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Biological and proteomic studies of *Schistosoma mansoni* with decreased sensitivity to praziquantel

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ABSTRACT

The impact of the laboratory induced *Schistosoma mansoni* with decreased PZQ sensitivity on the biological performance of its different developmental stages and the concomitant structural changes of adult worms' total proteins were investigated. PZQ exposed snails showed stoppage of cercarial shedding for eight weeks followed by progressive significant reduction of cercarial production along four successive weeks. In the vertebrate host, in comparison to *Schistosoma mansoni* susceptible isolate, inoculated cercariae with decreased PZQ sensitivity led to an evident decrease in male to female ratio associated with significant reduction in tissue egg counts and significant increase in dead egg percentage. Significant reduction in the fecundity was also determined. Interestingly, eggs from adult worms with decreased PZQ sensitivity showed two unique features as they found to be smaller and more spherical in addition to the observation of hourglass shaped miracidium in about 10% of the detected mature eggs. Proteomic analysis of adult worms with decreased sensitivity to PZQ using mass spectrometry revealed up-regulation of Ca^{2+} ATPase 2 and Hsp70. This study can point to the increase incidence of the neuroschistosomiasis due to the small size eggs of *Schistosoma mansoni* with reduced PZQ sensitivity. These worms can also impact the epidemiology in the field. The study can also provide help to elucidate underlying potential molecular mechanisms of resistance that could lead to possible strategies to reverse drug resistance.

1. Introduction

Schistosomiasis is one of the common chronic and debilitating parasitic diseases whose socioeconomic impact is only surpassed by malaria [1]. It affects approximately 290 million people worldwide, an estimated 93% of whom live in Africa [2,3]. Morbidity of the disease is mainly related to the density of eggs in the tissues [4]. Among Schistosoma species, *Schistosoma mansoni* (*S. mansoni*) is the most common species that infects humans mainly in Africa and South America. Its widespread distribution is permitted by the broad geographic range of susceptible species of the freshwater snail genus *Biomphalaria* that serve as obligatory hosts for its larval stages [5]. In Egypt, *Biomphalaria alexandrina* (*B. alexandrina*) is the intermediate host for *S. mansoni* [6,7].

There is no effective anti-schistosome vaccine until now and the disease is treated exclusively with praziquantel (PZQ), a drug that is developed in the 1970s [8] and shortly thereafter identified as the treatment of choice by the World Health Organization [9]. PZQ is active against all schistosome species with minimal side effects, administered orally and inexpensive [10]. Control programs in a variety of countries depending on mass drug administration (MDA) rely on PZQ as a cornerstone [11,12].

Repeated rounds of MDA using PZQ specially to school aged children for more than three decades have led to therapeutic failures of the drug at the curative dose, a single 40 mg/kg, in disease-endemic areas in African countries like Egypt [13] Senegal [14,15] and Kenya [16] raising the possibility of drug resistance. Moreover, failure of treatment especially in returning travelers from endemic areas may indicate that resistance to PZQ is a growing problem [17]. Resistance of *S. mansoni* to PZQ can be expressed in all stages of development and can be transmitted from the stages in the definitive host to those in the intermediate host [18].

Throughout the schistosome complex life cycle, the parasite undergoes remarkable changes in its morphology and biology. Different life cycle stages display adaptations to the movement between definitive and intermediate snail hosts and to parasitic and free-living

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environments. These biophysical approaches are of great importance on the parasite fitness, which is defined as survival, reproduction and transmission capability of the organisms between hosts [19]. Development, establishment and disappearance of drug resistance are likely linked to the fitness of the resistant parasites [20]. Reduce sensitivity to drug is usually associated with a fitness cost [21]. The magnitude of this cost could be estimated through studying the main biological parameters that influence resistance development such as cercarial production, infectivity, adult worms' reproductive success and fecundity.

Despite nearly 40 years of PZQ extensive use, much remains unknown about the drug precise mode of action, its metabolism in the host and its molecular targets [22]. In addition, resistance to PZQ has been found to have a quantitative feature, referring to the presence of different targets of the drug [23]. This raised questions relevant to the inter-relationship between the mechanisms of resistance development and the known mechanisms of drug actions.

Proteins are key players in many cellular processes. Their composition and interactions underlie the dynamic processes of life [24]. The ultimate aim of proteomics is to fully identify the proteins of interest in biological samples [25]. Mass spectrometry (MS) based proteomics [26] have become a reliable and essential tool for elucidating biological processes at the protein level. Tandem MS has achieved great successes in proteomics during the past 15 years and the novel, promising approach liquid chromatography-tandem mass spectrometry (LC–MS/MS) has enabled the identification of transporter proteins with high accuracy and sensitivity [27].

Couto et al. [28] have been able to use a process of drug pressure for selection of resistance in the laboratory. This allowed the authors of the present work to investigate the impact of a laboratory induced *S. mansoni* with decreased sensitivity to PZQ in the intramolluscan phase on the biological performance of its different developmental stages and how far its reproduction could be affected. Furthermore, using a proteomic approach, this work aimed at understanding how drug pressure affects the protein structure of parasite population with reduced sensitivity to the drug in a controlled setting, which could help to define potential mechanisms of drug resistance, and verify the presence or absence of a relationship between drug resistance and drug action. The findings of the current study could shed the light on the impact of the resistant parasite isolate in the field affecting the epidemiology and the control programs of the disease. Moreover, elucidate the potential mechanisms of resistance development could be necessary to determine the most efficient treatment strategy for infected populations.

2. Material and methods

2.1. Animals and parasites

Swiss strain albino mice, of both sexes, aged 4–6 weeks and each weighing 20–25 g previously infected with the Egyptian susceptible strain of *S. mansoni*, were purchased from Schistosome Biological Supply Center (SBSC), Theodor Bilharz Research Institute (TBRI), Giza, Egypt. Seven weeks post-infection, mice were sacrificed and perfused. Liver tissues were digested by overnight incubation in 4% (w/v) potassium hydroxide at 37 °C and the recovered eggs were used for maintenance of *S. mansoni* life cycle in laboratory snails.

Inbred Swiss albino mice raised and maintained at the Animal-unit, Department of Medical Parasitology, Faculty of Medicine, Alexandria University, Egypt, were used to confirm decreased sensitivity of the worms to PZQ, perform the biological study of *S. mansoni* developmental stages in the vertebrate host and to apply the proteomic assay on the adult worm stage. All work with laboratory animals was conducted in accordance with the Egyptian National Animal Welfare Standards and was approved by the Ethical Committee of the Faculty of Medicine, Alexandria University (Protocol approval number: 020732).

2.2. Snail source, maintenance and infection

Three hundred laboratory bred susceptible *B. alexandrina* juvenile snails about 3–4 mm were purchased from SBSC/TBRI, Giza, Egypt. They were maintained under suitable laboratory condition in Medical Parasitology Department, Faculty of Medicine, Alexandria University, Egypt, in transparent acrylic aquaria, each measuring 25 × 18 x 15 cm (length x width x height), each aquarium containing 50 snails. They contained five liters of well-aerated aged de-chlorinated tap water (DTW). Water was checked daily for one week to make sure that it was free of rotifer and other organisms. Water was changed twice a week and kept at 26–28 °C in an incubator. Fresh washed lettuce leaves were supplied as food every couple of days and soft chalk was added to all aquaria. Dead snails (lighter in color, immotile with retracted soft tissue) were regularly removed. Pieces of foam were placed in the containers for egg deposition [29]. The collected eggs from the digested liver of the purchased mice were exposed to light to stimulate miracidial release. The snails were exposed individually to eight- ten vigorously swimming freshly hatched miracidia under direct sunlight for 3–4 h and then were kept in the dark and maintained under previously described conditions [30,31].

2.3. Drugs

To induce parasites with decrease sensitivity to PZQ, PZQ powder (100 g of 99.5% pure, C19H24N2O2, white powder, molecular weight 312.40606 g/mol) was kindly provided by Alexandria Company for Pharmaceuticals and Chemical Industries, Egypt. PZQ was incorporated into mouse chow (purchased from the local marketing). The chow was ground up with calcium carbonate in ratio of 9:1. The ration was reconstituted with water until it become pasty. The snails were weighed individually to calculate the dose of the drug for each snail. The dose of PZQ to be administered per kg was incorporated into 100 mg food (the amount of food daily administered for each snail) [28]. The daily amount of food given each day was totally ingested by each snail, suggesting that the snails received all of the drugs offered in ration.

For *in vivo* confirmation of the development of reduced PZQ sensitive *S. mansoni*, 500 mg of PZQ powder was dissolved in one ml 70% ethanol and then suspended in phosphate buffer saline (seven milliliter) to make total volume of eight milliliter. Each mouse was given a single dose of PZQ (500 mg/kg) which was 0.2 ml of PZQ suspension [32].

