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ARTICLE *in* JOURNAL OF VIROLOGICAL METHODS · JULY 2009

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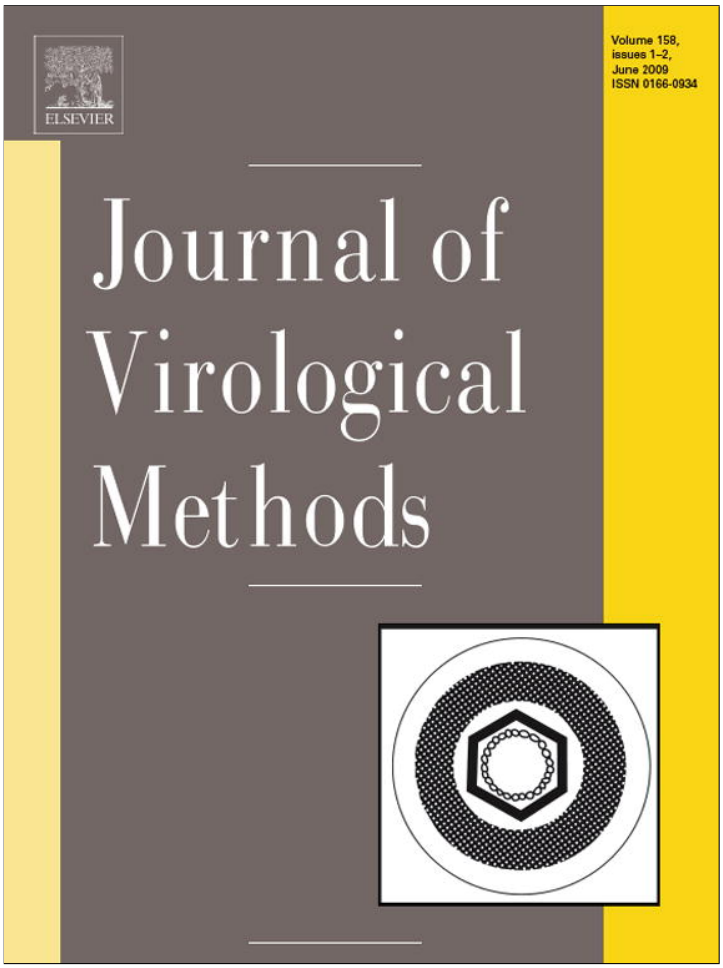

Journal of Virological Methods 158 (2009) 77–83

Contents lists available at ScienceDirect

Journal of Virological Methods

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

Sensitive and rapid detection of infectious pancreatic necrosis virus by reverse transcription loop mediated isothermal amplification

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Article history:
Received 12 November 2008
Received in revised form 15 January 2009
Accepted 21 January 2009
Available online 30 January 2009

Keywords:
IPN diagnosis
RT-LAMP
Aquabirnavirus
SYBR Green I
Fluorescent detection reagent (FDR)
Polyethylenimine (PEI)

A B S T R A C T

A new molecular diagnostic assay was developed for rapid and sensitive diagnosis of infectious pancreatic necrosis virus (IPNV) by using a one step, one tube reverse transcription loop-mediated isothermal amplification (RT-LAMP). A set of six LAMP primers was designed to amplify the target RNA by incubation with *Bst* DNA polymerase plus reverse transcriptase and the reaction was optimised at 65 °C for 60 min. Three different methods for detection of the amplified product by naked eye gave identical results to gel electrophoresis, which was run for confirmation. Negative results obtained with RNA from four other fish viruses confirmed the specificity of the test. The IPNV-RT-LAMP assay demonstrated superior analytical sensitivity compared to conventional RT-PCR conducted according to published methods ($1{:}10^{12}$ dilution of RNA extracted from an IPNV-infected cell culture supernatant vs. $1{:}10^{6}$ for the conventional RT-PCR). The feasibility of the RT-LAMP assay for detection of IPNV RNA in clinical specimen was authenticated using kidney tissue samples from experimentally IPNV-infected Atlantic salmon (*Salmo salar*) post-smolts. The results suggest that the RT-LAMP is a rapid and highly sensitive diagnostic assay for IPNV which lends itself well to use in aquaculture health management and disease control.

