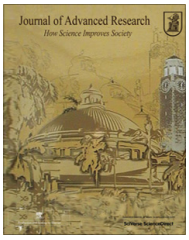
Journal of Advanced Research (2013) 4, 563–567

Cairo University

Journal of Advanced Research



SHORT COMMUNICATION

Application and evaluation of a molecular approach for detection of the schistosomicidal effect of Mirazid® (myrrh) in the murine model

Wael M. Lotfy [a,*], Aly M. Nageh [b], Neveen A. Hussein [b], Ashraf A. Hassan [b,c]

[a] *Parasitology Department, Medical Research Institute, Alexandria University, Egypt*
[b] *Department of Applied Medical Chemistry, Medical Research Institute, Alexandria University, Egypt*
[c] *Department of Laboratory Sciences, Faculty of Health Sciences, Jazan University, Saudi Arabia*

Received 14 July 2012; revised 8 August 2012; accepted 27 August 2012
Available online 25 October 2012

KEYWORDS

Mirazid;
Murine;
Schistosoma mansoni;
Antischistosomal;
PCR;
Diagnosis;
Treatment

Abstract The conventional PCR technique was used for studying the schistosomicidal effect of Mirazid® in the murine model. Results of the molecular study were compared with the parasitological results (ova and worm count). The used PCR technique was more sensitive than the Kato-Katz thick smears. Mirazid® showed some schistosomicidal effects against murine *Schistosoma mansoni*. However, it was not efficient enough to cure any of the studied mice.

Introduction

Schistosomiasis is a major public health problem. More than 200 million people in 74 countries currently have the disease; 120 million of them have symptoms while 20 million have severe illness [1]. Traditional diagnosis of *Schistosoma mansoni* infection involves direct microscopic detection of eggs in feces. However, such a diagnostic approach has some limitations that include a lack of sensitivity as the extent of egg shedding may fluctuate widely, and as many as three specimens may be required in some patients. The use of some stool concentration techniques may increase the diagnostic yield [2]. However, it seems that the sensitivity of parasitological methods diminishes when prevalence and intensity of infection are low, making these methods less appropriate for low-endemic areas and in post treatment situations [3]. Alternatively, the immunological detection of schistosome infection may be used. Such techniques may be useful but there are still problems with their sensitivity and specificity [4]. Polymerase Chain Reaction (PCR) based diagnosis of *S. mansoni* is a relatively new approach that is used for the detection of the parasite DNA in serum or fecal samples. The amplification reaction is capable of detecting as little as 1 fg of DNA, highly specific and is 10 times more sensitive than the Kato-Katz technique [5,6].

Praziquantel (PZQ) is considered the drug of choice for treatment of schistosome infections and a major advance in the treatment of most trematode and cestode infections. This pharmaceutical product is the first anthelminthic drug to fulfill the World Health Organization's requirements for

* Corresponding author. Tel.: +20 1008154959; fax: +203 428 3719.
E-mail address: waelotfy@alex-mri.edu.eg (W.M. Lotfy).

Peer review under responsibility of Cairo University.

Production and hosting by Elsevier

2090-1232 © 2012 Cairo University. Production and hosting by Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.jare.2012.08.012

population-based chemotherapy of a broad range of parasitic infections [7]. However, a relative resistance of the larval stages of *S. mansoni* to the drug is well documented [8]. Also, PZQ has been in use for more than 25 years [9], and concern is increasing that resistance has emerged in human parasites [10]. The situation is further complicated because the two other drugs for treatment of schistosomiasis either are no longer available (metrifonate) or are not effective against all species of schistosomes (metrifonate and oxamniquine) [11].

In 2001, a new antischistosomal drug, Mirazid® (the oleoresin extract from myrrh of *Comiphora molmol* tree, family: Burseraceae) was introduced into the Egyptian market in the form of gelatinous capsules produced by Pharco Pharmaceutical Company (Alexandria, Egypt). The extensive advertising efforts have encouraged physicians in private clinics to use Mirazid although it is not used by the Ministry of Health and Population (MoHP) in the national schistosomiasis control programs [12]. The chemistry of myrrh is not fully elucidated [13]. Reports on the drug anti-schistosomal effect in human or experimental animals are controversial [12].

The present study is a laboratory trial that aims at using and evaluating the conventional PCR technique for studying the schistosomicidal effect of Mirazid® in the murine model, and comparing the results of the molecular study with the parasitological results (ova and worm count).

