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Efficacy of praziquantel and a combination anthelmintic (Adecto®) in bath treatments against *Tagia ecuadori* and *Neobenedenia melleni* (Monogenea), parasites of bullseye puffer fish

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ABSTRACT

Diclidophoridae and Capsalidae are families of monogenean parasites that include some of the most pathogenic species for cultured finfish. In this study, the efficacy of praziquantel (PZQ) and a combination anthelmintic (PZQ, ivermectin, pyrantel pamoate and fenbendazole) commercialized under the name of Adecto® (Adler Pharma) were evaluated *in vitro* against adults and eggs of the diclidophorid *Tagia ecuadori* and the capsalid *Neobenedenia melleni*. Freshwater and formalin immersions were also evaluated against *T. ecuadori* for comparative purposes. In addition, the efficacy *in vivo* of Adecto® as a bath treatment to eliminate mixed infections as well as the median lethal concentration (LC_{50}) of this drug in juvenile, healthy bullseye puffer fish (*Sphoeroides annulatus*) were determined. Triglycerides, hemoglobin, total protein and glucose levels were measured in three groups of fish: uninfected, infected and infected/treated with Adecto®. At the doses tested, PZQ did not have a concentration-dependent effect. Thus, 2.5 mg/L PZQ was 100% effective against adults of *T. ecuadori* after 20 h, and 3 mg/L killed 87% of *N. melleni* after 12 h. Adecto® had a concentration-dependent effect. The concentration required to kill all parasites in the minimum time was 20 mg/L Adecto® (12 h for *T. ecuadori*, and 16 h for *N. melleni*). Neither PZQ nor Adecto® were effective at inhibiting egg hatching. Adults of *T. ecuadori* were highly tolerant to freshwater; mortality was < 40% after 24 h, whereas formalin was 100% effective against both adults and eggs in this species. *In vivo*, 20 mg/L Adecto® administered for 12 h was 100% effective against *T. ecuadori*; however, it was not 100% effective against *N. melleni*. Mixed infections provoked increases in hemoglobin and total protein levels in fish. Fish exposed to 20 mg/L Adecto® did not show signs of toxic effect after 24 h. For those fish, the 24-h LC_{50} of Adecto® was 30.8 mg/L. This study confirms that PZQ is effective against the parasitic phase of monogeneans but under prolonged exposure. Our results suggest that combination anthelmintics have the potential to kill all parasites in less time; however, alternative combinations should be investigated to find one that is effective under low concentrations to provide a greater safety margin.

1. Introduction

Monogenean ectoparasites are a significant threat for many maricultured fish species. The most pathogenic monogeneans belong to the families Capsalidae, Diplectanidae, Anoplodiscidae and Gyrodactylidae in the Monopisthocotylea group, and Microcotylidae, Heteraxinidae and Diclidophoridae in the Polyopisthocotylea group (Ogawa, 2015). The diclidophorid *Heterobothrium okamotoi*, is a species with high pathogenicity, fecundity and tolerance to chemical treatment which has seriously impacted the aquaculture industry in Japan (Ogawa, 2002). Likewise, capsalids such as *Neobenedenia* spp. have been responsible for disease and mortality of many species of farmed fish (Whittington, 2012). Hematological parameters may be significantly altered in severely infected fish due to blood loss or osmotic imbalance in their host (Hirazawa et al., 2016).

Monogeneans have a direct, typically short life cycle. Adult parasites continuously lay eggs that hatch into infectious, free-living larvae known as oncomiracidia which reach juvenile and adult stages once they settle onto fish hosts. In some species, eggs have filaments that entangle in net cages facilitating the infection process. All these life cycle related characteristics lead to high infection levels on fish within the culture systems.

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Table 1
PZQ bath treatments against marine monogeneans reported in the literature.

Parasite	Host	Concentration mg/L	Time (h)	Efficacy %	Reference
Benedenia seriolae	*Seriola dumerili*	1	1	100	Hirazawa et al., 2013
	Seriola lalandi	2.5	24	100	Sharp et al., 2004
Benedeniella posterocolpa	*Rhinoptera bonasus*	20	1.5	100	Thoney, 1990
Haliotrema sp. and *Euryhaliotrema* sp.	*Lutjanus guttatus*	4.5 (D)	14	100	Fajer-Ávila et al., 2007
		3.5 (V)	24	100	
		4.5 (V)	14	100	
Heterobothrium okamotoi	*Takifugu rubripes*	20	10	100	Hirazawa et al., 2000
Lepidotrema bidyana	*Bidyanus bidyanus*	10	48	99	Forwood et al., 2013
Microcotyle sebastis	*Sebastes schlegeli*	100	4 min	> 90	Kim and Cho, 2000
Neobenedenia girellae	–	2.5 and 5	5	> 95	Hirazawa et al., 2013
Sparicotyle chrysophrii	–	25	30 min	20	Sitjà-Bobadilla et al., 2006
		50	30 min	10	
		100	50 min	10	
Zeuxapta seriolae	*Seriola lalandi*	2.5	24	100	Sharp et al., 2004

D = Drontal™ Plus (50 mg praziquantel, 150 pyrantel embonate and 150 mg febantel).
V = Vermiplex™ Plus (2 mg ivermectin and 50 mg praziquentel).

