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Short communication

PCR effectiveness for sexing *Schistosoma mansoni* cercariae: application for sexing clonal cercarial populations

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Schistosoma mansoni is a digenean parasite known for its importance as a human pathogen. Compared with the other genera belonging to the digenean families, the *Schistosoma* genus is characterized by having acquired gonochorism from hermaphroditic ancestors [1]. Sex is genetically determined in the egg [2] and female schistosome is heterogametic (ZW) such as lepidoptera, reptiles and birds. A marked sexual dimorphism exists between male and female worms in the definitive host. However, no clear evidence for sexual dimorphism exists in the free larval stages (miracidia or cercariae) and in the intramolluscan stages (sporocysts). Morphological, histological and molecular methods have been developed in order to discriminate between male and female schistosome larvae. Differences in the number and pattern of the argentophilic papillae (chaetotaxy) were observed between male and female cercariae [3]. Histological studies were based on the fact that the female exhibits heterochromatin of the W chromosome in the interphase nuclei. Staining methods were applied on cercariae [4] and C-banding on miracidia, cercariae and sporocysts of *S. mansoni* [5]. Since 10 years, molecular methods have emerged in order to sex schistosome larvae and are always based on the heterosexual female-specific W chromosome. Three DNA repeated sequences were isolated and characterized from female W chromosome, D9 [6], W1 [7] and W2 [8], whose genomic sizes are ,respectively, 339, 476 and 715 bp. The W1 repeated sequence was shown to be female-specific in each stage of the life cycle in a Puerto Rican strain of the parasite [9]. These authors used a PCR technique with W1-specific primers on extracted DNA. The female specificity was confirmed by Southern blot. However, Grevelding [10] contested the female specificity of W sequences. This author showed, using extracted adult DNA, that the female-specific W1 sequence may occur in both genders of a Liberian strain, but reminds female-specific in a Puerto Rican strain of *S. mansoni*. The variability of the W gender-specificity was studied for many strains by Quack et al. [11]. This study revealed that W1 and W2 elements exist in both genders in numerous strains, Liberian, Old Kenya, New Kenya, Senegal, Campinas and an isolate of Puerto Rican strain. But, the Old Brazilian strain remained W1 and W2 female-specific. The presence of W sequences in males comes from genomic instability, probably originating from DNA recombinations during sporocyst development [12]. The PCR technique commonly uses extracted DNA. However, a PCR technique without DNA extraction (direct PCR) was developed for individual *S. mansoni* cercariae [13].

This paper aims to test the effectiveness of the direct PCR on cercariae from a W1 female-specific strain and to propose an optimized protocol in order to determine the sex of mollusc infection.

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The *S. mansoni* strain was isolated from man in Recife hospital (Brazil) in 1969. It was maintained in the laboratory using an albino variety of *Biomphalaria glabrata* from Brazil and Swiss OF1 mice. Monomiracidial infections were used and the sex of each mollusc infection was verified by infecting mice. Methods to infect molluscs and to infect and perfuse mice were described by Boissier and Moné [14]. Infected molluscs were individually placed in filtered spring water and exposed to artificial light to stimulate cercarial emission. Cercariae shed from each mollusc were collected with elongated Pasteur pipettes in glass containers filled with filtered spring water and washed twice. Then, they were individually isolated in 5 μl of filtered spring water and transferred in PCR reaction tube using a 20 μl micropipette with filter tips. The presence of only one cercaria in each tube was verified under a binocular microscope. Preliminary assays showed that frozen (-80°C) cercariae give better results that fresh ones. The PCR method was adapted from Grevelding et al. [13]. PCR reactions were carried out in a total volume of 25 μl containing 2.5 μl of buffer (10 mM Tris–HCl, pH 9.0 at 25°C, 50 mM KCl, 0.1% Triton X-100), 3 mM of $MgCl_2$, 0.5 μM of each oligonucleotide primer (W1a,b; [9]), 200 μM of each dNTP (Pharmacia) and 2 U of Taq polymerase (Promega, Madison, WI). The DNA was initially denatured for 5 min at 95°C. The PCR cycle was carried out for 1 min at 93°C, 30 s at 60°C and 30 s at 72°C, for a total of 40 cycles, followed by an extension polymerization reaction of 10 min at 72°C. The amplification was performed in a PTC 100 thermocycler (MJ-Research). Twenty microliters of each amplification were loaded on 1% agarose gels, separated by electrophoresis in $1 \times$ TBE, stained with ethidium bromide, and examined by illumination with UV. For each gel, control PCR reactions were performed with extracted female DNA, extracted male DNA and filtered spring water.

