Use of fluorescent probes as a useful tool to identify resistant *Schistosoma mansoni* isolates to praziquantel

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SUMMARY

The use of chemotherapy on a mass scale in endemic areas may lead to the appearance of resistant isolates through the mechanism of selective drug pressure. Studies have demonstrated that praziquantel (PZQ) is able to inhibit the excretory activity and to cause tegumental damage in *Schistosoma mansoni* adult worms. The use of the probe resorufin to evaluate excretory activity, as well as the probe Hoechst 33258 to detect tegumental damage in adult worms, may represent a method to identify resistant (or less susceptible) isolates. The purpose of the present work was to compare the changes caused by PZQ in the function of the excretory system and in the integrity of the tegument of adult worms from the LE isolate (susceptible to PZQ) and the LE-PZQ isolate (less susceptible to PZQ). Worms from the isolate LE-PZQ showed less severe tegumental lesions, in both *in vitro* and *in vivo* experiments, detected by labelling with Hoechst 33258 and continued to have a functional excretory system as shown by labelling with resorufin *in vitro*.

Key words: *Schistosoma mansoni*, drug resistance, praziquantel, fluorescent probes.

INTRODUCTION

Schistosomiasis is still a major health problem in many tropical and subtropical countries. It is estimated that at least 200 million people are currently infected with schistosomiasis and another 600 million people are at risk of infection (WHO, 2002). Currently, praziquantel (PZQ) is the drug of choice according to the World Health Organization, being effective against all 5 species of *Schistosoma* that infect humans (Doenhoff *et al*. 2002). Although the mechanism of action of this drug against *Schistosoma mansoni* is still not completely elucidated (Cioli and Picca-Matoccia, 2003), some of its effects on the parasite are well known, such as muscular contraction, tegumental damage and metabolic changes (Pax *et al*. 1978; Fetterer *et al*. 1980; Becker *et al*. 1980; Mehlhorn *et al*. 1981; Lima *et al*. 1994; Ribeiro *et al*. 1998; Oliveira *et al*. 2006; Kusel *et al*. 2006). The availability of a drug with low toxicity, oral single dose and administration with high effectiveness, allows the large-scale treatment of populations. However, the use of large-scale chemotherapy with successive treatments, as the main method for the control of schistosomiasis in endemic areas, could result in the appearance of resistant isolates, by means of selective drug pressure (Coelho *et al*. 1997).

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It has been recently suggested that the excretory system of *Schistosoma* has an important role in the parasite-host interaction and drug excretion. Sato *et al*. (2002, 2004) described the utilization of the fluorescent marker resorufin, a substrate for P-glycoprotein (PgP), for evaluation of the excretory activity of *S. mansoni*. PgP is a protein that exerts an important role in the absorption, elimination and distribution of many xenobiotics, including a variety of drugs (Schinkel and Johker, 2003). Oliveira *et al*. (2006), using resorufin, demonstrated that PZQ is able to inhibit the excretory activity of *S. mansoni* adult worms (LE isolate, which is susceptible to PZQ), and this activity can be restored when the worm is no longer in contact with the drug. Messerli *et al*. (2009) point to higher levels of Pgp being associated with reduced susceptibility to PZQ in *S. mansoni*.

The parasite-host interaction is extremely complicated, and the membrane surface is an interface between the two organisms (Oliveira *et al*. 2006). The literature shows that the *Schistosoma* tegument is an important target for anti-schistosomal drugs. William *et al*. (2001) observed a decrease in the magnitude of the damage caused by PZQ to the tegument of PZQ-resistant adult worms, when compared with the tegument of susceptible adult worms. Moreover, Oliveira *et al*. (2006) demonstrated that PZQ is able to cause damage to the tegument of susceptible adult worms, using the probe Hoechst 33258 (bisbenzimide), a hydrophilic probe, which becomes fluorescent only when it binds to

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the DNA after diffusing into subtegumental cells where there are tegumental lesions, thus acting as an indicator of membrane integrity.

