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Method

Application of Non-Fluorescent Dyes to Assess the Antischistosomal Effect of Antimalarial Drugs on *Schistosoma mansoni* Adult Worms

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SUMMARY: The possible emergence of praziquantel (PZQ)-tolerant and/or -resistant schistosomes requires the study and development of new antischistosomal drugs as alternatives to PZQ. The present study investigates the capability of 3 dyes—methylene blue (MB), neutral red (NR), and trypan blue (TB)—to assess the in vitro antischistosomal effect of antimalarial drugs on *Schistosoma mansoni* adult worms. *S. mansoni* adult worms were incubated in the medium alone as the control or in the medium supplemented with 10 μg/ml primaquine (PQ), artesunate (AR), or amodiaquine (AQ) for 5 days. Viabilities of the worms were observed following staining with MB, NR, or TB. The disparity of MB and NR staining among male and female adult worms treated with PQ, AR, and AQ correlated with the various levels of damage to the male and female worms. Furthermore, the severity of the damage to the adult worms treated with the 3 drugs appeared to be reflected in the TB staining status. The results indicate that the 3 non-fluorescent dyes can serve as useful complementary tools to assess the antischistosomal effect of antimalarial drugs.

INTRODUCTION

Extensive use of praziquantel (PZQ) in mass drug administration for the control of schistosomiasis has raised concerns about the possible emergence of PZQ-tolerant and/or -resistant schistosomes (1–3). Therefore, there is a need for new antischistosomal drugs as alternatives to PZQ. Many efforts over the past decade have sought to identify suitable alternatives. Artemisinin derivatives and current antimalarial drugs have potential antischistosomal activity (4–8). In vitro assessments of the related antischistosomal effects have relied mainly on microscopic observations of motility, morphological alternation, mortality, and egg production of schistosome adult worms treated with the candidate drugs.

Resorufin is a fluorescent dye. A recent study reported that resorufin was avidly taken up by the excretory system of *Schistosoma mansoni* adult worms. The authors suggested that resorufin might be valuable for the assessment of antischistosomal activity of test compounds (9). Non-fluorescent dyes, such as methylene blue (MB), trypan blue (TB), and neutral red (NR), have long been used to assess the viability of schistosome schistosomula, eggs, and miracidia (10,11). Nevertheless, to the best of our knowledge, the non-fluorescent dyes have rarely been used to screen for the antischistosomal effects of candidate compounds on schistosome adult worms.

The present study investigated the capability of MB, NR, and TB in the in vitro assessment of the antischistosomal effect of antimalarial drugs on *S. mansoni* adult worms. We investigated the viability of *S. mansoni* adult worms non-treated or treated with 10 μg/ml primaquine (PQ), artesunate (AR), or amodiaquine (AQ) for 5 days. The MB, NR, and TB staining characteristics of the adult worms treated with PQ, AR, and AQ were also determined.

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MATERIALS AND METHODS

Chemicals and media: AQ·HCl and PQ·$2H_3PO_4$ were purchased from MP Biomedicals (Fountain Parkway Solon, OH, USA) and Sigma-Aldrich (St. Louis, MO, USA), respectively. Stock solutions were made by dissolving the 2 drugs in deionized water (DW) at a concentration of 10 mg/ml as free bases. AR (LKT Laboratories, Inc. St. Paul, MN, USA) was dissolved in ethanol at a concentration of 10 mg/ml as a stock solution. Each drug was then added to NCTC 135 medium (pH 7.4; Sigma-Aldrich) containing 0.5% solution of antibiotics comprised of penicillin (5,000 units/ml) and streptomycin (5,000 μg/ml) (Gibco, Langley, OK, USA) to a concentration of 10 μg/ml. MB and NR dyes were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) and Nacalai Tesque Inc. (Kyoto, Japan), respectively. The dyes were each dissolved in DW at a concentration of 0.1% as a stock solution (MB, pH 4.1; NR, pH 3.1). TB (Merck, Darmstadt, Germany) was dissolved in DW at a concentration of 0.4% as a stock solution (pH 4.6).

Parasite strain: A Puerto Rican strain of *S. mansoni* (NIH-Sm-PR-1) was routinely maintained by an established method via passage through female ICR mice (Japan SLC, Inc. Hamamatsu, Japan) and *Biomphalaria glabrata* snails. Eight weeks after infection with 200 *S. mansoni* cercariae, adult worms were obtained by the

perfusion technique as previously described by Smithers and Terry (12), and washed twice with NCTC 135 medium.

