Aquaculture 432 (2014) 53–59

Contents lists available at ScienceDirect

Aquaculture

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

Incursions of *Cyprinid herpesvirus 2* in goldfish populations in Australia despite quarantine practices

Joy A. Becker [a,*], Alison Tweedie [a], Anneke Rimmer [a], Matthew Landos [a,b], Mark Lintermans [c], Richard J. Whittington [a]

[a] Faculty of Veterinary Science, University of Sydney, Camden, NSW, 2570, Australia
[b] Future Fisheries Veterinary Service Pty Ltd, Lennox Head NSW 2478, Australia
[c] Institute for Applied Ecology, University of Canberra, ACT 2601, Australia

ARTICLE INFO

Article history:
Received 13 December 2013
Received in revised form 10 April 2014
Accepted 15 April 2014
Available online 24 April 2014

Keywords:
Herpes
Virus
Goldfish
Biosecurity
Quarantine policy
Wild fish

ABSTRACT

The international trade in ornamental fish is considered a major factor for the transboundary spread of aquatic pathogens that can affect both wild and farmed fish populations. Nearly 18 million ornamental fish were imported into Australia in 2007, including approximately 3.9 million goldfish. Despite quarantine regulations during importation, there have been several incidents in Australia where exotic pathogens from ornamental fish have become established in farmed or free-living fish species. The exotic virus *Cyprinid herpesvirus 2* (CyHV2) was first found in Australia in 2003 in goldfish, suggesting that sub-clinically infected goldfish were passing through quarantine regardless of health certification and three weeks of quarantine. Repeated cross sectional surveys were conducted to determine whether CyHV2 has already established in farmed or wild ornamental fish in Australia. Goldfish populations were tested to OIE standard to detect 2% prevalence with 95% confidence assuming a test of 100% sensitivity and specificity. CyHV2 was found at retail outlets, farms and in several populations of wild goldfish in the ACT and Victoria. The prevalence and moderate to high viral loads in sub-clinically infected goldfish from different domestic populations suggested the introduction was not a recent event. This study demonstrated that CyHV2 has established in Australia and informed quarantine policy to revoke the requirement for goldfish exported to Australia to be certified free of CyHV2. The results provided clear evidence that an aquatic pathogen from imported ornamental fish can become established in farmed and wild populations. This is of particular significance to Australia as there are many endemic and ecologically sensitive populations of fish that may be severely affected by exotic pathogens. The incursion of CyHV2 in Australia should be considered a case study to inform pathway analysis for pathogen establishment.

1. Introduction

The international trade in ornamental pet fish is an efficient system for the translocation of aquatic pathogens (Peeler and Feist, 2011; Whittington and Chong, 2007). In Australia, around 18 million ornamental fish are imported each year, including approximately 4 million goldfish (*Carassius auratus*) (O'Sullivan et al., 2008). A further 7 million goldfish were sold from domestic breeders to supply the pet fish industry (O'Sullivan et al., 2008). Australia is one of a handful of countries in the world with stringent import controls for ornamental fish (Whittington and Chong, 2007). Controls include health certification at export premises and upon arrival, fish are subject to a visual health inspection and a quarantine period of 7 to 21 days depending on species. Despite quarantine regulations during importation, there have been several incidents in Australia where exotic pathogens from ornamental fish have affected wild and farmed populations (Lancaster et al., 2003; Stephens et al., 2004; Whittington et al., 1987). In particular, the international trade in goldfish has led to the establishment of significant pathogens with potential to affect aquaculture including atypical *Aeromonas salmonicida* responsible for goldfish ulcer disease (Trust et al., 1980) and *Cyprinid herpesvirus 2* (CyHV2) (Stephens et al., 2004), which was the focus of this study.

In general, herpesviruses are characterized by a high level of host specificity and infections are often persistent with few or no clinical signs (Hanson et al., 2011). The three species, *Cyprinid herpesvirus 1*, *Cyprinid herpesvirus 2*, and *Cyprinid herpesvirus 3* belonging to the genus *Cyprinivirus* are associated with both chronic infections and mass mortality events in common carp (*Cyprinus carpio*) or goldfish (Davison et al., 2013). Historically, the host species for CyHV1 and CyHV3 were considered to be common carp and koi carp (a variety of *Cyprinus carpio*), while CyHV2 was thought to be a pathogen of goldfish. It is now known that the three cyprinid herpesviruses are able to infect a much wider range of cyprinid species than previously assumed (Bergmann et al., 2010; Fichi et al., 2013; Hedrick et al., 2006). Crucian carp (*Carassius carassius*) (Fichi et al., 2013) and Prussian carp

* Corresponding author. Tel.: +61 2 9036 7731; fax: +61 2 9351 1618.
E-mail address: joy.becker@sydney.edu.au (J.A. Becker).

http://dx.doi.org/10.1016/j.aquaculture.2014.04.020
0044-8486/

(*C. gibelio*) are susceptible to infection with CyHV2 with the latter species showing haematopoietic necrosis resulting in mortality (Danek et al., 2012; Luo et al., 2013). CyHV3 can infect goldfish (Sadler et al., 2008), grass carp (*Ctenopharyngodon idella*), ide (*Leuciscus idus*) and ornamental catfish (*Ancistrus* sp.) (OIE, 2012). This is of importance to cyprinid aquaculture, which represents 40% of global production of aquatic animals with 24.2 million tonnes produced in 2010 (Food and Agriculture Organization of the United Nations, 2012). The international trade in ornamental koi carp and goldfish may facilitate the spread of these viruses without detection potentially placing local food fish production systems at risk.