The evaluation of the presence of tegumental damage and excretory activity in adult worms, after exposure to PZQ, using specific fluorescent markers, would be a novel method for identification of resistant *S. mansoni* isolates. In this work we have compared, using fluorescence microscopy, the changes caused by PZQ to the excretory system and to the tegument of adult normal (LE) worms with those less susceptible to PZQ (isolate LE-PZQ which was produced by use of drug pressure on infected *Biomphalaria glabrata* (Couto *et al*. manuscript submitted for publication preparation). This isolate LE-PZQ was termed resistant in accordance with the concept of resistance suggested by Coles and Kinoti (1997), i.e. "a population of *Schistosoma* is resistant when either a susceptible population shows a significant decrease in its response to a schistosomicide or it is significantly less sensitive than a fully susceptible population".

MATERIALS AND METHODS

Life cycle of the parasite and infection of animals

Swiss mice were infected subcutaneously with 100 *S. mansoni* cercariae (LE or LE-PZQ isolates). The LE isolate had been maintained for more than 50 years at the Centro de Pesquisas René Rachou (CPqRR)/FIOCRUZ (Pellegrino and Katz, 1968). The cercariae LE-PZQ were from an *S. mansoni* isolate submitted to treatments with PZQ at the intramolluscan stage. This isolate was obtained when infected *Biomphalaria glabrata* snails were submitted to 3 treatments with PZQ, each treatment administered on 5 consecutive days, with 1 week interval, for selection of less susceptible parasites to PZQ. The isolate LE-PZQ was passaged through mice and treated 45 days after infection with 400 mg/kg PZQ. It was observed that 52·3% of worms were recovered alive, whereas in the isolate without chemotherapeutical pressure (control) only 10% of worms were recovered alive (Couto *et al*. manuscript submitted for publication preparation). These values for worm recovery suggest that LE-PZQ isolate is resistant (Coles and Kinoti, 1997).

In vitro *and* in vivo *experiments*

Mice infected with LE or LE-PZQ cercariae, were perfused 45 days after infection, according to the technique described by Smithers and Terry (1965), using culture medium RPMI-1640 (Sigma Chemical Co., St Louis, MO, USA) with 0·2% heparin as perfusate. Four worm pairs were distributed into each well of tissue culture plates containing 6 wells (6-well plates). The worms of the LE and LE-PZQ isolates were put into separate wells. The methodologies for evaluation of the excretory system activity and tegumental damage have been previously described (Oliveira *et al*. 2006). Photographic records were taken with a digital camera (Canon EOS Digital Rebel XT).

Evaluation of the excretory system activity of adult worms LE and LE-PZQ with resorufin and incubation with PZQ in vitro

The worms were maintained for 30 min in 4 ml of RPMI culture medium containing an additional 5% foetal bovine serum (FBS – Gibco Limited, Paisley, Scotland, UK) and 100 μg/ml of the antibiotics penicillin/streptomycin (Sigma). Then, 10 μl of resorufin (Sigma – stored solution 10 mg/ml in medium) were added to each well, and incubated in 5% CO_2 at 37 °C for 30 min. After being washed 5 times with 2 ml of RPMI to remove the excess probe, 2 μg/ml of PZQ (Cestox® – Merck) were added to the wells containing worms (LE-PZQ or LE isolates), incubated in 5% CO_2, at 37 °C for 15 min. The worms were once again washed 5 times with culture medium and transferred to slides within a square demarcated with Vaseline to avoid overflow of the worms and medium. They were then observed under a fluorescence microscope (Karl Zeiss Axiostar Plus filter for Rhodamine – excitation/maximal emission of resorufin 571/585 nm). Where there is significant accumulation of resorufin into the excretory tubules, the resorufin appears bright red or yellow on a fainter red background.

Evaluation of tegumental damage in LE and LE-PZQ adult worms after incubation with PZQ in vitro*, using the probe Hoechst 33258*

The worms were maintained in 2 ml of RPMI culture medium containing 5% FBS and 100 μg/ml of the antibiotics penicillin/streptomycin. Then, 2 μg/ml PZQ were added and the worms incubated in 5% CO_2, at 37 °C for 1 h, after which they were washed 5 times with 1 ml of culture medium to remove the drug. Ten μl of the probe Hoechst 33258 (Sigma – stored aqueous solution 10 mg/ml) were added, and the worms were incubated in 5% CO_2 at 37 °C for 15 min. The worms were washed 5 times with culture medium, and transferred to slides demarcated with Vaseline before observation under the fluorescence microscope (Karl Zeiss Axiostar Plus filter for DAPI – excitation/maximal emission of Hoechst 33258 352/455 nm).