Formalin, copper sulphate, potassium permanganate, sodium chloride, sodium peroxycarbonate and trichlorfon are some of the chemotherapeutics used against monogeneans in aquaculture (Buchmann et al., 1987; Thoney, 1990); however, these chemicals may be toxic for fish and the surrounding aquatic ecosystems (Reardon and Harrell, 1990; Qin and Dong, 2004; Leal et al., 2016). An alternative treatment is Praziquantel (PZQ), which is a broad-spectrum anthelmintic commonly used in humans and domestic animals because it is safe, effective, economical and convenient (Wei et al., 2005; Cioli et al., 2014). Several studies have shown that PZQ administered either by bath or orally may reduce infections of monogeneans on fish (Forwood et al., 2013; Partridge et al., 2014). Efficacy > 80% has been observed with PZQ bath treatments in different regimes against monogeneans, with PZQ concentrations varying between 0.7 and 20 mg/L for periods of time ranging between 1 and 48 h (Table 1). Also, 100 mg/L PZQ for 4 min may be highly effective (Kim and Cho, 2000). Interestingly, it has been indicated that combination anthelmintics may be highly effective and offer additional benefits in the control of parasitic worms of terrestrial hosts (*e.g.* Bonneau et al., 2009; Taweethavonsawat et al., 2010); however, this subject has been poorly documented for monogeneans.

It is important to mention that PZQ treatments may dislodge monogeneans from fish but not always kill them and the dislodged parasites may continue to release viable eggs (Sharp et al., 2004; Hirazawa et al., 2013). The treatment tolerance of monogenean eggs is not usually evaluated, but there is some evidence indicating that eggs may be relatively more resistant to antiparasitic treatments. For instance, Umeda et al. (2006) observed that a 3-h treatment of sodium chloride solutions has antiparasitic effect against the oncomiracidia, but a 12-h treatment did not completely kill *Pseudodactylogyrus* eggs.

Considering the current interest in developing marine finfish aquaculture in northwestern Mexico, it is important not only to identify potential pathogens but also to implement strategies to control them. In this region, bullseye puffer fish (*Sphoeroides annulatus*) is a native species of commercial importance. Biotechnological studies have indicated that larvae and juveniles of *S. annulatus* can be intensively produced under hatchery conditions (Abdo-de la Parra et al., 2010). Interestingly, *S. annulatus* serves as host of several macroparasites (Fajer-Ávila et al., 2004; Morales-Serna et al., 2011), including two monogeneans: *Tagia ecuadori* (Diclidophoridae) and *Neobenedenia melleni* (Capsalidae). As seen in previous works, infections of monogeneans on *S. annulatus* may be performed in laboratory (*e.g.* Fajer-Ávila et al., 2008), providing a study model to evaluate antiparasitic treatments.

Therefore, the aim of this work was three-fold: 1) to evaluate *in vitro* the efficacy of PZQ and a combination drug containing PZQ, ivermectin, pyrantel pamoate and fenbendazole (Adecto®) against eggs and adults of *T. ecuadori* and *N. melleni*. Formalin and freshwater bath treatments against *T. ecuadori* were also performed for comparative purposes. 2) To evaluate *in vivo* the effect of administering Adecto® as a bath treatment for monogenean infections as well as the effect of these parasites on some hematological parameters of juvenile *S. annulatus*. 3) To determine the median lethal concentration (LC_{50}) of Adecto® to juvenile *S. annulatus*.

2. Materials and methods

2.1. Source of fish and parasites

Juvenile fish (*S. annulatus*) from single spawning batch were acquired from the Laboratory of Reproduction and Marine Finfish Hatchery at Centro de Investigación en Alimentación y Desarrollo, A. C. Unidad Mazatlán (CIAD-Mazatlán), Sinaloa, Mexico. A stock of these laboratory fish (n = 800) was held in a 5000-L tank supplied with flow-through-filtered seawater (35‰) and continuous aeration. Fish were fed a 3 mm pellet diet (basal diet, Skretting) to satiation, twice a day. This tank was the source of fish for all experiments.

Parasites (*T. ecuadori* and *N. melleni*) were obtained from naturally infected fish (*S. annulatus*) caught off Mazatlán and transferred to Aquatic Parasitology Laboratory at CIAD-Mazatlán. These fish were placed in a 400-L tank to collect parasite eggs and then perform a laboratory infection. This procedure consisted of attaching cotton threads to the aeration tube within the tank. Eggs of these parasites have filaments that entangle onto the threads. Two days after the co-location of the threads and wild fish, collection of threads tangled with eggs began. These were transferred to six 400-L tanks each containing 20 juvenile fish, which resulted in successful infection. Tanks were supplied with flow-through-filtered seawater (28 ± 0.5 °C, 35‰) and continuous aeration. We observed that the time period to complete parasite development from egg to adult was approximately 25 days for *T. ecuadori* and 15 days for *N. melleni*. Thus, we established our source of parasites for future experiments.

2.2. Chemicals

Adecto® (Adler Pharma) 600 mg tablets containing ivermectin (1 mg), praziquantel (50 mg), pyrantel pamoate (150 mg) and fenbendazole (150 mg) were purchased from the local market. Tablets were ground in a mortar prior to the treatment application. Praziquantel (100% powder formulation) was purchased from Sigma-Aldrich. Stock solutions of Adecto® and PZQ were prepared at a concentration of 12.5 mg/mL in 70% alcohol, and then aliquots were added to the test water to attain the target chemical concentration (Mitchell and