Development of Rapid Typing Methods for Teleost Pathogens

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ABSTRACT

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Infectious salmon anemia virus (ISAV), infectious pancreatic necrosis virus (IPNV), and nervous necrosis virus (NNV) are regarded as important fish pathogens in Atlantic Canada each with a proven or probable ability to decimate livestock. Disease management continues to rely primarily on regular surveillance using a variety of diagnostic methods. With the diversification of the aquaculture industry to include non-salmonid species there may be the potential for "cross-over" transmission of viral pathogens. Therefore, characterization of discrete strains of these viral pathogens will be important to define the significance of laboratory results for the purpose of disease management. In the current report, the use of reverse transcriptase polymerase reaction (RT-PCR) combined with denaturing gradient gel electrophoresis (DGGE) is described for the detection and differentiation of selected strains of ISAV, IPNV, and NNV.

RÉSUMÉ

L'anémie infectieuse du saumon (ISA), la nécrose pancréatique infectieuse (IPN) et la nécrose nerveuse virale (NNV) sont des maladies virales des poissons ayant des effets dévastateurs au Canada Atlantique, ou du moins en ayant le potentiel. La gestion de ces maladies repose principalement sur la surveillance régulière de l'état de santé des poissons au moyen de divers outils diagnostiques. Avec la diversification de l'industrie aquacole et l'exploitation d'espèces autres que les salmonidés, le potentiel de transmission inter-espèces de ces pathogènes pourrait augmenter. La caractérisation des souches distinctes de ces virus devient importante et aide à interpréter les résultats de laboratoire soumis aux gestionnaires. Dans le présent rapport, la technique de transcription inverse-polymérisation en chaîne de l'ADN (RT-PCR) combinée à l'électrophorèse en gradient de gel dénaturant (DGGE) est utilisée pour détecter et différencier des souches sélectionnées d'ISAV, IPNV et NNV.

INTRODUCTION

Infectious salmon anemia virus (ISAV), infectious salmon pancreatic virus (IPNV) and nervous necrosis virus (NNV) are regarded as the most important viral pathogens to finfish aquaculture in Atlantic Canada. ISAV has had the most serious impact on cultured Atlantic salmon of any disease and continues to be a challenge for the aquaculture industry (McGeachy and Moore 2003). Conversely, while IPNV and NNV have not adversely affected local salmon farming these viruses will become increasingly important during the development of intensive aquaculture of species such as haddock, halibut and cod. IPNV, or viruses demonstrating serological relatedness to IPNV, have been reported to cause disease in several fish species throughout the world (Reno 1999) including halibut (Rodger and Frerichs 1997). NNV has affected a number of different larval and juvenile marine fish in numerous regions of the world (Munday and Nakai 1997). An example in the past decade is the outbreak of NNV in Atlantic halibut in Norway (Dannevig et al. 2000). It has become apparent that a number of discrete strains exist for each of the three viral pathogens with potentially varying degrees of virulence to the exposed species (Blake et al. 2001; Sommer et al. 2004; Rolland et al. 1998). With the increasing diversification of aquaculture in the Maritime Provinces and the likely juxtaposition of different species, it will become increasingly important to differentiate between discrete strains of viruses to assist in the interpretation of diagnostic results. In addition, the ability to identify regionally important strains will be important in monitoring transmission between wild and cultured stocks of finfish both within and between species.

INFECTIOUS SALMON ANEMIA VIRUS (ISAV)

Infectious salmon anemia virus (ISAV) was first identified in Norway in 1984 (Thorud and Djupvik 1988) and later in Canada (Mullins et al. 1998; Lovely et al. 1999; Blake et al. 1999; Bouchard et al. 1999), Scotland (Rodger et al. 1998; Rowley et al. 1999), Chile (Kibenge et al. 2001a), and the United States (Bouchard et al. 2001). In Atlantic Canada, ISAV has cost the industry millions of dollars in direct costs and depopulation since 1996, not including costs incurred from surveillance efforts, research and indirect influences on supporting industries and local economy (Dr. S. McGeachy, NBDAFA, New Brunswick, pers.comm.). While surveillance programs are believed to have been effective in reducing the number of clinical outbreaks of the disease, ISAV remains the most economically important pathogen for regulatory authorities as reflected by mandatory depopulation of infected cages identified by a critical combination of diagnostic results. During the surveillance efforts however, contentions have arisen regarding the inability of diagnostic methodologies to discriminate between strains of ISAV. Where depopulation is an issue in disease management it is regarded as particularly important to be able to differentiate between strains of variable virulence. One approach to differentiating isolates is to define them at the genetic level.

The genome of ISAV consists of 8 segments of negative-stranded RNA (Mjaaland et al. 1997). Segment 2 is believed to encode a polymerase protein (Krossøy et al. 1999), segments 3 and 4 encode putative nucleoprotein and polymerase respectively (Ritchie et

al. 2001a), segment 6 encodes a hemagglutinin (Krossøy et al. 2001; Rimstad et al. 2001), and segments 7 and 8 encode putative non structural and matrix proteins (Ritchie et al. 2002; Cunningham and Snow 2000; Biering et al. 2002). Identification of these segments revealed the existence of nucleotide variation among isolates, especially in segment 6 (Krossøy et al. 2001; Rimstad et al. 2001; Kibenge et al. 2001b; Devold et al. 2001; Griffiths et al. 2001). The significance of the genetic variation has not been elucidated. Norwegian isolates include strains that can be differentiated by at least 13 sequence variants (Nylund et al. 2003) in the highly polymorphic region (hpr) of segment 6. The functional significance of this is not clear. However, it has been hypothesized that the shorter hpr versions found in various isolates may have arisen from a longer progenitor strain (Nylund et al. 2003; Cunningham et al. 2002; Mjaaland et al. 2002) and that these may exist as a commensal relationship with fish (Nylund et al. 2003). Cunningham et al. (2002) recently identified such a strain from an asymptomatic Scottish wild salmon and we have recently identified similar types of ISAV in asymptomatic aquaculture salmon in Atlantic Canada (Cook-Versloot et al. 2004). To date no disease has been associated with this strain but it remains to be seen whether this might indeed be an avirulent strain and whether or not it can change to a virulent form of the virus. Additionally, phenotypic differences have been observed in cell culture, with some strains of ISAV replicating on two cell lines whereas others could only replicate on one (Kibenge et al. 2000; Kibenge et al. 2001b). There is also anecdotal evidence for the existence of functional strain differences in that some ISAV outbreaks claim more fish than others, and some disease progression is acute or protracted (Mjaaland et al. 2002; Kibenge et al. 2006). Subclinical infections are also known to occur in wild salmonid species (Raynard et al. 2001) The virus has also been detected by RT-PCR in plaice and haddock (Molecular Biology Group, Research and Productivity Council, 921 College Hill Road, Fredericton, NB, Canada, E3B 6Z9, unpublished data). Although disease development is complex, involving virus, host and environment, these cumulative observations provide compelling evidence for the existence of discrete strains and make the study of strain differences important at the management level. Genetic strain differences are also relevant to epidemiological and relatedness studies. The ability to rapidly differentiate between viral strains will provide basic knowledge on virulence, epidemiology, and host specificity that can facilitate management decisions.

INFECTIOUS PANCREATIC NECROSIS VIRUS (IPNV)

Aquatic birnaviruses, such as infectious pancreatic necrosis virus (IPNV), are a genetically diverse group afflicting a number of different species (Wolf 1988). Aquatic birnaviruses are double stranded RNA viruses with 2 genomic segments (Dobos et al. 1979). The smaller genome segment B encodes a putative RNA dependent RNA polymerase (VP1) (Duncan et al. 1991). The larger genome segment A encodes the precursor protein (pVP2) to the major capsid protein (VP2), a non structural protein (NS) and a minor capsid protein (VP3) (Nagy et al. 1987). The larger segment also contains a second open reading frame encoding a minor protein (VP5) which has been detected in infected cells (Magyar and Dobos 1994). Based on their antigenic relatedness, aquatic birnaviruses have been segregated through reactivity to antisera into two groups, Serogroup A and Serogroup B, Serogroup A being the major serogroup (Caswell-Reno et

al. 1989; Nicholson 1993). The majority of aquatic birnaviruses belongs to Serogroup A which includes 9 serotypes: A1 (type strain West Buxton), A2 (type strain Sp), A3 (type strain Ab), A4 (type strain He), A5 (type strain Te), A6 (type strain Canada 1), A7 (type strain Canada 2), A8 (type strain Canada 3), and A9 (type strain Jasper). The distribution of these serotypes varies. Within Canada serotypes A6-A9 have been reported while in the United States serotype A1 is the predominant strain; serotypes A1-A3 have been reported in South America and Asia while serotypes A2-A5 are observed in Europe (Blake et al. 2001). Studies at the genetic level have identified differences between isolates. The VP2 gene has an approximately 93 amino acid variable region which includes two hypervariable domains (Heppell et al. 1995). Based on the analysis of the large ORF of genome segment A it was determined that the 3 major Canadian serotypes (A6-A8) were more closely related to isolates from Europe than to those from the United States (Blake et al. 2001). Given the number of different hosts affected by IPNV it is regarded as particularly important for fish disease management to differentiate between strains capable of producing clinical disease in one species and another. Such IPNV strain categorization will become increasingly important as aquaculture activity expands to include other species with the potential for horizontal transmission.

NERVOUS NECROSIS VIRUS (NNV)

Nodaviruses are responsible for disease in a variety of marine fish species world-wide. The disease, viral encephalopathy and retinopathy (VER), also known as viral nervous necrosis (VNN), is caused by a nodavirus. This nervous necrosis virus (NNV) pathogen has been responsible for high levels of mortality in larvae and juveniles (Munday and Nakai 1997). Within Eastern North America NNV has caused disease outbreaks in cod from Nova Scotia (Johnson et al. 2002), Newfoundland, and New Hampshire, in haddock from New Brunswick (Gagné et al. 2004) and has been isolated from wild winter flounder (Barker et al. 2002). The potential of NNV to affect local halibut also exists as reports have been made of nodavirus infection in Atlantic halibut in Norway (Grotmol et al. 1997; Grotmol et al. 2000). For this reason it is a pathogen of concern to Atlantic Canada where cod, haddock and halibut industries are being established and looking to grow. NNV is a nonenveloped icosahedral virus consisting of two RNA segments, RNA 1 and RNA 2. RNA 1 encodes an RNA-dependent RNA polymerase (protein A) and RNA 2 encodes a capsid protein (Mori et al. 1992). The capsid protein contains a region of high variability which is located in the T4 region (Nishiwawa et al. 1994). The variability observed in this capsid protein sequence has led researchers to organize isolates into clades based on phylogenetic analyses. The ability to rapidly and accurately type strains of nodavirus during surveillance will lead to better management of this pathogen.

The existence of genetic variation among isolates of ISAV, NNV and IPNV with different host backgrounds and disparate levels of virulence presents an opportunity for more effective epidemiology and tracking of discrete strains. Characterization of genetic variability may be achieved by a number of means including restriction fragment length polymorphism (RFLP) analysis and the direct sequencing of amplification products. However, these approaches can be time consuming and costly. The objective of this

project was to investigate the development of an alternative, faster and less costly method combining the reverse transcriptase polymerase chain reaction (RT-PCR) amplification of variable viral sequences and denaturing gradient gel electrophoresis (DGGE) (Myers et al. 1987). DGGE separates the double-stranded RT-PCR products based on their thermodynamic stability or melting temperature as determined by nucleotide sequence (the higher the GC content the higher the melting point). RT-PCR products are run in a linear ascending gradient of chemical denaturant. Once an amplification product has reached a point in the gradient of denaturing chemicals sufficiently strong to unwind the DNA, it will cease to move any further and be retained at a specific location within the gel. Fragments that differ in sequence, therefore, may be identified by comparison to known standards. This technique has been used to detect mutations in disease studies (Valero et al. 1994), separation of alleles for sequencing (Aldridge et al. 1998) and profiling of complex microbial populations (Muyzer et al. 1993). The DGGE technique is rapid, inexpensive, and can type multiple samples simultaneously. Here we describe the development of assays which detect genetically distinct ISAV, IPNV and NNV isolates and provide for the possibility of detecting other strains of these viruses that may be encountered in the future.

