Aquaculture 338-341 (2012) 23–28

Contents lists available at SciVerse ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aqua-online

# Validation of high throughput methods for tissue disruption and nucleic acid extraction for ranaviruses (family *Iridoviridae*)

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ARTICLE INFO

*Article history:*
Received 21 November 2011
Received in revised form 12 January 2012
Accepted 12 January 2012
Available online 27 January 2012

*Keywords:*
Automation
Homogenisation
Extraction
DNA virus
Nucleic acid
Iridovirus

ABSTRACT

Viruses in the family *Iridoviridae* cause severe disease and economic loss in a wide range of cultured, wild and ornamental fish species in many countries, justifying the development and validation of diagnostic tests. In an effort to improve the efficiency of test protocols, in this study manual and high throughput semi-automated methods for tissue homogenisation and nucleic acid purification were compared for the detection of *Epizootic Haematopoeitic Necrosis Virus* (EHNV). The effectiveness of these methods at releasing EHNV particles and obtaining DNA was evaluated by virus isolation in bluegill fry (BF-2) cells, enzyme-linked immunosorbent assay (ELISA) and quantitative polymerase chain reaction (qPCR). Samples were prepared using tissue from infected redfin perch (*Perca fluviatilis*). Antigen and DNA yield were greater after homogenisation by bead-beating than by manual grinding of tissues from experimentally infected fish, which contained low quantities of virus. Bead-beating was compatible with virus isolation. There was no difference between the manual and semi-automated methods using samples from naturally infected fish which contained large amounts of virus. There was no difference in DNA yield between manual and semi-automated nucleic acid extraction for experimentally infected fish, however nucleic acid yields were greater after manual extraction for samples from naturally infected fish. Semi-automated tissue homogenisation and nucleic acid extraction required the least amount of time and were the most cost effective. The results of this study can be used as a guide to the selection of sample preparation procedures for other ranaviruses and probably more widely.



## 1. Introduction

*Epizootic Haematopoetic Necrosis Virus* (EHNV) (Langdon, et al., 1986), in the family *Iridoviridae* and genus *Ranavirus*, is one of three endemic iridoviruses in Australia that affect fish, the others being Bohle iridovirus (Speare and Smith, 1992) and lymphocystis virus (Ashburner, 1975; Durham, et al., 1996). As a pathogen notifiable to the Office International des Epizooties (OIE), a range of diagnostic techniques have been developed for EHNV detection from infected fish tissues. Methods include polymerase chain reaction (PCR) assays (Marsh, et al., 2002; Pallister, et al., 2007), antigen capture enzyme-linked immunosorbant assay (ELISA) (Whittington and Steiner, 1993) and virus isolation in fish cell lines (Ariel, et al., 2009; Langdon, et al., 1986; Whittington, et al., 2010). The latter two methods have been fully validated for diagnostic purposes, are described in the Manual of Diagnostic Tests for Aquatic Animals (OIE-World Organisation for Animal Health, 2009) and are used internationally to facilitate trade in aquaculture products. The successful application of these methods requires efficient tissue homogenisation, and for PCR, the recovery of the target viral nucleic acids.

Research to define optimum tissue homogenisation for EHNV was completed in the early 1990s (Whittington and Steiner, 1993). A tube-based tissue grinding method led to a decrease in the turn-around time for obtaining results from laboratory submissions containing large numbers of fish (150–200) compared to more traditional mortar and pestle methods. Advances in technology have led to semi-automated and automated laboratory equipment being used in virological testing, for both tissue homogenisation and viral nucleic acid extraction. These new methods may present practical alternatives to traditional labour-intensive and operator-dependent procedures. Although previous reports identified the value of automated equipment in improving workflow compared to manual methods, the efficiency of tissue homogenisation (Hick, et al., 2010) and quality of extracted nucleic acid vary (Beuselinck, et al., 2005; Dundas, et al., 2008; Hourfar, et al., 2005; Knepp, et al., 2003; Petrich, et al., 2006; Rasmussen, et al., 2009). This may have an effect on the diagnostic accuracy of the particular technique being employed. Therefore before the widespread adoption of such technology a comparative study and validation of protocols is required.

