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Molecular and other diagnostic tools used to compare *Schistosoma haematobium* infections in pupils before and after praziquantel therapy

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ABSTRACT
Urogenital schistosomiasis is a neglected tropical disease (NTD) caused by the Trematode *Schistosoma haematobium*. In endemic areas, the infection is usually acquired from an early age. Drug of choice for treatment of all *Schistosoma* species is praziquantel. We compared *Schistosoma haematobium* infection rates obtained with polymerize chain reaction (PCR), polycarbonate track etched (PCTE) filters and urine dipstick test strip among 400 pupils from two schools in Makurdi before and after praziquantel therapy. *S. haematobium* eggs in urine samples using PCTE filters gave an overall prevalence of 22.7% before praziquantel therapy. Males had a prevalence (23.1%) higher than (22.3%) in females ($p>0.05$). Pupils under ten years old had 21.0% prevalence while age-group of 11-20 years recorded 23.4% ($p<0.05$). Pupils in Location "A" had a prevalence of (26.5%) higher than (19%) for those at location "B" ($p>0.05$). The PCR gave the highest prevalence (25.75%) before praziquantel treatment and least prevalence (3.5%) after treatment, indicating its highest sensitivity and specificity over PCTE filters and RDT dipstick. Since different diagnostic methods yielded different *S. haematobium* prevalence, it is necessary to use more than one method to increase the accuracy of diagnosis and for better interpretation of prevalence. Praziquantel is able to significantly reduce egg count and morbidities. Hence annual mass drug administration (MDA) with praziquantel should continue to be used for control of schistosomiasis in endemic areas of the country. However, infections should be avoided by all means to avoid debilitating effects of irreversible morbidities. .

Keywords: Urogenital schistosomiasis, Diagnosis, PCR, PCTE, urine dipstick, praziquantel

INTRODUCTION
Schistosomiasis is a neglected tropical disease of medical and veterinary importance. In endemic areas, schistosomiasis is usually acquired from an early age. School-aged children are at the greatest risk of acquiring schistosomiasis due to their play activities (Iwueze *et al*., 2018) and lack of hygiene; and the morbidities due to schistosomiasis affects children the most because children have lowered immunity compared to older people (Colley *et al*., 2014). Dawaki *et al*. (2016) reported highest prevalence in children of 10 –18 years perhaps due to their adventurous habits and increased contact with infested water at that age (Okwelogu *et al*., 2012; Khurana *et al*., 2018).

The drug of choice for treatment of all *Schistosoma* species is praziquantel; a single oral dose is highly effective, safe and is the key tool in the global schistosomiasis control strategy (WHO, 2019) but diagnosis of schistosomiasis is a very important step towards control and elimination. Epidemiological assessment of the community may be done to determine the prevalence of the disease (King, 2009) before treatment is embarked upon. In addition diagnosis of the infection in those treated helps to evaluate the efficacy of treatment administered. The current World Health Organization guideline for the diagnosis of urinary

recommends the examination of urine by microscopy to detect parasite eggs (WHO, 2019). However in cases of chronic infection with low egg production and low parasitemia, diagnosis based solely on egg count can be challenging and misleading. In addition, the analysis of a large number of samples could be laborious, time consuming and is further compounded by the fact that the parasite eggs disintegrate easily and as such must be detected within hours of sample collection for optimum results (Tamarozzi *et al*., 2021)

Sensitivity and specificity are two important measures for determining the accuracy of a clinical test, and their calculation requires comparison with a suitable gold standard test. The sensitivity of a test is the ability of the test to correctly identify patients with a particular condition (the true positive fraction), whereas specificity is the ability of the test to correctly identify individuals who do not have the condition (the true negative fraction) (Houmsou *et al*., 2011). The sensitivity of a diagnostic test is an important factor in determining its use in research and epidemiological surveys because tests with low sensitivity may miss certain infected individuals who thus remain undiagnosed but continue to contribute substantially to disease transmission; these individuals will continue to contaminate the environment thereby diminishing the efficiency of control efforts (Weerakoon *et al*., 2015).