2.4. Induction of reduced sensitivity to PZQ in the intramolluscan phase

Infected snails with *S. mansoni* were checked for cercarial shedding four weeks after infection. Two hundred shedding snails were divided equally into two groups; group A: infected PZQ unexposed snails were used as a source of cercariae susceptible to PZQ and group B: infected PZQ exposed snails. Each shedding snail in the exposed group (group B) was treated with 200 mg/kg PZQ twice per week for three un-successive weeks with one-week interval. During treatment, snails were individually maintained in glass beakers with DTW and the water was changed every 24 h after each treatment. The PZQ unexposed snails (group A) were kept under the same conditions of the treated groups, but without exposure to the drug.

2.5. Cercarial harvest

At the end of PZQ exposure of group B snails, fifty of the survived snails from each group served for cercarial harvest to check the cercarial response to PZQ *in vitro*, study their biological performance and to infect mice for assessing other *S. mansoni* life cycle stages. Snails were placed in a 200 ml-beaker containing water at 30 °C and kept under illumination at a distance of 50 cm from the light source. After 2 h, cercarial suspension was collected and cercariae in 50 µl of the suspension were stained by Lugol's iodine (1 g iodine, 2 g potassium

iodide in 100 ml sterile distilled water) and counted under a dissecting microscope. The average number of cercariae per ml was estimated.

2.6. Confirmation of PZQ reduced sensitivity status of S. mansoni

2.6.1. In vitro cercarial response to PZQ

The assay was performed on ordinary glass slides without using a cover slip. Six cercariae in 20 µl of DTW per drop, two drops per slide, and three glass slides for each group were examined (36 *S. mansoni* cercariae were evaluated for each group). Stock solutions of 0.1% DMSO in distilled water were used as drug solvent and as control, 10^{-6} M PZQ in 0.1% DMSO were prepared and stored for a maximum of two weeks in the refrigerator. In each drop on the slide, 20 µl of the PZQ solution were added to the cercariae. Cercariae from both groups were examined under ordinary microscope every ten minutes for one hour for their swimming activities. To avoid drying of the slides and the increase of the PZQ concentration and to stabilize the temperature during the 60 min examination period; slides were kept in a plastic box on a thin (3 mm) wet sponge. The temperature of the sponge was adjusted to 28 °C by adding warm water [33].

2.6.2. In vivo efficacy of PZQ against adult S. mansoni

Eighty mice were infected with 100 ± 10 cercariae/mouse by the paddling method described by Smithers and Terry [34]. They were equally subdivided into two groups: 40 mice were infected with cercariae shed from PZQ unexposed group of snails (group I) and the other 40 mice were infected with resistant cercariae shed from PZQ exposed group of snails (group II). Mice of each group were subdivided equally into two subgroups; subgroup a: infected untreated control group; subgroup b: infected PZQ treated group in which each animal was inoculated with single oral dose of 500 mg/kg. Drug was administrated against adult *S. mansoni* stage 42 days post infection. All mice were perfused from the hepatic and mesenteric vessels 49 days after cercarial challenge [34]. Total worm burden and percentage reduction was calculated and recorded.

2.7. Biological performance of different S. mansoni stages with reduced sensitivity to PZQ

2.7.1. Cercariae

The onset of the first cercarial shed after snail exposure to PZQ (group B) were monitored weekly. Afterwards, the re-shed cercariae from group B snails were evaluated for their cercarial count, activity and infectivity in mice simultaneously in comparison to the PZQ susceptible cercariae shed from group A snails.

2.7.1.1. Cercarial production. The total number of cercariae shed from individual PZQ unexposed (group A) and PZQ exposed (group B) snails were counted weekly for four successive weeks. Ten shedding snails from each group were separated individually in 25 ml beaker; containing 2 ml aged DTW, under direct sunlight for about two hours. After shedding, the snails were removed and the cercarial suspension was carefully shaken to obtain an even suspension for accurate count. Using a graduated pipette, 0.1 ml of this suspension was aspirated, stained with lugol's iodine to kill the cecariae to be easily counted using a dissecting microscope. The count was repeated three times for each snail then the mean total cercarial number/snail was calculated [35].

2.7.1.2. Cercarial activity. Evaluation of spontaneous swimming activities and presence of any attachment manner of susceptible and resistant cercariae that were shed from the PZQ unexposed (group A) and PZQ exposed (group B) snails were done under the ordinary microscope [36]. The free-swimming performance of un-attached cercariae was thoroughly examined for ten minutes starting immediately after shedding of the snails. Five replicate of cercariae were examined at the same time point for each group. This examination was repeated three times in three different sessions.

2.7.1.3. Infectivity in mice. Forty mice of subgroups Ia (infected with susceptible cercariae shed from PZQ unexposed group of snails) and IIa (infected with resistant cercariae shed from PZQ exposed group of snails), were used to determine the infectivity of cercariae shed from both groups of snails. The percentage of infected mice in each group was recorded.

2.7.2. Adult S. mansoni worms

Recovered adult worms from both subgroups of mice (subgroup Ia and subgroup IIa) were counted and sexed (Smithers and Terry, 1965), half of them was preserved for the proteomic assay while the others were additionally stained with Mayer's acid carmine stain which was prepared according to Pritchard and Kruse [37] and were examined under a light microscope equipped with camera lucida (x 10) for morphometric evaluation [38].

2.7.3. Eggs

2.7.3.1. Tissue egg count and size. Egg count in both liver and intestine obtained from the studied subgroups of mice (group Ia and group IIa) was done according to Cheever's technique in 1968 [39]. The mouse intestine and liver were digested by overnight incubation in 4% (w/v) potassium hydroxide at 37 °C. The digested tissue suspensions were thoroughly stirred and eggs were counted in 2 × 50 µl samples on microscope slides under 10× magnification. The result was presented as egg count per gram of tissue.

The fecundity of worms was measured as the mean number of intestinal eggs per female using the following equation [36]:

Fecundity = Number of eggs in one gram of intestine / Number of adult females

Ten eggs from digested tissues of each mouse from each group were examined under a light microscope equipped with perimeter scale (x10) to measure their diameters (vertical and horizontal diameters [38].

2.7.3.2. Oogram pattern. Three fragments, 10 mm each, of the middle part of the small intestine from the studied subgroups (Ia and IIa) were cut off longitudinally and processed for oogram. One hundred eggs were counted from each fragment and were classified into different stages of development; viable (immature and mature) or dead with different morphological features, according to Pellegrino et al. [40].

2.8. Proteomic study

2.8.1. Worm collection and preservation

This study was performed on *S. mansoni* adult susceptible and those with reduced sensitivity to PZQ (collected from mice of subgroups Ia and IIa, respectively). The harvested worms from each isolate were washed separately in phosphate buffered saline (PBS, pH = 7.4 ± 0.1, Media Lab) at 37 °C in order to remove any residual host proteins. After collection they were snap frozen and stored in liquid nitrogen (kindly provided by City for Scientific Research and Technology Applications, New Borg El Arab, Alexandria, Egypt) until use [41].

2.8.2. Lysis and protein extraction

Lysis buffer (pH 8.5) (Urea 4.2 g, Thiourea 1.5 g, Tris 78.7 g, Deionized water up to 10 Ml) (Sigma-Aldrich Chemie GmbH, USA and Bio-Rad, Hercules, CA, USA) was added into the tubes containing frozen *S. mansoni* adult worms. The worms were ground using a sterile glass rod. Cell disruption was achieved by sonication at 80 W for 10 s × 5 with intervals of 15 s, followed by the addition of lysis buffer to a final volume of 1 ml. Samples were maintained on ice during these procedures. The mixture was then centrifuged at 12,000 rpm for 40 min at 4 °C to remove insoluble material [42,43].This preparation method provides total cellular protein samples that are free of contaminating nucleic acids and free of protease activity. The protein extracts were

used for analysis of proteins by gel electrophoresis.

2.8.3. Determining protein concentration of cell lysates

The protein content in the supernatant was quantified by the Bradford method using Bio-Rad Protein Assay reagent (BioRad Laboratories, Inc., Hercules, USA), using bovine serum albumin (BSA) as the protein reference standard. Samples of aliquots were stored at −80 °C until use in proteomic analysis [44].

2.8.4. SDS-PAGE

This was done according to Link [44], Garfin [42] and Hong et al. [43]. All chemicals were purchased from Sigma-Aldrich Chemie GmbH, USA and Bio-Rad, Hercules, CA, USA. 8% SDS polyacrymide gel was prepared in duplicate. One was stained with SimplyBlue Safe stain (Invitrogen, UK) according to the manufacturers' instructions. Protein bands of interest were excised and digested for further analysis by mass spectrometry. The other one was unstained and proteins resolved with SDS-PAGE were transferred to nitrocellulose membrane for western immunoblotting. Protein molecular weight (MW) determination was done using standard protein MW markers (Bio Basic Inc, Canada) [42–44]. Analysis of the molecular weights of protein bands was done using TotalLab analysis software (Totallab TL100 v2009, Ireland) [45].

