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Population structure and dynamics of helminthic infection: schistosomiasis

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Summary

While disease and outbreaks are mainly clonal for bacteria and other asexually reproducing organisms, sexual reproduction in schistosomes and other helminths usually results in unique individuals. For sexually reproducing organisms, the traits conserved in clones will instead be conserved in the group of organisms that tends to breed together, the population. While the same tools are applied to characterize DNA, how results are interpreted can be quite different at times (see Part 4 this series). It is difficult to know what the real effect of any control program has on the parasite population without assessing the health of this population, how they respond to the control measure and how they recover, if they recover. This part of the series “*Advances in Molecular Epidemiology of Infectious Diseases*” will concentrate on one approach using pooled samples to study schistosome populations and show how this and other approaches have contributed to our understanding of this parasite family’s biology and epidemiology.

Biology of schistosomes

Schistosomiasis is a spectrum of conditions caused by infection with helminthic parasites of the genus *Schistosoma*. There are seven species that infect humans and three of these are responsible for the bulk of disease: *S. mansoni*, *S. haematobium* and *S. japonicum*. *S. mansoni* is present in the Americas and much of Africa. *S. haematobium* is found in Africa and parts of the Middle East, while *S. japonicum* is transmitted in East Asia. More than 200 million people are infected with one or more of these species, and large outbreaks, even epidemics have occurred in association with major water resource development projects (1). The complex biology of schistosomes distributes their development between a snail host specific to each species of parasite and humans. Other mammalian species may also be infected and maintain transmission, but are generally of less importance with the exception of *S. japonicum*. A number of domestic and wild animals may be major reservoirs for *S. japonicum*. The adult parasites inhabit the vascular system where females reside within a groove formed by the male’s body to produce 300 – 3000 eggs per day. Infection produces morbidity by the immune reaction to eggs swept along with the flow of blood back into the liver (*S. mansoni* and *S. japonicum*), in the wall of the colon or the urinary system (*S. haematobium*). Intensity and duration of infection along with host predisposition result in portal hypertension with esophageal varices and bleeding (*S. mansoni*, *S. japonicum*) or

hematuria, and hydronephrosis with non-functioning kidney (*S. haematobium*). Eggs embed in the intestinal wall or urinary bladder wall produce polyps or bleeding inflammatory masses. *S. haematobium* also can result in bladder cancer. When the vascular bed of the liver is overwhelmed, eggs are shunted to lungs or brain with resultant pulmonary hypertension or epilepsy. Aberrant migration may produce genital lesions or transverse myelitis and paralysis. These major morbidities occur in 5–10% of those infected. More subtle morbidities of stunted growth (2), anemia (3) and increased susceptibility to HIV (4) are common even in low intensity infections.

Asexual reproduction takes place in the snail (Figure 8.1). When stool or urine containing schistosome eggs reaches water, the change in osmolarity stimulates eggs to hatch. The infective form for the snail (miracidium) finds and penetrates the appropriate snail. Asexual reproduction of cells begins, and these eventually organize into multicellular forms (cercariae) that are released primarily in response to light. The cercariae will penetrate the skin of humans, and over a 4 week period migrate to the portal vein of the liver where they mate and move distally to vessels draining the intestines (*S. mansoni*, *S. japonicum*). Most *S. haematobium* adult worms will continue to migrate to vessels around the urinary system.

The standard diagnostic tool is direct microscopic examination of stool or urine for the presence of the parasite's eggs. Using the Kato-Katz method for feces (5) or urine filtration (6), this assessment can be made quantitative and expressed as the number of eggs per gram of feces or per 10 ml of urine. In areas with a high prevalence of *S. haematobium*, urine dipsticks for demonstrating the presence of blood correlate well with infection (7). During active infection, parasite antigens will circulate in the blood stream and some will be filtered in urine. The only commercial antigen detection test to date is based on the circulating cathodic antigen (CCA) (8) and only tests for *S. mansoni*. This has been developed into a qualitative point-of-care cassette (POC-CCA), which is rapid, easy to use (9), but may be difficult to interpret in low transmission areas (10). Though not commonly utilized, PCR of blood, stool or urine can be a sensitive detection method based on abundant (>10% of genome) repetitive sequences (11, 12), or a schistosome-specific ribosomal internal transcribed spacer (13).