Material and methods

The study was carried out on male Swiss albino mice of matching age (8 weeks) and weight (20 ± 2 g). Animals were obtained from Theodor Bilharz Research Institute (TBRI), Cairo, Egypt. The mice were kept in a controlled environment and maintained on water and stock commercial pellet diet *ad libitum*.

The mice were divided into three groups of ten animals each.

Group 1

Normal healthy control animals.

Group 2

S. mansoni infected mice sacrificed after 45 days of infection to avoid mortality of untreated mice.

Group 3

Mice treated with 600 mg Mirazid®/kg body weight for five consecutive days on empty stomach after 45 days of the *S. mansoni* infection. Animals of this group were then left for 27 days after the last treatment and sacrificed.

Each mouse was infected with 100 cercariae of TBRI laboratory strain of *S. mansoni* using the tail immersion technique [14].

Worm count

Adult *S. mansoni* worms were recovered from the hepatic portal system and the liver by the perfusion technique as described by Smithers and Terry, and the number of worms was then counted [15].

Counting of eggs in stool

Starting from the 28th day after infection, the animals were separated and feces passed by each animal were collected individually and examined by a modified Kato-Katz technique. A stool pellet was weighted, processed and examined by the Kato-Katz technique. The number of eggs per gram (epg) stool was calculated [16].

Extraction of DNA from stool

Extraction of DNA from stool samples was done using QIAamp® DNA stool mini Kit (QIAGEN, GmbH, Hilden, Germany).

Pcr

The PCR was done using a forward primer (5′-GAT CTG AAT CCG ACC AAC CG-3′) and reverse primer (5′-ATA TTA ACG CCC ACG CTC TC-3′) that were designed to amplify the 121-bp tandem repeat DNA sequence of *S. mansoni*. Briefly, for a 25 μL final volume of PCR mixture, 5 μL of DNA extract was used as template, 12.5 μL 2X PCR Master Mix (0.05 u/μL Taq DNA Polymerase, reaction buffer, 4 mM $MgCl_2$, 0.4 mM of each dNTPs), 1.5 μL of each primer and finally 4.5 μL of molecular biology grade water. The amplification reaction was carried out for 35 cycles, with each cycle consisting of a denaturation step at 95 °C for 40 s, an annealing step at 60 °C for 30 s and an extension step at 72 °C for 1 min. The first cycle had an extended denaturation step for 5 min and the reaction was ended with an extension step at 72 °C for 5 min. Amplified PCR products were analyzed by electrophoresis in 2.5% agarose gels and detected by staining with ethidium bromide [5].

Statistical analysis

All data were expressed as Mean ± SD. Statistical significance was determined by one way analysis of variance (ANOVA) accompanied by post hoc. The test was run on an IBM compatible PC using SPSS for windows statistical package (Version 17; SPSS Inc., Chicago, IL).

Results

The coproscopic examination of the two infected mice groups by the Kato-Katz technique revealed that all mice were passing *S. mansoni* eggs on the 45th day after infection. On the treated group, after treatment till perfusion of mice there was a reduction in egg count, which decreased from 236 ± 166.5 epg on the 45th day after infection to 7 ± 14.9 epg on the 77th day (Fig. 1). On that day 8 out of 10 mice (80%) were diagnosed negative by stool examination.

As regards the number of *S. mansoni* worms recovered from sacrificed mice of the infected groups (Table 1), the untreated group showed a higher number of worms as it was 29.3 ± 10.8 worms on the 45th day after infection, while the Mirazid® treated group showed 9 ± 6 worms on the 77th day after infection. This may indicate that there was a reduction of 69.3% in the Mirazid® treated group compared with the infected untreated group. The difference in worm count between the two groups

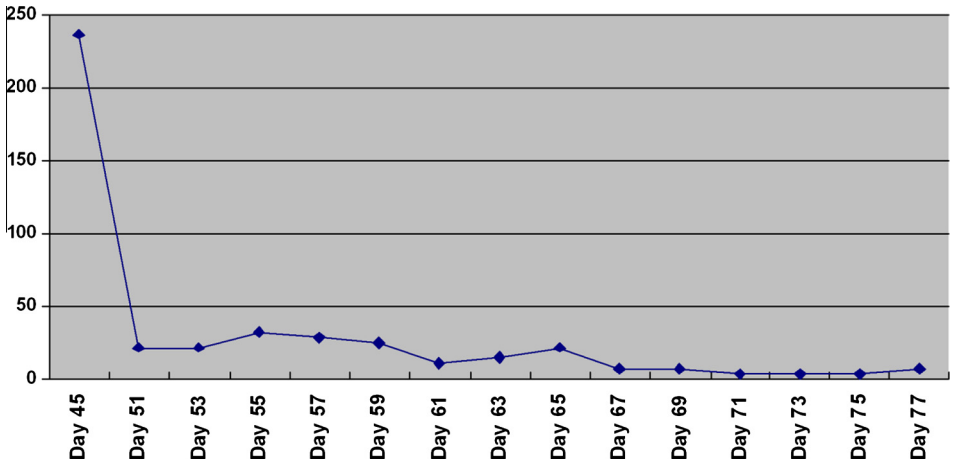


Fig. 1 Mean egg count in stool samples (epg) of the Mirazid® treated mice before and after treatment.

