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Establishment and Application of a SYBR Green I Real-Time Quantitative PCR for Detection of *Micropterus salmoides* Rhabdovirus

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NZ presented the concept, methodology and prepared original draft. RH and QL did validation. NZ and XZ did formal analysis. NZ, QL RH and GY conducted the investigation. WM and XD provided resources. NZ and RH reviewed and edited the manuscript. XD supervised the study. NZ and QL administered the prooject. XZ and NZ acquired resources.

Key words
***Micropterus salmoides* rhabdovirus, SYBR Green I, Detection method**

ABSTRACT

In order to develop a rapid and convenient detection assay of *Micropterus salmoides* rhabdovirus (MSRV), the SYBR Green I real-time quantitative PCR(RT-qPCR) assay was established. A pair of primers was designed according to the G protein gene of MSRV and a recombinant plasmid containing the target gene was constructed as a standard control. The correlation coefficient of the standard curve was 0.998, which indicated a good linear relationship between initial templates and Ct values. The established SYBR Green I RT-qPCR assay had a detection limit of 2.78×10^{1} copies/μL, which was 100 times more sensitive than the conventional PCR. Moreover, the coefficient of variations was less than 1% for both intra-assay and inter-assay, and no cross reaction was found in other aquatic viruses such as GCRV, KHV, ISKNV and NNV. 54 samples were detected positive from 104 clinical samples by the SYBR Green I RT-qPCR assay, while 22 samples were detected positive by the conventional PCR. The SYBR Green I RT-qPCR assay showed the characteristics of sensitivity and specificity. This method can provide reliable technical support for clinical diagnosis and epidemiological investigation of MSRV, which can effectively control the spread and epidemic of MSRV disease.

INTRODUCTION

Largemouth bass (*Micropterus salmoides*), commonly known as California perch, is native to the Mississippi River in California, USA, which has been welcomed by farmers and consumers with the advantage of delicious meat, rapid growth, wide temperature range and stress resistance (Bai and Li., 2013; Han *et al*., 2020). At present, largemouth bass has become an important freshwater aquaculture variety in China. However, in recent years, viral, bacterial and parasitic diseases have increased and caused great harm to largemouth bass aquaculture industry (Xia *et al*., 2018). Among them, rhabdovirus disease is one of the most pandemic and lethal disease, which mainly infects fry within 4cm in April and May. The clinical signs include irregular swimming and crooked body (Zhang *et al*., 2019). *Micropterus salmoides* rhabdovirus (MSRV) spreads rapidly and even more than 90% of fry could be killed quickly in a week according our investigation, which caused great economic losses to farmers. In addition, rhabdovirus is becoming more widespread, which has become one of the main obstacles to the development of largemouth bass aquaculture. So far, there is no suitable drug for the treatment of MSRV currently. Some studies have shown that subunit vaccine and live attenuated vaccine have good immune protection effect against MSRV of largemouth bass by injection or immersion (Zhang *et al*., 2018; Guo *et al*., 2020). In addition, ribavirin and 8-hydroxyquinoline showed a good therapeutic effect on MSRV infection *in vitro* and *in vivo* (Yang *et al*., 2021; Li *et al*., 2022). However, it is difficult to make suitable bait for feeding due to the small size of the fry, and there is also the risk of drug residue.

Rhabdovirus is a class of enveloped negative-stranded RNA viruses (Liu *et al*., 2020), which can infect mammals, birds, reptiles, fish, insects and plants (Kuzmin *et al*., 2009; Maclachlan and Dubovi, 2011). At present, more than twenty species of fish rhabdovirus have been reported, including *Siniperca chuatsi* rhabdovirus (SCRV), spring viraemia of carp virus (SVCV), hirame rhabdovirus (HIRRV), perch rhabdovirus (PRV), hybrid snakehead rhabdovirus (HSHRV), etc. (Zhang and Li.,

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1999; Padhi and Verghese, 2012; Dorson *et al*., 1984; Gui and Zhang, 2007; Zeng *et al*., 2014). MSRV was first discovered from largemouth bass fry by Lei *et al*. (2015) at a farm in Guangdong province, China (Lei *et al*., 2015). MSRV belongs to rhabdoviridae and *Perhabdovirus*, which consists of five structural proteins: nuclear protein (N), phosphorylated protein (P), matrix protein (M), glycoprotein (G) and RNA dependent RNA polymerase protein (L) (Walker *et al*., 2022). Glycoprotein constitutes the viral envelope and is the main protective antigen, which can stimulate organism producing neutralizing antibodies against viral infection (Ruan and Zhang, 2003). A carbon nanotubes-loaded glycoprotein subunit vaccine was evaluated to have protective effect against death caused by MSRV (Guo *et al*., 2020).

