Genetic Markers between *Biomphalaria glabrata* Snails Susceptible and Resistant to *Schistosoma mansoni* Infection

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The analysis of the genetic variability related to susceptibility to Schistosoma mansoni *infection in the vector of the genus* Biomphalaria *is important in terms of a better understanding of the epidemiology of schistosomiasis itself, the possible pathological implications of this interaction in vertebrate hosts, and the formulation of new strategies and approaches for disease control. In the present study, the genetic variability of* B. glabrata *strains found to be resistant or susceptible to S. mansoni infection was investigated using DNA amplification by random amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR). The amplification products were analyzed on 8% polyacrylamide gel and stained with silver. We selected 10 primers, since they have previously been useful to detect polymorphism among* B. glabrata *and/or* B. tenagophila. *The results showed polymorphisms with 5 primers. Polymorphic bands observed only in the susceptible strain. The RAPD-PCR methodology represents an adequate approach for the analysis of genetic polymorphisms. The understanding of the genetic polymorphisms associated to resistance may contribute to the future identification of genomic sequences related to the resistance/susceptibility of* Biomphalaria *to the larval forms of* S. mansoni *and to the development of new strategies for the control of schistosomiasis.*

Key words: *Schistosoma* - *Biomphalaria* - genetic variability - polymorphism - random amplified polymorphic DNA - polymerase chain reaction

Until mid 1980s the emphasis on the control of schistosomiasis was mainly directed to the combat against vector. After the discovery of more efficient and less toxic chemotherapeutics drugs (oxamniquine, praziquantel), the World Health Organization (WHO) started to emphasize morbidity control (WHO 1985, Dias et al. 1995, Lardans & Dissous 1998). Despite the fact that the emphasis directed to chemotherapy has reached favorable results, regarding reduction of morbidity, a significative reduction of the transmission was not obtained (Barbosa 1995, Lardans & Dissous 1998). As a result of those aspects, the control program proposed in 1993 by the WHO kept the emphasis on morbidity control. However other measures were associated, such as: improvement on sanitary qualities, water supply to population, vector control and sanitary education (WHO 1993, Dias et al. 1995).

An important aspect to be considered in the control program context is the emergent problem of resistance or tolerance of parasites to the main drugs used in schistosomiasis chemotherapy (Dias & Gonçalves 1992, Cioli 1993, Araújo et al. 1996, Conceição et al. 2000, Liang et al. 2001). Due to those aspects, the combat against vector, associated to other control measures, is again emphasized.

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The control of the vector has been tried by different methods, which include: the use of synthetical molluscicids, like niclosamide (WHO 1993), molluscicids of vegetal origin (Jurberg 1989, Schall et al. 1998), modifications in their natural habitat and use of predators or competitors of those vectors (Paraense 1987, Capron 1993, Lardans & Dissous 1998, Pointier & Augustin 1999, Pointier & Jourdane 2000, Amarista et al. 2001).

The genetical aspects, regarding the susceptibility of snail vector to infection by *Schistosoma mansoni*, were realized, in a pioneer way, by Newton (1955). The author associated the albino phenotype with a high pattern of susceptibility to the infection by Porto Rico strain of *Schistosoma mansoni* and proposed, for that phenotype, a polygenic inheritance pattern. Richards et al. (1992), employing techniques of classical genetic, proposed that susceptibility of *Biomphalaria glabrata* to infection by *S. mansoni* would present distinct patterns of inheritance dependable on the age of the snail. In that manner, the resultant phenotype would be determined by various genes in the young mollusk and by only one gene (monogenic) in the adult mollusk. In fact, up to the moment, the patterns of inheritance, regarding susceptibility, were not very well established.

Several techniques of molecular biology for studies of genetic variability in schistosomiasis vectors have been used (Knight et al. 1991, Millller et al. 1996, Spatz et al. 1999, Vidigal et al. 2000, 2001, Caldeira et al. 2000, Jannotti-Passos & Souza 2000, Knight et al. 2000, Souza & Jannotti-Passos 2001, Tuan & Bortolato 2001).

The introduction of the randon amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR) technique (Welsh & McClelland 1990, Williams et al. 1990) has am-

plified the possibilities of polymorphisms analysis, as it allowed the use of small arbitrary nucleotide segments without the need of a previous knowledge of genes and/or genomic sequences.