2.8.5. Western immunoblotting

In a second replicate SDS-PAGE gel, resolved proteins were transferred to a nitrocelluse membrane (Amersham Pharmacia Biotech, UK) according to Towbin et al. [46]. After visualizing the protein MW marker, the membrane was blocked with 5% skimmed milk in TBST (Tris-buffered saline with 0.5% V/V Tween 20) (Sigma, UK) overnight at 4 °C with gentle shaking on a roacker. Blocked membrane was washed three times with TBST (each five minutes with gentle shaking), then incubated with the primary antibody (rabbit anti-*S. mansoni* adult worm homogenate serum, kindly provided by Prof. Mike Doenhoff, University of Nottingham, UK) diluted 1:100 in TBST, for two hours at room temperature with gentle shaking. Nitrocellulose membrane was washed as above and then incubated with the secondary antibody (horse reddish perioxidase-conjugated goat anti-rabbit Ig G diluted 1:1000 in TBST, Sigma, UK) for two hours at room temperature. The immunoblot was developed using the 4-chloro-1- naphthol substrate (Sigma, UK) as described by the manufacturer [46,47].

2.8.6. Gel excision and digestion

Only two distinctive bands have been detected in the worms with reduced sensitivity to the drug in higher intensities relative to the susceptible and were, therefore, selected for subsequent proteomic analysis based on their relative intensity and their high molecular sizes (˜240 and 175 kDa). Most schistosome glycoproteins involved in PZQ resistance, such as P-glycoprotein and multidrug resistance-associated protein families have been shown to be of molecular weights > 100 kDa [48,49].The chosen SDS gel bands of interest were excised and cut into 1-mm cubes. These were then subjected to in-gel digestion, using a ProGest Investigator in-gel digestion robot (Genomic Solutions, USA) following the standard protocols. Briefly, the gel cubes were destained by washing with acetonitrile and subjected to reduction, with dithiothreitol, and alkylation, with iodoacetamide, before digestion with trypsin at 37 °C in 25 mM ammonium bicarbonate buffer. The peptides were extracted with 5% formic acid and concentrated down to 20 µl using a SpeedVac (Thermo Savant, USA) for further analysis by mass spectrometry (Sciex, Canada) [50].

2.8.7. Mass spectrometery

The peptides were then separated on an Acclaim PepMap 100 C18 trap and an Acclaim PepMap RSLC C18 column (Thermo Fisher Scientific, USA) using a nanoLC Ultra 2D plus loading pump and nanoLC as-2 autosampler (Exigent, Canada). The peptides were eluted with a gradient of increasing acetonitrile, containing 0.1% formic acid (5–40% acetonitrile in 16 min, 40–95% in a further 1 min, followed by 95% acetonitrile to clean the column, before re-equilibration to 5% acetonitrile). The eluent was sprayed into a Triple TOF (Time of Flight) 5600+ electrospray tandem mass spectrometer [51].

Analysis was done in information-dependent acquisition mode, performing cycles of 250 ms of MS followed by 100 ms MS/MS analyses on the 15 most intense peaks seen by MS. The MS/MS data file generated *via* the 'Create mgf file' script in PeakView (Sciex) was analyzed using the Mascot algorithm (Matrix Science), against an in-house database, NCBI/Swissprot.

2.9. Statistical analysis

The statistical package for social sciences (SPSS) software package version 20.0 statistical program was utilized for both data presentation and statistical analysis of the results. The Descriptive measures were used for the results included: count, percentage, arithmetic mean ($\bar{x}$), standard deviation (SD), minimum (Min.), maximum (Max), and median. F-test (ANOVA) was used for normally quantitative variables, to compare between more than two studied groups, and Post Hoc test (Tukey) for pair wise comparisons. Student *t*-test was used for comparison between means of different groups. Moreover, Mann-Whitney test: was used to calculate intensity of detected parasites in two groups (when the data did not follow normal distribution, a non parametric test was used).The level of significance chosen for this study was p equal to or less than 0.05 ($p \leq 0.05$) [52]. Percentage reduction was calculated for the studied parameters as follows; $P{=}C - V/C \times 100$ (where P: percentage reduction; C: mean number recovered from susceptible group; V: mean number recovered from reduced PZQ sensitivity group).

"The MS/MS data file was analysed using the Mascot algorithm (Matrix Science) against NCBInr/Swissprot database. Trypsin was used as the cleavage enzyme, carbamidomethyl as a fixed modification of cysteines and methionine oxidation as a variable modification. The peptide mass tolerance was set to ± 20 ppm and the fragment mass tolerance to ± 0.05 Da. The protein was accepted as identified if it had two or more peptides with Mascot Ion Scores above the Identity Threshold ($P < 0.05$)."

3. Results

3.1. Establishment of reduced sensitivity status of S. mansoni

In the snail exposed group (group B), the onset of the first cercarial shed was recorded at the 8th week post treatment with 200 mg/kg PZQ twice per week for three un-successive weeks with one-week interval.

3.1.1. In vitro cercarial response to PZQ

At the beginning of the experiment, the re-shed cercariae from PZQ exposed snails of group B showed no detectable differences regarding the cercarial activity in comparison to cercariae harvested from snails of group A. Immediately after adding PZQ, an initial increase in the cercarial activity of those of group B (prompt linear progressive and zigzag swimming motions) for about 20 s was observed.

After the first 10 min, 91.67% of cercariae from group B snails were completely unaffected with no recorded cercarial death while the remaining cercariae showed intermittent spins. In contrary, at the same duration point, 50.92% of cercariae from PZQ unexposed snails (group A) died. The cercarial death percentage in the same group increased up to 87.3% after 20 min of drug exposure with total cercarial death at 30 min examination point. On the other hand, increasing the time of drug exposure, the influence of PZQ on the harvested cercariae from PZQ exposed group (group B) showed slow and gradual enhancing effect.

At 30 min, up to 65.75% of cercariae harvested from group B (PZQ exposed snails) were still unaffected. The percentage of the affected

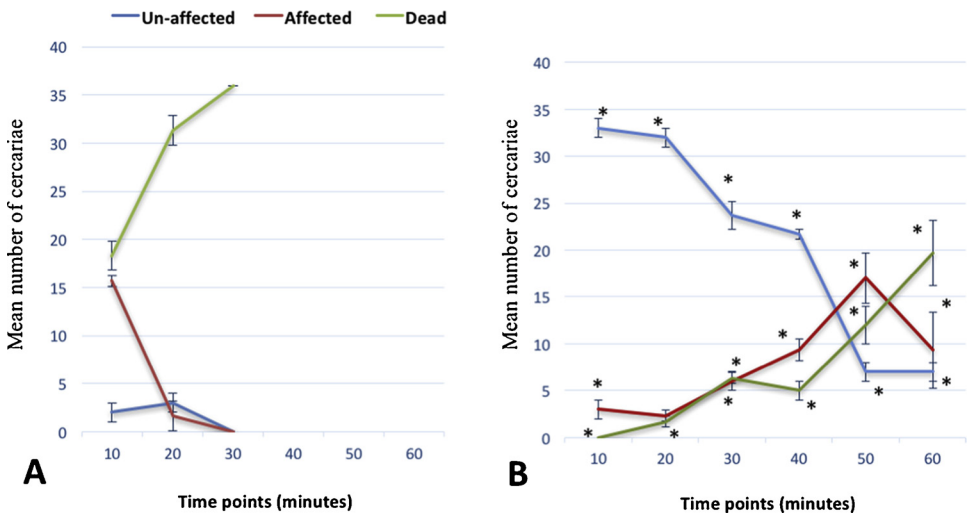

Fig. 1. *In vitro* effect of 5×10^{-6} M PZQ on the cercariae (n = 36) harvested from (A): PZQ unexposed control group (group A) and (B): PZQ exposed (group B) snails at a dose of 200 mg/kg PZQ twice per week for three unsuccessive weeks with one week interval. Lines represent mean count of un-affected, affected and dead cercariae after drug exposure every ten minutes for one hour. Error bars represent standard deviations of three cercarial sets per group. * *p* value for Chi square test.

cercariae was markedly increased at the points of examination of 50 and 60 min to reach about 80.55% in both time points, 33.33% and 54.64% out of them respectively reached complete death (Fig. 1).

3.1.2. In vivo efficacy of PZQ against adult S. mansoni

Oral treatment of mice infected with cercariae from PZQ unexposed snails with 500 mg/kg single dose of PZQ (subgroup Ib) resulted in a statistically significant reduction in the mean total worm load with a reduction of 89% in comparison to its control (subgroup Ia) (p = 0.004*). Meanwhile, marked reduction of the drug potency was detected against adult collected from PZQ treated mice infected with cercariae from PZQ exposed snails (subgroup IIb) in comparison to its control (subgroup IIa) with a total worm reduction of 31.1%. This result was statistically non significant (p = 0.172).

