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ULTRASTRUCTURAL CHANGES IN *Schistosoma mansoni* MALE WORMS AFTER *in vitro* INCUBATION WITH THE ESSENTIAL OIL OF *Mentha x villosa* Huds

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SUMMARY

Introduction: The essential oil *Mentha x villosa* (MVEO) has a wide range of actions, including antibacterial, antifungal, antiprotozoal and schistosomicidal actions. The present study aimed to investigate the ultrastructural changes of MVEO on the tegument of adult *Schistosoma mansoni*. **Materials and Methods:** Different concentrations of MVEO were tested on *S. mansoni* adult worms *in vitro*. Ultrastructural changes on the tegument of these adult worms were evaluated using scanning electron microscopy (SEM) and transmission electron microscopy (TEM). **Results:** The MVEO caused the death of all worms at 500 µg mL^{-1} after 24 h. After 24h of 500 µg mL^{-1} MVEO treatment, bubble lesions were observed over the entire body of worms and they presented loss of tubercles in some regions of the ventral portion. In the evaluation by TEM, *S. mansoni* adult worms treated with MVEO, 500 µg mL^{-1}, presented changes in the tegument and vacuoles in the syncytial matrix region. Glycogen granules close to the muscle fibers were visible. **Conclusion:** The ability of MVEO to cause extensive ultrastructural damage to *S. mansoni* adult worms correlates with its schistosomicidal effects and confirms earlier findings with *S. mansoni*.

KEYWORDS: Schistosomicidal activity; *Schistosoma mansoni; Mentha x villosa.*

INTRODUCTION

Schistosomiasis is a neglected disease widespread worldwide and poses a major public health problem. It is caused by parasitic trematode flatworms of the *Schistosoma* genus; moreover, *S. mansoni* is the only species found in Brazil[1,2].

The treatment of schistosomiasis is based on the use of praziquantel (PZQ); however, this drug seems ineffective against juvenile stages of *S. mansoni* and its extensive use in mass treatment of populations in schistosomiasis risk areas have favored the emergence of refractory strains of *S. mansoni* to conventional treatment with PZQ[3].

Therefore, the search for new drugs that can act against *S. mansoni* becomes relevant, and tools such as scanning electron microscopy (SEM) and transmission electron microscopy (TEM) have been employed to study the effects of compounds on the tegument of many helminths, especially *S. mansoni*[4]. In this context, the search for natural bioactive compounds against *S. mansoni* becomes an interesting alternative[2].

*Mentha x villosa*Hudson (Lamiaceae) has been used in traditional medicine due to its antiparasitic activity. It is known popularly as "hortelã-rasteira", "hortelã comum", or "hortelã-da-folha-miúda"[5]. Giamebil® is a commercial formulation presenting amebicidal (*Entamoeba histolytica*) and giardicidal (*Giardia lamblia*) activities, having as its active ingredient the dry extract from the leaves and stem of *M. x villosa*[6]. Recent studies have also demonstrated the efficacy of *M. x villosa* against *Trichomonas vaginalis*[7].

Essential oils (EOs) and extracts of aromatic plants have been recognized for many years as a great source of pharmaceutical agents and food additives[8]. Some studies show different biological effects caused by the *M. x villosa* essential oil (MVEO): antimicrobial[9], hypotensive and bradycardiac[10,11], cardiovascular[12-14], larvicidal[15], antinociceptive[16], cytotoxic, antitumor [17] and schistosomicidal activities [18],

Recent studies developed by our research group have demonstrated the *in vitro* schistosomicidal activity of MVEO[18]. However, there are no studies showing ultrastructural changes in *S. mansoni* adult worms after incubation with MVEO.

The aim of this study was to evaluate the ultrastructural changes in *S. mansoni* male worms after *in vitro* incubation with MVEO; the results shown here are supported by TEM and SEM.

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MATERIALS AND METHODS

Ethics statement: All experiments involving the use of experimental animals were performed in accordance to the ethical standards of the *Fundação Oswaldo Cruz* and were approved by the Animal Experimentation Ethics Committee (No. 06/2010).

