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Assessment of *in vitro* killing assays for detecting praziquantel-induced death in *Posthodiplostomum minimum* metacercariae

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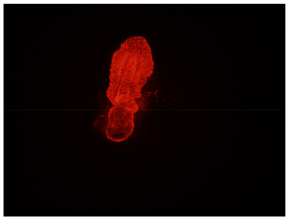
HIGHLIGHTS

- Fish intermediate hosts harbor trematode metacercariae that are infectious for vertebrates.
- In some systems, praziquantel treatment of fish may aid in disrupting parasite life cycles.
- Few *in vitro* tests are available to compare the drug sensitivity of metacercariae recovered from fish.
- Propidium iodide staining was a suitable method for detecting praziquantel-mediated death of metacerariae *in vitro*.

GRAPHICAL ABSTRACT



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ABSTRACT

Control of parasitic infections may be achieved by eliminating developmental stages present within intermediate hosts, thereby disrupting the parasite life cycle. For several trematodes relevant to human and veterinary medicine, this involves targeting the metacercarial stage found in fish intermediate hosts. Treatment of fish with praziquantel is one potential approach for targeting the metacercaria stage. To date, studies investigating praziquantel-induced metacercarial death in fish rely on counting parasites and visually assessing morphology or movement. In this study, we investigate quantitative methods for detecting praziquantel-induced death using a *Posthodiplostomum minimum* model. Our results revealed that propidium iodide staining accurately identified praziquantel-induced death and the level of staining was proportional to the concentration of praziquantel. In contrast, detection of ATP, resazurin metabolism, and trypan blue staining were poor indicators of metacercarial death. The propidium iodide method offers an advantage over simple visualization of parasite movement and could be used to determine EC_{50} values relevant for comparison of praziquantel sensitivity or resistance.

1. Introduction

Trematodes are parasitic flatworms with clinical importance in both human and veterinary medicine. Digenean trematodes have an indirect life cycle involving intermediate hosts. Adult trematodes live and mate within the definitive host while a mollusk

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serves as the first intermediate host. For many trematode species, cercariae leave the snail and penetrate a second intermediate host in order to form metacercariae that are infectious for the definitive host. The metacercarial cyst can reside in the tissues of the second intermediate host for extended periods of time until they are consumed in order to complete the life cycle. Various fish species frequently serve as second intermediate host since they are in close proximity to snails and are likely to be eaten by humans and other vertebrates.

Historically, trematode control has mainly focused on treatment of infected definitive hosts in addition to snail control. Overall, the fish second intermediate host is not commonly treated in order to remove metacercariae. Yet, targeting second intermediate hosts has value in terms of breaking the life cycle. For example, proper cooking of fish prevents transmission of *Clonorchis sinensis* and *Opisthorchi viverrini* (Keiser and Utzinger, 2005). In veterinary medicine and in aquaculture production systems, it becomes desirable to eliminate metacercariae, due to the unsightly appearance of metacercariae in fillets and potential health consequences of metacercariae in fish. This is true for *Posthodiplostomum minimum*, an intestinal trematode that infects Ciconiiformes and Charadriiformes (herons, gulls, terns, etc.), and uses Centrarchid fish as their second intermediate host. *P. minimum* metacercariae are often found in fish species such as bluegills (*Lepomis macrochirus*) and black crappies (*Pomoxis negromaculatus*) raised for recreational stocking efforts.

Praziquantel is one of the few agents with anti-trematode activity and is used widely in both humans and animals. In fish, praziquantel has been used to kill adult monogeneans and digenean trematodes (Shirakashi et al., 2012; Bader et al., 2017). Because there is also a need to eliminate metacercarial stages, numerous studies have assessed the use of praziquantel in fish including oral (Hardy-Smith et al., 2012), topical (Mitchell, 1995), and injectable treatments (Lorio, 1989). However, such studies are difficult to conduct since assessment of efficacy is based on counting metacercaria (Hardy-Smith et al., 2012; Plumb and Rogers, 1990). Counts alone are problematic since dead metacercariae can persist in fish tissues post-treatment. Movement is sometimes used to assess parasite viability, however, some encysted metacercariae have limited mobility (Asanji and Williams, 1975). Another disadvantage of such studies is that they do not allow fine scale detection of the minimum drug concentrations needed to kill the parasites and therefore cannot detect differences in praziquantel sensitivity among isolates or treatment regimens.

