Biomphalaria tenagophila: dominant character of the resistance to *Schistosoma mansoni* in descendants of crossbreedings between resistant (Taim, RS) and susceptible (Joinville, SC) strains

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The aim of the present work was to study parasitological, molecular, and genetic aspects in descendants of crossbreedings between a totally resistant Biomphalaria tenagophila *strain (Taim, RS) and another one highly susceptible (Joinville, SC) to* Schistosoma mansoni. *Descendants F_1 and F_2 were submitted to* S. mansoni *infection (LE strain). The susceptibility rates for individuals from Group F_1 were 0 to 0.6%, and from Group F_2 was 7.2%. The susceptible individuals from Group F_2 discharged a lower number of cercariae, when compared with the susceptible parental group, and in 2 out of 9 positive snails the cercarial elimination was discontinued. In order to identify genetic markers associated with resistance the genotype of parental snails and their offspring F_1 and F_2 were analyzed by means of the randomly amplified polymorphic DNA method. Nevertheless, it was not possible to detect any marker associated to resistance, but the results showed that in the mentioned species the resistance character is determined by two dominant genes.*

Key words: *Biomphalaria tenagophila* - dominant resistance character - *Schistosoma mansoni* - crossbreedings

A number of studies has been carried out aiming at determining and understanding the susceptibility and resistance levels of the mollusk genus *Biomphalaria* to *Schistosoma mansoni*. Several researchers while studying the relationship between *B. glabrata* and *S. mansoni* were able to observe that *B. glabrata* from various localities presented different susceptibility levels, as well as that some variation occurred in relation to the infectivity of *S. mansoni* to snails (Files & Cram 1949, Kuntz 1952, Barbosa & Barreto 1960, Paraense & Correa 1963).

Newton (1952) established for the first time that susceptibility is a hereditary character in *B. glabrata*. Richards (1970) demonstrated that in *B. glabrata* the resistance character of the mollusk to *S. mansoni* infection, which is acquired at the maturity phase, is monogenic and dominant. The relationship between parasite and host is very complex, and *S. mansoni* genetic factors interact in the infection process (Richards 1975). However, the snail genotype seems to exert a crucial and determinant role in this relation (Richards et al. 1992).

Some molecular strategies have been used to identify in the snail genome the regions that are associated with the resistance phenotype (Knight et al. 2000). One of those strategies consists in carrying out crossbreeding between resistant and susceptible snails, plus identification of the resistance markers, which are chosen among descendants F_1 and F_2. Recently, two markers (1.2 Kb and l.0 Kb) were identified by means of the randomly amplified polymorphic DNA (RAPD) technique, in a resistant *B. glabrata* strain (Knight et al. 1999).

As can be observed, almost all the studies dealing with genetic aspects of resistance to infection were carried out using *B. glabrata* snails. In this way, the nature of the gene (or genes) that is (are) able to confer resistance in *B. tenagophila* is poorly known.

B. tenagophila is a planorbid with a wide distribution in South America (Paraense 1984), and has epidemiological importance, since it maintains the life cycle of the trematode *S. mansoni* in some areas in Brazil. In spite of its dominance in some areas, *B. tenagophila* is found in nature with low rates of infection. However, it is responsible for the majority of the autochthonous cases of schistosomiasis in the state of São Paulo (Brazil), as well as for the foci of the disease in the states of Minas Gerais and Santa Catarina (Brazil) (Paraense 1986). This last focus is maintained by a highly susceptible population (Joinville, Santa Catarina, Brazil) of mollusks to *S. mansoni*. There is a *B. tenagophila* population, indigenous to the Ecological Station at Taim, state of Rio Grande do Sul, Brazil, that systematically has been found to be resistant to different *S. mansoni* strains, as shown in various laboratory experiments (Santos et al. 1979). The scope of the present work was to carry out crossbreedings between this resistant strain and a highly susceptible one (Joinville), as well as to study parasitological, molecular and genetic aspects of their offspring in the presence of *S. mansoni* infection.

The results obtained in this study could be the basis for future strategies on the control of schistosomiasis in areas where transmission of the disease is maintained by *B. tenagophila*.

