Infection, Genetics and Evolution 18 (2013) 100–105

Contents lists available at SciVerse ScienceDirect

Infection, Genetics and Evolution

journal homepage: www.elsevier.com/locate/meegid

# Regular treatments of praziquantel do not impact on the genetic make-up of *Schistosoma mansoni* in Northern Senegal

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ARTICLE INFO

*Article history:*
Received 8 October 2012
Received in revised form 3 May 2013
Accepted 6 May 2013
Available online 15 May 2013

*Keywords:*
Schistosomiasis
Drug resistance
Molecular epidemiology
Cure rates
Praziquantel
Transmission

ABSTRACT

The Senegal River Basin (SRB) experienced a major epidemic of intestinal schistosomiasis in the early nineties, after the construction of a dam for irrigation purposes. Exceptionally low cure rates following praziquantel (PZQ) treatment at the onset of the epidemic raised concerns about PZQ resistant strains of *Schistosoma mansoni*, although they could also be attributed to the intense transmission at that time. A field study in the same region more than 15 years later found cure rates for *S. mansoni* still to be low, whereas *Schistosomahaematobium* responded well to treatment. We collected *S. mansoni* miracidia from children at base-line prior to treatment, six months after two PZQ treatments and two years after the start of the study when they had received a total of five PZQ treatments. In total, 434 miracidia from 12 children were successfully genotyped with at least six out of nine DNA microsatellite loci. We found no significant differences in the genetic diversity of, and genetic differentiation between parasite populations before and after repeated treatment, suggesting that PZQ treatment does not have an impact on the neutral evolution of the parasite. This is in stark contrast with a similar study in Tanzania where a significant decrease in genetic diversity was observed in *S. mansoni* miracidia after a single round of PZQ treatment. We argue that PZQ resistance might play a role in our study area, although rapid re-infection cannot be excluded. It is important to monitor this situation carefully and conduct larger field studies with short-term follow-up after treatment. Since PZQ is the only general schistosomicide available, the possibility of PZQ resistance is of great concern both for disease control and for curative use in clinical practice.



## 1. Introduction

Schistosomiasis or bilharzia is a parasitic disease that mainly occurs in tropical and subtropical regions of the world and is caused by blood flukes of the genus *Schistosoma* (subclass Digenea); over 200 million people are infected, of which more than 90% live in Africa (Hotez and Kamath, 2009). *Schistosoma* species have a two-host life cycle with an asexual stage within a freshwater snail host and a sexual stage within the definitive mammalian host; parasite eggs are voided in the urine (eg *Schistosoma haematobium*) or faeces (eg *Schistosoma mansoni*). Despite the availability of adequate tools for diagnosis and treatment, schistosomiasis remains a major public health concern (Savioli et al., 2004). Due to alterations of the environment and increasing migration of man and their livestock, schistosomiasis continues to (re-) emerge. A dramatic example is the outbreak in Northern Senegal in the early nineties. The Diama dam on the Senegal River was constructed in 1985 to produce fresh water for rice and sugar cane agriculture and water supply for municipal use in Dakar. The subsequent ecological changes favored the spread of freshwater snails, followed by a major outbreak of intestinal schistosomiasis (Talla et al., 1990). Soon after, the restricted urinary schistosomiasis foci of the lower delta spread upstream (Verle et al., 1994), and many children can now be found with both urinary and intestinal schistosomiasis.