Vidigal et al. (1994) have used the technique of RAPD-PCR for studies of the genetic variability in *B. glabrata*. Larson et al. (1996) and Lewis et al. (1997), developed studies between *B. glabrata* strains, resistant and susceptible to infection by *S. mansoni*, managing to associate some markers to resistance and susceptibility. Similar studies were accomplished by Abdel-Hamid et al. (1999), using mollusks of *B. tenagophila* species.

In this article, the technique of RAPD-PCR was used for analysis of the genomic DNA, in *B. glabrata* strains, in an attempt to associate possible polymorphic markers related to susceptibility/resistance to infection by the *S. mansoni*. The comprehension of genetic basis of susceptibility/resistance of snails to *S. mansoni* may, as it was proposed by Rollinson et al. (1998), base the elaboration of new strategies for the control of schistosomiasis.

MATERIALS AND METHODS

Snails stocks - *S. mansoni*-resistant and -susceptible *B. glabrata* snails (BH strain) obtained by self-fertilization as described by Zanotti-Magalhães et al. (1997) were used. The mollusks were maintained in glass aquaria and fed with lettuce leaves and calcium carbonate.

Experimental infection - Each mollusk was challenged with 10 miracidia (BH strain) of *S. mansoni* according to Zanotti-Magalhães et al. (1997). They were placed in glass jars containing chlorine free water for 2 h at 25ºC. Susceptibility was determined after 30 days through cercariae release favoured by an incandescent lamp. Susceptibility to infection was monitored from 4 weeks up to 12 weeks post-exposition.

DNA extraction - The DNA was extracted from the foot of individual snails (resistant and susceptible strains), using lysis buffer containing 2% CTAB (Winnepenninckx et al. 1993, Abdel-Hamid et al. 1999) and incubated with proteinase K (2 µg/ml) for 2 h, following extraction with phenol, phenol:chloroform (1:1) and chloroform: isoamylalcohol (24:1) and incubation with ribonuclease A (350 µg/ml). The DNA was precipitated by adding isopropanol (2:3 v/v) and leaving overnight at room temperature. After centrifugation DNA was washed in 75% ethanol, 10 mM ammonium acetate for 30 min and recovered by centrifugation (12000 g) for 10 min (4ºC). The pellet was ressuspended in TE (10 mM de Tris, 1mM de EDTA, pH 8.0) and the DNA concentration and purity was determined by 2% agarose gel electrophoresis using the gel photodocumentation system (EDAS) DC 120 Zoom Digital Camera (Eastman Kodak, NY, USA) and spectrophotometrically (Spectrophotometer Ultrospec III, Pharmacia, UK) at absorbances of 260 and 280 nm.

DNA amplification by RAPD-PCR - The method of Simpson et al. (1993) was used, with small modifications. Two nanograms of genomic DNA obtained from the snails were amplified with a PTC 200 Peltier Thermal Cycler (MJ Research - USA) using RAPD-PCR. Each reaction was carried out in a final volume of 20 µl containing 1 unit Taq DNA polymerase (Gibco, BRL, MD, USA), 1X PCR buffer, 0.2 mM of each dNTP, and 7 pmol of each random primer (10 bp) (Gibco, BRL). Ten primers were used: 1 (5'-GGGTAACGCC-3'), 2 (5'-CTGATGCTAC-3'), 4 (5'-AGTGCTACGT-3'), 8 (5'- CTCTCCGCCA-3'), 9 (5'-CAGGCCCTTC-3'), 10 (5'-GGTCCCTGAC-3'), 12 (5'-AGGGAACGAG-3'), 14 (5'- GTTGCCAGCC-3') and 15 (5'-TGCCGAGCTG-3'). The amplification conditions were as follows: 1 cycle at 95ºC for 5 min, 2 cycles at 95ºC for 30 sec, at 30ºC for 2 min and at 72ºC for 1 min, and 33 cycles during which the annealing temperature was changed to 40ºC and the time of the extension step was increased to 5 min during the final cycle. The PCR products were stored at -20ºC.

Polyacrylamide gel electrophoresis and silver staining - Four microliters of each DNA amplification reaction was added to 2.5 µl sample buffer (0.125% bromophenol blue, 0.125% xylene cyanol, and 15% glycerol) and the mixture was submitted to 8% polyacrylamide (29/1 acrylamide-bisacrylamide) gel electrophoresis in TBE (2 mM Tris-borate, EDTA, pH 8.0) at 60 volts. The gels were fixed in 10% ethanol and 0.5% (v/v) acetic acid for 15 min, stained with 0.2% silver nitrate for 15 min, washed with deionized water for 5 min, and developed with 0.75 M NaOH and 0.1 M formaldehyde for 15 min (Santos et al. 1993).