3.2. Biological performance of different S. mansoni stages with reduced sensitivity to PZQ

3.2.1. Cercariae

The onset of the first cercarial shed from the PZQ exposed snail group (group B) was recorded at the 8th week post treatment.

3.2.1.1. Cercarial production/week/snail. The total number of cercariae shed from each snail of PZQ unexposed and exposed groups (groups A and B) were recorded weekly for four successive weeks starting from the point of regaining the snails' power of shedding (8 weeks post treatment). The highest production of cercariae/snail was reported in the first week with a mean number of 191.17 ± 19.84 in PZQ unexposed group (group A) and 112.33 ± 17.99 in PZQ exposed group (group B). The percentages of reduction of the cercariae produced by the PZQ exposed group of snails were progressively increased along the four weeks of examination to reach its highest reduction value of 70.62% at the fourth week after re-shedding. These findings were statistically significant all over the investigating weeks in comparison to PZQ unexposed group (p ≤ 0.05) (Fig. 2).

3.2.1.2. Cercarial activity. Close observation of susceptible cercariae and those with reduced sensitivity did not prove the presence of any attachment manners of cercariae to each other by either head or tail along the studied duration. Four types of motions were observed in cercariae shed from the two groups of the studied snails (A and B); **1: Prompt linear progressive swimming:** Cercarial tail undulated, first perpendicular to the body and then the body proceeded forwards or backwards in a linear progressive direction. Angular deviations in the direction of motion frequently occurred; **2: Prompt Zigzag swimming**: Movement started with the tail, which undulated vertical to the

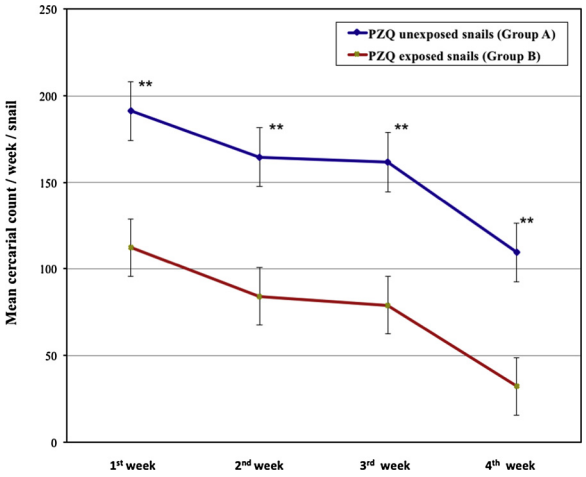

Fig. 2. Efficacy of laboratory induction of PZQ resistance in the intramolluscan phase on cercarial production/week/snail shed from PZQ exposed snail group (group B) using the regimen of 200 mg/kg PZQ twice per week for three unsuccessive weeks with one-week interval along four successive weeks of shedding. PZQ unexposed snails was served as control group (group A). Lines represent mean cercarial count per snail along the studied durations and error bars represent standard deviations of 10 snails per group, **P < 0.001, Mann–Whitney test.

position of the body, resulting in constant random changes in direction creating a zigzag pattern; **3: Pauses intervened by random movement:** Cercariae remained momentarily motionless with sparse sluggish head gliding action. This was interrupted by fast tail movements resulting in random directions; **4: Stationary pause**: There is cercarial immobility for pauses longer than normal.

On examination of susceptible cercariae shed from PZQ unexposed snails (group A), two swimming motion types were the most commonly noticed throughout the experiment; prompt linear progressive (1) and zigzag swimming motions (2). These two swimming patterns were temporarily interrupted by cercarial pausing and random motions (3). Regarding cercariae with reduced sensitivity shed from PZQ exposed snails (group B); straight line and zigzag swimming motions (1 and 2) were as detectable as random onward movements (3) throughout the period of examination. Moreover, stationary cercarial pauses (4) were exclusively identified in this group of snails.

3.2.1.3. Cercarial infectivity. The infectivity of PZQ susceptible cercariae and those with reduced sensitivity to PZQ was investigated by infecting mice. 95% of mice exposed to PZQ susceptible cercariae

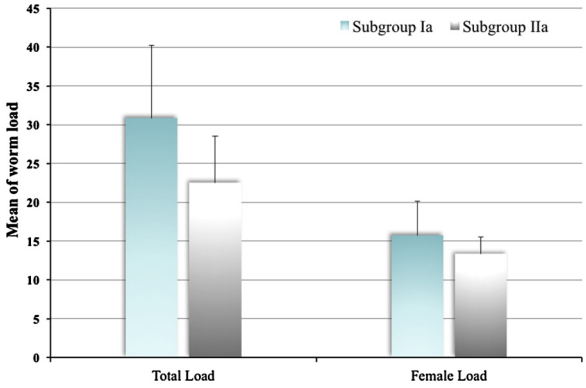

Fig. 3. Efficacy of laboratory induction of PZQ resistance in the intramolluscan phase on the total adult and female *S.mansoni* worm burden recovered from mice infected with PZQ resistant cercariae shed from PZQ exposed snails (subgroup IIa) in comparison to parasites recovered from mice infected with PZQ susceptible cercariae shed from PZQ unexposed snails (subgroup Ia). Columns represent mean counts for each subgroup and error bars represent standard deviations of 10 mice per subgroup, *P < 0.05, Mann–Whitney test.

were infected (subgroup Ia) while the infectivity rate was 90% in animals infected with cercariae with reduced sensitivity to PZQ (subgroup IIa). The difference in the infectivity powers between the studied groups was statistically non-significant ($P = 0.360$).

3.2.2. *S. mansoni* adult worms

Mice infected with PZQ cercariae with reduced sensitivity to PZQ (subgroup IIa) showed reduction of the total worm and female load with percentage reduction of 27.02 and 15.28, respectively. These reductions were statistically non significant ($p < 0.05$) (Fig. 3).

Regarding the morphometric study, no significant morphological changes were detected between adult female and male worms collected from animals infected with either susceptible cercariae or those with reduced sensitivity to PZQ (subgroup Ia and subgroup IIa). They showed regular tegument, uniform sized gut, normal pigment filling and normal reproductive organs.

The mean length of female and male worms collected from mice infected with PZQ susceptible cercariae (subgroup Ia) were 21.0 ± 3.30 cm and 14.0 ± 2.12 cm, respectively. The adult worms (female and male) harvested from mice infected with cercariae with reduced sensitivity to PZQ (subgroup IIa) showed statistically non significant increase in the perimetric measures with mean length of the female worms of 22.33 ± 2.66 with increase of 5.95% ($p = 0.492$) while the mean length of the male worms of 15.25 ± 1.6 cm with increase of 8.19% ($p = 0.247$) (Fig. 4).

3.2.3. *S. mansoni* eggs

3.2.3.1. Tissue egg counts and size. Statistically significant reduction was detected in the hepatic and intestinal counts of eggs harvested from mice infected with cercariae with reduced sensitivity to PZQ (subgroup IIa). The mean hepatic egg count in-subgroup IIa was 19,380 ± 6080 with percentage reduction of 44.18% while, the mean intestinal egg count was 23,167 ± 7440 with percentage reduction of 39.77% ($p \leq 0.05$) (Fig. 5).

Similarly, the fecundity of the female worms was reduced in the group of mice with reduced sensitivity (subgroup IIa). It was 24.38 ± 5.76 and 17.04 ± 3.84 in subgroups Ia and IIa, respectively with statistically significant reduction of 30.09% ($p \leq 0.05$).

In comparison to subgroup Ia, there was statistically significant reduction in the vertical diameters of *S. mansoni* eggs collected from mice infected with cercariae with reduced sensitivity to PZQ (subgroup IIa) with percentage reduction of 32% (Fig. 6A and B) and (Fig. 7) ($p \leq 0.05$). However, the difference in the horizontal diameters between the two groups was statistically non significant ($p < 0.05$) (Figs. 6C and 7).

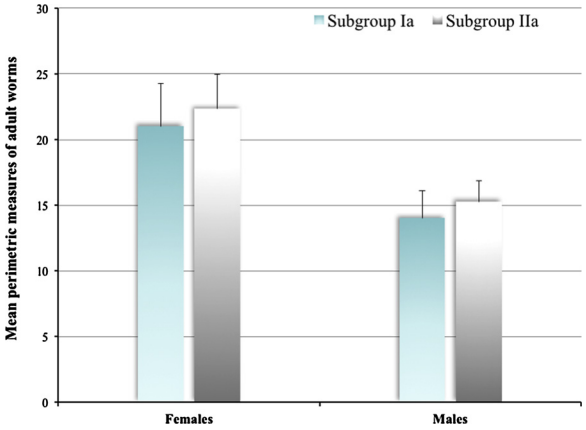

Fig. 4. Efficacy of laboratory induction of PZQ resistance in the intramolluscan phase on perimeters of the recovered adults *S. mansoni* female and male worms in centimeters (x 32 magnification) from mice infected with PZQ resistant cercariae shed from PZQ exposed snails (subgroup IIa) in comparison to parasites recovered from mice infected with PZQ susceptible cercariae shed from PZQ unexposed snails (subgroup Ia). Columns represent mean counts for each subgroup and error bars represent standard deviations of 10 mice per subgroup, *P < 0.05, Mann–Whitney test.