At present, the main detection methods for fish rhabdovirus are PCR and other molecular detection methods. Recently, recombinase polymerase amplification combined with lateral flow dipsticks targeting the nuclear protein were described for the detection of MSRV, which could detect the viral DNA of 170 copies/μl of the MSRV standard plasmid (Feng *et al*., 2022). However, fluorescence quantitative PCR has the advantages of fast detection, high sensitivity, real-time accuracy and so on. Therefore, in this study, SYBR Green I real-time quantitative PCR detection method was established for qualitative and semi-quantitative detection of MSRV, which provide technical support for prevention and control of MSRV.

MATERIALS AND METHODS

Primer design

The primer was designed using the software of Primer Premier 5 according to the G protein encoding gene of MSRV (GenBank No. MK397811.2). The primer sequence was as follows: MSRV-qF1: 5'-CACCAGCCACATCAATCCC-3'; MSRV-qR1: 5'-CCCGTCCGTCGCTTGA-3'. The amplified products were 179bp and sequenced by Sangon Biotech (Shanghai) Co., LTD.

Clinical samples and standard samples

Micropterus salmoides rhabdovirus (MSRV), grass carp hemorrhagic disease virus (GCRV), koi herpesvirus (KHV), infectious spleen and kidney necrosis virus (ISKNV) and nerve necrosis virus (NNV) were from our team. 104 clinical samples including 84 suspected infected samples (irregular swimming, or hemorrhage) and 20 suspected healthy samples (no clinical sign) were collected from largemouth bass farm in Hangzhou, Huzhou and Jiaxing city, China from April 2021 to April 2022. The G protein positive standard plasmid was synthesized by General Biosystems Co., Ltd. and verified by sequence analysis. The concentration of recombinant plasmid was determined by the equation: copies/μL=(ng/μL×10^9) × (6.02×10^{23})/DNA length × 660.

Optimization of reaction conditions

Gradient tests were carried out to optimize reaction cycles, reaction system and extension temperature. The optimal reaction system is selected as: 2×qPCR Super Mix 10 μL, MSRV-qF1/qR1 (10μmol/L) 0.6μL, cDNA template 1μL, RNA free water supplement to 20μL according to the screening criteria of highest relative fluorescence intensity (RFu), the lowest cyclic threshold (Ct) and a single peak melting curve. The reaction procedure is as follows: pre-denaturation at 95℃ for 20s; denaturation at 95℃ for 5s, annealing and extension at 60℃ for 30s, 40 cycles; followed by fluorescence signal acquisition.

Establishment of standard curve

The standard plasmids were extracted using Endo Free Mini Plasmid Kit II (TIANGEN Biotech (Beijing) Co., Ltd. China) and 10 times diluted from 1×10^8 to 1×10^1 copies/μL and amplified according to the optimized reaction system and conditions. Ct values were used to establish standard curve.

Sensitivity test

The minimum detection limit of SYBR Green I RT-qPCR was performed using 10 times diluted standard plasmids (1×10^7 ~ 1×10^1copies/μL) as templates. The sensitivity between SYBR Green I RT-qPCR and conventional PCR was compared with the same templates.

Specificity test

The nucleic acids of GCRV, KHV, ISKNV and NNV were extracted using TIANamp Virus DNA/RNA Kit (TIANGEN Biotech (Beijing) Co., Ltd. China), and the RNA was reversed into cDNA using Prime Script RT reagent Kit with gDNA Eraser (Takara, Japan). The specificity of SYBR Green I RT-qPCR was evaluated by using the templates including standard plasmid, ISKNV, KHV, GCRV, NNV and ddH_2O as negative control.

Reproducibility test

Intra-group parallel experiment was conducted with 10 times dilution of standard plasmids with 3 gradients (1×10^6~1×10^4 copies/μL) as the template, and each gradient was repeated 3 times. The standard plasmids were extracted in 3 batches, and the inter-group parallel experiment was conducted under the same conditions to analyze the repeatability. Differences between and within groups were calculated.

Application in the samples

The liver and spleen of the 104 samples were stored in sample protector for RNA/DNA (Takara, Japan) at -80℃ and the nucleic acids were extracted using TIANamp Virus DNA/RNA Kit (TIANGEN Biotech (Beijing) Co., Ltd. China), and the RNA was reversed into cDNA using PrimeScript RT reagent Kit with gDNA Eraser (Takara, Japan). Meanwhile, the samples were detected and compared by SYBR Green I RT-qPCR and conventional PCR (Lei *et al*., 2015).

