Aquaculture 420–421 (2014) 288–294

Contents lists available at ScienceDirect

Aquaculture

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

Ivermectin blocks the nuclear location signal of parvoviruses in crayfish, *Cherax quadricarinatus*

Kim Y. Nguyen [a], Kitikarn Sakuna [a,b], Robert Kinobe [c], Leigh Owens [a,*]

[a] Microbiology and Immunology, School of Veterinary and Biomedical Sciences, James Cook University, Townsville 4811, Queensland, Australia
[b] Faculty of Veterinary Science, Rajamangala University of Technology, Srivijaya 80240, Thailand
[c] Physiology and Pharmacology, School of Veterinary and Biomedical Sciences, James Cook University, Townsville 4811, Queensland, Australia

ARTICLE INFO

Article history:
Received 23 October 2013
Received in revised form 15 November 2013
Accepted 18 November 2013
Available online 27 November 2013

Keywords:
Ivermectin
Nuclear location signal
Parvoviruses
Crayfish
Cherax quadricarinatus

ABSTRACT

Parvoviruses have been responsible for major problems in the shrimp aquaculture for decades with few options for control apart from avoidance. As intranuclear viruses for some of their replication, parvoviruses need to use the cell's nuclear transport signals for entry into the nucleus. This study was conducted to see if ivermectin which has recently been shown to block importins *in vitro* would do so against two presumptive parvoviruses in a freshwater crayfish, *Cherax quadricarinatus*, model. Crayfish were shown to tolerate ivermectin at 7 μg/kg injected intramuscularly and survival appeared to be enhanced with increasing dose ($P \leq 0.1$). Ivermectin dramatically decreased hypertrophied nuclei caused by presumptive gill parvovirus by ~68% ($P \leq 0.001$) after 2 doses of 7 μg/kg reducing from 1591 to 505 affected cells in the gills. The reduction did not increase further with increasing doses. Also, ivermectin appeared to increase the survival of crayfish when challenged with *C. quadricarinatus* parvo-like virus (CqPV) to levels statistically equivalent to non-infected crayfish but did not appear to affect the number of viral infected cells. There was a negative correlation between the size of crayfish and their longevity ($P \leq 0.05$, $R^2 = 0.15$) with smaller crayfish dying faster when challenged with CqPV. This is the first *in vivo* testing of ivermectin against viruses and showed that ivermectins do dramatically block some parvoviruses, possibly by interactions with cellular importins. There may be a therapeutic role for ivermectins in viral reduction in broodstock in crustacean aquaculture.

1. Introduction

The penaeid parvoviruses *Penaeus monodon* Densovirus (PmonDNV, colloquially known as HPV) and *Penaeus stylirostris* Brevidensovirus (PstBNV, colloquially known as IHHNV) cause many disease issues in penaeids (see reviews Safeena et al., 2010 and Rai et al., 2012). Indeed parvoviruses cause major diseases in many animals including humans, dogs, cats, mink, pigs, cattle, crustaceans and insects. Parvoviruses are intranuclear in their replication and they need rapidly dividing cells in the S-phase to access the cellular DNA replication enzymes. Thus the parvovirus needs to transport their proteins into the nucleus using the cell's nuclear importing molecules, karyopherin also called importin, IMPα/β linked to their nuclear location sequences or signals (NLS). Recently, Owens (2013) identified many possible signals in these penaeid parvoviruses and indeed this current study was spawned from that analysis.

Recently, ivermectin and mifepristone were reported to have potent antiviral activity *in vitro* (Wagstaff et al., 2011, 2012) by preventing active nuclear transport of the integrase molecule of human immunodeficiency virus (HIV)-1. Mifepristone is a specific inhibitor of the nuclear import of the protein integrase, but ivermectin appears to act on IMPα/β-mediated nuclear import generally. This raises the intriguing possibility that ivermectin could be an anti-parvoviral agent if parvoviruses use IMPα/β to transit into the nucleus.

Ivermectin is an effective antiparasiticide used widely on animal farms including aquaculture against parasites such as sea lice *Lepeophtheirus salmonis* and *Caligus elongatus* (Davies and Rodger, 2000) and metacercariae of *Clinostomum marginatum* (Lorio, 1989).

