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A multiplex RT-PCR assay for the detection of fish picornaviruses

Sunil K. Mor[a,*], Nicholas B.D. Phelps[a], Marisa Barbknecht[b], Michael A. Hoffman[b], Sagar M. Goyal[a]

[a] Minnesota Veterinary Diagnostic Laboratory, Department of Veterinary Population Medicine, University of Minnesota, 1333 Gortner Avenue Street, Paul, MN 55108, USA
[b] Department of Microbiology, University of Wisconsin–La Crosse, 1725 State Street, La Crosse, WI 54601, USA

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A B S T R A C T

With the emergence of high profile fish diseases in the Great Lakes region, surveillance and regulatory inspections of fish populations have increased. This has resulted in a better understanding of known pathogens and isolation of many new pathogens of fish. In this study, a multiplex RT-PCR assay was developed for the detection of three newly discovered fish picornaviruses: bluegill picornavirus-1 (BGPV-1), fathead minnow picornavirus (FHMPV), and eel picornavirus-1 (EPV-1). This assay was found to be very sensitive with a detection limit of 81.9 pg/μl of extracted RNA from a pool of FHMPV and BGPV-1 and was able to detect 501 and 224 gene copies/μl of BGPV-1 and FHMPV, respectively. The assay was highly reproducible and did not cross react with other closely related pathogens. We believe that this new assay provides a rapid and cost effective tool for confirming cell culture isolates and conducting prevalence studies of these newly detected fish picornaviruses.

1. Introduction

The family Picornaviridae consists of 46 species divided into 26 genera: *Aphthovirus*, *Aquamavirus*, *Avihepatovirus*, *Avisivirus*, *Cardiovirus*, *Cosavirus*, *Dicipivirus*, *Enterovirus*, *Erbovirus*, *Gallivirus*, *Hepatovirus*, *Hunnivirus*, *Kobuvirus*, *Megrivirus*, *Mischivirus*, *Mosavirus*, *Oscivirus*, *Parechovirus*, *Pasivirus*, *Passerivirus*, *Rosavirus*, *Salivirus*, *Sapelovirus*, *Senecavirus*, *Teschovirus* and *Tremovirus* (Adams et al., 2013; Knowles et al., 2012; www.picornaviridae.com). Picornaviruses are small (~30–32 nm), non-enveloped, single-stranded, positive-sense RNA viruses with a genome size of approximately 7.0–9.5 kb. The single ORF encodes a polyprotein with three different regions P1, P2 and P3. The P1 region encodes viral capsid proteins (VP4-VP2-VP3-VP1) while P2 and P3 regions encode proteins involved in protein processing ($2A^{pro}$, $3C^{pro}$ and $3CD^{pro}$) and genome replication [2B, 2C, 3AB, 3B(VPg), $3CD^{pro}$, $3D^{pol}$], respectively (Racaniello, 2007).

Members of the family *Picornaviridae* are important pathogens associated with several diseases in humans and animals (Racaniello, 2007) including disorders of the gastrointestinal and respiratory tracts and of neural, hepatocellular and circulatory systems (Racaniello, 2007). Little was known about picornaviruses in fish until recent reports of novel picornaviruses in common carp (*Cyprinus carpio*; Lange et al., 2014), European glass eel (*Anguilla anguilla*; Fichtner et al., 2013), bluegill (*Lepomis macrochirus*; Barbknecht et al., 2014), and fathead minnows (*Pimephales promelas*; Phelps et al., 2014). These viruses are herein referred to as carp picornavirus-1 (CPV-1), eel picornavirus-1 (EPV-1), bluegill picornavirus-1 (BGPV-1) and fathead minnow picornavirus (FHMPV).

The BGPV-1 and EPV-1 were reported to cause morbidity and mortality in bluegills and European glass eels, respectively (Fichtner et al., 2013; Barbknecht et al., 2014). However, CPV-1 does not cause any clinical illness in carp and the disease causing potential of FHMPV is currently unknown (Lange et al., 2014; Phelps et al., 2014). Phylogenetic analysis of the four picornavirus (BGPV-1, CPV-1, EPV-1 and FHMPV) genomes reveals that these viruses can potentially belong to four new species in the family *Picornaviridae* (Barbknecht et al., 2014; Fichtner et al., 2013; Lange et al., 2014; Phelps et al., 2014).

