Detection of infectious haematopoietic necrosis virus and infectious salmon anaemia virus by molecular padlock amplification

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Abstract

A new method for the molecular detection of the fish pathogens, infectious haematopoietic necrosis virus (IHNV) and infectious salmon anaemia virus (ISAV), is described. By employing molecular padlock probe (MPP) technology combined with rolling circle amplification (RCA) and hyperbranching (Hbr), it is possible to detect RNA target sequence from these viruses at levels comparable with those detected by the polymerase chain reaction (PCR), but without prior reverse transcription. The use of MPP technology combined with RCA and Hbr for the detection of IHNV and ISAV in fish exhibited selectivity comparable with that of PCR while potentially reducing the time and cost required for analysis. The method described was used to detect as few as 10^4 DNA oligonucleotide targets and was sequence-specific at the single base level. Viral RNA could be detected directly, either alone or in the presence of non-viral RNA from fish tissue. This technology is applicable for detecting a variety of microbes, in addition to IHNV and ISAV, and is ideal for further integration into a biosensor platform for on-site diagnosis of pathogen infection in fish.

Keywords: detection, hyperbranching, infectious haematopoietic necrosis virus, infectious salmon anaemia virus, molecular padlock, rolling circle.

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Introduction

Each year infectious disease causes substantial economic losses for public and commercial aquaculture ventures that rely on the cultivation and/or harvest of marine and freshwater finfish. Two viruses of particular concern for salmonids are infectious haematopoietic necrosis virus (IHNV) and infectious salmon anaemia virus (ISAV). A member of the *Novirhabdovirus* genus of the *Rhabdoviridae* family, IHNV causes disease in a variety of species of Pacific salmon, *Oncorhynchus* spp., as well as in Atlantic salmon, *Salmo salar* L., and rainbow trout, *Oncorhynchus mykiss* (Walbaum) (Winton & Einer-Jensen 2002). Historically endemic among wild anadromous salmonids in the western portion of North America, IHNV has spread to Asia and Europe, most likely due to the movement of infected fish and eggs (Winton 1991). The IHN virus can be transmitted by contact with infected fish, feeding on infected carcasses, through water from infected stocks, or by egg-associated transmission to newly hatched fry (Roberts & Shepherd 1997). IHNV is now seen most frequently in freshwater, wild, feral and cultured salmonids, where, depending on viral dose and environmental conditions, mortality rates of infected stock range up to 100% (Wolf 1988; Bootland & Leong 1999; Williams, Blake, Sweeney, Singer & Nicholson 1999).

Infectious salmon anaemia virus is a pathogen that causes disease almost exclusively in farmed Atlantic salmon, resulting in significant losses to the

fish farming industry. Originally identified in Norway in 1984, the occurrence of ISAV has been confirmed in Canada, the Faroes, Scotland and the USA (Maine) (Mjaaland, Rimstad & Cunningham 2002) and has been reported in Chile (Kibenge, Garate, Johnson, Arriagade, Dibenge & Wadowska 2001). Until recently, ISAV was considered an unclassified virus of the family *Orthomyxoviridae* (Clouthier, Rector, Brown & Anderson 2002). However, as the only family member having teleosts as a host, ISAV is now classified as the only reported species of the new genus *Isavirus* (Anonymous 2002). ISAV transmission appears limited almost exclusively to contact with infected sea water. Although found primarily in farmed salmon, under experimental conditions ISAV has been shown to survive and replicate in several fish species, providing evidence for the existence of asymptomatic viral carriers (Nylund & Jakobson 1995); salmon lice, *Lepeophtheirus salmonis*, have also been implicated as vectors (Rolland & Nylund 1998). Mortality rates of ISAV-infected farmed Atlantic salmon stocks range up to 100% (Mjaaland *et al.* 2002). Currently, the only method for control of IHNV and ISAV is avoidance of exposure to the causative viruses coupled with eradication of infected fish stocks. Early virus detection and confirmation is crucial if significant economic losses are to be avoided. The utility of a rapid, sensitive and selective method for on-site molecular detection cannot be denied.

Diagnostic assays for pathogenic viruses currently available for use in finfish are based largely on tissue culture techniques, subsequently confirmed by antibody-based assays. While important for accurate diagnosis, these analyses can become cost-, labour- and time-intensive. The most sensitive tests currently available for the detection and molecular recognition of bacterial and viral pathogens in aquatic organisms involve the use of the polymerase chain reaction (PCR). PCR offers rapid diagnosis and relative ease of use but has several potential drawbacks, of which the most frequently cited is its sensitivity to contamination, resulting in non-specific amplification. For this reason, aquaculture organizations that make economically significant decisions based on testing results may be hesitant to rely solely on PCR. Additional-PCR studies and related analyses must therefore be conducted as confirmatory tests, increasing the cost associated with accurate diagnosis. Alternative pathogen detection methods are needed to provide rapid and more reliable detection and confirmation.

Molecular padlock probe (MPP) technology is an alternative molecular detection strategy that can be modified for use by individuals without specialized training to detect microbial pathogens quickly, accurately and reliably. MPPs are employed in an isothermal, non-PCR-based nucleic acid amplification system providing a high degree of selectivity with a low rate of false-positive results. As MPPs detect RNA and DNA directly, they can be used to report the presence, and potentially replication or gene expression, in a target organism. MPPs comprise a single-stranded linear probe oligonucleotide and a shorter, single-stranded target sequence. A specified number of bases at both the 5′ and 3′ ends of the probe, which are connected by a random linker sequence, are complementary to the 5′ and 3′ halves of the target sequence. Upon annealing and ligation, this yields a circular oligonucleotide construct with a single-stranded region of linker sequence and a double-stranded segment located at the MPP:target annealing region (Nilsson, Malmgren, Samiotaki, Kwiatkowski, Chowdhary & Landegren 1994). The circularized MPP (cMPP) can then undergo rolling circle amplification (RCA) where, with the addition of *Bst* polymerase and an appropriate primer, the target sequence is removed from the padlock and the circular oligonucleotide is amplified repeatedly, forming a concatamer of the original circular probe sequence. Further amplification can be accomplished via hyperbranching (Hbr), in which the incorporation of a second primer, complementary to a region on the amplified concatamer, initiates a new amplification reaction for each cycle of the original cMPP (Lizardi, Huang, Zhu, Bray-Ward, Thomas & Ward 1998). Under the appropriate conditions, the RCA/Hbr reaction can continue indefinitely, rapidly and accurately amplifying the padlock and thereby confirming the detection of a short, specific sequence of nucleic acid from the targeted organism.