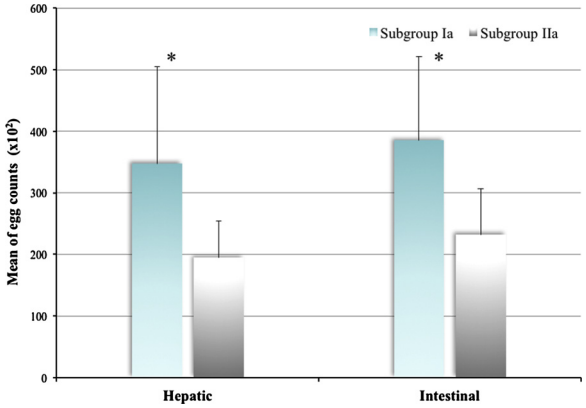

Fig. 5. Efficacy of laboratory induction of PZQ resistance in the intramolluscan phase on hepatic and intestinal tissue egg counts of *S.mansoni* in mice infected with PZQ resistant cercariae shed from PZQ exposed snails (subgroup IIa) in comparison to eggs recovered from mice infected with PZQ susceptible cercariae shed from PZQ unexposed snails (subgroup Ia). Columns represent mean hepatic and intestinal tissue egg counts ($\times 10^2$) per gram of tissue between the studied subgroups and error bars represent standard deviations of 10 mice per subgroup, *P < 0.05, Mann–Whitney test.

3.2.3.2. Oogram study. The mean percentage of mature eggs from mice infected with PZQ susceptible cercariae (subgroup Ia) was 33.92 ± 4.45 while it was 35.50 ± 29.24 in mice infected with cercariae with reduced sensitivity (subgroup IIa). This increase (4.45%) was statistically non significant ($p < 0.05$) (Fig. 8). Morphologically, mature eggs were oval, refractive with smooth boundaries and intact miracidium (Fig. 6D). While, exclusively in subgroup IIa, 10% of the miracidium showed hourglass appearance in which constriction of their embryo was obviously evident (Fig. 6E).

In comparison to mice infected with PZQ susceptible cercariae (subgroup Ia), there was a statistically non significant decrease by 7.2% in the immature eggs in subgroup IIa with the mean percentage of 59.17 ± 26.57 ($p < 0.05$).

In the studied subgroups (subgroup Ia and subgroup IIa), all forms of immature egg stages were detected. The majority of immature eggs in both groups were in the third stage, in which the embryo size

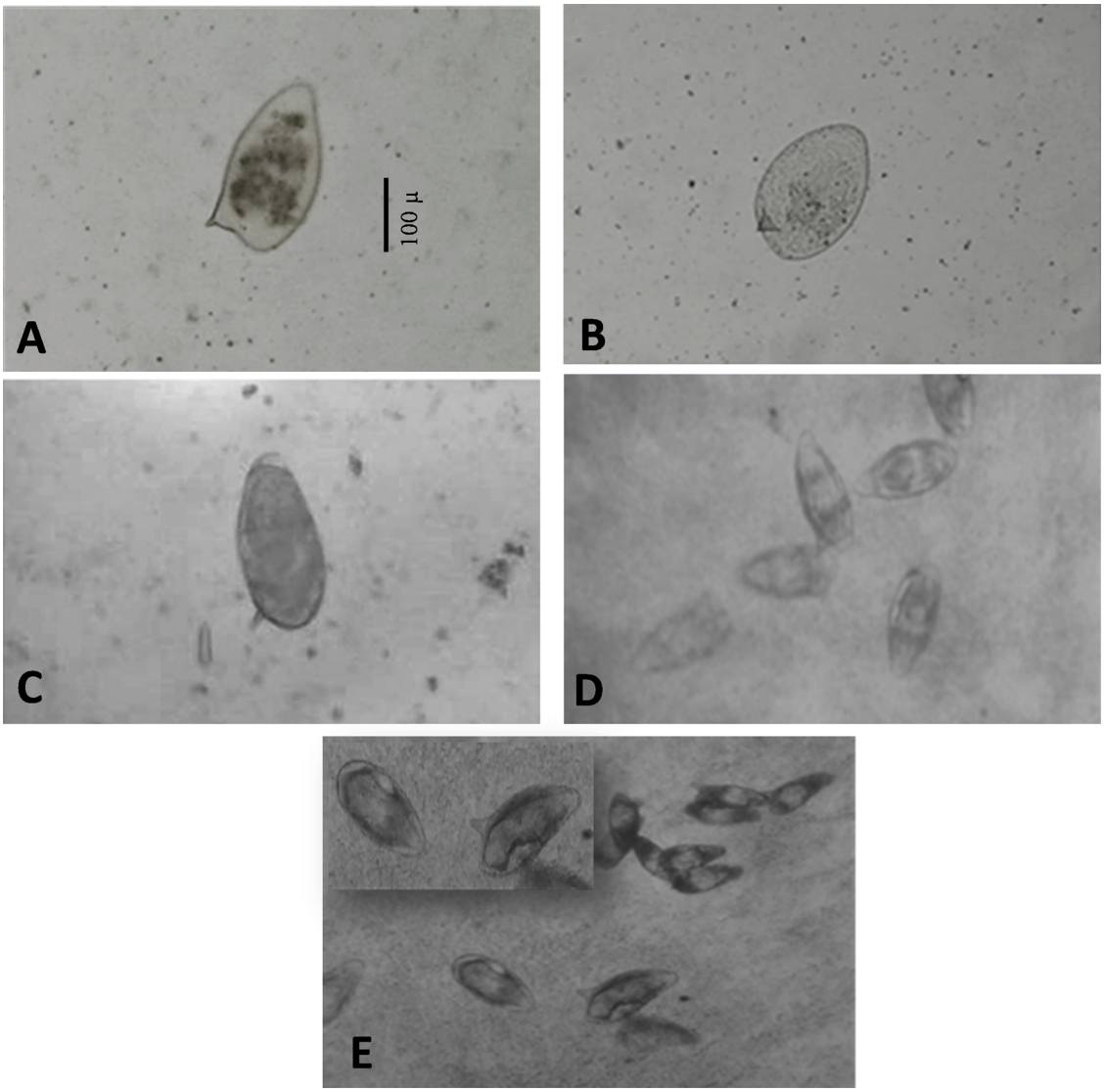

Fig. 6. Size and miracidial content of eggs of *S. mansoni* isolates (x100). (A and B): showing decrease in the vertical diameters of the isolate with reduced sensitivity to PZQ with converged lateral walls; (C): susceptible isolate with average diameters. (D): oval intact fully developed miracidium of susceptible isolate; (E): hourglass miracidium of the isolate with reduced sensitivity to PZQ.

corresponded to two thirds of the longitudinal diameter of the egg, and the fourth stage, where the developing embryo occupied nearly the whole of the egg shell.

Regarding the dead eggs, there was increase in the mean percentage of the dead eggs in the mice infected with cercariae with reduced sensitivity to PZQ (subgroup IIa). This increase was statistically significant with mean percentage of 2.32 ± 1.80 and 5.67 ± 3.56 in subgroups Ia and IIa, respectively ($p \leq 0.05$) (Fig. 8). The dead eggs appeared darkened, finely granular, semitransparent or with disintegrating miracidium. The darkened egg pattern was the most predominant one in both groups.

3.3. Proteomic assay

3.3.1. SDS-PAGE

This study was performed on adult *S. mansoni* susceptible and with reduced sensitivity to PZQ. The results of the SDS-PAGE were presented in Fig. 9A and Table 1. Two protein bands of MW of ˜241 and ˜175 kDa were detected in the less sensitive isolate but could not be seen in the susceptible in 8% SDS-PAGE gels (Fig. 9A, arrowed). These two bands were subsequently subjected to tandem mass spectrometry (MS/MS) to establish their identity. Analysis of the molecular weights of protein bands using Total lab analysis software revealed seven protein bands in the PZQ susceptible adult *S. mansoni* isolate with a molecular weight range of 45–246 kDa, while in the less sensitive isolate the number of bands were thirteen with a molecular weight range of 43–246 kDa.