Evaluation of tegumental damage in LE and LE-PZQ adult worms, after treatment with PZQ in vivo*, using the probe Hoechst 33258*

Mice infected with LE or LE-PZQ cercariae, were treated 45 days post-infection with 400 mg/kg PZQ.

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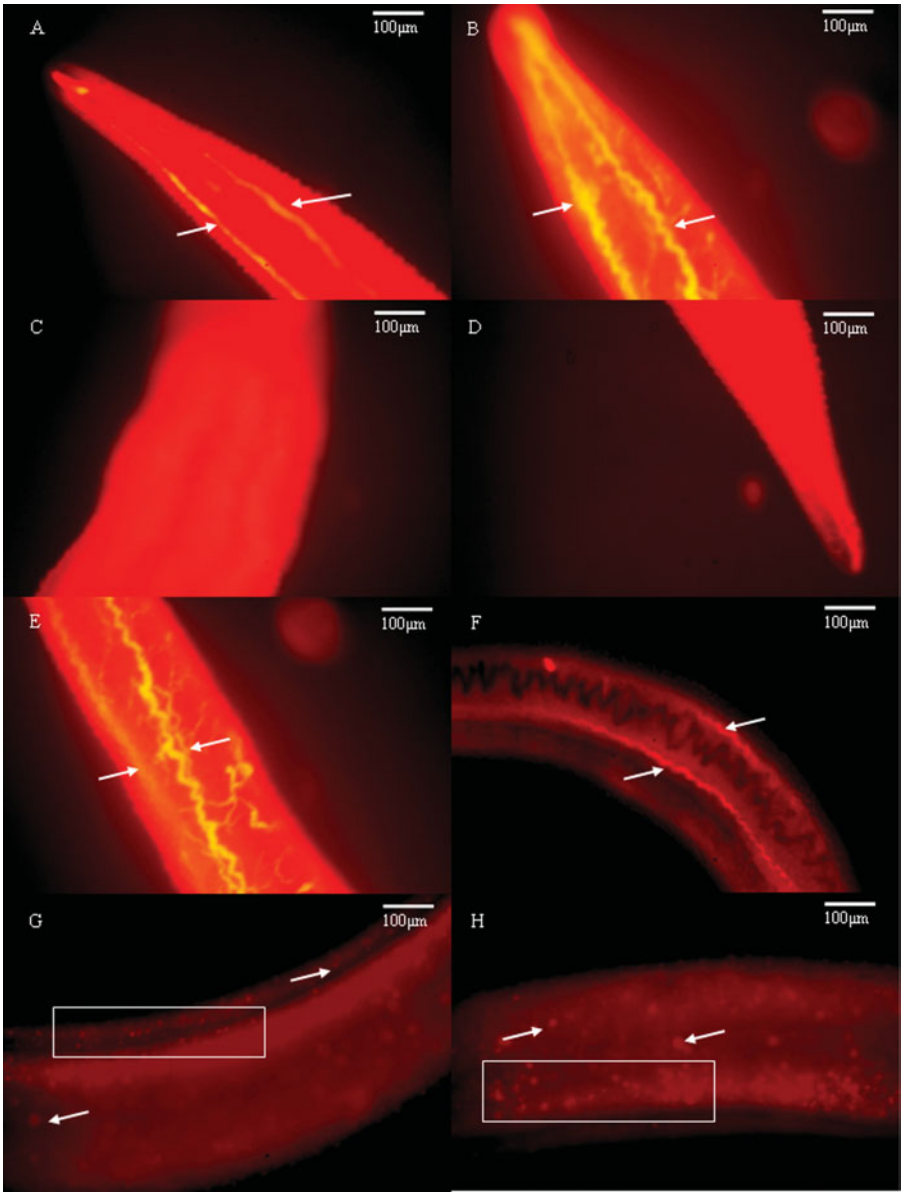