Treatment and resistance

Praziquantel is now the drug of choice (14, 15) and really the only drug available. It is effective against all schistosome species and is usually given as a single dose at a cost of \$0.20–0.30 per adult treatment (<http://www.who.int/schistosomiasis/strategy/en/>). Prior to the 1990's and the introduction of praziquantel at affordable prices, various drugs were used for each species and even for different geographic regions. Most provided 60–90% efficacy as judged by egg negative stool or urine examination. In particular, oxamniquine was very successful for *S. mansoni* in Brazil, but less so in much of Africa. Oxamniquine was eventually superseded because of cost and geographic and species limitations.

Drug resistance is a curious issue in schistosomiasis. In the laboratory it appears that resistance to a number of antischistosome drugs can be produced relatively easily within a few generations in a small laboratory population (16–18). In practice, drug resistance or the

propagation of resistance has been difficult to infer. Clinical resistance to the drug oxamniquine was clearly demonstrated in Brazil in the course of its widespread use for control (19). Parasites from individuals who remained egg positive despite repeated treatments were shown to also tolerate higher levels of the drug in vitro. This never became a broadly significant problem, since Brazil joined the rest of the world and switched to praziquantel in 1996. Recently, using classical genetic crosses and microsatellite linkage mapping, Valentim et al. (20) were able to identify a single gene that explained resistance to the drug in *S. mansoni*, as well as the drug's species-specificity.

Praziquantel is a superior antiparasitic drug with an efficacy of >80% defined as cessation of egg excretion. This still means that 20% may continue to excrete eggs after a single treatment. In part, this is thought due to the differential efficacy for juvenile vs adult worms. During the first 4 weeks after infection, the parasite is relatively resistant, so that cercariae penetrating <4 weeks prior to treatment will be less affected and continue to develop. In the laboratory, the ability to induce praziquantel resistance has been variable with failures (17, 21) and successes (18, 22, 23). Another confounder in evaluating drug efficacy is evidence that the drug may sterilize, but not kill some parasites (24). This may explain individuals who excrete no eggs, but still have positive POC-CCA tests (25). The public health significance of this, however, is not clear. Sterile worms attracting fertile mates and releasing antigens may even be protective.

Some degree of resistance to praziquantel in the course of treatment has been suggested broadly during several control programs (26, 27), in isolated case reports (28, 29) and small-scale research studies (30, 31). The evidence in field studies, however, remains weak. Failure of repeated treatments to clear infections has been reported in Egypt, Kenya and Senegal. The Senegal case was particularly disturbing, since "cure" rate was <40%. The Senegal case could rightly be described as an outbreak with essentially universal infection with some of the heaviest intensities ever seen for *S. mansoni*. The intensity in 40% of infections was >1,000 eggs per gram of feces in an area where *S. mansoni* infection was previously absent or rare. By WHO criteria, egg counts of 400 eggs per gram are considered heavy and usually represent ~10% of all infections in endemic areas. Normal response rates were seen in those who moved to non-endemic areas, and cure rates improved by examining treated individuals 6 weeks rather than 12 weeks after treatment. This suggested that the intensity of infection, the presence of juvenile worms and rapid reinfection best explained apparent low cure rates in most instances (26). Furthermore, cases of putative resistance have never extended out of a limited area. If these instances represent a type of heritable resistance, their degree of geographic limitation is unlike resistance in most global pathogens. Use of praziquantel for 40 years and in ways that seem designed to promote resistance has produced very little. It is still curious that more reports of resistance are not observed for praziquantel given the ease with which it can be selected for in laboratory studies. King (32) has suggested among other reasons praziquantel resistance may have fitness costs that limit its emergence in nature. Since there is only one drug in common use, the future development of resistance remains a serious concern.

Genotyping methods and tools

Genotyping methods for schistosomes have always reflected those applied to other eukaryotes. The appropriateness of markers follows the same criteria as for other organisms. They are judged by the degree of polymorphism, abundance, ease of typing, reproducibility and ease of interpretation. Isoenzyme analysis introduced in 1970 was used primarily to differentiate species and identify intraspecific geographic distribution (33). Restriction fragment length polymorphisms (RFLP) (34), random amplified polymorphic DNA (RAPD) (35) and mitochondrial markers (36) followed. While the other markers are primarily of historical interest, Mitochondrial markers continue to be used in phylogeny (37). For population studies, however, mitochondrial markers present a limited number of possible alleles, are not entirely neutral, and potential biparental inheritance in schistosomes may complicate interpretation of population data (38, 39).