MATERIALS AND METHODS

VIRAL ISOLATES

ISAV

Canadian isolates used in this study were isolated from aquaculture Atlantic salmon in Atlantic Canada (provinces of New Brunswick and Nova Scotia). Sequencing of the segment 6 highly polymorphic region (HPR) from New Brunswick isolates collected during the initial years of ISA's existence in Atlantic Canada indicated that there were 2 main groups of ISAV in New Brunswick (Kibenge et al. 2001b; Griffiths et al. 2001). Isolates NB280 and NB508 were included in this study as representatives from each group and to act as standard reference isolates. The standard used to represent the Nova Scotia strain was from a year 2000 clinical outbreak at a marine site. Isolates from Europe were also included in an effort to create a more international system. Strains from Europe included: Loch Nevis (Scotland) and Glesvaer (Norway) as standard reference strains. The Loch Nevis isolate was kindly provided by the FRS Marine Laboratory in Aberdeen, United Kingdom, and the Norwegian isolate by the National Veterinary Institute in Oslo, Norway.

IPNV

Isolates representing all 9 serotypes of serogroup A were kindly provided by Dr. B.L. Nicholson at the University of Maine and included: A1 (type strain West Buxton (WB)), A2 (type strain Sp), A3 (type strain Ab), A4 (type strain He), A5 (type strain Te), A6 (type strain Canada 1 (Can 1)), A7 (type strain Canada 2 (Can 2)), A8 (type strain Canada 3 (Can 3)), and A9 (type strain Jasper (Ja)). Also included in this study were: i) a clinical

isolate from salmon in Europe (Shetland), kindly supplied by the FRS Marine Laboratory in Aberdeen, United Kingdom; ii) 2 isolates identified by the Department of Fisheries and Oceans (DFO) as IPNV from brook trout (02:593) and rainbow trout (02:594) originating from a river in Nova Scotia, Canada; and iii) an isolate which tested positive as aquatic birnavirus isolated from an aquaculture salmon from Atlantic Canada (H4-6).

NNV

NNV isolated from local Eastern North American cod, (cod1999 (NS), cod71972002 (NS), cod (NH), cod (NFLD)), flounder (NB) and haddock (haddNB523-1), were used in this study. Note that acronyms contained in the names represent the province or state in which they originated; NS= Nova Scotia, Canada, NH= New Hampshire, USA, NFLD= Newfoundland, Canada, NB= New Brunswick, Canada. Additionally, isolates from Japanese striped jack (JP/06/SJ), Maltese sea bass (Mt/01/sba), and halibut (V9954), were obtained from the University of Stirling in Stirling, United Kingdom.

CELL CULTURE

ISAV strains were isolated and maintained using the salmon head kidney (SHK) cell line (Dannevig et al. 1995). Isolates obtained from fish tissue were isolated as previously described (Ritchie et al. 2001b). Isolates received as cell culture lysates were subpassaged on the SHK cell line by simply inoculating 0.1 ml per well onto SHK cells in 24 well tissue culture plates. Inoculated cells were incubated at 15^{o}C until a cytopathic effect (CPE) was noted.

IPNV strains were isolated and/or maintained using the chinook salmon embryo 214 (CHSE-214) cell line. Tissue, prepared as for ISAV isolations, or cell culture lysates were inoculated in 0.1 ml volumes/ well containing CHSE-214 cells and support MEM media with Hanks' salts containing 2mM glutamine and 1 x antibiotic-antimycotic. Inoculated cells were incubated at 15^{o}C until a cytopathic effect (CPE) was noted.

NNV strains were isolated and/or maintained using the striped snakehead (SSN-1) cell line. Tissue, prepared as for ISAV isolations, or cell culture lysates were inoculated in 0.1 ml volumes/ well containing SSN-1 cells and support Leibovitz's L-15 media with 2mM glutamine and 1 x antibiotic-antimycotic. Inoculated cells were incubated at 20^{o}C until a cytopathic effect (CPE) was noted.

IDENTIFICATION OF PRIMERS FOR PCR

Primers considered for PCR included those currently being used in diagnostic laboratories for disease surveillance or those newly designed from alignment of sequences from multiple isolates. When designing new primers efforts were made to design them around regions of variability with the primers lying in more conserved regions. Winmelt analyses (Bio-Rad Laboratories) were done of selected regions to

determine the primer on which the GC clamp (Myers et al. 1985), used in this study, should be added.

For ISAV, primers used in this study were designed from the sequences of segments 6, 7 and 8. Some of these primers can be seen in Table 1. Segment 8 primers were based on the work of Devold et al. (2000) with a few nucleotide changes and were included as a control because they target a conserved region and have been used for the surveillance program conducted for the New Brunswick Department of Agriculture, Fisheries and Aquaculture (NBDAFA) since 1997. Segments 6 and 7 primers were selected to flank variable regions identified following comparison of multiple international ISAV sequences retrieved from the GenBank database. For segments 6 and 7, multiple primer pairs and RT-PCR conditions were tested.

For IPNV, primers used in this study were selected from sequences of the VP2 coat protein (Table 2). Primer pair C were those as described by Williams et al. (1999).

For NNV, primers used in this study were selected from sequences of the NNV capsid protein or the RNA-dependent RNA polymerase (Table 3). Primer pairs B and C were as described by Nishizawa et al. (1994) and Johansen et al. (2002). The third set tested, primer pair A, had been previously used in our lab to detect local isolates.

RNA EXTRACTION, RT-PCR AND GEL ELECTROPHORESIS

Total RNA was extracted from cell cultures showing cytopathic effects using TRIzol® LS reagent (Invitrogen) according to the manufacturer's protocol. Pellets were suspended in DEPC-treated water (20-50 µl) and 2-4 µl used as template in RT-PCR. The RT-PCR reactions were done using Ready-to-Go™ RT-PCR beads (Amersham Pharmacia) as described by the manufacturer. Briefly, RNA was reverse transcribed in a total volume of 40 µl using 2.5 µg of random hexamer primers at 42°C for 30 min followed by 95°C for 5 min. The PCR primers were then added to a final concentration of 0.4 µM in a total volume of 50 µl. Final $MgCl_2$ concentrations in PCR were either 1.5 mM for all ISAV, IPNV, and NNV primer sets except for ISAV primers for segment 8 and NNV ANup and ANlow primers where 2 mM was used. Conditions for PCR amplification varied during optimization experiments. For ISAV final conditions for PCR amplification were as follows: 40 cycles of 94°C for 30 s, 62°C (segment 6 and 7 primers) or 65°C (segment 8 primers) for 45 s, and 72°C for 1 min 30 s. For IPNV final conditions for PCR amplification were as follows: 35 cycles of 94°C for 45 s, 55°C for 45 s, and 72°C for 1 min 30 s followed by 72°C for 7 min. For NNV final conditions for PCR amplification were: 35 cycles of 94°C for 45 s, 55°C for 45 s, and 72°C 45 s followed by 72°C for 7 min. After mixing with loading buffer, amplified products were run on 11% acrylamide TBE gels (Mini Protean II; BioRad) at 200 V for 1 hr to check for PCR product yield prior to DGGE analysis.

DENATURING GRADIENT GEL ELECTROPHORESIS

Denaturing gradient gel electrophoresis was performed using the DCode Mutation Detection System (BioRad) utilizing 1.5 mm thick x 15 cm long, 0.5 x TAE gels of varying percentages of urea-formamide mixture (7 M urea/40% formamide), 6-8 % polyacrylamide (using 40% acrylamide/ bis solution, 29:1 or 37.5:1) and a nondenaturing 4 % stacking gel. During the optimization process a broad gradient of denaturant was initially used (e.g. 0-80%, 20-80%, or 30-80%). By estimating the concentration of denaturant that terminated the migration of discrete RT-PCR products, subsequent conditions incorporated narrower ranges of denaturant to provide optimal resolution of fragments. Electrophoresis was conducted at 80 V, 60^{o}C for varying amounts of time (12-17 hrs).

SEQUENCE ANALYSIS

Isolate identification was confirmed by sequencing PCR products. The PCR products were purified using the Qiaquick PCR purification kit (Qiagen) and eluted in 50 µl of elution buffer. Three µl of purified PCR product were then added to 3.2 pmol of sequencing primer and 8 µl of Big Dye™ Terminator solution (PE Applied Biosystems) mixed 1:1 with *half*BD sequencing reagent (BioCan Scientific) and cycled 25 times at 96^{o}C for 10 s, 50^{o}C for 5 s, and 60^{o}C for 4 min in a total volume of 20 µl. Primers used for sequencing were the same as those used in PCR. Following the cycle sequencing reaction, the mixture was applied to Edge Biosystem gel filtration cartridges (MJ Research). The eluted product was heated to 95^{o}C and snap cooled on ice. Samples were then electrophoresed on an ABI 3100 Genetic Analyser and further examined using Sequencher (Genecodes).

RESULTS

ISAV

Multiple primers were tested for their ability to amplify isolates and to create amplicons capable of distinguishing ISAV isolates. Primers selected for initial testing targeted segments 6, 7 and 8 sequences. Primer pairs were selected for further RT-PCR/DGGE optimization based on their ability to amplify New Brunswick isolates and on product size, the latter criterion due to the optimal DGGE separation of fragments between 200-500 bp. Primer combinations were tested by RT-PCR of RNA from a panel of isolates representative of the 2 groups of New Brunswick ISAV mentioned by Kibenge et al. (2001b). RT-PCR produced amplification products of the expected size range in all instances.

Recently ISAV isolates from Nova Scotia have been identified which show greater sequence similarity to European strains than those found in New Brunswick (Ritchie et

al. 2001b; Kibenge et al. 2001b; Cook-Versloot et al. 2004). Accordingly, the selected primers for segments 6, 7 and 8 were included in RT-PCR analysis of isolates from New Brunswick, Nova Scotia, Norway (Glesvaer), and Scotland (Loch Nevis). Only segment 8 primer pair A (Table 1) amplified all of the isolates tested.

Due to the desire for broad specificity RT-PCR, additional segment 6 and 7 primers were selected for the purpose of DGGE profiling. All of the segment 6 primer pairs tested against typical strains from New Brunswick, Nova Scotia, Norway and Scotland yielded products of the expected size. Due to no obvious differences in efficiency, segment 6 primer pairs E and G were arbitrarily selected for further optimization along with segment 7 primer pairs B and C (Table 1). A 5' GC clamp was chosen after analysis of the sequence targeted by the primer pairs with Winmelt. The newly designed segment 6 and 7 primers with the inclusion of a GC clamp successfully amplified a selection of international ISAV isolates, designated here as standard reference strains. Due to the presence of insertions/deletions in the hpr of segment 6, primer pair G produced products of variable length ranging from 217 bp-265 bp that could be somewhat resolved by non-denaturing gel electrophoresis.

Amplification products from segments 6 and 7 were initially analyzed with a gradient of 20-80% denaturant. Narrower concentration ranges were tested to maximize resolution. Segment 7 primer pair B, MaF1GC/MaR1, and segment 6 primer pair G, HaFnewGC/1414mod, containing GC clamps, produced the best resolution and hence were selected as the segment 6 and 7 primer sets for ISAV typing. For segment 8, primer pair A, NBFA3/RA3, used in the surveillance program was retained for RT-PCR/DGGE analysis following addition of a GC clamp to the 5' primer. Gradients of denaturant were narrowed to optimize resolution and set at 35-55% for MaF1GC/MaR1 amplicons (454 bp) and 45-65% for HAFnewGC/1414mod amplicons (217-265 bp) and NBFA3GC/RA3 amplicons (251bp) in 6% acrylamide gels.

When the developed RT-PCR/DGGE assay was run on segment 6 for the five reference isolates, five distinct fragments were seen (Figure 1a). Different migration patterns correlated with nucleotide sequence differences (Figure 2a). The NB508 and Nova Scotian standards were only marginally resolved under these electrophoretic conditions. Accordingly, an effort was made to increase the resolution between these two isolates by decreasing the range of gradient to 10% (ie. a 50-60% gradient gel) and by varying the electrophoretic time and acrylamide percentages. Still, further improvement in resolution was not obtained. Nevertheless, the availability of well resolved RT-PCR products from segments 7 and 8 easily differentiated these two isolates (see Figures 1b and c).