1. Introduction

Infectious pancreatic necrosis virus (IPNV) is the aetiological agent of a well-characterized contagious disease of salmonid fish, that affects mainly young hatchery-reared salmonids but also Atlantic salmon smolts shortly after sea transfer (Reno, 1999; Smail et al., 1992). In disease outbreaks, cumulative mortalities may vary from less than 10% to more than 90% depending on virus strain, quantity, host age and environment (McAllister and Owens, 1995; Jarp et al., 1994). The disease has emerged as the most serious viral diseases in terms of its impact on Atlantic salmon (*Salmon salar*) production in the European Union (Ariel and Olesen, 2002; Skjelstad et al., 2003; Murray et al., 2003). IPNV is the prototype virus of the family Birnaviridae and belongs to the genus Aquabirnavirus (Delmas et al., 2005). Of particular relevance is its ability to persist in survivors through sexual maturation and to be shed with the sexual products with a consequent risk for vertical transmission (Bootland et al., 1991). According to the OIE (2006), IPN screening for disease control is based upon isolation of the virus in tissue culture followed by immunological identification, but reverse transcription-polymerase chain reaction (RT-PCR) protocols for IPNV have also been developed (Lopez-Lastra et al., 1994; Blake et al., 1995; Taksdal et al., 2001). Loop-mediated isothermal amplification (LAMP) has been developed as a novel method for nucleic acid amplification under isothermal conditions employing self-recurring strand-displacement DNA synthesis initiated by a specially designed set of target-specific primers (Notomi et al., 2000; Nagamine et al., 2001, 2002). Detection of several (including fish) animal RNA viruses, of high veterinary relevance has been reported (Parida et al., 2004; Gunimaladevi et al., 2005; Soliman and El-Matbouli, 2006; Dukes et al., 2006). In addition to agarose gel electrophoresis, several techniques were used for analysis of the LAMP products. The LAMP products can be detected visually by using SYBR Green I stain (Soliman and El-Matbouli, 2006), by monitoring the white insoluble precipitate of the by-product magnesium pyrophosphate in the reaction mixture (Mori et al., 2001), by using florescent detection reagent, FDR, (Yoda et al., 2007), or by using precipitation reaction after addition of cationic polymers to the LAMP amplicons (Mori et al., 2006).

The aim of the current study was to develop an accelerated, single tube, one step RT-LAMP assay for rapid detection of IPNV. The sensitivity and specificity of the assay were assessed along with its feasibility for use on fish kidney tissue.

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doi:10.1016/j.jviromet.2009.01.018

Table 1
Viruses assayed in IPNV-RT-LAMP experiments.

Virus	Strains/isolates/ serotype	Source
Infectious pancreatic necrosis (IPN)	VR-299	FLI[a]
Infectious pancreatic necrosis (IPN)	Abild (Ab)	FLI[a]
Infectious pancreatic necrosis (IPN)	Spjarup (Sp)	FLI[a]
Infectious pancreatic necrosis (IPN)	V-1244	VESO[b]
Infectious pancreatic necrosis (IPN)	NVI-015	Prof. Ø. Evensen[c]
Viral hemorrhagic septicaemia (VHS)	HE	FLI[a]
Infectious haematopoietic necrosis (IHN)	Oregon 69	FLI[a]
Infectious salmon anaemia (ISA)	Norwegian strain	FLI[a]
Sleeping disease virus (SDV)	S49P	FLI[a]

[a] Kindly provided by Dr. D. Fichtner, National Reference Laboratory for Fish Diseases, Institute of Infectology, Friedrich-Loeffler-Institute, Federal Research Institute for Animal Health, Isle of Riems Germany.
[b] Centre for Veterinary Contract Research and Commercial Services Ltd. (VESO), Oslo, Norway.
[c] Kindly provided by Prof. Ø. Evensen, Norwegian School of Veterinary Science, Oslo, Norway.

Fig. 1. Nucleotide sequence of the 191 bp IPNV amplicon (GenBank accession number AY379740, genome segment 2117–2308 bp) used for construction of the LAMP inner, outer and loop primers. The primer sequences are indicated in bold italic letters. Inner primers FIP and BIP comprise sequences within the amplicon; FIP is the complementary sequence of F1 (F1c) and F2, BIP is complementary sequence of B1 (B1c) plus the B2. LPF and LPB are the loop primers forward and backward, respectively. F3 and B3 are the outer primers.

2. Materials and methods

2.1. Viruses

IPN and non-IPN fish viruses strains employed in the current study (Table 1), were available for RNA extraction as Chinook salmon embryo (CHSE-214) tissue culture supernatants grown according to OIE (2006) recommendations. In the dilution experiment for determination of analytical sensitivity; frozen RTG-2 culture supernatant containing the Norwegian IPN virus strain NVI-015 (serotype Sp, titre approximately 1×10^6 $TCID_{50}$/ml) was used.

2.2. RNA extraction

Viral genomic RNA was extracted from 140 μl of each infected culture supernatant using the QIAamp Viral RNA Mini kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. Kidney tissue specimens were processed by thoroughly grinding small parts of the organs in liquid nitrogen with a mortar and pestle. Then 20 mg of the tissue powder was placed into RNase-free, liquid nitrogen-cooled 2 ml micro-centrifuge tube. RNA was then extracted using the RNeasy Mini kit (Qiagen, Hilden, Germany) as per manufacturer's instructions. A negative extraction control (sample contain sterile water) was performed to control for contaminations. Purified RNA was quantified by measuring the optical density at 260 and 280 nm. RNA samples were stored in aliquots at −80 °C until required.

