Cheap, rapid and efficient DNA extraction method to perform multilocus microsatellite genotyping on all Schistosoma mansoni stages

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Running headline: *Schistosoma mansoni* DNA extraction method

Cheap, rapid and efficient DNA extraction method to perform multilocus microsatellite genotyping on all *Schistosoma mansoni* stages

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Abstract

Schistosomes are endoparasites causing a serious human disease called schistosomiasis. The quantification of parasite genetic diversity is an essential component to understand the schistosome epidemiology and disease transmission patterns. In this paper, we propose a novel assay for a rapid, low costly and efficient DNA extraction method of egg, larval and adult stages of *Schistosoma mansoni.* One euro makes possible to perform 60 000 DNA extraction reactions at top speed (only 15 minutes of incubation and 5 handling steps).

Keywords : *Schistosoma mansoni* ; DNA extraction ; Microsatellites

Schistosomes (Plathyhelminth, Digenea) are endoparasites causing a serious human disease called schistosomiasis. Schistosomiasis ranks second only to malaria in terms of parasite-induced human morbidity and mortality, with more than 200 million people infected (Chitsulo et al. 2000; Crompton 1999). The quantification of parasite genetic diversity is an essential component to understand schistosome epidemiology and disease transmission patterns. This genetic diversity could be assessed either at the adult stage (Theron et al. 2004) or, more recently, at the larval stage (Shrivastava et al. 2005; Sorensen et al. 2006). The use of adult worms to quantify the genetic diversity in the definitive host is only relevant when worms can be directly recovered from naturally infected rodents (Theron et al. 2004). The quantification of parasite genetic diversity from intra-human (Brouwer et al. 2001; Curtis et al. 2002; Stohler et al. 2004) or intra-molluskan stages (Dabo et al. 1997; Eppert et al. 2002; Sire et al. 2001) requires a long time for a passaging through experimental hosts. However, mollusk or vertebrate experimental host may induce a bias due to this host selective pressure. Indeed, such laboratory passage may be predicted to result in genetic bottlenecking of the parasite population and impose selection pressures not encountered in field conditions. Firstly, exposure of individual snails to single miracidia results in only 5 to 50% of successful infections, depending on the parasite strain used (Theron et al. 1997), thus between 50% and 95% of the parasite genetic diversity is lost. Secondly, as far as the vertebrate host is concerned, it has been shown that passaging through experimental models decreases the parasite genetic diversity in comparison to field isolates (Loverde et al. 1985). To circumvent ethical, technical and epidemiological disadvantages of the use of experimental hosts, methods for genotyping larvae have been recently proposed (Shrivastava et al. 2005; Sorensen et al. 2006). Due to their small size (450 µm for

cercariae and 150 µm for miracidia), the main technical limitations of these studies were the available quantity of DNA to perform PCR amplifications. In 2005, Shrivastava et al. (2005) proposed a DNA extraction protocol allowing sufficient DNA for only one PCR reaction by larvae, thus for only one locus analyses. In 2006, Sorensen et al. (2006) proposed a more complex protocol, only tested on eggs and that required liquid nitrogen to disrupt the eggshell by heat shock and Instagen Matrix (Bio-Rad) to capture DNA. This last protocol permits multi-locus analyses but it requires a particular material and finally, the resulting analysis have been performed only on eggs. In this paper, we propose a novel assay for a very rapid, very low costly and efficient DNA extraction method of adult and free larval stages from individual *Schistosoma mansoni*. To investigate the efficiency of the method, we have performed DNA extraction of individual schistosomes from all life cycle stages (except intramolluskan stage) and used five microsatellite markers of various sizes (i) on 10 individual eggs derived from faeces of infected mice, (ii) on 10 individual miracidia obtained from eggs purified from the livers of infected mice, (iii) on 10 individual cercariae derived from monomiracidially infected mollusks, (iv) and finally, on 10 adults obtained from infected mice.

The *S. mansoni* strain was isolated from naturally infected mollusks collected in Guadeloupe (French West Indies) in December 2002. The intermediate host used was a Guadeloupean strain of *Biomphalaria glabrata* and the definitive host was the Swiss OF1 mouse strain. Detailed methods for the mollusk and mouse infections were previously described (Boissier and Moné 2000). The biological material (eggs, miracidia, cercariae and adults) was obtained around the parasite life cycle. (i) Eggs were recovered from faeces of two Swiss OF1 infected mice. Ten eggs were

individually isolated in 5 µl of NaCl 8% and transferred in a PCR reaction tube using a 20 µl micropipette. The presence of only one egg in each tube was checked under a binocular microscope. (ii) Miracidia were hatched from eggs purified from the liver of one OF1 infected mouse. Ten miracidia were individually isolated in 5 µl of spring water and transferred in a PCR reaction tube using a 20 µl micropipette. The presence of only one miracidium in each tube was checked under a binocular microscope. (iii) Mollusks were individually exposed to individual miracidium which all originated from the same mouse. Five weeks later, mollusks were individually placed in spring water and exposed to artificial light to stimulate cercarial release. Ten cercariae derived from mollusks were individually isolated in 5 µl of purified spring water and transferred in a PCR reaction tube using a 20 µl micropipette. The presence of only one cercariae in each tube was checked under a binocular microscope. (iv) One mouse was infected using 120 cercariae. Seven weeks later, the mouse was sacrificed and 10 worms were recovered and individually isolated.

