Development of a rapid, sensitive and specific diagnostic assay for fish 
Aquareovirus based on RT-PCR

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Abstract

A rapid, sensitive and highly specific detection method for Aquareovirus based on reverse-transcription polymerase chain reaction (RT-PCR) was developed. Based on multiple sequence alignment of the cloned sequences of a local isolates, the Threadfin reovirus (TFV) and Guppy reovirus (GPV), a pair of degenerate primers was selected carefully and synthesized. Using this primer combination, only one specific product, approximately 450 bp in length was obtained when RT-PCR was carried out using the genomic double-stranded RNA (dsRNA) of TFV, GPV and GCRV. Similar results were also obtained when Chum salmon reovirus (CSRV) and Striped bass reovirus (SBRV) dsRNA were used as templates. No products were observed when nucleic acids other than the dsRNA of the aquareoviruses described above were used as RT-PCR templates. This technique could detect not only TFV but also GPV and GCRV in low titer virus-infected cell cultured cells. Furthermore, this method has also been shown to be able to diagnose GPV-infected guppy (Poecilia reticulata) that exhibit clinical symptoms as well as GPV-carrier guppy. Collectively, these results showed that the RT-PCR amplification method using specific degenerate primers described below is very useful for rapid and accurate detection of a variety of aquareovirus strains isolated from different host species and origin.

Keywords: Aquareovirus; RT-PCR; Degenerate primers; Threadfin reovirus

1. Introduction

The genus Aquareovirus is one of the newer additions to the Family Reoviridae (Franci et al., 1991). The genome of the members from this genus is composed of 11 segments of double-stranded (ds) RNA. Aquareoviruses have been isolated from a wide range of aquatic animals including shellfish, crustaceans, and fish worldwide and are among the more frequently isolated groups of finfish viruses (Hetrick and Hedrick, 1993; Lupiani et al., 1995). Many of these viral isolates were obtained from apparently healthy fish, which were undergoing routine examination. However, some isolates of aquareoviruses were recovered from diseased fish which were experiencing mass mortalities. Previously, members of the genus Aquareovirus have been characterized into six genogroups (A–F) by reciprocal RNA–RNA hybridization, electrophoretype analysis and serology (Samal et al., 1991; Dopazo et al., 1992; Lupiani et al., 1993b; Lupiani et al., 1994; Dopazo et al., 1996; Subramanian et al., 1997). The latest report by the International Committee on Taxonomy of Viruses (ICTV) has replaced the genogroups A–F with their respective species, Aquareovirus A–F (ARV-A to ARV-F) and included other tentative species (Mertens et al., 2000). The type species is Striped bass reovirus (SBRV, species Aquareovirus A). Other members of the species Aquareovirus A, are Chum salmon reovirus (CSRV), Angelfish reovirus (AFRV) and Smelt reovirus (SRV) and many others. The members of the species Aquareovirus A are among the most diverse in terms of their geographical distribution ranging from temperate countries to the tropics. The species Aquareovirus B is represented by Chinook salmon reovirus (LBSV) and six other strains. Species Aquareovirus C has one member, the Golden shiner virus (GSRV). Recently, Attoui et al. (2002) showed that the genomes of the Grass carp reovirus
(GCRV) and Golden shiner virus were very similar to each other, indicating that GCRV is another member of the species Aquareovirus C. Members of the species Aquareovirus D is represented by Channel catfish reovirus (CCRV) while the species Aquareovirus E contains the Turbot reovirus (TRV). The last species, Aquareovirus F, have two members, the Chum salmon reovirus (PSRV) and the Coho salmon reovirus (SSRV) (Mertens et al., 2000).