In this study, we investigate the use of MPP technology coupled with RCA and Hbr for the detection of IHNV and ISAV. To our knowledge, this work represents the first application of MPP/RCA to the detection of viral fish pathogens. It also serves as the basis for ongoing studies in which MPP technology is being integrated into a surface-immobilized molecular sensing platform for the rapid and accurate on-site detection of viral and bacterial pathogens.

Materials and methods

Oligonucleotides

Table 1 shows the sequences of all oligonucleotides used in this study. Oligonucleotides were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA, USA) using standard phosphoramidite chemistry and subsequently purified by polyacrylamide gel electrophoresis. Synthetic target (splint) sequences were 3′-phosphorylated to prevent self-priming during the amplification process and probe sequences were 5′-phosphorylated. Corresponding to nucleotides 473–517 within the N gene, the IHNV splint sequence was chosen from the published genomic sequence of the IHNV genome (GenBank L40883) (Morzunov, Winton & Nichol 1995). The IHNV mismatch splint sequence incorporates a T → C substitution at base 492 to imitate a single nucleotide polymorphism. To induce properly oriented annealing for cMPP construction, a 90 base IHNV probe was designed based on the IHNV splint sequence. The 47 base ISAV splint sequence represents nucleotides 638–684 of the protein PB1 gene of the North American isolate of ISAV (GenBank AF095254) (Blake, Bouchard, Keleher, Opitz & Nicholson 1999). To represent a single-site mutation, the ISAV mismatch splint sequence contains a T → C substitution at base 660 of the published sequence. Appropriately, complementary to the splint sequence, a 95 base ISAV probe was designed to allow proper alignment with the splint for padlock probe construction. For RCA, a universal 18 base rolling circle primer was designed to complement bases within the single-stranded portion of the

formed cMPP and could therefore be used for both IHNV and ISAV amplification. A 15 base Hbr primer was designed to complement the single-stranded product of RCA. Both primers were universal and neither had a phosphorylated terminus.

Viral RNA isolation

The RNA used in these studies was identified as (i) purified, which was produced via cell culture; (ii) as part of the total RNA purified from virally-infected salmon kidney (provided by Micro Technologies, Richmond, ME, USA) or rainbow trout (infected) in which the presence of viral RNA was confirmed by reverse transcription-PCR (see *Results*); or (iii) from non-infected kidney as a negative control. IHNV RNA (mol. wt approximately 4×10^6 Da) was obtained by infecting *Epithelioma papulosum cyprini* (Fijan, Sulimanovic, Bearzotti, Muzinic, Zwillenberg, Chilmonczyk, Vautherot & de Kinkelin 1983) cells cultured in Earle's minimal essential medium (MEM with Earle's salts) supplemented with 5% foetal bovine serum (FBS) with IHN virus at a multiplicity of infection (MOI) of 0.1. Cultures were maintained at 19 °C under 5% CO_2. ISA virus was cultured at 19 °C under atmospheric conditions on CHSE 214 cells in Hanks' minimal essential medium (MEM with Hanks' salts) supplemented with 5% FBS at an MOI of 0.2 to yield ISAV RNA (mol. wt approximately 6×10^6 Da). Cultures were observed daily and the media collected for filtration when approximately 90% of the cells exhibited cytopathic effects (CPE). This normally occurred at 1 to 2-days post-infection for IHNV; CPE for ISAV

Table 1 Base sequences of MPPs, target oligonucleotides, RCA primers and RT-PCR primers

Oligonucleotide	Sequence
IHNV splint (target)	5′-GGGCACCACCAGACGCTACCTTCGCAGATCCCAACAACAAGCTTG-3′
IHNV probe (MPP)	5′-GTAGCGTCTGGTGGTGCCCATTCATGCATGGTCACTCTTCGTTCTAGTAC TCTAGTACGCTTAGCAAGCTTGTTGTTGGGATCTGCGAAG-3′
IHNV mismatch splint (target)	5′-GGGCACCACCAGACGCTAC**T**TTCGCAGATCCCAACAACAAGCTTG-3′
ISAV splint (target)	5′-AGGACCAAACTCGAGCCGAGAGCAGTGTTCACAGCAGGAGTTCCATG-3′
ISAV probe (MPP)	5′-CTCTCGGCTCGAGTTTGGTCCTAATCTCAGTCATGGTCACTCTTCGTT CTAGTACTCTAGTACGCTTAGCATGGAACTCCTGCTGTGAACACTG-3′
ISAV mismatch splint (target)	5′-AGGACCAAACTCGAGCCGAGAG**T**AGTGTTCACAGCAGGAGTTCCATG-3′
RCA	5′-ACGAAGAGTGACCATGCA-3′
Hyperbranching primer	5′-ACTCTAGTACGCTTA-3′
RT-PCR primers:	
IHN3	5′-GTTCAACTCAACGCCAACAGG-3′
IHN4	5′-TGAAGTACCCCACCCCGAGCATCC-3′
ISA1D	5′-GGCTATCTACCATGAACGAATC-3′
ISA2	5′-TAGGGGCATACATCTGCAT-3′

Region of probe complementary to splint is underlined; mismatched base in bold.

MPP, molecular padlock probe; RCA, rolling circle amplification; RT-PCR, reverse transcription-polymerase chain reaction; IHNV, infectious haematopoietic necrosis virus; ISAV, infectious salmon anaemia virus.

were usually seen at 10 to 12-days post-infection. After filter sterilization (0.2-μm membrane) of the media, purified RNA was extracted using TRI Reagent LS (Sigma, St Louis, MO, USA) as recommended by the manufacturer. RNA was extracted from control or infected tissue sources by homogenization in TRI Reagent (Sigma) as described by the manufacturer and stored at −20 °C until needed for cMPP construction.

