Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Elsevier hereby grants permission to make all its COVID-19-related research that is available on the COVID-19 resource centre - including this research content - immediately available in PubMed Central and other publicly funded repositories, such as the WHO COVID database with rights for unrestricted research re-use and analyses in any form or by any means with acknowledgement of the original source. These permissions are granted for free by Elsevier for as long as the COVID-19 resource centre remains active.

Journal of Virological Methods 202 (2014) 39–45

Contents lists available at ScienceDirect

Journal of Virological Methods

journal homepage: www.elsevier.com/locate/jviromet

Development and evaluation of reverse transcription loop-mediated isothermal amplification assay for the detection of the fathead minnow nidovirus

Qingli Zhang[a,b], Isaac Standish[b], Andrew D. Winters[b,c], Corey Puzach[d], Rachel Ulferts[e,1], John Ziebuhr[e,f], Mohamed Faisal[b,c,*]

[a] Yellow Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences, 106 Nanjing Road, Qingdao, Shandong 266071, China
[b] Department of Pathobiology and Diagnostic Investigation, College of Veterinary Medicine, Michigan State University, East Lansing, MI 48824, USA
[c] Department of Fisheries and Wildlife, College of Agriculture and Natural Resources, Michigan State University, East Lansing, MI 48824, USA
[d] La Crosse Fish Health Laboratory, United States Fish and Wildlife Service, 555 Lester Avenue, Onalaska, WI 54650, USA
[e] Centre for Infection and Immunity, Queen's University Belfast, 97 Lisburn Road, Belfast BT9 7BL, United Kingdom
[f] Institute of Medical Virology, Justus Liebig University Giessen, Schubertstrasse 81, 35392 Giessen, Germany

Article history:
Received 17 October 2013
Received in revised form 19 February 2014
Accepted 21 February 2014
Available online 1 March 2014

Keywords:
Fathead minnow nidovirus
Reverse transcription loop-mediated isothermal amplification
Real-time
Quantitative RT-LAMP
Calcein

ABSTRACT

Fathead minnow nidovirus (FHMNV) is a serious baitfish-pathogenic virus in North America. Studies to trace the spread of the virus and determine its host range are hampered by the absence of reliable diagnostic assays. In this study, a one-step, reverse transcription loop-mediated isothermal amplification (RT-LAMP) assay was developed that targets a region in the FHMNV spike protein gene. The assay was optimized, and the best results were obtained at 8 mM of Mg^{2+} with an incubation time of 40 min at 63 °C in the presence of calcein. The analytical sensitivity of the RT-LAMP method was estimated to be as low as 5 viral copies and was 1000-fold more sensitive than the conventional reverse transcription polymerase chain reaction (RT-PCR) method. The diagnostic sensitivity and specificity of the developed RT-LAMP assay versus the RT-PCR assay was 100% and 95.7%, respectively. A quantitative RT-LAMP of FHMNV with a high correlation coefficient ($r^2 = 0.9926$) was also developed and the result of quantitation of viral copies in tissue samples of infected fish showed that the viral loads of the infected fish tissue samples reached up to 4.7×10^{10} copies per mg. It is anticipated that the developed RT-LAMP and quantitative RT-LAMP methods will be instrumental for diagnosis and surveillance of FHMNV.

© 2014 Elsevier B.V. All rights reserved.

1. Introduction

In 1997, a bacilliform virus was isolated from fathead minnows (*Pimephales promelas*) exhibiting external hemorrhages and necrotic changes in visceral organs (Iwanowicz and Goodwin, 2002). The isolated virus, originally believed to belong to the family *Rhabdoviridae*, measured 130–180 nm in length and 31–47 nm in diameter. Additional phylogenetic analyses based on the complete genome proved that the fathead minnow virus isolate is a species within the genus *Bafinivirus*, subfamily *Torovirinae*, family *Coronaviridae*, order *Nidovirales* and was named the fathead minnow nidovirus (FHMNV, Batts et al., 2012). The detection of FHMNV and the marked pathology it causes raised concerns about potential losses in the multi-billion dollar baitfish industry and the potential for transmitting this deadly infectious agent to other water bodies, thereby threatening other native fish species.

In order to augment sport and commercial fisheries in the Laurentian Great Lakes basin, natural resource agencies propagate species such as the muskellunge (*Esox masquinongy*) in hatcheries and then release them into inland lakes to establish sustainable populations (Dexter and O'Neal, 2004). This entails feeding live baitfish, primarily fathead minnows, to these propagated fish. The risk of transmitting FHMNV to these hatchery-propagated fish and the losses it may cause remains to be determined.

Currently, the only available diagnostic protocol for FHMNV is the reverse transcription polymerase chain reaction (RT-PCR) developed by Batts et al. (2012). This assay was used successfully to confirm the presence of FHMNV in cultured cell lines that exhibited

* Corresponding author at: Department of Pathobiology and Diagnostic Investigation, College of Veterinary Medicine, Michigan State University, East Lansing, MI 48824, USA. Tel.: +1 517 884 2019; fax: +1 517 432 2310.
E-mail address: faisal@cvm.msu.edu (M. Faisal).
[1] Department of Infectious Diseases & Immunology, Virology Division, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands.

http://dx.doi.org/10.1016/j.jviromet.2014.02.018
0166-0934/© 2014 Elsevier B.V. All rights reserved.

Table 1
Primers used for reverse transcriptase loop-mediated isothermal amplification of the fathead minnow nidovirus.