Currently the most widely used genetic markers for the study of schistosome populations are microsatellites also termed short tandem repeats (STRs). Microsatellites are repeated sequences of typically 1–6 nucleotides. They are abundant and primarily located in non-coding regions of the genome. In the human genome these repeats occur on average every 15,000 bases (40), and hundreds have been identified in the genomes of schistosomes (41, 42, and others). Microsatellites tend to vary step-wise in size, so that a series of trinucleotide repeats vary by multiples of three and tetranucleotide repeats by four, etc. They can thus be easily scored by gel electrophoresis or automated sequencer. Microsatellites have high information content, since each locus can have 20 or more polymorphisms in a population. This is the result of a high mutation rate estimated at 10^{-4} /generation, which can complicate long-term comparisons.

Single nucleotide polymorphism and sequencing now dominate studies designed to identify genes associated with traits and diseases. SNPs occur every 300 – 1,000 bases in the human genome (43) permitting finer resolution than microsatellites, but they have lower information content, since their polymorphisms tends to be binary. Sequencing has been estimated to provide three orders of magnitude more information than typical microsatellite genotyping (44), but is limited by the number of miracidia and infrapopulations that can be processed. SNP analysis along defined genomic regions has been very useful for species differentiation and identification of species hybrids as have been seen in Senegal (45, 46) and even France (47). Microsatellites still remain one of the principle markers for population genetics studies due to their high information content, low cost, high throughput and sufficient resolution for most questions of population structure.

Sampling

Parasite developmental stage.

The first challenge in sampling schistosomes is to know what to sample, since parasite development is distributed between snail and human hosts or many additional vertebrates for *S. japonicum*. Each of these infrapopulations offers different stages and different distributions of genotypes. Sampling snail stages (sporocytes and cercariae) directly represents the immediate potential transmission to humans, but has many limitations with respect to the

total genetic composition in the human host. Snails generally have low infection rates (<1 to 5%), are infected with 1–3 individual parasites and have a short lifespan. This means that it is hard to comprehensively sample snails, and only the most recent snail exposures are present (48). The parasite population that is most important for morbidity and for transmission to snails is the one residing in the human host. Adult worms reside in the vascular system of their definitive hosts, so, of the stages present in humans, only eggs are practically accessible. At any given time, the majority of the parasite's genetic variation resides in the human hosts. The same is not true of stages in the snail. Despite our greater mobility, the biology and distribution of humans in a community is much better understood than the distribution of the intermediate host. There are few non-human definitive hosts for *S. mansoni*, and these are not essential for maintenance of disease in most endemic zones. In addition to being large, the best sample is one taken with the least selection bias or whose bias can be corrected for. For sampling, the distribution of humans is more easily accounted for than any other host. For sampling from humans, stool or urine eggs are abundant and readily available.

Compartments.

The interpretation of results for an epidemiologic study is dependent on how sampling is performed. In molecular epidemiology this means the sampling strategy should be dependent on the biology of the distribution of DNA. Schistosome population genetics involves at least three levels of sampling: gametes, infrapopulation and component population. The ideal number of eggs or gametes needed to represent each category is unknown and will differ depending on the structure of each particular population. A population with high variability requires more intense sampling than one with low variability. An adult with a light *S. mansoni* infection will eliminate ~5,000 eggs/day in their stool. The exact number of corresponding adult worms is not known, but an estimate based on studies in baboons (49) suggests this will be the product of 27 worm pairs compared to 80,000 egg from >400 pairs in a heavy infection. In any case, “the greatest accuracy for estimating population genetics parameters is achieved by employing a large sample size” in order to represent the gametic output (50).

Adult schistosomes live 5–40 years (51, 52) and are responsible for sexual reproduction in their human hosts. Each human host accumulates a complement of worms from the available pool by repeated infections (immigration) from variety of snails from a variety of locations and over years. The aggregate in one host is designated the **infrapopulation** (53), whereas all parasites from all hosts of one species within a geographic area are considered the **component population** (Figure 8.2). The component population can also be defined in a number of ways (vertebrate host, snail host, host characteristics, time) adding additional potential levels of analysis. These are not alternative or competing compartments, so the question is not which to use, but which aspects of biology and distribution are being queried. Parasite infrapopulations represent the distribution, characteristics and risk to individual hosts. They differ from the typical genetic definition of populations or subpopulations in that they are formed entirely by migration (repeated infection), and all progeny that will continue the cycle are exported. Thus, for a single infrapopulation there can be no expectation of Hardy-Weinberg equilibrium (HWE). Analyses of infrapopulations that rely on assumptions

of these proportions must be interpreted with caution. The component population is more typical of populations with an expectation of Hardy-Weinberg proportions by better conforming to the assumptions of this model. We (54) and others (55) find that the infrapopulation is an imperfect, random draw from the component population. Since the relationship among infrapopulations versus that among component populations addresses different questions, we termed differentiation comparisons between infrapopulations **Di** and between component populations **Dc**. We also calculate the differentiation of the individual infrapopulation from its component population or the **Dic**.

