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Analytical validation of a reverse transcriptase droplet digital PCR (RT-ddPCR) for quantitative detection of infectious hematopoietic necrosis virus

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ABSTRACT

Infectious hematopoietic necrosis virus (IHNV) is an important pathogen of salmonid fishes. A validated universal reverse transcriptase quantitative PCR (RT-qPCR) assay that can quantify levels of IHNV in fish tissues has been previously reported. In the present study, we adapted the published set of IHNV primers and probe for use in a reverse-transcriptase droplet digital PCR (RT-ddPCR) assay for quantification of the virus in fish tissue samples. The RT-ddPCR and RT-qPCR assays detected 13 phylogenetically diverse IHNV strains, but neither assay produced detectable amplification when RNA from other fish viruses was used. The RT-ddPCR assay had a limit of detection (LOD) equating to 2.2 plaque forming units (PFU)/μl while the LOD for the RT-qPCR was 0.2 PFU/μl. Good agreement (69.4–100%) between assays was observed when used to detect IHNV RNA in cell culture supernatant and tissues from IHNV infected rainbow trout (*Oncorhynchus mykiss*) and arctic char (*Salvelinus alpinus*). Estimates of RNA copy number produced by the two assays were significantly correlated but the RT-qPCR consistently produced higher estimates than the RT-ddPCR. The analytical properties of the N gene RT-ddPCR test indicated that this method may be useful to assess IHNV RNA copy number for research and diagnostic purposes. Future work is needed to establish the within and between laboratory diagnostic performance of the RT-ddPCR assay.

1. Introduction

Infectious hematopoietic necrosis virus (IHNV) is a major pathogen of rainbow and steelhead trout (*Oncorhynchus mykiss*), Chinook salmon (*O. tshawytscha*), sockeye and kokanee salmon (*O. nerka*) and other salmonid fish species (Wolf, 1988). IHNV has been detected in North America, Asia and Europe (OIE, 2015). In July 2016, the first occurrence of IHNV was reported in Nairobi, Kenya, in the Southern Hemisphere (OIE, 2016). Like all members of the family *Rhabdoviridae,* IHNV is a linear, non-segmented, single-stranded, negative-sense RNA virus. Phylogenetic analyses of IHNV isolates from many areas of the world where the virus is endemic or has been introduced have identified the existence of five major genogroups, namely, U, M, L, E, and J (Enzmann et al., 2005; Jia et al., 2014; Kurath et al., 2003; Nishizawa et al., 2006). The J genogroup is widespread in the salmon and trout farming regions of Asia and includes two subgroups, namely, J Nagano and J Shizuoka (Jia et al., 2014; Nishizawa et al., 2006).

A number of methods have been established for quantitative detection of IHNV. Plaque assay is the most established method for quantifying IHNV (Batts and Winton, 1989; Fendrick et al., 1982). While having the advantage of estimating titers of infectious virus, the plaque assay is prone to limitations such as variation in cell line sensitivity and assay turnaround time (Te et al., 2015), making the assay impractical for certain purposes (Purcell et al., 2006). Reverse transcriptase quantitative PCR (RT-qPCR) methods are also widely used to estimate gene copy number for RNA viruses (Raso and Biassoni, 2014), including for IHNV (Dhar et al., 2008; Liu et al., 2008; Overturf et al., 2001; Purcell et al., 2006; Purcell et al., 2013; Yue et al., 2008). However, the quantification of nucleic acids by qPCR depends on a calibration (standard) curve and there is no consistent standard

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material for every qPCR method. Marked variation in assay performance characteristics and in materials used as calibration standards may prevent agreement between different laboratories, even when testing identical material.

Recently, diagnostic assays based on droplet digital polymerase chain reaction (ddPCR) technology are increasingly being used for detecting nucleic acids of pathogens (Coudray-Meunier et al., 2015; Kiselinova et al., 2014; Rački et al., 2014; Sedlak et al., 2014; Yang et al., 2014), including RNA viruses (Coudray-Meunier et al., 2015; Rački et al., 2014; Sedlak et al., 2014). The ddPCR has many potential advantages over qPCR, as it provides an accurate, sensitive and specific measure of target DNA molecules without the need for a standard curve (Hayden et al., 2015). The ddPCR is an end-point measurement and the signal is measured only after finishing the PCR amplification reaction. The ddPCR has also been shown to have increased precision over qPCR (Hindson et al., 2013), and improved sensitivity to detect rare targets at low copy numbers (Hindson et al., 2011; Whale et al., 2012). To our knowledge, the study presented here is the first report of a RT-ddPCR assay for the detection of IHNV RNA.

The aim of this study was to evaluate the potential of RT-ddPCR for accurate quantification of IHNV RNA copy number. Performance of the RT-ddPCR method, using a commercialized droplet digital PCR platform, was compared with an established RT-qPCR assay for IHNV that targets the N gene. We compared the analytical properties of specificity and sensitivity (limit of detection) for both the RT-ddPCR and RT-qPCR assays, and evaluated agreement between the two tests. We also assessed the suitability of RT-ddPCR and RT-qPCR for quantitative detection of IHNV in tissues of infected juvenile rainbow trout (albino phenotype) and arctic char (*Salvelinus alpinus*).