Primer	Sequence
F3	ACGTAAAGAATCTCAGAGATGA
B3	TGAACTTTTGAGAGGTGACT
FIP (F1c + TTTT + F2)	CGTCTGCTGTTGTTTTGACAAACTTTTGTGCAGCAAACTTCAAAGC
BIP (B1c + TTTT + B2)	TTGCAGCACAGAATGAAGTGATTTTATGGCCATATCCTTAAGGGA
LF	GCGTTGTGCTTTTCTTCGATG
LB	CAAAGGATTCAACTCAAACATCGAC

syncytia formation, a characteristic cytopathic effect of this nidovirus. Herein, we report on the development of a rapid and sensitive reverse transcription loop-mediated isothermal amplification (RT-LAMP) assay for the detection of FHMNV in infected cell lines and fish tissues. In addition, a protocol to quantify FHMNV in infected fish tissue samples has been developed.

2. Materials and methods

2.1. Cell, virus and template RNA

Epithelioma papulosum cyprini cell line (EPC) was used throughout this study. The cells were allowed to grow at 25 °C in 150 cm^2 tissue culture flasks containing Eagle's minimum essential medium (MEM, Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS, Gemini Bio-Products, CA, USA) and adjusted to pH of 7.4 using 7.5% sodium bicarbonate until 80–90% confluent and then incubated at 20 °C until used.

The initial FHMNV strain used was originally isolated in the Aquatic Animal Health Laboratory of Michigan State University, East Lansing, Michigan, USA, from spontaneously infected juvenile muskellunge. The virus was allowed to replicate in EPC cells until the characteristic giant cells dominated the cell monolayer. Viral RNA was extracted from infected EPC cells using a QIAamp viral RNA kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. For RNA extraction from infected fathead minnow and muskellunge kidney and spleen tissue samples, the QIAamp® viral RNA Mini Kit (Qiagen) was used. cDNA were synthesized by the Reverse Transcription System (Promega, Madison, WI, USA) using random primers.

2.2. Primer design for the FHMNV RT-LAMP

It has been shown that, for the majority of nidoviruses with large genomes (26.3–31.6 kb), the gene sequence encoding for three protein domains (3CLpro, RdRp, and HEL1) is highly conserved while other genes are more variable (Nga et al., 2011; Lauber et al., 2012). To develop an RT-LAMP assay specific to FHMNV, the spike glycoprotein gene, which has been shown to be comparatively variable among other genes within nidovirus genomes (Nga et al., 2011; Lauber et al., 2012), was chosen as the target gene for this assay. Three pairs of RT-LAMP primers, including a pair of loop primers, were designed as described by Nagamine et al. (2002). The spike protein gene from the FHMNV genome (GenBank accession number GU002364) was submitted to the website (http://primerexplorer.jp/elamp4.0.0/index.html) and a set of RT-LAMP primers was generated by the Primer Explorer software, version 4.0 (Table 1 and Fig. 1).

2.3. Optimization of FHMNV RT-LAMP conditions

The RT-LAMP reaction was conducted as described by Tomita et al. (2008) and Zhang et al. (2013), with minor modifications. The reaction was carried out in a 20 μL reaction mixture containing 1.6 μM each of FIP and BIP primers; 0.8 μM each of LF and LB primers; 0.2 μM each of F3 and B3 primers; 1 × isothermal amplification buffer (20 mM Tris–HCl, 10 mM $(NH_4)_2SO_4$, 50 mM KCl, 2 mM $MgSO_4$, 0.1% Tween 20, pH 8.8); 6 mM $MgCl_2$; 1 M betaine; 1.4 mM each deoxynucleoside triphosphate (dNTP); 0.2 mM $MnCl_2$; 20 μM Calcein; 2U AMV Reverse Transcriptase (Invitrogen); 8U *Bst* DNA polymerase (New England Biolabs, Beverly, MA, USA); and 1 μL of template RNA. The mixture was incubated for 60 min in a Mastercycler gradient PCR machine (Eppendorf, Hauppauge, NY, USA) at 57, 60, 63, 66 or 69 °C followed by 80 °C for 20 min to terminate the reaction. Reaction tubes were collected at 10, 20, 30, 40, 50 or 60 min to determine the optimal reaction time for amplification at 63 °C as predetermined. Likewise, the optimal concentration of Mg^{2+} was determined by running the reaction in the presence of 4, 6, 8, 10, or 12 mM at 63 °C for 40 min. RT-LAMP amplification products (2.5 μL) were subjected to electrophoresis on a 2.0% agarose gel.

2.4. Specificity of FHMNV RT-LAMP assay

The specificity of the developed assay was tested against three other nidoviruses, the white bream virus (WBV, Schütze et al.,

Fig. 1. RT-LAMP target spike protein gene for the fathead minnow nidovirus (GenBank accession number GU002364). Nucleotide sequences used for primer design are indicated by boxes and arrows. F, forward primer; B, backward primer; LF, loop-forward primer; LB, loop-backward primer.