Five common shared peptide bands in both isolates were detected at molecular weights of approximately 246, 84, 55, 47 and 46 kDa. In susceptible isolate, the two bands which were not detected in the resistant one were of molecular weights of 117 and 52 kDa, while in the less sensitive isolate, eight bands not detected in susceptible one were seen at molecular weights of approximately 241, 194, 175, 115, 113, 83, 53 and 44 kDa (Table 1). Protein bands having same molecular weight, showed different band intensities between the studied isolates (lane %). Protein intensity of all shared bands was higher in the less sensitive isolate except for the ˜246 kDa band which was relatively higher in the susceptible one.

3.3.2. Western immunoblotting

Proteins resolved with SDS-PAGE were transferred onto a nitrocellulose membrane, which was probed with rabbit antibodies specific to *S. mansoni* adult worm proteins. Western immunoblots showed

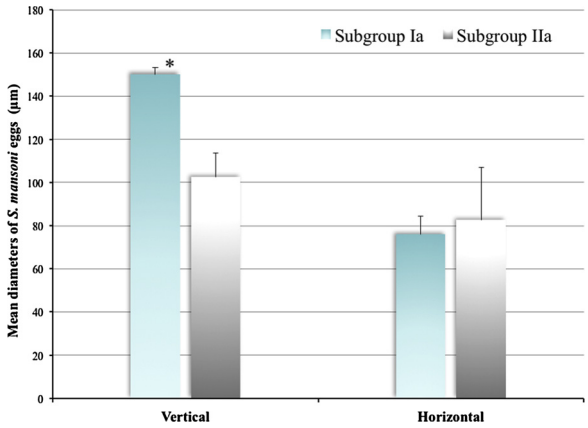

Fig. 7. Efficacy of laboratory induction of PZQ resistance in the intramolluscan phase on the vertical and horizontal diameters of *S.mansoni* eggs harvested from mice infected with PZQ resistant cercariae shed from PZQ exposed snails (subgroup IIa) in comparison to eggs recovered from mice infected with PZQ susceptible cercariae shed from PZQ unexposed snails (subgroup Ia). Columns represent mean egg diameters between the studied subgroups and error bars represent standard deviations of 10 mice per subgroup, *P < 0.05, Mann–Whitney test.

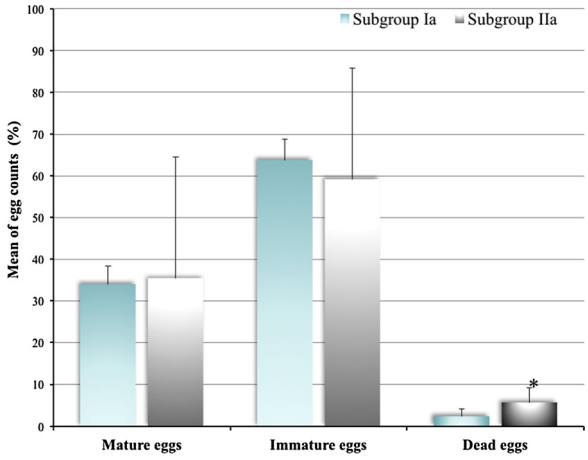

Fig. 8. Efficacy of laboratory induction of PZQ resistance in the intramolluscan phase on the quantitative and qualitative oogram patterns of *S.mansoni* eggs harvested from mice infected with PZQ resistant cercariae shed from PZQ exposed snails (subgroup IIa) in comparison to eggs recovered from mice infected with PZQ susceptible cercariae shed from PZQ unexposed snails (subgroup Ia). Columns represent mean count of eggs at their different developmental stages (mature, immature and dead) between the studied subgroups and error bars represent standard deviations of 10 mice per subgroup, *P < 0.05, Mann–Whitney test.

the same protein pattern detected by SDS-PAGE for both the susceptible and the less sensitive groups. Two protein bands of MW of ˜241 and ˜175 kDa subjected to MS/MS were also detected in the less sensitive isolate, while only trace reactivity at ˜241 could be seen (Fig. 9B, arrowed).

3.3.3. Tandem mass spectrometry (MS/MS)

The protein bands at ˜241 kDa and ˜175 kDa were manually and individually excised from SDS-PAGE gel and digested with trypsin. The resulting peptides were subjected to tandem mass spectrometry (MS/MS) for protein identification. Mascot search against the NCBInr database with the MS data for the ˜241 kDa band identified peptides for the *S. mansoni* calcium ATPase 2 protein with a Mascot score of 113 and a calculated mass of 112,539 Da (gi 3859490) (Table 2). The ˜241 kDa protein band probably corresponds to a *S. mansoni* Ca^{2+}-ATPase dimer.

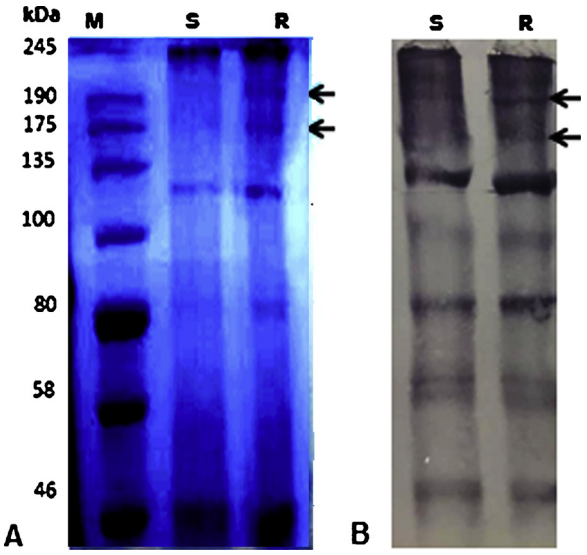

Fig. 9. Analysis of protein extracts from susceptible (S) and less sensitive (R) adult *S. mansoni* worm isolates using; A: 8% Simply Blue-stained SDS-PAGE; and B: Western immunoblotting. M, protein molecular weight marker. 0.1 mg BSA-equivalent protein was added to each lane). The bands at˜ 241 and 175 kDa are arrowed.

Table 1
Total lab analysis of SDS- PAGE of protein bands in susceptible and less sensitive worm isolates.

MW* in kDa	Susceptible worms		Less sensitive worms	
	Peak height	Lane %	Peak height	Lane %
246	115.2	0.58	109.6	0.21
241	–	–	122.7	0.57
194	–	–	165.24	1.28
175	–	–	175.13	2.23
117	183.5	1.83	–	–
115	–	–	188.85	1.11
113	–	–	190.60	24.88
84	186.9	1.27	189.74	1.23
83	–	–	189.12	1.10
55	159.7	0.47	165.68	1.44
53	–	–	164.38	0.48
52	156.22	0.76	–	–
47	107.54	1.06	110.24	1.23
46	101.81	0.88	107.50	1.46
44	–	–	110.85	1.55

*Mw: Molecular weight.

Eukaryotic Ca^{2+}-ATPase oligomers of a similar molecular size have been previously reported [53,54]. The protein band at ˜175 kDa band identified peptides for the *S. mansoni* HSP 70 with a Mascot score of 105 and a calculated mass of 68,331 Da (gi 10168) (Table 3). The protein band at ˜175 kDa probably corresponds to *S. mansoni* HPS 70 dimer [55].

4. Discussion

In the present study, some biological variances had been determined in the different developmental stages of *S. mansoni* with reduced sensitivity induced in laboratory at the intramolluscan phase. Herein, PZQ exposed snails showed stoppage of cercarial shedding for eight weeks after cessation of drug exposure followed by progressive statistically significant reduction of cercarial production along four successive weeks of examination. Mattos et al. [56] reported that, PZQ exposed sporocysts showed morphological and metabolic changes. Although the sporocyst remained alive and in motion, marked contraction of its

Table 2
MASCOT search output of tandem MS data from the ~241 kDa gel band.

gi: 3,859,490, calcium ATPase 2 [*Schistosoma mansoni*]

Mass: 112,539 Score: 113 Matches: 5 (5) Sequences: 5 (5)emPAI[a]: 0.24

Peptide match	Score	Expect	Rank	Unique
VPNTAGSGETGPR	70	0.00011	1	U
IGIFSEDEPTTGK	87	0.01	1	U
EFDALPIEEQR	37	0.037	1	U
ISAPVIFIDEILK	38	2.3e-05	1	U
FTDVPSTVGELK	53	0.042	1	U

Percentage sequence coverage: 6%. Matched peptides shown bold & underlined.

