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Susceptibility of a Brazilian wild rodent isolate of *Schistosoma mansoni* to praziquantel in mice

Michele Costa-Silva [a,c], Lucas de Andrade Barros [b], Juberlan Silva Garcia [a], Renata Heisler Neves [b], Rosângela Rodrigues-Silva [b], José Roberto Machado-Silva [c,1], Arnaldo Maldonado-Júnior [a,*,1]

[a] *Laboratory of Biology and Parasitology of Wild Mammal Reservoirs, Oswaldo Cruz Institute, Oswaldo Cruz Foundation, Av. Brasil 4365, Manguinhos, 21045-900 Rio de Janeiro, Brazil*
[b] *Laboratory of Helminth Parasites of Vertebrates, Oswaldo Cruz Institute, Oswaldo Cruz Foundation, Av. Brasil 4365, Manguinhos, 21045-900 Rio de Janeiro, Brazil*
[c] *Romero Lascasas Porto Laboratory of Helminthology, Department of Microbiology, Immunology and Parasitology, School of Medical Sciences, Biomedical Center, Rua Prof. Manoel de Abreu 444/5º Floor, Vila Isabel, 20511-070 Rio de Janeiro, Brazil*

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ABSTRACT

The therapeutic effects of praziquantel (PZQ) against a *Schistosoma mansoni* isolate derived from *Nectomys squamipes* (isolate R) and a susceptible isolate (BH) were analyzed in Swiss mice by fecal egg counting, adult worm reduction and oogram pattern. Infected mice were orally administrated with 62.5 mg/kg (group 1), 125 mg/kg (group 2), 250 mg/kg (group 3) and 500 mg/kg (group 4), each dose divided over 3 days (49, 50 and 51 days after infection). The data were analyzed using one-way analysis of variance (ANOVA). In regard to isolate R, no fecal eggs were observed with 250 mg/Kg and 500 mg/kg ($p < 0.05$), whereas BH excretion reached zero with all doses. Mean worm burden reduction was significantly ($p < 0.05$) higher at the two highest concentrations, regardless of isolate. At 62.5 mg/kg, the percentage of immature eggs varied from 17% (isolate R) to 38% (isolate BH). At 125 mg/kg, the percentage of immature eggs varied from 20% (isolate R) to 16% (isolate BH). At 250 mg/kg, immature eggs dropped significantly to 1% (isolate R) and 4% (isolate BH). At 500 mg/kg, no immature eggs were found in isolate R, whereas in BH was 8%. No dosage significantly ($p > 0.05$) affected the percentage of mature eggs, regardless of isolate. There was a large increase ($p < 0.001$) in the percentages of dead eggs in all treated groups of 62% and 64% in groups 3 and 4, respectively (isolate R). The percentage of dead eggs rose from 34% (group 1) to 58% (group 3) in isolate BH. Although group 4 showed lowest increase in the percentage of dead eggs (46%), it was higher ($p < 0.001$) compared to the 8% in the control. Our findings indicate that the wild isolate from *N. squamipes* is susceptible to PZQ.

1. Introduction

Human schistosomiasis mansoni, caused by the blood dwelling fluke *Schistosoma mansoni*, is one of the most widespread neglected tropical water-borne diseases (Chitsulo et al., 2000), because inhabitants in these regions have frequent contact with contaminated water (Sarvel et al., 2011).

Although *S. mansoni* is primarily a parasite of humans, other primates (Nelson, 1960; Standley et al., 2011), bovines (Barbosa et al., 1962; Modena et al., 2008) and rodents can serve as reservoir hosts (Théron et al., 1992; Alarcón de Noya et al., 1997). In Brazil, six aspects pertaining to the importance of *Nectomys squamipes* (water rat) in schistosomiasis transmission should be highlighted: (i) this species is semi-aquatic; (ii) its wide geographic range sometimes is coincident with *S. mansoni*-endemic areas (Bonvicino et al., 2002; D'Andrea et al., 2002); (iii) fecal sample examination shows steady release of viable eggs (Antunes et al., 1973; Silva et al., 1992; Maldonado et al., 1994) for 14 weeks after experimental infection (Souza et al., 1992); (iv) experimental studies show the susceptibility to several laboratory-bred *S. mansoni* stocks (Martinez et al., 2008); (v) neither natural nor experimental infections excessively threaten the life span and reproductive capacity of this rat (D'Andrea et al., 2000); and (vi) the existence of *N. squamipes* naturally infected by *S. mansoni* is a drawback for schistosomiasis control programs (Rey, 1993).

