMP assays. Crude DNA extracts from gill mucus, head mucus, dorsal mucus, anus mucus, blood, and peritoneal fluid collected at 96 hpi were subjected to LAMP assays. Fluorescence from the peritoneal fluid reaction was first visible to the naked eye after 30 min of amplification (Supplementary Fig. 1A). By 35 min, signals from head mucus, dorsal mucus, and blood were also detectable (Supplementary Fig. 1B). Real-time fluorescence monitoring further confirmed that peritoneal fluid produced the most robust and reliable signal (Supplementary Fig. 1C). Further LAMP assays of peritoneal fluid samples collected at different time points post-infection demonstrated consistently high positivity, achieving 100 % detection by 48 hpi. These results confirm peritoneal fluid as the most reliable sample type for rapid, on-site LAMP-based detection of LMBV.

3.4. Real-time LAMP amplification from peritoneal fluid peaked at as early as 15 min

Real-time fluorescence signals from LAMP amplification were monitored over 30 min using the WeD-1 device. Fluorescence intensity statistics were performed using ImageJ. As is shown in Fig. 4B, the positive control reached peak fluorescence intensity around 10 min. Peritoneal fluid and head mucus samples peaked at approximately 15 min, blood at 20 min, and anal mucus at 25 min. Gill and dorsal mucus samples failed to reach the exponential amplification phase, likely due to low viral loads or the presence of inhibitory substances.

4. Discussion

Largemouth Bass Ranavirus (LMBV) poses a significant threat to the sustainability of the largemouth bass aquaculture industry. Early pathogen detection and routine screening are crucial for preventing the spread of infections and maintaining aquaculture health. However, conventional PCR-based methods for LMBV detection require complicated procedures, extensive laboratory equipment, skilled personnel, and lengthy processing times, making them impractical for rapid on-site application. Therefore, there is an urgent need for a simpler, faster, and more cost-effective alternative suitable for widespread field deployment. Therefore, LAMP assays were introduced in this study because it offers several advantages, including high amplification efficiency, strong specificity, and compatibility with visual detection methods (Ahmadi et al., 2025; Saifuddin et al., 2024; Yang et al., 2024a). Therefore, the LAMP assay was employed in this study due to its high amplification efficiency, strong specificity, and compatibility with visual detection methods (Ahmadi et al., 2025; Saifuddin et al., 2024; Yang et al., 2024a).

In this study, we established a robust in vitro LMBV infection model in largemouth bass (*Micropterus salmoides*), enabling controlled evaluation of rapid diagnostic techniques. Following intraperitoneal injection of LMBV (10^6 $TCID_{50}$/0.1 mL), viral replication was confirmed in infected fish tissues, with characteristic pathological signs—such as distinct white liver lesions—evident by 96 h post-infection (hpi). The successful establishment of this infection model provided a reliable foundation for assessing diagnostic assays.

Previous studies have demonstrated that mucus samples can be used for the detection of LMBV pathogens in largemouth bass (Getchell et al.,

