Rev. Inst. Med. trop. S. Paulo
41 (5): 291-295, September-October, 1999.

GENETIC VARIATION BETWEEN SUSCEPTIBLE AND NON-SUSCEPTIBLE SNAILS TO *Schistosoma* INFECTION USING RANDOM AMPLIFIED POLYMORPHIC DNA ANALYSIS (RAPDs)

Abdel-Hamid Zaki ABDEL-HAMID(1), Jeanne Blanco de MOLFETTA(2), Vania FERNANDEZ(2) & Vanderlei RODRIGUES(2)

SUMMARY

Susceptibility of snails to infection by certain trematodes and their suitability as hosts for continued development has been a bewildering problem in host-parasite relationships. The present work emphasizes our interest in snail genetics to determine what genes or gene products are specifically responsible for susceptibility of snails to infection.

High molecular weight DNA was extracted from both susceptible and non-susceptible snails within the same species *Biomphalaria tenagophila.* RAPD was undertaken to distinguish between the two types of snails. Random primers (10 mers) were used to amplify the extracted DNA by the polymerase chain reaction (PCR) followed by polyacrylamide gel electrophoresis (PAGE) and silver staining.

The results suggest that RAPD represents an efficient means of genome comparison, since many molecular markers were detected as genetic variations between susceptible and non-susceptible snails.

KEYWORDS: Schistosomiasis; Snails; DNA; RAPD; PCR; PAGE

INTRODUCTION

Schistosomiasis or bilharziasis in its different forms is estimated to affect at least 200 million people and endanger another 600 million in 72 subtropical or tropical countries in Asia, Africa, The Caribbean and Latin America, creating public health problems of varying magnitudes WIRTH *et al*.[17]. It is responsible for retarding the advancement of work in rural areas.

Snail control is one of the most rapid and effective means available for reducing transmission of schistosomiasis, since there is a high degree of specificity of schistosomes, as well as of other trematodes, for their intermediate snail hosts. A schistosome miracidium might penetrate several species of snails, but its fate in the tissues of the snails is determined by biochemical and genetic adaptation to certain species. It might develop and produce cercariae in some species whereas they are walled off in others as a result of a strong host reaction which is the expression of an innate cellular internal defense mechanism MALEK[5]. NEWTON[8] considered that susceptibility or resistance of a snail to infection is an hereditary character. RICHARDS[9], RICHARDS & MERRITT[11] and NABIH & EL-ANSARY[7] confirmed this contention by extensive studies on the genetics of *B. glabrata* and *B. alexandrina* snails. They suggested that the specificity relationship between the parasite and its intermediate host snail is genetic. Moreover, RICHARDS[10] showed that resistance to infection in adult snails is governed by a single gene according to mendelian genetics. This suggestion has some support from the findings of KNIGHT *et al.*[3] and LARSON *et al.*[4] who described the occurrence of genetic polymorphisms between resistant and susceptible snails through the use of restriction fragment length polymorphism (RFLPs) in the rRNA gene as well as RAPD assays. In addition, comparative southern blot analysis of the resistant and susceptible snails lines using pBS11 (clone related with a *B. glabrata* albumen gland gene product) as a probe indicated the occurrence of *Bam*H1 and *Eco*R1 RFLPs between the snail lines which should be useful for future genetic linkage studies MILLER *et al*.[6].

Detection of genetic variation is essential to comparative genetic research endeavors. From a specific point of view, molluscan genetic polymorphisms appear to be important sources of inter-species variation in susceptibility of snails to *Schistosoma* infection. Molecular biology has added a powerful method for study genetic variations of mollusca known as RAPD "random amplified polymorphic DNA" WILLIAMS *et al*.[15]. The technique is based on random amplification of DNA fragments, via PCR, using short primers of arbitrary sequence.

The approach used is directed toward the identification of genetic variations between susceptible and non-susceptible snails within the same species *B. tenogophila*, aiming to isolate those genes and gene products, in subsequent studies, as factors in the susceptibility of snails to parasitic infection. Treatment of susceptible snails with specific mutagens may render than less susceptible and hence break the life cycle of schistosomiasis.