Although, a majority of the aquareovirus strains isolated and described so far seemed to be non-pathogenic in nature, some strains have been reported to infect fish and cause disease. In some cases the aquareovirus strains that cause disease are sometimes associated with secondary bacterial infections. The first report of such an occurrence was by Lupiani et al. (1989), who isolated a bacterium of the genus Vibrio from turbot (Scophthalmus maximus) infected with an aquareovirus. In another case, a bacterium of genus Moraxella was concomitantly isolated from fish suffering from Striped bass reovirus infection (Baya et al., 1990). In addition, Cusack and colleagues (2001) reported the presence of concurrent bacterial infections in some fish suffering from Atlantic halibut reovirus infection. Nevertheless, pathogenicity studies have shown that some strains of aquareovirus alone can cause mortality and/or induce observable histological changes, mainly in hepatic and renal tissues of infected fish. Among all the aquareovirus strains reported, the most pathogenic isolate is the Grass carp reovirus, species Aquareovirus C, from China, which causes severe hemorrhage in infected fish that eventually leads to death (Rangel et al., 1999). GCV is highly contagious and can cause high mortalities (50-95%) not only to its host, but also to black carp (Mylopharyngodon piceus), topmouth gudgeon (Pseudorasbora parva) (Li et al., 1997) and rare minnow (Gobio-cypsis rarus) (Wang et al., 1994). Other aquareovirus strains such as Chum salmon reovirus from Japan, Striped bass reovirus (SRV) from Canada, Angel fish reovirus (AFRV), all from the species Aquareovirus A, have also been reported to cause mortalities and losses to fish farmers (Lupiani et al., 1995). Therefore, the diseases caused by these pathogenic strains of aquareovirus may pose a major threat to aquaculture industries if no measures are taken to prevent its spread. Recently, another pathogenic aquareovirus strain was isolated and reported by Chang et al. (2002) from moribund threadfin fish. This virus was then characterized further by Seng et al. (2002) and designated as Threadfin reovirus (TFV). The TFV virions are non-enveloped,icosahedral, 75–80 nm in diameter and consist of a double-layer capsid. TFV has eleven double-stranded RNA (dsRNA) segments of approximately 25,000 Da in total molecular mass and separated into three size classes: large (L1, L2, L3), medium (M4, M5, M6) and small (S7, S8, S9, S10, S11). TFV was found to be resistant to heat, 56 °C for 3 h, ether resistant and non-sensitive to acidic (pH 3) and alkaline (pH 11) conditions. This virus caused syncytia in BF-2 fish cell line. TFV has also been shown to cause 100% mortality in infected threadfin fish (Eleutheroneura tetradactyla) and severe mortality (~65%) when introduced into sea bass (Lates calcarifer) (Seng et al., 2002). A closely related virus, Guppy reovirus (GPV) was also isolated from moribund guppy in the year 2001 from a fish farm in Singapore (Choo, 2001). It caused severe mortalities in infected guppy and could be a major threat to the local ornamental fish farming industry if left unchecked. Further investigations revealed that GPV was also present in seemingly healthy asymptomatic guppy suggesting a possibility of the establishment of a virus carrier state in guppy.

Despite the severity of diseases caused by several strains of aquareovirus, very few attempts have been made to quickly diagnose the virus upon a disease outbreak or to confirm the identity of reoviruses isolated during routine examinations. As these viruses have the potential to cause death in a wide variety of host fish, it is very important for veterinary authorities to have a reliable diagnostic tool for the detection of all strains of aquareoviruses as possible. Furthermore, a sensitive and specific diagnostic method is essential if dissemination of the virus is to be controlled, as no effective broad based vaccines currently exist for its prevention. Classical detection methods involved virus propagation in cell lines and detection via purification and electron microscopy, all which are laborious and time consuming. More current detection methods using immunoenzyme staining (Ye et al., 1989), staphylococcal co-agglutination (Yang, 1991) and immunofluorescence and enzyme-linked immunosorbent assays (Jiang et al., 1993) have also been developed. However, these methods have their limitations. For instance, the methods mentioned above were developed solely for the detection of one isolate of Aquareovirus, in this case, GCRV. More recently, an RT-PCR method was developed by Li et al. (1997), but this method was shown to be capable of detecting only one strain of GCRV, namely GCRV-861.

Due to these limitations, the development of a rapid, simple, sensitive and broad spectrum Aquareovirus detection method based on RT-PCR was carried out. The in vitro and in vivo studies showed that this RT-PCR assay was sensitive, specific and much less time consuming than traditional methods, making it useful for routine screening.

2. Materials and methods

2.1. Viruses

Threadfin reovirus was isolated from diseased threadfin at a fish farm in Singapore (Seng et al., 2002). Guppy reovirus was also locally isolated during a recent disease outbreak at one of the fish farms in Singapore (Choo, 2001). Grass carp reovirus (GCRV-873) was kindly provided by Dr. Q. Fang (Hydrobiology Institute, Wuhan Institute of Virology, Wuhan, Hubei, China). Striped bass reovirus was kindly provided in a suspension grown in Chinook salmon

embryo cell line (CHSE-214) by Dr. C.P. Dupoaz (Departamento de Microbiologia e Parasitologia, Facultade de Biologia, Universidade de Santiago de Compostela, Santiago de Compostela, Spain). Chum salmon reovirus was kindly provided in a suspension grown in Chinook salmon embryo cell line (CHSE-214) by Dr. J.R. Winton (Fish Health Section, Western Fisheries Research Center, Seattle, WA). In brief, both CIK and BF-2 cells were cultured in Eagles Minimal Essential Medium (MEM) supplemented with 1% fetal bovine serum, 2 mM l-glutamine, 100 IU penicillin ml−1 and 100 μg streptomycin ml−1 (Sigma).