Table 1 Number of *S. mansoni* worms recovered from sacrificed mice of the infected groups.

	Free male Mean ± SD (range)	Free female Mean ± SD (range)	Couple Mean ± SD (range)	Total Mean ± SD (range)	Worm reduction after treatment (*p*-value of T-test)
Untreated group	7.3 ± 2 (4–10)	3.8 ± 1.5 (2–7)	9.1 ± 4.1 (4–17)	29.3 ± 10.8 (14–48)	–
Mirazid® treated group	2.6 ± 1.5 (1–5)	1.1 ± 1.0 (0–3)	3.0 ± 1.9 (1–7)	9.0 ± 6.0 (3–22)	69.3% (0.001)

was significant. None of the treated mice showed complete cure as worms were recovered from all the treated mice, and at least one worm couple was recovered.

Concerning the results of using the PCR for detection of *S. mansoni* specific DNA sequences in murine fecal samples of the infected control group (Fig. 2A), all the fecal samples showed positive results by using feces from the 45th day after infection. On the other hand, all the fecal samples of the uninfected control group showed negative results (Fig. 2B). Regarding the results of the Mirazid® treated group (Fig. 2C), six fecal samples (60%) showed positive results for feces from the 77th day after infection. Interestingly, the four mice diagnosed negative by PCR were among the eight mice diagnosed negative by the microscopic technique.

Discussion

One of the main requirements for diagnosing *S. mansoni* is the development of a more sensitive assay. Consistent diagnosis of the disease still depends on the parasitological demonstration of the *S. mansoni* eggs in fecal samples, which is well accomplished by the Kato-Katz thick smears. Unfortunately, it seems that the technique sensitivity is less appropriate in low intensity conditions such as: low endemic areas, post-treatment, and for determination of incidence [17]. Thus, a more sensitive approach would be of great value in such situations. For studying of the schistosomicidal effect of Mirazid® in the murine model during the present work, in addition to the parasitological techniques including Kato-Katz thick smears and worm count, a PCR technique described by others was used [5,6]. According to the Kato-Katz results, Mirazid® succeeded to reduce the *S. mansoni* egg count in murine feces and gave a cure rate of 80%. By considering the results of the worm detection as the gold standard for judging the cure of mice after treatment (Table 1), it was found that although there was a significant reduction in worm count in the treated group none of the mice was completely cured. By using the qualitative PCR technique there was reduction in the number of the positive samples after treatment and a cure rate amounted 40% (Fig. 2C). This may indicate that the qualitative PCR technique is more sensitive than the Kato-Katz thick smears. Although at least worm couple was present in the treated mice, egg deposition was not confirmed and negative PCR in the mice may denote absence or very few eggs. Although, this is the first report of usage of this technique in diagnosis of murine *S. mansoni*, the present results are supported by previous work of others who reported a higher sensitivity of this PCR technique compared with the Kato-Katz thick smears for detection of the *S. mansoni* DNA in human fecal samples [18]. It is to be mentioned here that the results of the present study should be interpreted with caution because of the limited number of mice included in each group.

The used PCR primers were designed by Pontes et al. [5,6] to amplify a species specific highly repeated 121 bp sequence of *S. mansoni* DNA that comprises about 10% of the parasite genome. It was estimated that each *S. mansoni* cell have about 600,000 copies of this tandem repeat DNA sequence [5,6]. The high sensitivity of the approach enabled the detection of the parasite DNA in 2.16 epg of feces, which makes this technique 10 times more sensitive than the Kato-Katz examination. A detection limit of 1 fg of *S. mansoni* DNA was determined when pure DNA was used as PCR template [19].