Molecular padlock ligation reactions

Molecular padlock probe ligation reactions using complementary synthetic probe and splint were conducted in 50 μL of exonuclease I buffer (67 mM glycine–KOH, 6.7 mM MgCl$_2$, 10 mM 2-mercaptoethanol, pH 9.5) supplemented with 1 mM ATP. A quantity of 100 nM MPP and 400 nM splint were incubated with 40 U of T4 DNA ligase at 23 °C for 30 min. The reaction was terminated by heating at 65 °C for 15 min. Twenty units of exonuclease I (*Escherichia coli*) were subsequently added to degrade any non-ligated reactants. After incubation at 37 °C for 30 min, the exonuclease reaction was halted by heating at 80 °C for 15 min. MPP reaction mixtures were stored at −20 °C until used in the amplification process. All enzymes and buffers were purchased from New England Biolabs (NEB; Beverly, MA, USA). Modifications were made to the standard ligation reactions in specific experiments, depending on the parameters under investigation, as outlined below:

1 To determine the detection limits of probe for target, cMPPs were constructed using target samples that were serially diluted at 1:10. This yielded target concentrations from 400 nM to 400 aM, representing copy numbers approximately from 10^{13} to 10^4.
2 Circularized MPPs for the evaluation of MPP:splint pair specificity were constructed as above, but with splint sequences of varying complementarity.
3 Circularized MPPs constructed to cell culture-derived RNA targets were prepared using methods similar to those used for synthetic components, with the following modifications: 34 ng of purified RNA, yielding a 45- (IHNV) or 47- (ISAV) base target binding site concentration of 400 nM, was used as a target in a 25-μL reaction volume containing 100 nM MPP. MPP

ligations with RNA target were performed for 30 min at 37 °C in RNA buffer (10 mM Tris–acetate, 10 mM Mg acetate, 10 μM ATP, 1 μM dithiothreitol) containing RNasin (Promega, Madison, WI, USA). After ligation, samples with RNA target were simultaneously treated for 30 min with exonuclease 1 and RNase ONE RNase (Promega), which is not inhibited by RNasin, to remove non-ligated MPP components and excess RNA. The reactions were terminated by heating at 80 °C for 30 min.
4 RNA isolated from virally infected kidney tissue was used to evaluate the efficacy of MPPs as a diagnostic for the presence of viral infection. These cMPPs were made by combining 0–200 μg of infected-tissue RNA with 100 nM MPP in 50-μL aliquots using enzymes, buffers and conditions as described above for the construction of padlocks from virus RNA harvested from cell culture media.

The construction of cMPPs to IHNV and ISAV were confirmed via gel electrophoresis on 4% Nu-Sieve agarose (Cambrex Bioscience, Rockland, ME, USA). Gels were run at 1.5 V cm^{-1} for 30 min in TAE buffer (40 mM Tris–HCl, 20 mM acetic acid, 1 mM EDTA, pH 8.3) with 0.25 μg mL^{-1} ethidium bromide.

Rolling circle amplification with hyperbranching

Rolling circle amplification and Hbr reactions were carried out in 50-μL volumes. A volume of 5 μL of standard cMPP ligation products or 25 μL (to ensure MPP inclusion) of low target number or RNA/infected tissue MPP ligation products were combined with 1 μM each of rolling circle and Hbr primers in ThermoPol reaction buffer (20 mM Tris–HCl, 10 mM KCl, 10 mM (NH$_4$)$_2$SO$_4$, 2 mM MgSO$_4$, 0.1% Triton X-100, pH 8.8; NEB) supplemented with 1.25 μg T4 gene 32 protein (USB Corp., Cleveland, OH, USA), 0.2 mM dNTPs, and 8 U of *Bst* DNA polymerase (large fragment; NEB). A 1:5000 dilution of SYBR Green II (Molecular Probes, Eugene, OR, USA) was added to permit fluorescence monitoring of the development of RCA/Hbr product. The reaction was carried out at 65 °C in an iCycler iQ Real-Time Detection System, using Detection System Software Version 3.0 for Windows (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Fluorescence filters with excitation wavelength of

490 ± 10 nm and emission 530 ± 15 nm were used for detection. Readings were taken at 1-min intervals over an assay time of 2 h.

Rolling circle amplification was confirmed by eliminating the Hbr primer from the above reactions and running aliquots of the reaction products on a 1% alkaline agarose gel (1% agarose, 50 mM NaOH, 1 mM EDTA, pH 8). Electrophoresis was accomplished in alkaline buffer (50 mM NaOH, 1 mM EDTA, pH 8) at 3.5 V cm^{-1} for 3 h. Band visualization was accomplished by soaking the gel in neutralization solution (1 M Tris–HCl, pH 7.6, 1.5 M NaCl) containing 0.5 μg mL^{-1} ethidium bromide. Fluorescence averages from each lane were determined to show increasing amplification over time. Hbr was evaluated by analysing aliquots of the complete RCA/Hbr reaction products, as described above, on a 1% agarose electrophoresis gel in TAE buffer containing 0.25 μg mL ethidium bromide. Gels were run at 1.5 V cm^{-1} for 30 min.

Confirmational analysis via reverse transcription-PCR

Reverse transcription (RT)-PCR was performed as a confirmation of the presence of IHNV and ISAV in the virally infected RNA tissues used in this study. An RT-PCR assay was applied for the detection of IHNV RNA (Williams *et al.* 1999) using primer pair HN3/HN4. The presence of ISAV RNA was confirmed using primer pair ISAV1D/ISAV2 in an RT-PCR assay (Opitz, Bouchard, Anderson, Blake, Nicholson & Keleher 2000). See Table 1 for primer sequences.

Results

Construction and amplification of molecular padlocks

Figure 1 illustrates the results of construction of cMPPs and their subsequent rolling circle and Hbr amplifications. Panel A shows the result of circularization and ligation of the linear molecular padlocks for IHNV (lanes 1–3) and ISAV (lanes 5–7), depicted as an increased molecular size of the constructed product in lanes 3 and 7. The increase in molecular size indicates that the 5′ and 3′ ends of the linear MPP have identified the appropriate regions of the target sequence, i.e. ligation of the
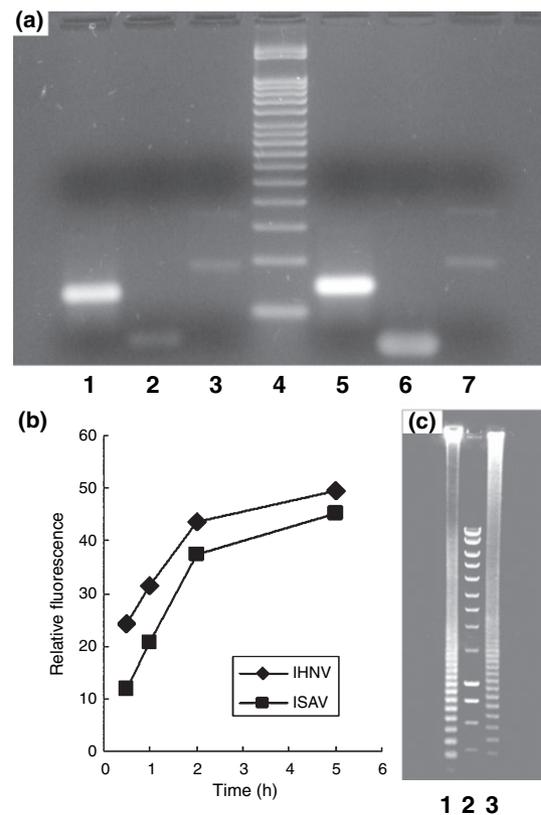