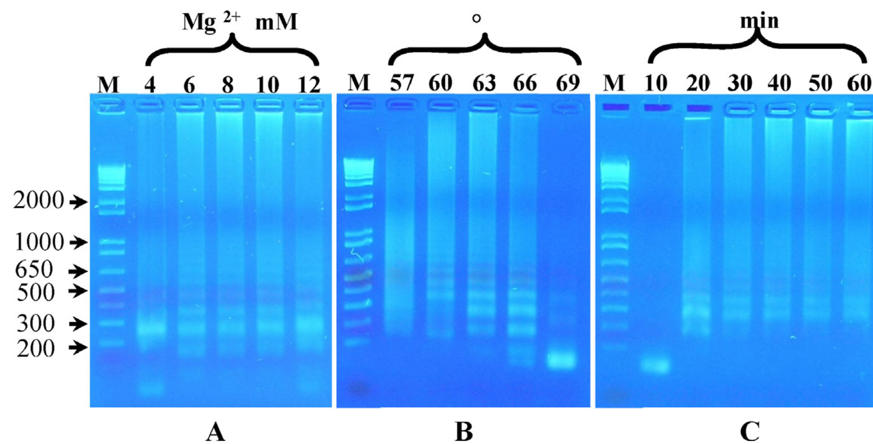

Fig. 2. Optimization of the RT-LAMP reaction for the detection of the fathead minnow nidovirus spike protein gene. (A) Effect of Mg^{2+} concentrations on the RT-LAMP reaction at 63 °C. (B) Effect of temperature on the RT-LAMP reaction using 8 mM Mg^{2+}. (C) Effect of reaction time on the RT-LAMP reaction using 8 mM $MgCl_2$, incubation at 64 °C plus DNA Ladder (Invitrogen, Carlsbad, CA, USA).

2006), which is the most closely related virus and only other known species within the genus *Bafinivirus* (Batts et al. (2012), as well as two viruses representing more distantly related nidoviruses: the human coronavirus 229E (HCoV-229E, genus *Alphacoronavirus*, subfamily *Coronavirinae*, family *Coronaviridae*) and Cavally virus (CavV, genus *Alphamesonivirus*, family *Mesoniviridae*) (Zirkel et al., 2011). In all cases, total RNA isolated from cells infected with WBV (EPC cells), HCoV-229E (HuH-7 cells) and CavV (C6/36 cells), respectively, was used as template. The cells were infected with the appropriate virus at a multiplicity of infection of 3 $TCID_{50}$ per cell and total RNA was isolated at 48 h p.i. Additionally, the specificity of the FHMNV LAMP assay was tested against three fish pathogenic rhabdoviruses isolated from infected cells: infectious hematopoietic necrosis virus (genus *Novirhabdovirus*), viral hemorrhagic septicemia virus genotype IVb (genus *Novirhabdovirus*), and the spring viremia of carp virus (genus *Vesiculorhabdovirus*). Additional fish pathogenic viruses used for specificity testing included the infectious pancreatic necrosis virus (genus *Aquabirnavirus*) isolated from infected cells and the genomic DNA of alloherpesvirus epizootic epitheliotropic disease virus (EEDV, salmonids herpesvirus 3), recovered from lake trout infected with EEDV. DNA and RNA of these viruses were used as template of the RT-LAMP using the primers designed for the detection of FHMNV under the predetermined conditions described above.

2.5. Analytical sensitivity of the FHMNV RT-LAMP assay

The detection limit of the RT-LAMP assay was analyzed with two kinds of templates. One of the templates was the total RNA extracted from the EPC cells infected by FHMNV. The other template was a plasmid pCR®2.1-TOPO® vector containing the target fragment from the FHMNV spike protein gene (designated as pCR®2.1-FHMNV). A 10-fold serial dilution of the total RNA (1.6 fg $\times 10^6$ to 10^{-1}) and the plasmid pCR®2.1-FHMNV (5×10^7 to 10^{-2} copies) were used as the template for qRT-LAMP under the conditions predetermined earlier. After the reaction, the products were analyzed on a 2% agarose gel and documented using LAS3000 Gel Imaging System (Fujifilm Medical Systems, Stamford, CT, USA).

2.6. Analytical sensitivity of the FHMNV RT-PCR assay

The detection limit of the developed FHMNV RT-LAMP assay was compared to the RT-PCR developed by Batts et al. (2012). A 10-fold serial dilution of the total RNA (1.6 fg $\times 10^6$ to 10^{-1}) was amplified by both the RT-PCR and FHMNV RT-LAMP. The SYBR® Green (Invitrogen) stained RT-PCR products were analyzed on a 1.5% agarose gel, while the RT-LAMP was performed under the optimized conditions described above.

2.7. Evaluation of the FHMNV RT-LAMP assay on samples

The newly developed RT-LAMP assay was used to determine the presence of FHMNV in 85 samples, including seven FHMNV isolates obtained using EPC cell lines from spontaneously infected fathead minnows; one isolate that was retrieved using EPC cell lines from spontaneously infected muskellunge; kidney and spleen tissue samples collected from 60 spontaneously infected hatchery propagated juvenile muskellunge (Wolf Lake State Fish Hatchery, Mattawan, MI, USA); eight kidney and spleen tissue samples from experimentally infected juvenile muskellunge; and nine kidney and spleen tissue samples experimentally infected fathead minnows. Total RNA of the samples was extracted and the optimized FHMNV RT-LAMP was performed as described above. Simultaneously, RT-PCR assays (as described by Batts et al., 2012) were performed on the same total RNA samples. Amplification products were electrophoresed and analyzed on a 2% agarose gel. The diagnostic sensitivity (DSe) and specificity (DSp) of the two methods, as defined by the World Organization for Animal Health (2011), were calculated as detailed in Zhang et al. (2013).

