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Reverse transcriptase loop-mediated isothermal amplification assay for infectious hematopoietic necrosis virus in *Oncorhynchus keta*

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ABSTRACT: A reverse transcriptase loop-mediated isothermal amplification (RT-LAMP) assay was developed for detecting infectious hematopoietic necrosis virus (IHNV) from chum salmon *Oncorhynchus keta* in South Korea with high specificity, sensitivity and rapidity. A set of 6 IHNV-specific primers was designed, based on the G-protein sequence of IHNV (PRT strain), recognizing 8 distinct sequences of the target RNA. The assay was optimized to detect IHNV at 63°C for 30 min. The limit of detection was 0.01 fg of RNA extracted from IHNV-infected CHSE-214 cells, compared with 1.0 fg for nested RT-PCR. The applicability of this RT-LAMP assay was further tested by comparison with nested RT-PCR using field samples. Of 473 samples tested, 191 samples (40.38%) were IHNV-positive by RT-LAMP, whereas 162 samples (34.25%) were IHNV-positive by nested RT-PCR. These results indicate that, because of its high sensitivity and rapidity, the RT-LAMP assay is useful for early diagnosis of IHN.

KEY WORDS: RT-LAMP · IHNV · Chum salmon · *Oncorhynchus keta*

INTRODUCTION

Infectious hematopoietic necrosis virus (IHNV) is a member of the genus *Novirhabdovirus* (family Rhabdoviridae) and has a linear single-strand, negative-sense RNA genome of ~11 000 nucleotides (nt). The IHNV genome consists of 6 genes encoding the nucleocapsid protein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G), nonvirion protein (NV) and polymerase (L) (Kurath et al. 1985, Morzunov et al. 1995). The G, N and L genes have been employed for investigating IHNV evolution, diversity and phylogenetic relationships among isolates worldwide (Garver et al. 2003, Kim et al. 2003, 2007, Enzmann et al. 2005, Nishizawa et al. 2006)

IHNV was first reported from fish hatcheries in Oregon and Washington, USA, in the 1950s (Rucker et al. 1953) and its worldwide distribution is thought to be due to movement of contaminated fish and fish eggs to Asian and European countries (Nishizawa et al. 2006, Kim et al. 2007). Consequently, the virus has been reported in various countries including Japan (Sano 1976), France (Baudin-Laurencin 1987), Italy (Bovo et al. 1987) and Germany (Enzmann et al. 1992). However, there was no IHNV occurrence in Korea until 1991, when the virus was recorded in juvenile rainbow trout *Oncorhynchus mykiss* and masu salmon *O. masou* in Kangwon Province, South Korea (Park et al. 1993). Since then, a series of IHN outbreaks have occurred in rainbow trout farms in various parts of Korea. In 2003, Korean rainbow trout farms experienced economic losses because of IHN in both juvenile and market-size fish (Kim et al. 2003). Three major virus genogroups designated U, M and L, indicating their correlation with the upper, middle and lower geographic areas in the Pacific Northwest of North America, were proposed (Garver et al. 2003, Kurath et al. 2003). More recently, 2 additional genogroups for European and Japanese (JRt) isolates were proposed (Enzmann et al. 2005, Nishizawa et al. 2006).

There are several technologies for detecting IHNV (Kim et al. 2001), including the conventional RT-PCR

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method. However, nested RT-PCR methods require a second amplification step that is prone to contamination. To solve these problems, the loop-mediated isothermal amplification (LAMP) assay was investigated as an alternative. LAMP was originally developed by Notomi et al. (2000) and can amplify very low numbers of target sequences to 10^9 copies under isothermal conditions within 1 h. This method depends on auto-cycling strand displacement DNA synthesis by the *Bst* DNA polymerase large fragment with high strand displacement activity, and a set of 2 specially designed inner primers and 2 outer primers. LAMP is highly specific because the target sequences are detected by 6 independent primers in the initial stage, followed by 4 independent primers in the later stages of the LAMP reaction. LAMP is also applicable for RNA detection by using a reverse transcriptase (RT) together with a DNA polymerase (Notomi et al. 2000). This technique can be carried out under isothermal conditions, which can be simply achieved with a water bath or a heating block. Expensive thermal cyclers used for PCR are not required (Notomi et al. 2000).