Figure 1 Circularized molecular padlock probe construction and amplification. Molecular padlock circularization and ligation is indicated by the shift in molecular weight of the ligated, exonuclease-treated construct (Panel a). Lane 1, infectious haematopoietic necrosis virus (IHNV) MPP. Lane 2, IHNV splint. Lane 3, ligated, exonuclease-treated IHNV MPP. Lane 4, 50-bp molecular weight ladder. Lane 5, infectious salmon anaemia virus (ISAV) MPP. Lane 6, ISAV splint. Lane 7, ligated, exonuclease-treated ISAV MPP. Panel b depicts the results of rolling circle amplification of IHNV and ISAV circularized MPPs. A volume of 10-μL aliquots of amplification reactions were taken over a 2-h time-period and analysed by 1% alkaline agarose gel electrophoresis. A digital image of the ethidium bromide-stained gel was acquired and the mean fluorescence intensity of each lane quantified using Photoshop software (Adobe Systems Incorporated, San Jose, CA, USA). Average fluorescence values of each lane were background-subtracted and the resulting values plotted with respect to time. Panel c shows the rolling circle amplification/hyperbranching (Hbr) reaction of IHNV and ISAV MPPs. A volume of 10-μL aliquots of a 2-h Hbr reaction were separated on 1% agarose. Lane 1, IHNV Hbr products. Lane 2, 100 bp molecular ladder. Lane 3, ISAV Hbr products.

juxtaposed ends of the probe created a cMPP. This conclusion is further supported by the absence of smaller oligonucleotides in these lanes when compared with the lanes containing control samples of MPPs (lanes 1 and 5) and targets (lanes 2 and 6).

A plot depicting the accumulation of fluorescent products over time from the RCA of the cMPP is shown in Panel B. RCA generated a large product that could not enter the native agarose gel matrix, even with very low (0.5%) agarose content; the amplification product is typically evident as a halo of fluorescence encircling the loading well only. However, when separated on a 1% alkaline-agarose gel, unbanded amplification product was detected, indicating that the denaturing gel maintained the RCA product as single-stranded oligonucleotides of multiple lengths, allowing separation through the gel matrix. The mass of the product increased with time, as seen in the graph, indicating continuous amplification. The indistinct pattern of the electrophoretic separation of the RCA product indicates that multiple padlock amplifications of a range of lengths occurred simultaneously (gel not shown). Hbr amplification of the concatameric RCA products is evident as a series of distinct bands in lanes 1 (IHNV) and 3 (ISAV) of Panel C. The Hbr primer initiated elongation at each concatamer of the cMPP along the extended RCA product. The strand-displacing property of the polymerase employed for amplification ensures that initial Hbr reactions are continuously displaced from the rolling circle backbone, creating Hbr amplification products with a range of distinct lengths. Amplification of the concatameric RCA product is indicated by the distinctive ladder pattern of the single-stranded Hbr products, with each band a multiple of the original length of the MPP.

Sensitivity of MPP for target

Circularized MPPs constructed by serially diluting the target sequence 10-fold prior to ligation were evaluated by RCA/Hbr amplification (Fig. 2). Target sequence concentration in the 50-µL padlock ligation reaction ranged from 400 nM, representing approximately 10^{13} copies of target, to 400 aM (approximately 10^4 targets). Measurement of fluorescence over 120 min showed that in samples with ligation target concentrations between 4 and 400 nM, RCA/Hbr amplification occurred virtually immediately, albeit at different rates depending on the target concentration, and largely ending by 60 min (see Inset, Fig. 2). In samples with ligation target concentration < 40 pM, a lag in the initiation of amplification was observed and products could not be detected until nearly 30 min after the start of the reaction.

Evidence of MPP sensitivity for decreasing target concentrations is shown in Fig. 2. These results (representative of multiple assays) indicate that both probes could reliably detect as few as approximately 10^5 targets, forming cMPPs which were subsequently amplified by RCA/Hbr.

Specificity of MPP:splint pair

Figures 3 and 4 demonstrate the specificity of the IHNV and ISAV MPPs for targets of varying complementarity. High specificity of each MPP for its target sequence is crucial if these probes are to be effective as diagnostic agents. Specificity of
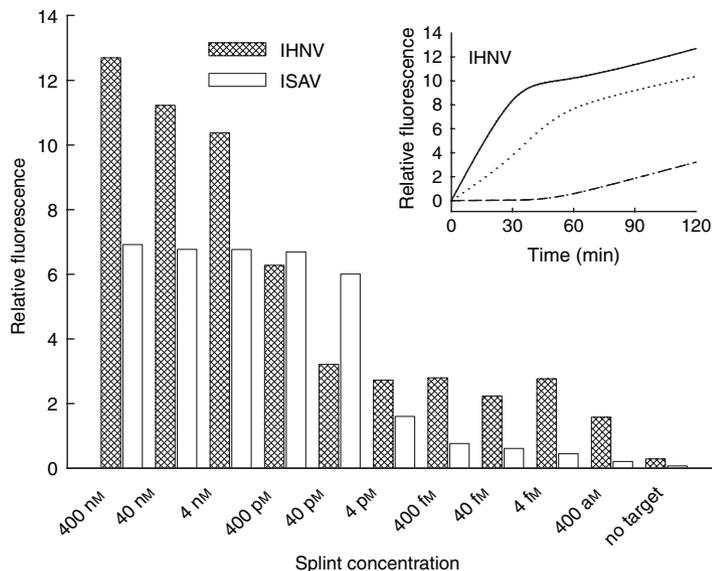

Figure 2 Sensitivity of molecular padlock probe (MPP) for decreasing target concentration. MPPs were constructed to a 10-fold serially-decreasing target (splint) concentration within the ligation reaction. Patterned bars indicate infectious haematopoietic necrosis virus (IHNV) splint as target; open bars indicate infectious salmon anaemia virus (ISAV) splint as target. The inset in the upper panel depicts the complete time course of IHNV splint-directed cMPP amplification by RCA at 400 nM (solid line), 4 nM (dotted line) and 40 pM (dashed line) of splint. Ligation and amplification reactions were run in parallel, but with different MPP sequences. Fluorescence values at time = 0 min were subtracted from all data sets. These data are representative of multiple analyses.