2.8. Quantitative FHMNV RT-LAMP assay

To determine viral load in infected fish tissues, the RNA extracted from the kidney and spleen tissue samples of the 77 fish used in 2.7 was amplified by quantitative RT-LAMP (qRT-LAMP). The qRT-LAMP assay was established by using ten-fold dilutions of FHMNV plasmid DNA (pCR®2.1-FHMNV) as standards and carried out in a final reaction volume of 20 μL, as described above. For real-time monitoring, the qRT-LAMP reactions were incubated at 63 °C for 40 cycles (one minute per cycle) with an Eppendorf realplex 2 (Eppendorf). The change of fluorescence value was monitored every minute at 520 nm.

3. Results

3.1. Determination of the conditions for FHMNV detection by RT-LAMP

The results demonstrated that we were able to optimize the RT-LAMP assay for the detection and quantitation of FHMNV in both infected cell culture and fish tissue samples. No clear bands

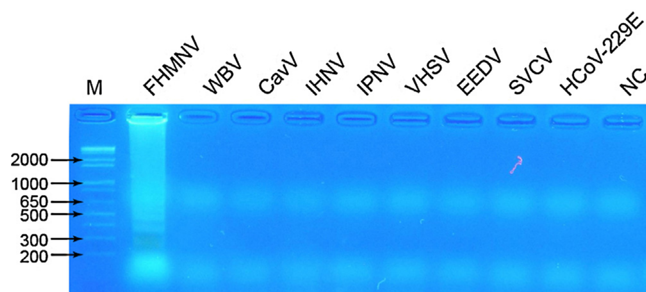

Fig. 3. Sybr green-stained agarose gel illustrating the specificity of amplification by the RT-LAMP assay using the primers designed for the detection of fathead minnow nidovirus (FHMNV). Amplification occurred only with the FHMNV but not with the white bream virus (WBV), the Cavally virus (CavV), the infectious hematopoietic necrosis virus (IHNV), infectious pancreatic necrosis virus (IPNV), viral hemorrhagic septicemia virus (VHSV), epizootic epitheliotropic disease virus (EEDV), spring viremia of carp virus (SVCV), or human coronavirus 229E (HCoV-229E). NC, negative control; M, 1Kb plus DNA Ladder.

were obtained when no additional $MgCl_2$ was added to the isothermal reaction buffer which contained 2 mM $MgSO_4$. The best results in terms of obtaining clear bands and maximizing amplification products were obtained when the Mg^{2+} concentration was at 8 mM (Fig. 2A). Therefore, we considered the optimal Mg^{2+} concentration to be 8 mM and further optimization experiments were performed at that concentration. The optimum temperature for RT-LAMP was found to be 63 °C, at which clear bands and maximum amplification products were obtained (Fig. 2B). Using the optimized Mg^{2+} concentration and incubation temperature, amplified products could be observed as early as 20 min after starting the reaction. An additional 10–20 min of reaction time, however, produced better amplification results (Fig. 2C). Based on these findings, further RT-LAMP assays were allowed a reaction time of 40 min at 63 °C with 8 mM Mg^{2+} final concentration.

3.2. Analytical specificity of the RT-LAMP assay for FHMNV

Positive results were only obtained when RNA from the FHMNV strains was used as template. RNA or DNA from other viruses including the closely related WBV was not amplified by the RT-LAMP (Fig. 3). Furthermore, both BLAST and EMBL searches revealed that the target sequence (189 bp) did not align with any other viral sequences available in these databases, including members of other nidovirus (sub)families, such as the equine torovirus (EToV, genus *Torovirus*), gill-associated virus (GAV *Okavirus*), infectious bronchitis virus (IBV *Gammacoronavirus*) and equine arteritis virus (EAV *Arterivirus*). Altogether, these results indicate that the RT-LAMP method is specific for amplification of FHMNV nucleic acid.

3.3. Comparison of the analytical sensitivities of RT-LAMP and RT-PCR

When the reaction was tested using 1 μL of 10-fold serial dilutions of plasmid pCR®2.1-FHMNV DNA (23.3 ng/μL equivalent to 5.4×10^9 copies/μL), the analytical sensitivity of the qRT-LAMP method was estimated to be as low as five copies of the plasmid (Fig. 4A). When the reactions were tested using 1 μL of 10-fold serial dilutions of RNA from EPC cells infected by FHMNV both for RT-LAMP and RT-PCR, the analytical sensitivities of the RT-LAMP method and RT-PCR were 1.6 fg and 1.6 pg total RNA, respectively (Fig. 4B). The DNA fragment produced by the RT-PCR was 278 bp (Fig. 4C).

Table 2
Detection of the fathead minnow nidovirus from infected EPC cell culture and infected fish tissue samples by both the newly developed RT-LAMP and conventional RT-PCR of Batts et al. (2012).

Detection results	RT-PCR positive	RT-PCR negative	Total
FHMNV-RT-LAMP positive	38	2[a]	40
FHMNV-RT-LAMP negative	0	45	45
Total	38	47	85

[a] Both negative samples by RT-PCR were from infected fish samples.

3.4. Application of the newly developed RT-LAMP assay for detection of FHMNV in tissue culture and fish tissue samples

Eight FHMNV isolates and 60 spontaneously infected fish samples were used simultaneously to compare between the RT-LAMP and RT-PCR methods. Results of both RT-LAMP and RT-PCR confirmed the identity of the eight isolates as FHMNV. RT-PCR results indicated that 13 of the 60 fish samples were infected by FHMNV and the rest of the samples were FHMNV-negative. The RT-LAMP assay showed that the RNA templates from the 13 FHMNV-positive samples as determined by the RT-PCR also produced positive RT-LAMP results. Furthermore, two of the 47 samples that were FHMNV-negative by RT-PCR yielded a positive result by the RT-LAMP (Tables 2 and 3). Therefore, the DSe and DSp of the RT-LAMP method compared to the RT-PCR method were 100% and 95.7%, respectively.