Crustaceans are very sensitive to ivermectin. Loss of action potential in the neuron, loss of motor function and eventual paralysis from avermectin in the brine shrimp *Artemia salina*, which contains neurotransmitter gamma-aminobutyric acid (GABA) receptors (Calcott and Fatig, 1984), have been documented. The mysid shrimp, *Mysidopsis bahia*, was sensitive at 96 h LC_{50} 0.022 μg/l (Wislocki et al., 1989), whilst the no-observed effect concentration (NOEC) was 4 ng/l, but the 96 h LC_{50} for pink shrimp *Penaeus duorarum* was 1.6 μg/l. The mysid, *Neomysis integer* showed a 96 h LC_{50} of 70 (44–96, 95% CI) ng/l, when immersed (Davies et al., 1997). Through the digestive tract of shrimp *Crangon septemspinosa*, ivermectin was toxic but not *via* the gills (Burridge and Haya, 1993). Shrimp could tolerate ivermectin in water at the maximum concentration 21.5 μg/l, but ivermectin was lethal at 96 h LC_{50} = 8.5 μg ivermectin/g of food. The shrimp's average weight was 2.76 g, and the feeding rate was 1% body weight per day. The

* Corresponding author.
E-mail address: leigh.owens@jcu.edu.au (L. Owens).

0044-8486/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.aquaculture.2013.11.022

NOEC was approximately 2.6 μg/g of food (Burridge and Haya, 1993). Given that ivermectin was toxic in crustacea but reported concentrations varied widely, it was necessary to examine the effect of concentrations of ivermectin in redclaw crayfish, *Cherax quadricarinatus*, the proposed crustacean model.

Two parvovirus-like cellular changes reported in *C. quadricarinatus* were used to investigate whether ivermectin could block their NLS. Gill parvovirus of *C. quadricarinatus* (Edgerton et al., 2000), herein called gill parvovirus, and parvo-like virus of *C. quadricarinatus* (Bowater et al., 2002) herein called CqPV were tested. Gill parvovirus produces signet-ring, hypertrophic nuclei in the gills without an inclusion body and is a mildly pathogenic, pre-existing cellular change in a population of crayfish (Rusaini et al., 2013) (Fig. 1a). CqPV produces basophilic, Cowdry type A intranuclear inclusions in the gills (Fig. 1b) and other systemic tissues, causes heavy mortality and infective tissue is available, so the virus can be administered on demand for experimentation. The aim was to see if ivermectin could influence the course of either parvo-like virus infection.

The study was designed in three parts. Part 1 was to establish concentrations of ivermectin that the crayfish would tolerate. Part 2 was to see if ivermectin at concentrations established from Part 1 could change the course of a weakly pathogenic gill parvovirus cellular change. Part 3 was to see if ivermectin could change the course of the highly pathogenic CqPV.

2. Materials and methods

2.1. Shared protocols: experimental animals

Grossly healthy crayfish (9–150 g) were obtained from commercial crayfish farms in northern Queensland and transported to the Aquatic Infectious Disease Facility (Fish Laboratory) of School of Veterinary and Biomedical Sciences (SVBS), James Cook University. The availability of crayfish was extremely limited and whatever crayfish were available had to be taken; thus size range was greater than desirable. During transporting, crayfish were kept within two layers of wet cloth in the Styrofoam boxes to decrease the temperature and activity of crayfish. Crayfish were maintained in 1000 l tanks with a recirculating biofilter system and aerator. Four days prior to commencement of the study, crayfish were divided into small groups of two or three crayfish to reduce cannibalism and kept in 50 l dark blue tanks on racks in a recirculating system at 26 ± 2 °C. All experimental crayfish were fed and monitored daily.

As there were tank/space constraints and a requirement by the ethics committee to limit the number of crayfish involved in experiments, crayfish were assigned randomly to treatments and treatments were assigned randomly to tanks. This meant that a perfect balance of the weight of crayfish in treatments and the replication of the treatment themselves were sacrificed to best fit the random design to maximise statistical power.

In this proof of concept stage, it was decided to deliver the potentially toxic ivermectin *via* injection to ensure all crayfish received the exact dose relative to their weight. All injections were performed using sterile 1 ml syringes and 26-gauge needles, and were discarded after each inoculation to minimise cross-infections.