Polymorphism analysis - Genetic variability of the susceptible and resistant strains was evaluated by analyzing the electrophoretic band patterns obtained on the gels and by determining the coefficient of similarity as described by Dice in 1945.

RESULTS

Susceptibility or resistance of *B. glabrata* snails to *S. mansoni* infection was studied starting 30 days post infection and continuing thereafter weekly up to 10 weeks after miracidia exposure. The obtained results pointed out that only 63% of the examined snails (100 snails) yielded high infection frequencies in contrast to about 37% of these snails were refractory.

A total of 10 primers were tested and polymorphic markers were obtained with 5 of them (primers 4, 8, 9, 10, 12). These primers were adopted for having been used in prior works, in which specific polymorphic markers were detected: among lineages from different regions of Brazil (Vidigal et al. 1994), between *B. glabrata* strains resistant and susceptible to *S. mansoni* infection (Larson et al. 1996), in *B. tenagophila* (Abdel-Hamid et al. 1999) and those made with the genetic material of the parasite *S. mansoni* (Sire et al. 1999).

The genomic DNA, amplified with primer 4 (5'-AGTGCTACGT-3'), as shown in Fig.1, presented a polymorphic band of nearly 4060 bp, present only in the susceptible lineages.

Fig. 2 presents the results of amplification with primer 8 (5'-CTCTCCGCCA-3'). According to Fig. 2, there is a band of 1.750 pb that occurs only in the susceptible lineage (well 6, 7 and 8).

The results presented in Fig. 3 arise from the electrophoresis polyacrylamide gel of material resultant from amplification of genomic DNA from *B. glabrata* with primer 9 (5'-CAGGCCCTTC-3'). According to Fig. 3 (wells 6, 7

and 8) a polymorphic marker of 1.100-bp was obtained, which was found only in the susceptible lineages.

Primer 10 (5'-GGTCCCTGAC-3') presented a polymorphic band, with approximately 1100-bp, present only in the susceptible lineage, according to Fig. 4, wells 6, 7 and 8.

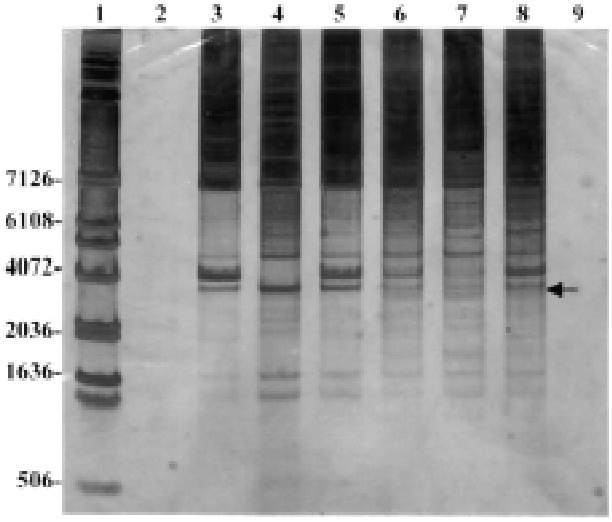

Fig. 1: random-amplified polymerase chain reaction from genomic DNA of *Biomphalaria glabrata* strains resistant and susceptible using arbitrary primer 4. Lanes - 1: 1-Kb DNA Ladder; 2: negative control (amplification without DNA); 3: R1 (resistant); 4: R2 (resistant); 5: pool (R1 and R2); 6: S1 (susceptible); 7: S2 (susceptible); 8: pool (S1 and S2); 9: negative control (amplification without DNA). Samples were analyzed by electrophoresis through a 8% polyacrylamide gel and visualized by silver staining. Polymorphic band is indicated by arrow.