When the RT-PCR products that had the same migration pattern under non denaturing conditions were run by DGGE for segment 7, four distinct migration patterns were seen (Figure 1b). The variation in migration could again be explained by nucleotide sequence variation (Figure 2b). Interestingly, isolates NB280 and NB508, which differed at 2 nucleotide positions (positions 70 and 106; Figure 2b) had seemingly identical migration patterns. Efforts to increase the resolution between these two isolates by varying electrophoresis time did not improve this resolution. Electrophoresis times of 12.5 hr, 14

hr, 15.5 hr and 17 hr all produced the same resolution for these 2 isolates (data not shown).

When the segment 8 RT-PCR products were run on DGGE (Figure 1c), three distinct migration patterns were observed: NB280 and NB508 migrated the shortest distance, Scottish and Nova Scotian isolates migrated further and the Norwegian isolate displayed the furthest migration. Migration patterns correlated with nucleotide variation detected by sequencing (Figure 2c). The NB280 and NB508 isolates were identical in sequence as were the Scottish and Nova Scotian isolates. Interestingly, although the Norwegian isolate only differed from the Scottish and Nova Scotian isolates by one nucleotide (position 144; Figure 2c), the change was from T to C and it had a noticeable effect on migration.

To further test the ability of this assay to type New Brunswick ISAV, five isolates from different sites in the Bay of Fundy were selected and run in the DGGE system against the five reference ISAV isolates for segments 6, 7, and 8 RT-PCR products. Segment 6 RT-PCR/DGGE analysis typed isolates NB877 and NB028 as reference standard NB280 whereas isolates NB049, NB002, and NB458 were typed as reference standard NB508 (Figure 3a), although confirmation by sequencing and/or the results of segment 7 and 8 RT-PCR/DGGE analysis was necessary as the segment 6 amplicons for these isolates also migrated closely to the Nova Scotia standard. Sequencing analysis of NB877, NB028, NB049, NB002, and NB458 (data not shown) confirmed the identifications made by the RT-PCR/DGGE assay. RT-PCR and DGGE analysis of these isolates based on segment 7 sequences (Figure 3b) typed these as New Brunswick strains because migration patterns were equivalent to those for reference standards NB280 and NB508, as did the analysis based on segment 8 sequences (Figure 3c).

For the purpose of economy, some preliminary experiments were conducted to determine the possibility of multiplex RT-PCR followed by DGGE for ISAV segments 6 and 7 (Figure 4). While both products were evident, the segment 7 amplicon appeared to have amplified more efficiently. Further optimization of PCR conditions by adjusting primer concentrations, etc. might resolve this issue. However, it may be that there are variable amounts of each target dependent upon infection phase and the transcription of viral segments. Despite possible amplification differences the multiplex RT-PCR/DGGE demonstrated utility in the characterization and differentiation of ISAV isolates, including a recently identified New Brunswick ISAV variant (NB1330) (lane 6). Although the segment 6 RT-PCR product for this isolate is difficult to distinguish from the Norwegian reference strain (Glesvaer) in DGGE, the segment 7 fragment migration was unique making unequivocal typing possible.

Table 4 summarizes the final conditions established for RT-PCR/DGGE of ISAV isolates (individual segments 6, 7 and 8).

IPNV

Three sets of primers (Table 2) were selected for RT-PCR development against a panel of IPNV isolates representing all 9 serotypes of serogroup A. At a Tm of 62^{o}C with inclusion of a GC clamp on the 5' primer, only the IPNV pair C primers (Williams et al. 1999) amplified the entire panel of isolates, although two isolates (He and Te-2) were not amplified efficiently. Reduction to a Tm of 55^{o}C improved this, however (data not shown). To observe the potential of primer pairs B and C amplicons to differentiate the isolates, RT-PCR products were run on a 20-80% DGGE gel. Comparative resolution of the amplicons and consideration of the number of serotypes which could be amplified led to primer pair C being selected for further DGGE assay development. Experimentation with the denaturing gradient percentages led to the adoption of an 8% acrylamide gel in a 45-65% gradient of urea/formamide for the resolution of IPNV strains (Figure 5), all of which were confirmed to be genetically unique by sequencing (Figure 6).

Table 4 summarizes the final conditions established for RT-PCR/DGGE of IPNV isolates.

NNV

Three sets of primers (Table 3) were tested for their ability to amplify a selection of NNV isolates. NNV primer pair A was designed from segment 1 and has been used to detect local NNV isolates by our group (unpublished data). Primer pairs B and C are those as described by Nishiwawa et al. (1994) and Johansen et al. (2002), respectively, targeting segment 2. GC clamps were added to the appropriate primers following Winmelt analysis. These primer sets containing 3' GC clamps were tested against a selection of international isolates, including cod1999 (NS), cod71972002 (NS), V9954, flounder (NB), Mt/01/sba, and JP/06/SJ (see materials and methods for isolate descriptions). Only the F2/R3GC primers amplified the entire panel of isolates (data not shown). Based on the ability to amplify more isolates and the fact that the primers surrounded the T4 region of the capsid protein, where genetic variability is prolific, primer pair B was selected for further assay development. Optimization of DGGE indicated that good resolution of primer pair B amplicons was achieved using 6 % acrylamide gels with a 40-65% gradient (Figure 7). While RT-PCR produced single fragments on non denaturing gels (data not shown), subsequent analysis by DGGE revealed multiple fragments for some of the NNV isolates, the fragment patterns being differentiable between isolates, however. Sequencing analysis confirmed the uniqueness of the isolates (Figure 8). Note that for 2 of the isolates (haddNB523-1 and JP/06/SJ) a few nucleotide positions could not be resolved with sequencing suggesting there was more than one nucleotide at these positions and more than one strain present in these samples. The presence of multiple fragments suggests this as well. In future the excision and sequencing of the individual fragments would be helpful in helping to elucidate the presence and sequence of multiple strains within samples.

Table 4 summarizes the final conditions established for RT-PCR/DGGE of NNV isolates.

DISCUSSION

We have investigated the use of RT-PCR/DGGE assays for the detection and identification of various strains of ISAV, IPNV, and NNV. Development of RT-PCR/ DGGE assays is a delicate balance between designing good PCR primers in conserved genomic regions and designing primers which flank a variable sequence amenable to analysis by DGGE. Ideally, when GC clamps are added to primers they create only one melting domain in the amplicon or melting domains with decreasing melting points, but which are not widely separated. This permits maximum resolution of possible sequence variants by allowing all nucleotide differences, which exist between primers, to influence migration and contribute to distinguishing isolates.

For ISAV we have described three different RT-PCR/DGGE systems based on segment 6, 7, and 8 sequences. Due to the low genetic variability in segment 8 amongst ISAV isolates, the assay using segment 8 primers rapidly distinguished New Brunswick isolates from a Nova Scotian and 2 European isolates. The assay using segment 7 primers distinguished New Brunswick isolates from a Nova Scotian, Norwegian and Scottish isolate. The reason for the inability to distinguish the two New Brunswick reference isolates based on segment 7 sequences, despite the fact that they differed at two nucleotide positions (positions 70 and 106), is unknown since the substitution of G (NB280) and A (NB508) would suggest that this was possible. The problem may lie with the proximity of these two base pairs to the GC clamp and their location in one of the higher melting domains. However, Winmelt analysis would suggest that movement of the GC clamp to the 3' primer might impede the detection of other nucleotide differences among ISAV isolates. Attempts to overcome this issue by adjusting electrophoresis times and acrylamide concentrations were not successful. However, combining the results of segment 7 DGGE with that of segment 6 makes it possible to resolve these 2 New Brunswick isolates.

Using RT-PCR/DGGE analysis of segment 6, it was possible to differentiate all five ISAV reference isolates corroborating previous suggestions that the variability in this segment may be enough to separate ISAV isolates (Krossøy et al. 2001). The NB508 sample and the Nova Scotia sample look the same, however, so care must be taken when analyzing gels. The use of segment 6 hpr sequences may not be the best region to analyze if the goal is to assay relatedness of strains as the differences here likely do not arise gradually by point mutations but rapidly by deletion events.

For IPNV we have described a system based on VP2 coat protein sequences that is capable of distinguishing strains representing all 9 serotypes of serogroup A. Strains 02:593 and 02:594 were the only strains tested that yielded RT-PCR products with common migration corroborating sequencing analysis, which showed these 2 isolates were identical in the region amplified. The latter isolates originated from brook trout and rainbow trout from the same river in Nova Scotia. It is interesting to note that these isolates did not match 100% with any of the type strains from Canada. Further sequencing analysis of a much larger region might reveal more on their relatedness to type strains.

For NNV we have described a system that is capable of distinguishing various local and foreign isolates based on capsid protein sequences. Multiple nucleotide identities at certain points in the sequences for haddNB523-1 and JP/06/SJ suggested the existence of sequence variants within the viral inoculum possibly explaining some of the multiple fragments observed in DGGE for these isolates. Sequencing analysis further revealed that all of the isolates were different by at least 1 clear nucleotide substitution with the possible exception of haddNB523-1 and cod (NH) which were only different at the two unresolved positions in haddNB523-1. Despite the ambiguity in the nucleotide sequence of haddNB523-1 and cod (NH), they were distinguished by DGGE which suggests that some difference must exist between haddNB523-1 and cod (NH) at the unresolved sequence positions in haddNB523-1. The flounder (NB) and cod (NH) were migrating closely and their sequences are almost identical except for a T to A difference, which was sufficient to separate them slightly under these conditions.

RT-PCR DGGE technology is more informative than RFLP analysis and more rapid and inexpensive than sequencing. The technique can be used to screen RT-PCR products for the rapid identification of new strains, and the presumptive confirmation of their identity to standard strains. However, care must be taken when drawing conclusions from DGGE since the existence of different nucleotide substitutions may have the same influence on migration. Therefore, it is prudent to run RT-PCR/DGGE on 2-3 different genetic sequences if small scale differences among strains need to be retained. Where differences in viral phenotype are associated with RT-PCR products of identical migration, confirmation by sequencing, the gold standard, is recommended.

With the burgeoning importance of diversified aquaculture in Atlantic Canada there will be a need to differentiate closely related strains that may be a cause of concern in one species but of little clinical importance in another. The ability to rapidly differentiate between viral strains is the foundation for studies on virulence, epidemiology, relatedness and host specificity that facilitate effective disease management. When proper standards are used the potential for RT-PCR/DGGE assays to quickly and affordably type and screen large numbers of isolates makes it a valuable tool in the monitoring of disease.

RECOMMENDATIONS

1. All existent ISAV, IPNV, NNV variants should be categorized with the currently developed system.
2. RT-PCR DGGE should be conducted periodically in any sampling program involving the analysis of large numbers of samples including both aquaculture and wild fish. Where differences in viral phenotype are associated with RT-PCR products of identical migration, confirmation by sequencing, the gold standard, is recommended.
3. More work should be done to develop multiplex reactions incorporating 2 or more primer pairs for the identification of isolates.

4. Standards should be run in one or few lanes to form a ladder. Samples should be run closely to these standard lanes to facilitate interpretation of their identity.
5. Other fish pathogens, such as *Vibrio* species, could be resolved using this type of assay, permitting greater differentiation of strains involved in opportunistic systemic infections and dermal lesions.

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Table 1. ISAV primers for segments 6, 7 and 8.