Botanical material: Fresh leaves of the species *M. x villosa* were used. They were gathered from the Medicinal Plants Garden of the *Laboratório de Tecnologia Farmacêutica*, *Universidade Federal da Paraíba* between April and June 2011. They were identified and authenticated by Dr. F. J. Abreu Matos (*Laboratório de Produtos Naturais, Universidade Federal do Ceará*) and Dr. Raymond Harley of the Royal Botanic Gardens, Kew, England. A voucher specimen was deposited in the Prisco Bezerra Herbarium of the Federal University of *Ceará* (N. 14996).

Preparation of samples: To extract MVEO, 10 kg of leaves were steam-distilled for 8 h. The oil obtained (0.1%) was dried over anhydrous sodium sulfate in the usual manner and stored at 4 °C. We used a gas chromatograph coupled to a mass spectrometer (Shimadzu QP-5000) under the following analytical conditions: capillary column, OV-5 (30m × 0.25 mm × 0.25 µm); injector (Ohio Valley Specialty Chemical, Inc.), 240 °C; detector, 230 °C; electron impact, 70 eV; gas drag, He; flow, 1.0 mL/min; split, 1/20; program temperature, 60 °C – 240 °C at 3 °C/min; and solution injection volume, 1 µL (1 µL of essential oil per 1 mL of ethyl acetate). The compounds were identified by comparing their mass spectra using the GC-MS database system (Nist 62 lib.) and the Kovats retention index. The compounds were dissolved in 100% dimethyl sulfoxide (DMSO)[18].

Praziquantel was commercially available through Sigma-Aldrich (Sigma chemical, St Louis, MO, USA) with purity of 99.9%.

Obtaining and maintenance of *S. mansoni* adult worms*:* The BH *S. mansoni* strain (*Belo Horizonte, Minas Gerais*, Brazil) was used throughout this study. This strain was maintained in *Biomphalaria glabrata* snails and Swiss Webster mice in a laboratory at the *Centro de Pesquisas Aggeu Magalhães* of *Fundação Oswaldo Cruz*. Female Swiss Webster mice weighing 20 ± 5 grams were used as the definitive host, and were infected transcutaneously with about 120 cercariae of the BH strain, -as previously described[18], using the tail immersion technique. The animals were exposed for 1 h to the cercariae and they were subsequently kept under controlled temperature and light conditions. Furthermore, they had access to food and water *ad libitum*[19].

After fifty-five days of infection, *S. mansoni* adult worms were recovered from the mice by perfusion, washed in RPMI 1640 medium buffered with HEPES (20 mM), pH 7.5, supplemented with penicillin (100 IU mL^{-1}), streptomycin (100 µg mL^{-1}), and 10% fetal bovine serum (Gibco), and placed in petri dishes containing 2 mL of sterile culture medium[20].

***In vitro* studies of *S. mansoni* adult worms:** To assess the damage to the tegument, adult worms of *S. mansoni* were recovered from the hepatic portal system of the infected mice and left for a period of 2 h to adapt to the culture medium. MVEO isolate and compound was added in varying concentrations: a) MVEO (5, 10, 100, 250, and 500 µg mL^{-1}). Then, the worms were incubated at 37 °C in an atmosphere containing 5% CO_2[18].

As controls, *S. mansoni* adult worms were incubated in the presence of 1.6% DMSO in RPMI 1640 (negative control) or exposed to 0.5 µg mL^{-1} PZQ (positive control). All experiments were performed with three replicates. The final volume in each well was 2 mL. The parasites were collected and monitored for routine processing with SEM and TEM at 24, 48, 72, 96, and 120 h intervals. The worms were considered dead when there was no motion detected after 3 minutes of observation. SEM and TEM were used as tools to evaluate the morphological changes in *S. mansoni* adult worms after *in vitro* exposure.

Transmission Electron Microscopy (TEM): *S. mansoni* adult worms in each group were fixed (2.5% glutaraldehyde in sodium cacodylate buffer 0.1 M, pH 7.4). After fixation, they were washed with sodium cacodylate buffer 0.1 M, pH 7.4, and postfixed with 1% osmium tetroxide (OsO_4), in the same buffer, for 2 h in the dark. Then, samples were washed, counterstained block with 5% uranyl acetate in water. Dehydration was performed in a series of increasing acetone (30, 50, 70, 90 and 3 x 100%) for 30 min, each at room temperature and followed by embedding in Embed 812/Araldite resin (Electron Microscopy Sciences, Hartfield, PA) at 70 °C for 48 h. Semi-thin sections were stained with toluidine blue for morphological observation, while ultrathin sections were subsequently contrasted in uranyl acetate for 1 h and lead citrate for 10 min, and observations done in TEM (TEM 100CXII JEOL).