2.3. Oligonucleotide primers

Several primer sets for the RT-LAMP assay targeting the NS/VP3 region of IPNV serotype Sp (GenBank accession number AY379740) were designed using the LAMP primer design support software program (PrimerExplorer ver.4; Net Laboratory, Tokyo, Japan). Primer sets from different IPNV genome regions were tested. The NS/VP3 encoding region of segment A was chosen as a suitable target because it provides a conserved region for IPNV serotypes Sp and Ab which are the most common serotypes in Europe. One set comprising six primers targeting this region was selected for the current RT-LAMP assay including an outer forward (F3) and an outer backward (B3) primer; a forward inner primer (FIP), a backward inner primer (BIP), as well as loop primers, forward and backward (LPF and LPB), respectively (Table 2). FIP primers contained complementary sequence of F1 (F1c), a TTTT spacer, and the F2 sequence. In the same way, BIP consisted of the complementary sequence of B1 (B1c), a TTTT spacer and B2 sequence (Fig. 1). The LPF primer was also used as an oligo-DNA probe (ODP) after fluorescent labelling of its 3′ end with ROX, in this case the primer set consisted of five primers (F3, B3, FIP, BIP and LPB) plus the oligo-DNA probe.

The conventional reverse transcription-polymerase chain reaction (RT-PCR) used IPNV specific primers DIAIPNF and DIAIPNR targeting a 224 bp sequence on the NS/VP3 region of segment A of IPNV serotype Sp (GenBank accession number AY379740) according to Taksdal et al. (2001).

2.4. IPN RT-LAMP assay

For optimisation of the RT-LAMP assay different incubation temperatures and different concentrations of primers, $MgSO_4$, eAMV RT enzyme and *Bst* DNA polymerase enzyme were tested. The final RT-LAMP assay was carried out in a 25 μl total reaction mixture volume

Table 2
Details of RT-LAMP and RT-PCR primers used in this endeavour. Primers were designated from the IPNV RNA segment A GenBank accession number (AY379740). RT-LAMP primers targeting 191 bp fragment of IPNV RNA (genome position 2117–2308 bp). RT-PCR primers targeting 242 bp fragment of IPNV RNA (genome position 2120–2343 bp).

Primer name	Primer sequence (5′-3′)	Genome position	Primer length
F3	CCAATCTGCGGTGTAGACAT	2117–2136	20 nt
B3	CATCAGCTCTCCCAGGTACT	2289–2308	20 nt
FIP	CCTCCTCGTCCACTCCTGGT-TTTT-CCATCGCAGCCCATGAAC	2185–2204/TTTT/2142–2159	42 nt
BIP	TGCGAAACACATCCCTGGCC-TTTT-TCTTGTTGGAGCCCTTTGC	2205–2224/TTTT/2264–2282	43 nt
LPF	CGATGAGTGGCAGCCCTT	2160–2177	18 nt
LPB	GATCCAGACCGGAACCCTG	2233–2251	19 nt
ODP	CGATGAGTGGCAGCCCTT-ROX	2160–2177	18 nt
DIAIPNF	ATCTGCGGTGTAGACATCAAAG	2120–2141	22 nt
DIAIPNR	TGCAGTTCCTCGTCCATCCC	2324–2343	20 nt

containing 1× Thermopol reaction buffer (20 mM Tris–HCl (pH 8.8), 10 mM KCl, 3.5 mM $MgSO_4$, 10 mM $(NH_4)_2SO_4$, 0.1% Triton X-100) (New England BioLabs, GmbH, Frankfurt, Germany) 1.6 M betaine (Sigma–Aldrich, GmbH, Schnelldorf, Germany), 1.4 mM of each dNTPs (Sigma–Aldrich, GmbH, Schnelldorf, Germany), 50 pmol each of inner primers FIB and BIP, 5 pmol each of outer primers F3 and B3, 25 pmol each of loop primers LPF and LPB, 8 U *Bst* DNA polymerase (New England BioLabs, GmbH, Frankfurt, Germany), 2 U (eAMV) enhanced avian reverse transcriptase (Sigma–Aldrich Logistic GmbH, Schnelldorf, Germany), 2 μl RNA template, and completed to 25 μl by PCR grade water. RNA template was omitted from one reaction as a negative control. The mixture was incubated at 65 °C for 1 h and then heated at 85 °C for 2 min to terminate the reaction.

2.5. Conventional IPN RT-PCR

A two-step RT-PCR was carried out according to Taksdal et al. (2001). In addition, a one step RT-PCR was performed by using the TITANIUM™ one-step RT-PCR kit (BD Clontech, Heidelberg, Germany): 1 μg of RNA was mixed with 10× one-step buffer, dNTPs mix, recombinant RNase inhibitor, thermostabilising reagent, GC-melt, oligo-(dT) primer, 45 μM of each DIAIPNF and DIAIPNR specific primers (Taksdal et al., 2001) and RT-TITANIUM™ Taq enzyme mix. The reaction conditions for the one-step procedure comprised: incubation at 50 °C for 1 h, then heated to 94 °C for 5 min; followed by 40 cycles of 95 °C for 30 s, 55 °C for 15 s, and 72 °C for 30 s. There was a final extension step of 72 °C for 5 min.