(1) Medicinal Chemistry Department. National Research Center, Dokki, Cairo, Egypt
(2) Department of Parasitology, Microbiology and Immunology. Faculty of Medicine of Ribeirão Preto. University of São Paulo, Ribeirão Preto, São Paulo, Brasil.
Correspondence to: Vanderlei Rodrigues. Departamento de Parasitologia, Microbiologia e Imunologia. Faculdade de Medicina de Ribeirão Preto, Av. Bandeirantes 3900, 14049-900 Ribeirão Preto, SP, Brazil. Tel.: 55-16-6023235, Fax: 55-16-6336631. E-mail: vrodrigu@fmrp.usp.br

MATERIAL AND METHODS

Snails maintenance

Adult *Biomphalaria tenagophila* snails (both susceptible and non-susceptible) were originally selected by autofertilization of mollusks from parental down to F4 generations with infection rates of 15% and 78% respectively with SJ strains of *S. mansoni* miracidia according to ZANOTTI-MAGALHÃES *et al.*[18] They were maintained, as stock cultures, in glass aquaria containing snail conditioned water (SCW-pH 7.8 at ~30 °C), with a density of 10 snails/L. The snails were fed lettuce leaves. The amount of the given food per day was approximately the quantity that would normally be consumed within 24 hours.

The experimental snails with shell diameter, 8±1mm, were obtained from the stock cultures and they were selected carefully on the basis of health and size.

Isolation of DNA

High molecular weight DNA was extracted from the foot of individual snails (both susceptible and non-susceptible) by the method of WINNEPENNINCKX *et al.*[16] as follows: snails tissues were dissected and the tissues were powdered in liquid nitrogen using a mortar and pestle and transferred to a centrifuge tube containing 15 ml of preheated (60 °C) CTAB buffer (2%w/v CTAB, 1.4 M NaCl, 0.2%v/v β-mercaptoethanol, 20 mM EDTA, 100mM Tris HCl pH 8, 0.1mg/ml proteinase K). After incubation at 60 °C for 30 minutes, the suspension was extracted with an equal volume of phenol:chloroform:isoamylalcohol (25:24:1). After centrifugation (8000 rpm, 10 min), the aqueous phase was incubated with 5 ml RNase for 1 hour at 37 °C. DNA was extracted with an equal volume of chloroform:isoamylalcohol (24:1). After centrifugation (8000 rpm, 10 min), the aqueous phase was transferred to a centrifuge tube. DNA was precipitated by adding ~2/3 vol isopropanol and gently inverting the tube and leaving overnight at room temperature DNA was then collected by centrifugation at 8000 rpm for 10 minutes. DNA was washed in 76% ethanol, 10 mM ammonium acetate for 30 minutes and recovered by centrifugation (8000 rpm, 10 min.). After air drying the DNA was dissolved in 20 µl deionized water, and the DNA concentration was estimated spectrophotometrically and also by using 2% agarose gel electrophoresis.

DNA amplification

The protocol used was described before by SIMPSON *et al*[13]. Essentially DNA samples from arbitrarily selected individual from both susceptible and non-susceptible snails were amplified using two different amounts (1 and 2 ng) of template DNA. Each reaction was undertaken in a final volume of 10 µl containing 0.8 units of taq DNA polymerase (Gibco BRL), 200 µM each dNTP, 1.5 mM $MgCl_2$, 50mM KCl, 10 mM Tris-HCl, pH 8.5, together with 6.4 pmole of primer. The primers 1(CTGCTGGGAC), 2(AGGGAACGAG), 3(GTGAGGCGTC), 4(GTTGCCAGCC) and 5(TGCCGAGCTG), were arbitrarily selected from laboratory stocks. The reaction mix was overlaid with 20 µl of mineral oil and, following an initial denaturation at 95 °C for 5 min, was subjected to two cycles through the following temperature profile: 95 °C for 30 sec for denaturation, 30 °C for 2 min for annealing, 72 °C for 1 min for extension, followed by 33 cycles for which the annealing step was altered to 40 °C. In the final cycle, the extension step was for 5 min.

Polyacrylamide gel electrophoresis

Following DNA amplification, 3 µl of each reaction was mixed with 3 µl of sample buffer (0.125% bromophenol blue, 0.125% xylene cyanol, 15% glycerol) and subjected to electrophoresis using 8% polyacrylamide gel (acrylamide-bisacrylamide 29/1) in TBE buffer (2mM EDTA, 10 mM Tris-borate, pH 8.0).

