Australian Journal of Basic and Applied Sciences, 2(3): 677-684, 2008
ISSN 1991-8178

Miracidial Assay as a Simple, Inexpensive Biological Test for Sensitivity to Praziquantel (Pzq) Using Field and Laboratory Pzq-susceptible and Insusceptible *Schistosoma Mansoni* Isolates

[1]S. Botros, A. Sabra and [2]S. William

[1]Pharmacology and [2]Parasitology Departments, Theodor Bilharz Research Institute, Giza, Egypt.

Abstract: Miracidial response of laboratory *S. mansoni* isolates and *S. mansoni* isolates from patients with different susceptibility to PZQ as a possible correlate of susceptibility to drug assessed by estimating the drug ED_{50} were investigated. Using flat bottom 24 wells microplate, a fixed number of miracidia (6-8) was placed in separate wells and 20 µL PZQ in different concentrations (10^{-3} M -10^{-6}) was added. Results revealed that, laboratory *S. mansoni* isolates with history of insusceptibility to PZQ (EE2, EE10 & ER5) possessing significantly increased drug ED_{50}s (200, 306 &167 mg/kg) compared to control (MOC and CD) isolates (82 &76 mg/kg) revealed significantly increased mean miracidial drug EC_{50} in one (EE2) out of the three PZQ insusceptible isolates (33.3%). Isolates retrieved from patients cured after the 1st dose of PZQ revealed drug ED_{50}s of 73 to 114 mg/kg with a mean of 90 mg/kg and drug EC_{50} of 32 to 665 $Mx10^{-6}$ with a mean of 312 $Mx10^{-6}$. On the other hand, isolates retrieved from patients needing more than a treatment with PZQ to be cured (ED_{50S} from 210 to 475 mg/kg with a mean of 346 mg/kg) revealed significantly increased drug EC_{50} (84 to 4435 $Mx10^{-6}$ with a mean of 2148 $Mx10^{-6}$) in five out of six patients (83%). Laboratory and field *S. mansoni* isolates insusceptible to PZQ, as denoted by increased drug ED_{50}, revealed increased miracidial drug EC_{50} in 33.3% and 83.0% respectively. Correlation analysis between drug ED_{50} *in vivo* and miracidial drug EC_{50} *in vitro* revealed positive correlation for both the laboratory and field isolates, yet correlation was only significant for field isolates.

Key words: *Schistosoma mansoni,* praziquantel ED_{50} and miracidial drug EC_{50}

INTRODUCTION

Praziquantel (PZQ) is the only drug available to treat schistosomiasis in many parts of the world (Cioli, 1998; 2000; Doenhoff, *et al.,* 2000; 2002). Unfortunately, resistance to PZQ may be emerging among schistosomes. Clinically, reduced cure rates and failure of treatment after PZQ in Senegalese, Kenyan and Egyptian patients have been reported (Fallon *et al.,* 1995; Stelma *et al.,*1995; Ismail *et al*., 1996). *In vivo* and *in vitro* studies on *S. mansoni* isolates retrieved from villagers not responding to treatment suggested that resistance to PZQ can occur (Stelma *et al.,* 1995; Ismail *et al*., 1996 & 1999; William *et al.,* 2001 a & b; William and Botros 2004). The conventional way to detect resistance in murine schistosomiasis established by Bruce, *et al.* (1987) was to place isolates retrieved from patients not responding to treatment in experimental animals. Bruce *et al.* (1987) in their protocol to detect resistance stated that a standardized protocol is required for use in the demonstration of schistosome resistance so that data from different laboratories and regions could be comparable. In their suggested protocol, they agreed for an: Outbred mice and that animal to be infected should be given a cercarial dose by tail immersion which usually ranges from 80 to 100 cercariae/mouse. At six to eight weeks after infection, groups of at least six mice should be dosed orally with test drug suspended in 25% glycerol and 1% Cremophor. They added that, mice should be examined post-mortem and the numbers and conditions of worms and the oogram changes noted in the control and treated mice should be recorded. The time of post-mortem examination suggested should allow for the death of all drug-damaged worms. They suggested 14 days after treatment as time of sacrifice when oxamniquine and praziquantel treatment was conducted and 56 days after treatment when oltipraz was given. They added that results should be expressed as percentage worm reduction in treated groups compared to controls. This suggested protocol requires long time, expensive materials and instruments. PZQED_{50} or the dose at which the worm burden is decreased by

Corresponding Author: S. Botros, Pharmacology Department, Theodor Bilharz Research Institute, Giza, Egypt.