the probes for their respective targets was evaluated by constructing splint sequences with imperfect complementarity to the MPP sequence (Table 2) and monitoring their efficacy via RCA/Hbr. As seen in Fig. 3, amplification of cMPPs constructed from cross-matched MPP/splint pairs (IHNV MPP/ISAV splint or ISAV MPP/IHNV splint) yielded no RCA/Hbr product. The complementarity of the IHNV MPP/ISAV splint pair is 32%; 15 of the 47 bases in the double-stranded region of the cMPP are complementary pairs. With respect to the location of the ligation site, nine of the 22 bp 5′ of the ligation site (41%) and six of the 25 bp 3′ of the ligation site (24%) are complementary. The ISAV MPP/IHNV splint pair is 34% complementary, with 15 of the 45 bases in the double-stranded region being either CG or AT pairs. Of these, 42% (eight of 19) are 5′ with respect to the ligation site, and 27% (seven of 26) are 3′ of the ligation site. To evaluate the utility of MPPs for the detection of single nucleotide polymorphisms, cMPPs were constructed using a splint with a single-base mismatch complementary to the 3′ end of the MPP. Figure 4 demonstrates that no RCA/Hbr product is generated by RCA/Hbr amplification of MPPs and non-complementary targets, indicating MPPs can be designed such that a high degree of complementarity is required for the production of cMPPs. The inability of these constructs, and the
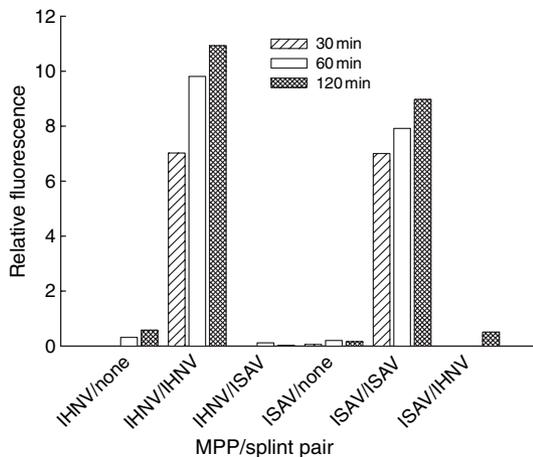
negative control, to yield product upon RCA/Hbr amplification analysis supports the conclusion that MPPs detect nucleic acid sequences with a high degree of specificity (Nilsson, Krejci, Koch, Kwiatkowski, Gustavsson & Landegren 1997).

Detection of viral RNA

In studies using 34 ng of either IHNV or ISAV purified viral RNA as the target, RCA/Hbr analysis indicated that both the IHNV and ISAV MPPs were able to detect their respective binding sites within the viral RNA. This result is illustrated in Fig. 5. Additional control studies using purified zebrafish, *Danio rerio* (Hamilton), or rainbow trout kidney RNA as a target for both IHNV and ISAV MPPs yielded no product when evaluated by RCA/Hbr (data not shown). Construction of cMPPs with 10-fold serially diluted target sequence (400 nM–4 fM) was also conducted in the presence of 100 μg of fish kidney RNA as background, a condition similar to that which might be encountered with a sample obtained from infected fish kidney, to evaluate effects of a large mass of non-species RNA on MPP:target interaction. As seen in Fig. 6, RCA/Hbr analysis shows that both IHNV and ISAV MPPs were able to form cMPPs, finding the
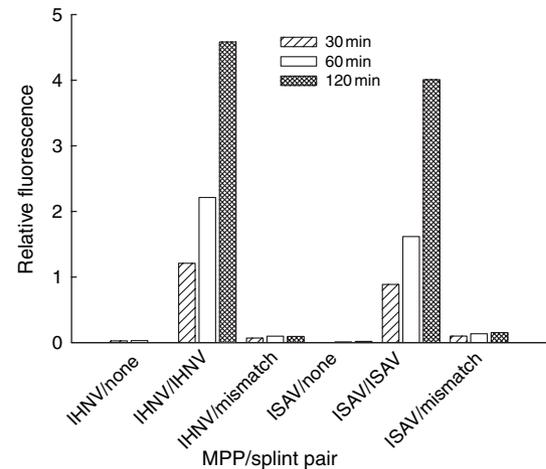

Figure 3 Specificity of molecular padlock probe (MPP)/splint pair. Circularized MPPs were constructed using targets of varying complementarity and amplified by rolling circle amplification/hyperbranching to determine ligation/circularization. Fluorescence was monitored for 120 min. Only padlocks made to the complementary target sequence exhibited amplification. Fluorescence values at time = 0 min were subtracted from all data sets. These data are representative of multiple analyses.

Figure 4 Detection of single-point mismatch. Molecular padlock probes (MPPs) were constructed to complementary target or target containing a single-base mismatch at the ligation site, and a no-target control (none). Ligation was determined by rolling circle amplification/hyperbranching amplification. Only circularized MPPs made to complementary target exhibited amplification. Fluorescence values at time = 0 min were subtracted from all data sets. These data are representative of multiple analyses.

Table 2 Probe-target alignment for circular molecular padlock construction

Oligonucleotide	Sequence
IHNV probe (MPP)	...TTACCCGTGGTGGTCTGCGATG*GAAGCGTCTAGGGTTGTTGTTCGAA...
ISAV splint (target)	5′-AGGACCAAACTCGAGCCGAGAG⁻CAGTGTTCACAGCAGGAGTTCCATG-3′
ISAV probe (MPP)	...TGGTTTGAGCTCGGCTCTC*GTCACAAGTGTCGTCCTCAAGGTACG...
IHNV splint (target)	5′-GGGCACCACCAGACGCTAC⁻CTTCGCAGATCCCAACAACAAGCTTG-3′
IHNV probe (MPP)	...CCCGTGGTGGTCTGCGATG**G**ˣAAGCGTCTAGGGTTGTTGTTCGAAC...
IHNV mismatch splint (target)	5′-GGGCACCACCAGACGCTAC**T**⁻TTCGCAGATCCCAACAACAAGCTTG-3′
ISAV probe (MPP)	...TCCTGGTTTGAGCTCGGCTCTC**G**ˣTCACAAGTGTCGACCTCAAGGTAC...
ISAV mismatch splint (target)	5′-AGGACCAAACTCGAGCCGAGAG**T**⁻AGTGTTCACAGCAGGAGTTCCATG-3′

*, ligation site; ˣ, point of non-ligation; mismatch base in bold.
MPP, molecular padlock probe; IHNV, infectious haematopoietic necrosis virus; ISAV, infectious salmon anaemia virus.
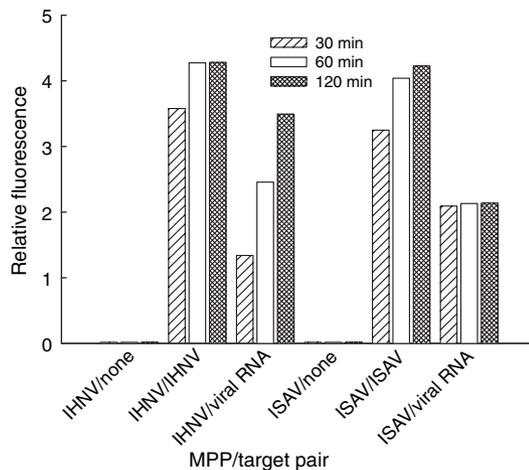