Staining of the gel

The gels were silver stained by fixing with 10% ethanol, 0.5% acetic acid for 15 min, stained with 0.2% silver nitrate for 15 min, washing with deionized water for 5 min and reduced with 0.75M NaOH/0.1M formaldehyde for 15 min according to SANTOS *et al*[12].

Analysis of bands

To calculate percentage band differences between susceptible and non-susceptible snails, the bands observed in a given lane were compared with those in the other lanes of the same gel, as described by VIDIGAL *et al*[14].

A taxon/character matrix was constructed on the basis of the presence/absence of bands. The total number of bands scored was 101. The matrix was then used to calculate Dice's similarity coefficient[2].

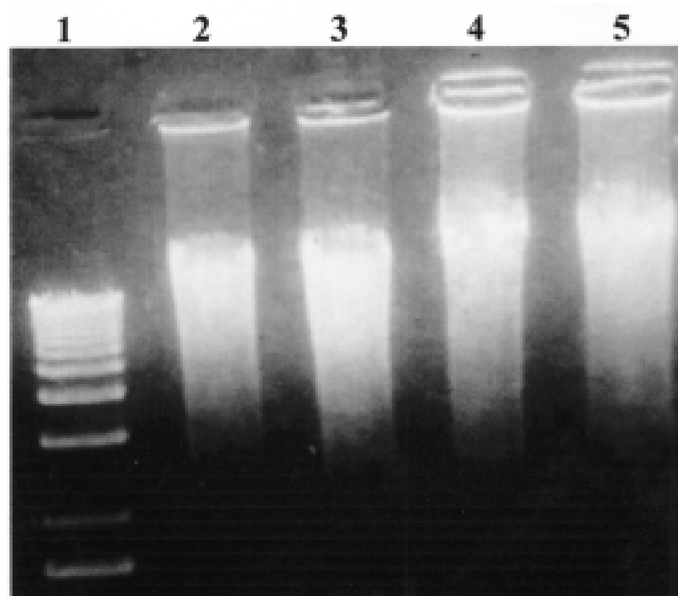


Fig. 1 – DNA isolated from individual *B. tenagophila* snails susceptible and non-susceptible to schistosome infection was analysed by agarose 2% gel electrophoresis. Lane 1: 1kb DNA marker, Lane 2 and 3 DNA of susceptible snails, Lane 4 and 5 DNA of non-susceptible individual snails.

RESULTS

RAPD analysis was performed using DNA extracted from two different types of snails of the *Biomphalaria tenagophila*. Four individuals from both susceptible and non-susceptible population were compared. Figure 1 shows gel electrophoresis of DNA isolated from individual susceptible or non-susceptible snails. The results support the view that the technique described by WINNEPENNINCKX *et al*[16], is useful for isolation of high molecular weight DNA from different snail populations.

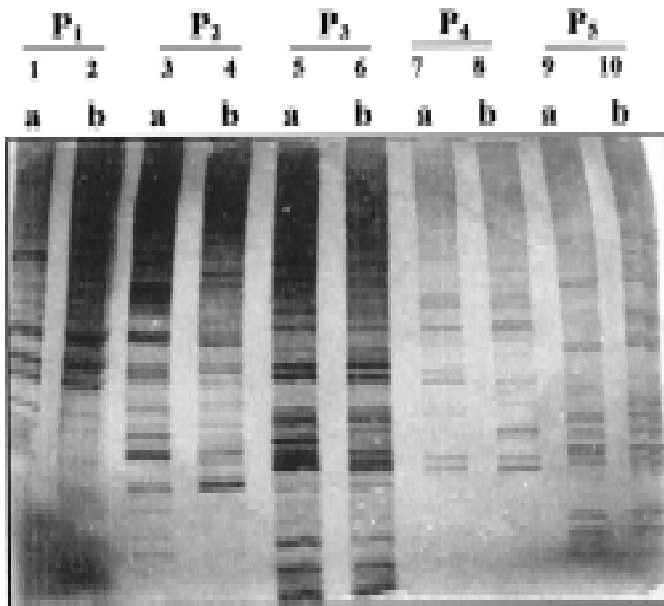
Figure 2, shows the results of amplifications of two different amounts (1 and 2) of template DNA with five primers used. Primer (1) CTGCTGGGAC, (2) AGGGAACGAG, (3) GTGAGGCGTC, (4) GTTGCCAGCC and (5) TGCCGAGCTG. The amplification obtained with the two amounts of genomic DNA gave basically identical patterns with the exception of the intensity of some bands.