The possible methods available to control schistosomiasis have long been recognized as being improved water supply, implementation of basic sanitation measures, improvement of socioeconomic conditions, snail control and chemotherapy for human infections (WHO, 1993). In addition, the Chinese control program against schistosomiasis selected treatment of animal hosts (bovines) with praziquantel (PZQ) (Chen, 2005). PZQ (2-cyclohexylcarbonyl-1,2,3,6,7,11b-hexahydro-4H-pyrazino{2,1-a}isoquinoline-4-one) is the drug of choice for both public health campaigns and clinical use (Moreira et al., 2007).

* Corresponding author. Fax: +55 21 25621276.
E-mail address: maldonad@ioc.fiocruz.br (A. Maldonado-Júnior).
[1] Brazilian Council of Science and Technology (CNPq) fellow.

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Investigations based on reduction of egg excretion have shown that the cure rate can reach 85–90% (Doenhoff et al., 2009). Laboratory studies have demonstrated that mice sex, strain and worm burden do not influence efficacy rates (Gonnert and Andrews, 1977). Experimental studies have investigated the antischistosomal activity of PZQ on schistosomes derived from humans (Araújo et al., 1996; Fallon and Doenhoff, 1994). However, to our knowledge the susceptibility of isolates derived from wild rodents has not been investigated.

2. Material and methods

2.1. Fieldwork area

The fieldwork was conducted in the municipality of Sumidouro (22°02′46″S; 42°41′21″W), a rural area of the Atlantic Forest situated in Rio de Janeiro state (Brazil). In this area, *N. squamipes* is the main non-human host of *S. mansoni* (Silva et al., 1992) and its levels of infection do not change even if the human population is treated with anti-schistosome drugs (D'Andrea et al., 2000).

The region has a humid-mesothermic climate; rainfall is seasonal and heaviest between November and March, whereas the dry months generally extend from May to August (D'Andrea et al., 2000). The capture sites were established along watercourses and flooded areas, according to the semi-aquatic habits of *N. squamipes* (D'Andrea et al., 2007). Fecal samples were collected from every trapped rat. To confirm infection, fecal samples were prepared on slides and microscopically examined, using the Kato-Katz smear technique (Katz et al., 1972). The miracidia obtained from eggs in positive fecal samples from three *N. squamipes* (isolate R) were used to infect laboratory-bred *Biomphalaria glabrata* snails.

2.2. Ethical approval

All animal experiments were conducted in accordance with valid international guidelines for animal experimentation (Ellery, 1985) and were in accordance with the ethical rules of the Oswaldo Cruz Foundation Animal Ethics Committee (L-0017/08) and Brazilian Institute of the Environment and Renewable Natural Resources (IBAMA License No. 02001.000384/00-12).

2.3. Mice and parasites

Swiss Webster (SW) mice were housed under standard caging conditions: polypropylene boxes (40 × 33 cm) with stainless steel screened covers in a room with controlled temperature (25 ± 1 °C) and humidity (60 ± 10%) and artificial dark/light cycle (lights on from 07:00 to 19:00). The animals had free access to water and food (Nuvilab CR-1, Paraná, Brazil).

2.4. Life-cycle maintenance

Cercariae of *S. mansoni* (isolate R) were obtained from infected *B. glabrata* snails following exposure to artificial light to induce cercariae shedding in our laboratory, while isolate BH was maintained in the Malacology Laboratory (Oswaldo Cruz Institute, Rio de Janeiro, Brazil), also using *B. glabrata* snails and SW mice as intermediate hosts, respectively (Paraense and Corrêa, 1981).

The procedures for experimental infections have been described previously (Freire et al., 2003). Briefly, female and male mice ($n = 28$) were subcutaneously infected with 50 cercariae (isolate R), while the control mice ($n = 25$) were exposed to isolate BH, susceptible to praziquantel (Drescher et al., 1993).

2.5. Treatment schedule

The mice were given praziquantel (Merck®) dissolved in 2% cremophor-EL (Sigma, Chemical Company, St. Louis, USA) by gastric gavage at total dosages of 62.5, 125, 250 and 500 mg/kg) each dose divided over 3 days (49, 50 and 51 days after infection) (Sabah et al., 1986). Infected untreated mice remained as control group.