2.2. Part 1. Tolerance to ivermectin; experimental design

After four days of acclimation, crayfish (64.0 ± 22.0 g) were divided into four groups (20 crayfish each): groups I, II, and III were intramuscularly injected with 0.6, 1.2, and 1.4 μg/ml ivermectin (IVOMEC Antiparasitic Injection for Cattle 200 ml, ivermectin 1% injectable, Vet-n-Pet Direct) solutions to make up doses of 3, 6, and 7 μg/kg, respectively and group IV controls were intramuscularly injected with the homogenising solution of phosphate buffered saline (PBS) and Tween 20 at a concentration of 5%. Each inoculum was divided into three portions and injected intramuscularly into the ventral side of the first, second and third abdominal segments of crayfish, away from the ventral nerve cord. The number of crayfish used for each treatment was 5, divided randomly either 2 or 3 crayfish/tank and dose treatments were randomised (Table 1). Each treatment group was spread randomly over 4 different racks. Thus, the total number of crayfish used for Part 1 was 80 (Table 2). The duration of observation between each injection was 20 days. This experiment was carried out with three injections (at days 1, 21 and 41) and terminated at 60 days.

2.3. Part 2. Effect of ivermectin on a pre-existing parvovirus

The concentration of ivermectin determined to have no toxic effect was the high dose (7 μg/kg) (see Results section) and therefore it was the therapeutic dose for testing of inhibition of the viral nuclear transportation.

2.3.1. Experimental animals

Crayfish were obtained from SVBS, JCU (Rusaini et al., 2013) and were affected by putative gill parvovirus (Edgerton et al., 2000). Five crayfish were examined by histopathology and all were heavily infected (Rusaini et al., 2013), thus it was assumed most crayfish would be infected and randomisation would be sufficient to limit vagaries in load. The weight of crayfish ranged from 9 to 45 g.

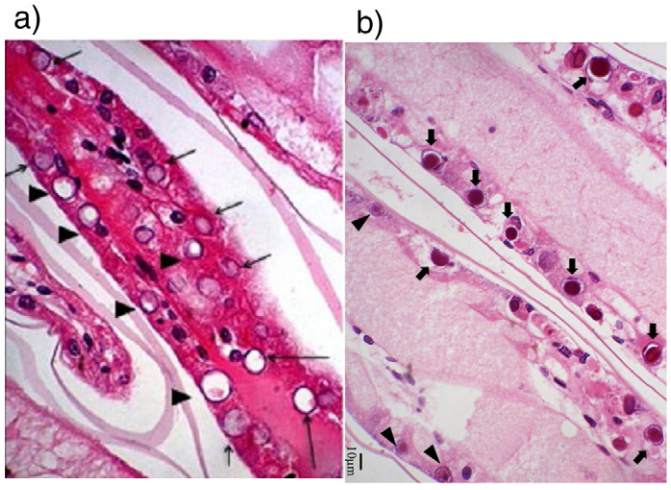

Fig. 1. (a). Different stages of hypertrophied nuclei caused by a putative parvovirus in gill of crayfish *Cherax quadricarinatus*. Early stage (short arrow), middle stage (arrowhead), and fully developed stage (long arrow). (b). The late-stage intranuclear inclusion bodies (INIBs) (fat arrows) and early-stage INIBs (narrow arrowheads) of *Cherax quadricarinatus* parvo-like virus observed in gill cells of crayfish. H&E. Scale bar = 10 μm.

Table 1
Part 1 (n); Part 2. A randomised design for examining the tolerance of crayfish to ivermectin (Part 1), and for testing the effect of ivermectin on pre-existing parvovirus infection in crayfish (Part 2). In Part 1, the first number is the dose of ivermectin in μg/kg. The numbers in parentheses ( ) indicate the number of crayfish in different tanks. In Part 2, the number after the semicolon is the number of doses of 7 μg/kg given to each crayfish. nc = no crayfish in Part 2.

Rack 1: (rows & columns)			Rack 2: (rows & columns)		
0 (2); 0	3 (2); 3	6 (2); 2	7 (2); 1	0 (3); 0	3 (3); 3
7 (3); 1	0 (3); 0	3 (3); 3	6 (3); 2	7 (2); 1	0 (2); 0
7 (2); 2	0 (2); nc	Filter	0 (3); 3	3 (3); nc	Filter
Rack 3: (rows & columns)			Rack 4: (rows & columns)		
6 (3); 2	7 (3); 1	0 (2); 0	3 (2); nc	6 (2); nc	7 (2); nc
3 (2); 3	6 (2); 2	7 (3); 1	0 (3); nc	3 (3); nc	6 (3); nc
6 (2); nc	7 (3); nc	Filter	3 (2); nc	6 (3); nc	Filter