Fig. 1. *In vitro* evaluation of the effect of PZQ on the excretory system of *Schistosoma mansoni* adult worms. (A and B) *S. mansoni* LE labelled with the probe resorufin and not exposed to PZQ. (C and D) *S. mansoni* LE labelled with the probe resorufin and exposed to 2 μg/ml PZQ. (E and F) *S. mansoni* LE-PZQ labelled with the probe resorufin and not exposed to PZQ. (G and H) *S. mansoni* LE-PZQ labelled with the probe resorufin and exposed to 2 μg/ml PZQ. Arrow: main tubule and ramifications of the excretory system. Each Scale bar represents 100 μm. In G and H, labelled regions of the excretory system are shown in a box as well as by an arrow.

After 2 h, mice were perfused according to the technique described by Smithers and Terry (1965), using RPMI-1640 culture medium containing 0·2% heparin. Four pairs of worms were placed into each well of 6-well culture plates. The worms of the LE and LE-PZQ isolates were put into separate wells with 2 ml of RPMI culture medium containing 5% FBS and 100 μg/ml of the antibiotics penicillin/streptomycin. Then, 10 μl of the probe Hoechst 33258 were added, and the worms were incubated in 5% CO_2 at 37 °C for 15 min. The worms were washed 5 times with culture medium, and transferred to slides demarcated with Vaseline before observation under the fluorescence microscope (Karl Zeiss Axiostar Plus filter for DAPI – excitation/maximal emission of Hoechst 33258 352/455 nm).

RESULTS

Labelling of the excretory system of adult worms (LE and LE-PZQ) with resorufin and incubation with PZQ

Due to the variability of results in female worms, we chose to evaluate only the excretory activity of males. The excretory system of adult male worms derived from LE and LE-PZQ cercariae, exposed to resorufin *in vitro* was brightly labelled by the probe with yellow or bright red fluorescence (Fig. 1A, B and E, F,

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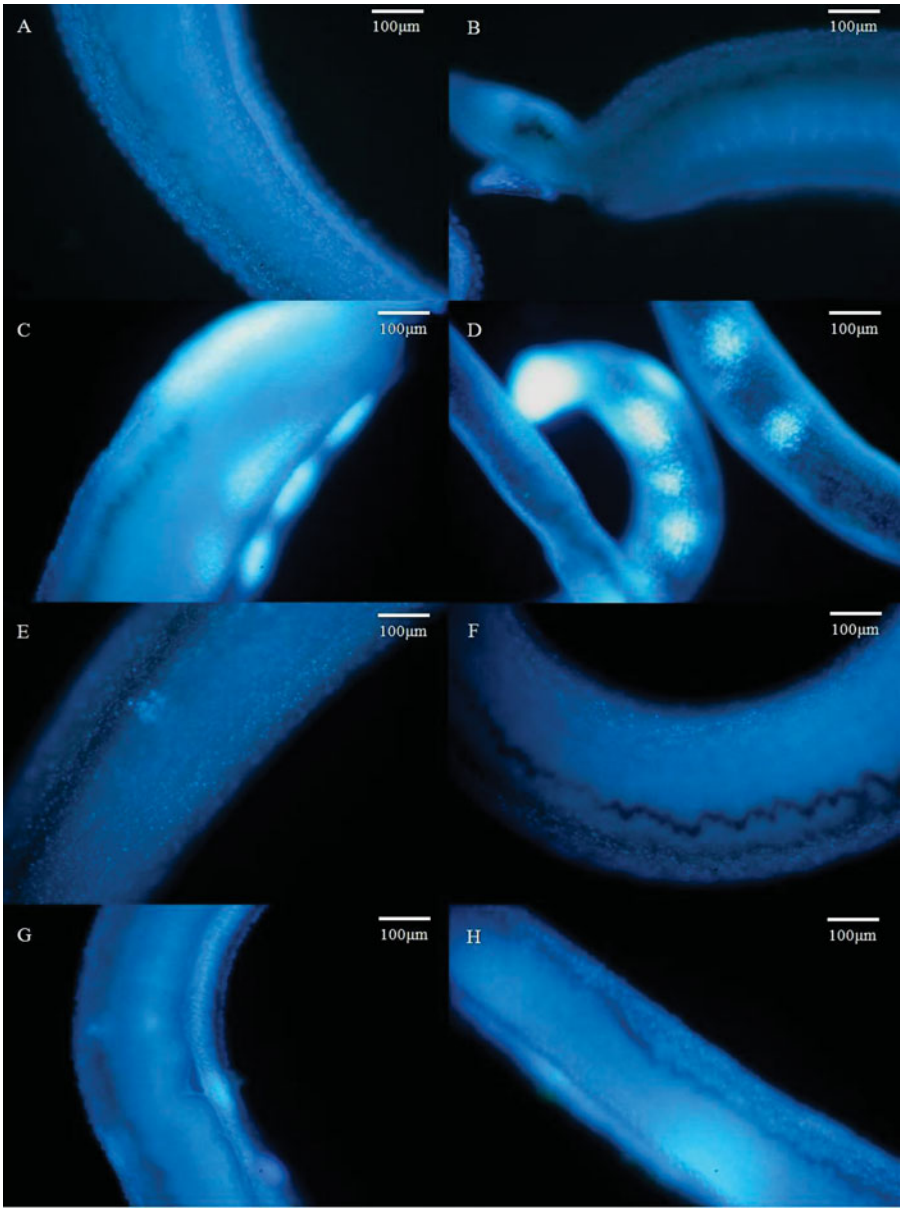