There is a great debate about the efficacy and even effectiveness of myrrh in the treatment of *S. mansoni*, both in labora-

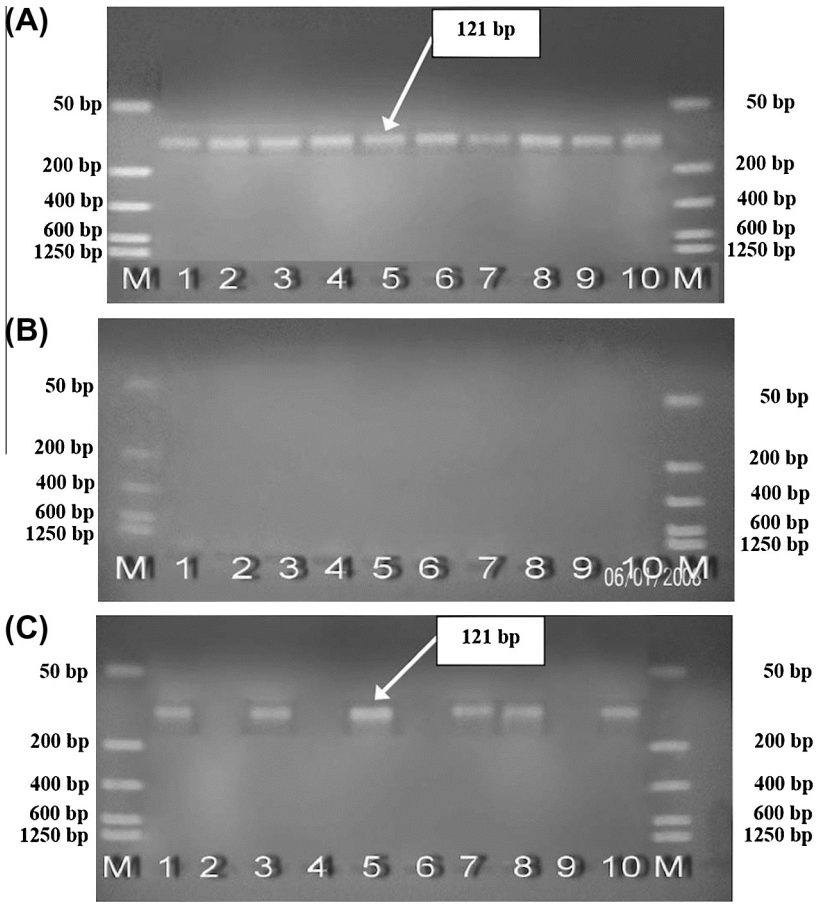


Fig. 2 Results of the PCR for murine fecal samples of the infected control [A], uninfected control [B] and Mirazid® treated [C] groups (M: molecular weight marker, lanes 1–10: samples).

tory and clinical settings. Badria et al. [20] reported the efficacy of myrrh in mice experimentally infected with *S. mansoni*. The oral administration of myrrh extract at 250 and 500 mg/kg body weight induced significant reductions in worm burdens, increased hepatic shift of worms and progressive reductions in the percentages of immature eggs deposited in the intestinal wall [20]. Massoud et al. [21] compared the efficacy of myrrh extract on different developmental stages of *S. mansoni* in experimentally infected mice. They reported that myrrh extract in a dose of 500 mg/kg body weight daily for five consecutive days resulted in a valuable schistosomicidal effect which was more evident in groups in which the drug was administered on 21st as well as on 45th days post infection [21]. However, other experiments negated the possibility of myrrh efficacy in the treatment of experimental schistosomiasis. The most striking results on the lack of therapeutic efficacy of myrrh against *S. mansoni* infected animals were obtained in a multicentre investigation conducted by Botros et al. [22]. Different derivatives of the myrrh resin, including the commercial preparation, Mirazid®, were tested using different doses against different strains. The worm reduction rates in mice infected with the Egyptian (CD) strain were negligible. High doses of Mirazid® solution were toxic for mice infected with the Puerto Rican (Mill Hill) strain of *S. mansoni* while lower doses induced modest or no worm reductions. In addition, no antischistosomal activity was observed in mice and hamsters infected with the Puerto Rican (NMRI) and Brazilian (LE) strains of *S. mansoni* treated with different concentrations of the crude extract of myrrh [22].

Conclusion

The used PCR technique was more sensitive than the Kato-Katz thick smears in post-treatment diagnosis of murine *S. mansoni* infection. Mirazid® showed some schistosomicidal effects against murine *S. mansoni* infection. However, it was not efficient enough to cure any of the studied mice. Thus, we believe that the re-evaluation of myrrh as a human schistosomicidal drug is a must because of its recommendation by some Egyptian physicians motivated by its natural origin.