In the present study, an RT-LAMP assay was developed for detecting IHNV from wild adult chum salmon *Oncorhynchus keta* and artificially hatched fry in Korea. A set of 6 LAMP-specific primers was designed, based on the G protein sequence of Korean IHNV (PRT strain, accession no. AY673684). The applicability of the RT-LAMP assay was evaluated by comparison with the nested RT-PCR assay using field samples.

MATERIALS AND METHODS

Viruses and cell culture. IHNV (PRT strain) was amplified in chinook salmon embryo (CHSE)-214 cells. Infectious pancreatic necrosis virus (IPNV) (VR-299, Jasper, Sp and Ab strains) and viral hemorrhagic septicemia virus (VHSV) were maintained in rainbow trout gonad (RTG)-2 cells with minimum essential medium-10 (supplemented with 10% fetal bovine serum, 200 mM L-glutamine, 50 IU ml^{-1} penicillin and 50 mg ml^{-1} streptomycin) at 15°C.

RNA extraction. To establish the optimal conditions for RT-LAMP, RNA was extracted from 500 µl of supernatant from IHNV-infected cell cultures. For diagnosis, RNA was extracted from 100 mg of fresh kidney and spleen taken from adult chum salmon and fry, homogenized in 750 µl of TRI Reagent® (Molecular Research Center), and incubated at room temperature for 5 min. In both RNA extractrions, 200 µl of chloroform was added to the homogenate and mixed by vortexing. The suspensions were incubated at room temperature for 10 min and centrifuged at 10 000 × *g* for 10 min. The upper clear solution was transferred into a new 1.5 ml microcentrifuge tube and the RNA precipitated using 200 µl of 100% isopropanol on ice for 10 min before centrifugation at 10 000 × *g* for 10 min. The RNA pellet was washed with 70% (v/v) ethanol, centrifuged at 5000 × *g* for 5 min, then dried at 95°C in the incubator for 2 min or until the ethanol had evaporated. Diethylpyrocarbonate-treated water (Bioneer) was added to make a final concentration of 100 ng $µl^{-1}$ and the RNA preparation was stored at –20°C until use. RT-LAMP and RT-PCR amplification were carried out using 2 µl of this RNA as template.

Primers for RT-LAMP assay. RT-LAMP primers for IHNV were designed based on the published sequence of the G gene of IHNV (PRT strain, accession no. AY673684) using the software Primer Explorer version 4 (http://primerexplorer.jp/e/lamp4.0.0/index.html; Eiken Chemical). The forward inner primer (FIP) consisted of the complementary sequence of F1c (22 nt), a TTTT linker and the sense sequence of F2 (18 nt). The backward inner primer (BIP) contained a sense sequence of B1c (22 nt), a TTTT linker and the complementary sequence of B2 (20 nt). The outer primers consisted of F3 (18 nt) and the complementary sequence of B3 (18 nt). To increase RT-LAMP sensitivity, loop primers were designed, which contained a loop forward primer (LF; 20 nt) and loop backward primer (LB; 25 nt). The details of the primers are listed in Table 1.

Optimization of RT-LAMP conditions. For optimization of the temperature and time conditions used for the RT-LAMP assay, reactions were carried out at 58, 60, 63 or 65°C for 30, 45 or 60 min. The influence of Mg^{2+}, betaine, dNTPs, *Bst* DNA polymerase and *Avian myeloblastosis virus* (AMV) reverse transcriptase was also tested. The LAMP reactions were performed in 25 µl of total reaction mixture containing: 2 µM each FIP and BIP, 0.2 µM each F3 and B3, 2 µM LF and LB primers, 1× thermopol-supplied reaction buffer, with 0.6 to 1.4 M betaine (Sigma-Aldrich), 2 to 10 mM $MgSO_4$ (Sigma-Aldrich), 1 to 1.4 mM dNTPs mix (Takara), 6 to 12 U of *Bst* DNA polymerase (large fragment; New England Biolabs) and 0.25 to 0.75 U of AMV reverse transcriptase (Promega). Extracts from uninfected CHSE-214 cell cultures and reaction mixture without template were included as negative controls. RT-LAMP products were analyzed by 2% agarose gel electrophoresis. The time required to complete the LAMP reaction protocol was ~60 min.