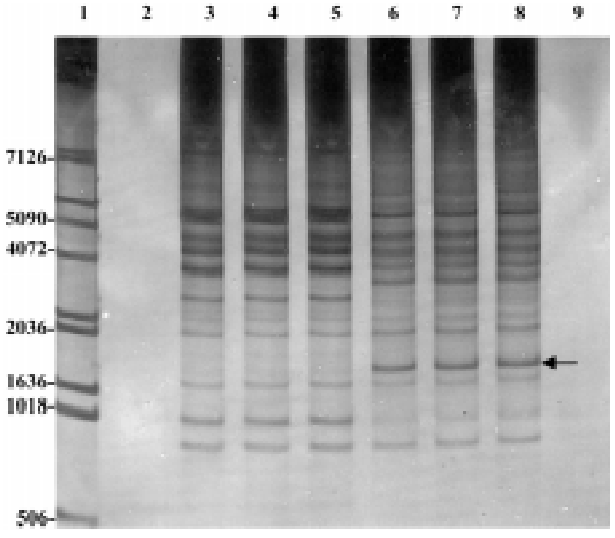

Fig. 2: random-amplified polymerase chain reaction from genomic DNA of *Biomphalaria glabrata* strains resistant and susceptible using arbitrary primer 8. Lanes - 1: 1-Kb DNA Ladder; 2: negative control (amplification without DNA); 3: R1 (resistant); 4: R2 (resistant); 5: pool (R1 and R2); 6: S1 (susceptible); 7: S2 (susceptible); 8: pool (S1 and S2); 9: negative control (amplification without DNA). Samples were analyzed by electrophoresis through a 8% polyacrylamide gel and visualized by silver staining. Polymorphic band is indicated by arrow.

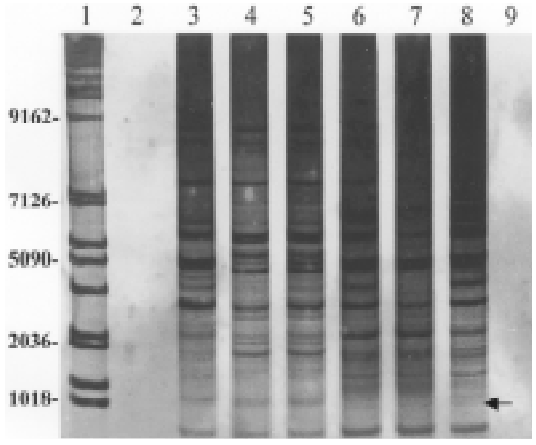

Fig. 3: random-amplified polymerase chain reaction from genomic DNA of *Biomphalaria glabrata* strains resistant and susceptible using arbitrary primer 9. Lanes - 1: 1-Kb DNA Ladder; 2: negative control (amplification without DNA); 3: R1 (resistant); 4: R2 (resistant); 5: pool (R1 and R2); 6: S1 (susceptible); 7: S2 (susceptible); 8: pool (S1 and S2); 9: negative control (amplification without DNA). Samples were analyzed by electrophoresis through a 8% polyacrylamide gel and visualized by silver staining. Polymorphic band is indicated by arrow.

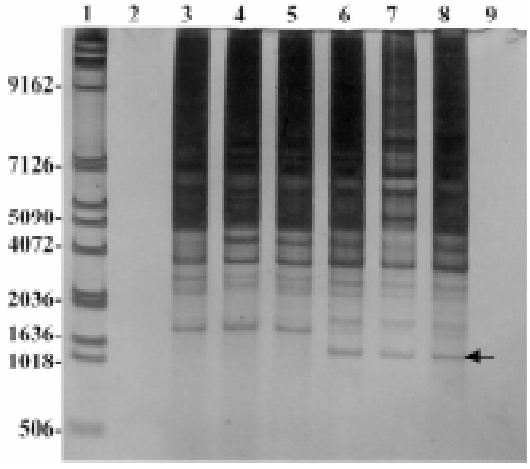

Fig. 4: random-amplified polymerase chain reaction from genomic DNA of *Biomphalaria glabrata* strains resistant and susceptible using arbitrary primer 10. Lanes - 1: 1-Kb DNA Ladder; 2: negative control (amplification without DNA); 3: R1 (resistant); 4: R2 (resistant); 5: pool (R1 and R2); 6: S1 (susceptible); 7: S2 (susceptible); 8: pool (S1 and S2); 9: negative control (amplification without DNA). Samples were analyzed by electrophoresis through a 8% polyacrylamide gel and visualized by silver staining. Polymorphic band is indicated by arrow.

Fig. 5 corresponds to the electrophoresis in gel of polyacrylamide resultant from the amplification of genomic DNA with primer 12 (5'-AGGGAACGAG-3'). It is verified, with that primer, the acquisition of a band of nearly 800 bp (wells 6, 7 and 8), whose occurence is only restricted to snails of susceptible lineage.