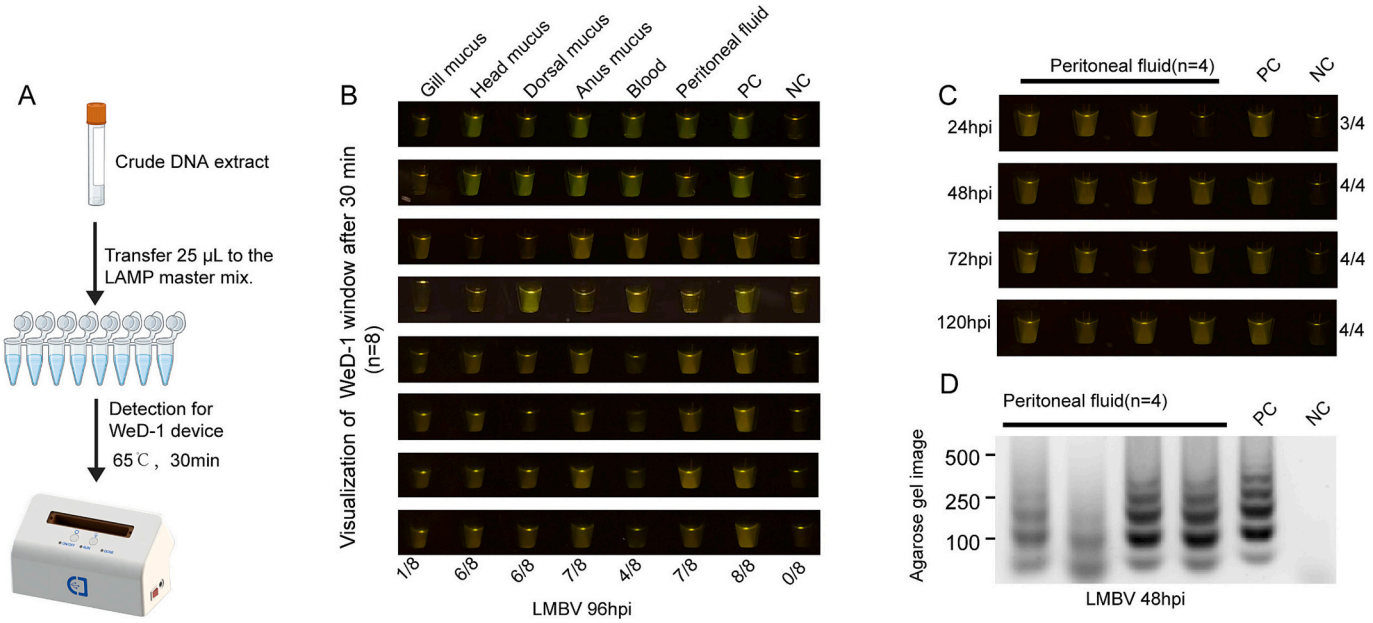

Fig. 3. LAMP detection performance using crude DNA extracts and the WeD-1 device.
(A) Workflow illustrating the LAMP assay using crude extracts. Samples were mixed with the LAMP MasterMix and amplified at 65 °C for 30 min using the WeD-1 device.
(B) Detection results from crude DNA extracts at 96 hpi, showing positive detection rates for each sample type. Peritoneal fluid and anal mucus exhibited the highest positivity (7/8), followed by head and dorsal mucus (6/8), blood (4/8), and gill mucus (1/8). Positive control (PC) and negative control (NC) yielded 8/8 and 0/8, respectively.
(C) Detection rates in peritoneal fluid samples collected at 24, 48, 72, and 120 hpi, with consistent detection from 48 hpi onwards.
(D) Representative gel image of LAMP amplification products from peritoneal fluid at 48 hpi, showing multiple DNA ladder-like bands characteristic of positive LAMP reactions.