Standard diagnostic tests for viral infections e.g., virus isolation in cell cultures and electron microscopy are lengthy procedures and lack the sensitivity and specificity needed during outbreak investigations and surveillance. Hence, methods are needed for the rapid detection and confirmation of this emerging group of picornaviruses in fish. Molecular methods, such as reverse

* Corresponding author. Tel.: +1 612 625 8263.
E-mail address: kumars@umn.edu (S.K. Mor).

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transcription-polymerase chain reaction (RT-PCR), can help identify existing and novel viral agents in fish. Species-specific RT-PCR assays for fish picornaviruses have been developed for the purpose of confirmatory diagnosis (Barbknecht et al., 2014; S. Mor, unpublished). However, a single-assay for all known fish picornaviruses can be valuable for surveillance testing or if the agent is unknown. This study was undertaken to develop a multiplex one-step RT-PCR (mRT-PCR) assay for the detection of three picornaviruses e.g., BGPV-1, FHMPV, and EPV-1.

2. Materials and methods

2.1. Viruses

The FHMPV was isolated at the Minnesota Veterinary Diagnostic Laboratory (MVDL) and BGPV-1 was isolated at the University of Wisconsin–La Crosse. At the MVDL, both viruses were cultured in Epithelioma papulosum cyprini (EPC) cells at 20 °C. The inoculated cells were examined daily under a light microscope for the appearance of virus-induced cytopathic effects. After six days of incubation, the cells were frozen and thawed three times followed by centrifugation at 2000 × *g* for 15 min at 4 °C. The supernatants were collected, aliquoted, and stored at −80 °C. The EPV-1 was not available for culture in this study because of import regulations. Neither the sequence nor the isolate of CPV-1 was available for this study. Supernatant from non-infected EPC cells was used as a negative control.

2.2. Primer design

The primers used in this study were self-designed with oligo Perfect Designer software (www.lifetechnologies.com). Specific primers for each of the three viruses were designed from the 3D gene (RNA dependent RNA polymerase) region using respective sequences of BGPV-1 (NC_018506), EPV-1 (NC_022332) and FHMPV (NC_023437) available in GenBank (Table 1).

2.3. Multiplex RT-PCR

RNA was extracted from infected cell culture supernatants using the QIAamp viral RNA mini kit (Qiagen, Valencia, CA); manufacturer's instructions were followed and final elution was done in 40 μl elution buffer. The mRT-PCR was performed using Qiagen One Step RT-PCR kit in Mastercycler® epRealplex 2 (Eppendorf, Hamburg, Germany). The reaction mix (12.5 μl) consisted of 1X RT-PCR reaction buffer, 200 μM of each dNTP, 0.5 μM of each primer, 1 μl of enzyme blend, and 2 μl of extracted RNA. Thermal cycling conditions for RT-PCR were: reverse transcription at 50 °C for 30 min and *Taq* activation for 15 min at 95 °C followed by 35 cycles with denaturation at 94 °C for 30 s, annealing at 53 °C for 30 s, and elongation at 72 °C for 45 s. Final elongation was done at 72 °C for 10 min. PCR products were visualized on 1.2% agarose gel in Tris-acetate-EDTA buffer by electrophoresis. The position of the bands on agarose gel at 821 and 1043 bp confirmed the presence of FHMPV and BGPV-1, respectively.

To confirm that the PCR was specific for BGPV-1 and FHMPV; both bands were excised from the gel and purified using QIAquick gel extraction kit. Purified products were sequenced with forward and reverse primers at the University of Minnesota Genomic Center. Forward and reverse sequences were aligned together using Sequencher software 5.1 (www.genecodes.com) followed by BLAST analysis (www.ncbi.nlm.nih.gov).

Table 1
Primers designed from 3D gene and used in multiplex RT-PCR.

Virus	Primers	Product size (bp)	Accession number of reference sequence
EPV-1	EPV-1_mul_F 5′CAATGGCACATGGAGAGTTG 3′ EPV-1_mul_R 5′ CCTTGTTCGATTTCCTCCAA 3′	558	NC_022332
FHMPV	FHMPV_mul_F 5′ACAATGAGRAGTTTCATGCC 3′ FHMPV_mul_R 5′AATCACGGTCTCATGAAGTCT 3′	821	NC_023437
BGPV	BGPV-1_mul_F 5′GGCACTTGGAAAGACGAGAG 3′ BGPV-1_mul_R 5′ ATTGTCAAACGGTCCGAGTC 3′	1043	NC_018506

EPV-1 – eel picornavirus-1; FHMPV – fathead minnow picornavirus; BGPV-1 – bluegill picornavirus-1.