First indentified in Japan in 1992, CyHV2 has caused mortality events in farmed goldfish in Taiwan (Chang et al., 1999), USA (Goodwin et al., 2006b), UK (Jeffery et al., 2007) and Australia (Stephens et al., 2004). Outbreaks of CyHV2 are often associated with high mortality in all age classes and typically occur in spring and autumn or during handling events with sudden drops in temperature, such as shipping and holding of goldfish at wholesalers (Davison et al., 2013; Goodwin et al., 2009). The virus is difficult to isolate in cell culture (Jung and Miyazaki, 1995) and laboratory methods demonstrating continual cultivation have only recently been published (Ito et al., 2013). Several molecular-based assays have been developed to identify infected fish (Goodwin et al., 2006a; Waltzek et al., 2009). The intensity of CyHV2 infection in apparently healthy goldfish can be as high as 10^7 to 10^9 copies μg^{-1} host DNA and was commonly found to be in the range of 10^3 to 10^5 copies (Goodwin et al., 2009).

In Australia, CyHV2 was first identified in 2003 at a goldfish farm in Western Australia (Stephens et al., 2004). It was suggested that sub-clinically infected goldfish were passing through quarantine and coming into contact with domestic stocks through live fish trading (Stephens et al., 2004). An opportunistic survey of sick or moribund fish collected from retail outlets in Sydney revealed that CyHV2 was present in 17% of goldfish (Whittington et al., 2009). However, since retail outlets contained an assortment of imported and domestic fish sharing the same environment, the source of the virus could not be identified. Therefore, the objective was to ascertain if CyHV2 was indeed passing through quarantine undetected and if so, to determine whether the virus has become established in domestic stocks.

2. Materials and methods

2.1. Selection of the goldfish populations and animals

The study involved repeated cross sectional surveys of farmed and wild goldfish populations in Australia. Also, we conducted opportunistic surveys of retail outlets located in New South Wales (NSW) and wild goldfish from Victoria (VIC). For the cross sectional surveys, a sample size was set at 150 individuals to detect 2% prevalence with 95% confidence assuming a test of 100% sensitivity and specificity. For all surveys, populations were defined as groups of goldfish that were in close contact (e.g. sharing water, same farm location) and were collected at the same time or within the same season. Where possible, samples were collected four times each year to account for possible seasonal variations in prevalence of infection in order to increase the likelihood of detecting infections.

Four goldfish farms were involved in the survey, all of which used outdoor pond or tank-based rearing facilities (Table 2).These farms supply over 90% of the domestic production (by quantity) of goldfish to wholesale and retail outlets in Australia. For each collection at Farm 1, 30 ponds were selected using a table of random numbers and then five fish were collected using a dip net at the edge of the pond. This sampling strategy was used for two collections at Farm 2. The other two collections at Farm 2 consisted of between four and seven fish collected from 37 and 21 outdoor tanks chosen from a table of random numbers. Farm 3 consisted of many small cages held within three large ponds (Table 2). Three to five fish were collected by dip net from all cages in each pond to reach the sample size of at least 150. At Farm 4, five out of 12 ponds were selected using a table of random numbers and approximately 25 fish were collected by dip net from each one.

Wild fish were collected opportunistically by means of electrofishing and netting techniques in areas of known feral goldfish populations in the Australian Capital Territory (ACT) for the cross sectional survey. Wild goldfish from Victoria were opportunistically collected during annual native fish monitoring programs by fisheries biologists.

Retail outlets located in NSW with a prior relationship with Future Fisheries Veterinary Service Pty Ltd were visited on two occasions and asked to participate in the opportunistic survey. Three of eight retail outlets agreed to participate and periodically placed dead or moribund goldfish in a freezer for collection and testing for CyHV2.

2.2. Detection and Confirmation of CyHV2

Upon death, fish were kept on ice or immediately frozen, transported to the laboratory and stored at −80 °C until the time of processing. Kidney, liver and spleen were dissected from each fish using aseptic techniques. The tissue was homogenized and clarified by bead beating followed by centrifugation as described by Rimmer et al. (2012). Nucleic acids were extracted from a 50 μl aliquot of the clarified tissue homogenate (1:10 w/v) of individual fish using a MagMax-96 viral isolation kit (Ambion, USA) according to manufacturer's instructions.

A quantitative polymerase chain reaction (qPCR) assay targeting a specific sequence of the DNA polymerase (DNApol) gene of CyHV2 was used to detect the virus (Rimmer et al., 2012). Briefly, amplification was performed in 25 μl reactions containing 5 μl of template DNA, 0.125 μl of forward primer and reverse primer (100 pmol/μl), 12.5 μl of Quantitect SYBR Green Master Mix (Qiagen) and 7.25 μl of molecular grade water. The primers C1153 and C1154 were used for the initial screening assay and were designed to exclude detection of CyHV3 (Table 1). Thermocycling was performed on a MX3000 Multiplex Quantitative PCR System (Stratagene) with reaction conditions: hotstart activation and denaturation at 95 °C for 15 min, followed by 40 cycles of 95 °C denaturation for 30 s, 62 °C annealing for 30 s and 72 °C extension

Table 1
Description of the primers used in this study to detect CyHV2.

Oligonucleotide		Product size (base pairs)	Gene	Reference
Name	Sequence (5'–3')			
C1153	GTCGGTTGGACTCGGTTTGT	121	DNAPol[1]	Whittington et al., 2009
C1154	CATAGTACGCCTTGAGACTC			
C1109	CCCAGCAACATGTGCGACGG	401	DNAPol[1]	Whittington et al., 2009
C1158	GACCAAGTAGTCAGATGCTG			
C1281	GGACTTGCGAAGAGTTTGATTTCTAC	366	Helicase	Waltzek et al., 2009
C1282	CCATAGTCACCATCGTCTCATC			

[1] DNAPol = DNA polymerase.