Haematuria, proteinuria and leucocyturia have been known to be associated with urinary schistosomiasis (Houmsou *et al*., 2011; Rodrigue Roman *et al*., 2016). These can be detected with a dipstick and are strongly indicative of *S. haematobium* infection even in the absence of eggs shed in urine (Rodrigue Roman *et al*., 2016). However, haematuria is not always specific for schistosomiasis particularly in low-prevalence settings, and as such additional parasitological tests may be needed to confirm diagnosis (Krauth *et al*., 2015), and leucocyturia is broadly indicative of the presence of parasitic infection. Polycarbonate Track Etched (PCTE) filters can be used to detect *S. haematobium* eggs in urine; it has the added advantage of being used to determine *S. haematobium* infection intensity (Houmsou *et al*., 2016). Advances have been made in the use of molecular techniques in detecting urinary schistosomiasis infection. It is more expensive but saves time, large samples can be handled and it has been shown to have high sensitivity. Detection of *S. haematobium*-specific DNA in urine samples has been successful with urine sediments on Whatman filter papers (Ibironke *et al*., 2011) and the detection of *S. haematobium*-specific DNA in *Schistosoma* eggs and miracidia have been described by (Huyse *et al*., 2013). However, Dra1 PCR diagnosis has the disadvantage of being unable to detect low infection intensity and low amount of parasite DNA (King and Bertsch, 2013).

The present study was aimed at Comparing *Schistosoma haematobium* infection rates, in school children at Makurdi Nigeria, before and after praziquantel MDA using different diagnostic methods.

MATERIALS AND METHODS

Study Area: The study was conducted in Kanshio and North-bank communities in Makurdi, Benue State Nigeria. The annual state-wide mass drug administration (MDA) with praziquantel against schistosomiasis organized by Benue State Epidemiology Unit was ongoing as at the time of the study between August 2018 and May 2019. Thus two (2) schools, each from these two communities close to natural water bodies where pupils were yet to be treated in Makurdi were selected for the study (Figure 1).

Study population: All pupils (N=386) registered in two schools coded 'A' (N_A=190) located at Kanshio, and 'B' (N_B=196) located at North bank communities made up the study population.

Sample Size: Sample size formula $n = \frac{N}{1+N(e^2)}$ (Rao and Scott, 1992) where *n* is minimum sample size, *N* is study population, *e* is error term, 0.05 at 95% confidence interval, was used to determine the sample size for the study. Minimum sample size $n = \frac{386}{1+(386\ x\ 0.05^2)} = \frac{386}{1+0.965} = \frac{386}{1.965} = 196.4 \equiv 200$. The first 200 volunteers from each of the two schools were selected giving a sample size of 400 for the study.

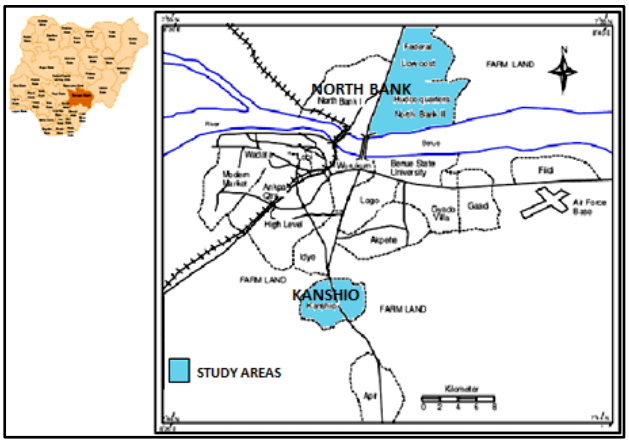

Figure 1: Map of Nigeria (inset) showing Benue State, and Kanshio and North Bank communities of Makurdi

Ethical considerations: Prior to the commencement of the study, ethical clearance NHREC/01/01/2007 was obtained from Federal Ministry of Health, National Health Research Ethics Committee while informed consent was obtained from pupils' parents or guardians.

Health awareness talk on the importance of annual MDA to reduce morbidities and for schistosomiasis control was given to staff and pupils of the two schools. Pupils were also encouraged to cease the practice of urinating in natural water bodies in order to stop the transmission of schistosomiasis. Pupils' ages were obtained through oral interviews with them.

Urine sample Collection: Urine samples were collected between during break-time (10 am - 11am) which also coincides with the peak period for shedding of *Schistosoma* eggs within the human host (Cheesbrough, 2010), then taken immediately in an ice packed cooler to Biological Science Laboratory, University of Agriculture, Makurdi for analysis.