1 METAFSKTVD	EVLHHFESDP	ENGLSDEQIK	KQTAKFGPNE	LPAEESKAIW
51 QLVLEQFDDL	LVKILLMAAI	ISFVLALFEE	NEESISAFVE	PLVILLILIA
101 NAVIGVWQER	NAESAIEALK	EYEPEIAKVF	RKSHYGIQRI	KARELVPGDI
151 VEVSVGDKVP	ADMRIIKIMS	TTLRVDQSIL	TGESVSVIKF	TDAVPDPRAV
201 NQDKKNILFS	GTNIAAGKAR	GIVVSTGLMT	EIGKIRNQMM	DTEPDKTPLQ
251 QKLDEFGQQL	SKVISIICVA	VWAINIGHFN	DPAHGGSWLK	GAIYYFKIAV
301 ALAVAAIPEG	LPAVITTCLA	LGTRRMAAKN	AIVRSLPSVE	TLGCTSVICS
351 DKTGTLTTNQ	MSVCRMFIFS	KADDKAPEVH	HFEITGSKYA	PEGEVFLNGQ
401 KVESGEYDGL	VEVANICAMC	NDSAIDYNET	KHVYEKVGEA	TETALCCLVE
451 KMNVYKTSKS	GLSKKDLSMV	CNHQIQAMWN	KEFTLEFSRD	RKSMSVYLQV
501 KPAFASK**VPN**	**TAGSGETGPR**	MFVKGAPEGV	LDRCTFVRIG	NKKVPMTPPL
551 KAEIVKHVAS	YGTGRDTLRC	LALATCDAPV	NKAQMDLEDS	TKFVKYEQNL
601 TFVGVVGMLD	PPRMEVFDSI	ARCRKSGIRV	IMITGDNKAT	AEAICRR**IGI**
651 **FSEDEPTTGK**	SFTGR**EFDAL**	**PIEEQR**EACR	RARLFARVEP	MHKSKIVEFL
701 QEDGEVSAMT	GDGVNDAPAL	KKAEIGIAMG	SGTAVAKSAA	DMVLADDNFN
751 SIVLAVEEGR	AIYNNMKQFI	RYLISSNIGE	VVSIFLTAAL	GLPEALIPVQ
801 LLWVNLVTDG	LPATALGFNP	PDLDIMERPP	RNIKDPLISG	WLFFRYVAIG
851 VYVGCATVGA	AAWWFSLYPK	GPQLNYYQLT	HHMQCLAEKD	NFHGIDCHIF
901 ENPKPMTMAL	SVLVLIEMFN	ALNSLSENQS	LVAMPPWVNI	WLVLAMIVSM
951 SLHFLILEVE	FLSKVFQITP	LSLEEWFMVI	K**ISAPVIFID**	**EILK**LIARR**F**
1001 **TDVPSTVGEL**	**K**			

[a] emPAI, the exponentially modified protein abundance index.

Table 3
MASCOT search output of tandem MS data from the ~175 kDa gel band.

gi: 10,168, hsp70 homologue (619 AA), partial [*Schistosoma mansoni*]

Mass: 68,331 Score: 105 Matches: 5 (5) Sequences: 5 (5)emPAI[a]: 0.42

Peptide match	Score	Expect	Rank	Unique
VEIIANDQGNR	64	0.015	1	
DAGAIAGLNVLR	59	0.027	1	U
IINEPTAAAIAYGLDKK	74	0.0014	6	U
STAGDTHLGGEDFDNR	63	0.0034	1	
FEELNADLFR	71	0.0036	1	U

Percentage sequence coverage: 10%. Matched peptides shown bold & underlined.

FQHGK**VEIIA**	**NDQGNR**TTPS	YVAFTDSERL	IGDGAKNQVA	MNPTNTVFDA
TRLIGRRFDD	PSVQSDMKHW	PFEVTQVGGK	LKICVEYKGE	KKMFSPEEIS
SMVLTKMKEV	AESYLGRTVS	DAVITVPAYF	NDSQRQATK**D**	**AGAIAGLNVL**
RIINEPTAAA	**IAYGLDKK**VG	GERNVLIFDL	GGGTFDVSIL	TIEDGIFEVK
STAGDTHLGG	**EDFDNR**MVDH	FVKEFQKKYN	KDNRGNKRAL	RRLRTACERA
KRTLSSSAQT	NLEIDSLCDG	IDFYTVITRA	R**FEELNADLF**	**R**GTLDPVEKA
LRDAKMDKSQ	IHDIVLVGGS	TRIPKVQKLL	QDFFNGKELN	KSINPDEAVA
YGAAVQAAIL	SGDKCEAVQD	LLLLDVAPLS	LGLETAGGVM	TALIKRNTTI
PTKQTQTFTT	YSDNQPGVLI	QVFEGERALT	KDNNLLGKFE	LSGIPPAPRG
TPQIEVTFDI	DANGILNVSA	VDKGTGKQNK	ITITTNDKGRL	SKEEIERMVA
DADKYKAEDE	KQRDRVSAKN	SLESYVYTMK	QQVEGELKEK	IPESDHQVII
SKCEDTISWL	DVHQSAEKHE	YESKREELEK	VCAPIITKVY	QAGGMPGGMH
EASGAGGGSG	KGPTIEEVD			

[a] emPAI, the exponentially modified protein abundance index.

musculature, damages to the tegument and larger delimited area of its protective acidic vesicles were observed. These results could partially explain the interruption/reduction mechanism of cercarial shedding in snails exposed to PZQ. Furthermore, it is well known that PZQ is effective against cercariae. Therefore, snails exposure to PZQ destroyed mature and developing cercariae within the daughter sporocysts but had no apparent effect on daughter sporocysts; this may account for the eventual resumption of cercarial production [57].

Regarding cercarial activity, Coles [58], reported that upon exposure of susceptible cercariae to sub-lethal dose of PZQ, repeated body contractions without complete paralysis obviously occurred. In the present work, the examined cercariae were harvested from the PZQ exposed snails at the first shed after stoppage of drug exposure. The emerged PZQ less sensitive cercariae showed more predominance of

momentarily motionless with sparse sluggish head gliding action interrupted by fast tail movements than the susceptible ones with exclusively identified stationary cercarial pauses. These findings could be attributed to the fact that, intermittent locomotion is usually expected to increase energetic costs because of additional expenditure for acceleration and deceleration. Moreover, organism's endurance also can be improved by partial recovery from fatigue during pauses. Perceptual benefits can arise because pauses increase the capacity of the sensory systems to detect relevant stimuli [59].These facts and the recorded compensatory cercarial pauses additionally explained the retained cercarial power of infectivity which was reported in this study.

In the vertebrate host, although the decreased sensitivity to the drug had no effect on the total number of adult worms, an evident decrease in male to female ratio was reported in the current work. In mice infected with susceptible cercariae without drug pressure, adult sex ratios of *S. mansoni* were shown to be biased toward males despite the equal sex ratios of infecting cercariae. The adult male bias was attributed to the greater male infectivity of miracidia for snails and cercariae for mice [60]. On the other hand, Liang et al. [61] demonstrated differential susceptibilities to PZQ between male and female cercariae in the PZQ-susceptible and PZQ-resistant isolates of *S. mansoni*, using tail loss as a measurement of PZQ action. In PZQ-susceptible isolates, male cercariae have higher tail-shedding rates than that of female ones, but this phenomenon was not observed in the PZQ-resistant isolates.. This indicated that outgoing infective male cercariae after intra molluscan induction of resistance in this study could be much lower than infective female cercariae, which consequently explained the detected lower male to female adult worms' ratio.

In the current study, worms with decrease sensitivity to PZQ showed statistically significant reduction in tissue egg counts associated with significant increase in dead stage. Significant reduction of worms' fecundity was also determined. These could be attributed to the imbalance of adult male to female ratio. However Mati and Melo [62] reported that using total egg counts, including mature and dead eggs, may be inappropriate to consistently evaluate the fecundity of female schistosomes; authors referred to an early developmental stage of the viable immature egg as the most appropriate for measurement of worm fecundity. Herein, hourglass shaped miracidium was exclusively observed in 10% of the detected mature eggs of the worms with reduced sensitivity. Previously, it was reported that upon *in vitro* miracidial exposure to 3×10^{-6} M praziquantel, the middle part of the miracidia contracted, giving the appearance of an unequal dumbbell. These miracidia were completely non-infective to *B. glabrata* snails [58]. Currently, this unfamiliar form of miracidia enhances the authors' curiosity to investigate their infectivity power to the invertebrate snail host.

Interestingly, eggs from adult worms with reduced sensitivity in this work were found to be smaller and more spherical than those obtained from susceptible isolate due to significant reduction in the vertical diameters without significant difference in the horizontal diameters of the eggs. The reduction of the egg size could be of great impact, because small sized eggs may be lodged into distal ectopic tissues in the body especially the brain and the spinal cord causing mansonic neuroschistosomiasis (MN). At first regarded as a rare condition, spinal cord MN has been increasingly diagnosed in patients from endemic areas for schistosomiasis [63]. This raises a question about a link between the emergence of PZQ resistant *S. mansoni* and the increasing number of patients with MN. In this context, Pinto-Almeida et al. [64] obtained smaller sized *S. mansoni* eggs with smaller lateral spine from mice infected with PZQ resistant parasite. In their study, mice infected with PZQ resistant parasite showed some neurological manifestations. Moreover, William et al. [21] reported that, infection with less sensitive isolates also produced significantly less circulating schistosomal antigen in mice, suggesting that a decrease in the host immune response elicited by these worms could be one of the factors contributing to the alteration of parasite tropism.