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MATERIALS AND METHODS

Snails - *B. tenagophila* Taim (from the Ecological Station at Taim, state of Rio Grande do Sul, Brazil) is the resistant strain to *S. mansoni* infection used in this experiment. This strain has been maintained at the laboratory of the Schistosomiasis Research Unit, Federal University of Minas Gerais, Brazil, for more than 20 years, without any kind of selection favoring the resistance character, since these snails are naturally resistant to *S. mansoni*. The albino *B. tenagophila* strain from Joinville, state of Santa Catarina, Brazil, has been maintained at the same laboratory, and was kindly provided by Dr Paraense in October 2002. This strain is highly susceptible to *S. mansoni* infection.

Crossbreedings - Ten *B. tenagophila* couples were used in this study. In order to obtain crossbreeding, a susceptible *B. tenagophila* specimen (albino) from Joinville was placed into a plastic recipient containing 100 ml dechlorinated water, together with a resistant *B. tenagophila* specimen from Taim. The snails measured approximately 5 mm in diameter, and were sexually immature. The snail couples were kept together for 50 days. After that, the couples were isolately maintained for obtention of F_1 generation. In this experiment, eggs of albino and pigmented parental snails were collected for 4 weeks. It must be emphasized that the eggs obtained from pigmented parental snails were separately maintained, in order to confirm that they were generated from crossbreeding only after obtention of F_2 generation. On the other hand, when eggs were obtained from albino parental snails, soon at the first week it was possible to identify whether there was an effective crossbreeding or not. These eggs were observed by means of a stereomicroscope. When the snails were pigmented, their embryos showed ocular spots at the base of each antenna, thus indicating that F_1 individuals were generated by crossbreeding. For obtention of F_2 descendants, 10 snail couples were used. F_2 generation was obtained by crosses between F_1 individuals according to the previously described procedures.

Parasites - The *S. mansoni* strains used were LE (Belo Horizonte, MG) and SJ (São José dos Campos, SP), both kept under laboratory conditions for 35 and 20 years, respectively.

Snail infection - The parental snails and their offspring F_1 and F_2, aged 3 months approximately, were individually exposed to 25 miracidia, according to Pellegrino and Katz (1968). In order to assess the susceptibility of F_1 and F_2 descendants in the presence of *S. mansoni*, two distinct experiments were carried out at different periods.

Methods for analysis of snail infection - Aiming at verifying the emergence of *S. mansoni* cercariae, the snails were individually kept in small Snap-cap glass recipients, with dechlorinated water, and exposed to artificial light for 2 h. From then onwards the snails were observed weekly under the stereomicroscope up to 30-90 days after exposure to miracidia. The negative snails were squeezed between slide and coverslip in search of sporocysts. The positive snails had their respective number of cercariae counted as follows: the material containing the larvae and fixed in formol was centrifuged at 1000 rpm for 1 min, the supernatant was discarded and the cercariae resuspended in 2 ml water. After homogenization, a sample of 0.2 ml was collected, and the cercariae present in the sample were counted under stereomicroscope. The number of cercariae so obtained was multiplied by ten for assesssment of the total number shed by the snail.

DNA extraction and RAPD analysis - After the parasitological test, the snails were submitted to DNA extraction, according to the procedure described by Vidigal et al. (1994). The DNA sample of each individual was amplified using final concentrations of l5 µg. A pool with DNA from F_2 susceptible individuals was performed, due to the low DNA concentration obtained after extraction. The final volume of the reaction was 13 µl, containing the following reagents: 10 × buffer, 2 mM Mg Cl_2, 0.2 mM of each dNTPs, 15 µg from primer, 5 U of Taq Polimerase (Cenbiot-RS), 15 µg DNA. Reaction mix was covered with 20 µl of mineral oil and placed into the thermocycler "Minicycler MJ Research" for PCR. Amplification conditions were as follows: 92ºC-2 min, 40 cycles of 92ºC-1 min, 35ºC-1 min, 72ºC-2 min and the last cycle of 72ºC-5 min. After reactions, the amplified products were submitted to electrophoresis in 1.5% agarose gel. In principle, 24 primers (most of them from "Operon Technologies") were utilized.

Band analysis - For each amplified DNA sample, the presence or absence of each band was observed, and DNAs from parental generation utilized as standard amplification.

RESULTS AND DISCUSSION

Crossbreeding between Taim and Joinville populations was confirmed by means of a recessive phenotypic marker (albinism character). The specimens of F_1 generation, in result of crossbreeding between those two populations, presented with pigmentation, whereas in F_2 generation there were pigmented and albino individuals. Paraense (1955) used the albino lineage aiming at observing the reproductive behavior in *Biomphalaria*. These studies demonstrated that the snails are able to perform self-fertilization, but in the presence of one or more individuals they prefer crossbreeding.

