Aquaculture 334–337 (2012) 39–44

Contents lists available at SciVerse ScienceDirect

Aquaculture

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

In vitro and *in vivo* efficacy of anthelmintic compounds against blood fluke (*Cardicola forsteri*)

Paul Hardy-Smith [a,1], David Ellis [b,c,1], John Humphrey [a], Mathew Evans [c], Daryl Evans [c], Kirsten Rough [b], Victoria Valdenegro [c], Barbara Nowak [c,*]

[a] *Panaquatic® Health Solutions Pty Ltd, 26A Liddiard Street, Hawthorn, Victoria, 3122, Australia*
[b] *ASBTIA Australian Southern Bluefin Tuna Industry Association, PO Box 1146, Port Lincoln, South Australia 5606, Australia*
[c] *NCMCRS, University of Tasmania, Locked Bag 1370, Launceston 7250 Tasmania, Australia*

ARTICLE INFO

Article history:
Received 22 September 2011
Received in revised form 19 December 2011
Accepted 20 December 2011
Available online 30 December 2011

Keywords:
Southern Bluefin Tuna
Ranching
Fish parasite
Treatment

ABSTRACT

Blood fluke, *Cardicola forsteri*, infects Southern Bluefin Tuna, particularly during ranching. Efficacy of four anthelmintics was tested against this parasite. There was an agreement between *in vitro* and *in vivo* results. Praziquantel was the only effective anthelmintic. It was the most potent anthelmintic in decreasing fluke responsiveness *in vitro*, with concentrations ranging between 1.5 μg/mL and 200 μg/mL stopping adult fluke response within less than 5 min. *In vivo*, both the higher (150 mg/kg) and the lower (75 mg/kg) dose praziquantel treatment resulted in a significant reduction of the number of flukes present in the hearts. A significant effect of treatment on the mean number of blood fluke eggs per cm^2 of tuna myocardium was observed, with fish treated with either of the two doses of praziquantel having at least 6 times lower numbers of eggs in their hearts. Control fish and fish treated with praziquantel (both doses) and lower dose Closal had very low average number of eggs per cm^2 of gill and were significantly lower than in fish treated with fenbendazole. While this research shows that praziquantel is the treatment of choice against blood fluke, *C. forsteri*, further research is needed to determine optimum dose, best treatment application method, palatability and any potential side effects.

1. Introduction

Ranching of Southern Bluefin Tuna (SBT) *Thunnus maccoyii* commenced in Australia in 1991 as a result of value adding to the wild catch of SBT. Since then SBT ranching has developed into the second most productive (both by volume and value) aquaculture finfish industry in Australia. The industry is based on the capture of wild fish which are conditioned over a few months and sold principally to the Japanese sashimi market (Farwell, 2001). The fish are towed in pontoons from the capture site in the Great Australian Bight to ranching sites near Port Lincoln, South Australia. After approximately 2–9 months fish are harvested and marketed both domestically and internationally (Aiken et al., 2006; Colquitt et al., 2001). There are few health problems during ranching, possibly due to the age of the fish at capture and the short ranching time (Deveney et al., 2005; Nowak, 2004).

Blood fluke, *Cardicola forsteri* (Trematoda Aporocotylidae) was described from ranched SBT (Cribb et al., 2000), which is the final host, while a marine polychaete *Longicarpus modestus* is the intermediate host (Cribb et al., 2011). This parasite mostly infects SBT during ranching as the intensity and prevalence of infections are very low in the wild (Aiken et al., 2007), and at transfer (Aiken et al., 2006), and an antibody response against *C. forsteri* increases only after transfer to the ranching zone from the wild (Aiken et al., 2008). Epizootics of this blood fluke have been reported in ranched SBT (Aiken et al., 2006, 2008, 2009; Colquitt et al., 2001; Cribb et al., 2000; Dennis et al., 2011; Kirchhoff et al., 2011) with the prevalence of infection reaching 100% within 2 months post-transfer to ranching pontoon (Aiken et al., 2006; Kirchhoff et al., 2011). Although in the past infections with *C. forsteri* did not appear to result in clinical disease in the host (Aiken et al., 2006, 2008; Colquitt et al., 2001), more recently an increase in the intensity of infection was reported, which coincided with peaks of tuna mortalities (Dennis et al., 2011; Hayward et al., 2010). Based on the association between the increased intensity of *C. forsteri* and the mortalities in SBT, clinical trials to investigate the efficacy of anthelmintics in the treatment of *C. forsteri* were undertaken.