Sample size.

Some approaches taken for genetic sampling of schistosome populations, have been necessarily restrictive and potentially biased. One approach has been to isolate eggs from infected hosts, use these to infect snails and then laboratory animals. This sampling scheme estimates parasite population parameters by selecting a small percentage of eggs from a small number of individuals and using these to infect laboratory-bred animals. At the first step, less than 1% of eggs is collected, then <50% of the eggs will hatch (56, 57). Parasite strains are often specific for certain snail strains (58), so infection of a non-adapted snail with a non-adapted parasite population could produce selection and bias. If an investigator were to go further and infect laboratory animals, these show preference for some genotypes (59). When the population is sampled indirectly from laboratory-passaged populations, successive filters greatly reduce the sample size and introduce a significant degree of bias.

Another approach to the problem of sampling is to genotype single miracidia hatched from eggs. While avoiding the filtering effects of animal passage and laboratory rearing, this approach greatly limits the number of samples that can be processed from an infrapopulation and the number of infrapopulations that can be sampled within a component population. Nevertheless, one study concluded that as few as five eggs per host was adequate for the population studied (60). Others have sampled a maximum of 60–70 eggs for each of 60 hosts (55), but it is difficult to confirm whether either of these numbers is adequate. Schistosome diversity is such that in a study of parasite diversity in mother and child pairs, the cumulative number of *cox 1* haplotypes never approached a limit despite genotyping more than 1,600 miracidia from >100 infrapopulations (61). We empirically tested the effect of limited sampling of infrapopulations by taking random samples from two small communities where stool had been obtained from most of the detectable infections. We then compared the accuracy and precision of random samples taken from the whole population. We observed that 50% of samples were within 10% of the true value only when at least 35–40% of the infrapopulations were included (62).

An egg-by-egg approach has an additional limitation, the small amount of DNA obtained. Whole genome amplification (WGA) can overcome this problem (63, 64), and while WGA is generally very faithful, it can introduce amplification bias (65, 66) and does increase cost. By contrast, our approach has been to collect the pool or aggregate of eggs from the whole stool of infected individuals, isolate the DNA and genotype this “total” infrapopulation DNA to obtain allele frequencies. Sampling the full complement of eggs from the stool of infected humans provides the most direct assessment of the infecting population. It produces a large

sample of the infrapopulation and because of processing efficiency, allows the greatest number of infrapopulations to be assayed. Sampling small numbers can result in strong bias for important population measures, which are sensitive to sample size, such as N_e (67). Some suggest that adequate samples should consist of up to 10% of the N_e (68). Differentiation, diversity and effective population size are powerful analytic tools in population genetics for which aggregate samples are appropriate and have significant advantages in scale and representativeness.

Genotyping microsatellites in pooled samples produces some technical distortions that are easily overcome. Some strategies can be associated with bias in the construction of the pools, but schistosome eggs are naturally aggregated in stool or urine. Stuttering during amplification can occur due to the same mechanism that produces microsatellite polymorphisms in vivo, especially for dinucleotide repeats. We have shown that stuttering for tri- and tetrameric markers is not a common problem (see Figure 8.3). Larger alleles tend to be slightly less efficiently amplified, but unequal amplification can be corrected. When corrected and uncorrected values were used to calculate differentiation indices, there was no difference in their values in our own work (69, 70) or that of others (71). Peaks not at the expected repeat interval and peaks with heights less than the arbitrary cut off of 100 pixels were eliminated from the analysis. The program Peak Scanner v. 2 (<http://peak-scanner-software.software.informer.com/>) will label peaks with height, area and size when selected. A table of the selected peak heights and areas is also generated for analysis.