DISCUSSION

The genomic DNA amplified with primer 4 (5'-AGTGCTACGT-3'), according to Fig. 1, presented a polymorphic band, of approximately 4060 bp, between the re-

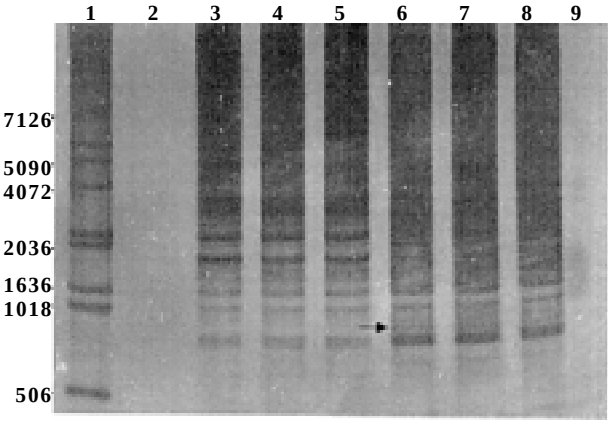

Fig. 5: random-amplified polymerase chain reaction from genomic DNA of *Biomphalaria glabrata* strains resistant and susceptible using arbitrary primer 12. Lanes - 1: 1-Kb DNA Ladder; 2: negative control (amplification without DNA); 3: R1 (resistant); 4: R2 (resistant); 5: pool (R1 and R2); 6: S1 (susceptible); 7: S2 (susceptible); 8: pool (S1 and S2); 9: negative control (amplification without DNA). Samples were analyzed by electrophoresis through a 8% polyacrylamide gel and visualized by silver staining. Polymorphic band is indicated by arrow.

sistant and susceptible lineage and that marker occurred only in the susceptible lineages. Vidigal et al. (1994), employing an equivalent sequence of oligonucleotides found, in individuals of *B. glabrata* species, collected in field, polymorphic bands in snails of different places in Brazil. In the present study, descendants from snails maintained in the laboratory for more than 20 years were used. Thus, although resistant and susceptible strains were studied, we could expect that the genetic variability was low, since laboratory strains are submitted to much less intense selective pressures than field isolates are, i.e., natural conditions. This fact may explain the high coefficient of similarity observed for the strains studied here (Table) and also the expressive quantitative difference in genetic variability obtained by Vidigal et al. (1994) with an oligonucleoride sequence similar to primer 4.

Sire et al. (1998) employed a series of aleatory primers for the analysis of genetic diversity of *S. mansoni* adult worms originated of cercarie, obtained from a natural population of *B. glabrata*, in a site of transmission at Guadeloupe Island. Among the 18 tested primers, 3 of them allowed the detection of polymorphic markers. One of those oligonucleutide sequences (5'-CTCTCCGCCA-3') was used in this project (primer 8) and resulted, after amplification of genomic DNA, in the obtaining of a polymorphic marker of 1750 pb, whose occurrence may only be verified in the susceptible lineage of the mollusk, according to Fig. 2. The amplification of genomic sequence of the parasite and the vector mollusk from the same primer, presents two immediate occurrences: the parasite and the vector may present similar genomic sequences which could, in its turn, be implied in the definition and establishment of the relation parasite/host, and, as a second consequence, care and caution should be adopted in the amplification of the genomic DNA of the vector mollusks infected, in order to avoid contamination of the samplers with genetic material of the larval forms of the parasite.

With primer 9 (5'-CAGGCCCTTC-3'), which corresponds to primer OPA-01 employed by Larson et al. (1996), a polymorphic marker of 1.100-bp was found (Fig. 3). That band was repeatedly found only in the susceptible lineages. Larson et al. (1996) obtained, with primer OPA-01 a band of 400 bp, characteristic of susceptible lineage (M-line). That lineage was obtained by Newton, in 1955 and combines the albino phenotype with a high susceptibility to infection by Porto Rico strain from *S. mansoni*. The distinct origins of M-line and BH strains, employed in the current work, could justify the acquisition of distinct polymorphic bands from the amplification with a primer with equal sequence of nucleotides. In other words, the susceptible lineages in question present specific markers but they are different among themselves.

With primer 10 (5'-GGTCCCTGAC-3'), which corresponds to primer OPA-06, employed by Larson et al. (1996), a polymorphic band was obtained, with nearly 1100-bp present (and repeatedly obtained) only in the susceptible lineage (Fig. 4). Such results were agreeing with the ones obtained by Larson et al. (1996) in the susceptible lineage (M-line). It seems it is a matter of a specific marker in the susceptible lineage.