Anthelmintics have been used to treat farmed fish species against monogeneans, digeneans and cestodes. A number of different classes of these compounds were considered as potential candidates for these trials including an isoquinolone, three benzimidazoles and a salicylanilide. Praziquantel, an isoquinolone, causes spastic paralysis in trematodes (flukes) as well as damaging the parasite tegument (Taylor et al., 2007). Praziquantel has been used in fish against

* Corresponding author. Tel.: +61 3 63243814; fax: +61 3 63243804.
E-mail address: B.Nowak@utas.edu.au (B. Nowak).
[1] These authors contributed equally to this paper.

0044-8486/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
doi:10.1016/j.aquaculture.2011.12.037

monogeneans (Schmahl and Mehlhorn, 1985; Sharp et al., 2004; Sitjà-Bobadilla et al., 2006; Williams et al., 2007), digeneans (Björklund and Bylund, 1987) and intestinal cestodes (Sanmartin Duran et al., 1989). Importantly, it is the drug of choice to treat schistosomiasis in man (Doenhoff et al., 2008). Albendazole, fenbendazole and triclabendazole are all benzimidazoles, a class of anthelmintic considered generally to be safe compounds which are used widely in livestock (Taylor et al., 2007). Closantel, a salicylanilide, was also considered for these trials. Albendazole, fenbendazole, triclabendazole and tetramisole have been tested as a treatment against a wide range of fish parasites, triclabendazole and fenbendazole were effective against monogeneans, albendazole was effective against flagellate *Hexamita salmonis* infection in rainbow trout (Tojo and Santamarina, 1998a, 1998b, 1998c; Tojo et al., 1992).

The aim of this study was to determine the *in vitro* and *in vivo* efficacy of anthelminthic drugs against *C. forsteri* infection of SBT and to assess the reliability of *in vitro* screening.

2. Materials and methods

2.1. In vitro trial

2.1.1. Heart processing and live fluke isolation

Up to 150 hearts were collected and stored in a large icebag during SBT commercial harvests. Hearts were dissected lengthwise from apex to base and 3–5 cuts were made in the internal surface to increase the flushing area. Ten to fifteen hearts were placed in individual 2 L bags, along with any remaining blood and approximately 300 mL of saline solution was added to the bags, which were then shaken. Heart flushes and blood were then poured into 500 mL plastic containers and allowed to settle for 20 min. Supernatant was removed and the remaining liquid (approximately 60 mL) decanted into 4–12 Petri dishes, diluted with saline and left to settle for 2 min. Live *C. forsteri* were observed using a dissecting microscope and transferred to a storage solution (saline solution + 25 μg/mL gentamicin sulphate) for the duration of fluke isolation.

2.1.2. Live fluke preparation and testing

To assess toxicity of anthelmintics to flukes, the effect of different concentrations of the tested compounds on responsiveness of the adult flukes was determined over time. *C. forsteri* were deemed responsive if any movement was observed following 3–5 s of manual plate shaking. Live *C. forsteri* were isolated from hearts and transferred with a pipette in 100 μL aliquots from the storage solution into a 12-well plate, separated into groups of 10–20 flukes and washed three times in 2 mL Dulbecco's Modified Eagle's Medium (DMEM). The plates were then incubated at 20 °C for 1 h. After 1 h any nonresponsive individuals were removed from the test. Remaining live flukes were re-incubated under the same conditions and any flukes that seemed to have lost responsiveness were replaced with responsive flukes.