50% has been used to assess of sensitivity to drugs (Cioli *et al.,* 2004). Although, it is an accurate measure, allowing comparison between different isolates with different drug sensitivity yet, it is still a lengthy and costly experiment. A simple, quick and economic assay that can detect resistance to treatment in schistosomiasis has been always the dream of researchers in endemic areas. The *in vitro* response of miracidia to PZQ revealed that not only miracidia are more sensitive to PZQ than adult worms but also cercariae (Coles, 1979 & Xiao *et al.,* 1985). A simple rapid test in a trial to detect PZQ resistance in *S. mansoni*, involving effect and change in the shape of miracidia upon exposure to PZQ was evaluated in China by Kenworthy, *et al*. (2003). This test involved not only the use of miracidia but also eggs, since these are the stages of the parasite life cycle which can easily be obtained from the faecal material of infected humans.

This work investigates the possibility to detect resistance to PZQ using a cheap, simple inexpensive biological assay which is the *in vitro* miracidial response to antischistosomal drugs using miracidia of laboratory *S. mansoni* isolates insusceptible and susceptible to PZQ. Also miracidia retrieved from villagers responding and not responding to PZQ treatment will be examined the same way in a trial to find out whether or not, this test could be applied in infected humans.

MATERIALS AND METHODS

S. mansoni isolates:

Laboratory isolates:

Five *S. mansoni* isolates were used, two of these isolates were PZQ-susceptible (CD and MOC) and three were PZQ-insusceptible (EE2, EE10 and ER5). The PZQ- insusceptible isolates were obtained from villagers not cured after three doses of PZQ (40 mg/kg, 40 mg/kg & 60 mg/kg). The PZQ susceptible *S. mansoni* isolate (MOC) was obtained before treatment from an Egyptian villager cured after the first dose of PZQ (Ismail, *et al.,* 1996). The second sensitive *S. mansoni* isolate (CD), was an isolate which has never been exposed to PZQ. These isolates were maintained at the Schistosome Biological Supply Centre, Theodor Bilharz Research Institute, Egypt by repeated passages through *Biomphalaria alexandrina* snails and mice since 1996.

Field isolates:

Field *S. mansoni* isolates from *S. mansoni* infected villagers were collected from four Governorates of Egypt; Gharbia (7 isolates), Kafr El-Sheikh (12 isolates), Giza (16 isolates) and El-Behera (3 isolates) during the period of 2002-2004.

Animals:

Swiss albino mice (CD-1), weighing 18-20 g were used. Animals were kept under standard conditions at Schistosome Biological Supply Center (SBSC) of Theodor Bilharz Research Institute (TBRI). Animal experiments were conducted in accordance with international valid animal ethics guidelines. Mice were infected each with 80±10 cercariae from each of the isolates to be tested using the body immersion technique (Liang, *et al.,* 1987).

Drug:

For *in vivo* studies, PZQ (Shin Poong Pharmaceutical Company, South Korea) was used as freshly prepared suspension in 2% Cremophor El in doses of 12.5mg/kg, 25mg/kg, 50 mg/kg, 100mg/kg and 200mg/kg. Animals were dosed orally by gastric gavage.

For *in vitro* studies, PZQ was prepared as a 10 mM stock solution in dimethyl sulfoxide (DMSO). A stock solution of 10^{-2} M was prepared at first and serial dilutions of 10^{-3}M, 10^{-4}M, 10^{-5}M, 10^{-6}M were prepared.