2.6. Analysis of the amplification products

RT-LAMP products were analysed either by naked eye using different methods of visual inspection or by agarose gel electrophoresis.

2.6.1. Visual read-out methods

(1) *SYBR Green I method*: 1 μl of 1:10 diluted SYBR Green I nucleic acid gel stain 10,000× concentrations in DMSO (Cambrex Bio-Science, Rockland Inc., ME, USA) was added to the reaction tube after completion of the reaction. After thorough mixing any colour change recognised by the naked eye was noted.
(2) *Fluorescent detection reagent (FDR) method*: The RT-LAMP assay was performed as described above except for the addition of 1 μl of FDR (Eiken Chemical Co. Ltd.) to the mixture before the reaction. After completion of the amplification, the result was noted after visual observation under UV (wavelength 254 nm) illumination.
(3) *Using fluorescent labelled probe and addition of cationic polymers method:* The RT-LAMP assay was carried out as described above except that the forward loop primer (LPF) was replaced by 1 pmol of ROX labelled probe. At completion of the reaction, the solution was centrifuged at 6000 rpm for 10 s, and 0.2 μmol of low-molecular weight (MW = 600) (PEI) polyethylenimine (Wako chemicals GmbH, Neuss, Germany) was added. The resultant LAMP amplicon-PEI complex, which contains the fluorescently labelled probe, was precipitated as an insoluble pellet by centrifugation of the tube at 6000 rpm for 10 s. The emitted fluorescence was detected by naked eye under a conventional UV illuminator or by the aid of a fluorescence microscope, and noted.

2.6.2. Agarose gel electrophoresis analysis of the RT-LAMP and RT-PCR products

The RT-LAMP and RT-PCR amplification products were analysed by gel electrophoresis with 2% agarose in Tris acetate–EDTA buffer, TAE (0.04 M Tris acetate, 1 mM EDTA), stained with ethidium bromide and visualized on an UV transilluminator. A Superladder-low 100 pb ladder (ABgene, Hamburg, Germany) and TrackIt™ 100 bp DNA ladder (Invitrogen GmbH, Karlsruhe, Germany) were used.

2.7. RT-LAMP product confirmation by restriction enzyme digestion

The specificity of the RT-LAMP assay was authenticated by digestion of RT-LAMP products with *Fok I* restriction enzyme. The RT-LAMP products were first purified from excess enzymes, salts, primers and dNTPs by using high-pure PCR product purification kit (Roche Molecular Biochemicals, Mannheim, Germany) as per manufacturer's instructions. After spectrophotometrical measurement, 1 μg of the RT-LAMP products was incubated with *Fok I* restriction enzyme (New England BioLabs, GmbH, Frankfurt, Germany) at 37 °C for 2 h, and subsequently analysed by agarose gel electrophoresis.

2.8. IPNV-RT-LAMP assay specificity

The analytical specificity of the RT-LAMP was evaluated by running the assay with RNA from viral hemorrhagic septicaemia (VHS) virus, infectious haematopoietic necrosis (IHN) virus, infectious salmon anaemia (ISA) virus and sleeping disease virus (SDV). RNA from control CHSE-214 cells was used as a negative control to determine any non-specific amplification.

2.9. IPNV-RT-LAMP assay limit of detection

Ten-fold serial dilutions of 1 μg purified RNA from the IPNV (isolate NVI-015) RTG-2 supernatant was tested by RT-LAMP and by both one-step and two-step conventional RT-PCR method to determine the lower detection limit of each assay. Result of the RT-LAMP sensitivity was analysed by agarose gel electrophoresis as well as by visual inspection as described above.

2.10. IPNV-RT-LAMP performed on kidney samples

The ability of the RT-LAMP assay to detect IPNV in clinical specimens was evaluated by testing 24 Atlantic salmon kidney samples from post-smolts that had survived IPNV infection experiment (Ramstad et al., 2007) including 5 non-IPNV-exposed control fish. All samples were tested by both RT-LAMP and conventional RT-PCR. Parallel kidney samples had previously been tested by IPNV antigen ELISA (Test-Line Clinical Diagnostics, Brno, Czech Republic; Rodàk et al., 1988) in the originator laboratory.

3. Results

Successful RT-LAMP of IPNV RNA was achieved by incubation of the designated IPN-specific primers, 8 U of *Bst* DNA polymerase and 2 U of eAMV reverse transcriptase enzyme with the target RNA at 65 °C for 60 min. After amplification; a visibly distinct ladder-like pattern was observed on agarose gel electrophoresis (Fig. 2).