Segment 6 Primers			
Primer Pair	Amplicon[a,b] Size	Primer Name	Primer Sequence
E	~ 380 bp	1070mod*	5’ GGGGGYGTWTATCCTTCTGAT 3’
		1414mod	5’ ACAGWGCWATCCCAAAACCTG 3’
G**	~ 235 bp	HaFnew*	5’ TKGTKAAAGAWTTTGACCARACA 3’
		1414mod	5’ ACAGWGCWATCCCAAAACCTG 3’
Segment 7 Primers			
Primer Pair	Amplicon[b] Size	Primer Name	Primer Sequence
B**	454 bp	MaF1*	5’ CKGAACAAGGWGGAAAAGTGGT 3’
		MaR1	5’ TAGCAAGTTCATCAAGGAAAATG 3’
C	257 bp	MaF2*	5’ ARGAGAAACAACTTCATGGACA 3’
		MaR1	5’ TAGCAAGTTCATCAAGGAAAATG 3’
Segment 8 primers			
Primer Pair	Amplicon[b] Size	Primer Name[c]	Primer Sequence
A**[c]	251 bp	NBFA3*	5’ GAGGAATCAGGATGCCAGGACG 3’
		RA3	5’ GAAGTCGATGAACTGCAGCGA 3’

* GC clamp added to selected primers:5’ cgcccgccgcgccccgcgcccgtcccgccgccccccgcccg 3’

** Indicates primer pairs ultimately selected for ISAV RT-PCR/DGGE typing.

Note: a) size here is only approximate due to variable length of hpr for different isolates; size here was calculated from isolate NB508.

b) includes the 40 bp GC clamp.

c) based on those of Devold et al. 2000 with a few nucleotide changes.

Table 2. IPNV primers tested in RT-PCR.

Primer Pair	Amplicon[b] Size	Primer Name	Targeted Gene	Primer Sequence
A	620 bp	VP2 up*	Coat protein	5’ TCCGCCTAGAGGACGAGAC 3’
		VP2 low1	Coat protein	5’ CCAGCCACGGTCAGGATTGA 3’
B	731 bp	VP2 up*	Coat protein	5’ TCCGCCTAGAGGACGAGAC 3’
		VP2 low2	Coat protein	5’ TGGGACAGGATCATCTTGGC 3’
C**	246 bp	WB1*[a]	Coat protein	5’ CCGCAACTTACTTGAGATCCATTATGC 3’
		WB2[a]	Coat protein	5’ CGTCTGGTTCAGATTCCACCTGTAGTG 3’

* GC clamp added to selected primers:5’ cgcccgccgcgccccgcgcccggcccgccgcccccgccc 3’ (WB1); 5’ cgcccgccgcgccccgcgcccgtcccgccgcccccgcccg 3’ (VP2 up).
** Indicates primer pair ultimately selected for IPNV RT-PCR/DGGE typing.
Note: a) Described in Williams et al. 1999.
b) Includes the 40 bp GC clamp.

Table 3. NNV primers tested in RT-PCR.

Primer Pair	Amplicon[b] Size	Primer Name	Targeted Gene	Primer Sequence
A	370 bp	1bF	Polymerase	5’ TGAGACTGACTATAGCAAATTCGAC 3’
		3bR*	Polymerase	5’ GATCTGAGACCGTCGTCACC 3’
B**	467 bp	F2[a]	Capsid protein	5’ CGTGTCAGTCATGTGTCGCT 3’
		R3*[a]	Capsid protein	5’ CGAGTCAACACGGGTGAAGA 3’
C	304 bp	AnUp[c]	Capsid protein	5’ CTGAAGATACATTCGCTCCAA 3’
		AnLow*[c]	Capsid protein	5’ TATCCCATAGCCCCCAGTG 3’

* GC clamp added to selected primers:5’ cgcccgccgcgccccgcgcccgtcccgccgcccccgcccg 3’ (R3 and 3bR); 5’ cgcccgccgcgccccgcgcccggcccgccgcccccgccc 3’ (AnLow).
** Indicates primer pair ultimately selected for NNV RT-PCR/DGGE typing.
Note: a) Described in Nishiwawa et al. 1994.
b) Includes the 40 bp GC clamp.
c) Described in Johansen et al. 2002.

Table 4. Summary of final conditions established for RT-PCR/DGGE assays for ISAV, IPNV, and NNV. For RT-PCR conditions, see the materials and methods section.

Virus	Primer Pair	DGGE conditions
ISAV	G (segment 6)	Gels: gradient of 45-65% in 6% acrylamide gels Voltage: 80 V Time: 17 hrs Temperature: 60^{o}C
	B (segment 7)	Gels: gradient of 35-55% in 6% acrylamide gels Voltage: 80 V Time: 17 hrs Temperature: 60^{o}C
	A (segment 8)	Gels: gradient of 45-65% in 6% acrylamide gels Voltage: 80 V Time: 17 hrs Temperature: 60^{o}C
IPNV	C	Gels: gradient of 45-65% in 8% acrylamide gels Voltage: 80 V Time: 17 hrs Temperature: 60^{o}C
NNV	B	Gels: gradient of 40-65% in 6% acrylamide gels Voltage: 80 V Time: 17 hrs Temperature: 60^{o}C

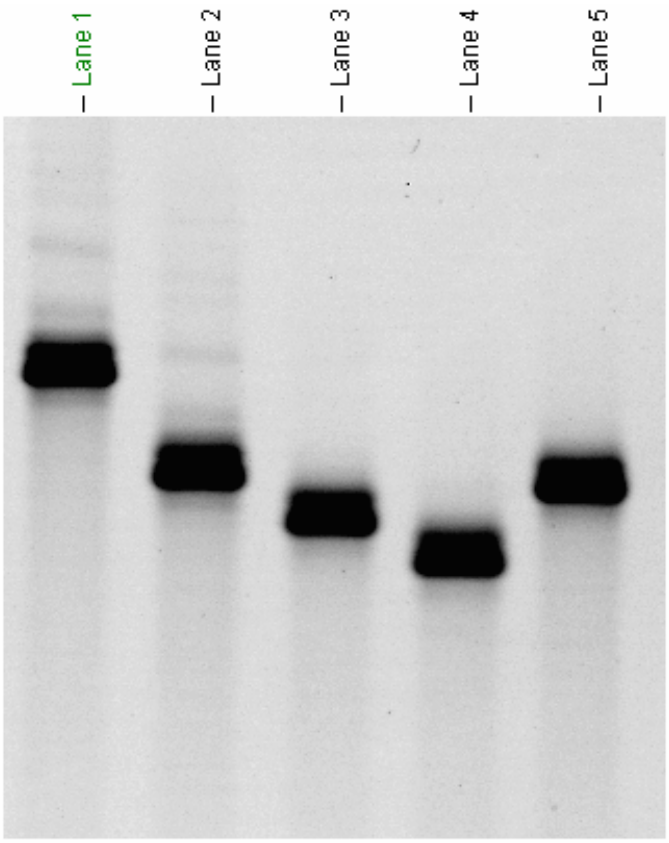

Figure 1a. DGGE analysis of segment 6 HaFnewGC/1414mod RT-PCR products for ISAV reference isolates: NB280 (lane 1), NB508 (lane 2), Norway (lane 3), Scotland (lane 4), Nova Scotia (lane 5). Note: NB= New Brunswick. RT-PCR products were electrophoresed in a 6% acrylamide, 45-65% denaturant gel at 80 V, 17 hrs, 60^{o}C.

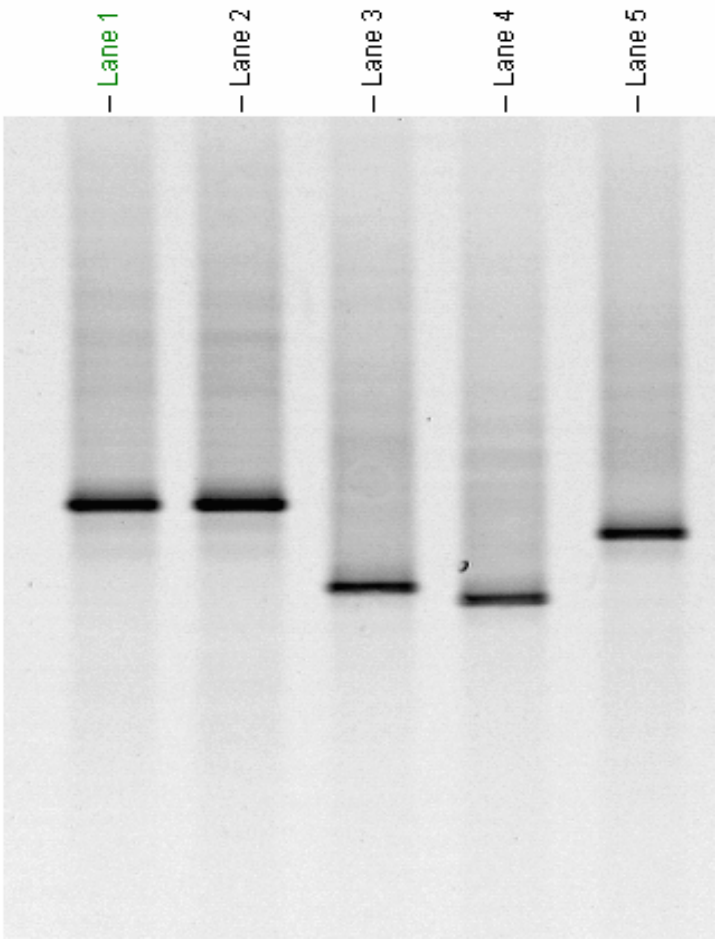

Figure 1b. DGGE analysis of segment 7 MaF1GC/MaR1 RT-PCR products for ISAV reference isolates: NB280 (lane 1), NB508 (lane 2), Norway (lane 3), Scotland (lane 4), Nova Scotia (lane 5). Note: NB= New Brunswick. RT-PCR products were electrophoresed in a 6% acrylamide, 35-55% denaturant gel at 80 V, 17 hrs, 60^{o}C.

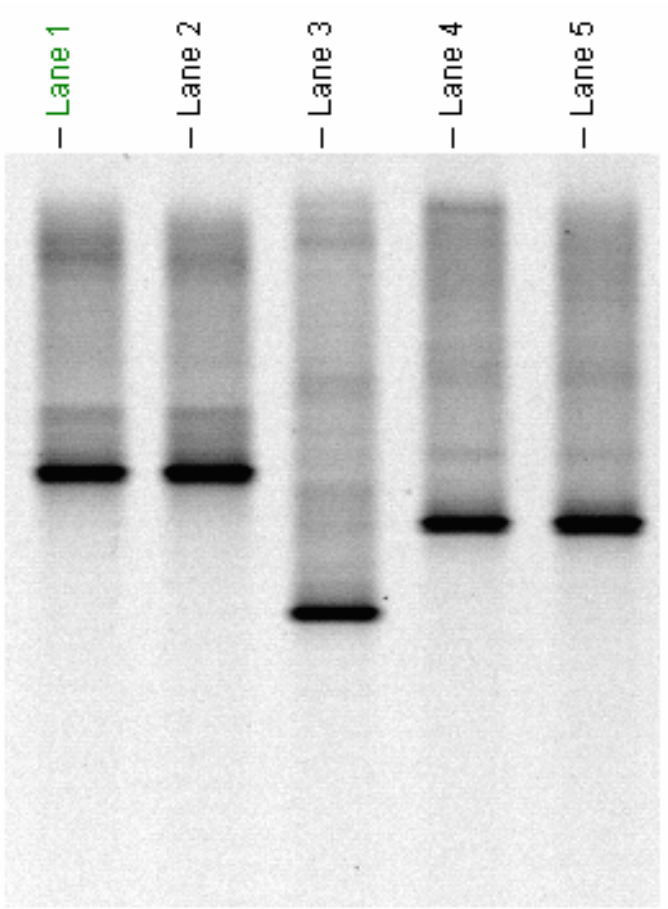

Figure 1c. DGGE analysis of segment 8 NBFA3GC/RA3 RT-PCR products for ISAV reference isolates: NB280 (lane 1), NB508 (lane 2), Norway (lane 3), Scotland (lane 4), Nova Scotia (lane 5). Note: NB= New Brunswick. RT-PCR products were electrophoresed in a 6% acrylamide, 45-65% denaturant gel at 80 V, 17 hrs, 60^{o}C.