2.2. Cell culture and virus inoculation

Two cell lines were used in the experiment, (BF-2; ATCC CCL91) and Grass carp kidney cells (CIK) which was kindly provided by Dr. Q. Fang. BF-2 cell line was used to propagate TFV as previously described by Seng et al. (2002). Similarly, GPV was also propagated in BF-2. The CIK cell line was used to propagate GCRV as described by Wang et al. (1994). TFV was also grown in CIK cell line as preliminary investigations showed that it also propagated well in the CIK cell line. In brief, both CIK and BF-2 cells were cultured in Eagles Minimal Essential Medium (MEM) supplemented with 10% fetal bovine serum (FBS), 2 mM l-glutamine ml−1 and 200 IU penicillin ml−1 and 100 μg streptomycin ml−1 (Sigma).

2.3. Determination of virus titer

Virus infectivity was assayed using 96-well microplates (IWAKI) containing monolayers of BF-2 or CIK cells. The titer was determined by using the 50% tissue culture infective dose (TCID50) assay with endpoints calculated by the method of Reed and Muench (1938). The virus titer of TFV and GPV was determined using BF-2 cells while GCRV virus titer was determined using CIK cells.

2.4. Virus purification and viral RNA extraction

Purified TFV, GPV and GCRV were obtained from infected cell culture supernatants by centrifugation and genomic dsRNA was extracted using the High PureTM Viral RNA Kit (Boehringer Mannheim) as described by Seng et al. (2002). The dsRNA was resuspended in DEPC-treated water and stored at −80°C until use. Cell culture supernatants were first pelletted by centrifugation at 78 000 × g for 1.5h and total RNA was extracted from the pellet using 1.0 ml of Trizol® (Life Technologies, Gibco-BRL), homogenized, and kept at −80°C until use. The upper aqueous layer containing the RNA was removed to a fresh tube, recovered by precipitation with isopropyl alcohol and washed with 70% ethanol. The RNA was then resuspended in 10 μl of DEPC-treated water and kept until use.

2.5. Cloning and sequencing of TFV and GPV S6 gene segment

The synthesis of cDNA was carried out according to the method of Lambden et al. (1992). The cDNAs obtained were then sequenced by TAQ dye primer cycle sequencing on an ABI PRISM® 377 DNA Sequencer (Perkin-Elmer). The complete full-length sequence (2056 bp) of the S6 gene of TFV was then submitted to GenBank and given an accession number (AY 235426). This TFV fragment was then later used for analysis in the selection of the appropriate pair of primers for RT-PCR detection. The partial sequences of the S6 gene of GPV was also determined using the method described by Lambden et al. (1992) and was then submitted to the GenBank and was given an accession number (AY 235427).

2.6. Experimental guppy fish infection

A group of 50 female guppy (Poecilia reticulata) were injected with 20 μl of 1010 TCID50 ml−1 of GPV per fish. Similarly, a group of 20 female guppy were injected with BF-2 cell culture supernatant and used as a negative control. Another group of 20 female guppy received no injections and were also used as negative controls. Fish mortalities were recorded daily through day 35. On the 2nd, 3rd, 4th, 5th, 6th, 7th, 14th, 28th and 35th day post-injection, a group of five fish were killed and specific tissues excised and total RNA was extracted from these tissues as described below. Similarly, tissues from five fish from the negative control groups were collected and pooled on the 2nd, 7th, 28th and 35th day post-treatment and processed as described above.

2.7. Total RNA extraction from virus infected cultured cells and tissues

Predetermined virus titers of TFV, GPV and GCRV were serial ten-fold diluted to the appropriate concentrations and inoculated onto 24-well microplates containing monolayered cultured cells and incubated at 25°C for 24 h. After 24 h, total RNA was extracted from virus-infected cells using 1.0 ml of Trizol® (Life Technologies, Gibco-BRL)/well and kept in 1.5 ml centrifuge tubes at −80°C until further processed according to the manufacturer’s instructions. Briefly, samples were thawed at room temperature and 200 μl of chloroform was added to each tube and mixed. The tubes were then left at room temperature for 3 min before centrifugation at 12 000 × g for 15 min at 4°C. The upper aqueous layer containing the RNA was removed to a fresh tube, recovered by precipitation with isopropl alcohol and washed with 70% ethanol. The RNA was then resuspended in 10 μl of DEPC-treated water and kept at −80°C until use. Similarly, total RNA from 250 μl of cell culture supernatant harboring viruses at different titers was extracted using 1.0 ml of Trizol® and processed as described above.

Samples of the liver and spleen tissues from a group consisting of five individual guppy at various time intervals were obtained from GPV infected and non-infected (control) fish. Tissues were added directly to 1 ml of Trizol® (Life Technologies, Gibco-BRL), homogenized, and kept at −80°C before being processed in accordance to the manufacturer’s
instructions. The precipitated RNA was then re-dissolved in 100 μl of DEPC-treated water and kept at −80 °C until use.