To confirm that the RT-LAMP products were amplified from the target region, the products were digested with *Fok I* enzyme. The *Fok I* enzyme digestion produced fragments of 108 and 84 bp as predicted from the amplicon structure, and the typical ladder like structure of the LAMP product was disappeared. The negative (no template) control of both RT-LAMP and restriction digestion were negative (Fig. 2).

Colour changes were noted on visual inspection of the IPNV-RT-LAMP reaction tube after addition of the diluted SYBR Green I: positive samples turned green, while negative samples remained orange (Fig. 3E and F, respectively). When prepared with the FDR,

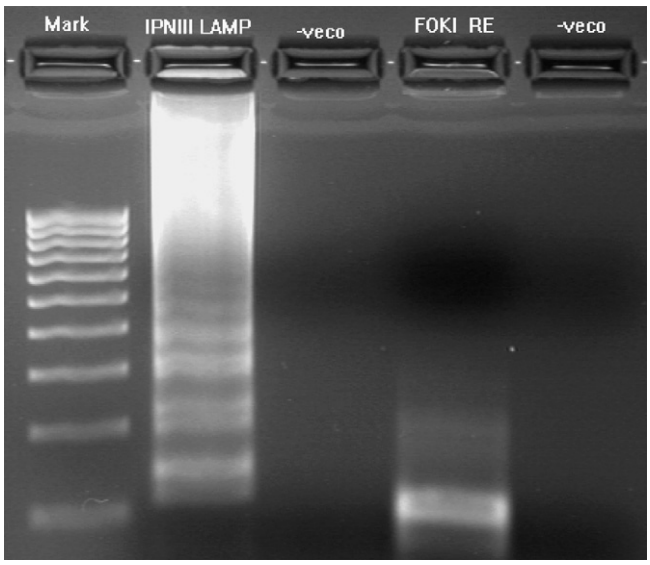

Fig. 2. Agarose gel electrophoresis demonstrates RT-LAMP product of IPNV and restriction digestion analysis of the amplified product. The reaction was carried out at 65 °C using 6 primers set. Lane Mark: 100 bp molecular weight marker (ABgene), lane IPNIII LAMP: the ladder-like structure of the IPNV-RT-LAMP product; lane -veco: negative (no template) control of RT-LAMP reaction; lane FOKI RE: result of RT-LAMP product digestion with *FOK I* restriction enzyme; disappearing of the ladder-like structure and appearance of two bands at molecular weight 84 and 108 bp; lane -veco: negative (no template) control of the digestion reaction.

positive samples emitted strong green fluorescence while the negative samples did not emit green fluorescence (Fig. 3D and C, respectively). After use of the ROX labelled probe with PEI the pellet in positive reaction vials emitted red fluorescence, while no pellet was formed and no emission of red fluorescence occurred in the negative controls (Fig. 3B and A, respectively). The visual inspection results were comparable with the gel electrophoresis results.

The IPNV-RT-LAMP did not amplify RNA extracted from IHN, VHS, ISA, SDV, or non-infected CHSE-214 cell culture (data not shown).

Amplification of 10-fold serial dilutions of IPNV RNA by RT-LAMP and conventional RT-PCR revealed that the RT-LAMP could detect IPN viral RNA to a dilution of 1 in 10^{12}, while the RT-PCR lost the amplification signal after a dilution of 1 in 10^{6} (Fig. 4).

The RT-LAMP assay detected IPNV RNA from 19 infected kidney samples (Fig. 5), that also tested positive by conventional RT-PCR (Fig. 6), while there was no amplification product from the 5 kidney samples from non-infected fish. Negative tissue controls were also negative with both RT-LAMP and RT-PCR, as were the negative extraction controls and the no template reaction controls. The results from all tissue samples were in agreement with the antigen ELISA results obtained at VESO Vikan.

4. Discussion

This is the first study documenting a rapid and technically simple procedure for the diagnosis of IPNV infection based on RT-LAMP technology. Conventional RT-PCR protocols usually require more than 6 h to amplify and confirm IPNV infection, compared to only 1 h required for the new RT-LAMP assay when using visual readout. The RT-LAMP is carried out in a single tube and only requires a simple water bath or a heating block to provide a constant temperature of 65 °C (Notomi et al., 2000). The success of the RT-LAMP assay relies on the specificities of the primer sets designed (Yoda et al., 2007). By reviewing the IPN literatures and the available sequences in the GenBank, it was found that the serotypes Sp and Ab are the most common IPNV serotypes in Europe. Consequently, all available sequences from serotypes Sp and Ab in the GenBank were compared and aligned against the other IPNV strains sequences such as VR-299, He, Jasper and West Buxton using DNAstar software. Different RT-LAMP primer sets were constructed and evaluated from each conserved region specific for Sp and Ab serotypes. While several primer sets provided a positive LAMP reaction, the used RT-LAMP primer set showed consistently the best performance and a highly effective and sensitive detection of the IPNV RNA. The use of six primers in the RT-LAMP assay ensures for a very high degree of specificity, as these six primers recognise eight distinct regions on the target DNA (Notomi et al., 2000; Nagamine et al., 2002).