2.6. Assessment of the therapeutic effects

2.6.1. Worm burden determination

Two weeks after treatment, the mice from both isolates were euthanized using a CO_2 chamber. Adult worms recovered by hepatic and mesenteric perfusion were counted and sexed under a stereomicroscope (Smithers and Terry, 1965). The worm burden (infectivity) was determined as the percentage of maturation of cercariae into adult worms recovered from the portal system and mesenteric veins (Freire et al., 2003). The percentage reduction of worm burden in each drug treated group (Cioli et al., 2004) was calculated according to the following equation:

$$\text{Reduction}\,(\%) = \frac{\text{No. of worms in control group} - \text{No. of worms in treated group})}{(\text{No. of worms in control group})} \times 100$$

The mean of worm reduction related for each isolate PZQ dose was calculated and then the effective dose required to kill 50% of the worms (ED_{50}) was estimated. Data were analyzed using GraphPad Prism 4 computer software using the sigmoidal curve, with 5% significance.

2.6.2. Parasitological studies

Stool pellets were collected weekly from the mice at 6 weeks until euthanasia and microscopically examined to confirm the establishment of infection and fecal egg dynamics. Eggs per gram of feces (epg) were determined in individual samples/animal/day, using the Kato-Katz thick smear technique (Katz et al., 1972).

2.6.3. Egg developmental stages (oogram pattern)

The whole small intestine was removed from every mouse, opened lengthwise and separated into two equal sections (proximal and distal). A sample (1 cm long) from the last section was taken and crushed between two glass slides to obtain a thin preparation (Machado-Silva et al., 1991). The percentage of eggs at various developmental stages (immature, mature and dead) was determined by light microscopy (Pellegrino et al., 1962).

2.7. Statistical analysis

Data analysis was performed using the Graph Pad Instat statistical program. Biological data were analyzed using one-way analysis of variance (ANOVA). Measurements with p-values $\leqslant 0.05$ were considered significantly different.

3. Results

3.1. Fecal egg release

A similar kinetics of fecal egg elimination was detected for both R and BH isolates (Fig. 1). Eggs were first detected in the feces at week 6, with mean numbers peaking between weeks 7 and 8 and declining thereafter, which was coincident with drug administration. There was a dose-dependent decrease in fecal egg count. Praziquantel was significantly ($p = 0.003$) effective against isolate R at a dose of 500 mg/kg, at which egg excretion reached zero

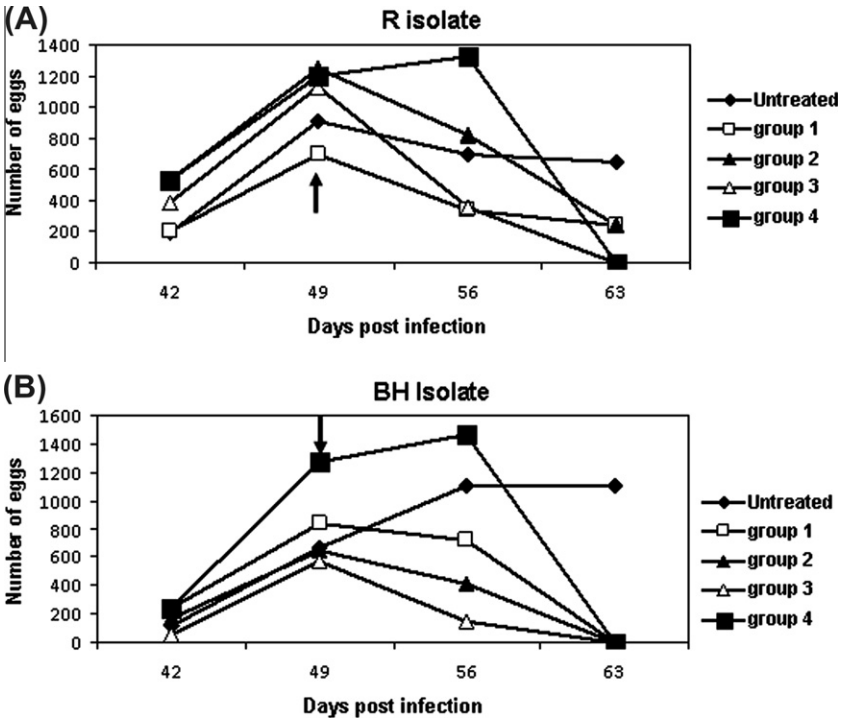

Fig. 1. Kinetics of egg elimination in stools after treatment with praziquantel (PZQ) administered at different dosages to Swiss Webster mice infected with (A) rodent (R) or (B) Belo Horizonte (BH) isolates of *Schistosoma mansoni*.

by the end of the study. With respect to isolate BH, all dosages terminated fecal egg output. Egg-laying from the corresponding untreated controls was significantly higher ($p < 0.001$) than from the treated group.