The tissue homogenisation technique of bead-beating employing the FastPrep System (MP Biosciences, USA), designed for high-throughput laboratories, utilises reciprocal motion to disrupt tissues/cells through the beating of robust beads against the sample. Capable of homogenising up to 48 samples simultaneously, the FastPrep

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doi:10.1016/j.aquaculture.2012.01.012

System can be applied to many different types of animal tissue (Arimura, et al., 2005; Jewell, et al., 2005; Klempa, et al., 2005; Scupham, et al., 2006). Similarly, semi-automated platforms incorporating magnetic beads allow extraction of nucleic acids and have the potential to overcome some of the practical problems posed by manual DNA/RNA extraction using phenol/chloroform steps or silica column-based kits. These methods can increase throughput, and reduce contamination rates, labour-inputs, operator-dependent variability and the amount of washed/sterile equipment needed.

Previous studies have analysed the efficiencies of manual homogenisation methods for the recovery of infectious pancreatic necrosis virus (IPNV) (family *Birnaviridae*) from fish (Hedrick, et al., 1986; Smail, et al., 2003) and more recently mechanised and automated homogenisation and nucleic acid extraction methods have been described for preparation of fish tissues for optimal detection of betanodovirus (family *Nodaviridae*) (Hick, et al., 2010). Both IPNV and betanodoviruses possess a ribonucleic acid (RNA) genome and are much smaller in size (60 nm and 30 nm in diameter respectively) than EHNV, which is a large DNA virus 120–200 nm in diameter. The performance of extraction methods may differ depending on the physicochemical properties of the virus. Therefore the objective of this study was to compare traditional manual tissue homogenisation and nucleic acid extraction procedures with semi-automated methods and to use the information obtained to develop a method for optimal tissue disruption and nucleic acid extraction from EHNV-infected fish tissues. Three different tissue homogenisation procedures were evaluated based on the yield of DNA measured by quantitative polymerase chain reaction (qPCR), the amount of antigen assessed by ELISA and infectivity determined on cultured cells. The analysis included samples from naturally and experimentally infected fish and a comparison of time and cost for each homogenisation and nucleic acid extraction procedure. The result was an optimised method for processing samples to determine EHNV infection status suitable for high throughput testing.

## 2. Materials and methods

### 2.1. Viral inoculum

EHNV (strain 86/8774) (Whittington and Steiner, 1993) was propagated in bluegill fry (BF-2) cells with an incubation temperature of 22 °C. End point viral titration was performed in 96-well microtitre plates containing monolayers of BF-2 cells. Virus was serially diluted in 10 fold steps in minimal essential medium (MEM) supplemented with 2% foetal bovine serum and a commercial antibiotic–antimycotic solution (antibiotic–antimycotic 100×, Gibco) (MEM + 2%FBS + 2× antibiotic–antimycotic) to a final dilution of $1{:}10^{-10}$ and inoculated at a rate of 100 μL/well, with eight replicates per dilution. Seven days post inoculation the 50% tissue culture infective dose ($TCID_{50}$) was calculated (Reed and Muench, 1938).

### 2.2. Experimentally infected fish

Two wild adult redfin perch (*Perca fluviatilis*) of mean mass 121.5 ± 2.5 g and mean total length 207.5 ± 2.5 mm were maintained separately in 100 L aquaria at 27 °C with biological filtration. Redfin perch were fed lamb liver three times a week. The fish were anaesthetised in benzocaine (60 mg $L^{-1}$) and 0.1 mL of viral inoculum containing $10^5$ $TCID_{50}$ was administered intraperitoneally. After onset of disease, the liver, spleen and kidney were aseptically dissected from each fish, weighed and stored at −80 °C in separate sterile tubes. Subsequently, liver and spleen were harvested from twenty healthy EHNV-naïve silver perch (*Bidyanus bidyanus*) of mean mass 78.9 ± 3 g and mean total length 186.8 ± 2.2 mm, which were being maintained in a recirculating facility and fed a commercial diet. Fish were euthanised by immersion in benzocaine solution (>100 mg $L^{-1}$).