3.5. Quantitative RT-LAMP assay

For the quantitative detection of FHMNV in samples, a standard curve was generated for FHMNV qRT-LAMP by plotting a graph between different concentrations of pCR®2.1-FHMNV plasmids ranging from 10^7 to 10^2 copy numbers to cycle threshold (*Ct*) value through real-time monitoring of the amplification. A high correlation coefficient ($r^2 = 0.9926$) was obtained by the qRT-LAMP (Fig. 5A and B). Based on the *Ct* value of all RNA samples, the quantitation of viral copies in the kidney and spleen of 77 clinical samples was extrapolated by employing the standard curve. The viral loads of the positive clinical samples varied within the range of 4.3×10^2 to 4.7×10^{10} copies per mg tissue (Table 3).

4. Discussion

Early detection of FHMNV is important for the effective health management of this emerging infection. The developed single tube, accelerated RT-LAMP combines reverse transcription and amplification in one step, amplifies target nucleic acids efficiently, is both time- and cost-effective, and represents a valuable diagnostic tool for the detection and titration of FHMNV. This is useful for compliance with current regulations within the bait industry requiring timely and accurate health certifications for the transport and use of fish in public waters.

As a first step, we optimized the assay conditions to run at 8 mM Mg^{2+} concentration in the amplification buffer and incubation at 63 °C for 40 min. These conditions are consistent with those found for other single-stranded RNA viruses such as the infectious hematopoietic necrosis virus (Gunimaladevi et al., 2005), the rift valley fever virus (Peyrefitte et al., 2008) and the Crimean Congo hemorrhagic fever virus (Osman et al., 2013). The results of the temperature optimization showed that *Bst* DNA polymerase effectively amplified the nucleic acid templates at temperatures of 63–66 °C, which is consistent with the classic reports on LAMP (Notomi et al., 2000; Mori et al., 2001, 2006). The effective amplification of FHMNV nucleic acid by RT-LAMP in a wide temperature range should greatly benefit the future application of the method under

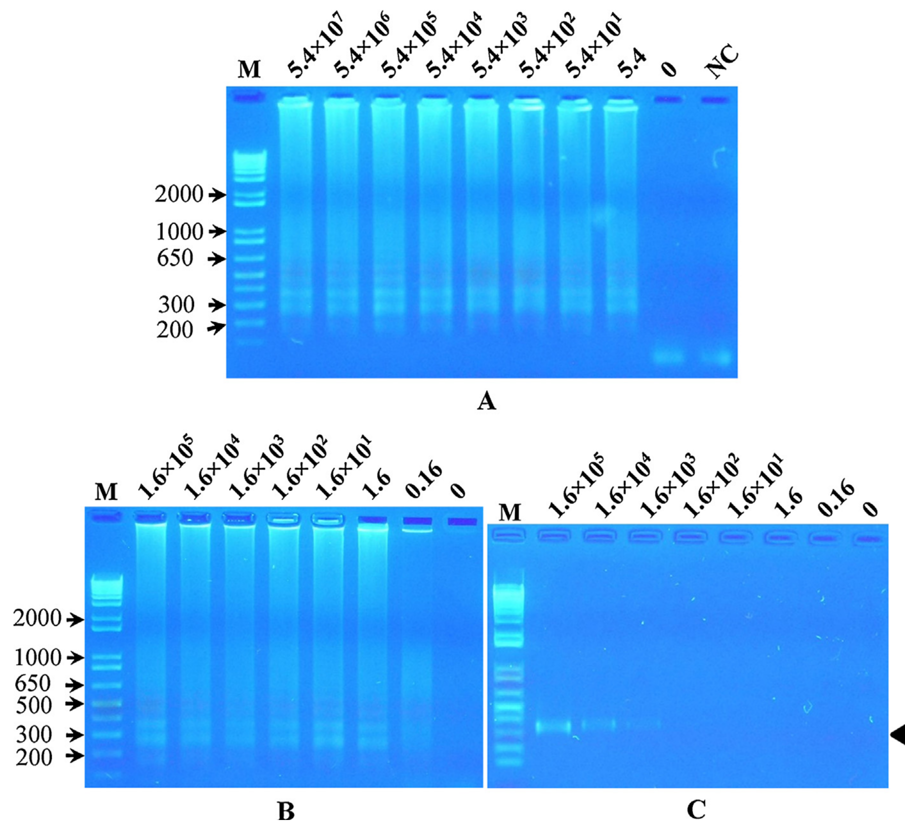

Fig. 4. Sensitivity of RT-LAMP and RT-PCR detection methods for the fathead minnow nidovirus (FHMNV). (A) Sensitivity of RT-LAMP detection of pCR®2.1-FHMNV plasmid containing the targeted DNA fragment from the spike protein gene of FHMNV. In lanes 1–9 the reaction was conducted using 10-fold serial dilutions of the plasmid (pCR®2.1-FHMNV) DNA of 5.4×10^7 to 0 copies, respectively, while lane 10 served as a negative control. (B) Sensitivity of RT-LAMP detection of RNA from EPC cells infected by FHMNV. In lanes 1–8 the reaction was conducted using 10-fold serial dilutions of RNA from FHMNV infected fish of 1.6×10^5 to 0 fg, respectively. (C) Sensitivity of RT-PCR detection of RNA from FHMNV infected fathead minnow tissues. In lanes 1–8, the reaction was conducted using 10-fold serial dilutions of RNA from FHMNV infected fish of 1.6×10^5 to 0 fg, respectively. The sizes of the DNA fragment produced by the RT-PCR were 278 bp, as shown by the arrowhead. M, 1 Kb plus DNA Ladder.