Nested RT-PCR for IHNV detection. cDNA synthesis was conducted with 1 µg of total RNA using an M-MLV reverse transcriptase kit (Bioneer) following the manufacturer's instructions. Five µl of cDNA were mixed with 20 µM each of primers EXT-1 and EXT-2 (Table 2) (Troyer & Kurath 2003) and transferred into an AccuPower® PCR PreMix tube (Bioneer); the reaction

Table 1. Oligonucleotide primers used for reverse transcriptase loop-mediated isothermal amplification detection of infectious hematopoietic necrosis virus in chum salmon *Oncorhynchus keta*. BIP: backward inner primer; FIP: forward inner primer; LB: loop backward; LF: loop forward

Primer	Position	Sequence (5′–3′)
F3	110–127	CAGCCAAACCGTCCAACC
B3	299–316	TCGTTTCCGACCGACAGG
FIP	179–200/TTTT/128–145	CAGAGTGCATCCCTCGGGGTAG/TTTT/CGACACCGCAAGCGAATC
BIP	220–241/TTTT/269–288	ATGCCTCTCAACTGAGATGCCC/TTTT/ATGTGGGATAGGCAATCAGC
LF	158–177	TGAAGAGCGGGTTTGACCAG
LB	243–267	AGGATCTTCGATGATGAGAATAGGG

volume was made up to 20 µl with DEPC-treated water (Bioneer). PCR amplification was conducted with an initial cycle at 95°C for 5 min followed by 30 cycles of 95°C for 30 s, 50°C for 30 s and 72°C for 60 s. A final extension step was conducted at 72°C for 7 min. The expected PCR product was 672 bp. A nested PCR amplification was carried out using 5 µl of the first PCR products mixed with 20 µM each of primers INT-1 and INT-2 (Table 2) (Troyer & Kurath 2003), transferred into an AccuPower® PCR PreMix tube (Bioneer) and made up to a final volume of 20 µl with DEPC-treated water. PCR reaction conditions were the same as described above. The expected PCR product was 462 bp in size.

Comparison of sensitivity between RT-LAMP and nested RT-PCR assays. The sensitivity of the 2 assays was determined using 10-fold dilutions (100 pg to 0.001 fg) of RNA extracted from IHNV-infected CHSE-214 cell cultures as template with the optimized conditions for the RT-LAMP and the nested RT-PCR as described above.

Specificity of the RT-LAMP assay. Specificity was tested using 100 ng of RNA extracted from IPNV- (VR-299, Ab, Jasper and Sp strains) and VHSV-infected RTG-2 cell cultures as non-target templates.

Evaluation of the RT-LAMP assay with field samples. The applicability of the RT-LAMP assay to detect IHNV was evaluated by comparing its ability to detect IHNV in field samples with that of nested RT-PCR. From 2006 to 2009, samples of adult wild chum salmon and artificially hatched fry were randomly collected from the Namdae River basin and hatcheries at Yangyang City, located on the east coast of South Korea. The kidney and spleen from individual adult fish and pools of 5 fry were used for RNA extraction as described above. A total of 473 samples were analysed using RT-LAMP and nested RT-PCR as described above.

Table 2. Oligonucleotide primers used for nested reverse transcriptase PCR detection of infectious hematopoietic necrosis virus in chum salmon *Oncorhynchus keta*

Primer	Orientation	Position	Sequence (5′–3′)
EXT-1	Sense	563–583	AGAGATCCCTACACCAGAGAC
EXT-2	Antisense	1235–1255	GGTGGTGTTGTTTCCGTGCAA
INT-1	Sense	623–643	TCACCCTGCCAGACTCATTGG
INT-2	Antisense	1085–1105	ATAGATGGAGCCTTTGTGCAT

RESULTS

Optimization of RT-LAMP reaction conditions

When RT-LAMP was conducted at 58, 60, 63 and 65°C using 100 pg of RNA, product was detected only at 63°C (Fig. 1). Moreover, RT-LAMP products were seen as multiple bands of different sizes on 2% agarose gel following run times of 30, 45 or 60 min when using 10 ng and 100 pg of RNA (Fig. 2). Therefore, the optimal time was determined as 30 min for detecting IHNV by RT-LAMP in this study.