In vivo assessment of PZQ ED_{50} in animals:

CD-1 Swiss albino mice were infected with *S. mansoni* cercariae (80/mouse) shed out of snails infected with each of the laboratory and field *S. mansoni* isolates under investigation. At least 60 mice/isolate were used. Seven weeks post-infection, mice infected with each of the test isolates were divided into six groups. Five of these groups received PZQ orally in rising doses of 12.5 mg/kg, 25 mg/kg, 50 mg/kg, 100 mg/kg and 200 mg/kg for five consecutive days, while the sixth group was left without treatment to serve as infected untreated control. Two weeks post-treatment, mice were sacrificed, perfused and the worm burden counted (Duvall and DeWitt, 1967). The PZQ ED_{50} (The dose at which the worm burden was decreased by 50%) and the significance between different ED_{50} values were calculated using a computerized program "Pharm/PCS"

Version 4.2 (Pharmacologic calculation system) by a plot of the percent reduction in worm count (versus infected untreated controls) against the amount of the drug administered.

In vitro assessment of PZQ EC_{50} using miracidia:

To assess PZQ EC_{50} using miracidia of animals, liver and intestine of mice infected with PZQ-susceptible (CD and MOC) and -insusceptible (EE2, EE10 and ER5) *S. mansoni* isolates (individual liver and intestine/mouse/isolate/day) were digested in cold normal saline. For the assessment of PZQ EC_{50} using miracidia retrieved from eggs of patients, stool samples of seven, 12, 16 and three *S. mansoni* infected patients living at Gharbia, Kafr El-Sheikh, Giza and El-Behera governorates respectively were examined. Using Kato-Katz, 100-300gm stool samples, were placed in normal saline for 24 hours. Stool samples were filtered through coarse mesh sieves to separate fibers and undigested food particles. To recover eggs from either digested animal liver and intestine samples or patient stool samples, egg homogenate was furtherly filtered through standard testing sieves with 355, 150, 90 & 38 micrometer mesh diameter (Fisher Scientific Co., USA). Dechlorinated water was then added and samples were exposed to direct light for 2-3 hours for hatching of eggs to recover the miracidia.

The response of freshly hatched miracidia to PZQ was tested in 24 wells flat- bottom micoroplates. The wells were numbered and fixed number of miracidiae in fixed volume of dechlorinated water (100 µL) was added to each well. The miracidial behavior was observed before and after the addition of 20 µL of different PZQ concentrations to each well. A stock solution of 10^{-2} M was prepared at first and then serial dilutions of 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6} M PZQ were prepared. Each concentration was tested three times. The number of living miracidia and consequently the percentage mortality was calculated 30 minutes post PZQ addition. PZQ EC_{50} (the concentration at which 50% of miracidia were recorded to be dead) and the significance between different EC_{50} values were calculated using computerized program "Pharm/PCS" Version 4.2 (Pharmacologic calculation system) by a plot of the percent of miracidial mortality (versus living miracidia) against the concentration of the drug.

Statistical analysis:

Statistical analyses of the results were carried out using unpaired Student's *t*-test. The data were considered significant if $p<0.05$.

RESULTS AND DISCUSSIONS

Results:

Response of laboratory and field S. mansoni isolates to PZQ using drug ED_{50} after placement of isolates in mice and drug EC_{50} using miracidia after hatching of eggs.Laboratory S. mansoni isolates:

Table 1 shows the relationship between PZQ ED_{50} (*in vivo)* and miracidial EC_{50} (*in vitro)* using miracidia retrieved from eggs of mice infected with PZQ susceptible and insusceptible *S. mansoni* isolates. The two PZQ susceptible (MOC and CD) *S. mansoni* isolates showed ED_{50}s less than 85mg/kg. On the other hand, isolates with history of insusceptibility to PZQ (EE2, EE10 and ER5) showed ED_{50}s ranging from 167-306mg/kg. The mean PZQ EC_{50} of 6 animals examined over 6 days observation period was found to be significantly increased in one (EE2) out of the three PZQ insusceptible *S. mansoni* isolates showing significantly increased drug ED_{50} in mice infected with the same isolates. When individual mouse PZQ EC_{50} for each of the test isolates was compared to that of control isolate over the different observation days, sporadic significant differences were observed for some of the PZQ insusceptible isolates, with no fixed reproducible profile over the several examination days.