Figure 2a. Sequence analysis of RT-PCR products generated by segment 6 primers for ISAV isolates. GC clamp sequence not included here. Nucleotide 1-23: HAFnew primer (italics); 217-237: 1414mod primer (italics). Note: NB= New Brunswick. Gaps (deletions) in the hpr are denoted by colons (:). The bottom line denotes the consensus sequence. Differences are marked by an asterisk (*).

```
NB280          #1            ---------- ---------- ---G-GC-C- -A-------- T--T-AA--A
NB508          #1            ---------- ---------- ---G-GC-C- -A-------- T--T-AA--A
NORWAY         #1            ---------- ---------- ---A-CT-A- -T-------- C--A-TT--C
SCOTLAND       #1            ---------- ---------- ---A-CT-A- -T-------- C--A-TT--C
NOVA SCOTIA    #1            ---------- ---------- ---A-CT-A- -T-------- C--A-TT--C
                             ..................................................
               #1            TNGTNAAAGA NTTTGACCAN ACARGSYTMG GWAACACAGA YACWCWWATM
                              +  +      +        +    * ** *   *         *  * **  *

NB280          #51           -----A---C -A-A-GCA-- A--------- -CTG-C---- --GGC--A--
NB508          #51           -----A---C -A-A-GCA-- A--------- -TTG-T---- --GGC--A--
NORWAY         #51           -----G---G -A-C-TTG-- T--------- -TCA-T---- --CAG--G--
SCOTLAND       #51           -----G---G -A-C-TTG-- C--------- -TCA-T---- --CAG--G--
NOVA SCOTIA    #51           -----G---G -G-C-TTG-- C--------- -TCA-C---- --CAG--G--
                             ..................................................
               #51           ATGAGRGAGS TRGMAKYRCA HAAGGAGATG AYYRGYAAAC TTSRSAGRAA
                                  *   *  * * ***   *           *** *       ***  *

NB280          #101          ---T-----C -----C-A-- GAG-A--T-- --T-:-:::: ::::::::--
NB508          #101          ---T-----C -----C-A-- GAG-A--T-- --:-:::::: :::::::-:
NORWAY         #101          ---C-----T -----G-T-- GGG-A--C-- :-A------- -------:::
SCOTLAND       #101          ---C-----T -----A-:-- T:C-G::::: :::::::::: :::::----:
NOVA SCOTIA    #101          ---C-----T -----G-T-- GGG-A--C-- :-A------- -------:::
                             ..................................................
               #101          CATYACAGAY GTAAAVAWCA KRSTRGAYGC TAWTCCCACC TCAGCTGTTG
                                *     *      * *   *** *  *   * * *            ***

NB280          #151          ---------- -G----A--- ---------T -T----CC-- ----------
NB508          #151          :::::::::: :::::::::: ::::-----A -A::--TT-- ----------
NORWAY         #151          ::::::---- -A:::::::: :::::::::: :::::::::: ::::::::::
SCOTLAND       #151          :::::::::: ::::--G--- -:::::::::: :::::::::: ::::------
NOVA SCOTIA    #151          ::::::---- -A:::::::: :::::::::: :::::::::: :::--::---
                             ..................................................
               #151          GTGTAAACCA ARTAGARCAA CCGTCCACCW CWGTGCYYAG CAACATCTTC
                             *****       *    *             *  ***  **        **

NB280          #201          --C------- -A--G----- ---------- ---------- ----------
NB508          #201          --C------- -A--G----- ---------- ---------- ----------
NORWAY         #201          :::------- -T--A----- ---------- ---------- ----------
SCOTLAND       #201          --T------- -T--A----- ---------- ---------- ----------
NOVA SCOTIA    #201          --T------- -T--A----- ---------- ---------- ----------
                             ..................................................
               #201          ATYTCTATGG GWGTRGCAGG TTTTGGGATN GCNCTGT
                               *         *  *              +   +
```

Figure 2b. Sequence analysis of RT-PCR products generated by segment 7 primers for ISAV isolates. GC clamp sequence not included here. Nucleotide 1-22: MAF1 primer (italics); 392-414: MAR1 primer. Note: NB= New Brunswick. The bottom line denotes the consensus sequence. Differences are marked by an asterisk (*).

```
NB280         #1    ---------- ---------- --C-----C- -------A-C ---G------
NB508         #1    ---------- ---------- --C-----C- -------A-C ---G------
NORWAY        #1    ---------- ---------- --T-----T- -------G-T ---A------
SCOTLAND      #1    ---------- ---------- --T-----T- -------G-T ---A------
NOVA SCOTIA   #1    ---------- ---------- --T-----T- -------G-T ---A------
                    ..................................................
              #1    CNGAACAAGG NGGAAAAGTG GTYTTCAGYC TTACTGARAY GGGRTCATGT
                     +         +           *     *         * *    *

NB280         #51   --C--G-TT- -C--A--T-G T-----A-GT --A-----C- -C--------
NB508         #51   --C--G-TT- -C--A--T-A T-----A-GT --A-----C- -C--------
NORWAY        #51   --T--C-GC- -C--G--C-A C-----T-AC --G-----A- -T--------
SCOTLAND      #51   --T--C-GC- -T--G--C-A C-----T-AC --G-----A- -T--------
NOVA SCOTIA   #51   --T--T-GC- -C--G--C-A C-----T-AC --G-----A- -T--------
                    ..................................................
              #51   GTYTCBTKYT AYGGRGAYGR YGAACCWGRY GARGGGTCMT GYGAACTTGC
                      *  * **   *  *  * * *     * **   *     *   *

NB280         #101  C----GA--- -----T---- ----T--T-- ---G------ ----------
NB508         #101  C----AA--- -----T---- ----T--T-- ---G------ ----------
NORWAY        #101  A----AG--- -----C---- ----C--C-- ---C------ ----------
SCOTLAND      #101  A----AG--- -----T---- ----C--C-- ---C------ ----------
NOVA SCOTIA   #101  A----AG--- -----T---- ----C--C-- ---C------ ----------
                    ..................................................
              #101  MTCTGRRAAC ATGGAYTTTC CAAGYTGYCC TCTSGGGAAT GGAGATGACT
                    *    **         *         *  *      *

NB280         #151  -----C---- G-----G--A ---------- -------T-- G-----C-A-
NB508         #151  -----C---- G-----G--A ---------- -------T-- G-----C-A-
NORWAY        #151  -----T---- A-----C--G ---------- -------A-- A-----T-G-
SCOTLAND      #151  -----T---- A-----C--G ---------- -------A-- A-----T-G-
NOVA SCOTIA   #151  -----T---- A-----A--G ---------- -------A-- A-----T-G-
                    ..................................................
              #151  TCTGTYTGTC RCTGGCVCTR AGCACAATGA GATGGTCWGG RATGACYARG
                         *     *     *  *                   *   *     * *

NB280         #201  ---------- ---------- ---------- --T------- -T--------
NB508         #201  ---------- ---------- ---------- --T------- -T--------
NORWAY        #201  ---------- ---------- ---------- --C------- -C--------
SCOTLAND      #201  ---------- ---------- ---------- --C------- -C--------
NOVA SCOTIA   #201  ---------- ---------- ---------- --C------- -C--------
                    ..................................................
              #201  AGAAACAACT TCATGGACAG ATTCATTGGA AGYTTTGTTC AYTGTACACC
                                                     *         *

NB280         #251  ---G-----C ----C---TG GA--TT--T- C-A--A--GC --T-----A-
NB508         #251  ---G-----C ----C---TG GA--TT--T- C-A--A--GC --T-----A-
NORWAY        #251  ---C-----T ----T---CG AG--AA--G- T-G--G--AT --C-----G-
SCOTLAND      #251  ---C-----T ----T---CG AG--AA--G- T-G--G--AT --C-----G-
NOVA SCOTIA   #251  ---C-----T ----T---CA AG--AA--G- T-G--G--AT --C-----G-
                    ..................................................
              #251  AGTSATGATY TGGTYGTAYR RRAAWWTGKC YARGARAARY CAYCACAARA
                       *     *     *   ** **  **  *  * *  *  **   *     *

NB280         #301  ----T--C-- ---T-----A --C--GT--- -------T-- C-----C---
NB508         #301  ----T--C-- ---T-----A --C--GT--- -------T-- C-----C---
NORWAY        #301  ----C--T-- ---A-----T --T--AC--- -------C-- A-----T---
SCOTLAND      #301  ----C--T-- ---A-----T --T--AC--- -------C-- A-----T---
NOVA SCOTIA   #301  ----C--T-- ---A-----T --T--AC--- -------C-- A-----T---
                    ..................................................
              #301  TGGTYTGYCA CACWTGCCCW GAYGARYACA AGTTCAGYGA MAAGGAYGAG
                      *  *      *     *   *  **           *   *     *
```

```
NB280         #351          A-------A- ---------- A-----A--G --T----C-- ----------
NB508         #351          A-------A- ---------- A-----A--G --T----C-- ----------
NORWAY        #351          T-------G- ---------- G-----G--A --C----T-- ----------
SCOTLAND      #351          T-------G- ---------- G-----G--A --C----C-- ----------
NOVA SCOTIA   #351          T-------G- ---------- G-----G--A --C----C-- ----------
                            ..................................................
              #351          WTGCAGGGRT ACTATGAGGA RTGTCTRGAR GCYTCTAYTG ACATTTTCCT
                            *       *            *     *  *   *    *

NB280         #401          ---------- ---------- ---------- ---------- ----------
NB508         #401          ---------- ---------- ---------- ---------- ----------
NORWAY        #401          ---------- ---------- ---------- ---------- ----------
SCOTLAND      #401          ---------- ---------- ---------- ---------- ----------
NOVA SCOTIA   #401          ---------- ---------- ---------- ---------- ----------
                            ..........................................................
              #401          TGATGAACTT GCTA
```

Figure 2c. Sequence analysis of RT-PCR products generated by segment 8 primers for ISAV isolates. GC clamp sequence not included here. Nucleotide 1-22: NBFA3 primer (italics); 191-211: RA3 primer (italics). Note: NB= New Brunswick. The bottom line denotes the consensus sequence. Differences are marked by an asterisk (*).

```
NB280         #1            ---------- ---------- ---------- ---------- -A--------
NB508         #1            ---------- ---------- ---------- ---------- -A--------
NORWAY        #1            ---------- ---------- ---------- ---------- -G--------
SCOTLAND      #1            ---------- ---------- ---------- ---------- -G--------
NOVA SCOTIA   #1            ---------- ---------- ---------- ---------- -G--------
                            ..................................................
              #1            GAGGAATCAG GATGCCAGGA CGCGGATGGT GGAGAGGAAA ARTGGGCAAT
                                                                          *

NB280         #51           ---------- ---------- -C-------- A--------- ------C---
NB508         #51           ---------- ---------- -C-------- A--------- ------C---
NORWAY        #51           ---------- ---------- -A-------- G--------- ------T---
SCOTLAND      #51           ---------- ---------- -A-------- G--------- ------T---
NOVA SCOTIA   #51           ---------- ---------- -A-------- G--------- ------T---
                            ..................................................
              #51           GGTGTATGGT ATGATTTCAC CMGACATGGC RGAGGAGAAG ACGATGYTGA
                                       *          *                    *

NB280         #101          ----G----- A--------- ---------- ---------- ---T--G--T
NB508         #101          ----G----- A--------- ---------- ---------- ---T--G--T
NORWAY        #101          ----C----- G--------- ---------- ---------- ---C--A--A
SCOTLAND      #101          ----C----- G--------- ---------- ---------- ---T--A--A
NOVA SCOTIA   #101          ----C----- G--------- ---------- ---------- ---T--A--A
                            ..................................................
              #101          AGGASCTGAA RACAATGCTA CACAGCAGGA TGCAGATGTA TGCYCTRGGW
                               *      *                                  *  *  *

NB280         #151          --A------- -------A-- G-A------- ---------- ----------
NB508         #151          --A------- -------A-- G-A------- ---------- ----------
NORWAY        #151          --G------- -------G-- A-C------- ---------- ----------
SCOTLAND      #151          --G------- -------G-- A-C------- ---------- ----------
NOVA SCOTIA   #151          --G------- -------G-- A-C------- ---------- ----------
                            ..................................................
              #151          GCRAGTTCGA AAGCCCTRGA RAMTTTAGAA AAGGCCATCG TCGCTGCAGT
                              *              *   * *

NB280         #201          ---------- ---------- ---------- ---------- ----------
NB508         #201          ---------- ---------- ---------- ---------- ----------
NORWAY        #201          ---------- ---------- ---------- ---------- ----------
SCOTLAND      #201          ---------- ---------- ---------- ---------- ----------
NOVA SCOTIA   #201          ---------- ---------- ---------- ---------- ----------
                            ..................................................
              #201          TCATCGACTT C
```