References

[1] Chitsulo L, Engels D, Montresor A, Savioli L. The global status of schistosomiasis and its control. Acta Trop 2000;77:41–51.
[2] Garcia LS, Shimizu RY, Palmer JC. Algorithms for detection and identification of parasites. In: Murray PR, editor. Manual of clinical microbiology. Washington, D.C.: American Society for Microbiology Press; 1999. p. 1336–54.
[3] Doenhoff MJ. Is schistosomicidal chemotherapy subcurative? Implications for drug resistance. Parasitol Today 1998;14:434–5.
[4] Doenhoff MJ, Chiodini PL, Hamilton JV. Specific and sensitive diagnosis of schistosome infection: can it be done with antibodies? Trends Parasitol 2004;20:35–9.

[5] Pontes LA, Dias-Neto E, Rabello A. PCR detection of *Schistosoma mansoni* DNA in human fecal and serum samples. Am J Trop Med Hyg 2002;66:157–62.
[6] Pontes LA, Oliveira MC, Katz N, Dias-Neto E, Rabello A. Comparison of polymerase chain reaction and the Kato-Katz technique for diagnosing infection with *Schistosoma mansoni*. Am J Trop Med Hyg 2003;68:652–6.
[7] Wegner DHG. Trial designs for multicentre clinical studies of investigational phases IB to III with Praziquantel. Arzneimittelforschung 1981;31:566–7.
[8] Silva LM, Menezes RM, de Oliveira SA, Andrade ZA. Chemotherapeutic effects on larval stages of *Schistosoma mansoni* during infection and reinfection of mice. Rev Soc Bras Med Trop 2003;36:335–41.
[9] King CH, Mahmoud AA. Drugs five years later: praziquantel. Ann Intern Med 1989;110:290–6.
[10] Doenhoff MJ, Pica-Mattoccia L. Praziquantel for the treatment of schistosomiasis: its use for control in areas with endemic disease and prospects for drug resistance. Expert Rev Anti Infect Ther 2006;4:199–210.
[11] Borrmann S, Szlezak N, Faucher JF, Matsiegui PB, Neubauer R, Binder RK, et al. Artesunate and Praziquantel for the treatment of *Schistosoma haematobium* infections: a double-blind, randomized, placebo-controlled study. J Infect Dis 2001;184:1363–6.
[12] Barakat R, El morshedy H, Fenwick A. Efficacy of myrrh in treatment of human schistosomiasis mansoni. Am J Trop Med Hyg 2005;73:365–7.
[13] Sheir Z, Nasr AA, Massoud A, Salama O, Badra GA, El-Shennawy H, et al. A safe, effective, herbal antischistosomal therapy derived from myrrh. Am J Trop Med Hyg 2001;65:700–4.
[14] Oliver L, Stirewalt MA. An efficient method for the exposure of mice to cercariae of *Schistosoma mansoni*. J Parasitol 1952;38:19–23.
[15] Smithers SR, Terry RJ. The infection of laboratory hosts with cercariae of *Schistosoma mansoni* and the recovery of worms. Parasitology 1965;55:695–700.
[16] Katz N, Chaves A, Pellegrino J. A simple device for quantitative stool thick smear technique in *Schistosomiasis mansoni*. Rev Inst Med Trop Sao Paulo 1972;14:337–40.
[17] Engels D, Nahimana S, De Vlas SJ, Gryseels B. Variation in weight of stool samples prepared by the Kato-Katz method and its implications. Trop Med Int Health 1997;2:265–71.
[18] Allam AF, Kader O, Zaki A, Shehab AY, Farag HF. Assessing the marginal error in diagnosis and cure of *Schistosoma mansoni* in areas of low endemicity using Percoll and PCR techniques. Trop Med Int Health 2009;14:316–21.
[19] Rabello A, Pontes LA, Dias-Neto E. Recent advances in the diagnosis of *Schistosoma mansoni* infection: the detection of parasite DNA. Mem Inst Oswaldo Cruz 2002;97:171–2.
[20] Badria F, Abou-Mohamed G, El-Mowafy A, Massoud A, Salama O. Mirazid: a new schistosomicidal drug. Pharm Biol 2001;39:127–31.
[21] Massoud AM, El Ebiary FH, Abou-Gamra MM, Mohamed GF, Shaker SM. Evaluation of schistosomicidal activity of myrrh extract: parasitological and histological study. J Egypt Soc Parasitol 2004;34:1051–176.
[22] Botros S, William S, Ebeid F, Cioli D, Katz N, Day TA, et al. Lack of evidence for an antischistosomal activity of myrrh in experimental animals. Am J Trop Med Hyg 2004;71:206–10.