Assays have been developed for estimating viability of various cell types. For example, ability to metabolize resazurin (Alamar Blue) has been used as a surrogate measurement of viability (Rampersad, 2012). Similarly, ATP production can be used to detect and quantify cell death (Cree and Andreotti, 1997). In addition to measures of metabolism, membrane integrity can also be used to differentiate live and dead cells. Trypan blue is commonly used to detect dead cultured cells (Bhuyan et al., 1976). Similarly, propidium iodide enters cells with compromised membranes and intercalates into DNA (Gould et al., 2008). Thus, there are numerous methods for conducting killing assays that involve indirect measurements of cellular metabolism or membrane integrity. Yet, these methods have not been adequately exploited for the study of trematode metacercariae.

P. minimum metacercariae are commonly found in wild-caught or extensive-culture-reared Centrarchid fish, with numerous metacercariae commonly recovered from the liver and kidneys (Lane et al., 2015). In the present study, we evaluated *in vitro* methods for evaluating praziquantel-induced death of metacercariae using *P. minimum* as a model.

2. Materials and methods

2.1. Parasites

Fish (*Lepomis macrochirus*) were obtained by legal methods from ponds near Ames, Iowa, USA. All procedures were in accordance with applicable laws and were approved by the Institutional Animal Care and Use Committee (IACUC). Liver and posterior kidneys were removed and *P. minimum* metacercariae were extracted with the aid of a dissection microscope. Metacercariae were placed in 24 well plates with each well containing 300 μL RPMI, 10% bovine serum, 60 μg/ml penicillin, and 100 μg/mL streptomycin. For parasite killing assays, metacercariae were co-incubated with dilutions of praziquantel (Bimeda, Le Sueur, MN) or propylene glycol which served as a vehicle control. Dead (control) metacercariae were generated by killing parasites in a heat block for 15 min at 60 °C. Assays were conducted both for metacercariae within cysts as well as metacercariae removed from cysts. Following addition of praziquantel, plates were incubated overnight at room temperature prior to assessment. One way analysis of variance was used to compare viability assay results among multiple groups using GraphPad Prism statistical software. If ANOVA indicated evidence of differences among the means of experimental groups, Tukey's multiple comparison test was used as a post-hoc test to make pairwise comparisons among groups.

2.2. Propidium iodide

Propidium iodide assays were conducted by co-incubating metacercariae with 50 μg/ml propidium iodide (Sigma) in the dark for 15 min. Metacercariae were removed from the well and imaged on glass microscope slides using a fluorescence microscope. Exposure time and camera sensitivity were calibrated with control parasites. Percent of parasite tissue area fluorescing was determined with image analysis software (Halo FL v1.0, area quantification module). In this approach, the analysis software identifies the percentage of total parasite area that is stained. At least 5 metacercariae were used for each experimental condition and experiments were conducted a minimum of two times. Dose response curves were constructed by applying a four parameter logistic regression assuming a variable slope model.

2.3. Trypan blue

Trypan blue staining was performed by adding 300 μg of trypan blue (Fisher Scientific) to each well, followed by incubation for 3 min at room temperature. Parasites were washed three times with PBS followed by imaging on glass microscope slides using a light microscope. Percent of parasite tissue staining was determined with image analysis software (Halo FL v1.0, area quantification module). At least 5 metacercariae were used for each experimental condition and experiments were conducted a minimum of two times.

2.4. Resazurin metabolism

For resazurin metabolism assessment, 30 μL of 10× Alamar Blue (Fisher Scientific) was added to each well and incubated for 1 or 24 h at room temperature. Following incubation, 50 μL of culture media was transferred to a 96 well plate and fluorescence (570 nm excitation, 590 nm emission) and absorbance values (570 nm and 600 nm) were recorded with a plate reader (Spectramax M2, Molecular Devices) at 1 and 24 h. A minimum of 5 metacercariae were used for each experimental condition and experiments were conducted a minimum of two times.