Administration of Praziquantel Drug Treatment: After carrying out the laboratory analysis of urine samples, all pupils who tested positive were treated with Praziquantel at dosage level of 40 mg/kg body weight (Zwang *et al*., 2017).

Urinalysis with urine dipstick test strips: Each urine sample was tested with urine dipstick test strip (One-Step dipstick, 10 parameters manufactured by Dongbang Acuprime, DFI Co. Ltd.) which is a rapid basic diagnostic tool used to detect pathological changes in patient's urine in standard urinalysis. Specific interest in the present study was on detection of leucocyturia, micro-haematuria, and proteinuria but the strip can as well detect nitrite, pH, specific gravity, ketone, bilirubin and glucose in urine. The test was done according to the manufacturer's instructions, and results for haematuria, proteinuria and leucocyturia recorded.

Urinalysis with polycarbonate track etched (PCTE) filters: Each urine specimen was suctioned with a 10 ml syringe and filtered through 13 mm-diameter PCTE filters after shaking. The filters were then removed with a pair of un-toothed forceps, placed on a glass slide, stained with two drops of Lugol's Iodine and examined under x10 and x40 light microscope (Houmsou *et al*., 2016) for the presence of *Schistosoma* eggs, easily identified by their oval shapes and terminal spines.

Molecular Analysis

DNA extraction: Five micro litres of urine was poured into specimen bottles and maintained in a cold chain for diagnosis using Polymerase Chain Reaction (PCR). Molecular analysis was done as described by (Kane and Rollinson, 1994). In the molecular laboratory, DNA extraction was carried out on urine samples using Phosphate Bromide Solution (PBS).

Collection of urine pellets from urine sample (washing): Five millilitres (5 ml) of urine was spun in test tube at 5, 000 rpm for 10 minutes. The deposits (pellets) that precipitated

were rinsed in 5 ml of Phosphate Bromide Solution (PBS). The pellets were then spun again in 5 ml of PBS; and decanted into an eppendorf tube using a disposable pipette. Within the eppendorf tube, the pellets were spun in an eppendorf centrifuge at 10, 000 g for 5 minutes. The supernatant was discarded with the aid of a 5 ml micropippete. The deposit pellets were stored overnight at -80 °C.

DNA extraction protocol using phenol chloroform: One hundred and fifty micro litres (150 µl) of extraction buffer was added to the same volume (150 µl) of urine pellets and incubated overnight. Equal volume of 150µl phenol pH 8.0 was added, and mixed with a gentle mixer for 10 minutes. After gentle mixing, the eppendorf tubes containing the solution were centrifuge for 10 minutes at 13, 000 rpm. The aqueous layer was carefully reduced to another eppendorf tube and half of the volume of phenol and the same volume of chloroform isoamyl alchohol added again. The aqueous layer was carefully reduced to another eppendorf tube and half of the same volume (150 µl) of 3M sodium acetate was added. It was then left overnight at -20 °C for the DNA to precipitate.

Polymerase chain reaction (PCR): After extraction the precipitate was centrifuged for 10 minutes after which the pellets were washed twice with 20 µl of 20 % ice cold ethanol. For each wash, the pellets were centrifuged at 13, 000 rpm for 10 minutes.

Preparation of PCR mix: Primers used targeted Cyclooxygenase 1 (COX 1) and Internal Transcribed Spacer (ITS) regions of the nucleus where used. For primer targeting ITS DNA region, ETTS1 (forward) primer was used – sequence (direction 5^1 to 3^1) TGCTTAAGTTCAGCGGGGT; and ETTS2 (reverse) primer – sequence AACAAGGTTTCCGTAGGTGAA. For primer targeting cox1 DNA region Cox1_Schist_5^1 (forward) TCTTTRGATCATAAGCG; cox1 DNA region Cox1_Schist_3^1 (reverse) TAATGCATMGGAAAAAAACCA.

Polymerase chain reaction cycling steps and conditions: For PCR targeting ITS DNA region: Initial denaturation was done for one (1) cycle at 95 °C for 5 minutes; Denaturation was done at 95 °C for 30 seconds, annealing was done at 56 °C for 1 minute, extension was done at 72 °C for 1 minute. Denaturation, annealing and extension were repeated for 35 cycles; final extension was done for one (1) cycle at 72 °C for 7 minutes. For PCR targeting cox1 DNA region: Initial denaturation was done for one (1) cycle at 94 °C for 5 minutes; Denaturation was done at 94^0C for 30 seconds, annealing was done at 52 °C for 1 minute, extension was done at 72 °C for 1 minute. Denaturation, annealing and extension were repeated for 35 cycles; final extension was done for one (1) cycle at 72 °C for 7 minutes.