The genotyping of aggregated stool eggs is designed to answer specific kinds of questions that are most important for public health, i.e. differentiation of populations, their dynamics of growth or decline, heterogeneity and effective population size. Some standard genetic indices and effects cannot be measured, such as, Hardy-Weinberg proportions, F_{is} , null alleles and doubtlessly others. However, family structure, “who’s sleeping with whom” and many other features of populations are not essential to the questions being asked in public health related to schistosomes. Due to asexual reproduction in the snail host, there is also potential for a greater degree of clonality in infrapopulations than seen in animals that only reproduce sexually. Yet, in the vertebrate host, there is high diversity, clonal siblings are uncommon (72) and diversity is higher within than among hosts. Théron et al. (73) conclude that the asexual reproduction in the snail does not influence the diversity of adult worms in the definitive host. While eliminating relatedness is essential for genetic association studies, some degree of relatedness is part of all populations. The frequency of sickle-trait in Ghana or Cleveland is not determined by eliminating siblings from the analysis. Waples and Anderson [Waples, 2017 #289] caution that purging of siblings risks reducing sample size, decreased statistical power and precision and incorrect assignment. At an extreme, eliminating relatedness in a population where 50% of individuals are clones would lead to a distorted picture of its past and present composition and its potential. Pooled samples provide good estimates of allele frequencies that compare favorably with those as measured by discrete genotypes with 5–10% error (70, 74). There is little benefit to obtaining more accurate allele frequencies for biased or inadequate samples.

Public Health Implications of Schistosome Population Structure

Regardless of the approach taken, certain characteristics are central to understanding parasite dynamics: differentiation (how different are two populations), and diversity (how much heterogeneity is there in the gene pool, how resistant to genetic drift). Knowledge of these aspects of the parasite community can be used to assess parasite reproduction, selection, response to control measures and persistence.

For schistosomes, many papers have described a cross-sectional analysis of local populations using genetic markers, especially microsatellites. Universally they conclude that schistosome populations are highly diverse (55, 72, 75–79). There is also a high degree of parasite variability in individual human infections despite clonal reproduction in the snail host (73, 80). The results of the effect of treatment, however, differ substantially from site-to-site and depending on the investigational approach. We have studied schistosome populations in nearly all (96%) infected individuals in two small rural communities of Brazil through repeated treatments over five years and two rounds of community-wide treatment (54, 81, 82). We also used an analytic approach based on allele frequencies from aggregated eggs to assess the nature of transmission in an urban area not recognized as endemic (48, 83).

The two study villages in rural Bahia state were selected for their small size and for being geographically discrete. The larger community (Jenipapo population 482) and the smaller community (Volta do Rio population 367) were comprehensively surveyed and parasites from all identified infections included in an analysis of population structure. They were separated from each other by 6 km, from major towns by 10–12 km and all located along the same river (Jiquiriçá). Importantly, essentially all homes had flush toilets, but 37% (larger village) and 10% (smaller village) of these discharged to the river. Day-to-day egg production is known to be highly variable, so an early question was how stable were allele frequencies. Stools from three different days were examined for 96% of participants, and 30% of those found to be infected by Kato-Katz thick smear had eggs on all three samples. Each was genotyped and compared. The mean D_i between different runs of the same sample established a baseline of 0.007. The variation in egg output could be as high as 80 fold, but the mean D_i between samples from the same person collected on three different days was just 0.010. Thus, there was little variation in the composition of genotypes excreted over the span of a week, and samples from a single day will be representative of that infrapopulation. In addition, this implies that the daily variations are not due to variation in the egg output of specific pairs or groups, but more likely due to host factors that globally regulate egg output, egg release or egg detection.

Infrapopulations were moderately differentiated. The mean D_i between hosts was 0.083 and 0.096 in Jenipapo and Volta do Rio, respectively. The aggregate of parasites present in an individual host, therefore, is a poor sample of the available genetic potential of the component population. The complete sampling of detectable infections for two component populations provides an important comparator for other studies. It also emphasizes that p values only indicate whether samples are different and depend on the number of samples collected. Since thousands of eggs contributed to each determination, p values were

universally significant at <0.05 and confidence intervals were very narrow. We, therefore, relied on the effect size as measured by Cohen's D (84–87) to decide whether samples were not only different, but whether that difference was important. Cohen's D is the standardized mean difference between two groups.

The parasites in the two communities should be considered two different component populations if there is limited gene flow between them. They are separated by only 6 km along the same river and a paved highway. All of the children from one community go to school in the other, and the regional health clinic with a nurse, doctor and pharmacy is located in one community. Component population allele frequencies can be determined by calculating the allele numbers for each infrapopulation (allele frequency X eggs per gram X stool weight), summing these for each community and, finally, calculating the D_c based on total number of alleles per marker per community. The D_c was 0.045, which is not 0.05, the standard definition of moderate differentiation, nor is it 0.007, the baseline value for differentiation in infrapopulations, but it clearly indicates there is an obstacle to gene flow between these communities. This is to say that in these communities where everyone has a flush toilet, people tend not to leave feces with their neighbors down the road, and the septic tanks at the school and the clinic function. Consistent with this we observed that few residents moved between the communities. Importantly, it reinforces the observation that in many areas, transmission dynamics are local and in this region, at least, schistosomiasis exists on a fragmented landscape.