Fig. 2. *In vitro* evaluation of the effect of PZQ on the tegument of *Schistosoma mansoni* adult worms. (A and B) *S. mansoni* LE not exposed to PZQ and labelled with the probe Hoechst 33258. (C and D) *S. mansoni* LE exposed to 2 μg/ml PZQ and labelled with the probe Hoechst 33258. (E and F) *S. mansoni* LE-PZQ not exposed to PZQ and labelled with the probe Hoechst 33258. (G and H) *S. mansoni* LE-PZQ exposed to 2 μg/ml PZQ and labelled with the probe Hoechst 33258. The fluorescent areas indicate intense lesions. Each Scale bar represents 100 μm.

respectively. After PZQ treatment, in each well, the majority of the male worms of the LE isolate showed complete inhibition at a PZQ concentration of 2 μg/ml (Fig. 1C and D) whereas very few (25%) of the isolate LE-PZQ showed inhibition. Thus, it was observed that the majority of the LE-PZQ worms exposed to resorufin and incubated with PZQ *in vitro*, showed some fluorescence in the excretory system although of less intensity compared with the worms not exposed to PZQ (Fig. 1G and H). This result indicates a reduction in the susceptibility of the worms LE-PZQ to PZQ.

Evaluation of the tegumental damage of adult worms after exposure to PZQ in vitro *and* in vivo *using the probe Hoechst 33258*

The fluorescent regions indicate tegumental damage. The worms derived from the LE (Figs 2A and B and 3A and B) and LE-PZQ isolates (Figs 2E and F and 3E and F) that were not exposed to the drug *in vitro* or *in vivo* did not show tegumental damage. In each well, the majority of the male and female worms of the LE isolate, exposed to 2 μg/ml *in vitro* or worms obtained from mice 2 h after administration of