Initial trials of this assay produced the characteristic ladder-like pattern of LAMP products up on the gel electrophoresis. This ladder-like pattern is due to the formation of a mixture of stem-loop DNA products with varying stem length, and cauliflower-like structures of multiple loops formed by annealing between alternately inverted repeats of the target sequence in the same strand (Thai et al., 2004). The identity of the amplified product was confirmed by *Fok I* restriction enzyme digestion.

To support a close-to-the-field application of the IPNV-RT-LAMP assay, three different methods were successfully used to read the amplification result by the naked eye. First, using of SYBR Green I stain, visual detection proved possible due to the high-binding affinity of SYBR Green I to double stranded DNA combined with the high-amplification efficiency of the LAMP assay (Karlsen et al., 1995; Iwamoto et al., 2003). Secondly, using the FDR, the LAMP reaction produces a by-product (pyrophosphate ions) that binds with and removes manganese from the calcein, which is part of the FDR, leading to emission of fluorescence from the calcein molecule, which in its original state is combined with manganese ions and

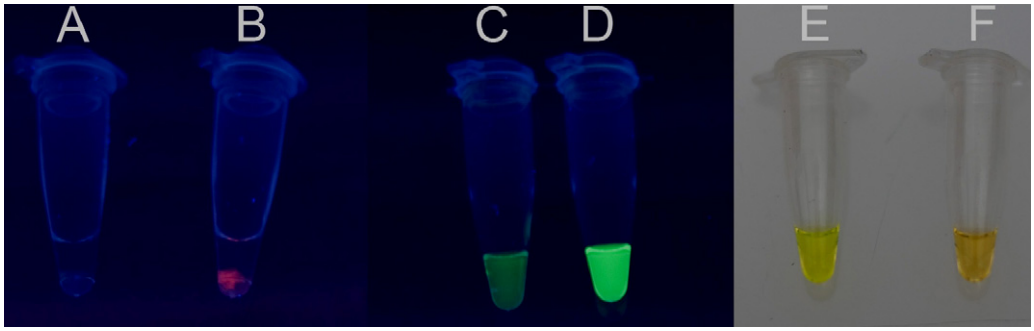

Fig. 3. Visual detection of IPNV-RT-LAMP product by using different naked eye detection methods: A = negative sample by using ROX labelled probe and PEI; there is neither pellet nor red fluorescence. B = positive sample by using ROX labelled probe and PEI; pellet emitted strong red fluorescence. C = negative sample by using FDR, did not emitted green fluorescence. D = positive sample by using FDR, emitted strong green fluorescence. E = positive sample with green colour by using SYBR Green I stain. F = negative sample with orange colour by using SYBR Green I stain.

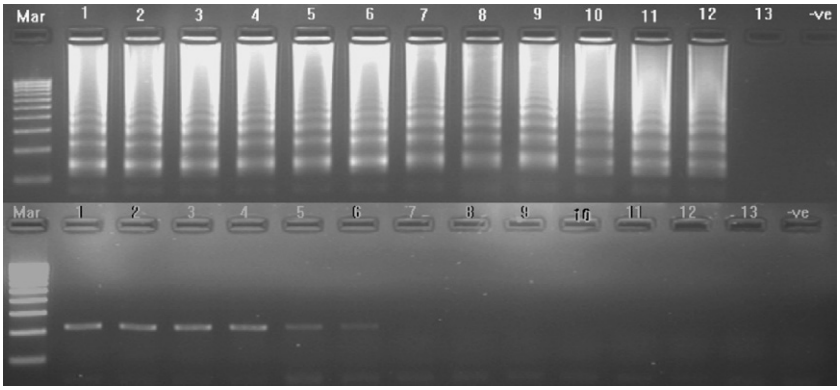

Fig. 4. Sensitivity of the RT-LAMP and RT-PCR assays determined by agarose gel electrophoresis of the RT-LAMP/RT-PCR products from 10-fold serially diluted sample of 1 μg purified IPNV RNA. The amplification shows a ladder-like pattern of the RT-LAMP product and the expected 224 bp segment of the RT-PCR amplicons. The RT-LAMP assay was detected purified IPNV RNA till the dilution of 10^{12}, while the RT-PCR was detected purified IPNV RNA till the dilution of 10^{6}. Lane Mar = 100 bp DNA molecular weight marker (ABgene); lanes 1–13: 10-fold serial dilution of the purified genomic RNA of IPNV; lane -ve: negative (no template) reaction control.

remains quenched (Imai et al., 2007; Yoda et al., 2007). The third visual inspection method was by using cationic polymers such as the low-molecular weight PEI to form insoluble amplicon-PEI complex containing the hybridized fluorescently labelled probe, which emitted fluorescence by its exposure to UV light (Mori et al., 2006). Low-molecular weight PEI was selected because it can form an insoluble complex with DNA of high-molecular weight, as the LAMP product, while it cannot form insoluble complex with a single-stranded anionic polymer with a low-molecular weight such as the oligo-DNA probe (Mori et al., 2006). All samples that tested positive by visual inspection were positive also when analysed by gel electrophoresis; there were no samples that were negative by visual examination but tested positive by gel electrophoresis.