Incubation of *S. mansoni* adult worms with antimalarial drugs and staining with MB, NR, or TB: Adult male and female worms of *S. mansoni* were randomly allocated into 4 groups of 9 worms each: non-treated control and 10 μg/ml each of PQ, AR, and AQ. The worms in each group were further allocated into 3 subgroups for the individual staining experiment using the 3 dyes. Each group and subgroup were incubated for one day in a 35 mm plastic dish (Sumitomo Bakelite Co., Ltd. Osaka, Japan) containing 2 ml of NCTC 135 medium alone in a 5% CO_2 incubator at 37°C. Subsequently, 3 male and female worms in each group were transferred to a new plastic dish containing 2 ml of NCTC 135 medium alone or supplemented with PQ, AR, or AQ. The dishes were continuously incubated in a 5% CO_2 incubator at 37°C for 5 days with replacement of the non-supplemented control medium or PQ, AR, or AQ-supplemented media after 3 days incubation. At the end of the incubation, the movements and morphologies of the worms were observed under a model SMZ 800 stereoscopic microscope (Nikon Corporation, Tokyo, Japan). The status of the adult worms (alive or dead) was determined according to the criteria of Mitsui et al. (5).

Subsequently, 40 μl of the stock MB or NR solution, or 100 μl of the stock TB solution was added to each dish (final concentrations: 0.002%, 0.002%, and 0.02%, respectively) and incubated in a 5% CO_2 incubator at 37°C for 30–45 min. The medium was aspirated out of each dish and 1 ml of phosphate-buffered saline (PBS, pH 7.4) was added. Two hundred microliters of PBS containing 1% formalin was added to each dish to fix the worms and the dish was left at room temperature for more than 30 min. The male and female worms were each placed in 30 μl of PBS containing 1% formalin mounted on a regular microscope slide (76 × 26 × 0.9–1.2 mm; Matsunami Glass Industries, Ltd., Osaka, Japan) and sandwiched with a coverslip (18 × 18 mm; Matsunami Glass Industries, Ltd.). Images of the worms were captured using a modelBX43 microscope (Olympus Corporation, Tokyo, Japan) equipped with an Alpha NEX-5 digital camera (Sony Corporation, Tokyo, Japan).

Ethical statement: The present investigation was approved by the Ethics Review Committee for Animal Experimentation of Nagasaki University School of Medicine. All animal experiments were performed in the Laboratory Animal Center for Biomedical Research at Nagasaki University School of Medicine in accordance with the animal experimentation guidelines.

RESULTS

Effects of antimalarial drugs on the movement and morphology of *S. mansoni* adult worms: The movements and morphologies of worms that were non-treated or treated for 5 days with PQ, AR, or AQ are summarized in Table 1. Non-treated male and female worms in the control group retained moderate contracting and extending movements. No morphological damage was observed. Furthermore, the movements and morphologies of male and female worms in the PQ-treated group were similar to those in the control group. In the AR-treated

Table 1. Viability of *Schistosoma mansoni* adult worms incubated with the antimalarial drugs at a concentration of 10 μg/ml for 5 days

Treatment	Viability of adult worms[1)]	
	Male (n = 9)	Female (n = 9)
Control	+++	+++
Primaquine	+++	+++
Artesunate	+++	++
Amodiaquine	+	+

[1)]: Criteria for the viability of worms according to the movement and morphology: (+++, moderately contracting and extending, transparent color of tegument; ++, occasional and sluggish movement, non-transparent color of tegument; +, no spontaneous movement, elongation or severe swelling with localization of black content). n = Total number of worms in 3 staining experiments each using 3 worms.