### 2.3. Naturally infected fish

Four dead wild redfin perch of mean mass 52.7 ± 6.8 g and mean total length 157.5 ± 6 mm were collected from Blowering Dam on the Tumut River, NSW. These fish were part of an opportunistic survey for EHNV in Australian fish. EHNV was detected in each fish by routine cell culture, PCR and restriction endonuclease analysis (REA) (Marsh, et al., 2002).

### 2.4. Sample preparation

To ensure even distribution of virus, liver from experimentally infected redfin perch was diced into cubes and passed through a strainer with a mesh size of 2 mm. This material was diluted by combining 1 g with 3 g of silver perch liver to provide enough material for replication. Aliquots consisting of 0.1 g of combined liver were randomly selected and allocated to six different processing procedures (Table 1). For naturally infected redfin perch 0.1 g liver aliquots were allocated to four of these processing procedures (Table 1). Finally, to assess the compatibility of the bead-beating tissue homogenisation method with virus isolation, aliquots consisting of 0.1 g of naive silver perch liver were spiked with EHNV by the addition of 100 μL of the virus from cell culture from a 10 fold dilution series with a total of four replicates per dilution. Spiked aliquots also were homogenised by bead-beating with the FastPrep System (MP Biosciences, USA) and nucleic acids were extracted using a MagMax-96 Viral Isolation Kit (Ambion, USA) and a MagMax Express-96 magnetic particle processor (Applied Biosystems, USA).

#### 2.4.1. Tissue homogenisation

Clarified tissue homogenates at a dilution rate of one part tissue plus nine parts medium (1:10 W/V) were prepared using three methods as described below. The supernatant was retained for analysis for each method.

*2.4.1.1. Tube with a fitted pestle.* This method was modified from Whittington and Steiner (1993). In a 1.5 mL tube liver was roughly minced with fine scissors followed by grinding with a fitted pestle. Phosphate buffered saline (PBS) (0.9 mL) was added to give a 1:10 w/v tissue homogenate and stored at 4 °C. Tissue homogenates were vortexed for 20 s every 30 min for 2 h following the addition of three 3 mm diameter glass beads. For clarification tubes were centrifuged at 900×*g* for 10 min in a microcentrifuge at room temperature.

*2.4.1.2. Mortar and pestle.* Tissue homogenates were prepared by grinding liver with 0.9 mL homogenising medium (MEM + 2%FBS +

**Table 1**
Tissue homogenisation and nucleic acid extraction procedures performed on liver aliquots. Initial values are for aliquots derived from experimentally infected redfin perch (*Perca fluviatilis*) and data in parenthesis are for aliquots derived from naturally infected redfin perch.

| Procedure | Homogenisation method | Nucleic acid extraction method | Number of samples tested by qPCR | Number of samples tested by ELISA |
|---|---|---|---|---|
| 1 | TP[a] | SC[b] | 12 | 6 |
| 2 | TP | MB[c] | 12 | 6 |
| 3 | MP[d] | SC | 12 (10) | 6 (5) |
| 4 | MP | MB | 12 (10) | 6 (5) |
| 5 | BB[e] | SC | 12 (10) | 6 (5) |
| 6 | BB | MB | 12 (10) | 6 (5) |

[a] TP = tube with a fitted pestle.
[b] SC = spin column (High Pure Viral Nucleic Acid Kit).
[c] MB = magnetic beads (MagMax Express-96).
[d] MP = mortar and pestle.
[e] BB = bead-beating with FastPrep system.