Praziquantel (PZQ) is the drug of choice to treat schistosomiasis because of the few side effects, the low cost and it is the only drug that is effective against all human schistosome species (Doenhoff et al., 2002). Whereas cure rates for *S. mansoni* usually lie between

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http://dx.doi.org/10.1016/j.meegid.2013.05.007

**Table 1**
*Schistosoma mansoni* infection intensity (eggs/g) of the children enrolled in the study followed through time. Shaded columns indicate from which time points schistosome populations have been sampled and the darker squares the samples genotyped.

| | Timing | | | | | |
|---|---|---|---|---|---|---|
| **Child ID** | **S0** | **S1** | **S2** | **S3** | **S4** | **S5** |
| 1 | 70 | 0.0 | 1200.0 | 220.0 | 447.0 | 1332.0 |
| 3 | 26.7 | 6.7 | 600.0 | 7.0 | 13.0 | 84.0 |
| 9 | 146.7 | 0.0 | 646.7 | 0.0 | 187.0 | 852.0 |
| 11 | 53.3 | 0.0 | 673.3 | 7.0 | 193.0 | 660.0 |
| 53 | 246.7 | 0.0 | 320.0 | 20.0 | 360.0 | 132.0 |
| 65 | 366.7 | 0.0 | 733.3 | 13.0 | 13.0 | 24.0 |
| 49 | 100 | 0.0 | 1126.7 | 27.0 | 570.0 | 2698.0 |
| 15 | 400 | 33.3 | 247.0 | 53.0 | 107.0 | 12.0 |
| 45 | 146.7 | 0.0 | 944.0 | 160.0 | 1053.0 | 660.0 |
| 46 | 1360 | 6.7 | 247.0 | 93.0 | 547.0 | 36.0 |
| 73 | 13.3 | 0.0 | 420.0 | 20.0 | 600.0 | 480.0 |
| 31 | 20 | 0.0 | N/A | | | |
| 85 | 70 | 0.0 | 1200 | 220 | 447 | 1332 |

S0 = baseline survey (survey and double treatment).
S1 = six weeks post-baseline (survey only).
S2 = six months post-baseline (survey and double treatment).
S3 = six weeks post-S2 (survey only).
S4 = one year post-baseline (survey and single treatment).
S5 = two years post-baseline (survey and single treatment).

70% and 90% (Gryseels et al., 2006), the observed cure rate at the onset of the Senegalese epidemic reached only 18–32% (Stelma et al., 1995). Such a low figure had never been reported elsewhere before and the emergence of resistance was feared. Several alternative explanations have been put forward related to intense transmission and/or the recent nature of the focus, e.g. rapid re-infection, immunological naivety of the human population, and a high number of immature worms (Gryseels et al., 2001, 1994), which are tolerant to PZQ (Sabah et al., 1986). A meta-analysis including PZQ treatment studies from various endemic countries showed that cure rates from Senegal were consistently lower than expected, even when initial infection intensity, follow-up time and sensitivity of diagnosis were accounted for (Danso-Appiah and De Vlas, 2002). Laboratory experiments showed that *S. mansoni* isolated from snails in the epicentre of the *S. mansoni* epidemic were significantly less responsive to PZQ as compared to Kenyan and Puerto Rican strains. They were however fully responsive to the drug oxamniquine (Fallon et al., 1995, 1997), supporting the possibility of PZQ resistance in these Senegalese strains (Fallon et al., 1997). Conclusive evidence for any of the above scenarios has not been obtained so far.

It has been suggested that cure rates may not be a good proxy for drug efficacy against schistosomiasis and soil-transmitted helminths (Gryseels et al., 1994; Montresor, 2011). The standard Kato Katz technique for the diagnosis of *S. mansoni* is not sufficiently sensitive to detect light infections, and cure rates are dependent on baseline/pre-treatment infection intensities. We now have molecular tools to genetically characterize parasite populations. By quantifying neutral genetic variation, we can infer changes in

parasite population diversity, size and structuring. Observed variations between pre- and post-treatment populations could in turn be linked to drug pressure and therefore serve as a proxy for intervention efficacy. Here we specifically test with neutral microsatellite markers if and how natural schistosome populations within human hosts change when exposed to repeated PZQ treatments. Genetic diversity of miracidial offspring sampled from each individual host was quantified and used as a proxy for the genetic diversity of the adult worms within that host. We hereby assume that if treatment were effective in eliminating (most of the) adult worms, a significant decrease in genetic diversity of the offspring will be observed.