Scanning Electron Microscopy (SEM): The worms were incubated for 24 h and, after their death, they were washed with sodium cacodylate buffer (pH = 7.2), fixed with 2.5% glutaraldehyde (pH = 7.4) during 24 h, and then fixed with 1% osmium tetroxide for 1 h. The samples were dehydrated by an increasing amount of ethanol solution, dried in a critical point dryer, and then mounted on stubs and coated with gold using a sputter coater. The material was examined under a JEOL - 5600 LV microscope.

RESULTS AND DISCUSSION

The tegument of *S. mansoni* is an important structure for its survival since it is involved in nutrient absorption, secretion of metabolites, osmotic balance, and parasitic defense against the host immune system; this structure is an important target for drug action[21]. Some studies have documented damages to the tegument of *S. mansoni* caused by synthetic[22-24] and natural[25-26] antischistosomal compounds.

Ultrastructural analysis was performed on male worms for two reasons: females are frequently in contact with the host microenvironment and studies in the literature have shown that soft tissue alterations are more pronounced in males than in female worms[27].

Ultrastructural analysis of MVEO-induced surface damage in *S. mansoni*

Scanning Electron Microscopy (SEM): Control groups were not affected for up to five days of observation and all worms exhibited vigorous activity. It can be seen that male worms of *S. mansoni* in the control group presented the tegument covered with tubercules and tiny projections (spines). The back was long and contained the gynecophoral canal (gc). The area between the oral and ventral suckers did not have

any tubercles (tu), spines (sp) or sensory papillae (Fig. 1A-1B). The presence of a large number of tubercles with typical spines (Fig. 1C) as well as sensory papillae (st) (Fig. 1D) was observed.

In assessing the viabitility of the worms treated with PZQ, it was observed the death of all worms after 24h of incubation. Using SEM, it was identified that the *S. mansoni* adult worms treated with PZQ (0.5 μg mL^{-1}) showed spiraled body (Fig. 1E). In the tegument there was destruction of tubercules and spines, and many regions with ulceration (Fig. 1F).