A polymorphic band of approximately 800 pb was found in the susceptible lineage of the mollusks *B. glabrata* after the amplification of the genomic DNA with primer 12 (Fig. 5), whose sequence of oligonucleutideos (5'-AGGGAACGAG-3') was employed by Abdel-Hamid et al. (1999) for the analysis of the genetic variability between resistant and susceptible lineages of *B. tenagophila*. The authors did not detect, with that primer, any polymorphic marker between the resistant and susceptible cepas. It is concluded that the primer may amplify a very preserved area of the genoma *B. tenagophila* and, apart from allowing the detection of a marker of the susceptible lineage, that primer presents itself potentially interesting for taxonomic studies among the sympatric

TABLE

Dice's similarity coefficient (*a*) between resistant and susceptible strains of *Biomphalaria glabrata* snails

Primers	P4	P8	P9	P10	P12
No. of shared bands between resistant and susceptible strains (a)	20	20	30	17	25
No. of bands in susceptible but not in resistant strains (b)	1	1	1	1	1
No. of bands in resistant but not in susceptible strains (c)	0	0	0	0	0
Similarity coefficient (S)	0.98	0.98	0.98	0.97	0.98

a: S = 2 a/2 a + b+ c

species which include *B. glabrata* and *B. tenagophila*.

Some studies have used isoenzymes and pigmentation analysis to define specific susceptibility markers in *B. glabrata* (Mulvey & Vrijenhoek 1981, Mulvey & Wooddruff 1985), but no marker was safely associated with resistance or susceptibility. Even through no clear association has been found in the present work between a specific marker and *B. glabrata* susceptibility, identical results have been obtained by Larson et al. (1996), which led to the identification of a 1100 bp marker shower only in the susceptible strains. Therefore, we presume this is a specific marker to the susceptible strain.

Knight et al (1999) analyzed through RAPD-PCR F1 and F2 generations and the F1 versus parental backcross progeny. This approach led to the identification of two markers, one 1200 bp band with OPM-04 primer and 1000 bp band with OPZ-11 primer. Those markers were found only in non-susceptible strains. This is an indication that markers of segregation may be found in *B. glabrata* resistant to *S. mansoni* infection.

A possible existence of transposable elements in the snails opens new perspectives of approach for transfection of sequences with incorporation to the snails genome (Richards et al. 1992). The markers or genomic sequences associated to resistance could be useful for the production of transgenic snails. The perspective of acquisition of transgenic snails strains resistant to *S. mansoni* infection implies the exceeding of many theoretical difficulties, especially the experimental ones. In spite of that, such methodological approach presents interesting perspectives (Lardans & Dissous 1998), which, in association with other measures, will allow the elaboration of new strategies for the control of schistosomiasis.

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REFERENCES

Abdel-Hamid A-Z, Molfetta JB, Fernandez V, Rodrigues V 1999. Genetic variation between susceptible and non-susceptible snails to *Schistosoma* infection using random amplified polymorphic DNA analysis (RAPDs) *Rev Inst Med Trop São Paulo 41*: 291-295.

Amarista M, Niquil N, Balzan C, Pointier JP 2001. Interspecific competition between freshwater snails of medical importance: a Venezuelan example. *Life Science 324*: 143-148.

Araújo N, Souza CP, Janotti-Passos LK, Simpson AJG, Dias Neto E, Pereira TR, Cerutti, CJ, Alencar FEC, Dietze R, Katz N 1996. Suscetibilidade aos agentes quimioterápicos de isolados de *Schistosoma mansoni* oriundos de pacientes tratados com oxamniquine e praziquantel e não curados. *Rev Soc Bras Med Trop 29*: 467-476.

Barbosa FS 1995. Determination and control of schistosomiasis. *Mem Inst Oswaldo Cruz 90*: 155-159.

Caldeira RL, Vidigal THDA, Matinella L, Simpson AJG, Carvalho OM 2000. Identification of planorbids from Venezuela by polymerase chain reaction amplification and restriction fragment length polymorphism of internal transcriber spacer of the RNA ribosomal gene. *Mem Inst Oswaldo Cruz 95*: 171-177.

Capron A 1993. La bilharziose: du papyrus à l'ordinateur. *Le généraliste 1411*: 8-18.