Table 2. Characteristics of staining of *Schistosoma mansoni* adult worms continuously incubated with antimalarial drugs at a concentration of 10 μg/ml for 5 days

Dyes	Stained parts of the body of *S. mansoni* adult worms and their color	
Drugs	Male (n = 3)	Female (n = 3)
Methylene blue		
Control	wb: deep blue, it: unstained	ant. body: bright blue, ov: blue, vt: light blue, mid. and pos. it: unstained
PQ	wb: deep blue, it: unstained with lbc	ant. body: bright blue, ov: blue, vt: light blue, mid. and pos. it: unstained
AR	wb: deep blue, it: unstained	ant. body: bright blue, ov: blue, mid. and pos. body: deep green with lbc
AQ	wb: light blue and light brown, it: unstained with lbc	ant. body: partially stained light blue, mid. and pos. body: dark green with lbc
Neutral red		
Control	wb: deep red, it: dark red	ant. it: dark red, ov: pink, vt: deep red, mid. and pos. it: dark red
PQ	wb: deep red, it: dark red	ant. it: dark red, ov: pink, vt: deep red, mid. and pos. it: dark red
AR	wb: deep red, it: dark red	ant. it: dark red with lbc, ov: pink, mid. and pos. vt: deep red and brown, pos. it: unstained
AQ	wb: deep red, it: blebs	ant. body: partially stained deep red, ov: unstained, mid. and pos. body: dark brown with lbc, mid. and pos. it: unstained
Trypan blue		
Control	wb: unstained	wb: unstained
PQ	wb: unstained	wb: unstained
AR	wb: unstained	half body: dark blue
AQ	wb: partially stained dark blue	wb: dark blue

n = Number of worms. PQ, primaquine; AR, artesunate; AQ, amodiaquine; wb, whole body; it, intestinal tract; ov, ovary; vt, vitellaria; lbc, localization of black content; ant., anterior; mid., middle; pos., posterior.

group, the movements and morphologies of male worms were also similar to those in the control group. However, female worms moved occasionally and sluggishly, and their tegument was not transparent. In the AQ-treated group, male and female worms moved only slightly or not at all without needle stimulation. In addition, the male worms shrank with severe blebs being evident and the female worms elongated with localized black content.

Staining of *S. mansoni* adult worms treated with antimalarial drugs with non-fluorescent dyes: After *S. mansoni* adult worms were not treated (control) or treated with PQ, AR, or AQ, they were stained with MB, NR, or TB. The images of staining of the non-, PQ-, AR-, and AQ-treated *S. mansoni* adult male and female worms are shown in Figs. 1–3 and summarized in Table 2.

Staining with MB: When non-treated male and female worms were stained with MB, the whole body of the males was stained deep blue and the intestinal tract was unstained, while the anterior part and ovary of the female worms stained bright blue and blue, respectively (Fig. 1A and E). The vitellaria became light blue and the middle and posterior intestinal tract was unstained. The staining status of PQ-treated male and female worms was similar to that of the non-treated worms (Fig. 1B and F). In the AR-treated group, the staining status of the male worms was similar to that of the non-treated male worms, whereas the anterior part of the female worms was stained bright blue with localization of black content, and the middle and posterior parts were stained deep green with localization of black content (Fig. 1C and G). When AQ-treated male and female worms were stained with MB, the whole body of the males was stained light blue and light brown, whereas the anterior part of the female worms was partially stained light blue or unstained, and the middle and posterior parts were stained dark green with localization of black content in the intestinal tract (Fig. 1D and H).

Staining with NR: When non-treated male and female worms were stained with NR, the whole body of the males was stained deep red and their intestinal tract