Figure 5 Molecular padlock probe (MPP) detection of purified viral RNA. Circularized MPPs were constructed using purified viral RNA or synthetic DNA oligonucleotide splint as target. Fluorescence values at time = 0 min were subtracted from all data sets. These data are representative of multiple analyses.

correct target from within the total RNA mass over a range of target concentrations. This result is in agreement with the data shown in Fig. 2 (cMPP construction to 10-fold serially diluted target sequence). These studies also support the results of the purified-RNA specificity experiments (Fig. 5), showing that MPPs directed to specific regions of IHNV RNA or ISAV RNA will form cMPPs only when the complementary viral RNA sequence is identified, even in the presence of a vast excess of background RNA.

In preparation for determining the utility of MPPs for detection of viral RNA from diseased hatchery stocks, kidney and liver tissues were harvested from the mortalities of an experiment in which 500 g rainbow trout were injected with a lethal dose of IHNV. This tissue was evaluated by RT-PCR analysis and agarose gel electrophoresis to confirm IHNV infection, using previously confirmed non-infected tissue as a control. Archival kidney tissue from fish previously infected with ISAV was reconfirmed in the same manner. The electrophoresis gel of this analysis is shown in Fig. 7. Positive RT-PCR analysis for IHNV using primer pair HN3/HN4 yielded a single band at 371 bp, as seen in lane 7; using primer pair SA1D/SA2, a positive result for ISAV infection is indicated by the presence of a band at 475 bp in lane 8. These results confirmed that the procured tissues were indeed infected with the expected virus.

Figure 8 displays the utility of MPPs for detection of viral RNA sequence from rainbow trout (IHNV) or salmon (ISAV) kidney. RNA extracted from virus-infected kidney tissue was used as the target for constructing cMPPs. RCA/Hbr amplification analysis showed that MPPs could detect the appropriate RNA target from within the total RNA extracted from the kidney of IHNV and ISAV infected fish. These results were predicted by the simulated target (splint) studies described earlier and indicate specifically that the presence of kidney RNA within a sample does not inhibit the MPP from detecting its target. The RNA sample extracted and purified from the infected kidney contains both tissue RNA and viral RNA. The percentage of RNA that is actually of viral origin is not known; therefore, the lower limit of detection of viral RNA cannot be determined directly in this study. However, both IHNV and ISAV MPPs were able to identify their viral targets in samples containing as little as 50 μg of total RNA extracted from infected kidney tissue.

Discussion

Diagnostic assays are used to identify the presence and agent of clinical disease, as opposed to
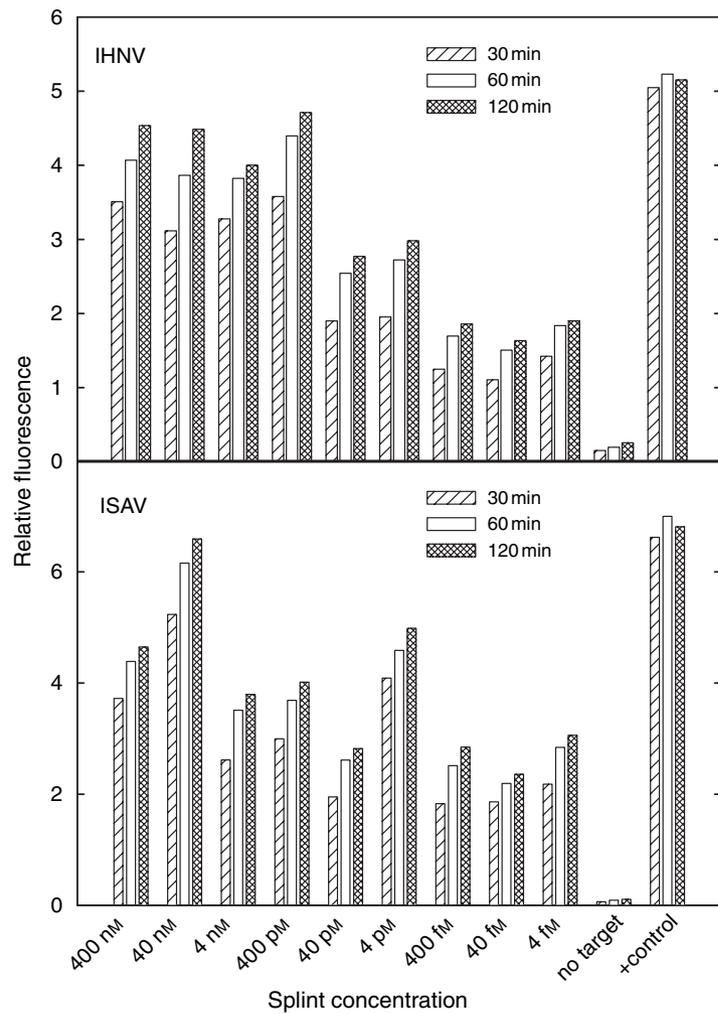
Figure 6 Sensitivity to decreasing target concentration in the presence of RNA background. Circularized molecular padlock probes (MPPs) were constructed to serially diluted target sequence for infectious haematopoietic necrosis virus (IHNV; upper plot) and infectious salmon anaemia virus (ISAV; lower plot) in the presence of 100 μg non-viral RNA as background. Control sample is 400 nM target, without RNA background. The presence of a large mass of non-viral RNA in the ligation reaction did not inhibit the MPP from finding its complementary target. Fluorescence values at time = 0 min were subtracted from all data sets. These data are representative of multiple analyses.