field conditions. With 16 ng RNA (approximately 10^6 copies of virus, determined by quantitative RT-LAMP) from EPC cells infected with FHMNV as template, clear positive bands can be observed even when the incubation time is 20 min due to the addition of loop primers, which proved to accelerate the LAMP reaction (Nagamine et al., 2002; Yang et al., 2012) and also showed the advantage of rapid RT-LAMP.

The data generated by testing several viruses (the closely related WBV, two other nidoviruses, and other common fish-pathogenic viruses), along with the non-alignment of the FHMNV target sequence with any viral sequences currently available in public databases demonstrate that the RT-LAMP assay developed in this study is specific for FHMNV. We plan to regularly validate and, if needed, further improve the specificity of the assay when novel closely related nidoviruses are discovered. The developed assay is estimated to detect as little as five copies, which is considered very sensitive. These results are in line with previously described LAMP assays developed for a number of viruses, such as the severe acute

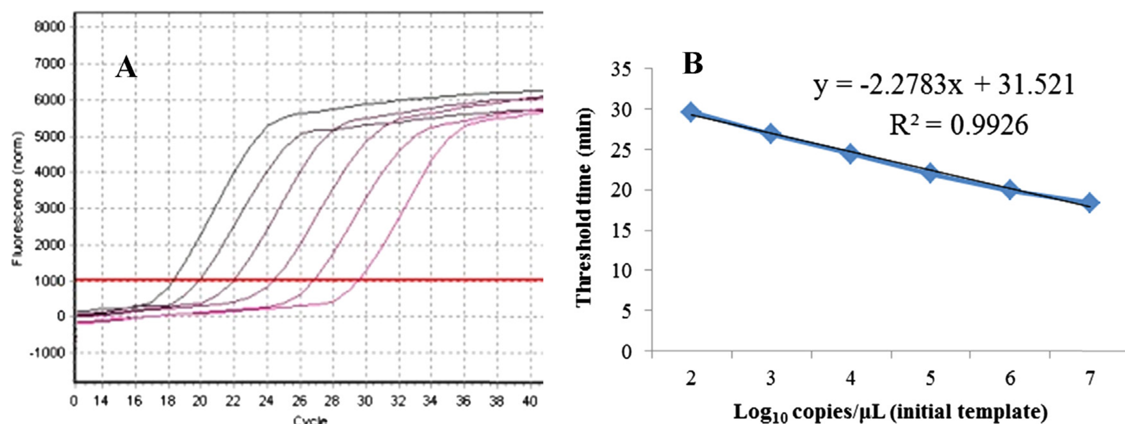

Fig. 5. The amplification plots and standard curve of the serial 10-fold dilution plasmids by qRT-LAMP. (A) The amplification plots of qRT-LAMP of serial 10-fold dilution plasmids. Copies of pCR®2.1-FHMNV plasmids: 5.4×10^7, 5.4×10^6, 5.4×10^5, 5.4×10^4, 5.4×10^3 and 5.4×10^2 (from left to right). (B) Standard curve and standard curve equation for the fathead minnow nidovirus-specific qRT-LAMP assay generated from the amplification plots between the serial 10-fold diluted pCR®2.1-FHMNV plasmid and *Ct* value.

Table 3
Comparison of results of conventional RT-PCR (Batts et al., 2012) assay and the newly developed qRT-LAMP for the detection of the fathead minnow nidovirus in fish tissue samples of spontaneously and experimentally infected muskellunge and fathead minnows. Virus load is expressed as copies/mg tissues.

#	RT-PCR	qRT-LAMP #copies	#	RT-PCR	qRT-LAMP #copies	#	RT-PCR	qRT-LAMP #copies
1[a]	–	None	27	–	None	53	–	None
2	–	None	28	–	None	54	–	None
3	–	None	29	–	None	55	–	None
4	–	None	30	–	None	56	–	None
5	–	None	31	–	None	57	–	None
6	–	None	32	+	3.1×10^4	58[d]	–	6.1×10^4
7	–	None	33	–	None	59	–	None
8	+	2.4×10^4	34	–	None	60	–	None
9	+	9×10^3	35	–	None	61[b]	+	4.7×10^{10}
10	+	1.2×10^5	36	–	None	62	+	3.8×10^4
11	+	6.7×10^3	37	–	None	63	+	4.3×10^7
12	–	None	38	–	None	64	+	3.6×10^5
13	–	None	39	–	None	65	+	3.9×10^6
14	–	None	40	+	1.6×10^4	66	+	4.8×10^3
15	–	None	41	+	1.5×10^4	67	+	2.0×10^5
16	+	1.6×10^4	42	+	2.7×10^4	68	+	4.3×10^2
17	+	1.3×10^4	43	+	2.6×10^4	69[c]	+	1.2×10^5
18	–	None	44	–	None	70	+	3.5×10^6
19	–	None	45	–	None	71	+	1.8×10^5
20	–	None	46	–	None	72	+	8.6×10^4
21	–	None	47	–	None	73	+	1.6×10^6
22	–	None	48	–	None	74	+	2.6×10^4
23	–	None	49	–	None	75	+	1.6×10^4
24	–	None	50[d]	–	4.1×10^3	76	+	1.1×10^5
25[d]	–	2.7×10^3	51	–	None	77	+	9.2×10^3
26	+	6.4×10^4	52	–	None			
PC	+	4.0×10^4	NC	–	None			

PC represents positive control and NC represents negative control.