Individual amplifications of both susceptible and non-susceptible snails with primer 5 TGCCGAGCTG are illustrated in Figure 3. There are very limited differences in the amplified bands of the individual population. The only reproducible differences we have noticed are between the two types of snails (susceptible and non-susceptible).

The total number of bands scored was 101, with the average number of bands scored per lane being 10.1. The values that differentiates susceptible from non-susceptible snails vary from 4.4% to 9.0% dependent on the primers used, while the values that reveal closer phylogenetic relationships between the two populations within the same species *Biomphalaria tenagophila* are varying from 91% to 95.6% (Table 1).

DISCUSSION

In order to achieve satisfactory amplification, we found DNA extraction using phenol:chloroform:isoamyl alcohol, re-extract the aqueous phase obtained with chloroform:isoamyl alcohol, and treatment DNa with Rnase are necessary. The yield of DNA revealed that this technique promise to be useful in the genetic studies of snails.



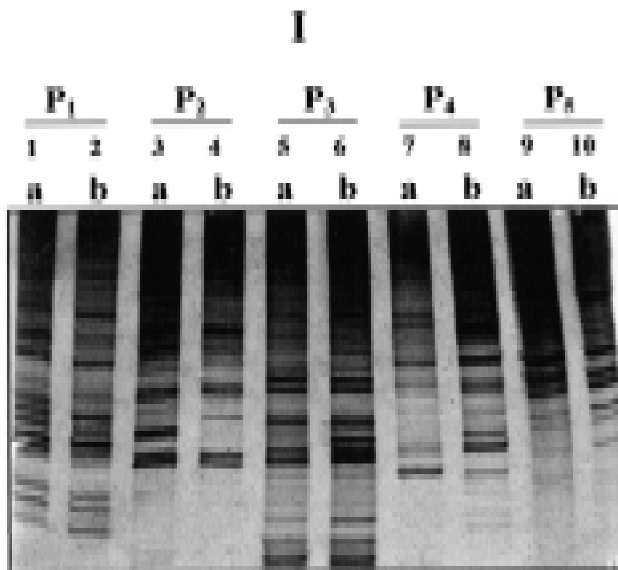


Fig. 2 – RAPD profiles produced from *B. tenagophila* snails susceptible (a) and non-susceptible (b), using 1ng (I) and 2ng (II) of template DNA and primers (1) CTGCTGGGAC, lanes 1 and 2, (2) AGGGAACGAG, lanes 3 and 4, (3) GTGAGGCGTC, lanes 5 and 6, (4) GTTGCCAGCC lanes 7 and 8, (5) TGCCGAGCTG lanes 9 and 10. The PCR products were resolved by electrophoresis through a 8% polyacrylamide gel followed by silver staining.

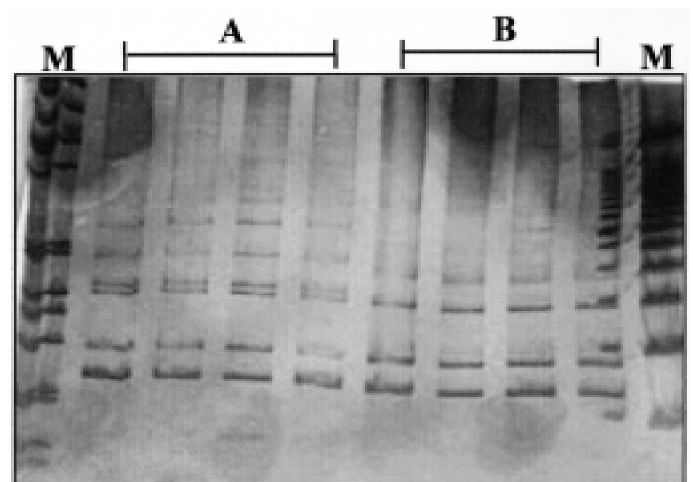


Fig. 3 – Comparison of the RAPD profiles produced from four individuals of (*B. tenagophila*) snails susceptible (A) and non-susceptible (B) using primer (5) TGCCAGCTG. The PCR products were resolved by electrophoresis through a 8% polyacrylamide gel followed by silver staining. M: 1kb DNA marker.