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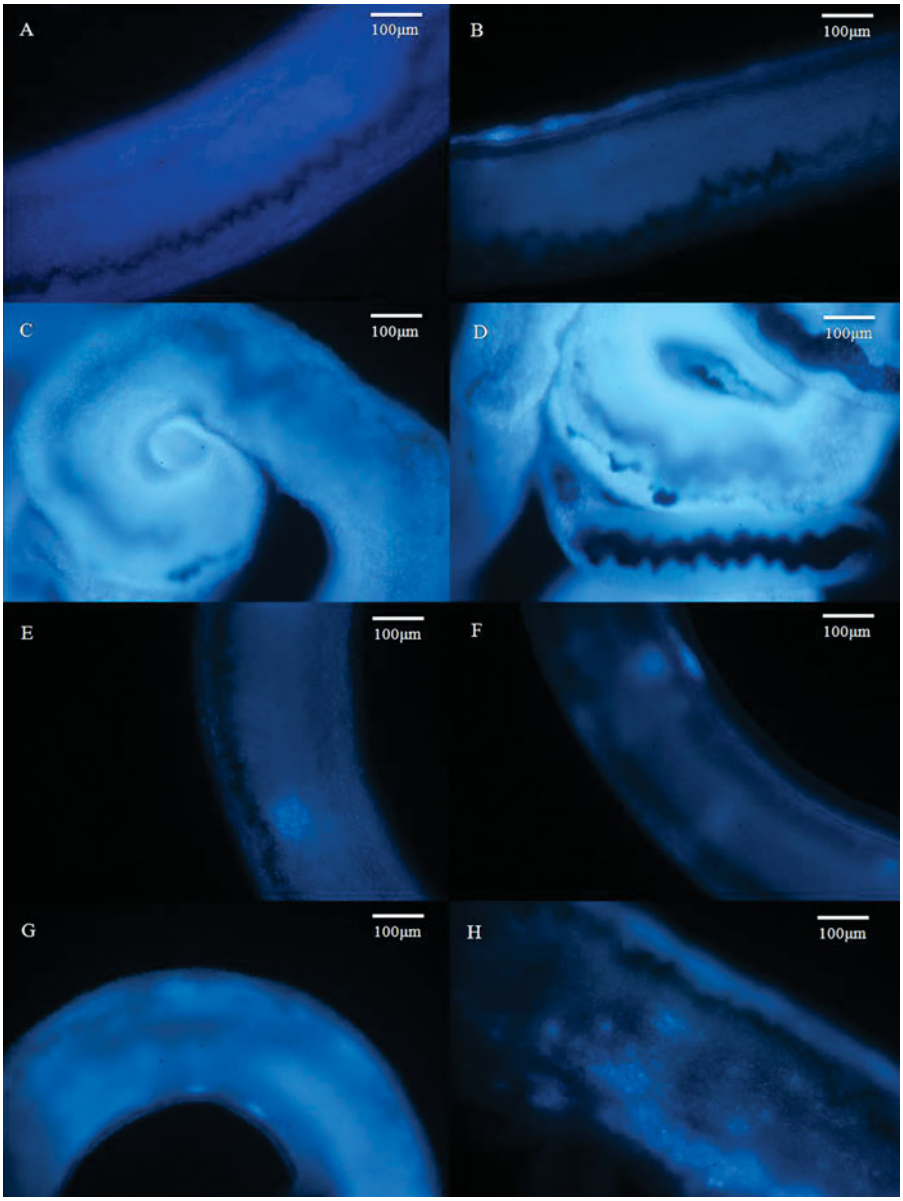

Fig. 3. The tegument of *Schistosoma mansoni* adult worms observed *in vivo*. (A and B) *S. mansoni* LE not treated with PZQ and labelled with the probe Hoechst 33258. (C and D) *S. mansoni* LE treated with 400 mg/kg PZQ and labelled with the probe Hoechst 33258 of PZQ. (E and F) *S. mansoni* LE-PZQ not treated with PZQ and labelled with the probe Hoechst 33258. (G and H) *S. mansoni* LE-PZQ treated with 400 mg/kg and labelled with the probe Hoechst 33258. The fluorescent areas indicate intense lesions. Each Scale bar represents 100 μm.

PZQ (single oral dose of 400 mg/kg) showed intense damage in the tegument (Figs 2C and 3C and D) but very few (25%) of the LE-PZQ worms showed tegumental damage. Thus, in each well, the majority of the LE-PZQ male and female worms presented significantly less intense damage in their teguments in comparison with the LE worms (Figs 2G and H and 3G and H). These results corroborate the findings concerning the excretory activity, indicating a reduction in the susceptibility of the LE-PZQ to PZQ.