## 2. Material and methods

### 2.1. Ethics statement

This study was part of the EU-FP6 CONTRAST study looking at re-infection rates post-treatment, for which approval was obtained from the ethical committees of the Ministry of Health in Dakar, Senegal, and the NHS-LREC of Imperial College London, England. All parents and teachers gave oral consent for urine and stool examination and the data were anonymized prior to analysis. All schistosomiasis positive children were treated with praziquantel (40 mg/kg) throughout the study (Table 1) even if they were not included in the study cohort. Treatment of all children in the village was carried out one and two years after baseline (i.e. S4 and S5, respectively; Table 1).

### 2.2. Treatment and data collection

The village of Nder is situated on the Western side of Lac de Guiers, about 30 km from Richard Toll, and counts about 500 inhabitants. They mainly depend on the lake for their water-related activities. The study started in April 2007, with the collection of urine and stool samples from 107 children aged 5–15 years on three consecutive days, followed by two PZQ treatments three weeks apart (S0; Table 1). Follow-up surveys and treatments were conducted on the same cohort of children (see Table 1 for treatment and survey regime). The children received a maximum of five treatments in total over a period of 13 months. *S. mansoni* infections were diagnosed using the Kato Katz technique (Katz et al., 1972) with duplicate thick smears for each stool sample collected on three consecutive days; *S. haematobium* infections by filtration of 10 mls of urine sampled on three consecutive days. The initial prevalence was 100% for *S. mansoni* and 97% for *S. haematobium*, with geometric mean infection intensities of 102 eggs/g (epg) and 14 eggs/10 ml, respectively. For detailed infection data and study design see Webster et al. (in press).

### 2.3. Parasite collection and molecular analysis

For this molecular study *S. mansoni* eggs were filtered from positive stool samples using a Pitchford and Visser funnel (Pitchford and Visser, 1975), concentrated and hatched in bottled mineral water. Using a binocular microscope individual miracidia were pipetted onto Whatman FTA® classic cards in a volume of 3 µl of water. The cards were allowed to dry and transported to the lab for molecular analysis. A 3.0 mm disc was removed with a Harris Micro Punch from the Whatman cards at the center of which the sample was loaded and the DNA was either purified using the manufacturer's instructions (only the samples from 2007) or eluted and purified using the Nucleospin® Tissue kit (Macherey–Nagel). The latter samples were eluted in 100 µl elution buffer, vacuum dried using a Univapo 150 ECH (Sanyo Biomedical Equipment) and resuspended in a volume of 20 µl MilliQ $H_2O$. This latter procedure allows for multiple analyses on a single sample, in contrast to the classical FTA assay where a single FTA punch can only be used once (Van den Broeck et al., 2011). Samples were genetically characterized using a multiplex microsatellite PCR with nine loci (see Van den Broeck et al. (2011) for technical details). Allele sizes were manually verified using GENEMAPPER v4.0 (Applied Biosystems).

**Table 2**
Statistical comparison of genetic diversity indices of parasite populations from S0 (baseline), S2 (six months post-baseline), and S5 (two years post-baseline) using FSTAT (Goudet, 2001; two-sided $p$ test; 2000 permutations). *AR*: allelic richness; $H_e$: expected heterozygosity; $F_{IS}$: inbreeding coefficient and $F_{ST}$: fixation index.

| Sampling time | *AR* | $H_e$ | $F_{IS}$ | $F_{ST}$ |
|---|---|---|---|---|
| S0 | 2.38 | 0.40 | 0.06 | 0.001 |
| S2 | 2.47 | 0.41 | −0.001 | 0.017 |
| S5 | 2.52 | 0.43 | 0.08 | −0.007 |
| $p$-value | 0.12 | 0.06 | 0.25 | 0.16 |

### 2.4. Quality control

The software package MICRO-CHECKER v2.2.3 (Van Oosterhout et al., 2004) was used to test for scoring errors, allelic dropouts (i.e. only one of the two alleles present at a heterozygous locus was amplified) and null alleles (i.e. non-amplified allele due to mutation in primer target sequence). Genotyping errors were quantified by re-amplifying at least 10% of all samples (Van den Broeck et al., 2011). Since SMD43 and SMD11 appeared to suffer from null alleles, analyses were performed with and without these loci. All other loci had an estimated error rate ranging between 0% for SMD89 and 4.29% for L46951.