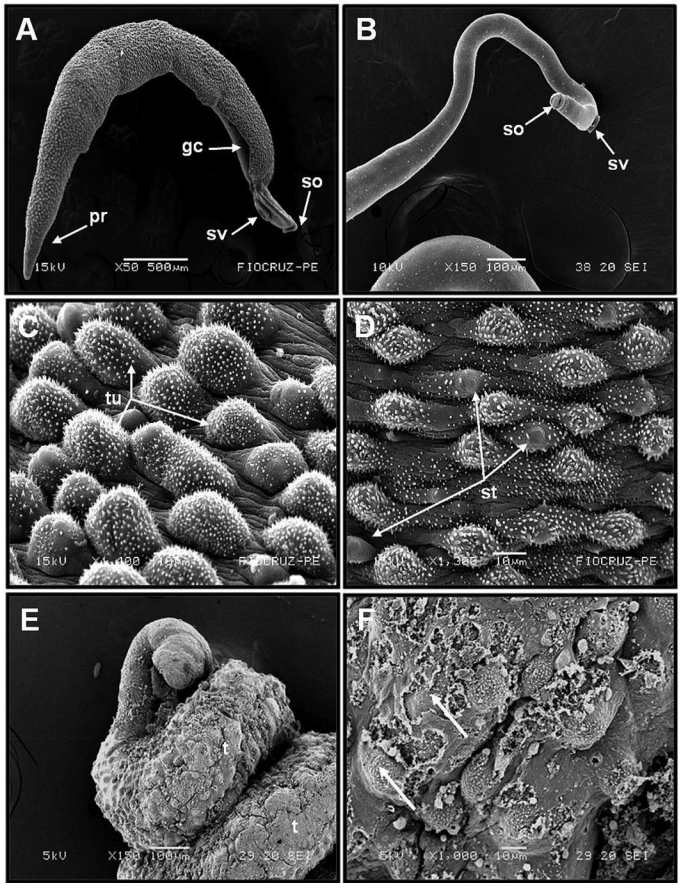

Fig. 1 (A-D) Electromicrographs of adult worms of *S. mansoni* without treatment. **(A)** Gynecophoric canal (gc), thinner portion of the worms located in the posterior region (pr), **(B)** while in the anterior region are located the oral (so) and ventral (sv) suckers. **(C)** In the tegument of male worms the presence of tubercles (tu) with spines was observed. **(D)** The presence of a large number of tubercles with typical spines, randomly distributed throughout the body (st) was identified. **(E-F)** Electromicrographs of adult male worms of *S. mansoni* treated with PZQ (0.5 μg mL^{-1}). **(E)** Adult worms presenting winding body and extensive destruction of the tegument (t). **(F)** Severe damage on the tegument with loss of spines and extensive ulceration with muscle exposition (arrows).

After 24 h of MVEO (500 μg mL^{-1}) treatment, bubble lesions were spread over the entire body of the worms (Fig. 2A), and the worms showed loss of tubercules in some regions of the ventral portion (Fig. 2B). After 48 h of incubation at 250 μg mL^{-1}, death of worms was observed, destroyed the oral sucker had been destroyed and the ventral sucker contracted (Fig. 2C). Tegument lesion severity increased after 72 h of MVEO treatment (100 μg mL^{-1}), which caused the basal membrane to become unprotected (Fig. 2D). Lower concentrations (5 and 10 μg mL^{-1}) were unable to cause mortality of *S. mansoni* adult worms after 120 h of exposure; however, changes in the tegument of the worms were recorded. At a concentration of 10 μg mL^{-1}, tegument erosion can be visualized at higher magnification (Fig. 2E) and in the worms treated with 5 μg mL^{-1} there was destruction of some tubercules (Fig. 2F).

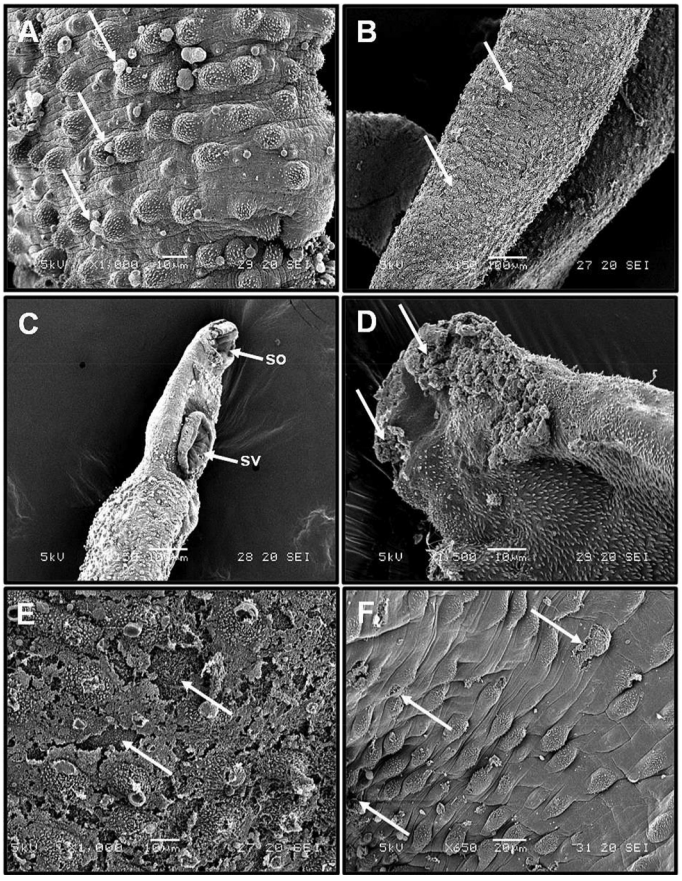

Fig. 2 (A-F). Electromicrographs of *S. mansoni* adult male worms of treated with different concentrations of MVEO. **(A)** After 24 h of MVEO (500 μg mL^{-1}) treatment, bubble lesions were spread over the entire body of the worms (arrow). **(B)** Ventral portion of the adult worms of *S. mansoni* after 24h of incubation with MVEO (500 μg mL^{-1}). The loss of tubercules in some regions was observed (arrows). **(C)** Anterior region of the adult male worms 48 h after incubation with 250 μg mL^{-1} of MVEO. Destruction of the oral (os) and ventral (vs) suckers. **(D)** Tegument lesion severity increased (arrows) after 72 h of MVEO treatment (100 μg mL^{-1}). **(E)** Tegument erosion (arrows) can be visualized at a higher magnification with no spines after 96 h of exposure to 10 μg mL^{-1} of MVEO. **(F)** Destruction of some tubercules after 120 h of incubation with 5 μg mL^{-1} of MVEO (arrows).