2.8. Primers

Two degenerate primers (AQ1 and AQ2) were designed based on the C-terminal end-terminal sequences of the segment 6 gene of TFV (AY 235428), GPV (AY 235427) and published sequences of the inner coat protein gene (S6) of GCRV (ARV-C) (AF 239175). These sequences were also aligned with other representative Aquareovirus species. ARV-A represented by Chum salmon reovirus (AF 418299), ARV-C represented by Golden shiner reovirus (GSRV) (AF 403403) and Grass carp reovirus-A (AF 403392) using the Clustal W computer program. The primer pair was selected based on conserved regions located within the inner coat protein gene. The primers were then used for RT-PCR amplification, AQ1 as the sense (upstream) primer and AQ2 as the antisense (downstream) primer. The AQ2 primer hybridizes to nucleotide positions 1331–1350 of the TFV inner coat protein sequences (AY 235428) while the AQ1 primer hybridizes to nucleotide positions 1351–1350 of the TFV inner coat protein sequences (AY 235428).

2.9. RT-PCR assay

cDNA synthesis was carried out using the Superscript First-Strand System for RT-PCR (Life Technologies, Gibco BRL) with slight modifications. The RNA was heated at 98 °C for 2.5 min with 0.25 mM of AQ1 primer and 10 mM dNTPmix and quickly cooled on ice for 5 min. A mixture of reverse transcriptase (RT) buffer (20 mM Tris–HCl, pH 8.4, 50 mM KCl), 5 mM MgCl₂, 10 mM DTT and (RNaseOUT)-recombinant RNase inhibitor (40U) was then incubated at 50 °C for 50 min. Reverse-transcription was then terminated by heating the final mixture at 70 °C for 15 min. The RT product was then subjected to RNase H (2U) digestion at 37 °C for 20 min and stored at −20 °C until further use. For the PCR, 5 μl of the cDNA products was added to 45 μl of PCR reaction mix containing 10 mM Tris-HCl (pH 9.0 or 25 °C), 2.0 mM MgCl₂, 50 mM KCl and 0.1% Triton X-100, 200 μM dNTPs (Promega), 0.3 μM of each primer AQ1 and AQ2 and 2U Taq DNA polymerase (Promega). PCR amplification was then carried out as follows: 2 min at 94 °C for pre-denaturation, followed by 30 cycles each consisting of denaturation at 94 °C for 30 s, annealing at 50 °C for 30 s and extension at 72 °C for 1 min, with a final extension 72 °C for 5 min by using a DNA thermal cycler (I-Cycler, BioRad). From each reaction tube, 15 μl of PCR products was separated by electrophoresis on a 1.4% agarose gel in TBE buffer and stained with ethidium bromide (Sambrook et al., 1989).

3. Results

3.1. Primer sequences

A multiple sequence alignment of the C-terminal sequences (>1000 bp from the 5’ end-terminal sequences) of dsRNA gene segment 6 from various species of Aquareovirus; Chum salmon reovirus (species ARV-A), Golden shiner reovirus (GSRV), Grass carp reovirus (GCRV-A), Grass carp reovirus (GCRV), all three strains belonging to species ARV-C, and Guppy reovirus and Threadfin reovirus, of unknown species, revealed that despite being placed in different species, all strains exhibit some degree of conservation in their segment 6 gene nucleotide sequences (Fig. 1). By this rationale, two sites of conserved regions were selected carefully and primers were designed for use in RT-PCR for the detection of various Aquareovirus strains. The downstream (antisense) primer, AQ2, was selected from the conserved region 2 (underlined) as shown in Fig. 1. Closer analysis of the sequences in region 2 (Fig. 1) indicated that fifteen out of twenty nucleotides (nt) from the 3’ end of all six Aquareovirus strains were conserved. When the TFV sequence was compared to GPV or GSRV, there were only two nucleotide mismatches found. Comparison of the TFV sequence to CSRV, GCRV-A and GCRV sequences revealed only three nucleotide mismatches. An upstream primer, AQ1, was selected from the conserved region 1 (boxed) (Fig. 1). The sequence of the upstream (sense) primer, AQ1, is indicated Table 1. In this region, 15 out of 20 nucleotides (nt) from the 5’ end were common to all the Aquareovirus species compared here (Fig. 1). The last 8 nt from the 3’ end of primer AQ1, exactly match the nucleotide sequences present in all six different strains of Aquareovirus. The primers, AQ1 and AQ2, designed here, were mainly meant for the detection of TFV, GPV and GCRV and were not intended for the detection of CSRV, GSRV and GCRV-A due to the unavailability of these sequences when the experiment was carried out in the early months of the year 2001. However, two Aquareovirus strains, CSRV and striped bass reovirus were blindly tested using the RT-PCR assay described here and surprisingly a specific band was observed for both strains. In order to fully accommodate for the detection of CSRV, GSRV and GCRV-A in addition to TFV, GPV and GCRV, an increase in the degeneracy of the AQ2 primer from the R residue (Table 1, underlined) to D is required. For the sense primer, AQ1, the S residue needs to be changed to N, while the C (Table 1, underlined) needs to be changed to A. The question