Statistical correlation analysis between miracidial PZQ EC_{50} (*In vitro*) and PZQ ED_{50} (*In vivo*) revealed a positive yet insignificant ($r = +0.551$) correlation (Fig. 1).

Field S. mansoni isolates:

Table (2) shows the PZQ ED_{50} and miracidial EC_{50} of patients from different governorates of Egypt. Miracidial EC_{50} of the seven patients examined at Gharbia governorate ranged from 24.1 to 3390.7 $Mx10^{-6}$ .ED_{50} of PZQ in animals was examined for three of those patients. The three patients positive for *S. mansoni* turning negative after the first treatment with PZQ in a dose of 40mg/kg. ED_{50}'s revealed of 81 , 114 and 80 mg/kg after placement of the isolates in mice. Miracidial EC_{50} for the same patients were 3390.7, 480 and 48 $Mx10^{-6}$ respectively.

Table 1: Relationship between $PZQEC_{50}$ and $PZQED_{50}$ in animals infected with PZQ-susceptible (MOC&CD) and insusceptible (EE2, EE10 and ER5) *S. mansoni* isolates.

S. mansoni isolates	Individual animal EC_{50} /day for 6 days							
	1st day	2nd day	3rd day	4th day	5th day	6th day	Mean $PZQEC_{50}$ M x10^6	$PZQED_{50}$ mg/kg
MOC	144.0	185.0	78.0	21.4	76.4	114.2	103.2±23.5	82
CD	6.5	1.0	409.0	106.0	132.0	20.0	112.4±63.4	76
EE2	541.0	359.9	559.0 *	325.0	92.0	23.0 *	316.0±91.0*	200 *
EE10	15.8 *	223.0	389.0	450.5	65.5	24.0	193.0±78.4	306 *
ER5	6.7 *	6.7 *	56.2	230.0	259.9	112.0	112.0±55.3	218 *

*Significant difference from control MOC *S. mansoni* isolate at P>0.05

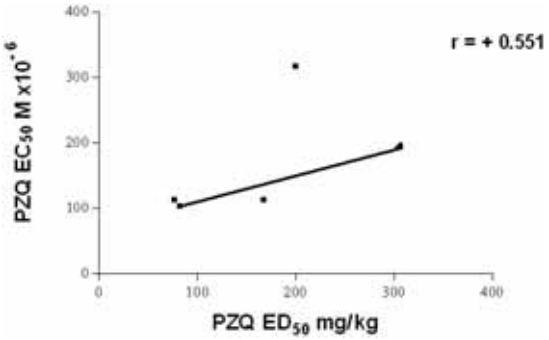

Fig. 1: Correlation analysis between PZQ ED_{50} after placement of isolates in mice and PZQ ED_{50} using miracidia retrived from eggs of mice infected with PZQ sensitive (MOC & CD) and insensitive (EE2, EE10 & ER5) S. *mansoni* isolates.

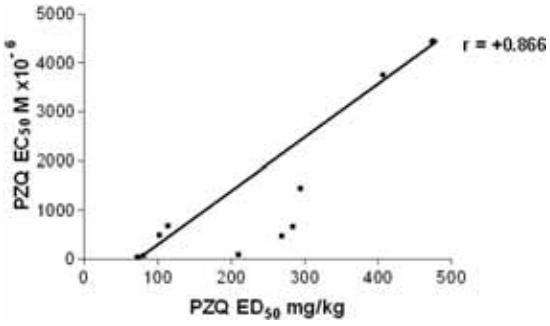

Fig. 2: Correlation analysis between PZQ ED_{50} after placement of isolates in mice and PZQ ED_{50} using miracida retrived from patients infected with PZQ sensitive and insensitive S. *mansoni* isolates.