Transmission Electron Microscopy (TEM): The ultrastructural evaluation of *S. mansoni* adult worms by TEM revealed the presence of spines, characteristical matrix syncytial, and circular and longitudinal muscles in the subtegumentary region of the worms (Fig. 3A). The TEM analysis of *S. mansoni* adult worms treated with PZQ showed many changes like vacuoles in tuber, presence of vesicles in the syncytial matrix, and mesenchymal vacuolization (Fig. 2B).

In the evaluation by TEM, the *S. mansoni* adult worms treated with MVEO (500 μg mL^{-1}) presented changes in the tegument and presence

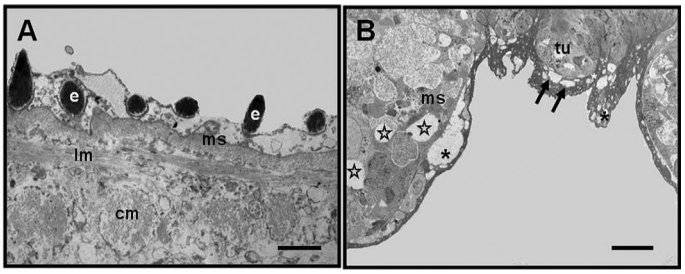

Fig. 3 (A-B). Electromicrographs of *S. mansoni* adult worms visualized by TEM. (A) In the control group, observe the spines (e). In the tegument, it is possible to identify the matrix syncytial (ms) and, in the subtegumentary region, it is possible to visualize the circular (cm) and longitudinal (lm) muscles. (B) In the group treated with PZQ (0.5 μg mL^{-1}) vacuoles (arrows) are observed in the tubercules (tu), presence of vesicles (asterisks) in the matrix syncytial (ms) and vacuolated mesenchymal (stars). Bars = 1μm.

of vacuoles in the syncytial matrix region. It was visible the presence of glycogen granules close to the muscle fibers (Fig. 4). Essential oils are highly enriched with compounds termed terpenoids that possess several biological properties such as schistosomicidal activity[21-25,28-30]. In recent years, a number of studies have been developed through *in vitro* screening using essential oils, extracts, and bioactive compounds from medicinal plants[27,29-32,] to identify a leading substance that can be used in preclinical trials for the treatment of experimental schistosomiasis [33-36].

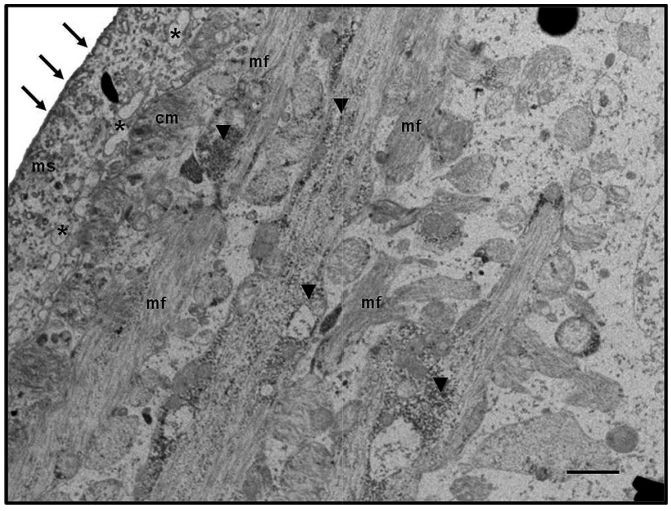

Fig. 4. Electromicrographs of adult worms of *S. mansoni* treated with MVEO (500 μg mL^{-1}). Observe changes in the tegument (arrows) and vacuoles (asterisks). In the matrix syncytial (ms), it is even possible to identify accumulation of glycogen granules (arrowheads) around muscle fibers (mf) and circular muscle (cm). Bar = 1μm

Generally, there was a marked difference between the morphology of worms treated with PZQ compared with MVEO, and compounds used individually. In the macroscopic examination, the *S. mansoni* adult worms, when exposed to PZQ, presented muscle contractions causing them to stay retracted or twisted. However, this behavior was not observed in the worms treated with MVEO or their constituents.