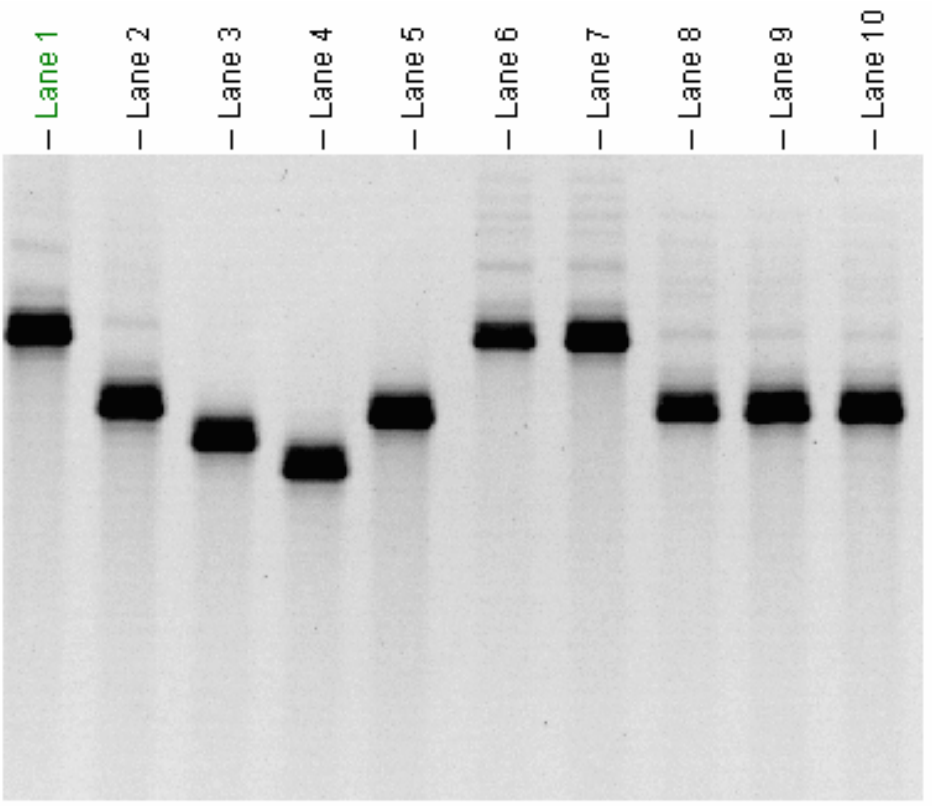

Figure 3a. Typing of New Brunswick ISAV isolates using segment 6 RT-PCR/DGGE analysis. Reference isolates: NB280 (lane 1), NB508 (lane 2), Norway (lane 3), Scotland (lane 4), Nova Scotia (lane 5). Test isolates: NB877 (lane 6), NB028 (lane 7), NB049 (lane 8), NB002 (lane 9), NB458 (lane 10). RT-PCR products were run in a 6% acrylamide, 45-65% denaturant gel at 80 V, 17 hrs, 60^{o}C.

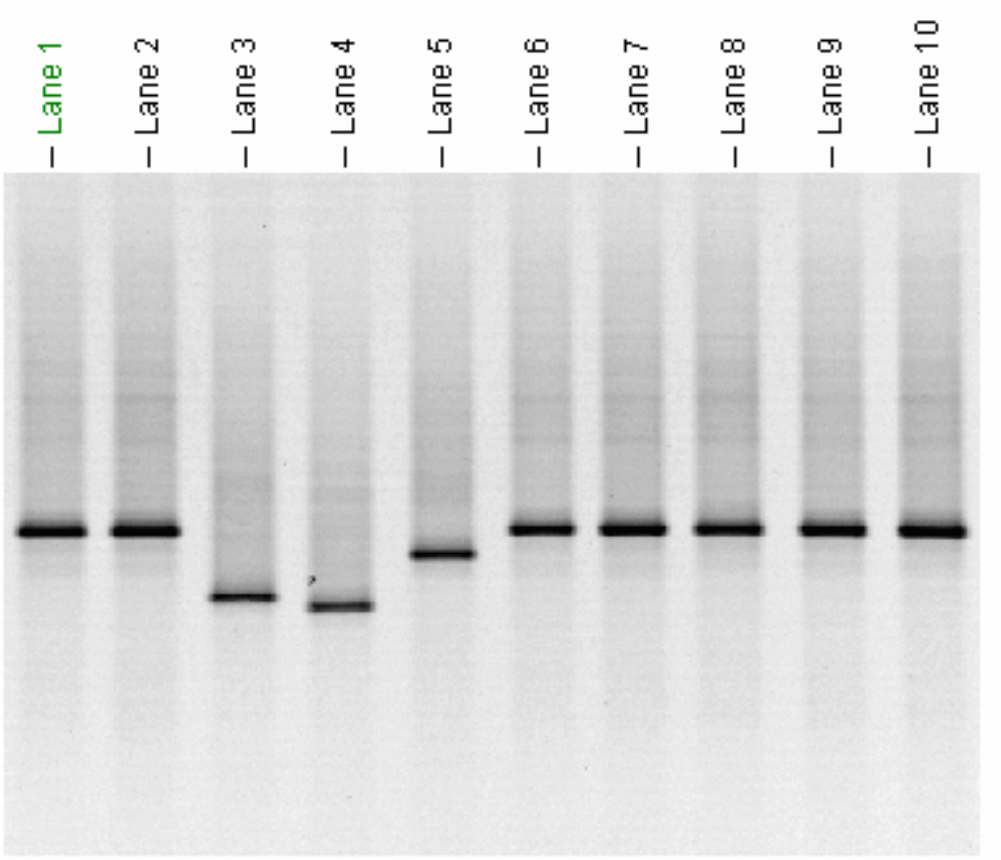

Figure 3b. Typing of New Brunswick ISAV isolates using segment 7 RT-PCR/DGGE analysis. Reference isolates: NB280 (lane 1), NB508 (lane 2), Norway (lane 3), Scotland (lane 4), Nova Scotia (lane 5). Test isolates: NB877 (lane 6), NB028 (lane 7), NB049 (lane 8), NB002 (lane 9), NB458 (lane 10). RT-PCR products were run in a 6% acrylamide, 35-55% denaturant gel at 80 V, 17 hrs, 60^{o}C.

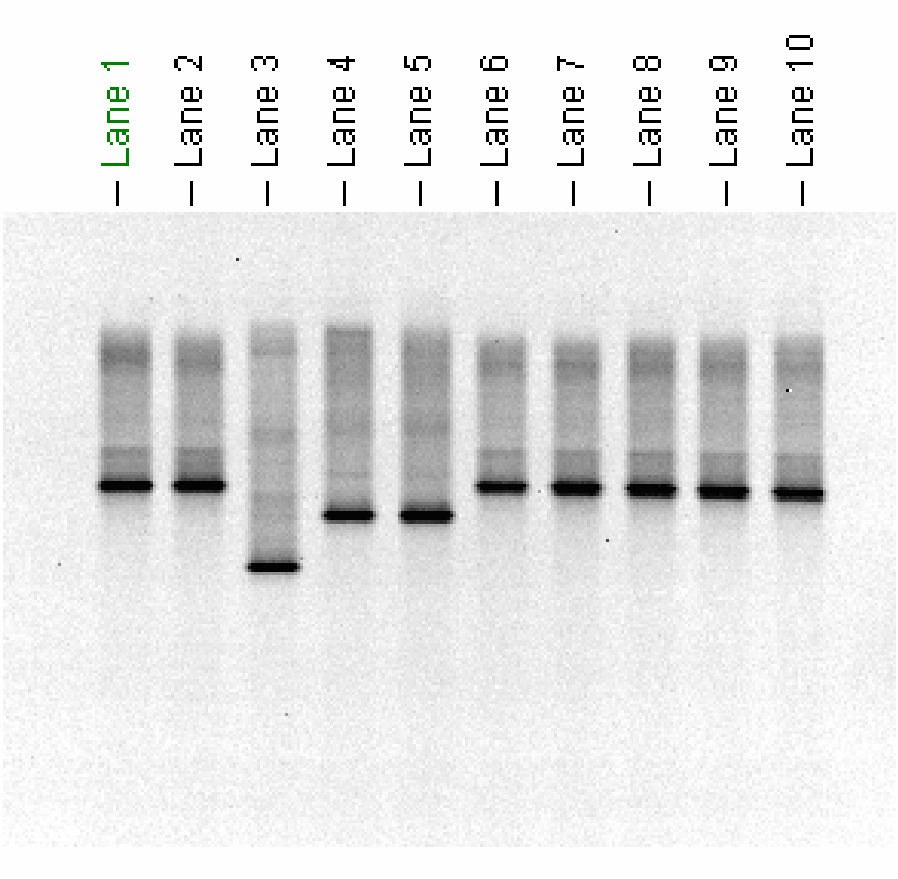

Figure 3c. Typing of New Brunswick ISAV isolates using segment 8 RT-PCR/DGGE analysis. Reference isolates: NB280 (lane 1), NB508 (lane 2), Norway (lane 3), Scotland (lane 4), Nova Scotia (lane 5). Test isolates: NB877 (lane 6), NB028 (lane 7), NB049 (lane 8), NB002 (lane 9), NB458 (lane 10). RT-PCR products were run in a 6% acrylamide, 45-65% denaturant gel at 80 V, 17 hrs, 60^{o}C.

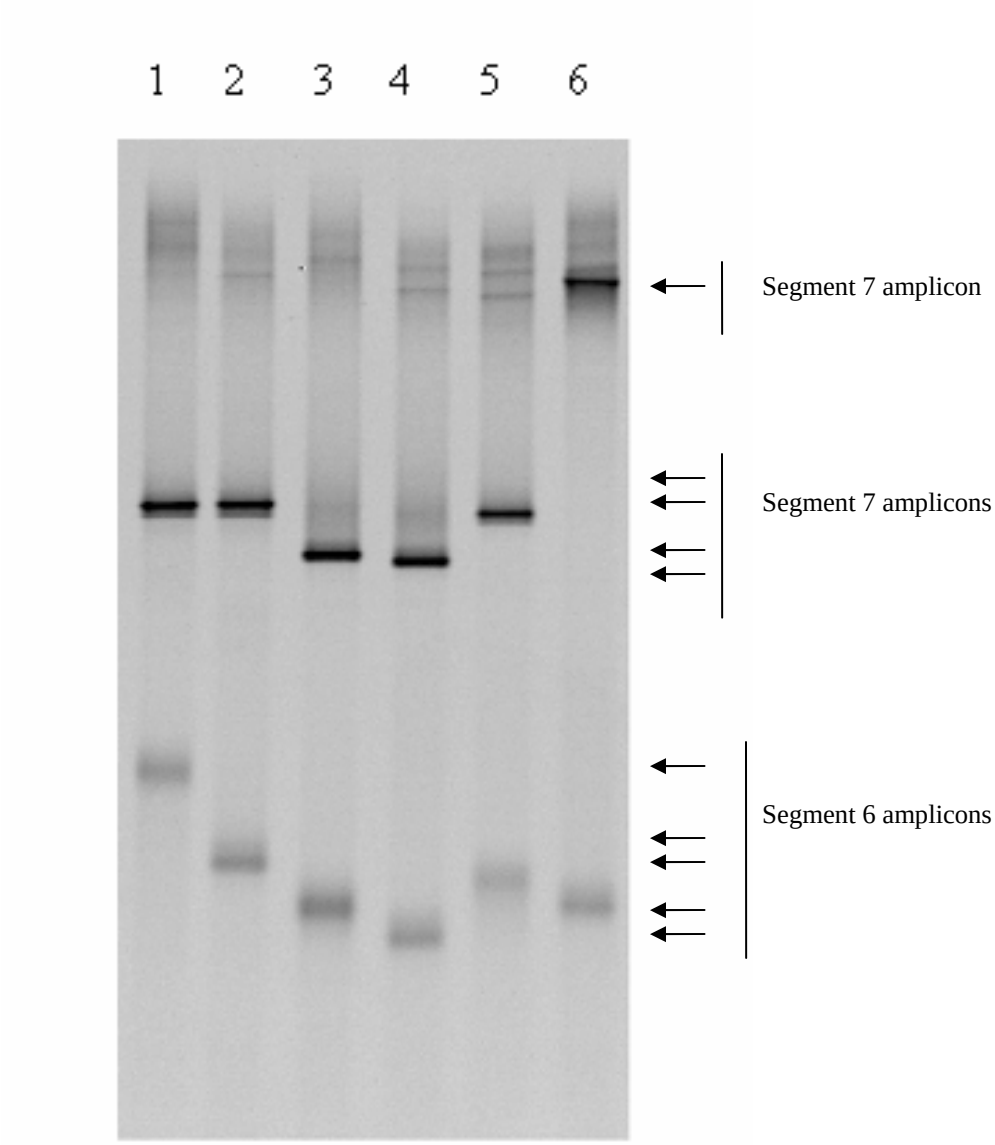

Figure 4. DGGE analysis of multiplex RT-PCR products for international ISAV isolates amplified with segment 6 HaFnewGC/1414mod and segment 7 MaF1GC/MaR1primers in a single tube. Lane 1: NB280; lane 2: NB508; lane 3: Glesvaer; lane 4: Loch Nevis; lane 5: Nova Scotia; lane 6: NB1330. RT-PCR products were electrophoresed in a 6% acrylamide, 35-65% denaturant gel at 80V, 17 hr, 60^{o}C.