Miracidial $PZQEC_{50}$ of 12 patients examined at Kafr El-Sheikh governorate ranged from 3.7 to 4435 $Mx10^{-6}$. ED_{50}'s of PZQ assessed in animals were examined for isolates from three patients only. One patient showing negative stool examination for *S. mansoni* eggs after two treatments with PZQ in a dose of 40 & 40 mg/kg, revealed PZQ ED_{50} of 294 mg/kg when the isolate from this patient was placed in animals. The second patient was not examined after treatment and the third patient was found to be passing eggs after the first treatment with PZQ in a dose of 40mg/kg. PZQ ED_{50} for the two patients were 475 and 284 mg/kg respectively, while EC_{50} for the same patients were 1438.2, 4435 and 648.3 $Mx10^{-6}$ respectively.

Miracidial $PZQEC_{50}$ of 16 patients examined at Giza governorate ranged from 15.0 to 8863.3 $Mx10^{-6}$. ED_{50}'s of PZQ in animals were examined in two of these patients only. One of the two patients diagnosed as positive for *S. mansoni* turning negative after the first treatment with PZQ in a dose of 40mg/kg, revealed PZQ ED_{50} of 73 mg/kg after placement in animals. The second patient passing eggs after the first treatment with PZQ in a dose of 40mg/kg showed PZQ ED_{50} of 210 mg/kg. The miracidial $PZQEC_{50}$'s were 32 and 84 $Mx10^{-6}$ respectively.

Table 2: Response to PZQ of miracidia out of eggs of *S. mansoni* infected villagers from different governorates (Gharbia, Kafr El –Shiekh, Giza and El-Behera) of Egypt.

Serial no. of patient	Sex	Age	Number of ova /g stool		Worms ED_{50}		Miracidium mg/kg	EC_{50} $Mx10^{-6}$
			Before treatment	After treatment				
				1st	2nd	3rd		
Gharbia governorate								
1	M	52	780	-ve				2609.8
2	M	47	400	-ve			81	3390.7
3	M	46	408	-ve				1150.9
4	M	17	480	-ve				1687.9
5	F	46	156	-ve				24.1
6	M	32	1200	-ve	-ve		114	480.5
7	M	54	492	-ve			80	31.8
X								**1339.4**
Kafr El –Shiekh governorate								
1	F	7	852	-ve				94.4
2	M	7	696	-ve				82.6
3	M	7	24	12				337.0
4	F	7	288	-ve				768.0
5	F	7	432	12				270.0
6	M	7	60	-ve				799.5
7	M	7	3360	12				3.7
8	F	7	144	-ve				16.12
9	M	9	396	36	46		294	1438.2
10	M	7	432	46				74.3
11	M	9	552	not			475	83.8
12	F	9	876	300	-ve	-ve	284	648.3
X								**384.7**
Giza governorate								
1	F	18	138	18				346.9
2	F	13	714	-ve				3792.0
3	F	26	324	-ve			130	71.0
4	M	65	234	-ve				420.1
5	M	18	138	-ve				454.0
6	M	16	132	-				36.1
7	F	16v	438	336				2139.3
8	M	50	50	-ve				1338.0
9	M	11	528	-ve				8863.3
10	M	12	42	-ve				36.3
11	M	16	168	-				352
12	M	16	528	-				1752.0
13	M	15	294	-				15.0
14	M	15	1362	-				60.3
15	M	17	252	-				2221.0
16	M	15	150	304	-	-	210	4435.2
X								**1645.8**
El-Behera governorate								
1	F	-	600	-	534	-ve	407	475.0
2	F	-	3612	-	468	-ve	269	37439.5
3	M	-	996	-ve	-ve	-ve	113	665.3
X								**12859.9**

Miracidial $PZQEC_{50}$ examined for three patients at El-Behera governorate revealed that, one patient turning negative after the first treatment with PZQ in a dose of 40mg/kg showed ED_{50} of 113mg/kg. The rest of two patients needing two treatments of PZQ in a dose of 40mg/kg &40mg/kg to be cured showed ED_{50},s of 407 and 269 mg/kg. Miracidial $PZQEC_{50}$ for the same patients were 665, 3744.0 and $475Mx10^{-6}$ respectively.