Toxicity testing was undertaken in 3 mL DMEM containing 25 μg/mL gentamicin sulphate per well. This medium was chosen on the basis of *in vitro* models for helminths, particularly schistosomes (Keiser, 2010) and our own experience with maintaining *C. forsteri* adults *in vitro* (F. Van Ede, P. Crosbie and B. Nowak unpublished). While the use of blood or blood serum has been suggested, it is not recommended for *in vitro* studies (Keiser, 2010), most likely as it would introduce another source of variability into multiple *in vitro* experiments. For each anthelmintic, multiple concentrations were assessed to determine their effects on fluke responsiveness, with each concentration having 3 replicate wells, and each replicate well containing 5 flukes, which were responsive at the start of the experiment. Wells were assigned randomly to the anthelmintic concentrations, and 10 μL of each drug concentration was added to wells. Four different anthelmintics were tested: praziquantel, fenbendazole, tetramisole chloride and closantel (Table 1). Depending on the treatment, 10 μL of dimethyl sulfoxide (DMSO) or distilled H_2O was added to control wells. All reagents were purchased from Sigma Aldrich Pty Ltd. The commercial praziquantel preparation is a racemate composed of equal parts of "levo" R(−) and "dextro" S(+) isomers (Cioli and Pica-Mattoccia, 2003). Pilot experiment showed that 0.3% DMSO had no effect on responsiveness of the blood flukes for at least 50 h (results not shown). Plates were incubated at 20 °C, and regularly checked for the number of responsive flukes. The final time point for assessment of the effect of treatment was 48 h.

2.2. In vivo treatment

2.2.1. Anthelmintics and experimental doses

Four compounds, Prazifish (All Farm Animal Health), Closal® (Coopers Animal Health), Panacur 100® (Intervet Schering Plough Animal Health) and Fasinex 240® (Novartis Animal Health), were used individually and in combination in this trial. Two compounds (Prazifishl and Closal®) were used at two different dose rates. The dose given to each SBT under each treatment regime is shown in Table 2. Treatment was timed to coincide with increasing fluke burdens in the SBT and prior to the mortality peak observed approximately 6–8 weeks post transfer to the grow out cage. SBT were treated 27 days after transfer from the towing pontoon to the ranching pontoon and a total of 63 days post capture. All treatments were administered as a single dose to a total of twenty SBT for each treatment group.

None of these compounds are registered in Australia for treatment of fish destined for human consumption. Each treatment was administered by a registered veterinarian. In addition, a license condition of aquaculture farms in South Australia is that any use of an unregistered compound, even under veterinary direction, must be given Ministerial approval.

2.2.2. Experimental fish and administration of anthelmintic

Individual fish were caught using a baited barbless hook and handline and placed in a padded capture cradle. A moistened towel was placed over the eyes of the SBT. The length of the SBT was measured and two identification tags were inserted. A Kruuse No. 26 Small Animal plastic stomach tube was used for stomach tubing. Prior to insertion a fiberglass stylet was inserted into the tube to provide increased rigidity. A small amount of vegetable oil was then applied to the tip of the tube and the tube inserted through the mouth using a piece of hard PVC piping (the "bite pipe") to protect the softer stomach tube from the teeth of the SBT. Once the tip of the stomach tube had entered the oesophagus the fibreglass stylet was withdrawn as the tube was advanced into the stomach. Location of the tube within the stomach was confirmed by stomach ingesta tracking back out of the tube. The syringe containing the appropriate concentration of anthelmintic was then attached to the end of the stomach tube and the treatment administered. A further 10–15 ml of diluted saline was then flushed through the stomach tube to ensure no treatment was left within the dead space of the stomach tube. The stomach tube near where the syringe attached was then crimped and the tube withdrawn back out through the "bite pipe". The fish was then released into the treatment pontoon. Tuna were medicated based on their individual size at the dose rates shown in Table 2. Mortalities were monitored post treatment. Twenty five percent of fish were not recovered at the end of the trial, with the loss attributed to a combination of mortality, poaching and/or predation by seals.

2.2.3. Sample collection

Twenty four days after treatment, all SBT remaining in the treatment pontoon were euthanised. The treatment to which the fish was assigned was identified using the tags inserted at the time of treatment. Immediately after euthanasia, a sample of gill filaments