Individuals from F_1 and F_2 generations and parental snails were submitted to infection. In principle,the parental snails were infected with two *S. mansoni* strains (LE and SJ). As can be seen in Table I, *B. tenagophila* from Taim, RS was found to be resistant to *S. mansoni* (SJ and LE strains). These results were already expected, since that strain showed resistance to infection in various studies previously carried out (Santos et al. 1979). *B. tenagophila* from Joinville, SC presented susceptibility rates of 68.5 and 65.6% in relation to *S. mansoni* SJ and LE strains, respectively. These data corroborate the results obtained by Freitas et al. (1985), who demonstrated that *B. tenagophila* Joinville was susceptible to both *S. mansoni* strains (SJ and LE). It was observed that susceptible parental snails showed a mortality rate higher than the resistant parental snails. These data suggest that *S. mansoni* infection leads to high mortality in susceptible snails. Taking into account that *B. tenagophila* Joinville showed similar susceptibility to both *S. mansoni*

strains, our experiments were carried out using only LE strain, which was readily available at our laboratory.

According to observations related to F_1 generation in the presence of infection, we can notice that this generation was almost 100% resistance to the LE strain of *S. mansoni* (Table II). In the first experiment, out of 170 infected F_1 individuals, 150 survived. From these surviving individuals only one was positive and shedded a mean of 15 cercariae/snail. Thus, the surviving F_1 individuals showed a susceptibility rate of 0.6% to *S. mansoni* (LE strain). The mortality rate of this group was 11.7. In order to confirm these results, a second experiment was carried out with 50 infected specimens from F_1 generation. From this total, 44 individuals survived, but none of them shed cercariae. The mortality rate of this group was 12%. Previous experiments carrying out crossbreedings between *B. tenagophila* Taim, RS and susceptible specimens from Belo Horizonte, MG, produced F_1 descendants with 4.1% positivity rate to SJ strain (Santos et al. 1979). Freitas et al. (1985) were able to obtain F_1 generation (with 0 and 4.5% susceptibility to LE strain) as a result of crossbreeding between *B. tenagophila* Joinville and *B. tenagophila* Taim.

F_2 generation individuals were also submitted to infection with *S. mansoni* (LE strain) (Table III). Due to homogeneity of the samples resulted from two experiments that we carried out, the data were placed together into a group, therefore increasing the total samples. One hundred twenty five out of 160 infected individuals survived. Among the surviving individuals, only 9 were able to shed cercariae. The susceptibility rate of this group was 7.2%, and the mortality rate was 21.88%. It was observed that the mean cercarial production among susceptible F_2 individuals was lower than that obtained in the parental group (Joinville) (Figure), and 2 out of 9 individuals from group F_2 had the cercarial production discontinued. A comparison between the two groups, taking into account the mean of shedded cercariae, showed high statistically significant differences ($p < 0.01$), according to the Student's t test.

In the intermediate host, the phenotype of resistance to *S. mansoni* is very complex. Richards (1970) demonstrated that in *B. glabrata* the resistance character of the mollusk to *S. mansoni* infection, which is acquired at the maturity phase, is monogenic and dominant. However, there are some specimens of *B. glabrata* that present with

TABLE I

Test of susceptibility of the lineages *Biomphalaria tenagophila* Taim, RS and Joinville, SC to *Schistosoma mansoni* (SJ and LE strains)

Strain of *B. tenagophila*	Strains of *S. mansoni*	Infected snails	Surviving snails	Mortality rate %	Number of infected snails eliminating cercariae	Positive snails surviving %
Taim, RS	SJ	50	42	16	0	0
	LE	50	48	4	0	0
Joinville, SC	SJ	60	35	41.6	24	68.5
	LE	60	32	46.6	21	65.6

TABLE II

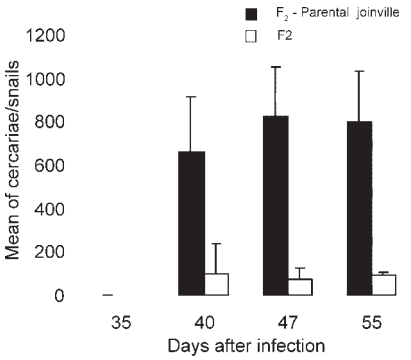
Mortality rate and susceptibility rates of *Biomphalaria tenagophila* Joinville, *Biomphalaria tenagophila* Taim, and F_1 generation, when submitted to infection with LE strain of *Schistosoma mansoni*