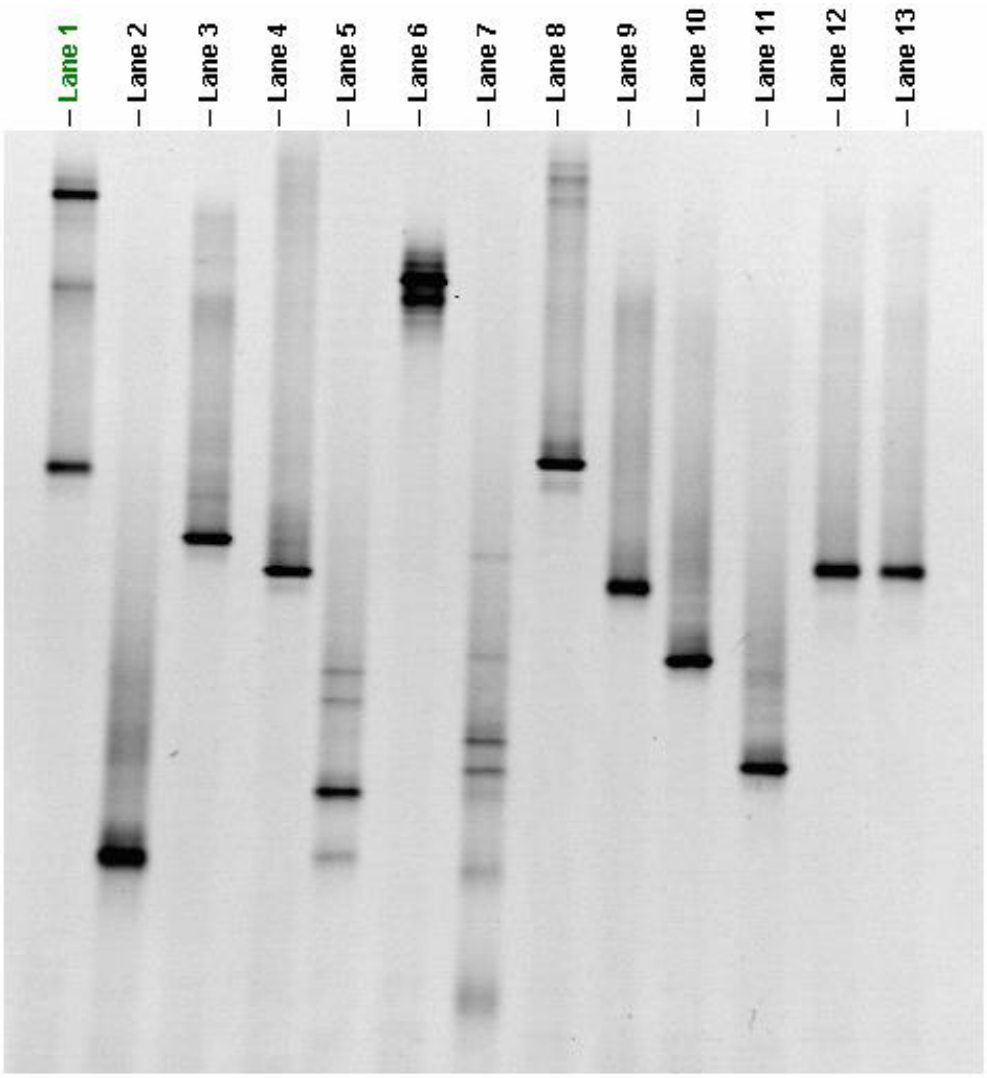

Figure 5. DGGE analysis of WB1GC/WB2 RT-PCR products for international IPNV isolates. Lane 1: Ja (type strain Jasper, serotype A9); lane 2: C2 (type strain Canada 2, serotype A7); lane 3: Sp (type strain Sp, serotype A2); lane 4: C1 (type strain Canada 1, serotype A6); lane 5: He (type strain He, serotype A4); lane 6: Ab (type strain Ab, serotype A3); lane 7: C3 (type strain Canada 3, serotype A8); lane 8: Te-2 (type strain Te-2, serotype A5); lane 9: Wb (type strain West Buxton, serotype A1); lane 10: Shetland (European clinical salmon isolate); lane 11: H4-6 (Atlantic Canada salmon aquatic birnavirus isolate); lane 12: 02:593 (Nova Scotia, Canada brook trout IPNV isolate); lane 13: 02:594 (Nova Scotia, Canada rainbow trout IPNV isolate). RT-PCR products were electrophoresed in a 8% acrylamide, 45-65% denaturant gel at 80V, 17 hr, 60^{o}C.

Figure 6. Sequence analysis of IPNV RT-PCR products generated by WB1GC/WB2 primers. Primer and GC clamp sequences have been omitted. IPNV isolates: Ja (type strain Jasper, serotype A9); C2 (type strain Canada 2, serotype A7); Sp (type strain Sp, serotype A2); C1 (type strain Canada 1, serotype A6); He (type strain He, serotype A4); Ab (type strain Ab, serotype A3); C3 (type strain Canada 3, serotype A8); Te-2 (type strain Te-2, serotype A5); Wb (type strain West Buxton, serotype A1); Shetland (European clinical salmon isolate); H4-6 (Atlantic Canada salmon aquatic birnavirus isolate); 02:593 (Nova Scotia, Canada brook trout IPNV isolate); 02:594 (Nova Scotia, Canada rainbow trout IPNV isolate). The bottom line denotes the consensus sequence. Differences are marked by an asterisk (*).

```
Ja          #1    ---------A ---------G -----T---- ---------- ---------T
C2          #1    ---------- ---G------ ---------- ---------- ----------
Sp          #1    ----A----- ---------- ---------- ---------- ----------
C1          #1    ---------- ---T-----C ---------- ----TG---- ----------
He          #1    -A-----AT- ---G-----G -----T--T- -T--TG-G-- A---C-T---
Ab          #1    ----A----- ------TT-C -----T---- ---------- ----------
C3          #1    -C-------- ---------- ---------- -T-------- ----------
Te-2        #1    ---------- ---------C ---------- ----TG---- ----------
Wb          #1    ---------A ---------- -----T---- ---------- A---------
Shetland    #1    ----A----- ---------- ---------- ---------- ----------
H4-6        #1    ---------- ---------- ---------- ---------- ----------
02:593      #1    ---------A ---G------ -----T---- ---------- A--------T
02:594      #1    ---------A ---G------ -----T---- ---------- A--------T
                  ..........................................................
            #1    TTCCCGAGAC TGGACCAGCA AGCATCCCGG ACGACATAAC GGAGAGACAC
                   *  *  ***    *  ** *      *  *   *  ** *   *   * *  *

Ja          #51   --AC------ ---------- A--------- ---------- -A--------
C2          #51   ---------- ---------- ---------- ---------- -A-----C--
Sp          #51   ---------- ---------- ---------- ---------- -C--A-----
C1          #51   ---------- ---------- ---------- ---------- -G--A-----
He          #51   ---C-G---- -G-----A-- A--C-----T C----A--T- -A--G-----
Ab          #51   ---------- -------T-- ---C------ ---------- -G--------
C3          #51   ---------- ---------- ---------- C--------- -A-----C--
Te-2        #51   ---------- ---------- ---------- ---------- -G--A-----
Wb          #51   --AC------ ---------- A--------- ---------- -C--------
Shetland    #51   ---------- ---------- ---------- ---------- -C--------
H4-6        #51   ---------- ---------- ---------- ---------- -A-----C--
02:593      #51   --AC------ ---------- ---------- ---------- -C--A-----
02:594      #51   --AC------ ---------- ---------- ---------- -C--A-----
                  ..........................................................
            #51   ATCTTAAAAC AAGAGACCTC GTCATACAAC TTAGAGGTCT CMGACTCAGG
                    ** *      *     *   *  *     * *    *  *   *  *  *

Ja          #101  ---------- ---------- -------A-- T--T--A--C ----------
C2          #101  ---------- --A------- ----G--C-- ---------G C-T--T----
Sp          #101  ------CA-- -----T--T- -------G-- ---------- C--A------
C1          #101  ------TG-- --------T- -------G-- ---------- ----------
He          #101  C--C--CA-A --G--G---- -T--A--G-- ---------G C-CA-A----
Ab          #101  ------TG-- -----T--T- -------A-- ------A--- -----A----
C3          #101  ---------- --A------- ----G--C-- ---------G C-T--T--C-
Te-2        #101  ------TG-- --------T- -------A-- ----A----- ----------
Wb          #101  ---------- ---------- -------G-- T--C--A--C --A-------
Shetland    #101  ------CA-- -----T--T- -------G-- ---------- C--A----C-
H4-6        #101  ---------- --A------- ----G--T-- ---------G C-T--T----
02:593      #101  ---------- ---------- -------A-- T--C--A--C ----------
02:594      #101  ---------- ---------- -------A-- T--C--A--C ----------
                  ..........................................................
            #101  AAGTGGGCTT CTTGTCTGCT TCCCTGGRGC ACCAGGCTCA AGGGTCGGTG
                  *  *  ** *   *  *  *   *  *  *   *  ** *  * * ** *  *
```

```
Ja              #151            -C-------- ---------- ---------- ---------- ----------
C2              #151            ---------- ---------- ---------- ---------- ----------
Sp              #151            -A-------- ---------- ---------- ---------- ----------
C1              #151            ---------- ---------- ---------- ---------- ----------
He              #151            -A-------- ---------- ---------- ---------- ----------
Ab              #151            ---------- ---------- ---------- ---------- ----------
C3              #151            ---------- ---------- ---------- ---------- ----------
Te-2            #151            ---------- ---------- ---------- ---------- ----------
Wb              #151            -C-------- ---------- ---------- ---------- ----------
Shetland        #151            -A-------- ---------- ---------- ---------- ----------
H4-6            #151            ---------- ---------- ---------- ---------- ----------
02:593          #151            -C-------- ---------- ---------- ---------- ----------
02:594          #151            -C-------- ---------- ---------- ---------- ----------
                                ..................................................
                #151            CT
                                 *
```