The influence of varying the concentrations of $MgSO_4$, betaine, dNTPs, *Bst* DNA polymerase and AMV reverse transcriptase for RT-LAMP reaction were determined using 100 pg of RNA as template. As the concentration of $MgSO_4$ increased from 2 to 10 mM, the intensity of the LAMP products also increased and remained high from 6 to 10 mM $MgSO_4$ (Fig. 3A). The optimal $MgSO_4$ concentration was hence determined to be 6 mM. Under the optimal $MgSO_4$ concentration, the RT-LAMP reactions were not affected by the different concentrations of betaine (0.6 to 1.4 M). Thus, the optimal concentration of betaine was determined to be 0.6 M (Fig. 3B). RT-LAMP products were observed with all the tested concentrations of the dNTP mix (1 to 1.4 mM) (Fig. 3C). Therefore, the optimal dNTP concentration was determined to be 1 mM for IHNV detection.

The effect of *Bst* DNA polymerase concentration was tested at 6, 8, 10 and 12 U. The RT-LAMP products were observed at all concentrations tested (Fig. 4A). The AMV reverse transcrip-

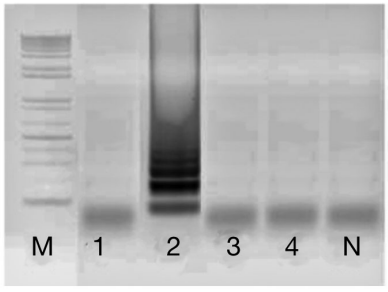

Fig. 1. Optimization of temperature for reverse transcriptase loop-mediated isothermal amplification (RT-LAMP) reactions using 100 pg of RNA extracted from infectious hematopoietic necrosis virus (IHNV)-infected chinook salmon embryo (CHSE)-214 cells. Lane M: 100 bp DNA marker; Lanes 1 to 4: LAMP carried out at 65, 63, 60 and 58°C, respectively; Lane N: negative control

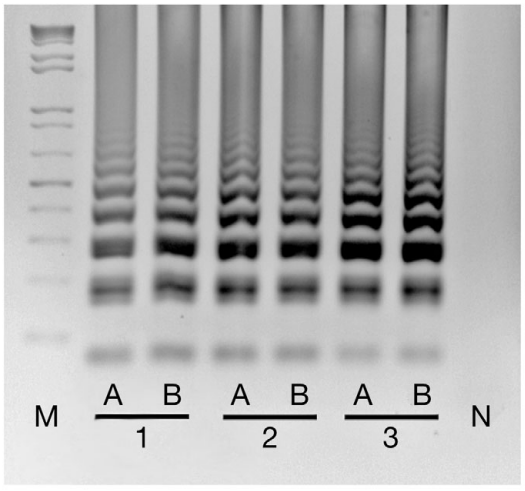

Fig. 2. Determination of RT-LAMP reaction time using (A) 10 ng and (B) 100 pg of RNA extracted from IHNV-infected CHSE-214 cells. Lane M: 100 bp DNA marker; Lanes 1 to 3: LAMP carried out for 30, 45 and 60 min, respectively; Lane N: negative control

tase concentration was also tested at 0.25, 0.5, and 0.75 U. The RT-LAMP products were observed at 0.25 and 0.5 U AMV and their intensity decreased at 0.75 U (Fig. 4B). Therefore, the optimal *Bst* DNA polymerase and AMV reverse transcriptase concentrations were determined to be 6 and 0.25 U, respectively.