DISCUSSION

In the experiments examining the changes caused by PZQ in the excretory system of *S. mansoni,* our findings corroborate the results obtained by Oliveira *et al*. (2006), showing that PZQ was able to inhibit the excretory activity of *S. mansoni* adult worms (susceptible LE isolate). Compared to the parasites of the LE isolate, PZQ was not able to completely inhibit the excretory activity of the worms from the LE-PZQ isolate. After PZQ treatment of these resistant worms, (LE-PZQ isolate) it was possible to observe a clear labelling with the probe resorufin, although less intense than that observed in the untreated control. The control LE-PZQ parasites showed accumulation of resorufin into the excretory ducts as a yellow or bright red fluorescence on a red background. After treatment with PZQ there was some bright red fluorescence detectable in the

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parasite (arrowed and put in a box for clarity). As described by Sato *et al*. (2002), we chose to work only with male parasites, due to variation in the responses of females to resorufin. Resorufin is a putative substrate of PgP. The SMDR2, a PgP homologue, has already been described in *S. mansoni* by Bosch *et al*. (1994). These PgP, as well as the proteins associated with the resistance to multi-drugs (MRP), are ABC carriers (ATP – Binding Cassette) and are associated with a resistance phenotype to drugs, as well as frequently expressed drug-resistant tumour cells, with the function to reduce cytotoxicity of drugs in the cytoplasm of those cells (Kusel *et al*. 2006). In this way, the super-expression of these homologous PgPs with *S. mansoni* may be responsible for the resistance of this parasite to the drug. Messerli *et al*. (2009) obtained results indicating that high levels of SMDR2 may be associated with the reduced susceptibility to PgP in *S. mansoni*.

We can assert as a hypothesis that the labelling of the excretory system of LE-PZQ worms, even after exposure to the drug *in vitro*, demonstrates the continued excretory activity of the worm. If PZQ is excreted through the excretory system of the schistosome, the possible resistance mechanism would be related to a rapid excretion of PZQ in LE-PZQ isolate, thus impairing its lethal activity on the parasite. PZQ might be more damaging to the LE isolate worms if their excretory activity was inhibited by the drug. We suggest that PgP or homologous proteins present in the epithelium of the excretory system have an important role in the elimination of various drugs. This suggestion is in accordance with that of Schinkel *et al*. (2003).

It is well known that PZQ is able to cause intense damage to the surface of the adult worm (Becker *et al*. 1980; Mehlhorn *et al*. 1981; Oliveira *et al*. 2006). The damage generated by PZQ causes either the immediate death of the parasite, or could lead to the exposure of antigens which, after recognition by the specific immune response, would consequently eliminate the parasite (Harnett and Kusel, 1986; Doenhoff *et al*. 1987; Modha *et al*. 1990). The magnitude of the tegumental lesions of adult worms has already been suggested as a possible parameter to differentiate susceptible and resistant isolates to PZQ (William *et al*. 2001).

A sensitive tool for identification of tegumental lesions in *S. mansoni* is the fluorescent probe Hoechst 33258 (Oliveira *et al*. 2006). This probe has already been used in various studies, not only in adult worms (Lima *et al*. 1994; Oliveira *et al*. 2006), but also in schistosomula (Kusel *et al*. 2007), sporocyst (Mattos *et al*. 2006), and cercariae (Thornhill *et al*. 2009). The probe Hoechst 33258, which binds DNA of cells, is a very sensitive indicator of membrane integrity, since even the worms of the LE and LE-PZQ isolates – not exposed to PZQ *in vitro* or *in vivo* – showed slight labelling with the probe, leading to some variability among the groups. These small labelled regions, observed in the worms of the control group, are a consequence of the method used for worm recovery (Oliveira *et al*. 2006). However, the distinct labelling by Hoechst 33258 in worms of the LE isolate exposed to PZQ *in vitro* and *in vivo* is very clear, confirming the capacity of PZQ to cause tegumental damage in *S. mansoni* worms of the control group (susceptible to PZQ). However, in worms of the LE-PZQ isolate exposed to the drug *in vitro* and *in vivo,* only small fluorescent regions could be observed. According to William *et al*. (2001), the worms that are less susceptible to the effects of PZQ on the tegument *in vivo*, are also less susceptible to the same effects of the drug *in vitro*. Ismail *et al*. (1996) also demonstrated that worms resistant to the effects of PZQ *in vivo* showed a significant reduction in the responses of PZQ *in vitro*.

In conclusion, we have shown that resistant adult worms (produced from *S. mansoni*-infected *B. glabrata* snails subjected to PZQ drug pressure) exhibit less tegumental damage and less inhibition of the excretory system than normal parasites. Further work on cercariae and other larval stages of normal and resistant isolates would be valuable.

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