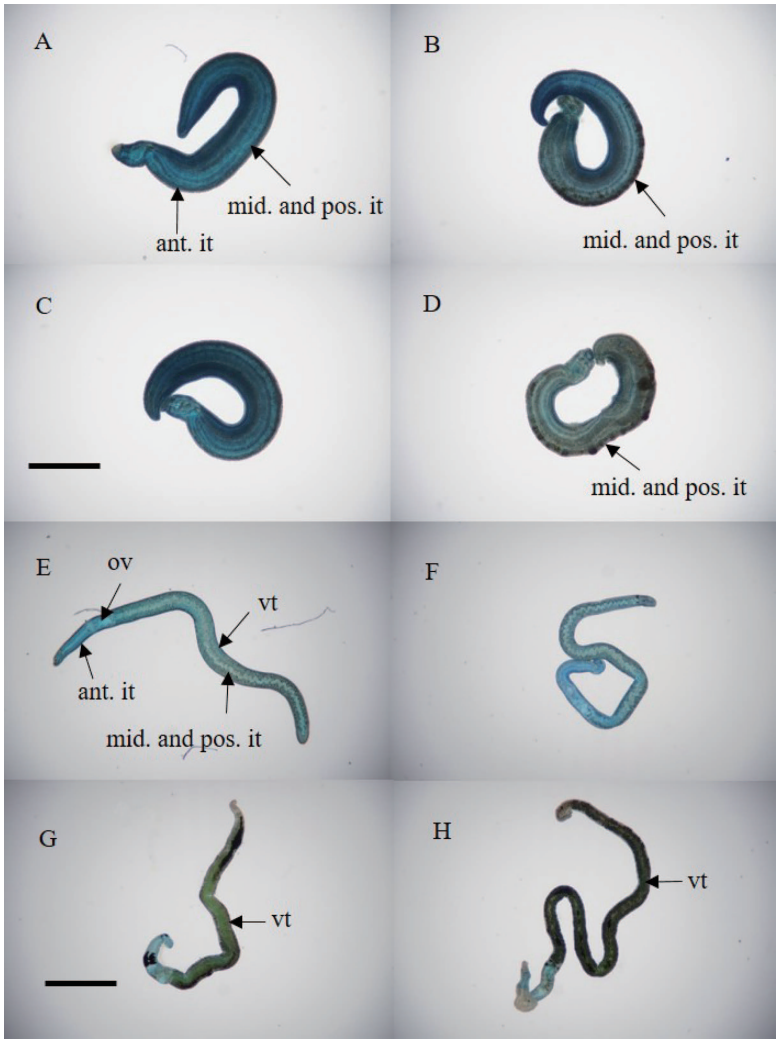

Fig. 1. (Color online) Methylene blue staining of *Schistosoma mansoni* adult worms incubated with antimalarial drugs. *S. mansoni* adult worms were incubated with none (male: A, female: E), 10 μg/ml primaquine (male: B, female: F), artesunate (male: C, female: G) and amodiaquine (male: D, female: H) for 5 days and stained with methylene blue. it, intestinal tract; ov, ovary; vt, vitellaria; ant., anterior; mid., middle; pos., posterior. Scale bar = 1 mm.

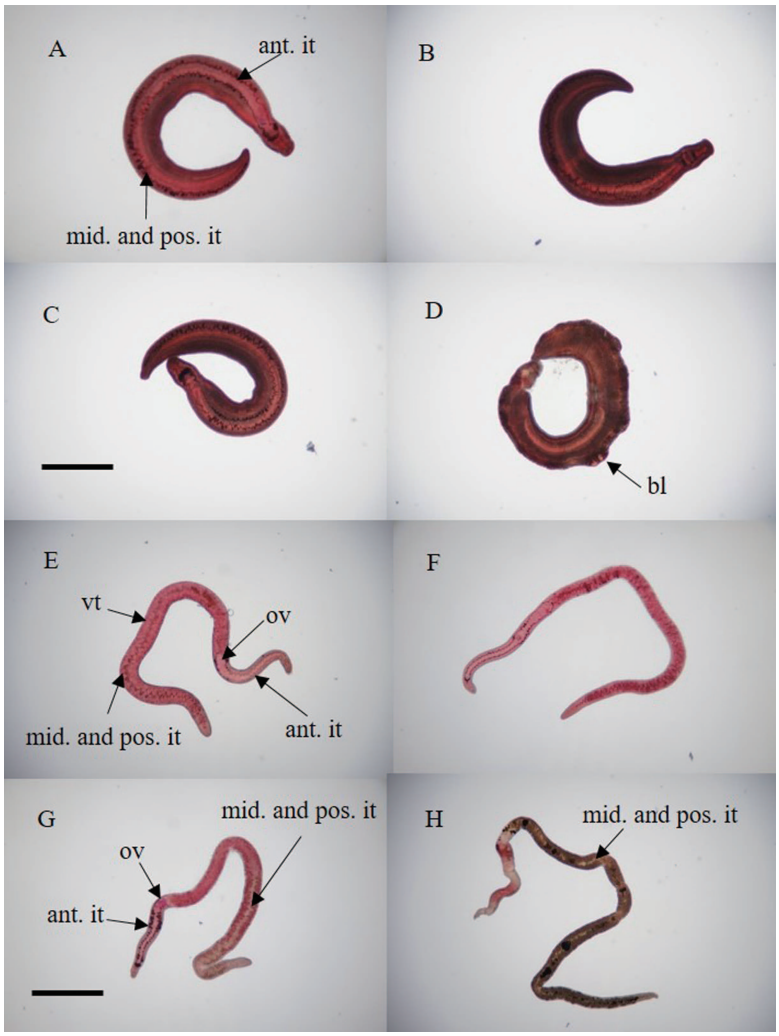

Fig. 2. (Color online) Neutral red staining of *Schistosoma mansoni* adult worms incubated with antimalarial drugs. *S. mansoni* adult worms were incubated with none (male: A, female: E), 10 μg/ml primaquine (male: B, female: F), artesunate (male: C, female: G) and amodiaquine (male: D, female: H) for 5 days and stained with neutral red. bl, blebs; it, intestinal tract; ov, ovary; vt, vitellaria; ant., anterior; mid., middle; pos., posterior. Scale bar = 1 mm.