Gel electrophoreses: Gel electrophoreses was carried out using 2 % Agarose in 0.5x TBE buffer ran at 80 Volts for 1 hour. Afterwards, the results (images) were viewed, captured, and interpreted accordingly.

Statistical Analysis: Data obtained was analyzed for statistical significance. Chi-square test was used to verify the homogeneity of the disease in the different schools. The diagnostic performance of the different tests for urinary schistosomiasis (microhaematuria, proteinuria, leucocyturia, PCR, sedimentation, centrifugation and transparency) was compared against the diagnostic performance of PCTE filters (standard test for diagnosing urinary schistosomiasis) by calculating for sensitivity and specificity.

Sensitivity = $\frac{a}{a+b}$, where a=true positive, b = false negative

Sensitivity = $\frac{c}{c+d}$, where c=true negative, d = false positive

RESULTS AND DISCUSSIONS

The PCTE filters gave an overall prevalence of 22.7% urogenital schistosomiasis infection among the pupils before praziquantel mass therapy (Table 1) with males having a higher prevalence of 23.1% than 22.3% in females ($p>0.05$) (Table 1). Also, subjects aged less than ten years had prevalence of 21.0 % but those aged 11-20 years had highest prevalence of 23.4% ($p>0.05$). Subjects in School “A” also had higher prevalence of 26.5% than subjects in School “B” with 19.0% ($p>0.05$). Egg detection in urine with PCTE filters (Plate 1), and

micro-haematuria with RDT strips were indications of urogenital schistosomiasis among pupils under study.

Table 1: Diagnosis of urogenital schistosomiasis with PCTE before praziquantel therapy

		Pupils Examined		Pupils Positive	
		No.	%	No	%
Gender	Male	212	53.0	49	23.11
	Female	188	47.0	42	22.34
	Total	400	100	91	22.75
	χ^2=0.538, *df*=1, *p*>0.05				
Age-group (years)	<10	105	26.3	22	20.95
	11-20	295	73.7	69	23.38
	Total	400	100	91	22.75
	χ^2= 24.274, *df*=2, *p*<0.05				
Location	School "A"	200	50.0	53	26.50
	School "B"	200	50.0	38	19.00
	Total	400	100	91	22.75
	χ^2=2.472, *df* =1, *p*>0.05				

Table 2 revealed that PCR test gave the highest prevalence (25.75%) before MDA with praziquantel but had the least prevalence after MDA. Other tests results with PCTE, microhaematuria, proteinuria and Leucocyturia before and after MDA with praziquantel are also shown in Table 2. Gel electrophoresis results showing that *S. haematobium* were amplified at 121 bed pairs, and similarly at 121 base pairs are shown in Plates 2 and 3 respectively.

Table 2: Diagnosis of urogenital schistosomiasis before and after praziquantel treatment

Diagnostic parameters	Infection before MDA		Infection after MDA	
	No.	%	No.	%
Molecular analysis with PCR	103	25.75	14	3.50
Urinalysis with PCTE filters	91	22.75	29	7.25
Urinalysis with RDT strips:				
Microhaematuria	96	24.00	52	13.00
Proteinuria	82	20.50	44	11.00
Leucocyturia	62	15.50	40	10.00
		χ^2=27.743, *df*=4, *p*<0.05		

Urine samples examined (n=400)

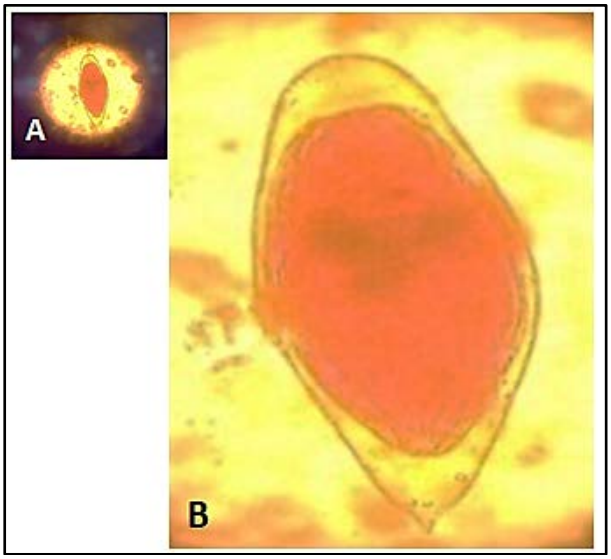

Plate 1: Ovoid egg of *Schistosoma haematobium* as seen under a compound microscope [A]; and zoomed to show characteristic terminal spine [B]