The same DNA extraction procedure was used, for either adult or larval stages. Before DNA extraction, individual eggs, miracidium, cercariae or adult worms were individually vacuum-dried for 15 minutes in a Speedvac evaporator. Then, 20 µl of NaOH (250 mM) was added to each tube. After a 15 minutes incubation period at 25°C, the tubes were heated at 99°C for 2 minutes. 10 µl HCl (250 mM), 5 µl of Tris-HCl (500 mM) and 5 µl Triton X-100 (2%) were added and a second heat shock at 99°C for 2 minutes was performed. The products were stored at -20°C. The PCR amplifications were performed in duplicate using 5 microsatellite markers (Table 1). The PCR reactions were carried out in a total volume of 20 µl containing 4 µl of 5x buffer (10 mM Tris-HCl, pH 9.0 at 25°C, 50 mM KCl, 0.1% Triton X-100), 0.2 µM of each

oligonucleotide primer, 200 µM of each dNTP (Promega), 1 unit of GoTaq polymerase (Promega, Madison, Wisconsin), 1 µl of extracted DNA and DNase-free water qsp 20 µl. The PCR programme consisted in an initial denaturation phase at 95°C for 5 min, followed by 40 cycles at 95°C for 30 s, annealing temperature for 20 s (Table 1), 72°C for 30 s, and a final extension at 72°C for 10 min in a thermocycler (Bio-Rad, Hercules, USA). For each marker, the forward PCR primer was 5' fluorescein labelled (Proligo, Cambridge, UK) allowing a precise analysis in an automated DNA sequencer. The microsatellite PCR products were diluted in Sample Loading Solution (Beckman Coulter, Villepinte, France) with a red labelled size standard (CEQ^{TM} DNA size standard kit, 400, Beckman Coulter) and electrophoresed using an automatic sequencer (CEQ^{TM} 8000, Beckman Coulter) with CEQ^{TM} 8000 sequence analysis software. The sizes of the alleles were calculated with the fragment analyzer package.

From the 10 eggs, we obtained, during the first amplification, 52% of success. A second amplification on the same extracted DNA gave 92% of success. From the 10 miracidia, we obtained, during the first amplification, 90% of success. A second amplification on the same extracted DNA gave 100% of success. From the 10 cercariae, during the first PCR amplifications performed, we obtained 98% of success. A second amplification of the same extracted DNA gave 100% of success. From the 10 adults, during the first PCR amplifications performed, we obtained 98% of success. A second amplification of the same extracted DNA gave 100% of success. The amplification failures were independent from the locus tested. Furthermore, it is likely that 100% of DNA had been extracted because after one or two PCR reactions, all expected PCR products gave at least one result in one microsatellite marker. DNA extraction methods are generally complex and time consuming, or quick and usually more expensive due to

the use of commercial kits. Table 2 shows a comparison between our method and the two previous ones (Shrivastava et al. 2005; Sorensen et al. 2006). Our DNA extraction protocol is efficient on all parasite stages and makes it possible to obtain an extracted DNA for PCR amplification at top speed (15 minutes of incubation), with few handling steps (5) and at a very low cost (1 Euro is sufficient to perform more than 60 000 DNA extraction reactions). This extraction procedure yields 40 µl of DNA from individual egg, miracidium, cercaria or adult that allows for 40 PCR amplifications, according to our protocol. This method could be perform in 96-well microplates allowing several hundreds DNA extractions in one hour.

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193 Table 1: Microsatellite markers used to test the extraction procedure.

Locus	Genbank accession number	Annealing temperature	Amplicon size (bp)	Number of alleles detected	Authors
SMDO11	AF325698	60°C	351-383	7	Curtis et al. 2001
SMC1	AF325694	56°C	287-303	5	Curtis et al. 2001
SMD57	AF202967	52,5°C	278-300	11	Durand et al. 2000
R95529	R95529	52,5°C	229-274	3	Durand et al. 2000
SMBR16	LO4480	59,5°C	337-341	4	Rodrigues et al 2007

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195 Table 2: Comparison of DNA extraction procedures.

Protocol from	**Sorensen et al. (2006)**	**Shrivastava et al. (2005)**	**Present study**
Tested parasite stages	egg	miracidium, cercaria	egg, miracidium, cercaria, adult
Incubation time	20 min	2 h 25	15 min
Number of steps	8	6	5
Number of reactions with 1 Euro	7	300	60 000
Number of allowed PCR reactions	25	1	40

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