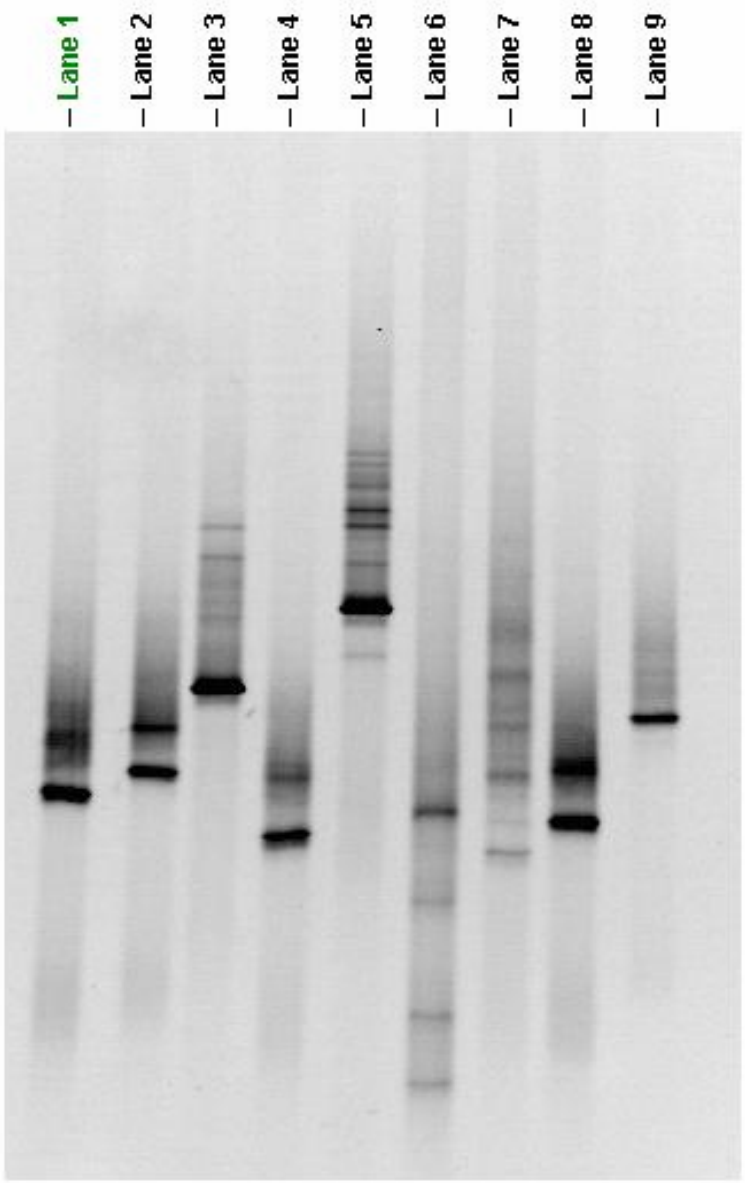

Figure 7. DGGE analysis of F2/R3GC RT-PCR products for international NNV isolates. Lane 1: cod 1999 (NS); lane 2: cod7197 2002 (NS); lane 3: halibut V9954; lane 4: flounder (NB); lane 5: Maltese sea bass Mt/01/sba; lane 6: Japanese striped jack JP/06/SJ; lane 7: haddockNB523-1; lane 8: cod (NH); lane 9: cod (NFLD). Note that acronyms contained in the names represent the province or state in which they originated; NS= Nova Scotia, Canada, NH= New Hampshire, USA, NFLD= Newfoundland, Canada, NB= New Brunswick, Canada. RT-PCR products were electrophoresed in a 6% acrylamide, 40-65% denaturant gel at 80V, 17 hr, 60^{o}C.

Figure 8. Sequence analysis of NNV RT-PCR products generated by F2/R3GC primers. Primer and GCclamp sequences have been omitted. NNV isolates: Eastern North American cod (cod1999 (NS), cod71972002 (NS), cod (NH), cod (NFLD)), flounder (NB) and haddock (haddNB523-1). Note that acronyms contained in the names represent the province or state in which they originated; NS= Nova Scotia, Canada, NH= New Hampshire, USA, NFLD= Newfoundland, Canada, NB= New Brunswick, Canada. Additionally, isolates from Japanese striped jack (JP/06/SJ), Maltese sea bass (Mt/01/sba), and halibut (V9954), are included here. Note that the JP/06/SJ isolate contains more nucleotides in the amplified region than the other isolates. Best fit alignment by the nucleotide alignment program Sequencher (Genecodes) creates gaps designated by colons (:) when these sequences are compared. The bottom line denotes the consensus sequence. Differences are marked by an asterisk (*).

```
cod1999(NS)      #1    ---------- ---------- ---------- ---------- ----------
cod71972002(NS)  #1    ---------- ---------- ---------- ---------- ----------
halibutV9954     #1    -------G-- ---------T -----T--C- ----A--T-- -------TT-
flounder(NB)     #1    ---------- ---------- ---------- ---------- ----------
Mt/01/sba        #1    -------T-- AT-G--C--T -----T---- ----A--T-- ---G--T---
JP/06/SJ         #1    ----C--T-- C--T-----C --G------- ----A--T-- G--C--C---
haddNB523-1      #1    ---------- ---------- ---------- ---------- ----------
cod (NH)         #1    ---------- ---------- ---------- ---------- ----------
cod(NFLD)        #1    ---------- ---------A --------C- ---------- -------TT-
                       ..........................................................
                 #1    GGAGTGTACG TCTCAGTGTG CCATCCCTTG AGACGCCCGA AGATACAACC
                           *  *   ** *  *  *   *  *  *      *  *   *  *  ***
cod1999(NS)      #51   -----G---- ---------- ---------- ---------- ----------
cod71972002(NS)  #51   ---------- ---------- ---------- ---------- ----------
halibutV9954     #51   ---------- -------G-- ---------- -----C---- -T---C----
flounder(NB)     #51   ---------- ---------- ---------- ---------- ----------
Mt/01/sba        #51   -----C---A -G--A-AA-- TT-C--G--- ---------- -TT-:CA---
JP/06/SJ         #51   --------TA CT----AG-C G------C-- ---------A -::T-:A---
haddNB523-1      #51   ---------- ---------- ---------- ---------- ----------
cod (NH)         #51   ---------- ---------- ---------- ---------- ----------
cod(NFLD)        #51   --------T- --T------- ---------- --T------- ----C-T---
                       ..........................................................
                 #51   GCTCCAATCC TAACCCTCGG ACCACTCTAC AACGATTCCC TCGCAGCCAA
                           *  ** *** * ** * ** *  **     *  *   *  ******
cod1999(NS)      #101  ---------- ---------- ---------- ---------- ----------
cod71972002(NS)  #101  ---------- ---------- ---------- ---------- ----------
halibutV9954     #101  ---------- -C-G---A-- A----T---- ----T----C --G--T----
flounder(NB)     #101  ---------- ---------- ---------- ---------- ----------
Mt/01/sba        #101  -T-------- -C--G--C-- ---------A ---AT-C--A -C---G----
JP/06/SJ         #101  -G-TT-CAC- GG-TT---T- --AT--T--- G---T-G--C ---------C
haddNB523-1      #101  ---------- ---------- ---------- ---------- ----------
cod (NH)         #101  ---------- ---------- ---------- ---------- ----------
cod(NFLD)        #101  ---------- -C-------- ---------- ---------- --G-------
                       ..........................................................
                 #101  C:G::A::TT TTAAATCGAT CC::TC:CTT :GGCGCTACT CAACTCGACA
                        * ** ***  ** **  **  * ** **  * *  ** *  *  **  *   *
cod1999(NS)      #151  ---------- ---------- ---------- --T------- ----------
cod71972002(NS)  #151  ---------- ---------- ---------- --T------- ----------
halibutV9954     #151  ----C--T-- C--------- --TT------ --T------- -G-----T--
flounder(NB)     #151  ---------- ---------- ---------- --C------- ----------
Mt/01/sba        #151  -T--C--T-- T-----A--- -:TC--GC-G --C--T---- -G-----T--
JP/06/SJ         #151  -------T-C -AAC--T--- -TTGTCAC-: --CAAA---T -GC----T--
haddNB523-1      #151  ---------- ---------- ---------- --N------- ----------
cod (NH)         #151  ---------- ---------- ---------- --C------- ----------
cod(NFLD)        #151  -------T-- ---------- ---------- --T--A--T- ----------
                       ..........................................................
                 #151  TCGCTCCCGA AGGAGCCGTC TACACA:TTA GAYCGGCCGC TTTCCATCGA
                        *  *  * * ****  *     ******* *   ****  **  **    *
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cod1999(NS)     #201          ---------- ---------- ---------- ---------- ----------
cod71972002(NS) #201          ---------- ---------- ---------- ---------- ----------
halibutV9954    #201          C----G---- --C------- ----C----- ----GTT--C ----------
flounder(NB)    #201          ---------- ---------- ---------- ---------- ----------
Mt/01/sba       #201          C----GC--T --------A- ---------- ---TGTT--- -----CA-C-
JP/06/SJ        #201          ---------T ---GTG--C- -C--C----- G---GTG--C -----CC--C
haddNB523-1     #201          ---------- ---------- ---------- ---------- ----------
cod (NH)        #201          ---------- ---------- ---------- ---------- ----------
cod(NFLD)       #201          ---------- ---------- ----C----- ----GT---- ----------
                              ..........................................................
                #201          TTACAATCTG GGAACTGGTG ATGTTGACCG TGCCACCTAT TGGCATGTGA
                              *    **  *   ****  *   *  *      *  ****  *      ** **
cod1999(NS)     #251          ---------- ---------- ---------- ---------- ----------
cod71972002(NS) #251          ---------- ---------- ---------- ---------- ----------
halibutV9954    #251          -------T-- ---C---G-- ---------- ---------- C---------
flounder(NB)    #251          -------T-- ---------- --------C- ---------- ----------
Mt/01/sba       #251          ----GT-T-- ---A---G-T --C------- -A--C----- --G------C
JP/06/SJ        #251          G---GAN--- ---AG-C--T CAGGT----- -T----AC-- -G-------A
haddNB523-1     #251          ---------- ---------- --------C- ---------- ----------
cod (NH)        #251          ---------- ---------- --------C- ---------- ----------
cod(NFLD)       #251          -------T-- -------G-- ---------- ---------- ----------
                              ..........................................................
                #251          AGAAAGTAGC TGGGAATACG GGAACACCTG CGGGGTGGTT TCACTGGGGG
                              *   **+*      ** ** * *****   *   *  * **   ***      *
cod1999(NS)     #301          ---------- ---------- ------T--- ---------- ----------
cod71972002(NS) #301          ---------- ---------- ------T--- ---------- ----------
halibutV9954    #301          --------T- ---------- ------C--- -------C-- -C--------
flounder(NB)    #301          ---------- ---------- ------T--- ---------- ----------
Mt/01/sba       #301          A-C------- -C-------- G--G--C--- G-T------- -C-----C--
JP/06/SJ        #301          --G-----TG -C--T----- G-----C--- GTT--G-CAC -C-----C--
haddNB523-1     #301          ---------- ---------- ------N--- ---------- ----------
cod (NH)        #301          ---------- ---------- ------T--- ---------- ----------
cod(NFLD)       #301          ---------- ---------- ------C--- ---------- ----T-----
                              ..........................................................
                #301          CTATGGGACA ATTTCAACAA AACATTYACA CAGGGCGTTG CTTACTATTC
                              * *     **  *  *      *  *  *    ***  * ***  *  *  *
cod1999(NS)     #351          ---------- ---------- ---------- ---------- ----------
cod71972002(NS) #351          ---------- ---------- ---------- ---------- ----------
halibutV9954    #351          ---TGC---- --TC------ ---------- ---------- ----------
flounder(NB)    #351          ---------- ---------- ---------- ---------- ----------
Mt/01/sba       #351          ---TG----- --CC-T--A- --C------- T--T-----T G---------
JP/06/SJ        #351          C--------A ---C-G--A- ---------- G-CT------ ----------
haddNB523-1     #351          ---------- ---------- ---------- ---------- ----------
cod (NH)        #351          ---------- ---------- ---------- ---------- ----------
cod(NFLD)       #351          ---------- ---------- ---------- ---------- ----------
                              ..........................................................
                #351          TGACCAGCAG CCAAGACAGA TCTTGCTGCC AGTGGGCACG C
                              *  ***   *   ** *  *    *        * **     * *
```