It is still a matter of debate whether the resistance development is predominantly affected by the associated changes of the organisms' functional performance or the concomitant structural changes in the organisms could be important factors in triggering and maintaining the establishment. In the current study, proteomic analysis of the laboratory induced PZQ less sensitive *S. mansoni* adult worms isolate was done in comparison to its PZQ-sensitive one by SDS-PAGE and western immunoblotting. The detected protein bands of both isolates in 8% SDS-PAGE were analyzed using Total Lab Analysis software program. The two selected bands (at ˜ 241 and 175 kDa), that were only detected in the isolate with reduced sensitivity to PZQ by SDS-PAGE, were further subjected to mass spectrometry (MS) to identify their identities. Western blotting revealed only a trace of reactivity at˜ 241 in the susceptible isolate, while a distinct reactive band at this molecular size was clearly detected in the worms with reduced sensitivity. This could indicate higher expression of the ˜ 241 protein in the less sensitive group. Western immunoblotting is known to be more sensitive than Coomassie-stained SDS-PAGE gels in detecting small quantities of proteins [65]. Mascot search for the two bands against the NCBInr database identified peptides for the *S. mansoni* Ca^{2+} ATPase 2 and Hsp 70, respectively.

Fieldand laboratory isolates that exhibit reduced susceptibility of schistosomes to PZQ is on rise, this is true, but the real wonder is the mechanisms of this resistance development. The findings of this work revealed up-regulation of Ca^{2+} ATPase 2 and Hsp 70 in *S. mansoni* less sensitive isolate which could clearly verify the interrelationship between resistance development and the known PZQ mechanisms of action. It has been reported that PZQ mediates its anti-schistosomal effects through a rapid influx of calciuminto the worm, followed by calcium-dependent muscle contraction and paralysis. Ca^{2+} ATPase 2 could thus be intimately linked in opposing this PZQ-induced contraction.

The Ca^{2+} ATPases or calcium pumps are integral membrane proteins that couple the hydrolysis of ATP to the transport of Ca^{2+} ions across the membranes. They transport excess Ca^{2+} away from the cytosol, either out of the cell or into the sarco/endoplasmic reticulum. The control of low cytosolic calcium concentrations appears to be very important in *S. mansoni* cell physiology as a whole and in muscular contractility in particular [66,67]. Several subclasses of Ca^{2+} ATPase have been identified, the best characterized are those localized to sarco/endoplasmic (SERCAs). In *S. mansoni*, SERCAs are encoded by the SMA1 and SMA2 genes [68,69]. Cunha and Noel [70], described the link between the role of SARCAs in regulation of $Ca2^{+}$ homeostasis and the emergence of PZQ resistance in *S. mansoni.*

Furthermore, though the molecular target of PZQ has not been rigorously defined, substantial evidence suggests that PZQ interacts with schistosome voltage-gated Ca^{2+} channels [71]. These channels are membrane protein complexes that form Ca^{2+}-selective pores gated by depolarization playing a critical role in regulation of Ca^{2+}homeostasis. They provide the pathway for the Ca^{2+}influxes that underlie excitation-contraction coupling triggering the sensitivity to PZQ [72]. Paudel and Carlson [73] reported that an ionic ATPase activity is, in fact, associated with phosphorylase kinase and is regulated in parallel with its protein kinase activity. Phosphorylation of voltage-gated Ca^{2+} channel subunits by protein kinases (PKC) no longer confers sensitivity to PZQ [74]. This may indicate that overexpression of SMA2 could induce PZQ resistance through activation of PKC that block and modulate the properties of voltage-gated Ca^{2+} channels. Aragon et al. [75] drew attention to the crucial role of these channels in worms' susceptibility to PZQ. The authors reported that, using quantitative real time PCR, no evidence that the gene encoding β subunits of these channels is differentially regulated in juvenile schistosomes that are refractory to the drug. Moreover, it has been demonstrated that drug resistance in schistosomes might involve efflux pumps such as members of ATP-binding cassette transport proteins, including P-glycoprotein and multidrug resistance-associated protein families. The activity of these

pumps can be inhibited by calcium channel blockers such as phenothiazines or verapamil [76].

Herein, an additional protein band of *S. mansoni* Hsp70 was evidently over-expressed in the resistant isolate. Hsp70 proteins are one of the most abundant families of proteins expressed by all living organisms, both eukaryotic and prokaryotic [77,78]. They were found to be expressed in all life cycle developmental stages of *Schistosoma* species [79,80]. They are highly conserved and constitutively expressed molecules whose chief functions are as chaperone proteins that aid in (re) folding nascent and denatured proteins following exposure to stress [81]. Cellular stress other than heat, such as exposure to UV light, toxins, hypoxia and oxidative stress can induce their up-regulation [82]. In the present work, over-expression of *S. mansoni* Hsp 70 in the laboratory induced isolate with reduced sensitivity to PZQ could be due to the stressful drug pressure in the intramolluscan phase. This finding is in agreement with Aragon et al. [75] who stated that, schistosomes exhibited an increase in heat shock related transcript following exposure to PZQ. Additionally, they reported that treatment with PZQ might result in a molecular response similar to that observed when schistosomes undergo oxidative stress.

The relationship of hsp70 in the development of resistant schistosomes to the drug mechanisms of action is a curious one. Guanine nucleotide binding proteins(G proteins) are a family of proteins that act as molecular switches inside cells. G proteins and their G protein-coupled receptors(GPCRs) are involved in transmitting signals from a variety of stimuli outside a cell to its interior [83]. Chan et al. [84] demonstrate that PZQ is a GPCR ligand and suggest that the efficacy of this clinically important anthelmintic is based on beneficial effects of GPCR engagement with PZQ modulating signaling events in both host and parasite. Interestingly, it was reported that, up-regulation of Hsp70 could attenuate signaling from specific GPCRs as a part of the stress response to foster survival [85].

In a trial towards understanding the mechanisms of action of PZQ, Aragon et al. [75] explained the known lack of efficacy of PZQ against juvenile schistosoma worms. They demonstrated that, from 28 to 37 days post infection (timing coincides with juvenile worms development); schistosomes exhibited an increase in heat shock related transcripts following exposure to PZQ. At day 40-post infection, however, schistosomes could no longer respond transcriptionally to heat shock following drug exposure, indicating the onset of PZQ susceptibility against mature worms. In accordance to this finding, Hines-Kay et al. [86] reported that juvenile worms could be maintained and survived under PZQ exposure by increasing the transcription of stress response genes that have cytoprotective properties against apoptotic machinery at the pre- and post-mitochondrial level.

Metal ions and a calcium ionophore are among the stimuli that affect the transcription of some chaperone proteins. They need metal ions for ATP binding and hydrolysis, and for chaperone activity. Calcium-dependent auto-phosphorylation *in vitro* has been reported for proteins in the Hsp70 family, but its role *in vivo* remains unclear [87,88]. Given that the initial effects of PZQ on schistosomes include the influx of calcium ions, additional investigations are required to functionally characterize the interactions of calcium ions with *Schistosoma* Hsp70.

Having in mind the above-mentioned potential mechanisms, we assume that PZQ exposure stress-induced up-regulation of Hsp70 are likely involved in reducing the sensitivity of *Schistosoma* to PZQ. Biyani et al. [89] also revealed that, elevated expression of stress related proteins were observed in the resistant *Leishmania donovani* parasites to pentavalent antimonials.

To sum up, the present work revealed certain biological and structural changes as being associated with reduced sensitivity to PZQ in *S. mansoni*. Reduction in many life history traits including cercarial production, fecundity and total egg burden were in parallel with increase the number of dead eggs and adult male to female worm ratio imbalance. These traits can refer to reduced reproductive fitness of the less susceptible *S. mansoni* worms to PZQ, which may have implications for their future adaptation, transmission and pathogenicity. This could potentially explain why PZQ resistant *S. mansoni* have not been widely spread in spite of continuous therapeutic pressure in areas where resistant strains were previously identified [90]. However, PZQ resistance threatens the effectiveprevention and treatment of a wide range of infectious diseases. Results of this study may help to elucidate underlying potential molecular mechanisms of resistance which could in turn lead to insights into possible strategies to reverse drug resistance or develop alternative therapies.

Future experiments may seek to compare differential expression of proteins in the proteomes of the induced PZQ-less sensitive and PZQ-susceptible worms at a large scale utilizing a quantitative gel-based proteomic approach involving two-dimensional gel electrophoresis and MS/MS.

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Declaration of Competing Interest

No competing interests declared.

Appendix A. Supplementary data

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