was stained dark red. The body of the female worms was stained as follows: anterior intestinal tract, dark red; ovary, pink; vitellaria, deep red; and middle and posterior intestinal tract, dark red (Fig. 2A and E). The staining status of PQ-treated male and female worms was similar to that of the non-treated worms (Fig. 2B and F). In the AR-treated group, the staining status of the male worms was similar to that of the non-treated male worms, whereas the anterior part of the female worms was stained deep red with localization of black content along the intestinal tract, and the ovary was stained pink (Fig. 2C and G). In addition, the middle and posterior vitellaria of the female worms were stained deep red and brown, respectively, and the posterior intestinal tract of the worms was not stained. In the AQ-treated group, NR stained the whole body of the male worms deep red with severe blebs along the intestinal tract, whereas the anterior part of the female worms was partially stained deep red or unstained, and the middle and posterior parts were stained dark brown with localization of black content along the intestinal tract (Fig. 2D and H).

Staining with TB: The whole body of the male and female worms in the control and PQ-treated groups was unstained with TB (Fig. 3A, B, E, and F). While the body of the male worms was unstained, the body of the AR-treated female worms was partially stained dark blue with TB (Fig. 3C and G). In the AQ-treated group, the body of the male worms was partially stained dark blue with TB, while the whole body of the female worms was stained dark blue (Fig. 3D and H).

DISCUSSION

We attempted to determine whether the antischistosomal effect of antimalarial drugs on schistosome adult worms is reflected in the staining status of worms treated using drugs with non-fluorescent dyes (MB, NR, and TB). MB and NR are cationic dyes that are absorbed by living cells, but not by dead or damaged cells. In contrast, TB is an anionic dye that is absorbed by dead or damaged cells, but not by living cells. Thus, the 3 dyes make it possible to distinguish between living and dead