The lack of cross-reactivity with the RNA extracted from the fish pathogenic viruses IHN, VHS, ISA, SDV, or from uninfected CHSE-214 cell culture confirms the specificity of the developed assay. The assay produced the characteristic ladder like structure with the IPNV strains Sp and Ab RNA while there were no amplification prod-

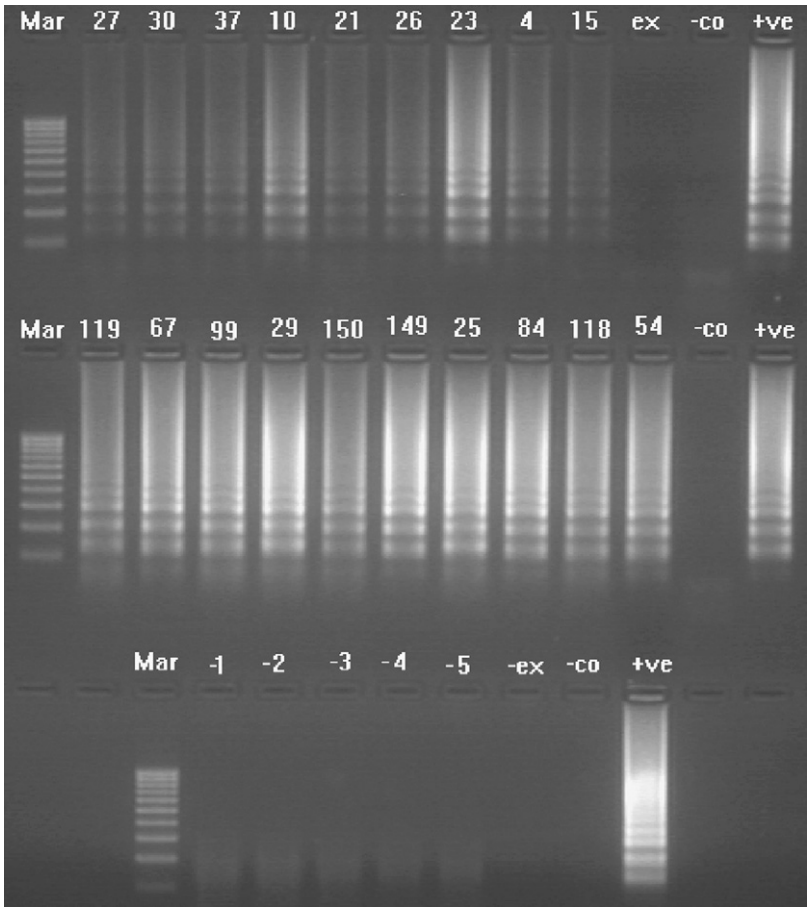

Fig. 5. Agarose gel electrophoresis illustrating the practicability of the RT-LAMP assay to detect IPNV RNA from infected fish tissues. Lane Mar = 100 bp DNA molecular weight standard (ABgene), numbers above each lane is the archive number of each case; lane ex: negative extraction control; lane -co: negative (no template) RT-LAMP reaction control; lane +ve: IPNV positive control. Lanes -1, -2, -3, -4, -5 are the non-infected fish samples.