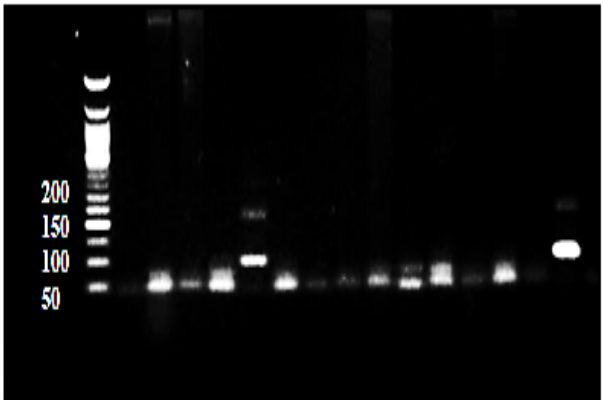

Plate 2: Gel electrophoresis result of *S. haematobium* amplified at 121 bed pairs.

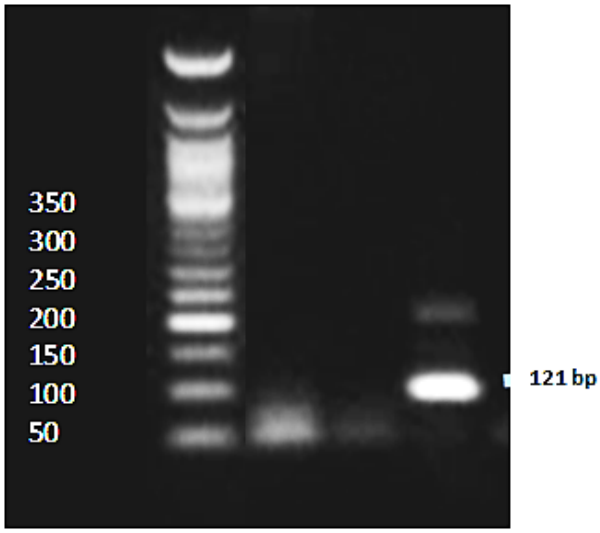

Plate 3: Gel electrophoresis showing *S. haematobium* amplified at 121 base pairs

Schistosoma haematobium prevalence in the present study was higher in males than in females in this study, it has been reported that differences in gender related prevalence in a study could be as a result of water contact activities of resident of the area. Hajissa *et al*. (2018) reported no significant difference in schistosomiasis prevalence between males and females in Sudan but Gyuse *et al*. (2010) reported higher prevalence in females than males in Osun State, Nigeria due to higher water contact activities by females than males. Moira *et al*. (2010) reported higher *S. haematobium* prevalence in males than in females reporting also that, even after drug treatment, males had significantly higher reinfection rates than their female counterparts due to their higher degree of exposure to infected water.

Schistosoma haematobium prevalence was significantly higher in school children aged between 10 and 20 years of age. This differences in age- prevalence could be due to the fact that children aged 10 - 20 years tend to be more adventurous and as a result visit water bodies with their peers, to swim and to fish (Chikwendu *et al*., 2019, Okwelogu *et al*., 2012), also fishing is a common water contact activity in the section of River Benue at Makurdi, thereby making them more vulnerable to *S. haematobium* infection.