inspection methods, which certify the disease-free status of a population (Office Internationale des Epizooties 2003). Diagnostic assays require speed and specificity, two of the important characteristics of MPP technology. The current guidelines determining the officially recognized criteria for confirmation of IHNV and ISAV infection in fish, as established by the OIE, require monitoring clinical, pathological, histopathological and haematological changes, as well as detection of viral particles via virus isolation (VI), indirect immunofluorescent antibody testing (IFAT) and RT-PCR (Office Internationale des Epizooties 2003). Five laboratories participating in a blind study of 400 ISAV-infected kidney samples representing four populations of farmed Atlantic salmon to compare the precision (Nerette, Dohoo, Hammell, Gagne, Barbash, MacLean & Yason 2005b) and accuracy (Nerette, Dohoo & Hammell 2005a) of these

methods concluded that none can be used as a 'gold standard' for viral detection. Found to be highly accurate and highly precise, VI studies are typically expensive to conduct and, although possible to accomplish in as little as 1 week, may take 30–45 days to complete. The results of VI are thus usually too delayed to be useful in making immediate management decisions regarding infection of fish stocks. IFAT has been determined to be useful as a low-cost initial screening method, but the interpretation of IFAT is highly subjective, leading to variation in the results both within and between laboratories. IFAT also exhibits low sensitivity (65–75%), which is thought to be related to the protein being detected, as some epitopes are not present until late in the stages of the disease. These results suggest that IFAT is best paired with another, more reliable method.
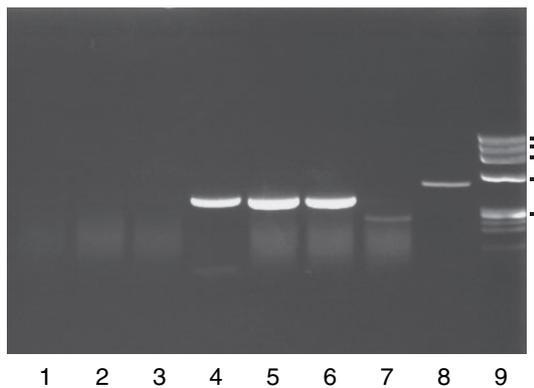
Figure 7 Reverse transcription-polymerase chain reaction (RT-PCR) confirmation of viral infection of kidney tissue. RT-PCR analysis of uninfected (control) and infected kidney tissue confirmed the presence of IHNV or ISAV infection; products were analysed by 1% agarose gel electrophoresis. Lanes 1–3, uninfected tissue amplified with primer pair IHN3/4 as a negative control for infectious haematopoietic necrosis virus (IHNV). Lanes 4–6, infected tissue amplified with primer pair IHN3/4. Lane 7, uninfected tissue amplified with primer pair ISA1D/2 as a negative control for infectious salmon anaemia virus (ISAV). Lane 8, infected tissue amplified with primer pair ISA1D/2. Lane 9, ΦX174 DNA-Hae III molecular weight markers (the marked bands represent 1353, 1078, 872, 603 and 310 bp). Bands at approximately 371 bp in lanes 4–6 are positive identification of IHNV infection. Band at approximately 475 bp in lane 8 is positive identification of ISAV infection.

Figure 8 Molecular padlock probe (MPP) detection of viral RNA from infected kidney tissue. Circularized MPPs were constructed to RNA extracted from virally infected fish kidney; uninfected kidney tissue was compared as a negative control. Fluorescence monitoring of rolling circle amplification/hyper-branching amplification indicates that MPPs directed against infectious haematopoietic necrosis virus (IHNV) and infectious salmon anaemia virus (ISAV) can detect viral RNA target sequence in tissue samples from infected fish. Fluorescence values at time = 0 min were subtracted from all data sets. These data are representative of multiple analyses.

As no vaccine or treatment is available for IHNV and ISAV infections, early detection is critical to control the spread of disease. Even so, most of the molecular diagnostic tools established for virus detection in fish have been designed for use with diseased fish containing high virus loads (Mjaaland *et al.* 2002). More sensitive diagnostic methods are necessary to detect the presence of low levels of viral infection, such as may be seen very early or late in the course of the disease. RT-PCR is considered by many laboratories to be the method of choice for detection of low levels of viral nucleic acid and ideally could serve as a confirmation of IFAT results. While applicable, RT-PCR may have significant limitations in practice. In the precision and accuracy studies directed toward ISAV, which are referenced above, Nerette *et al.* (2005a,b) found statistically significant differences in RT-PCR sensitivity across populations, indicating that, while sensitive, RT-PCR has a higher false-positive rate than either IFAT or VI. Repeatability and reproducibility of RT-PCR clearly depended on the laboratory performing the analysis. Furthermore, RT-PCR analysis depended strongly on interpret-
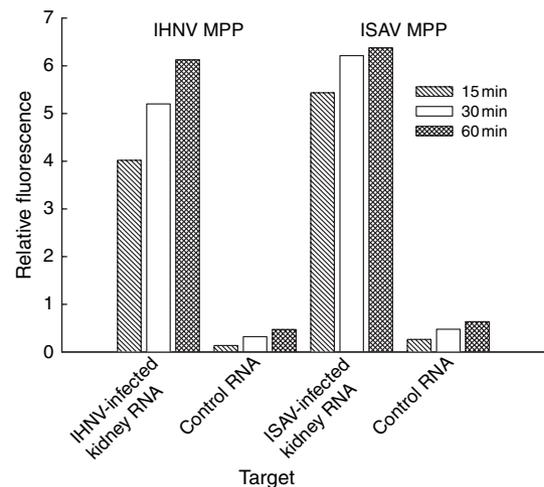
ation for definition of a positive result. These studies concluded that none of the three tests currently in use detect with consistent sensitivity and specificity across populations (Nerette *et al.* 2005a), and that both IFAT and RT-PCR need to be standardized with respect to protocol and interpretation before they can be used as precise methods for determining viral infection across populations (Nerette *et al.* 2005b). Less subjective analytical methods for the detection of IHNV and ISAV would clearly be welcome.

An alternative isothermal amplification approach has recently been investigated for detecting viral fish pathogens. Loop-mediated isothermal amplification (LAMP) of DNA (Notomi, Okayama, Masubuchi, Yonekawa, Watanabe, Amino & Hase 2000; Gunimaladevi, Kono, LaPatra & Sakai 2005) has been investigated as an analytical substitute for PCR. Using six amplification primers, LAMP yields a large, branching, multilooped product that can be detected by several methods. Recent studies suggest that LAMP is as sensitive as PCR for the amplification of cDNA of Koi herpesvirus in *Cyprinus carpio* L. (Gunimaladevi, Kono, Venugopal & Sakai 2004) and red seabream iridovirus (Caipang,

Haraguchi, Ohira, Hirono & Aoki 2004). Although LAMP has been evaluated for RNA detection (Notomi *et al.* 2000), it requires an initial RT step.