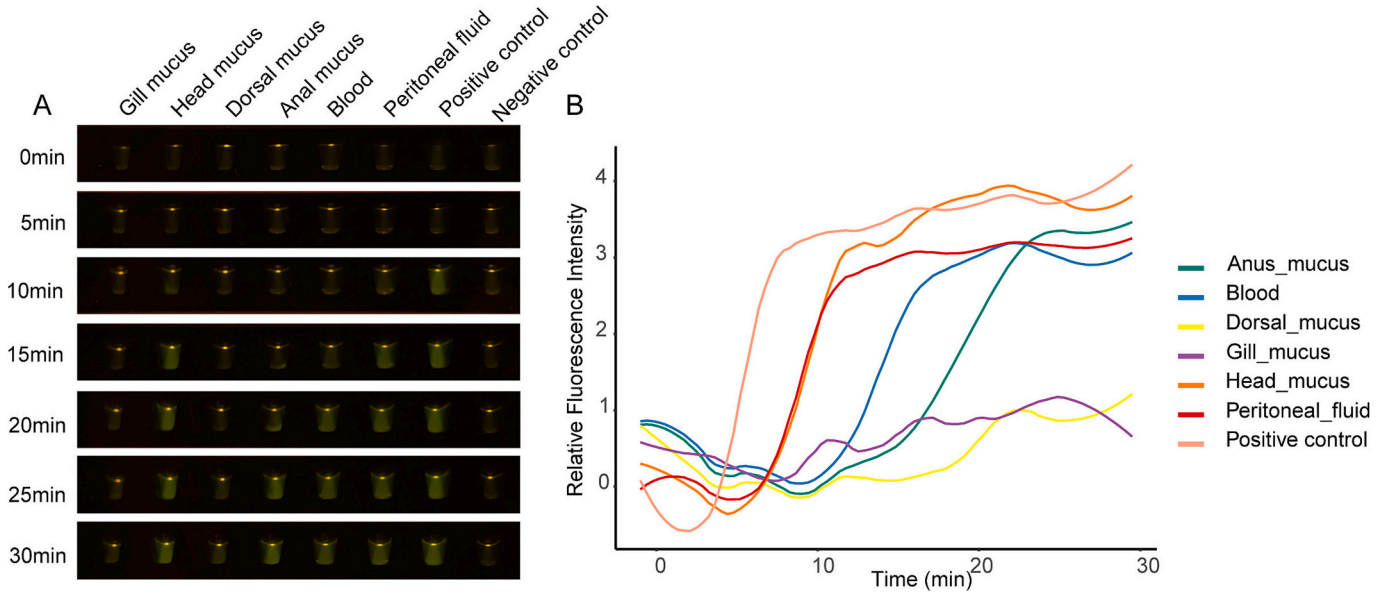

Fig. 4. Real-time fluorescence monitoring of LAMP amplification using WeD-1.
(A) Representative fluorescence detection curves of crude DNA extracts from gill mucus, head mucus, dorsal mucus, anal mucus, blood, and peritoneal fluid at 96 hpi. (B) Fluorescence intensity profiles indicating peak times for each sample type. Peak fluorescence occurred earliest in positive controls (~10 min), followed by peritoneal fluid and head mucus (~15 min), blood (~20 min), and anal mucus (~25 min). Gill and dorsal mucus samples did not exhibit exponential amplification. Fluorescence intensity was quantified from grayscale image analysis using ImageJ software.

2007; Leis et al., 2018). Using minimally invasive sampling techniques, we demonstrated that mucus from various anatomical regions, as well as blood and peritoneal fluid, contained detectable viral DNA suitable for diagnostic purposes. Notably, peritoneal fluid consistently exhibited the highest viral loads (ranging from 10^5 to 10^{10} copies/mL) and provided optimal sensitivity for pathogen detection. This finding highlights the potential of peritoneal fluid as the most effective sample type for early LMBV diagnosis.

To facilitate rapid, simplified detection, we employed a purification-free loop-mediated isothermal amplification (LAMP) protocol combined with a portable fluorescence detection device (WeD-1). Unlike conventional PCR and previous nucleic acid-based methods (Guang et al., 2024; Guo et al., 2022; Jiang et al., 2023; Jin et al., 2020; Li et al., 2022; Zhu et al., 2020; Zhu et al., 2022), our protocol does not require sample homogenization, nucleic acid purification, or specialized laboratory infrastructure. Crude lysates derived directly from peritoneal fluid samples yielded strong fluorescent signals and high positivity rates in LAMP assays, achieving 100 % detection accuracy within 48 hpi. Furthermore, real-time fluorescence monitoring indicated that positive amplification signals could be detected within approximately 15 min, enabling rapid diagnostic decisions.

Collectively, our study presents several significant advancements: (Zhu et al., 2020) we developed a purification-free LAMP protocol enabling rapid and direct detection of LMBV using minimally invasive samples, greatly simplifying field diagnostics; (Pohl et al., 2024) we identified peritoneal fluid as an optimal sample source for highly sensitive and early-stage viral detection; and (Willner, 2010) we validated a portable and cost-effective fluorescence-based diagnostic platform (WeD-1) capable of delivering accurate, user-friendly results within 40 min. This streamlined approach provides a powerful tool for aquaculture health management and has significant potential to be adapted for the rapid detection of other aquatic pathogens.

CRediT authorship contribution statement

Chaoguang Wei: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation. **Chenwei Yu:** Resources, Methodology, Investigation. **Jinjie Zu:** Visualization, Investigation. **Xinyang Li:** Visualization, Investigation, Formal analysis. **Yilan Bao:** Validation, Investigation. **Feibiao Pang:** Methodology. **Zhichao Wu:** Writing – review & editing. **Dan Xu:** Writing – review & editing, Project administration. **Jinzhao Song:** Project administration, Methodology, Formal analysis. **Peng Hu:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Methodology, Funding acquisition.

Declaration of competing interest

The authors declare no competing interests.

Data availability

Data will be made available on request.

Acknowledgements

The schematic illustrations in Fig. 1A, Fig. 2A and Fig. 3A were created or modified using images from BioRender.com. This study was supported by the National Key Research and Development Program of China (2023YFD2402100).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2025.743190.

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