Comparison of RT-LAMP and nested RT-PCR limits of detection

To compare the limits of detection, RT-LAMP and nested RT-PCR were carried out using 10-fold serial dilutions of RNA extracted from IHNV-infected CHSE-214 cell cultures as template. The RT-LAMP was able

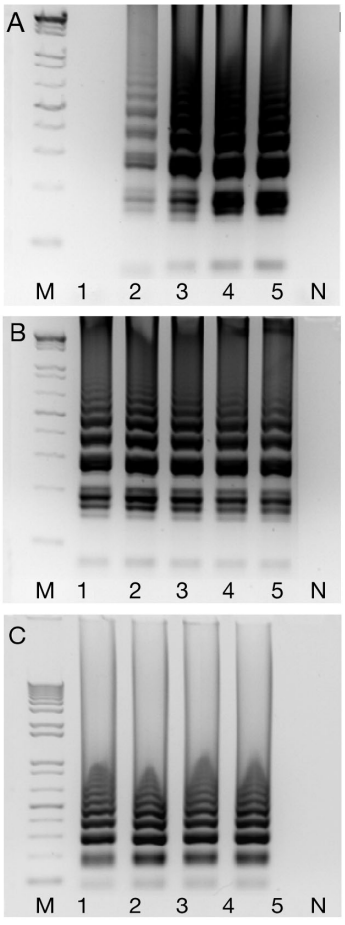

Fig. 3. Effects of $MgSO_4$, betaine and dNTP concentrations on RT-LAMP reactions using 100 pg of RNA extracted from IHNV-infected CHSE-214 cells. Lane M: 100 bp DNA marker; Lane N: negative control; (A) Lanes 1 to 5: $MgSO_4$ concentrations of 2, 4, 6, 8 and 10 mM, respectively. (B) Lanes 1 to 5: betaine concentrations of 0.6, 0.8, 1.0, 1.2 and 1.4 M, respectively. (C) Lanes 1 to 4: dNTP mix concentrations of 1, 1.2, 1.4 and 1.6 mM, respectively

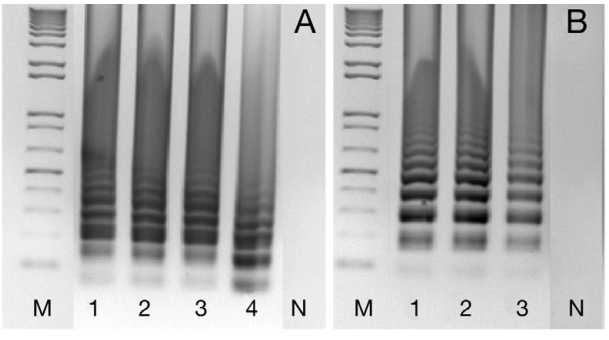

Fig. 4. Effects of *Bst* DNA polymerase and *Avian myeloblastosis virus* (AMV) reverse transcriptase concentrations for RT-LAMP reactions using 100 pg of RNA extracted from IHNV-infected CHSE-214 cells. Lane M: 100 bp DNA marker; Lane N: negative control. (A) Lanes 1 to 4: *Bst* DNA polymerase concentrations of 6, 8, 10 and 12 U, respectively. (B) Lanes 1 to 3: AMV reverse transcriptase concentrations of 0.25, 0.5 and 0.75 U, respectively