Statistical correlation analysis between miracidial PZQ EC_{50} (*In vitro*) and PZQ ED_{50} (*In vivo*) revealed a positive correlation (Fig. 2). That is whenever, there is a more need for PZQ to cure the animals (increased drug ED_{50} *in vivo*). There has been also, an increased drug need to kill miracidia (increased drug EC_{50} *in vitro*), ($r = +0.866$).

Discussion:

The standardization of simple, inexpensive biological test for the diagnosis of drug resistance in schistosomiasis to be used in endemic areas with limited resources is a key requirement. To test for how far miracidial response to PZQ is a reliable assay to judge resistance to treatment in infected humans, the test has been investigated first using *S. mansoni* isolates kept in the laboratory with different susceptibility to PZQ. The susceptibility /insusceptibility to PZQ has been decided for these isolates using estimates of drug ED_{50}, which is the effective dose required to kill 50% of the adult worms in mice infected with these isolates. Although estimation of drug ED_{50} as an indicator of susceptibility /insusceptibility to the drug after placement of isolates to be tested in experimental animals expresses well the state of susceptibility to the drug, yet it is a lengthy expensive experiment, in which at least 6 groups encompassing at least 6 animals / group harbouring the PZQ susceptible or insusceptible *S. mansoni* isolates are subjected to rising doses of PZQ. Sacrifice of the animals is usually 9-10 wks post infection at least two weeks after the treatment which should not be carried out before 7 weeks of infection, to guarantee worm maturity and hence drug effect. Miracidial response to PZQ on the other hand is a simple, cheap biological test in which the result could be obtained the same day of the experiment.

In this work, laboratory *S. mansoni* isolates were investigated, two of them (CD&MOC) were the control PZQ susceptible *S. mansoni* isolates because they were not at any time knowingly been subjected to PZQ. The three laboratory PZQ insusceptible (ER5, EE10 & EE2) *S. mansoni* isolates were isolates established in the laboratory with eggs from patients who had been treated three times (2x40 and 1x60 mg/kg) but were still not cured (passing eggs) after the third drug dose (Ismail, et al., 1996). After placement of isolates in mice, these isolates revealed significantly increased drug ED_{50}s of 167, 200 & 306 mg/kg. Using these laboratory isolates to test the miracidial response to PZQ as a test for detecting resistance as drug ED_{50} estimates, the test only showed significantly increased miracidial drug EC_{50} in one (EE2) out of three laboratory PZQ-insusceptible *S. mansoni* isolates with proven insusceptibility to PZQ, possessing increased drug ED_{50} when compared to values recorded in the control PZQ-susceptible isolates. Meanwhile, when this assay was examined for several days using individual animals in a trial to test for its reproducibility, miracidial drug EC_{50}s were not found to be significantly increased all over the examination days. Failure of reproducibility could be related to use of different animals with different frequencies of sensitive/insensitive parasites. This phenomenon was recorded previously (William and Botros, 2004), where correlation between *in vivo* drug estimates and *in vitro* worm muscle tension and Ca^{2+} uptake assay were different when the values were examined on a mean basis compared to individual values because of failure of expression of resistance in all the worms as a result of different frequencies of sensitive/insensitive parasite in the individual sample. Failure to show increased miracidial drug EC_{50} all over the several examination days in mice harbouring laboratory PZQ insusceptible *S. mansoni* isolates may be because not all animals comprising the group harbouring PZQ-insusceptible isolate were carrying the same frequency of resistant organisms. Meanwhile, the limited number of laboratory isolates examined makes it difficult to reach such a conclusion. Liang, *et al.* (2001) reported that differences between susceptible and resistance isolates does not encompass only worm response to the drug but also it showed clearly upon use of eggs, miracidia and cercariae. In the work of Liang, *et al.* (2000) resistance using miracidial response to PZQ was judged in an objective way one minute after the addition of 10^{-6}M PZQ, where change in the morphology in fixed and stained specimens was the parameter to detect resistance.