3.2. Worm burden reduction

Table 1 shows the worm burden reduction and oogram pattern in relation to the dosage of praziquantel administered to the infected mice. Untreated control mice infected with isolate R yielded higher mean number (31 ± 4; 62%) than BH (25 ± 3; 50%). The data on worm burden reduction revealed that treatment efficacy was dose-dependent. At low doses of 62.5 mg/kg, the mean worm burden reduction and percentage were lower for both isolate R (25 ± 4; 19%) and isolate BH (17 ± 5; 32%). When 125 mg/kg was administered, the efficacy against isolate R increased (23 ± 5; 26%), as it did against isolate BH (11 ± 2; 56%). The treatment efficacy was significantly higher ($p < 0.05$) at the two highest praziquantel concentrations tested: 250 mg/kg (9 ± 3; 71% and 6 ± 2; 76%) and 500 mg/kg (4 ± 1; 87% and 1 ± 1; 96%), for isolate R and BH, respectively.

The ED_{50} was higher in isolate R (181 mg/kg de PZQ) than isolate BH (105 mg/kg de PZQ).

It should be noted that treatment caused a dose-dependent reduction in the male-female ratio only in isolate R. The male/female ratio decreased from 4:1 (untreated group) to 1:1 (group 4) (Table 1).

3.3. Egg developmental stages

In the oogram assay, both treated and untreated mice presented eggs at all stages of development, although therapeutic efficacy was dependent on the treatment schedule. At the lowest dose (62.5 mg/kg) of PZQ, the percentage of immature eggs was 17% (isolate R) and 38% (isolate BH). At the next higher dose (125 mg/kg), isolate R showed a slight increasing in the percentage of immature eggs (20%), whereas the rate for isolate BH declined from 38% to 16%. When 250 mg/kg was given, immature eggs dropped

Table 1
Parasitological effects of different dosages of praziquantel against isolates (R and BH) of *Schistosoma mansoni* harbored in mice.

Parameters	Groups									
	R isolate					BH isolate				
	Untreated	1	2	3	4	Untreated	1	2	3	4
Worm recovery	31 ± 4	25 ± 4	23 ± 5	9 ± 3*	4 ± 1*	25 ± 3	17 ± 5	11 ± 2*	6 ± 2*	1 ± 1*
Male–female ratio	4:1	3:1	2:1	3:1	1:1	1:1	1:1	1:1	1:1	1:1
Worm reduction (%)	–	19	26	71*	87*	–	32	56*	76*	96*
Oogram										
Immature	32 ± 6	17 ± 2	20 ± 6	1 ± 0.5*	0*	39 ± 4	38 ± 15	16 ± 6	4 ± 2*	8 ± 4
Mature	47 ± 5	48 ± 4#	38 ± 6	37 ± 7	36 ± 1	53 ± 4	28 ± 5#	47 ± 2	38 ± 13	46 ± 7
Dead	21 ± 2#	35 ± 3	42 ± 6*	62 ± 7*	64 ± 1*#	8 ± 1#	34 ± 11	37 ± 4	58 ± 13*	46 ± 6*#

Groups: 1 (62.5 mg/kg), 2 (125 mg/kg), 3 (250 mg/kg), 4 (500 mg/kg).
Oogram (%): egg developmental stages.
* Significant difference ($p < 0.05$) when compared with the respective untreated group.
# Significant difference ($p < 0.05$) when compared between each dosage regarding BH and R isolate.

significantly to 1% (isolate R) and 4% (isolate BH). At the highest dose tested (500 mg/Kg), no immature eggs were found in isolate R, whereas in BH the rate was 8%.

As shown in Table 1, the percentage of mature eggs was less than in the corresponding untreated control at 125, 250 and 500 mg/kg (isolate R). In regard to isolate BH, this percentage occurred only at the lowest dosage. There was a sharp increase in the percentages of dead eggs in all treated groups, with significant ($p < 0.0001$) increases to 62% and 64% in groups 3 and 4, respectively (isolate R). The percentage of dead eggs rose from 34% (group 1) to 58% (group 3) in isolate BH. Although group 4 showed lowest increase in the percentage of dead eggs, to 46%, it was significantly higher ($p < 0.05$) compared to the 8% in the control group (Table 1).

There was no correlation between the number of worms recovered and the oogram pattern or fecal egg output.

4. Discussion

PZQ has become the drug of choice for schistosomiasis due to its effective pharmacological properties, lack of significant toxicity and relatively low cost (for a review, see Doenhoff et al., 2008). To our knowledge, this is the first study of the effects of PZQ against a naturally occurring Brazilian rodent isolate of *S. mansoni*. Such investigation is important because Sigmondontinae rodents act as reservoirs of *S. mansoni*, which hampers schistosomiasis control programs (Rey, 1993; Peralta et al., 2009). Another contribution of this study is that the isolate used is derived from a region where there is a positive correlation between the prevalence of infection in the rodent and human populations (D'Andrea et al., 2000).