2. Material and methods

2.1. Virus

The IHNV strain Ch20101008 (passage 3) was isolated from a diseased brook trout (*Salvelinus fontinalis*) in 2010 in Jilin province in China (Jia et al., 2014; Jia et al., 2013). The Ch20101008 isolate was propagated in *Epithelioma papulosum cyprini* (EPC) cell line (Fijan et al., 1983) at 15 °C following OIE methods (OIE, 2015). The titer of the Ch20101008 isolate was determined by plaque assay using monolayers of EPC cells as previously described (Batts and Winton, 1989). To evaluate analytical specificity of the RT-ddPCR and RT-qPCR assays, cell culture supernatant from a total of 13 IHNV strains representing the global diversity of IHNV was obtained, along with isolates of other fish viruses including, spring viremia of carp virus (SVCV), viral hemorrhagic septicemia virus (VHSV), infectious pancreatic necrosis virus (IPNV), infectious salmon anemia virus (ISAV), hirame rhabdovirus (HIRRV) and salmonid alphavirus (SAV) (Table 1). The ability of both assays to detect IHNV in the presence of other fish viruses was tested by mixing an equal volume of IHNV cDNA (2.5 µl) with cDNA from one of the six viruses listed above.

2.2. RNA isolation

Viral RNA was isolated using the RNeasy Mini Kit (QIAGEN, CA, USA) according to the manufacturer's instructions. For viral supernatant, 300 µl was extracted with a final elution volume of 50 µl. For tissues, the material was weighed and approximately 30 mg was homogenized in RLT buffer (Qiagen, Germany) using a bead lysing matrix (MP Biomedicals, Germany), followed by elution in 50 µl. The remainder of the procedure was as described by the manufacturer. The negative and blank controls for the extraction procedure consisted of non-infected fish tissues, non-infected cell culture supernatant or nuclease-free water. The isolated RNA was stored at −80 °C until used.

2.3. Reverse transcription

Reverse transcription was initiated by incubating 8 µl RNA with 1 µl random primer, 1 µl 10 mM dNTP and 10 µl RNAse free water at 65 °C for 5 min followed by 5 min on ice. Next, 4 µl 5 × First-strand buffer, 1 µl RNaseOUT™ (Invitrogen, USA), 1 µl 10 mM DTT (Invitrogen, USA) and 1 µl 200 U/µl of SuperScript™ III RT (Invitrogen, USA) were added to a final volume of 20 µl. Tubes were incubated at 25 °C for 5 min, 50 °C for 60 min, and then at 70 °C for 15 min. The cDNA was stored at −80 °C until used.

2.4. Primers and probes

The RT-qPCR and RT-ddPCR assays used a published set of primers and probe that targets a conserved region (positions 798–879) of the IHNV N gene (Table 2; Purcell et al., 2013). An arbitrary tag sequence probe was also included in the RT-qPCR assay that would bind to a site on the artificial positive control (APC) plasmid to detect any contamination due to plasmid DNA (Snow et al., 2009)

2.5. RT-ddPCR

IHNV was quantified using the QX100™ Droplet Digital™ PCR system (Bio-Rad, Pleasanton, CA). The RT-ddPCR reaction mixture consisted of 10 µl of a 2 × ddPCR supermix (Bio-Rad, USA), each IHNV specific primer and probe at 500 nmol, and 2.5 µl of sample cDNA in a final volume of 20 µl. Each sample was applied in three technical replicate tubes to a final 20 µl reaction volume; copy number of an individual sample was based on the mean of these three replicate measures. The entire reaction mixture was loaded into a disposable plastic cartridge (Bio-Rad, USA) together with 70 µl of droplet generation oil (Bio-Rad, USA) and placed in the droplet generator (Bio-Rad, USA). After processing, the droplets generated from each sample were transferred to a 96-well PCR plate (Bio-Rad, USA). PCR amplification was carried out on the T100 thermal cycler (Bio-Rad, USA) using a thermal profile of beginning at 95 °C for 10 min, followed by 40 cycles of 94 °C for 30 s and 60 °C for 45 s, 1 cycle of 98 °C for 10 min, and ending at 12 °C. After amplification, the plate was loaded on the droplet reader (Bio-Rad, USA) and the droplets from each well of the plate were read automatically at a rate of 32 wells/hour.

The RT-ddPCR data were analyzed with QuantaSoft™ analysis software (Bio-Rad, USA), and presented as the number of copies per µl of RT-ddPCR mixture. The raw quantitative output (RNA copy number in the input sample) was normalized to either IHNV N gene copies/µl (viral supernatant) or copies/g (tissue samples). Positive droplets, containing amplification products, were discriminated from negative droplets by applying a fluorescence amplitude threshold in the QuantaSoft software (Bio-Rad, CA, USA). The threshold was set manually at the highest point of the negative droplet cluster, as visualized using both the fluorescence amplitude vs. event number and the histogram of events vs. amplitude data streams, on the FAM channel. A sample was considered positive by the assay if detectable amplification was observed in at least 2 of 3 technical replicates.

2.6. RT-qPCR

The RT-qPCR assay was performed using a 7500 Fast Real-Time PCR system (Applied Biosystems, CA, USA). Each sample was tested in triplicate in a final 20 µl reaction volume; copy number of an individual sample was based on the mean of these three replicate measures. Each reaction contained 10 µl of 2 × QuantiNova™ (QN; Qiagen, Germany) Probe PCR master Mix, 0.2 µl of QN ROX Reference Dye, each IHNV specific primer and FAM® labeled probe at 500 nmol, the VIC® labeled arbitrary tag sequence probe at 200 nmol, and 2.5 µl of sample cDNA in a final volume of 20 µl. The thermocycler parameters included 2 min at 95 °C, followed by 5 s at 95 °C, 45 s at 60 °C for 40 cycles. The IHNV