Molecular padlock probes must be specific, sensitive, robust and straightforward in their application if they are to find applications in field tests for the detection of viral pathogens in fish tissue. Our studies support MPP technology coupled with rolling circle and Hbr amplification as a reasonable basis for the development of accurate tests for the detection of IHNV and ISAV associated with fish tissues. MPPs exhibit specificity and sensitivity levels similar to RT-PCR, but with fewer of the limitations of this method. MPPs can be designed to detect RNA directly, without reverse transcription, reducing the potential concern that genomic DNA is carried over into the RNA preparation. Furthermore, the use of MPP technology eliminates the need for PCR, thereby removing the possibility of a false-positive reaction from contamination via PCR products. The physical construction of a MPP favours a high degree of specificity. The dual recognition of the juxtaposed MPP ends for a target sequence ensures specificity of detection similar to PCR (Baner, Nilsson, Isaksson, Mendel-Hartvig, Anson & Landegren 2001) by requiring an exact match in base pairing if ligation is to occur. Studies have shown that MPPs can be used to identify point mutations of DNA in solution and in fixed cells, as evidenced by a lack of ligation at the site of the mutation (Christian, Pattee, Attiz, Reed, Soreasen & Tucker 2001; Zhong, Lizardi, Huang, Bray-Ward & Ward 2001). We have confirmed that the IHNV and ISAV MPPs evaluated in this study also do not ligate when matched to targets with a varying degree of non-complementarity, including a single-base mismatch at the ligation site. The ability to detect single nucleotide polymorphisms is analytically beneficial as it provides the ability to distinguish among specific strains of a given species through unique MPP:target pairs. If specificity at this level is not desirable, but variation in target sequence is known to occur with high frequency, MPPs can be constructed with a range of recognition/ligation sites to accommodate this variance and avoid false-negative responses. The identity of specific viral RNA can be confirmed by complementary methods, but as with PCR, a positive finding can be interpreted as presence of a specific virus without further analysis. Inclusion of recognition regions that are more highly conserved across

a range of viral strains, or use of a pool of strain-specific MPPs, reduces the likelihood that specific viral strains or variants will be missed. Furthermore, complementary target site identification by MPPs was not affected by the presence of large quantities of non-viral RNA within the test sample. This property is essential because viral RNA will always be found and isolated in the presence of an abundance of host RNA from cells of an infected tissue. Neither abrogation of MPP:target pairing nor loss of target specificity resulted from excess non-target nucleic acid mass in the MPP assay.

Molecular padlock probe technology does not exhibit a high degree of susceptibility to cross-contamination. In this study, exonuclease I was used to degrade single-stranded nucleic acid sequence prior to the initiation of RCA, ensuring that the only nucleic acid within the sample was cMPP. Furthermore, *Bst* polymerase does not exhibit strand displacement on single-stranded oligonucleotides; thus, non-circular, unligated probe cannot serve as an effective amplification template. This ensures that only the fully-circularized, ligated MPP will be amplified into detectable, high-molecular weight concatenated product.

The MPPs used in this study showed sensitivity for as little as 400 aM complementary target (splint), representing approximately 10^4 copies of target oligonucleotide sequence. This result is in agreement with RCA/Hbr studies designed to detect oligonucleotide sequences in other microbes (Hafner, Yang, Wolter, Stafford & Giffard 2001; Zhang, Zhang, Le & Konomi 2001). The ability to distinguish low levels of viral infection becomes significant when considering viral infection at a cellular level. In animal cells, viral replication occurs over several hours to a few days, potentially resulting in the synthesis of approximately 10^3–10^5 virions per cell (Chinchar 1999). The molecular probes under study are thus theoretically able to detect viral infection of a single cell. It is also clear that the sensitivity of the molecular probe for its complementary target is not inhibited by high-background levels of RNA. Thus, cMPPs can be constructed from RNA obtained from tissue punches, a standard method of sample collection, without sacrificing sensitivity.

For a method to be effective as an on-site field test for the detection of IHNV and ISAV in fish kidney tissue, the technology employed must be rapid, cost-effective and require minimal technical ability, while maintaining sensitivity and specificity.

Assays employing MPP technology can be designed to fulfil these requirements. In the laboratory, the detection of IHNV and ISAV in fish kidney tissue through the use of circularized molecular padlocks can be accomplished in < 4 h. A simple ethanol precipitation step yields RNA adequately purified for cMPP construction in 45 min or less. With the addition of MPP, appropriate buffer and DNA ligase, MPPs can be constructed in 30 min. Further purification via the incorporation of exonuclease and RNase is accomplished in 30 min using a simple temperature-controlled incubator or heat block. RCA and Hbr occur simultaneously, yielding detectable results in as little as 15–60 min, depending on viral load in the tissue. RCA with Hbr is an isothermal process, eliminating the need for thermal cycling apparatus required for PCR. Although the MPP/RCA method described here was carried out in a photometric thermocycler for ease of operation with 96-well format microplates, the few temperature changes essential to the method have been performed with much simpler apparatus in single tubes (not shown). Measurement of SYBR Green II signal associated with RCA/Hbr products in solution requires specialized instrumentation to measure fluorescence, however, measurements may be made in single samples using one of the several inexpensive single-sample filter-based fluorometers now available commercially. Several types of miniaturized systems for cost-effective fluorescence measurement are currently under development.

As the circularized probes are continuously, linearly amplified via one primer-annealing event, the risk of overwhelming non-specific amplification due to cross-contamination is reduced substantially. Although RCA of cMPPs proceeds linearly, Hbr produces significant numbers of unlinked concatamers of the original padlock probe simultaneously with the RCA. Elevated SYBR II nucleic acid stain fluorescence was a clear indication of specific target viral RNA sequence in the tissue being tested. With as few as 10^5 purified viral RNA target sites, the RCA/Hbr amplification process produced specific signal in < 2 h. Discrimination between positive and negative tests is an empirical determination based on the duration of the assay and the subsequent relative fluorescence signal from negative controls, positive controls and test samples. Positive tests typically gave rise to greater than fivefold increases in fluorescence relative to negative controls over the assay time, even at femtomolar-target concentrations.

This study reports the application of MPP technology as a detection assay for IHNV in rainbow trout and ISAV infections in Atlantic salmon. To our knowledge, this is the first report of the application of amplified molecular padlocks for the identification of virus in fish. MPPs paired with RCA/Hbr amplification combine the specificity and selectivity of ligated, targeted probes with the generation of an amplified DNA concatamer that is easily detected, while bypassing several of the issues that can give rise to false results associated with RT-PCR. Our results indicate MPPs to be an accurate and adaptable diagnostic identification method. This technology is currently in development as a portable, solid state format that is more applicable to on-site testing, with the goal of enabling fish growers to detect the presence of IHNV and ISAV at their own facilities. The robustness and simplicity of this technology, combined with its sensitivity and specificity, suggest that MPPs combined with RCA/Hbr amplification will prove to be a competitive methodology for the development of an on-site field test for the detection of IHNV and ISAV in fish stocks.

Acknowledgements

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