[a] Samples 1–60 are from spontaneously infected muskellunge.
[b] Samples 61–68 are from experimentally infected juvenile muskellunge.
[c] Samples 69–77 are from experimentally infected fathead minnows.
[d] Notice samples 25, 50, and 58 were negative by RT-PCR but tested positive by qRT-LAMP.

respiratory syndrome coronavirus (Hong et al., 2004), noroviruses (Yoda et al., 2007; Suzuki et al., 2011), human astrovirus (Wei et al., 2013), and influenza A virus (H7N9, Ge et al., 2013). On the other hand, the analytical sensitivities of the RT-LAMP method and RT-PCR were 1.6 fg and 1.6 pg of RNA extracted from FHMNV-infected EPC cells, respectively. This suggests a 1000-fold higher analytical sensitivity of the RT-LAMP assay than that of the RT-PCR assay. This finding is in agreement with the reports of Chen et al. (2011) for the *Scylla serrata* reovirus; Arunrut et al. (2011) for the Laem-Singh virus; and Wang et al. (2013) for the hepatitis delta virus genotype I, which reveals that RT-LAMP is more sensitive than the conventional RT-PCR for these viruses. This increased sensitivity makes the RT-LAMP a better choice than the conventional RT-PCR for the detection of FHMNV, particularly when virus titers are very low.

A standard curve was constructed using serial 10-fold dilutions of the pCR®2.1-FHMNV plasmid with reference to the *Ct* value. Based on the standard curve, an equation was calculated using regression analysis comparing the *Ct* value to the standard copy number. In the range of 10^7–10^2 plasmid copies, the correlation coefficient was high ($r^2 = 0.9926$), which indicates that the qRT-LAMP is appropriate as a quantitation tool. However, when numbers of plasmid decrease to less than 100 copies, the correlation coefficient declines significantly ($r^2 = 0.9437$, data not shown). Previous reports also demonstrated that it is difficult to determine the exact correlation of virus quantity and *Ct* value at very low concentrations of template (Suzuki et al., 2011; Wei et al., 2013).

In conclusion, we report the development of an RT-LAMP assay for the detection of FHMNV from cell culture and fish tissues. This novel assay has the advantage of being rapid, sensitive, and specific. Moreover, the qRT-LAMP established in this study provides a low-cost quantitation method for FHMNV.

Acknowledgements

The authors would like to thank Michelle R. Gunn and Elena V. Millard for their excellent help. The authors also thank Mr. Daniel Bjorklund for editing the figures. This work was supported by the State Scholarship Fund from the China Scholarship Council (Beijing, The People's Republic of China) for QZ and a grant from the United States Fish and Wildlife Service (USFWS) (Grant No. USFWS F12AP00632) for MF.

References

Arunrut, N., Seetang-Nun, Y., Phromjai, J., Panphut, W., Kiatpathomchai, W., 2011. Rapid and sensitive detection of Laem-Singh virus by reverse transcription loop-mediated isothermal amplification combined with a lateral flow dipstick. J. Virol. Methods 177, 71–74.

Batts, W.N., Goodwin, A.E., Winton, J.R., 2012. Genetic analysis of a novel nidovirus from fathead minnows. J. Gen. Virol. 93, 1247–1252.

Chen, J., Xiong, J., Cui, B., Yang, J., Mao, Z., Li, W., Chen, X., Zheng, X., 2011. Rapid and sensitive detection of mud crab *Scylla serrata* reovirus by a reverse transcription loop-mediated isothermal amplification assay. J. Virol. Methods 178, 153–160.

Dexter, J.L., Richard Paul, O.N., 2004. Michigan Fish Stocking Guidelines II: With Periodic Updates. Michigan Department of Natural Resources, Fisheries Division.

Ge, Y., Wu, B., Qi, X., Zhao, K., Guo, X., Zhu, Y., Qi, Y., Shi, Z., Zhou, M., Wang, H., Cui, L., 2013. Rapid and sensitive detection of novel avian-origin influenza A (H7N9) virus by reverse transcription loop-mediated isothermal amplification combined with a lateral-flow device. PLoS ONE 8, e69941.

Gunimaladevi, I., Kono, T., LaPatra, S.E., Sakai, M., 2005. A loop mediated isothermal amplification (LAMP) method for detection of infectious hematopoietic necrosis virus (IHNV) in rainbow trout (*Oncorhynchus mykiss*). Arch. Virol. 150, 899–909.

Hong, T.C., Mai, Q.L., Cuong, D.V., Parida, M., Minekawa, H., Notomi, T., Hasebe, F., Morita, K., 2004. Development and evaluation of a novel loop-mediated isothermal amplification method for rapid detection of severe acute respiratory syndrome coronavirus. J. Clin. Microbiol. 42, 1956–1961.

Iwanowicz, L.R., Goodwin, A.E., 2002. A new bacilliform fathead minnow rhabdovirus that produces syncytia in tissue culture. Arch. Virol. 147, 899–915.