Multiple studies suggest that schistosomes are parasites of opportunity. They do not select hosts by demographic characteristics. Their populations are not structured by sex or infection intensity. In terms of age, Betson et al. (61) found that parasites from mother-child pairs were drawn from the same genetic pool. Barbosa et al. (54) also found no structuring by age. By contrast, Van den Broeck et al. (55) did see weak inverse correlations between age and inbreeding ($r^2=0.112$) and between age and observed heterozygosity ($r^2=0.169$). Discrepancies may reflect local differences in the population structure and/or methodological differences. The strong overall implication is that for the most part, infection risk is geographical and behavioral and not associated with other features of individual hosts.

Immigration

Schistosomiasis transmission is generally considered a problem of rural areas, and in Brazil, for example, major cities are not usually considered endemic for transmission. Yet infected individuals can be identified at low prevalence in major cities of the endemic regions of Brazil (88–90) and Africa (91, 92). The public health response should differ depending on the relative contribution of immigration or local transmission. Brazil has undergone a comparatively recent demographic transition from rural to urban, such that the country is now $>80\%$ urban. The city of Salvador is the state capital of Bahia and Brazil's fourth largest city. It has grown 300% over the last 30 years to >3 million. Overall, the prevalence of schistosomiasis is $<2\%$, but these infections are not uniformly distributed. In the neighborhood of São Bartolomeu, 30% of school-aged children were found to be infected in 2004 (93). This area is defined geographically by major roads and an urban park with a

reservoir that drains into a river whose outlet is surrounded by homes. The total population at the time of the study was 6,000, so we were only able to examine all residents in the three most heavily infected sectors of the community (n=1,225). The rapid growth of Salvador might suggest that a major component of schistosomiasis in São Bartolomeu represents the arrival of infections from rural districts. We observed that only 12% of the population had been born outside of Salvador. These had come from 35 districts throughout the endemic area of the state. One expectation from a large amount of recent immigration of parasites from many different regions would be that average D_i would be large. Average D_i for São Bartolomeu was 0.063, perhaps smaller, but not larger than in the rural area [Blanton, 2015 #112]. Further evidence was provided by comparison of stool samples collected in 2004 compared to those in 2011 representing the component populations at these times. Here the D_c was 0.007, essentially no differentiation. Although these rural and urban parasite populations likely began by immigration at some point, it was the local conditions that supported their persistence. The implication for public health is that if schistosomiasis could be eliminated in this and other foci, immigration would not soon result in its reintroduction. Additionally, given the fragmented genetic landscape for this infection, if resistance to praziquantel were to arise in one community, it would not easily be carried to its neighbors.

Resistance

In Brazil, we have encountered on occasion individuals who repeatedly fail to clear or even reduce their infections. On close questioning, it was clear that they had never actually taken the medication. For those who did take medication, most cases of persistence are associated with heavy infections, and in our studies, the initial intensity of infection for those with persistent infections was twice that of those who cleared with the first dose. In the first year of the study for both rural communities, 11% (36) of those treated were still egg-positive 4–6 weeks later. Jost's D for these persistent cases compared to their own pre-treatment infrapopulations or to infrapopulations that cleared with the first treatment was between 0.005 and 0.008, essentially no differentiation. To account for the possibility that some of these cases had not taken praziquantel, those whose egg counts did not fall by >50% were removed from the analysis with no change in Jost D values. This analysis is equivalent to assaying a population the day after a selective sweep. There has not been time for mating and crossing over to transfer a resistant gene or genes onto other multilocus backgrounds. Assuming resistance arises from one or a few organisms, the removal of susceptible adults should result in a less diverse population that is differentiated from the parent population (Figure 8.4). The D_c between persistent and susceptible groups was small. In other words, the persistent and susceptible parasites derive from the same pool. This is not direct proof of a lack of resistance, but under a set of reasonable assumptions, it contributes evidence that persistence here is not due to resistance to praziquantel. Subsequent rounds of treatment could not be assessed for resistance since only zero, one or two individuals continued to produce eggs. Gower et al. (94) also compared *S. mansoni* population structure before and after repeated treatments and likewise observed no evidence of selection. This approach can be applied to help understand those instances where response appears to be low due to praziquantel resistance.