In this work, we have tried to record both change in miracidial morphology upon the addition of PZQ "objective" and also quantitate resistance by estimation of drug EC_{50} using miracidia as a parameter allowing comparison between the sensitivity of several isolates. We could not judge resistance using change in miracidial morphology to judge resistance. The miracidia were found vulnerable and more sensitive to PZQ than adult worms not allowing for any change in morphology to be recorded. To test for the applicability of miracidial response to PZQ as a simple, biological test to examine for resistance in infected humans, we have examined stool samples from different villagers at different governorates of Egypt with different response to PZQ over the period 2002 - 2004. The investigation in infected humans started by examining the miracidial response of patients to PZQ after hatching of eggs. We have tried to place as much as possible of the isolates examined for their miracidial response in animals to estimate in parallel to drug miracidial EC_{50}, the drug ED_{50} *in vivo*. Not all the isolates successfully completed their development in snail-mice and consequently limited number of isolates were investigated for their ED_{50} in parallel to the response of miracidia to PZQ (miracidial EC_{50}). For those successfully examined for their drug EC_{50}'s using miracidia and drug ED_{50} *in vivo* after placement of isolates in mice, the data revealed significantly increased drug EC_{50} in patients needing more than a PZQ treatment to be cured in 5 out of 6 patients upon testing the response of patients miracidia to PZQ. At the

same time the same patients showed significantly increased drug ED_{50}s (269, 284, 294, 407& 475mg/kg) when compared to patients responding to first dose of PZQ with drug ED_{50}s of 73, 80, 103 &114mg/kg. Meanwhile, correlation analysis between estimates of drug ED_{50} *in vivo* and EC_{50} *in vitro* using miracidia proved to be positive and significant contrary to what has been recorded for the laboratory isolates where the positive correlation recorded was not significant. Although the significant correlation recorded between drug ED_{50} in animals and EC_{50} using miracidia in the infected villagers could be due to the higher number of isolates examined (10 isolates versus 5 laboratory isolates), yet data reveals that this test is more promising in infected humans because, it was found in harmony with drug ED_{50} estimates in 83% of the samples, while for laboratory isolates, this percentage was only 33.3%. It would have been of great support to the findings if the examination of miracidia response to PZQ in infected humans was examined for the same patient over several days to look for reproducibility, but this chance was not possible for the investigators.

Miracidial response to PZQ tested by estimation of drug EC_{50} using miracidia after hatching of eggs from infected humans with different response to PZQ and in mice harbouring *S. mansoni* isolates with different susceptibility to the drug proved to be more promising in infected humans. Significant positive correlation was recorded between drug ED_{50} *in vivo* and EC_{50} *in vitro* for patients with different response to the drug. Failure of this correlation to be significant for laboratory isolates may be due to the less numbers of isolates tested. Meanwhile unreproducibility of the data over several examination days could be due to different animals examined over the different examination days with different percentage frequency of sensitive/insensitive parasites.

ACKNOWLEDGMENTS

This investigation received financial support from the INCO-II Programme of the European Commission (contract ICA4-CT-2001-10079).

REFERENCES

Bruce, Y.I., L.C. DE Souza, Y.S. Liang, C.C. Gerald, 1987. Drug resistance in schistosomiasis: A review. Mem. Inst. Oswaldo Cruz, Rio de Janeiro, 82: 143-150.

Cioli, D., 1998. Chemotherapy of schistosomiasis: an update. Parasitology Today, 14: 418-422.

Cioli, D., 2000. Praziquentel: is there real resistance and are there alternatives? Curr. Opin. Infec. Disease, 13: 659-663.

Cioli, D., S.S. Botros, K. Weatcroft-Francklow, A. Mbaye, V. Southgate, L. Tchuem Tchunte, L. Pica-Mattocia, A. Troiani, S.H. Seif el-Din, A.A. Sabra, J. Albin, D. Engels and M.J. Doenhoff, 2004. Determination of ED_{50} values for praziquantel in praziquantel-resistant and -susceptible *Schistosoma mansoni* isolates. International Journal for Parasitology, 34: 979-987.