RESULTS

Standard curve of SYBR Green I RT-qPCR

The concentration of standard plasmids was 2.78×10^{10} copies/μL according to the formula. Standard plasmids with a gradient of 2.78×10^{1} ~ 2.78×10^{8} copies/μL were selected for SYBR Green I RT-qPCR amplification using the optimized reaction system. The results showed that Ct value had a good linear relationship with the concentration of standard plasmids (Fig. 1A). The linear regression equation was Y= -3.3429x + 37.029, R^2 was 0.9975, and the amplification efficiency was 99.1%. The melting curve showed that the melting temperature (Tm) was 82.65℃, and a specific single peak with no primer dimer and non-specific products (Fig. 1B).

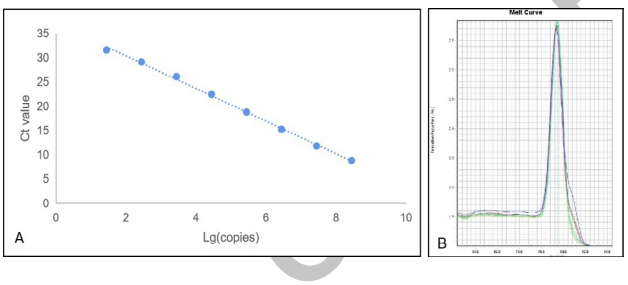

Fig. 1. Standard curve (A) and melting cure (B) of the SYBR Green I RT-qPCR.

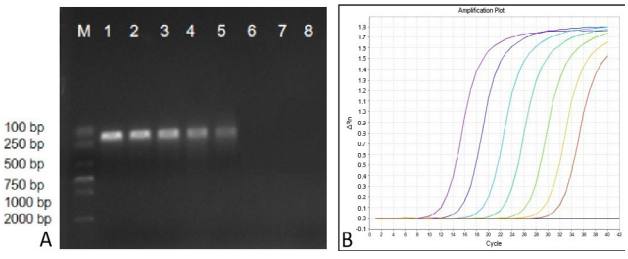

Fig. 2. Sensitivity test results of the PCR (A) and SYBR Green I RT-qPCR (B). M: DL2000 marker, 1: 2.78×10^{7} copies; 2: 2.78×10^{6} copies; 3: 2.78×10^{5} copies; 4: 2.78×10^{4} copies; 5: 2.78×10^{3} copies; 6: 2.78×10^{2} copies; 7: 2.78×10^{1} copies; 8: 2.78×10^{0} copies.

Sensitivity test

The minimum content of positive plasmid detected by conventional PCR was 2.78×10^{3} copies/μL (Fig. 2A), while that detected by SYBR Green I RT-qPCR was 2.78×10^{1} copies/μL (Fig. 2B). The sensitivity of SYBR Green I RT-qPCR was 100 times higher than conventional PCR. The results showed that the SYBR Green I RT-qPCR established in this experiment was highly sensitive.

Specificity test

The results showed that only the standard plasmid had specific amplification, and no amplification curve in other pathogens including GCRV, KHV, ISKNV, NNV and negative control (Fig. 3), which indicated that the method had good specificity.

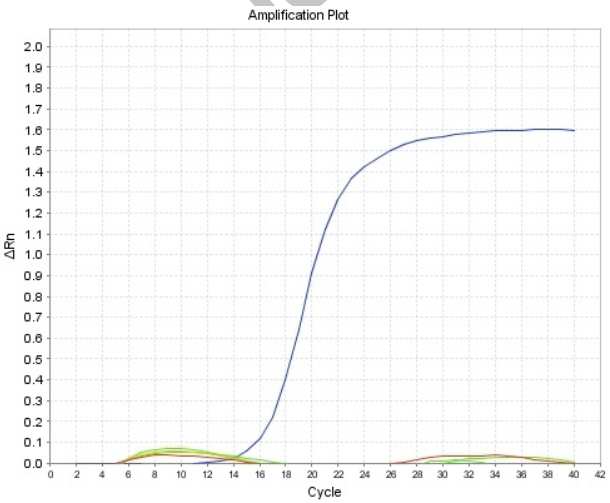

Fig. 3. Specificity analysis of the SYBR Green I RT-qPCR.

Repeatability test

The coefficient of variation within and between groups were less than 1%, indicating that the method had good stability and repeatability (Table I). Several positive samples were sequenced to verify the result.

Samples detection results

104 suspected samples including suspected infected and suspected healthy were detected with the established MSRV SYBR Green I RT-qPCR and conventional PCR. 54 samples were detected positive by SYBR Green I RT-qPCR, with the detection rate of 51.92%. While 22 samples were detected positive by conventional PCR, with the detection rate of 21.15% (Table II). The SYBR Green I RT-qPCR can be used for the detection of MSRV in clinical samples and more sensitive than conventional PCR method.