### 2.5. Theoretical expectations

While it has been recognized that gene dynamics in schistosomes is complex due to their indirect life-cycle (Prugnolle et al., 2005), we here focus on a simplified case, namely the sexually generated offspring from a dioecious population of size $N$, sampled prior to migration. We assume that praziquantel randomly kills worms in the treated population and that there is no immigration in the population, so that $N_2$ (the size of the reproducing population after treatment) is a random subset of $N_1$ (the population before treatment), with $N_2 \leqslant N_1$ and $\Delta N = N_1 - N_2$. A small $N_2$ (successful treatment) will result in a correlative reduction of the allelic diversity and expected heterozygosity (Cornuet and Luikart, 1996). While the observed heterozygosity is expected to be rather insensitive to $\Delta N$, this is not the case for the inbreeding coefficient $F_{IS}$ (in the offspring population prior to migration). Under random mating, we expect $F_{IS} = 1/(-2N-1)$ (equation 29 in Balloux (2004), with the number of subpopulations $n$ set to 1). When $N_2$ is small, the effect of size reduction $\Delta N$ is reflected in $F_{IS}$. Since $N_1$ and $N_2$ are effective population sizes, excess variance in reproductive success and deviations from equal sex ratio, which has been described for *S. mansoni* (Webster et al., 1999), will further increase the possibility to detect a bottleneck using $F_{IS}$. Low effective population sizes will also result in alleles becoming fixed randomly (i.e. genetic drift), causing genetic differentiation ($F_{ST}$) to increase between parasite populations after treatment, and between pre- and post-treatment populations. Re-infection following treatment could also result in higher $F_{ST}$ values between pre-and post-treatment populations if it occurs from genetically differentiated source populations. We therefore assess the impact of PZQ treatment on the following population statistics: allelic richness (*AR*), gene diversity ($H_e$), inbreeding coefficient ($F_{IS}$) and genetic differentiation ($F_{ST}$).

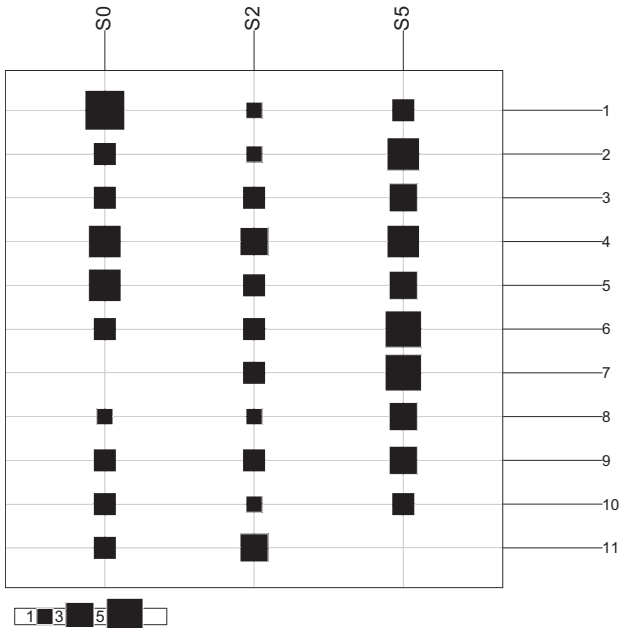


**Fig. 1.** Assignment of the parasite genotypes collected from child ID 49 to the eleven clusters inferred by K-means clustering, for survey 0, 2 and 5. The sample sizes are represented by black squares.