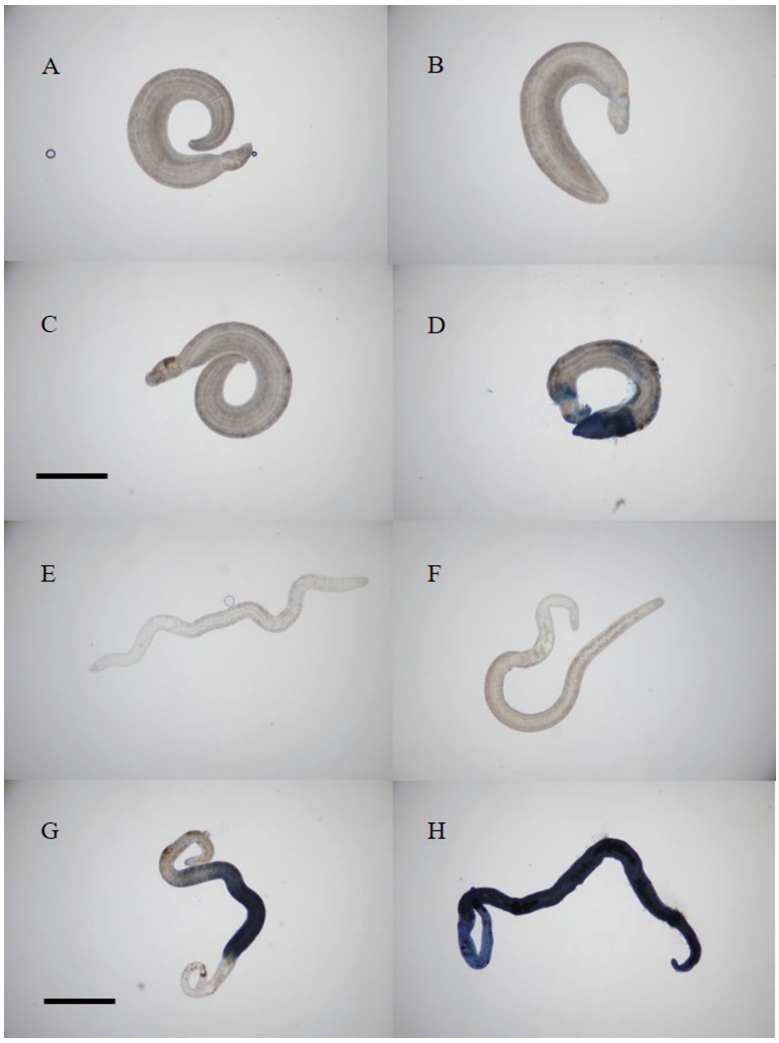

Fig. 3. (Color online) Trypan blue staining of *Schistosoma mansoni* adult worms incubated with antimalarial drugs. *S. mansoni* adult worms were incubated with none (male: A, female: E), 10 μg/ml primaquine (male: B, female: F), artesunate (male: C, female: G) or amodiaquine (male: D, female: H) for 5 days and stained with trypan blue. Scale bar = 1 mm.

or damaged cells caused by drug treatments.

The in vitro effect of currently available antimalarial drugs on *S. mansoni* adult worms was previously assessed at a concentration of 10 μg/ml (7). Since 10 μg/ml was considered to be the critical concentration needed to reveal an in vitro antischistosomal effect of antimalarial drugs on schistosome adult worms, the present study used the same concentration to assess the in vitro effect of PQ, AR, and AQ on *S. mansoni* adult worms using MB, NR, and TB. In the experiment using AR, the medium contained 0.1% ethanol. This concentration of 0.1% ethanol did not affect the viability or morphology of *S. mansoni* adult worms in vitro culture as compared with ethanol-free media (7). The next step was to determine the concentration of the 3 dyes for the staining of adult worms. When non-treated adult male and female worms were incubated with 0.01% MB or NR for 30–45 min, the worms were deeply stained blue and red, respectively. Hence, the concentration of the dyes was reduced to 0.002% to lessen the non-specific staining of adult worms and to reduce surplus uptake of the dyes by worms, which would lead to damage or death. Consequently, the present study stained adult worms with MB or NR at a final concentration of 0.002% throughout. TB was used at 0.02% to stain adult worms.

In the control and PQ-treated groups, the male and female worms were morphologically normal (Table 1) and stained deep blue and light blue, respectively, with MB (Fig. 1A, B, E, and F), and stained deep red with NR (Fig. 2A, B, E, and F). In the AR-treated group, the male worms appeared normal (Table 1) and were stained deep blue with MB and deep red with NR, respectively (Figs. 1C and 2C). In contrast, female worms were sluggish (Table 1) and stained deep green with MB and deep red and brown with NR, respectively (Figs. 1G and 2G). In the AQ-treated group, the male and female worms were morphologically damaged (Table 1) and stained light blue and brown, and dark green, respectively, using MB (Fig. 1D and H), and deep red and dark brown, respectively, using NR (Fig. 2D and H). These results showed that the disparity of the staining status of adult male and female worms treated with PQ, AR, and AQ with MB and NR correlated with the various levels of damage to the male and female worms (Table 1). In

addition, the uptake of NR to the intestinal tracts of the AR- and AQ-treated female worms was clearly inhibited as compared with non-treated worms (Fig. 2E, G, and H)

The bodies of the male and female worms in the control and PQ groups were unstained with TB (Fig. 3A, B, E, and F). On the other hand, all the AR-treated female worms were partially stained dark blue, whereas the male worms were unstained (Fig. 3C and G). Furthermore, the body of the male worms in the AQ-treated group was partially stained deep blue (Fig. 3D), while the female worms were wholly stained deep blue (Fig. 3H). These results suggest that the strength of the damage to tissues and cells of the adult worms treated with the 3 drugs may be reflected in the TB staining state of the worms.

The present study demonstrates that MB, NR, and TB are useful tools for the assessment of the antischistosomal effect of antimalarial drugs on *S. mansoni* adult worms. Moreover, since these non-fluorescent vital dyes are simple and convenient to use, and inexpensive to purchase, they can serve as useful complementary tools to assess the viability of adult worms, and should be valuable in the development of new antischistosomal drugs.

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Conflict of interest None to declare.

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