Cioli D, Pica-Mattoccia L, Archer S 1993. Drug resistance in schistosomes. *Parasitol Today 9*: 162-169.

Conceição MJ, Argento CA, Corrêa A 2000. Study of *Schistosoma mansoni* isolates from patients with failure of treatment with oxamniquine. *Mem Inst Oswaldo Cruz 95*: 375-380.

Dias LCS, Gonçalves ER 1992. O *Schistosoma mansoni* diz não às drogas. *Ci Hoje 14*: 22-25.

Dias LCS, Marçal-Júnior O, Glasser CM 1995. Control of schistosomiasis transmission. *Mem Inst Oswaldo Cruz 90*: 285-288.

Dice LR 1945. Measures of the amount of ecological association between species. *Ecology 26*: 297-302.

Jannotti-Passos LK, Souza CP 2000. Susceptibility of *Biomphalaria tenagophila* and *Biomphalaria straminea* to *Schistosoma mansoni* infection detected by low stringency polymerase chain reaction. *Rev Inst Med Trop São Paulo 42*: 291-294.

Jurberg P 1989. Plantas empregadas como moluciscidas: uma visão crítica. *Mem Inst Oswaldo Cruz 84*: 76-83.

Knight M, Brindley PJ, Richards CS, Lewis FA 1991. *Schistosoma mansoni*: use of a cloned ribosomal RNA gene probe to detect restriction fragment length polymorphisms in the intermediate host *Biomphalaria glabrata*. *Exp Parasitol 73*: 285-294.

Knight M, Miller NA, Patterson CN, Rowe CG, Michaels G, Carr D, Richards CS, Lewis FA 1999. The identification of markers segregating with resistance to *Schistosoma mansoni* infection in the snail *Biomphalaria glabrata*. *Proc Natl Acad Sci 96*: 1510-1515.

Knight M, Ongele E, Lewis FA 2000. Molecular studies of *Biomphalaria glabrata*, an intermediate hosts of *Schistosoma mansoni*. *Int J Parasitol 30*: 535-541.

Lardans V, Dissous C 1998. Snail control strategies for reduction of schistosomiasis transmission. *Parasitol Today 14*: 413-417.

Larson SE, Andersen PL, Miller AN, Cousin CE, Richards CS, Lewis FA, Knight M 1996. Use of RAPD-PCR to differentiate genetically defined lines of *B. glabrata*, an intermediate host of *S. mansoni*. *J Parasitol 82*: 237-244.

Lewis FA, Knight M, Richards CS 1997. A laboratory-based approach to biological control of snails. *Mem Inst Oswaldo Cruz 92*: 661-662.

Liang Y-S, Coles GC, Doenhoff MJ, Southgate VR 2001. In vitro responses of praziquantel-resistant and susceptible *Schistosoma mansoni* to praziquantel. *Int J Parasitol 31*: 1227-1235.

Miller AN, Ofori K, Lewis FA, Knight M 1996. *Schistosoma mansoni*: use of a subtractive cloning strategy to search for RFLPs in parasite-resistant *Biomphalaria glabrata*. *Exp Parasitol 84*: 420-428.

Mulvey M, Vrijenhoek RC 1981. Population structure in *Biomphalaria glabrata*: examination of hypothesis for the patchy distribution of susceptility to schistosomes. *Am J Trop Med Hyg 31*: 1195.

Mulvey M, Woodruff DS 1985. Genetics of *Biomphalaria glabrata*: linkage analysis of genes for pigmentation, enzymes, and resistance to *Schistosoma mansoni*. *Biochem Genet 22***:** 877.

Newton WL 1955. The establishment of a strain of *Australorbis glabratus* which combines albinism and high susceptibility to infection with *Schistosoma mansoni*. *J Parasitol 41*: 526-528.

Paraense WL 1987. Control of schistosomiasis mansoni: an outlook from current expectation. *Mem Inst Oswaldo Cruz 82*: 1-12.

Pointier JP, Augustin D 1999. Biological control and invading

freshwater snails. A case study. *Life Sci 322*: 1093-1098.

Pointier JP, Jourdane J 2000. Biological control of the snail hosts of schistosomiasis in areas of low transmission: the example of the Caribbean area. *Acta Trop 77*: 53-60.

Richards CS, Knight M, Lewis FA 1992. Genetic of *Biomphalaria glabrata* and its effect on the outcome of *Schistosoma mansoni* infection. *Parasitol Today 8*: 171-174.