In order to test the effectiveness of the PCR method, a group of 16 monomiracidially-infected molluscs was analyzed. At 6 weeks post-infection, 36 cercariae of each mollusc were isolated to perform a W1-PCR reaction and 200 cercariae were used to infect a mouse. For ten molluscs, none of the 36 cercariae showed any band, and infection and perfusion of mice showed that all the mollusc infections were male. For the six molluscs left, the 36 cercariae showed, respectively, 22, 24, 31, 31, 34 and 35 times a band. This PCR product, around 470 bp, corresponds to the W1 sequence isolated by Webster et al. [7]. Controls with extracted female DNA were positive and controls with male DNA or filtered spring water were negative. Infection and perfusion of mice showed that all the mollusc infections were female. The effectiveness of the PCR method for the female cercariae, expressed in percentage of positive female cercariae is 81.9%, ranging from 61.1 to 97.2%. No explanation is available to explain that some female cercariae did not give any band.

The optimal number of cercariae necessary to determine the sex of a mollusc infection may be calculated as follows. When one considers the worst effectiveness of 61.1% in sexing cercariae, we can calculate the probability to be mistaken in sexing clonal cercarial population. This probability is $(0.39)^5 \times 100 = 0.9\%$, $(0.39)^4 \times 100 = 2.3\%$, $(0.39)^3 \times 100 = 5.9\%$, when 5, 4 and 3 cercariae are tested, respectively. Consequently, when considering the threshold of 5%, 4 may be the optimal number of cercariae that permits the determination of the sex of cercariae from a monomiracidial mollusc infection.

In order to test this hypothesis, a group of 104 monomiracidially-infected molluscs was analyzed, using only four cercariae from each infected mollusc. At 5 weeks post-infection, four cercariae of each mollusc were isolated to perform W1-PCR reactions and 200 cercariae of each mollusc were used to infect a mouse. For 58 molluscs, none of the four cercariae showed any band, and infection and perfusion of mice showed that all the mollusc infections were male. Among the 46 molluscs left, 21 showed a band for each of the four cercariae, 17 showed a band for three cercariae, seven showed a band for two cercariae and one showed a band for one cercaria. Controls with extracted female DNA were positive and controls with male DNA or filtered spring water were negative. Infection and perfusion of mice showed that all the mollusc infections were female. The effectiveness of the PCR method for the 184 female cercariae tested for this group was 81.5%. It confirmed the result obtained for the first group ($P > 0.05$) and showed that the method is reproducible. This protocol, which uses only four cercariae per mollusc, gives 100% of success in sex determination of clonal cercariae obtained by monomiracidially-infected molluscs using a Brazilian strain of *S. mansoni*. The use of the vertebrate mice may be avoided.

These PCR effectiveness and protocol optimization for sexing clonal cercariae provide both scientific and ethical interests for all the W1 female-specific strains. Scientifically, this protocol permits a rapid method for sex determination; it allows to perform experiments as soon as the first day of shedding. It may enhance our comprehension on the early host-parasite relationships, existing between each schistosome sex and its mollusc host during the first 2 months of the patent period. Ethically, the use of molecular biology for cercarial sexing may avoid the vertebrate host sacrifice, which was necessary to detect the sex of the parasite.

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