### 2.6. Data analysis

Parasite genetic diversity per host was computed as the expected heterozygosity ($H_e$) and the allelic richness (AR) using FSTAT v2.9.3 (Goudet, 2001). Paired *t*-tests were performed to compare these parameters estimated per locus for each host before (S0) and after treatment (S2) (STATISTICA v9.0). We furthermore tested whether *AR*, $H_o$, $H_e$, $F_{ST}$, and $F_{IS}$ estimated per host differed between sampling times (i.e. before (S0), six months after (S2) and two years after treatment (S5); Table 2; two-sided p-values were obtained after 2000 permutations). This was done in FSTAT using the option "comparisons among groups of samples" where miracidia from each host were treated as a sample and each sampling time as a group.

Pairwise differentiation between hosts was estimated using pairwise $F_{ST}$ following Weir and Cockerham (Weir and Cockerham, 1984) in FSTAT (4000 permutations). K-means clustering coupled with Bayesian Information Criterium (BIC) (Jombart et al., 2010) as implemented in the adegenet package (Jombart, 2008) for R (R Development Core Team, 2011) was used to study the differentiation among hosts and among surveys. The principal component analysis (PCA) was not scaled, the 60 first PCs and four discriminant functions were retained; the proportion of conserved variance was 0.997. Ten independent runs of K-means were used. The number of clusters was assessed by means of successive K-means clustering with increasing number of clusters. The 'optimal' number of clusters was selected on the basis of the lowest associated BIC (i.e. after which the BIC increases or decreases by a negligible amount).

## 3. Results

After the second round of double treatment (time point S3), *S. mansoni* prevalence and infection intensities remained high (67%; 9.8 epg) with a cure rate of 34.1%, while *S. haematobium* was fully cleared. Detailed results on *S. haematobium* and *S. mansoni* infection following each treatment have been described by Webster et al. (in press). Here we present the data for the subset of children from which *S. mansoni* populations have been genotyped (Table 1).

### 3.1. Dataset

Only miracidia with at least six successfully scored loci were included in the analysis, leading to a total of 434 miracidia (91%) divided in 17 samples collected from 12 children at different time points (see Table 1). The sample size ranged between 14 and 45 miracidia per child per time point (mean 26). Nine samples were collected at S0 (214 miracidia; baseline), five at S2 (140 miracidia; 6 months post-baseline) and three at S5 (80 miracidia; two years post-baseline).

### 3.2. Genetic diversity before and after treatment

We detected no significant effect of treatment on the genetic diversity of *S. mansoni* populations. This was true for all summary statistics investigated (*AR*, $H_e$, $F_{IS}$ and $F_{ST}$; Table 2), which showed no significant difference between the three sampling times S0, S2 and S5 (day 0; 6 months later; 2 years later). Paired *t*-tests comparing *AR*, $H_e$, $F_{IS}$ per locus and per child separately before (S0) and after treatment (S2) were not significant either. The number of private alleles was higher in populations from S5 (8) than those of S0 (2) and S2 (0).

### 3.3. Genetic differentiation before and after treatment

K-means clustering coupled with BIC found 11 distinct clusters in the complete dataset without prior boundary definition (overall $F_{ST}$ between clusters was 0.17); these clusters could not be assigned to the individual children. Each child harbored parasite genotypes from almost all clusters. There was no significant shift in the genetic composition of the parasite population at the host level or survey level before and after treatment as indicated by the chi-square test (all $p$ values >0.01). This is also illustrated in Fig. 1 showing the assignment of the parasite genotypes collected from child ID 49 to the eleven clusters inferred by K-means clustering, for S0, S2 and S5. When comparing all 17 samples (host level) with each other, there were only four pairwise $F_{ST}$ values significant after Bonferroni correction, two between children from the same survey (S0), and two between children from S0 and S2. Parasite populations collected from the same child before and after treatment were never significantly different.