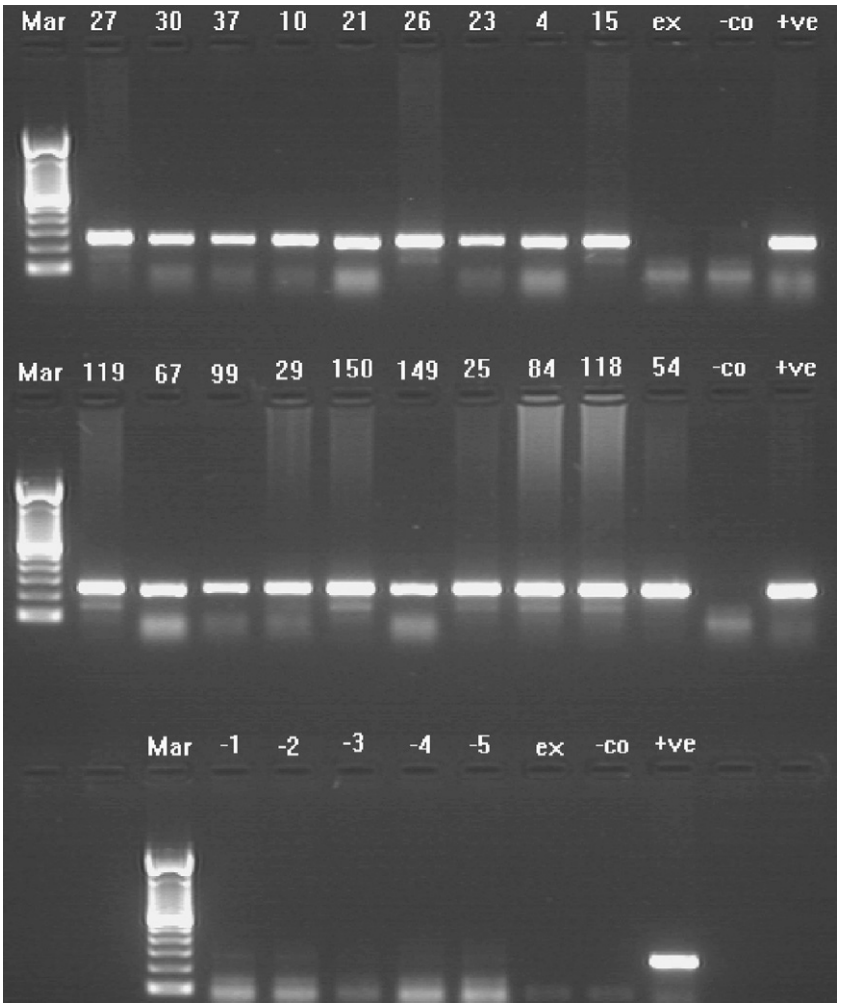

Fig. 6. Agarose gel electrophoresis illustrating the practicability of the RT-PCR assay to detect IPNV RNA from infected fish tissues. Lane Mar = 100 bp DNA molecular weight standard (Invitrogen), numbers above each lane is the archive number of each case; lane ex: negative extraction control; lane -co: negative (no template) RT-PCR reaction control; lane +ve: IPNV positive control. Lanes -1, -2, -3, -4, -5 are the non-infected fish samples.

ucts appeared by using RNA extracted from the IPNV strains VR-299, He, Jasper and West Buxton (data not shown) which confirm the specificity of the developed assay to IPNV strains Sp and Ab.

The titration experiment showed that the RT-LAMP assay has a considerably higher analytical sensitivity than the conventional RT-PCR, as IPNV RNA was detected at a dilution of 10^{12} (~0.001 fg RNA) while the conventional RT-PCR (Taksdal et al., 2001) detected IPNV RNA at a dilution of 10^{6} (~1 pg RNA) only. The genome size of IPN virus segment A and B is about 5.881 kb (Duncan et al., 1987, 1991) indicating that the 0.001 fg and 1 pg detected by the IPNV-RT-LAMP and RT-PCR assays correspond to approximately 1.6 and 1.6×10^{5} IPN virions, respectively. The RT-PCR assay of Taksdal et al. (2001) was chosen for comparison because the primers used in this study are from the same IPNV genomic region, namely the NS/VP3 region of segment A of serotype Sp. This new RT-LAMP assay is potentially more sensitive than other IPNV gene amplification assays as it detected 0.001 fg of purified IPNV RNA, while Lopez-Lastra et al. (1994) and Wang et al. (1997) detected 1 pg and 15 fg of purified IPNV RNA, respectively. It also showed superior sensitivity than the nested PCR developed by Suzuki et al. (1997), which detected 1 fg of purified IPNV RNA.

The principal potential of the RT-LAMP assay to be used on clinical specimen was successfully validated by detection of IPNV RNA from the infected kidney samples while there was no amplification products in case of using samples from non-infected fish or negative extraction controls.

From the previous findings it is concluded that the developed RT-LAMP assay is highly sensitive, specific and very rapid for detection of IPNV RNA. Using any of the visual inspection methods tried in this study, the assay can be used as an easy molecular diagnostic test for rapid diagnosis of IPNV infection. This method, especially when supplied as a kit with RNA extraction- and ready to use reagent mixes has an interesting potential for improving routine IPNV control measures in the aquaculture industry such as for example brood fish testing, for population testing prior to movement of live fish to IPNV-free establishments, and for rapid differential diagnosis during unidentified disease outbreaks.

Acknowledgments

This work was financially supported by Centre for Veterinary Contract Research and Commercial Services Ltd. (VESO), Oslo, Norway. We thank Dr. D. Fichtner, National Reference Laboratory for Fish Diseases, Germany, for providing us with the reference strains of VHS, IHN, ISA, SDV and IPN viruses, and Prof. Ø. Evensen, Norwegian School of Veterinary Science for providing IPNV strain NVI-015 supernatant. Thanks are also due to Ms. Anne Ramstad, VESO Vikan for provision of clinical samples.

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