Table 1
Dice's similarity coefficient (*) between susceptible and non-susceptible *B. tenagophila* snails

Gel analysis	Primers P1	P2	P3	P4	P5
Number of marked bands in susceptible snails	14	12	12	8	7
Number of marked bands in non susceptible snails	11	10	12	8	7
Number of shared bands between susceptible and non susceptible snails (a)	11	9	11	6	7
Number of bands in susceptible but not in non-susceptible snails (b)	3	3	1	2	0
Number of bands in non-susceptible but not in susceptible snails (c)	0	1	1	2	0
Similarity coefficient (S)	0.79	0.69	0.85	0.60	1.0

(*) S=2a/2a+b+c

The findings showed that there are intrapopulation of differences in the same species *Biomphalaria tenagophila* between susceptible and non-susceptible snails. Thus, when tissues from several individuals of the same population are pooled, one can obtain a pattern which is to some extent distinctive of the strain.

Since there is a relatively restricted genetic variation between susceptible and non-susceptible snails within the same species, it is indeed possible that the susceptibility of snails to parasitic infection is genetically controlled.

As can be seen in Figures 2 and 3 RAPD primers were useful for distinguishing snail populations. They were also particularly useful for discriminating between different isolates of the same species. Reports and observations from this study suggest that RAPDs should be highly useful for phylogenetic analysis among closely related individuals. This suggestion is in agreement with the both BARRAL *et al*.[1] and VIDIGAL *et al*.[14] who indicated that RAPD markers are a highly resolving and helpful tool for investigation of variability. They provide a simple technology which can be used to rapidly distinguish species, strains and sexes in laboratory conditions. Also, SIMPSON *et al*.[13] proved that RAPD is undoubtedly a powerful approach for analysis of genetic variation and the identification of genetic markers. So, RAPDs is of particular value in the study of genetic variation of snails that would allow the design of specific primers for genome analysis. RAPD is finding increasing use because of its technical simplicity.

The availability of isogenic snail lines has made it possible to use molecular tools to determine the degree of genetic variability between them. By comparing genome DNA of susceptible and non-susceptible snails, we could find either a component that the susceptible ones produced as shown with primers (1, 2, 3 and 5) or lacked as in case of primer (4) that would make them nonsusceptible. In subsequent studies, it might be possible to insert (or delete) this component in susceptible snails by treatment with specific mutagens. These genetically altered varieties could then be released into areas endemic for Schistosomes, following treatment of these areas to remove most or all of the genetically unaltered susceptible snails. This concept will lead to genetic control of Schistosomiasis.

RESUMO

Variação genética entre moluscos susceptíveis e não susceptíveis à infecção pelo *Schistosoma* através da análise do DNA polimórfico amplificado aleatóriamente (RAPDs)

A susceptibilidade de moluscos à infecção por certos trematódeos e a sua capacidade como hospedeiro para o contínuo desenvolvimento é o problema mais deslumbrante nas relações parasita hospedeiro. O presente trabalho, focaliza nosso interesse na genética dos moluscos para determinar quais genes ou produtos gênicos são especificamente responsáveis pela susceptibilidade do molusco à infecção.

DNA de alto peso molecular, foi extraído de ambos moluscos susceptíveis e não susceptíveis da espécie *Biomphalaria tenagophila*. Iniciadores aleatórios com 10 pares de bases foram usados na amplificação aleatória (RAPD) de ambos os DNAs e análise por eletroforese em gel de poliacrilamida e coloração com prata.

Os resultados mostram que a amplificação aleatória do DNA representa um eficiente caminho para a comparação dos genomas desde que marcadores moleculares foram detectados como variantes genéticos entre os moluscos susceptíveis e não susceptíveis.

ACKNOWLEDGEMENTS

This work received financial support from Third World Academy of Sciences (TWAS) and Conselho Nacional de Pesquisas (CNPq). We thank Mrs. Olinda Mara Brigato Trevilato, for maintenance of snails.

REFERENCES

1. BARRAL, V.; THIS, P.; IMBERT-ESTABLET, D.; COMBES, C. & DELSENY, M. - Genetic variability and evaluation of Schistosoma genome analyzed by using random amplified polymorphic DNA markers. **Molec. Biochem. Parasit., 59**: 211-222, 1993.

2. DICE, L.R. - Measures of the amount of ecological association between species. **Ecology (Brooklyn), 26**: 297-302, 1945.