Table 1

<table>
<thead>
<tr>
<th>Primers used for RT-PCR amplification</th>
<th>Designation</th>
<th>Nucleotide position (nt)</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>AQ1 (sense)</td>
<td>1351-1350</td>
<td>5’TTRCCTATATCTGTGATTTC-3'</td>
<td></td>
</tr>
<tr>
<td>AQ2 (antisense)</td>
<td>1754-1773</td>
<td>5’TCTGGAAGCGAATMAENAGACG-3'</td>
<td></td>
</tr>
</tbody>
</table>
Fig. 1. Multiple alignment of the C-terminal nucleotide sequences of gene segment 6 from some Aquareovirus strains. Sequences above 1000 bp from the 5' end terminus of gene segment 6 of various Aquareovirus strains were aligned using the DNASIS ver. 2.5 (Hitachi Software Engineering Company Ltd.) computer program. Sequences used are: TFV (accession number: AY 235428); GPV (AY 235527); CSRV (AF 418299); GSRV (AF 403403); GCRV-A (AF 403392); GCRV (AF 239175). Asterisks mean identical sequences among all strains. Two conserved regions, region 1 (boxed) and region 2 (underlined) were used for the design of primers for the RT-PCR assay.
Fig. 1. (Continued)
then arises as to whether this increase in degeneracy to accommodate the detection for all the *Aquareovirus* strains described here will still result in high specificity, remains to be tested. Nonetheless, based on the sequence alignment data (Fig. 1), using the currently described primers, AQ1 and AQ2, and TFV, GPV and CSRV genomes as template, the predicted RT-PCR product is 443 bp in length. When the AQ1 and AQ2 primers are applied to GSRV, GCRV-A and GCRV genome, an RT-PCR product of 434 bp is expected.

### 3.2. Detection of aquareoviruses by RT-PCR

RT-PCR was carried out using various *Aquareovirus* dsRNA as templates (Fig. 2). Results utilizing this pair of degenerate primers (AQ1 and AQ2) revealed that a single specific product of approximately 450 bp was detected when genomic dsRNA of Threadfin reovirus was used (Lanes 2 and 6). The same 450 bp band was also observed when GPV, GCRV, CSRV and SBRV genomic dsRNA were used as the template for RT-PCR (Lanes 3, 5, 7 and 8, respectively). However, the intensity of the CSRV and SBRV bands was less (Lanes 7 and 8) compared to others.

### 3.3. Sensitivity of the RT-PCR assay

After RT-PCR, a positive band of approximately 450 bp was still detected when 100 pg of purified Threadfin reovirus dsRNA was used as template (Fig. 3A). A similar result was recorded when purified Guppy reovirus dsRNA was used (Fig. 3B). Using purified dsRNA of Grass carp reovirus as template, the detection limit was much better, with products still visible even at 10 pg (Fig. 3C).

### 3.4. Detection of Aquareovirus by RT-PCR without culturing onto BF-2 or CIK cells

When the RT-PCR assay was carried out using total RNA extracted from 250 μl of cell culture supernatant harboring viruses at various titers, the lowest virus titre at which the specific amplicon (∼450 bp) was observed is at $10^5$ for TFV and GPV and $10^6$ for GCRV (Fig. 4A-C, respectively).

### 3.5. Enhancement of detection limits of Aquareovirus by RT-PCR after 24 h-culture onto BF-2 or CIK cells

Upon inoculation of TFV for 24 h onto BF-2 cells followed by RT-PCR using primers, AQ1 and AQ2, the correct

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**Fig. 1.** (Continued)

**Fig. 2.** Detection of aquareoviruses by RT-PCR. M: 100 bp DNA ladder (Promega); Lane 1: BF-2 RNA; Lanes 2 and 6: TFV dsRNA; Lane 3: GPV dsRNA; Lane 4: CIK RNA; Lane 5: GCRV dsRNA; Lane 7: CSRV dsRNA; Lane 8: SBRV dsRNA. Arrow indicates the expected size of the specific product (∼450 bp).
amplicon (∼450 bp) could be detected when the initial inoculum virus titer was as low as 10^{-3} TCID_{50} (Fig. 5A). In contrast, the 450 bp product was only observed when TFV at 10^7 TCID_{50} was inoculated onto CIK cells after 24 h (Fig. 5D). The positive PCR amplicon could be detected when GPV at 10^{-3} TCID_{50} was inoculated onto BF-2 cells and cultured for 24 h (Fig. 5B). Similarly, when GCRV at 10^{-3} TCID_{50} was inoculated onto CIK cells for 24 h, the correct PCR product (Fig. 5C) was observed. However, the intensity of the GCRV PCR amplicon was much greater than for TFV and GPV at dilutions of 10^{-1} to 10^{-3} TCID_{50}. During this 24 h incubation period, no CPE was observed in cells that were inoculated with different viruses at various titers using microscopic examination.