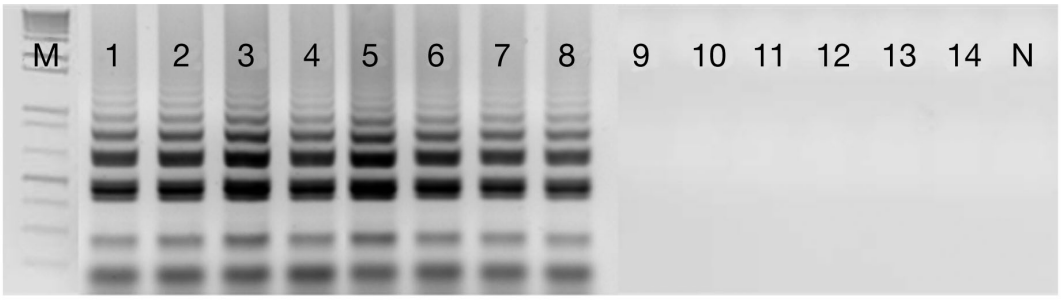

Fig. 5. Sensitivity and specificity of RT-LAMP for detection of IHNV in chum salmon *Oncorhynchus keta*. Lane M: 100 bp DNA marker; Lanes 1 to 9: 10-fold dilutions of RNA extracted from IHNV-infected CHSE-214 cells (100 pg to 0.001 fg); Lane 10: VHSV-infected RTG-2 cells; Lane 11: IPNV (VR-299 strain)-infected RTG-2 cells; Lane 12: IPNV (Sp strain)-infected RTG-2 cells; Lane 13: IPNV (Ab strain)-infected RTG-2 cells; Lane 14: IPNV (Jasper strain)-infected RTG-2 cells; Lane N: negative control

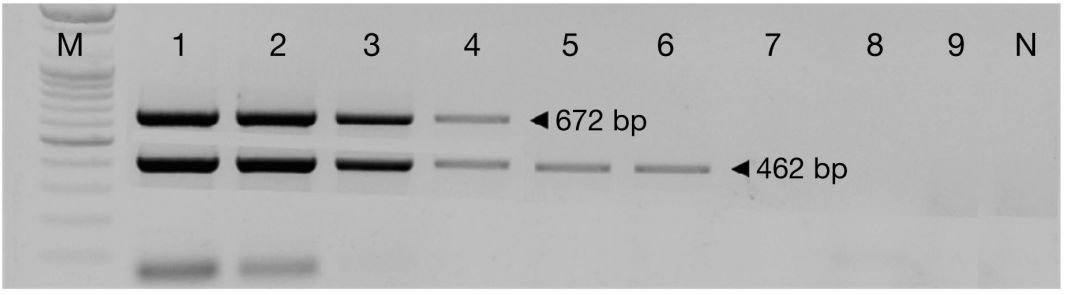

Fig. 6. Sensitivity of nested RT-PCR for detection of IHNV in chum salmon *Oncorhynchus keta*. Lane M: 100 bp DNA marker; Lanes 1 to 9: 10-fold dilutions of RNA extracted from IHNV-infected CHSE-214 cells (100 pg to 0.001 fg); Lane N: negative control

to detect template at 0.01 fg RNA (Fig. 5, Lane 8), whereas the nested RT-PCR was able to detect template at 1.0 fg (Fig. 6, Lane 6). Thus, the sensitivity of the LAMP method was 100 times higher than that of nested RT-PCR in this study.

Specificity of RT-LAMP detection

RNA from 2 other salmonid viral pathogens (IPNV and VHSV) was tested to assess the specificity of RT-LAMP. No amplification was observed (Fig. 5, Lanes 10 to 14).

Evaluation of RT-LAMP method with field samples of chum salmon

A total of 473 chum salmon samples were randomly collected from Namdae River, Yangyang City, South Korea from 2006 to 2009, including 113 pools of 5 artificially hatched fry and 360 wild adult chum salmon. The IHNV detection rates from the tested samples are summarized in Table 3. Out of 473 samples, 191 (40.38%) were IHNV-positive with the RT-LAMP assay, including 76 pooled fry samples and 115 adult samples (Fig. 7). By nested RT-PCR assay, 162 (34.25%; 53 pooled fry samples and 109 adult samples) of the 473 samples were IHNV-positive (Fig. 8).

DISCUSSION

Fish disease diagnosis is based on the appearance of clinical signs with confirmation by isolation and identification of the aetiological agent. To facilitate disease

Table 3. Comparison of the prevalence of infectious hematopoietic necrosis virus (IHNV) in chum salmon *Oncorhynchus keta* by nested reverse trancriptase (RT)-PCR and RT loop-mediated isothermal amplification (RT-LAMP)

Year	Sample type	Number of samples	Prevalence	
			Nested RT-PCR	RT-LAMP
2006	Adult	80	23 (28.75%)	23 (28.75%)
2007	Fry	32	21 (65.63%)	32 (100.00%)
	Adult	80	3 (3.75%)	9 (11.25%)
2008	Fry	32	23 (71.88%)	32 (100.00%)
	Adult	80	17 (21.25%)	17 (21.25%)
2009	Fry	49	9 (18.37%)	12 (24.49%)
	Adult	120	66 (55.00%)	66 (55.00%)
Total		473	162 (34.25%)	191 (40.38%)