Lauber, C., Ziebuhr, J., Junglen, S., Drosten, C., Zirkel, F., Nga, P., Morita, K., Snijder, E., Gorbalenya, A., 2012. Mesoniviridae: a proposed new family in the order

Nidovirales formed by a single species of mosquito-borne viruses. Arch. Virol. 157, 1623–1628.

Mori, N., Motegi, Y., Shimamura, Y., Ezaki, T., Natsumeda, T., Yonekawa, T., Ota, Y., Notomi, T., Nakayama, T., 2006. Development of a new method for diagnosis of rubella virus infection by reverse transcription-loop-mediated isothermal amplification. J. Clin. Microbiol. 44, 3268–3273.

Mori, Y., Nagamine, K., Tomita, N., Notomi, T., 2001. Detection of loop-mediated isothermal amplification reaction by turbidity derived from magnesium pyrophosphate formation. Biochem. Biophys. Res. Commun. 289, 150–154.

Nagamine, K., Hase, T., Notomi, T., 2002. Accelerated reaction by loop-mediated isothermal amplification using loop primers. Mol. Cell. Probes 16, 223–229.

Nga, P.T., Parquet, M.d.C., Lauber, C., Parida, M., Nabeshima, T., Yu, F., Thuy, N.T., Inoue, S., Ito, T., Okamoto, K., Ichinose, A., Snijder, E.J., Morita, K., Gorbalenya, A.E., 2011. Discovery of the first insect nidovirus, a missing evolutionary link in the emergence of the largest RNA virus genomes. PLoS Pathog. 7, e1002215.

Notomi, T., Okayama, H., Masubuchi, H., Yonekawa, T., Watanabe, K., Amino, N., Hase, T., 2000. Loop-mediated isothermal amplification of DNA. Nucleic Acids Res. 28, E63.

Osman, H.A.M., Eltom, K.H., Musa, N.O., Bilal, N.M., Elbashir, M.I., Aradaib, I.E., 2013. Development and evaluation of loop-mediated isothermal amplification assay for detection of Crimean Congo hemorrhagic fever virus in Sudan. J. Virol. Methods 190, 4–10.

Peyrefitte, C.N., Boubis, L., Coudrier, D., Bouloy, M., Grandadam, M., Tolou, H.J., Plumet, S., 2008. Real-time reverse transcription loop-mediated isothermal amplification for rapid detection of Rift Valley Fever Virus. J. Clin. Microbiol. 46, 3653–3659.

Schütze, H., Ulferts, R., Schelle, B., Bayer, S., Granzow, H., Hoffmann, B., Mettenleiter, T.C., Ziebuhr, J., 2006. Characterization of white bream virus reveals a novel genetic cluster of nidoviruses. J. Virol. 80, 11598–11609.

Suzuki, Y., Narimatsu, S., Furukawa, T., Iwakiri, A., Miura, M., Yamamoto, S., Katayama, H., 2011. Comparison of real-time reverse transcription loop-mediated isothermal amplification and real-time reverse transcription polymerase chain reaction for detection of noroviruses in municipal wastewater. J. Biosci. Bioeng. 112, 369–372.

Tomita, N., Mori, Y., Kanda, H., Notomi, T., 2008. Loop-mediated isothermal amplification (LAMP) of gene sequences and simple visual detection of products. Nat. Protoc. 3, 877–882.

Wang, C., Shen, X., Lu, J., Zhang, L., 2013. Development of a reverse transcription loop-mediated isothermal amplification (RT-LAMP) system for rapid detection of HDV genotype 1. Lett. Appl. Microbiol. 56, 229–235.

Wei, H., Zeng, J., Deng, C., Zheng, C., Zhang, X., Ma, D., Yi, Y., 2013. A novel method of real-time reverse transcription loop-mediated isothermal amplification developed for rapid and quantitative detection of human astrovirus. J. Virol. Methods 188, 126–131.

World Organization for Animal Health (OIE), 2011. Principles and methods of validation of diagnostic assays for infectious diseases. In: Manual of Diagnostic Tests for Aquatic Animals. OIE, Paris, France (chapter 1.1.2).

Yang, J.L., Ma, G.P., Yang, R., Yang, S.Q., Fu, L.Z., Cheng, A.C., Wang, M.S., Zhang, S.H., Shen, K.F., Jia, R.Y., Deng, S.X., Xu, Z.Y., 2012. Simple and rapid detection of Salmonella serovar Enteritidis under field conditions by loop-mediated isothermal amplification. J. Appl. Microbiol. 109 (5), 1715–1723.

Yoda, T., Suzuki, Y., Yamazaki, K., Sakon, N., Kanki, M., Aoyama, I., Tsukamoto, T., 2007. Evaluation and application of reverse transcription loop-mediated isothermal amplification for detection of noroviruses. J. Med. Virol. 79 (863), 326–334, Erratum In: J. Med. Virol.

Zhang, Q.L., Yan, Y., Shen, J.Y., Hao, G.J., Shi, C.Y., Wang, Q.T., Liu, H., Huang, J., 2013. Development of a reverse transcription loop-mediated isothermal amplification assay for rapid detection of grass carp reovirus. J. Virol. Methods 187, 384–389.

Zirkel, F., Kurth, A., Quan, P.-L., Briese, T., Ellerbrok, H., Pauli, G., Leendertz, F.H., Lipkin, W.I., Ziebuhr, J., Drosten, C., Junglen, S., 2011. An insect nidovirus emerging from a primary tropical rainforest. mBio 2, e00077-11.