3.6. Limits of detection of Aquareovirus via various detection methods

By culturing TFV, GPV and GCRV onto their permissive cell lines for 24 h, CPE could only be detected by microscopic examination when 10^0–10^2 TCID_{50} ml^{-1} of virus was inoculated (Table 2). By extracting RNA from supernatant containing virus followed by RT-PCR a 100-fold increase in detection limit for TFV and GPV (from 10^0 to 10^2) and only 10 fold increase for GCRV (from 10^0 to 10^2) was achieved compared to microscopic examination at 24 h. However, upon culturing of TFV, GPV and GCRV in their most permissive cell lines for 24 h followed by total RNA
Of supernatant harboring 10^6 TCID_50 ml^(-1) amplicon was also detected in the positive control (RNA 35, the intensity of the amplicon was more or less similar to the control fish (Lane 1). GPV infection could be detected as early as day 2 (Lane 2) post injection of GPV into the peritoneal cavity of the guppy. The intensity of the 450 bp prod-

Table 2

<table>
<thead>
<tr>
<th>Virus</th>
<th>After 24 h culture (CPE examination via microscope)</th>
<th>Before culture (RT-PCR)</th>
<th>After 24 h culture (RT-PCR)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TFV</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>GPV</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>GCRV</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

3.7. Time course detection of Aquareovirus in tissues of experimentally infected guppy

The correct amplicon (∼450 bp) was detected by RT-PCR when total RNA from liver and spleen tissues of the infected fish were prepared and used as templates (Fig. 6). No amplicons were detected in the negative (cell-culture injected) control fish (Lane 1). GPV infection could be detected as early as day 2 (Lane 2) post injection of GPV into the peritoneal cavity of the guppy. The intensity of the 450 bp product gradually increased and peaked at day 5 (Lane 5), but no mortalities were encountered. At days 6, 7, 14, 28 and 35, the intensity of the amplicon was more or less similar in the live virus-infected guppy (Lane 6-10). The 450 bp amplicon was also detected in the positive control (RNA extracted from supernatant harboring 10^6 TCID_50 ml^(-1) of GPV) via RT-PCR (Lane 11). RT-PCR assay using total RNA extracted from the spleen and liver of one sole mori-bund guppy at day 35 (Lane 12) displayed the most intense amplicon. No amplicons were observed in the other negative control (non-injected) guppy when RT-PCR was conducted (data not shown).

4. Discussion

Since the first reported case of Aquareovirus in golden shiner in 1977, many isolates of Aquareovirus have been isolated from diseased as well as healthy looking fishes during routine screening (Brady and Plumb, 1991). To date, most detection of aquareoviruses in infected fish has so far been carried out using several classical methods. These methods include: propagation of the virus in a susceptible cell line, observations of virus using electron microscopy, immunoenzyme staining (Ye et al., 1989), staphylococcal coagglutination (Yang, 1991), immunofluorescence and enzyme-linked immunosorbent assay (ELISA) (Jiang et al., 1993). Most of these techniques are time consuming or require special equipment and may not be very sensitive. With the current advancement of molecular techniques, Subramanian et al. (1993) developed a detection method based on nucleic acid hybridization for the detection of Striped bass reovirus in fish tissues. Unfortunately, this method was only applied to detect one individual isolate, the Striped bass reovirus (species Aquareovirus A) and furthermore it was also laborious and time consuming to carry out. More recently, Li et al. (1997) developed an RT-PCR method to detect for Grass carp reovirus (species Aquareovirus C). However, the two pairs of primers they chose could only detect one of the two strains of GCRV, namely strain GCRV-861 and not GCRV-873. Thus, a more universal set of primers is needed to ensure that a wider spectrum of Aquareovirus strains from various species (ARV-A to ARV-F) could be detected in infected or carrier fishes.