Longitudinal population structure following repeated treatment

Increasingly, schistosomiasis control efforts have become aimed at its eventual elimination or local eradication, a goal not often achieved. All approaches include treatment of infected children and/or adults with praziquantel, but these treatments have to be repeated due to reinfection. The composition of the parasite population following repeated treatments may reveal some of the bases for persistence validate current approaches or point the way forward to more effective measures. A huge obstacle is that much of schistosome biology is hidden. Individual adult worms and lineages are not visible; their movements across the landscape cannot be followed; circulation of the parasite in the intermediate host population is difficult to measure; overall reproductive success may not be well reflected in the census numbers of parasites, and prevalence and intensity cannot suggest future reproductive capacity. Population genetics today has been applied most actively to animal breeding and conservation. Conservation genetics in particular has provided useful indicators for the health of endangered populations, future prospects for survival and the effect of management efforts. Its analytic inversion, extinction genetics (95), can be useful in the evaluation of population status and their potential for elimination based on repeated treatments.

The study of the consequences of repeated treatment on schistosome population genetics has produced disparate outcomes (31, 78, 96). This is not surprising given that there is differing biology between species, differing ecology in which hosts and parasites are embedded and different approaches to treatment and study design. At our rural site, after 3–4 rounds of community-wide treatment over a five-year period, prevalence decreased in the two communities from 45 and 35% to 5.9 and 5.6%, in the larger and smaller communities, respectively. Total community-wide egg burden was reduced by >10 fold. Some measures of diversity responded more quickly to control efforts than measures of differentiation. The D_i was unchanged unless total community egg production reduced by at least five fold. By contrast, N_e , which is related to diversity, showed a striking decline by 15–20 fold after the first round of treatment in both communities. After the second treatment, we observed a further reduction in N_e such that N_e in both communities was near or below 1,000. N_e is an important quantity in that it suggests how resilient a population is to environmental stresses. Franklin (97, 98) proposed that an N_e of 500 is associated with the eventual long-term elimination of a population, while Lande (99) proposed that 5,000 was sufficient for predicting extinction. A lower value of 50 has been suggested as predictive of short-term extinction (68, 100). Repeated treatments of schistosome populations will be one way of assessing the significance of these values or provide new ones that may indicate the likelihood of elimination in the short-term. Changes in N_e itself can indicate the effectiveness or failure of control measures.

Norton (96) in a school-based study did observe differences in component population composition after a single treatment, however, statistical significance was not achieved for all analyses, sample sizes differed for estimates of allelic richness and different groups of children were compared in different years. Most other studies have failed to show an impact of treatment on parasite genetic structure with school-based approaches. In Senegal, Hyse et al. (31) observed little change in diversity in one village after five rounds of treatment in two years. Individuals older than 15 years had not been treated, however. A similar result was

observed following a decade of school-based treatment in Kenya (78). In our studies where all those found to be infected were treated, we observed that a change in diversity requires a 10 fold decrease in community-wide egg production. Although the treatment approach and analytic methods employed were quite different from our own (some studies examined only 12 children), the lack of change in diversity may have been a consequence of rapid reinfection from an extensive snail reservoir, the extreme biomass of parasites in the area and/or lack of drug coverage for the whole population. School-aged children represent less than 20–30% of a Brazilian population. This age group produces a disproportionate number of eggs and may be more prone to contaminate the environment, but in our experience, in Brazil the majority of eggs are still produced by the non-school-aged population. School-based studies are a way to reduce morbidity in the most heavily infected group, but transmission is likely difficult to interrupt and population structure difficult to change without more extensive coverage.

Rapid reinfection versus failure to eliminate worms can also be distinguished by analysis of individual infrapopulations over time (81). This requires a wide enough sampling of individual infrapopulations in order to make valid comparisons post-treatment. We confirmed egg- negativity and genotyped apparent reinfections. Mean infrapopulation differentiation between treatment periods was moderate (0.055 – 0.077), indicating that a substantially new population of egg-laying parasites had been acquired by individuals, rather than persistence of the pre-treatment population. This does not eliminate the possibility that adult worms persisted but were infertile. This possibility should have little consequence for public health, however.