2.4. Sensitivity

The extracted RNA was quantified in NanoDrop 2000 spectrophotometer. RNA from individual viruses and from a pool of both viruses were diluted serially (10^{-1}–10^{-8}) in nuclease-free water to test the sensitivity of mRT-PCR. The amplified PCR products of BGPV and FHMPV were cloned into a plasmid vector (Zero-Blunt® PCR cloning kit; Invitrogen, NY, USA) according to the manufacturer's instructions. The plasmid DNA was extracted from vector-cloned PCR products of BGPV-1 and FHMPV, which were also quantified in NanoDrop 2000 spectrophotometer. The quantified plasmid DNA of each virus was diluted serially (10^{-1}–10^{-10}) in nuclease-free water to calculate the lowest copy number detection limit of mRT-PCR for BGPV-1 and FHMPV. The copy numbers were calculated by using the formula: (X g/μl DNA/[plasmid length in base pairs × 660]) × 6.022×10^{23} = Y molecules/μl where X is concentration of plasmid DNA of cloned PCR product.

2.5. Specificity

To determine the specificity of the newly developed mRT-PCR, we used viral hemorrhagic septicemia virus (VHSV), spring viremia of carp virus (SVCV), undescribed fish astrovirus, undescribed fish calicivirus, turkey picornavirus, and porcine kobuvirus. Also included in the study were eight strains of picornaviruses isolated recently at the MVDL from fathead minnows (Phelps et al., 2014).

2.6. Reproducibility

The mRT-PCR was repeated three times on three different days to verify reproducibility. In addition, a blinded panel of RNA samples (Table 2) and primer sets were tested at the University of Wisconsin–La Crosse according to the newly developed protocol to assess inter-laboratory variation.

3. Results

3.1. Multiplex RT-PCR

The reaction conditions were optimized with respective specific primers. Using these primers, both FHMPV and BGPV-1 were amplified and 1.2% gel electrophoresis identified specific bands of 821 and 1043 bp, respectively (Fig. 1). We did not have access to EPV-1 and hence no amplification was observed with the EPV-1 primer set. Both bands were excised, purified and sequenced with their respective primers. BLAST analysis confirmed these results and matched with FHMPV and BGPV-1. There was no amplification in the negative control EPC cell suspension.

Table 2
Blinded panel of RNA samples and primers sent to the Wisconsin laboratory.

Sample code	Original samples ID
S1	FHMPV 2912-8
S2	-1
S3	-2
S4	-3
S5	BGPV-1
S6	-1
S7	-2
S8	-3
S9	BGPV-1 + FHMPV
S10	-1
S11	-2
S12	-3
S13	Fish calicivirus
S14	Fish astrovirus
S15	SVCV
S16	VHSV
S17	Porcine kobuvirus
S18	Turkey picornavirus
Primer name	Primer ID
PF1	EPV-1_F
PR1	EPV-1_R
PF2	BGPV-1_F
PR2	BGPV-1_R
PF3	FHMPV-F
PR3	FHMPV-R

EPV – eel picornavirus-1; FHMPV – fathead minnow picornavirus; BGPV-1 – bluegill picornavirus-1; SVCV – spring viremia of carp virus; VHSV – viral hemorrhagic septicemia virus.

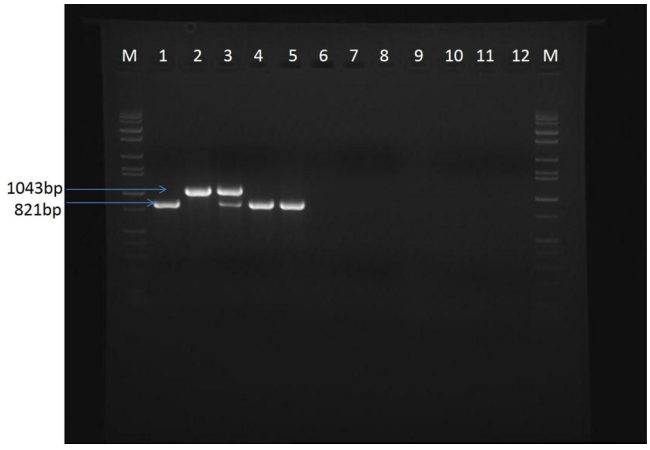

Fig. 1. A 1.2 percent gel electrophoresis showing sensitivity and specificity of multiplex RT-PCR. Lanes: M = molecular marker; 1 = fathead minnow picornavirus; 2 = bluegill picornavirus-1; 3 = bluegill + fathead minnow picornavirus; 4–5 = fathead minnow picornavirus positive field isolate; 6 = viral hemorrhagic septicemia virus; 7 = spring viremia of carp virus; 8 = fish astrovirus; 9 = fish calicivirus; 10 = turkey picornavirus; 12 = porcine kobuvirus.