Table I. Repeatability test results of the SYBR Green I RT-qPCR.

Plasmid concentration (copies/μL)	N	Variation within groups		Variation between groups	
		Mean±SD	Variable coefficient (CV%)	Mean±SD	Variable coefficient (CV%)
10^3	3	26.00±0.12	0.48	26.35±0.21	0.88
10^4	3	22.42±0.06	0.25	22.44±0.14	0.60
10^5	3	18.81±0.13	0.67	18.74±0.17	0.80

Table II. Apparent epidemiological status for MSRV, number of sampled fish and results of both conventional PCR and qPCR.

Larger mouth bass	No. sampled fish	Conventional PCR		qPCR	
		Positive	Negative	Positive	Negative
Suspected infected	84	22 (26.19%)	62 (73.81%)	54 (64.28%)	30 (35.72%)
Suspected healthy	20	0 (0%)	20 (100%)	0 (0%)	20 (100%)
Total	104	22 (21.15%)	82 (78.85%)	54 (51.92%)	50 (48.08%)

DISCUSSION

MSRV has strong infectivity, epidemic and pathogenicity, and resulted a high mortality rate. The mortality rate of largemouth bass fry caused by MSRV reached into 100% in some major farms of Zhejiang province, China in 2020-2021. At present, no drug can be effective for this disease. Therefore, strengthening virus detection in the fry stage and selecting high-quality healthy largemouth bass fry can effectively reduce the outbreak of the disease. Meanwhile, strict control of cross-area transportation of fry carrying MRSV is the main means to cut off the transmission route of MSRV. Therefore, it is very important to establish and apply a method for rapid detection of MSRV in order to prevent virus infection and reduce economic losses of farmers.

At present, the main virus detection methods are cytological, immunological and molecular biology diagnosis technology. Virus isolation and identification are time-consuming and technically difficult. Immunological diagnosis is complicated and not suitable for the detection of a large number of samples (Zhang *et al*., 2015). Currently, molecular biology diagnosis technology is mostly used for detection. Compared with conventional PCR, fluorescence quantitative PCR is characterized by high sensitivity, good specificity, real-time accuracy, etc. SYBR Green I, as a fluorescent dye bound to double-stranded DNA, can release fluorescence signal by combining with double-stranded PCR products in the process of fluorescence quantitative PCR reaction and thus be detected by instruments (Shi *et al*., 2009). SYBR Green I is the most common fluorescent quantitative detection dye with low price and relatively simple primer design. In addition, non-specific amplification products and primer dimers can be distinguished by fusion curve analysis, which has been widely used in pathogen detection (Song *et al*., 2014).

Studies have found that G proteins between different rhabdovirus were low homology (Zhang *et al*., 2011), so primers designed based on G protein genes have good specificity. In this study, a pair of specific primers were designed for MSRV G protein, and a SYBR Green I RT-qPCR method was established through optimization of reaction conditions. When using 10 times diluted standard plasmid ($2.78\times10^1 \sim 2.78\times10^8$copies/μL) as template, the established SYBR Green I RT-qPCR method had a good linear relationship, and R^2 was 0.9975. The detection limit was 2.78×10^1 copies/μL, which was similar to previous studies. Liang *et al*. (2019) established the TaqMan fluorescence quantitative PCR method for *siniperca chuatsi* virus, and the minimum detection limit was 10^2 copies/μL. Liu *et al*. (2014) established TaqMan fluorescence quantitative PCR for detection of HSHRV with a detection limit of 10^1 copies/μL. Gao *et al*. (2002) established the RT-PCR of cyprinus spring viremia virus with detection limit of 10^3 copies/μL. No specific amplification was found when the SYBR Green I RT-qPCR was used to detect GCRV, KHV, ISKNV and NNV, suggesting that the method has strong specificity. The coefficients of variation within and between groups were less than 1%, which ensured the repeatability of fluorescence quantitative detection. The positive rate of SYBR Green I RT-qPCR was 51.92% (54/104), which was higher than that of conventional PCR (21.15%, 22/104).

CONCLUSION

In conclusion, the SYBR Green I RT-qPCR method has the advantages of sensitive, specific and stable for MSRV early rapid detection, which has a great significance in prevention and control of the disease. This method can provide reliable technical support for MSRV clinical monitoring, and effectively controlling the spread and epidemic of MSRV.

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Data availability

Data will be made available on request.

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IRB approval

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Ethical statement

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Statement of conflict of interest

The authors have declared no conflict of interest.

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