With this idea, the development of an RT-PCR assay that could detect a wider range of Aquareovirus strains was undertaken. The assay required the design of a pair of novel degenerate primers to accommodate the degeneracy exhibited by most of the Aquareovirus sequences used in this study. This approach of using degenerate primers was chosen as studies by Legeay et al. (2000), Wyn-Jones et al. (2000) and Okuda & Hanada (2001) have demonstrated that degenerate primers can be used in RT-PCR for detection of viruses successfully. Hence, utilizing the degenerate primer pair described here (AQ1 and AQ2), an RT-PCR assay was developed and tested. Test indicated that this RT-PCR method, utilizing degenerate primers, could detect threadfin reovirus, Guppy reovirus, Grass carp reovirus, Striped bass reovirus as well as Chum salmon reovirus. In all the RT-PCR reactions that were carried out only one main product at ∼450 bp was clearly observed although the product in CSRV and SBBRV were less intense compared to the rest. This product size was relatively close with the predicted amplification products of 443 bp for TFV, GPV and CSRV and 434 bp for GCRV based on sequence analysis. No products were obtained when nucleic acids other than the genomic dsRNA of the specified aquareoviruses mentioned above were used as RT-PCR templates. The lower intensity of CSRV and SBBRV could be attributed to the inherent degenerate primers used here. As shown in Table 1 and Fig. 1, the degenerate primers AQ1
Fig. 5. Enhancement of detection limits of Aquareovirus by RT-PCR after 24 h-culture onto BF-2 or CIK cells. Cells were inoculated with virus at different doses (TCID50) and incubated at 25°C for 24 h before total RNA was extracted and RT-PCR performed. M: 100 bp DNA ladder (Promega); virus inoculation dose: (TCID50 ml⁻¹) onto the cells: 10⁴ (Lane 1); 10³ (Lane 2); 10² (Lane 3); 10¹ (Lane 4); 10⁰ (Lane 5); 10⁻¹ (Lane 6); 10⁻² (Lane 7); 10⁻³ (Lane 8); purified dsRNA of TFV (Lane 9). (A) BF-2 cells infected with TFV; (B) BF-2 cells infected with GPV; (C) CIK cells infected with GCRV; D: CIK cells infected with TFV. Arrow indicates the correct amplicon of (∼450 bp) after RT-PCR.

Fig. 6. Time course detection of Aquareovirus in tissues of experimentally-infected guppy by RT-PCR. Each fish was injected intra-peritoneally with 20 µl of 10¹⁵ TCID50 ml⁻¹ GPV. At various time intervals liver and spleen tissues were excised from the fish and total RNA extracted followed by RT-PCR. M: 1 kb DNA ladder (Promega); Lane 1: BF-2 cell culture supernatant injected fish; Lanes 2–7: 2–7 days post-injection (dpi); Lane 8: 14 dpi; Lane 9: 26 dpi; Lane 10: 35 dpi; Lane 11: total RNA isolated from supernatant of cell culture cells infected with GPV; Lane 12: moribund virus-injected day 35 guppy. Arrow indicates the correct amplicon of (∼450 bp) after RT-PCR.

CSRV, SBRV and GCRV could be detected. This is also the first RT-PCR assay that could detect aquareoviruses from different species, SBV, and CSRV (both from the species ARV-A), and GCRV (species ARV-C) besides TFV and GPV (unknown species). Based on sequence analysis alone, it can be predicted that the primers AQ1 and AQ2 could also be used to detect the presence of another Aquareovirus strain, golden shiner reovirus (GSRV), species ARV-C, but this remains to be verified.

When the RT-PCR method developed above was tested for its sensitivity using three strains, TFV, GPV, and GCRV, as little as 100 pg of the purified dsRNA from TFV and GPV and 10 pg of purified dsRNA from GCRV was sufficient to be detected. The detection limits shown here were much better in comparison to the nucleic acid hybridization method developed by Subramanian et al. (1993) which could only detect as little as 5 ng of dsRNA from purified Striped bass reovirus (species ARV-A) and Lupiani et al. ‘s (1993a) genetic probe which when used in a dot blot assay was only able to specifically detect 50 ng of dsRNA from purified Turbot reovirus, species ARV-E and not other strains of Aquareovirus.

Li et al. (1997) reported that they could detect GCRV using RT-PCR by utilizing only as little as 0.10 pg of purified GCRV dsRNA. Results from the RT-PCR assay described here indicated the detection limit for GCRV was at 10 pg, 100-fold less sensitive than that found by Li et al. (1997). The lower sensitivity could simply be due to the specific primers being used or the different conditions employed by Li et al. (1997) in their RT-PCR assay.

Davis et al. (1994) described a method that combined the amplification power of cell culture with the objectivity and specificity of enzyme-linked immunosorbent assay (ELISA) for the detection of infectious pancreatic necrosis...
virus (IPNV), another dsRNA virus. The combined assay did not greatly reduce the total laboratory time (2 weeks); however, they showed that the method was simple and reliable for screening fish populations sub-clinically infected with IPNV. Iwamoto et al. (2001) demonstrated the use of a combined cell-culture and RT-PCR method for rapid detection of piscine nodaviruses with great success. Hence, in the present study, an assessment of the suitability of the RT-PCR amplification technique in combination with cell culture for rapid detection of piscine aquareoviruses at low virus titers was carried out.