Schistosoma haematobium infection in participants was diagnosed by the detection of eggs in urine using PCTE filters. Egg output in children is known to be high in children less than ten years, and to peak at ages 10-20 years and thereafter decline in young adults of 20 years and above (Pereira *et al*., 2010). For this reason, children aged 10-20 years are the main target of mass chemotherapy with praziquantel for the control of urinary schistosomiasis (Pereira *et al*., 2010). It is also important to note that if schistosomiasis is not treated in children even though egg output may become significantly reduced or stop completely as they grow older, secondary morbidities due to *S. haematobium* may linger leading to complications like female genital schistosomiasis in females (Masong *et al*., 2021) and

infertility in males, kidney and bladder cancers; morbidities which at an older age, may not be reversed by simple praziquantel treatment (Ossai *et al*., 2014). Our findings were in agreement with Moira *et al*. (2010) who opined that children are more susceptible to schistosomiasis than adults. In a case of experimental exposure to cercariae, adults were found to be less susceptible leading to the suggestion that adults have acquired a form of resistance to *S. haematobium* infection due to childhood exposure. Shehata *et al*. (2018) also reported significantly higher *S. haematobium* prevalence and infection intensities in children in Zambia.

The presence of parasite eggs in urine as determined using PCTE filters dropped significantly after treatment with praziquantel, as did the prevalence as determined by PCR. The presence of blood in urine (microhaematuria) also reduced significantly following praziquantel MDA. Leucocyturia also reduced after MDA; however the reduction was not significant. The significant drop in *S. haematobium* prevalence is an indication that the drug praziquantel is highly effective against urinary schistosomiasis.

The significant reduction in microhaematuria is proof that praziquantel is effective in reducing blood loss due to schistosomiasis. Microhaematuria and proteinuria are important morbidity due to urinary schistosomiasis and occurs as a result of the sharp terminal spines of *S. haematobium* eggs puncturing the urinary bladder of those infected (Knopp *et al*., 2018). The loss of blood and proteins is the reason for protein deficiency anaemia and malnourishment associated with the disease; in some cases blood loss is so severe it appears as visible blood in urine in form of macro-haematuria (Houmsou *et al*., 2008). Following praziquantel treatment a significant reversal of blood loss in urine occurred (see Table 2) showing that praziquantel may not only be effective against the *Schistosoma* worms but could also reduce morbidity due to infection with the worms. Since the reduction in proteinuria was not significant, it follows that certain chronic morbidities may not be reversible even with treatment, so chronic infections with *S. haematobium* should be avoided at all cost. Other preventive measures like health education, water sanitation and hygiene, avoiding waters with infected intermediate snail hosts should be implemented, and not relying solely on a yearly MDA with praziquantel. In the present study we also observed that leucocyturia did not reduce significantly eight (8) weeks after treatment with praziquantel. According to Ochodo *et al*. (2016) and Rodrigue Roman *et al*. (2016), the presence of white blood cells in urine is indicative of infection. Ossai *et al*. (2014) opined that secondary bacterial infections can occur as a result of schistosomiasis, which could cause the body to release leucocytes to fight the infection. Praziquantel drug treatment alone may be unable to reverse secondary bacterial infections resulting from schistosomiasis infections. With this in mind, routine treatment should be done faithfully in endemic communities to avoid morbidities which may not be easily reversed.

After praziquantel treatment, we noticed that egg positive samples detected by PCTE filters were significantly higher in number than positive samples as detected by PCR. This could mean that although subjects continued to pass out eggs in urine, the eggs were actually non-viable, and so were not detected by PCR; thus showing PCR as a superior method capable of distinguishing true infection from false positives. Adewale *et al*. (2018) observed a significant drop in intensity (egg count) and in urinary schistosomiasis prevalence in school aged children 6 months after praziquantel treatment with a single dose of praziquantel in Lagos, Nigeria. However, our results were at variance with Shehata *et al*. (2018) who reported only a slight (non-significant) decrease in schistosomiasis prevalence following praziquantel treatment in Zambia, which was followed by a significant increase in prevalence and intensity the following year. Such a result demonstrates the need for integrated control measures in the control of schistosomiasis as opposed to relying strictly on chemotherapy for the control of the disease particularly in areas with high infection and reinfection rates. Shehata *et al*. (2018) opined that as long as reinfection following treatment is not checked, praziquantel treatment would be a futile annual routine.

Conclusion

This study has demonstrated that different diagnostic methods may yield different *S. haematobium* prevalence and as such, it may be necessary to combine more than one method to increase the accuracy of diagnosis and for proper understanding and interpretation of prevalence. The PCR method has proved to be of high sensitivity and specificity. Since Praziquantel is able to significantly reduce egg count and morbidities like microhaematuria, annual MDA with praziquantel should continue to be used for control of schistosomiasis in endemic areas of the country but infections should be avoided when and where ever possible.

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