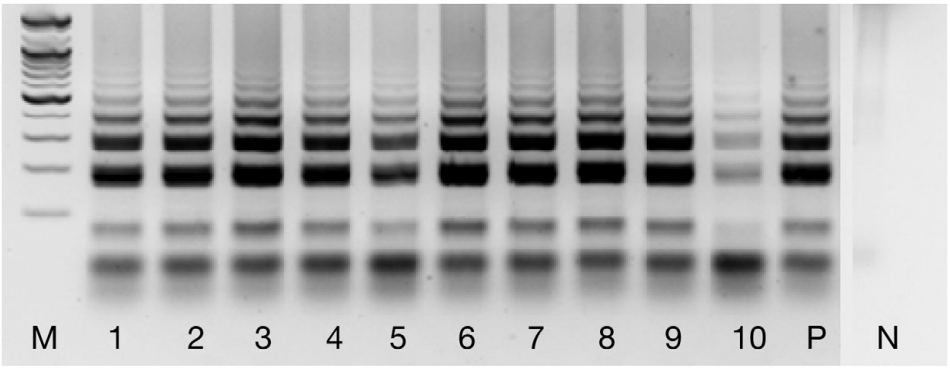

Fig. 7. Detection of IHNV in chum salmon *Oncorhynchus keta* field samples by RT-LAMP. Lane M: 100 bp DNA marker; Lanes 1 to 10: pooled fry samples; Lane P: 10 fg of RNA extracted from IHNV-infected CHSE-214 cells; Lane N: negative control

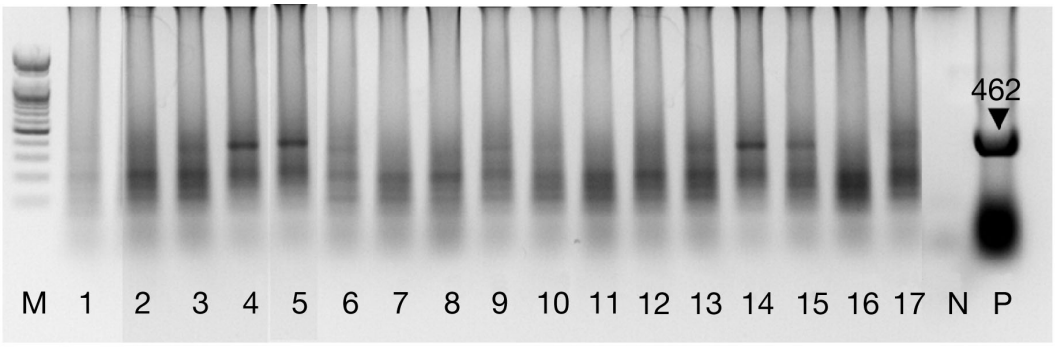

Fig. 8. Detection of IHNV in chum salmon *Oncorhynchus keta* field samples by nested RT-PCR. Lane M: 100 bp DNA marker; Lanes 1 to 17: pooled fry samples; Lane N: negative control; Lane P: 10 fg of RNA extracted from IHNV-infected CHSE-214 cells

control and management the use of rapid and sensitive methods is desirable. LAMP is a novel method of DNA amplification that has already been applied to detection of several pathogens in aquaculture (Savan et al. 2005). These include IHNV (Gunimaladevi et al. 2005, McCarthy et al. 2006), infectious salmon anemia virus (ISAV) (McCarthy et al. 2006), VHSV (Soliman & El-Matbouli 2006), spring viraemia of carp virus (SVCV) (Liu et al. 2008), IPNV (Soliman et al. 2009), turbot reddish body iridovirus (TRBIV) (Zhang et al. 2009), white spot syndrome virus (WSSV) (Kono et al. 2004), koi herpesvirus (KHV) (Gunimaladevi et al. 2004), yellow head virus (YHV) (Mekata et al. 2006) and Taura syndrome virus (TSV) (Kiatpathomchai et al. 2007). According to these studies, the sensitivity of the LAMP assay is higher than the conventional PCR assay.