Summary

Results from the study of population genetics of schistosomes has several important implications for the elimination of the parasite. Schistosomiasis mansoni is a locally transmitted disease. Some studies do suggest a large amount of gene flow over a wide area (80), but most studies suggest a predominance of focal transmission on the scale of village or neighborhood (72, 75, 79, 81). While schistosomiasis may be introduced to an area by migration, its persistence in an area owes more to local transmission. Even when the life cycle is in wild rodents, the genetic distribution of schistosomes is described as a fragmented landscape (101, 102), which reflects the degree of spatial differentiation and obstacles to gene flow. Humans are highly mobile day-to-day, but these studies suggest they tend to leave their feces at home. Parasite genotypes do not show preference based on host characteristics (54, 55), so individuals are at equal risk upon exposure. It is possible to drive prevalence, intensity and most importantly total egg burden down to low levels with intensive praziquantel alone, but very hard to eliminate the parasite in this way. Unpublished studies from our ongoing project in rural Brazil show unrelenting declines in *S. mansoni* populations out to 6 years even with lapses in yearly treatment. Each year the percent decline is significantly less even as we reach single digit prevalence. This does not appear to be the result of development of significant resistance. With directly observed drug administration, except for the first round, treatment was followed by only 1–2 persistent cases. Note that despite the report of resistance in Egypt in 1996 (27), the continued intense use distribution of praziquantel has remained effective and shown continued decline in

prevalence even in the same village (103). In population genetics, a fragmented landscape impedes gene flow and makes it difficult for mutations to spread throughout the population. In addition, driving the census population down and eventually the N_e as well increases genetic drift, and increasing drift blunts the force of selection. In the Brazilian populations we study, intense application of praziquantel over identifiable component populations that includes all detectable infections is the recommended approach. Comparative studies of African populations should be conducted and may contribute to explaining their relative refractoriness to control programs. However, saving the obvious for last, adequate sanitation combined with awareness that urine also transmits disease where there is *S. haematobium* is the final push needed to effect not just an amelioration of morbidity, but the elimination of transmission.

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KEY POINTS

- There is little day-to-day variation in intrapopulation allele frequencies
- There is moderate inter-host parasite differentiation
- Individual hosts incompletely sample the component population
- There is an obstacle to gene flow over short distances (km)
- Parasites do not select hosts
- Immigration may establish populations, but is a minor factor in persistence
- Resistance is likely a minor factor for persistence in most populations

Figure 8.1. Schistosome life cycle.

Developmental stages for the three major species, the three intermediate host snail genera and the human host. **Eggs** in urine (**H**- *S. haematobium*) or stool (**M**-*S. mansoni*, **J**-*S. japonicum*) hatch on contact with fresh water. The newly hatched **miracidium** finds the appropriate snail host where asexual reproduction occurs to produce **cercariae**. Cercariae are released from snails and penetrate the skin of the human host (also other animals for *S. japonicum*) and migrate to the hepatic portal vein system. Mated worms take up residence in veins near the intestines (*S. mansoni*, *S. japonicum*) or the urinary system (*S. haematobium*).

Adult worms reproduce sexually and make eggs that continue the cycle. [Adapted with permission from (3)].

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Figure 8.2. Schistosome subpopulation structures.

Infrapopulations: Each small box represents the multilocus individual schistosomes (colored dots) from an infected person. **Component Population:** The large box is the combined multilocus individual parasites from all humans in the community. New infections conceptually are taken from the human component population, since this over time returns to the snail component population where human infections actually arise.

Figure 8.3. Electropherograms for multiplexed amplifications of *S. mansoni* eggs from stool. DNA from infected individuals was first amplified using primers for one marker and then up to four amplifications from different markers were combined and processed for microsatellite genotyping on an automated sequencer. a) Results for one infrapopulation and a set of four microsatellite markers. Top – all markers combined, Middle – two markers with labeled peak characteristics for SM13-410 including size (bp), peak height and peak area, Bottom – three markers showing only trimeric marker SM13-410 yielding peaks with three bp spacing. Peaks with spacing not a multiple of three are not recorded. b) Results for the same marker set as a) from three different individuals with different intensities of infection. Allele frequencies are calculated as the peak height or area divided by the sum for the peaks for that allele. Using height or area gave comparable results. Tabs under each peak in the electropherogram identify height, area and fragment size.

No Selection

Selection

Figure 8.4. Assessing for selection by population differentiation post-treatment with praziquantel.

Schistosome infrapopulations are highly variable. If persistent populations arise from stochastic processes (e.g. refractory juvenile parasites, parasites in a protected location), the day after a selective sweep (praziquantel treatment) will leave a residual largely derived from the parent population (small pre-post Di). Assuming that resistance arises on one or a few multilocus backgrounds, the day following a selective sweep will leave a population that

is more similar to itself than to the parent population (large pre-post Di). Colored discs represent different classes of multilocus genotypes.

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