3. KNIGHT, M.; BRINDLEY, P.J.; RICHARDS, C.S. & LEWIS, F.A. - *Schistosoma mansoni*: use of a cloned ribosomal RNA gene probe to detect restriction fragment length polymorphisms in the intermediate host *Biomphalaria glabrata*. **Exp. Parasit., 73**: 285-294, 1991.

4. LARSON, S.E.; ANDERSON, P.L.; MILLER, A.N. *et al.* - Use of RAPD-PCR to differentiate genetically defined lines of an intermediate host of *S. mansoni, B. glabrata.* **J. Parasit., 82**: 237-244, 1996.

5. MALEK, E.A. - Susceptibility of tropicorbid snails from Louisiana to infection with *Schistosoma mansoni.* **Amer. J. trop. Med. Hyg., 16**: 715-717, 1967.

6. MILLER, A.N.; OFORI, K.; LEWIS, F. & KNIGHT, M. - *Schistosoma mansoni*: use of a subtractive cloning strategy to search for RFLPs in parasite-resistant *Biomphalaria glabrata.* **Exp. Parasit., 84**: 420-428, 1996.

7. NABIH, I. & EL-ANSARY, A. - Genetic studies on freshwater snails "specific intermediate hosts for schistosomiasis". II. Isolation and base composition determination of deoxyribonucleic acid. **Cell. molec. Biol., 26**: 455-458, 1980.

8. NEWTON, W.E. - The comparative tissue reaction of two strains of *Australorbis glabratus* to infection with *Schistosoma mansoni.* **J. Parasit., 38**: 362-370, 1962.

9. RICHARDS, C.G. - Genetics of a molluscan vector of schistosomiasis. **Nature (Lond.), 227:** 806, 1970.

10. RICHARDS, C.S. - Influence of snail age on genetic variation in susceptibility of *Biomphalaria glabrata* for infection with *Schistosoma mansoni.* **Malacologia, 25**: 493-503, 1984.

11. RICHARDS, C.S. & MERRITT JR., J.W. - Genetic factors in the susceptibility of juvenile *Biomphalaria glabrata* to *Schistosoma mansoni* infection. **Amer. J. trop. Med. Hyg., 21**: 425-434, 1972.

12. SANTOS, F.R.; PENA, S.D.J. & EPPLEN, J.T. - Genetic and population study of a Y-linked tetranucleotide repeat DNA polymorphism with a simple non-isotopic technique. **Hum. Genet., 90**: 655-656, 1993.

13. SIMPSON, A.J.G.; NETO, E.D.; STEINDEL, M. *et al*. - The use of RAPDs for the analysis of parasites. In: PENA, S.D.J.; CHAKRABORTY, R.; EPPLEN, J.T. & JEFFREYS, J., ed. **DNA fingerprinting: state of the sciences.** Boston, Birkhauser, 1993. p. 331-337.

14. VIDIGAL, T.H.; NETO, E.D.; CARVALHO, O.S. & SIMPSON, A.J. - *Biomphalaria glabrata*: extensive genetic variation in Brazilian isolates revealed by random amplified polymorphic DNA analysis. **Exp. Parasit., 79**: 187-194, 1994.

15. WILLIAMS, J.G.K.; KUBELIK, A.R.; LIVAK, K.J.; RAFALSKI, J.A. & TINGEY, S.V. - DNA polymorphism amplified by arbitrary primers are useful as genetic markers. **Nucleic Acids Res., 18**: 6531-6535, 1991.

16. WINNEPENNINCKX, B.; BACKELJAU, T. & DE WACHTER, R. - Extraction of high molecular weight DNA from molluscs. **Trends Genet., 9**: 407, 1993.

17. WIRTH, H.P.; CASANOVA, C.; MEYENBERGER, C. *et al*. - Hepatosplenic schistosomiasis: case report and clinical review. **Schweiz. med. Wschr.**, **123**: 1991-1995, 1993.

18. ZANOTTI-MAGALHÃES, E.M.; MAGALHÃES, L.A & CARVALHO, J.F. - Relação entre patogenicidade do *Schistosoma mansoni* em camundongos e susceptibilidade do molusco vetor. IV. Infecciosidade dos miracídeos. **Rev. Saúde públ. (S. Paulo), 31**: 488-494, 1997.

Received: 07 May 1998
Accepted: 17 September 1999