## 4. Discussion

More than two decades after the outbreak of intestinal schistosomiasis in northern Senegal, we now have new tools to study the impact of treatment on *S. mansoni* populations. Microsatellite markers allow to study the population genetic structure of schistosomes, and to infer the demographic fluctuations through time. We genotyped parasite populations from twelve children sampled at different time points with nine microsatellite loci. Special care was taken to maximize data quality by means of re-genotyping and detailed quality control (Van den Broeck et al., 2011). Thorough data-analysis demonstrated no significant change in the genetic diversity and structure of parasite populations after repeated PZQ treatment. Pooling parasites according to child or survey did not influence this outcome.

These results are in stark contrast with those reported by Norton et al. (2010) who compared *S. mansoni* populations from two Tanzanian schools before and after treatment, using seven DNA microsatellite markers (of which six have also been used in the present study). They found a significant decrease in genetic diversity six

months after a single round of PZQ treatment, and the parasite populations before and after treatment were significantly differentiated. The latter was suggested to be the result of re-infection. A similar reduction in genetic diversity was observed in parasite populations from the untreated pre-school children, demonstrating that PZQ can have a strong and long-lasting effect on *S. mansoni* population structure. So why do we not find a similar impact of PZQ in the present study?

### 4.1. Re-infection or PZQ resistance?

Drug failure can be excluded because of the successful elimination of *S. haematobium*. Additionally, the second treatment three weeks later should have eliminated the immature worms that may have survived the first treatment (Renganathan and Cioli, 1998). As such, the present results can either be explained by rapid re-infection (intense transmission), or by resistance of the Senegalese *S. mansoni* strains to PZQ, or a combination of these two. Of note, here we consider a population resistant when it is significantly less responsive to treatment than a fully susceptible population, following Coles (2006).

In the first scenario, re-infection should have been very fast and intense to restore the genetic diversity within six months after the first two treatments. Despite the high transmission in the Tanzanian study, the decrease in genetic diversity was still clearly detectable six months after a single treatment (Norton et al., 2010). *F* statistics in this study showed that parasite populations from the same child sampled at the start and six months later (S2), were not significantly different from each other. The number of private alleles only increased in the last survey (S5), two years after the start of the study, suggesting that re-infection might be mainly important on a longer timescale. The absence of new alleles in S2, together with the continued high diversity, could suggest that (part of) the parasite population from S2 survived double treatment.

The possibility of PZQ resistant strains in northern Senegal has been raised before, as an alternative explanation for the low cure rates at the onset of the epidemic in the early nineties (Danso-Appiah and De Vlas, 2002; Ernould et al., 1999; Stelma et al., 1995). At the time, no conclusion could be reached due to many confounding factors of intense transmission and/or the recent nature of the focus (see Section 1). Today, the epidemiological situation has changed. The infection intensities of *S. mansoni* have decreased considerably, with current figures in Nder about 5-fold lower compared to those in 1996 (Picquet et al., 1998). Snail abundance and snail infection are also much lower (personal data) compared to the year-round high numbers at that time (De Clercq et al., 1999). As such, the above confounding factors are less likely to play a prominent role in the current context.

### 4.2. Additional factors

We observed a drastic drop in egg production six weeks after treatment followed by a rapid increase six months later (Table 1). Aside from rapid re-infection, it is possible that this could be explained by a temporary cessation of egg production induced by PZQ (Polman et al., 2002; Webster et al., in press). Other factors that can be involved are treatment history and the high number of mixed infections. The village Nder has been involved in several longitudinal studies with mass treatments in e.g. 1996 and 1997 (Picquet et al., 1996, 1998), and more recent treatments in 2003 and 2006 (our studies). The intense treatment in northern Senegal might have imposed a selection for PZQ-resistant parasites.