The adult worms incubated for 24 h with MVEO (500 μg mL^{-1}) showed damaged tegument and exposed musculature in some worms. These findings were identified by LORSUWANNARAT *et al*.[37] when testing plumbagin (100 μg mL^{-1}) in *S. mansoni* adult worms. EISSA *et al*.[24] paid attention to this same finding when evaluating the effect of miltefosine (10 μg mL^{-1}) on *S. mansoni* adult worms; however, the authors made observations after 120 h of incubation. LIMA *et al*.[4], when assessing the effects of allicin on the tegument of *S. mansoni*, describe the occurrence of ulceration on the parasite tegument after 120 minutes of incubation with 20 μg mL^{-1} of MVEO. Previously, BERTÃO *et al*[38] also evaluated the effects of miltefosine on *S. mansoni* adult worms by testing 200 μM and found the same results; however, they used only 12 h of incubation.

In the present study, after 48 h of incubation with 250 μg mL^{-1} of MVEO, morphological changes in the oral sucker and ventral suckers of *S. mansoni* adult worms were observed. Comparable to our findings, ALBUQUERQUE *et al*.[22] describe similar changes by treating *S. mansoni* with (Z)-3-(4-chloro-benzyl)-5-(4-nitro-benzylidene)-imidazolidine-2,4-dione (120 μg mL^{-1}); however, this data was observed following five days of incubation. OLIVEIRA *et al*.[39] draw attention to the occurrence of damage in the oral sucker of *S. mansoni* adult worms after exposure to the essential oil of *Baccharis trimera* (130 mg mL^{-1}) after 24 h of exposure. NEVES *et al*.[2] only observed the contraction of the ventral sucker when evaluating a derivative of thioxo-imidazolidine (100 μM) on *S. mansoni* adult worms after 3 h of incubation. KEISER *et al*.[40] also reported erosion of the tegument of female worms after exposure to mefloquine (10 μg mL^{-1}) after 1 h of incubation. Our results show that the lowest concentration of MVEO (5 and 10 μg mL^{-1}) caused less damage to soft tissue compared to the highest concentrations. The worms incubated in these concentrations generally showed destruction of tubercules with no spines. The same finding was observed by MANNECK *et al*.[41] when assessing the effects of mefloquine (10 μg mL^{-1}) on the tegument of *S. mansoni* adult worms. Recently, NEVES *et al*.[2], while evaluating a thioxo-imidazolidine, determined that after less than 1 h, adult *S. mansoni* vesicles showed that the increased number of these vesicles was proportional to the time of evaluation.

The mechanism by which MVEO exerts its *in vitro* anti-*S. mansoni* action is unclear. However, it has been reported that, because of the great different classes of compounds, usually essential oils may have no specific cellular target. Essential oils are typical lipophilic compounds, thus, the chemical substances of the oil, may pass through the cell wall, tegument, and cytoplasmic membrane damaging their structures and cellular membranes, which may lead to cellular lysis[23]. Regarding the therapeutic benefits of essential oils, so far, there are no studies that can give us a clear idea, or be accurate, about the mode of action. However, some effects are associated with loss of ions and reduction of membrane potential, as well as collapse of the proton pump and depletion of the ATP pool[23]. Furthermore, it has to be kept in mind that essential oils are complex mixtures of volatile constituents biosynthesized by plants[28]. The present study showed that these MVEO are capable of producing a range of ultrastructural changes in the *S. mansoni* tegument. Therefore, considering the anti-*S. mansoni* action of MVEO, it may be possible that the activity of its main constituents can be modulated by molecules present in the essential oil.

CONCLUSIONS

The ability of MVEO to cause extensive ultrastructural damage to

S. mansoni adult worms correlates with its schistosomicidal effects and confirms earlier findings with *S. mansoni*.

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