3.2. Sensitivity and specificity

Sensitivity was tested by making serial ten-fold dilutions of extracted RNA. With individual primers, specific and clear bands were observed with RNA dilutions up to 10^{-3} (72.1 pg/μl) and 10^{-4} (9.47 pg/μl) for BGPV-1 and FHMPV, respectively. In the multiplex reaction, specific and clear bands were observed with an RNA dilution of 10^{-3} (81.9 pg/μl) for both viruses when pools of BGPV-1 and FHMPV were tested. The FHMPV band was also observed in up to 10^{-4} RNA dilution indicating that individual and multiplex primers sets were equally sensitive in detecting both viruses for at least 10^{-3} dilutions of extracted RNA.

The extracted RNA was transcribed into plasmid and sensitivity was also tested by making serial ten-fold dilutions of the plasmid. This assay was able to detect 501 and 224 plasmid copies/μl of BGPV-1 and FHMPV, respectively. No amplification of viruses other than FHMPV and BGPV-1 was observed indicating high specificity of the new assay. Eight newly isolated FHMPV were tested and a single specific band for FHMPV was observed in all cases.

3.3. Reproducibility

The assay produced consistent results when repeating the test on three different days. In addition, the same results were obtained when the test was performed at another laboratory by different personnel.

4. Discussion

The newly developed fish picornavirus mRT-PCR is very sensitive with detection limits of 501 and 224 gene copies/μl of BGPV-1 and FHMPV, respectively. The sensitivity of mRT-PCR assay was almost the same as that of RT-PCR with individual primers, which indicates that one primer set does not affect the sensitivity of the other. The assay was specific for amplification of fish picornaviruses; no other tested virus was amplified and non-infected EPC cells were also negative. The finding of a single and specific band on testing eight newly isolated FHMPVs in our laboratory also indicates that the mRT-PCR meets diagnostic requirements.

There are two previous reports on testing BGPV-1 and FHMPV by using RT-PCR assays. Barbknecht et al. (2014) reported a specific RT-PCR for BGPV-1, targeting the 180 bp region from 3′UTR and detected BGPV in 29% of waters sampled in Wisconsin. In a preliminary study on FHMPV, an RT-PCR assay was developed for screening of surveillance samples for FHMPV (S. Mor, unpublished). This assay was used by McCann (2012) for a virus survey of fathead minnows in Wisconsin; five of 15 lots were positive for FHMPV. Both of these studies indicate the need to screen bodies of water from different areas for the rapid detection of fish picornaviruses to better understand their prevalence, distribution, ecology, and epidemiology. The mRT-PCR developed in this study should prove useful for this purpose; however, further validation of field samples or experimentally exposed fish may be warranted. Since this mRT-PCR targets 1043 bp for BGPV-1 and 821 bp for FHMPV, the amplified product can be used for further sequence analysis and comparison with previously reported BGPV-1 and FHMPV.

Surveillance and regulatory testing using virus isolation is widespread and considered by many to be the gold standard for virus detection in fish. Given the non-specific nature of this approach, confirmatory testing is often required to identify the virus present. Although the host species of BGPV-1, EPV-1, and FHMPV are not known to overlap, the host range and epidemiology of these viruses remains largely unknown. Having a single picornavirus confirmatory assay would be a valuable addition to a diagnostic lab's toolkit to save cost and time. Furthermore, the nucleotide and amino acid variations in the 3D gene will help identify new variants of viruses, if any. This information should be helpful in phylogeographical analyses of these viruses. Although we were unable to test isolates of EPV-1 in the mRT-PCR assay development, we are confident that this new test will detect EPV-1 based on primer design. To the best of our knowledge this is the first study describing the development of an mRT-PCR for the sensitive and specific detection of fish picornaviruses and has the potential to be used as a confirmatory and screening test for these viruses.

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