In the present study, the RT-LAMP assay was developed for the detection of IHNV in chum salmon *Oncorhynchus keta*. Three sets of LAMP primers were able to amplify a 206 bp sequence of the G gene of IHNV PRT strain under optimized conditions at 63°C for 30 min. The $MgSO_4$, betaine, dNTPs, DNA polymerase and reverse transcriptase concentrations were optimized as these can influence RT-LAMP reactions as previously described (Notomi et al. 2000, Fukuta et al. 2003, Nie 2005). At least 4 mM $MgSO_4$ was needed for a visible reaction and 6 to 10 mM produced similar optimal results. The presence of 0.5 to 1.5 M betaine could elevate the overall rate of the reaction and also increase target selectivity with a significant reduction in amplification of inappropriate sequences as previously reported by Notomi et al. (2000). With betaine, the amplification of short target fragments (<300 bp) was not affected, but non-specific amplification, as reported by Baskaran et al. (1996), was reduced. The levels of *Bst* DNA polymerase (6 U) and AMV reverse transcriptase (0.25 U) were also optimized for use under optimal $MgSO_4$, betaine and dNTPs concentrations.

The RT-LAMP technique for detecting IHNV was previously reported in Japan (Gunimaladevi et al. 2005). In their experiment, IHNV-specific LAMP

primers were based on the G protein of the WRAC strain (accession no. L40883) isolated from chinook salmon *Oncorhynchus tschawytscha* belonging to the American genogroup M (Enzmann et al. 2005). However, all of the Korean IHNV isolates belong to the JRt genogroup (Kim et al. 2007) and there is no report on the occurrence of the M genogroup in Korea at the present time. Thus, we selected the PRT strain for designing RT-LAMP primers to detect the G protein of IHNV from chum salmon in Korea. This strain was isolated from masu salmon *O. masou* in Korea (Park et al. 1993). These loop primer sets can hybridize to the stem-loop forms and hence the present RT-LAMP assay can recognize 8 regions of the G gene of IHNV with a set of 6 primers. The reaction could be completed within 30 min at 63°C in the present study, whereas conventional RT-PCR required 2.5 to 3 h to complete. Moreover, RT-LAMP detected as little as 0.01 fg of RNA extracted from IHNV-infected CHSE-214 cells, compared with 1.0 fg for nested RT-PCR. No cross-amplification with IPNV and VHSV was observed, all of which indicated that this RT-LAMP assay can specifically detect IHNV in Korea. In addition, the nested RT-PCR assay requires an additional step for completing the reaction, whereas RT-LAMP requires only one step, limiting the risk of cross-contamination. The RT-LAMP assay is simple and easy to perform and only requires the use of a conventional laboratory water bath or a heating block.

The applicability of the IHNV RT-LAMP assay was evaluated with field samples and the results were compared with those obtained using a nested RT-PCR. The results showed that 191 out of 473 samples (40.38%) were IHNV-positive using RT-LAMP, whereas the detection rate decreased when using nested RT-PCR (34.25%; 162 of 473). These results indicate that RT-LAMP would be more suitable than the nested RT-PCR assay for early field diagnosis and/or surveillance programs because it is more sensitive, does not require expensive equipment such as a thermal cycler and is less time-consuming.

IHNV causes significant mortalities both in wild and cultured salmonids (Wolf 1988, Bootland & Leong 1999, Williams et al. 1999). Although rainbow trout are known to be the most susceptible fish species, several other salmonid species are also susceptible to IHNV infection (Wolf 1988, Winton 1991, Bootland & Leong 1999), with fry being more susceptible than adult fish. Surviving fry or adults can become sub-clinical carriers (St-Hilaire et al. 2001) and early detection of sub-clinical infection is very important for preventing the spread of IHNV.

In conclusion, considering the prevalence and economic losses due to IHNV infection in salmonids, a simple, cheap, rapid, sensitive and specific method is desirable for early diagnosis in fish farms. The RT-LAMP assay described here fulfills these requirements, at least for the Korean strains of IHNV. It could be used as a routine assay for targeted surveillance for early detection of IHNV infection in salmonid farms, especially where expensive diagnostic instruments are not available.

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