As demonstrated in this study, cytopathic effect (CPE) caused TFV, GPV and GCRV could only be detected 24 h after culture when the initial virus inoculum is very high (10^7–10^8 TCID_{50} ml^{-1}) via microscopic examination. By using the RT-PCR method developed here, utilizing total RNA extracted from culture supernatant-containing viruses as the template, the presence of aquareovirus infection could be detected when 10^9 TCID_{50} ml^{-1} of TFV and GPV and 10^8 TCID_{50} ml^{-1} of GCRV was inoculated into susceptible fish cell lines. This represented a mere 100-fold enhancement in detection limits as compared to CPE detection via microscopic examination at 24 h.

Further enhancement of detection limits was achieved via the combination of 24 h virus-infected cell culture followed by RT-PCR. This method allowed for the detection of TFV, GPV and GCRV even when the initial inoculum of the virus was as low as 10^3 TCID_{50} ml^{-1}. This represented a 10-fold increase in detection limits when compared to detection by observation of CPE at 24 h. Typically, CPE caused by Aquareovirus infection can be detected within 3–7 days post-inoculation onto susceptible fish cell lines. After 7 days post-inoculation, the lowest virus inoculum titer, which resulted in CPE, is at 10^5 TCID_{50} ml^{-1}. Hence, these results clearly demonstrated that the 24 h culture of aquareoviruses in highly permissive cell lines followed by the RT-PCR described here is the best choice to conveniently detect these viruses from suspected samples harboring low viral titers.

The validity of the RT-PCR method for detecting Aquareovirus in fish was carried out in artificially GPV-infected guppy. Results indicated that even as early as 2 days post-inoculation (dpi), GPV could be detected in the combined liver and spleen tissues of the infected fish. Only one specific product at ~450 bp was detected. This clearly demonstrated the specificity of the RT-PCR method when used in vivo. By 4–5 dpi, peak GPV viral RNA was observed but no mortalities were encountered. The presence of less intense GPV band from the 6h–35h dpi may suggest that the virus was slowly being shed from the fish, but the fact that it remained in the fish even at 35 dpi is highly suggestive that a virus carrier state had been established in the GPV injected guppy. These data is in agreement with Meyer’s (1983) results, which showed that rainbow trout injected with American oyster reovirus (13p2V), strain (ARV-A) did not result in any death but produced sub-clinical infections in the adults as well as the juvenile fish. At 35 dpi, RT-PCR of the liver and spleen tissues of a sole moribund guppy revealed a very intense product suggestive that GPV was the main cause of death of the fish. A homogenate of the liver and spleen tissues from this moribund fish when inoculated onto BF-2 cells developed CPE. 3-day post inoculation (data not shown). This result further supports that GPV infection was the actual cause of death of the moribund fish at day 35. Despite the low mortality rates of the artificially GPV-infected guppy observed in this study (2%), the confirmation that asymptomatic guppy could harbor the virus for long-periods of time (≤35 days post injection), is of great significance. In general, losses attributed to aquareoviruses are not routine and are usually associated with sub-optimal culture conditions (e.g. overcrowding, poor water quality) that suppresses immunity or due to secondary bacterial infections. Hence, the persistence of the virus, GPV in infected guppy could mean that when certain conditions are favorable for the virus, the virus could quickly gain its virulence and cause clinical disease that would eventually lead to mass mortality. This scenario is analogous to the virus being a “ticking time bomb”. In addition, without any diagnostic test for Aquareovirus, fishes that harbor the virus but show no clinical signs could be exported unknowingly to a foreign country and spread the virus into a new community of fishes. Thus, the RT-PCR detection method developed above for the detection of Aquareovirus is of high relevance to the aquaculture industry.

In conclusion, a simple, rapid, sensitive and highly specific RT-PCR assay using a pair of degenerate primers, suitable for routine diagnostic use, was developed for the detection of Aquareovirus in fish. This procedure could be used to detect various strains of Aquareovirus, namely CSRV and SBRV both from the species (ARV-A), GCRV (ARV-B), and TFV and GPV (unknown species). In addition, a combined cell culture and RT-PCR method was also described in this study that was able to detect samples harboring very low titers of Aquareovirus. Hence, the RT-PCR assay described here will be a useful tool in the isolation and characterization, and epidemiology studies of various aquareoviruses as well as studies of their transmission modes (vertical and/or horizontal transmission). This study also showed that Guppy reovirus was detectable not only in tissues from moribund experimentally infected guppy but also from virus carrier fish as well. Hence, the RT-PCR assay developed here clearly demonstrated its usefulness by its ability to specifically detect fish infected with Aquareovirus which displayed clinical symptoms as well as aquareovirus infection in seemingly healthy but virus carrier fish. The ability of diagnosing such viral infections early is of great importance to the aquaculture industry in order to circumvent outbreaks caused by aquareoviruses in the near future.
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