Obtaining an isolate from wild rodents in laboratory mice was the first step for further testing of its susceptibility to schistosomicides. It is interesting that the isolate derived from *N. squamipes* was adapted to SW mice based on the kinetics of fecal egg-output and perfusion data, which revealed maturation between 56 and 60% at 8 weeks in the C3H/He mouse strain (Freire et al., 2003).

The gold standard for diagnosing schistosomiasis infection is microscopic detection of eggs from stools. Moreover, changes in patterns of schistosome egg elimination are mostly used to determine the drug's effectiveness, in terms of a cure rate and/or egg reduction rate (Doenhoff et al., 2008). Analysis of fecal samples may not reveal eggs due to a number of factors, including the level of infection, which influences the sensitivity limits of the examination technique (Berhe et al., 2004; Gonçalves et al., 2006). In this study, aiming to improve the limitation of diagnosis, multiple fecal examinations were performed (Wilson et al., 2006).

The examination of fecal material using the Kato-Katz thick smear technique demonstrated an initial rise in the egg-output by weeks 6–7, which declined after the mice were given PZQ. These results are consistent with reports from other therapeutic assays (Lescano et al., 2004).

Under laboratory conditions, this efficacy against mature infection can also be assessed by worm burden reduction and oogram changes (Pellegrino et al., 1977; Botros et al., 2004; Araújo et al., 2008). It is worth mentioning that all dosages used here are included as PZQ subcurative doses, while 600 mg/kg is a curative one (Chaiworaporn et al., 2005). The praziquantel dosage protocols in the present study caused a significant worm burden reduction and modifications in the oogram pattern, with the best efficacy attained with the highest dose (500 mg/kg).

In this study, PZQ was effective against isolate R and BH. Isolate BH has been found to be sensitive to PZQ (Drescher et al., 1993). Moreover, differences in susceptibility to PZQ have also been reported among isolates of different geographic origin (Fallon et al., 1997) and between long-maintained laboratory isolates and those from natural human infections (Melman et al., 2009). A major consideration for such difference is that field isolates from Brazil are more diverse than long-maintained laboratory isolates (Rodrigues et al., 2002). One potential explanation is that field isolates decrease variability due to loss of alleles after passage in murine hosts (Loverde et al., 1985). The genetic bottleneck may be the reason for such findings (Bech et al., 2010). The time lapse for each isolate that has been maintained under laboratory conditions is probably the underlying reason for such differences between isolates.

Our *in vivo* study showed a higher sensitivity of male worms from isolate R, inferred from the male-female- ratio data, which were male-biased (4:1, untreated group) and decreased to a 1:1 male/female ratio in mice exposed to a higher dosage (500 mg/kg). Our finding support previous observations (Gonnert and Andrews, 1977), but other studies have shown preferential killing of female worms by PZQ (Delgado et al., 1992).

Examination of the oograms showed a lower percentage of immature and mature eggs and a higher percentage of dead eggs than in the control animals. Nevertheless, there are no doubts about the effects of PZQ on adult worms (Doenhoff et al., 2008). The effect of a double dose of PZQ on mature eggs has been reported (Giboda and Smith, 1994). This is an important issue because only mature eggs can cross gut tissue to be excreted with the host's feces (Giboda and Smith, 1994). The possible explanation is that high doses of PZQ could be acting on the reproductive system of female worms. In this situation, eggs could display low development capacity or the dosages used here were enough to act on the mature eggs, as previously reported (Giboda and Smith, 1994). This impairment could lead to the death of the embryo within the egg. Morphological studies to assess changes in the reproductive system (Shaw and Erasmus, 1988; Neves et al., 2004) of adult worms are ongoing. Interestingly, morphological studies through scanning confocal laser microscopy for determination of egg viability (Holtfreter et al., 2011) and functional criteria such as labeling the eggs with the Hoechst 33258 fluorescent probe (Sarvel et al., 2006) and intestine histopathology (Conceição et al., 2008) can provide important information regarding this issue. Our findings indicate that the wild isolate from *N. squamipes* is susceptible to praziquantel. Although public health officials consider the best schistosomiasis control strategy to be treatment of the infected human population, the epidemiologic significance of the presence of infected wild water rat hosts needs to be considered.

Conflicts of interest statement

The authors have no conflicts of interest concerning the work reported in this paper.

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