Rollinson D, Stothard JR, Jones CS, Lockyer AE, Souza CP, Noble LR 1998. Molecular characterisation of intermediate snail hosts and the search for resistance genes. *Mem Inst Oswaldo Cruz 93*: 111-116.

Santos FR, Pena SDJ, Epplen JT 1993. Genetic and population study of a Y-linked intranucleotide repeat DNA polymorphism. *Hum Gen 90***:** 655-656.

Schall VT, Vasconcelos MC, Souza CP, Baptista BF 1998. The molluscidal activity of crown of Christ (*Euphorbia splendens* va. *Hislopii*) latex on snails acting as intermediate hosts of *Schistosoma mansoni* and *Schistosoma haematobium*. *Am J Trop Med Hyg 58*: 7-10.

Simpson AJG, Dias Neto E, Steindel M, Caballero OLSD, Janotti-Passos LK, Pena SDJ 1993. The use of RAPDs for the analysis of parasites. In SDJ Pena, R Chakraborty, JT Epplen, AJ Jeffreys (eds), *DNA Fingerprinting: State of the Science*, Birkhauser, Verlag Basel, Switzerland, p. 331-337.

Sire C, Durand P, Pointier J, Théron A 1999. Genetic diversity and recruitment pattern of *Schistosoma mansoni* in a *Biomphalaria glabrata* snail population: a field study using random amplified polymorphic DNA markers. *J Parasitol 85*: 436-441.

Souza CP, Jannotti-Passos LK 2001. Resistance of *Biomphalaria occidentalis* from Várzea das Flores Dam, Minas Gerais, to *Schistosoma mansoni* infection detected by low stringency polymerase chain reaction. *Mem Inst Oswaldo Cruz 96*: 381-383.

Spatz L, Vidigal THDA, Caldeira RL, Dias Neto E, Cappa SMG, Carvalho OS 1999. Study of *Biomphalaria tenagophila tenagophila, B. t. guaiabensis and B. occidentalis* by polymerase chain reaction amplification and restriction enzyme digestion of the ribosomal RNA intergenic spacer regions. *J Moll Stud 65*: 143.

Tuan R, Bortolato PC 2001. Genetic markers from *Biomphalaria tenagophila* (Gastropoda: Pulmonata: Planorbidae) obtained by the double stringency polymerase chain reaction technique. *Mem Inst Oswaldo Cruz 96*: 435-436.

Vidigal THDA, Caldeira RL, Simpson AJG, Carvalho OS 2000. Further studies on the molecular systematics of *Biomphalaria* snails from Brazil. *Mem Inst Oswaldo Cruz 95*: 57-66.

Vidigal THDA, Caldeira RL, Simpson AJG, Carvalho OS 2001. Identification of *Biomphalaria havanensis* and *Biomphalaria obstructa* populations from Cuba using polymerase chain reaction and restriction fragment length polymorphism of the ribosomal RNA intergenic spacer. *Mem Inst Oswaldo Cruz 96*: 661-665

Vidigal THDA, Dias Neto E, Carvalho ODS, Simpson AJG 1994. *Biomphalaria glabrata*: extensive genetic variation in Brazilian isolates revealed by random amplified polymorphic DNA analysis. *Exp Parasitol 79*: 187-194.

Welsh J, McClelland M 1990. Fingerprinting genome using PCR with arbitrary primers. *Nuc Acids Res 18*: 7213-7218.

Williams JGK, Kubelik AR, Livak J, Rafalski JA, Tingey SV 1990. DNA polymorphics amplified by arbitrary primers are useful as genetic markers. *Nuc Acids Res 18*: 6531-6535.

Winnepenninckx B, Backeljau T, Wacbier R 1993. Extraction of high molecular weight DNA from mollusca. *Trends in Genetics 9*: 407.

WHO-World Health Organization 1985. *Epidemiology and Control of Shistosomiasis*, Technical Report Series 728, Geneva, Switzerland.

WHO-World Health Organization 1993. *The Control of Schistosomiasis*, Technical Report Series 830, Geneva, Switzerland.

Zanotti-Magalhães EM, Magalhães LA, Carvalho JR 1997. Relação entre patogenicidade do *Schistosoma mansoni* em camundongos e a susceptibilidade do molusco vetor. IV. Infecciosidade dos miracídios. *Rev Saúde Pública 29*: 265-270.