Experiment	*B. tenogophila*	Infected snails	Surviving snails	Mortality rate %	Number of infected snails eliminating cercariae	Positive snails surviving %
	Taim	64	60	6.25	0	0
1	Joinville	35	12	65.5	7	58.3
	F_1	170	150	11.7	1	0.6
	Taim	30	26	13.3	0	0
2	Joinville	30	17	43.3	10	58.8
	F_1	50	44	12	0	0

TABLE III

Mortality rate and susceptibility level of *Biomphalaria tenagophila* Joinville, *Biomphalaria tenagophila* Taim, and F_2 generation when submitted to infection with LE strain of *Shistosoma mansoni*

B. tenogophila	Infected snails	Surviving snails	Mortality rate %	Number of infected snails eliminating cercariae	Positive snails surviving %
Taim	94	79	15.9	0	0
Joinville	94	45	52.13	29	64.4
F_2	160	125	21.88	9	7.2



Mean of cercariae produced by the parental group *Biomphalaria tenagophila* Joinville and by the F2 group.

a peculiar behavior in relation to the parasite. In this case, the resistance character seems to be determined by various genes (Jones et al. 2001). The aim of our work was to study *B. tenagophila,* and up to now we did not know how was the transmission process of the resistance character in this species. F_1 individuals were found to be almost 100% resistant to infection, thereby showing that the gene (or genes) involved in resistance has (have) a dominant character. Nevertheless, what we cannot affirm in this study is whether resistance is associated or not to an unique gene, since a typical mendelian segregation could not be seen among F_2 individuals. However, we cannot discard the possibility that, among the dead F_2 snails, some of them could be susceptible (mortality rate of 21.8%). Considering such hypothesis, it could be that the number of susceptible snails was greater than the one that was obtained, and in this way the resistance character determined by an unique gene would be confirmed, since we could obtain the expected mendelian proportion (25% of susceptible individuals).

According to the results obtained, the most plausible hypothesis is that resistance in this species is determined by two dominant genes. Taking into account that the parental lineages are endogamic and, therefore, homozygote for the majority of their loci, we can consider that the resistant Taim lineage is double dominant homozygote for the genes which control resistance, whereas the susceptible Joinville lineage is double recessive homozygote for the same loci. Thus, in F_1 generation all the individuals are double heterozygote and, therefore, resistant. After analysing the data depicted in Table III, and after testing such hypothesis, we could see that the results obtained were those expected for this model. According to mendelian segregation related to two loci, the expected number of susceptible individuals in F_2 generation was, approximately, 8 (1/6 from the total of 125 F_2 individuals). Taking into account the susceptibility rate, which can be considered incomplete penetration (approximately 65%), we would expect to detect 5 susceptible F_2 individuals, but we detected 7 specimens that remained positive. Although the results obtained corroborated the suggested model (susceptible character conditioned by a double recessive homozygosis with an incomplete penetration), we observed that the calculated χ^2 value has a probability relatively low ($p = 0.07$). This result suggests a possible interaction of other modulator (genetic and/or environmental) factors. These modulator factors may be partially acting in susceptible F_2 individuals since, as it was previously observed, these individuals presented a decrease in cercarial production. Thus, we stress the need for further studies in order to confirm or not whether resistance in *B. tenagophila* is really associated to two dominant genes.

Another scope of this work was to identify molecular markers, which could be associated to resistance. To this end we used the RAPD technique, and 24 primers were tested, but no molecular marker could be detected. Recently, our staff was able to detect a molecular marker, which is typical of Taim lineage and presents a mendelian behavior (Rosa et al. 2004). This marker, although not associated to resistance, is represented by the presence of a band with 350 base pairs of the ITS region in the ribosomal mitocondrial DNA detected by means of PCR-RFPL technique, using the D*del* I enzyme. This marker will be very important in monitoring a possible biological control. Our idea would be changing the genetic inheritance of the local susceptible strain by the genetic inheritance of the introduced resistant strain. In this case, we consider that further studies are necessary to elucidate our proposition.

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