Coles, G.C., 1979. The effect of praziquantel on *Schistosoma mansoni*. J. Helminthol., 53: 31-33.
Doenhoff, M.J., G. Kimani and D. Cioli, 2000. Praziquantel and the control of schistosomiasis . Parasitol. Today, 16: 346-366.

Doenhoff, M.J., J.R. Kusel, G.C. Coles and D. Cioli, 2002. Resistance of *Schistosoma mansoni* to Praziquantel: is there a problem? Trans. Roy. Soc. Trop. Med., 96: 465-469.

Duvall, R.H. and W.B. De Witt, 1967. An improved perfusion technique for recovering adult schistosomes from laboratory animals. Am. J. Trop. Med. Hyg., 16: 483-486.

Fallon, P.G., R.F. Sturrock, A. Capron, M. Niang and M.J. Doenhoff, 1995. Diminished susceptibility to praziquantel in a Senegal isolate of *Schistosoma mansoni*. Am. J. Trop. Med. Hyg., 53: 61-62.

Ismail, M.M., A.A. Metwally, A. Farghally, J.I. Bruce, L.F. Tao and J.L. Bennett, 1996. Characterization of isolates of *Schistosoma mansoni* from Egyptian Villagers that tolerate high doses of praziquantel., Am. J. Trop. Med. Hyg., 55: 214-218.

Ismail, M., S. Botros, A. Metwally, S. William, A. Farghaly, L.F. Tao, T.A. Day and J.L. Bennett, 1999. Resistance to praziquantel: direct evidence from *Schistosoma mansoni* isolated from Egyptian villagers. Am. J. Trop. Med. Hyg., 60: 932-935.

Kenworthy, J.D., P. Ye, G.C. Wu, H. Y.U., Y.Y. Shi, H. Li, G.C. Coles, 2003. Field evaluation of a test for Praziquantel resistance in *Schistosoma* SP. Vet. Parasitol., 113: 83-87.

Liang, Y.S., J.I. Bruce and D.A. Boy, 1987. Laboratory cultivation of schistosome vector snails and maintenance of schistosome life cycle. Proceeding of the first Sino-American symposium., 1: 34-48.

Liang, Y.S., G.C. Coles and M.J. Doenhoff, 2000. Detection of praziquantel resistant in schistosomes. Trop. Med. Inter. Helth., 5: 72.

Liang, Y.S., J.R. Dai, A. Ning, D.B. Yu, X.J. Xu, Y.C. Zhu and G.C. Coles, 2001. Susceptibility of *Schistosoma japonicum* to Praziquantel in China. Trop. Med. Int. Health., 6: 707-714.

Stelma, F.F., I. Talla, S. Sow, A. Kongs, M. Niang, K.B. Polman, A.M. Deelder and B. Gryseels, 1995. Efficacy and side effects of praziquantel in an epidemic focusy of *Schistosoma mansoni*. Am. J. Trop. Med. Hyg., 53: 167-170.

William, S. and S. Botros, 2004. Validation of sensitivity to praziquantel using *Schistosoma mansoni* worm muscle tension and Ca^{2+} -uptake as possible *in vitro* correlates to *in vivo* ED_{50} determination. International Journal for Parasitology., 34: 971-977.

William, S., S. Botros, M. Ismail, A. Farghally, T.A. Day and J.L. Bennett, 2001a. Praziquantel-induced tegumental damage *in vitro* is diminished in schistosomes derived from Praziquantel-resistant infections. Parasitology, 122: 63-66.

William, S., A. Sabra, F. Ramzy, M. Mousa, Z. Demerdash, J.L. Bennett, T.A. Day and S. Botros, 2001b. Stability and reproductive fitness of *Schistosoma mansoni* isolates with decreased sensitivity to praziquantel. International Journal for Parasitology., 31:1093-1100.

Xiao, S.H., B.A. Catto, and L.T. Webster. 1985. Effects of Praziquantel on different developmental stages of *Schistosoma mansoni in vitro* and *in vivo*. Journal of the infectious Diseases., 151: 1130-1137.