Mixed infections can lead to direct competition and mating interaction between schistosome species (Southgate et al., 1998). Such interactions have already been documented in Senegal, with ectopic elimination of eggs (Ernould et al., 1999; Huyse et al., 2009; Meurs et al., 2012), and the occurrence of hybrids between human and animal schistosome species (Huyse et al., 2009). Ernould et al. (1999) found cure rates to be much lower in the Senegalese village with mixed infection compared to villages with single infections. The high prevalence of ectopic *S. mansoni* eggs in urine samples (31%) indicated heterologous pairing between *S. mansoni* and *S. haematobium*. Ten months after treatment, *S. haematobium* infection remained low, while *S. mansoni* egg excretion was seven times higher than at the start of the study. Besides the possibility of PZQ resistance, the authors hypothesized that this was due to heterologous pairing: elimination of *S. haematobium* after treatment 'released' the *S. mansoni* females for mating with the unpaired *S. mansoni* males that survived treatment. A similar scenario might occur here, as the number of mixed infections in Nder increased from 23% in 1996 (De Clercq et al., 1999) to 97.2% in the current study (Webster et al., in press).

### 4.3. Implications and future perspectives

For almost a decade several African countries have been enrolled in mass treatment initiatives aiming at the broad scale control of morbidity due to schistosomiasis. These campaigns provide annual PZQ treatment of all school children. However, even four treatments in a single year were unable to control *S. mansoni* infections in Nder, suggesting that a single treatment per year might not be sufficient. Comparative studies are needed to find out whether Senegal represents a special, isolated case. A meta-analysis by King et al. (2011) showed that the observed cure rates for *S. mansoni* were higher after two treatments with PZQ compared to a single treatment, but the optimal timing interval for the second treatment remains uncertain. This might depend on local parameters such as the transmission season and the maturation rate of the specific strains. Laboratory experiments showed that the Senegalese isolates have a slower maturation rate than the isolates from Kenya and Puerto Rico (with a prepatent period of up to 10 days longer (Fallon et al., 1997)). It might therefore be an option to administer the second treatment 4–8 weeks rather than 3 weeks after the initial treatment.

In order to better quantify the role of re-infection in the continued high genetic diversity of the *S. mansoni* populations after repeated PZQ treatment, future studies should include a larger study cohort together with shorter follow-up times and in depth snail surveys and cercariae genotyping. Increased genomic coverage will provide a better insight in the impact of PZQ treatment on the genetic make-up of schistosome populations, while it can also identify genomic regions that are potentially under selection.

## 5. Conclusions

We could not find an effect of repeated PZQ treatment on the genetic diversity and population structure of *S. mansoni* in Senegal. Besides the possibility of rapid re-infection, this could suggest that some strains may survive repeated PZQ treatment. More field data from the SRB coupled with in depth molecular studies are needed to confirm the results, together with laboratory experiments to assess the possibility of PZQ resistance in this region. Repeated praziquantel dosing as suggested by King et al. (2011) should be explored.

## Acknowledgements

TH is currently a Postdoctoral Fellow of the Research Foundation – Flanders (FWO-Vlaanderen). We would like to thank the Senegalese field team (M. Diop, A. Fall, N. Sy, M. Wade and A. Ya-

gue) for their great help with the fieldwork, and the Senegalese children for their enthusiastic participation in this project. We thank one anonymous referee for useful comments on a previous version of this paper. The molecular part of this work was financed by a project Grant of Research Foundation – Flanders (G0552.10) and the sample collection was organized through the EU-FP6 project "CONTRAST": A multidisciplinary alliance to optimize schistosomiasis control and transmission surveillance in sub-Saharan Africa (FP6 STREP contract no: 032203, http://www.eu-contrast.eu). TJ and FB were supported by the European Research Council (ERC) Grant 260801-BIG_IDEA; TJ is currently supported by the NIGMS MIDAS initiative and the Medical Research Council. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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