

DOT-ELISA FOR THE DETECTION OF IgM AND IgG ANTIBODIES TO *SCHISTOSOMA MANSONI* WORM AND EGG ANTIGENS, ASSOCIATED WITH EGG EXCRETION BY PATIENTS.

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SUMMARY

Human schistosomiasis, caused by *Schistosoma mansoni*, is highly prevalent in Brazil and usually diagnosed by time consuming stool analysis. Serological tests are of limited use in this disease, mainly for epidemiological studies, showing no discrimination between previous contact with the parasite and active infections. In the present study, we standardized and compared a Dot-ELISA for IgM and IgG antibodies against *S. mansoni* antigens from eggs and worms with a routine IgG and IgM immunofluorescence assay using similar antigens, in the study of sera from 27 patients who had quantified egg stool excretion. The positivity obtained for IgG Dot-ELISA was 96.3% and 88.9% for IgM Dot-ELISA with worm antigen and 92.6% and 90.9% with egg antigen. The IFI presented similar positivities using worm antigen, 92.6% (IgG) and 96.3% (IgM), and lower results with egg antigen, 77.8% (IgG and IgM). The patients studied were divided into two groups according to their egg excretion, with greater positivity of serological tests in higher egg excreters. When comparing the quantitative egg excretion and the serological titers of the patients, we detected a correlation only with IgM Dot-ELISA, with $r=0.552$ ($p=0.0127$). These data show that Dot-ELISA can be used for the detection of specific antibodies against *S. mansoni* in sera from suspected patients or in epidemiological studies and, with further purification of egg antigen and larger samples, IgM Dot-ELISA could be a possible tool for rough estimates of parasite burden in epidemiological studies.

KEYWORDS: Schistosomiasis; DOT-ELISA; Egg excretion; Diagnosis.

INTRODUCTION

Human schistosomiasis, an endemic parasitic disease frequently found in Brazil, is usually diagnosed by direct demonstration of eggs of the causative helminth *Schistosoma mansoni* in stools^{9, 10}. These tests are time consuming, requiring trained personnel

and patient cooperation. Despite the specificity of the test, multiple samples are required for the diagnosis of infected patients with few parasites^{6, 10}. Detection of specific anti-*S. mansoni* antibodies should be a good alternative for the diagnosis of patients with light or

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recent infections who frequently present a low parasitic burden, and many reports of these tests have been described^{1, 2, 8}. The specificity or sensitivity of the serological tests are dependent on antigen source or preparation, demanding strict standardization for good reproducibility between different laboratories¹². The standard technique in the indirect immunofluorescence assay using intact worms or eggs in cryostat preparations as antigens⁸. Recently, the immunoenzymatic assay on microplates adsorbed with worm and egg extracts has shown higher sensitivity but low specificity and reproducibility due to unequal unstable antigenic preparations allied to cross reactions with other helminthic infections¹². A simplified modification of the immunoenzymatic assay, using antigens adsorbed on nitrocellulose membranes and developed with insoluble and permanent stains, has been introduced for many serological tests^{1, 7}. These tests are also of low cost and require less complex equipment than the usual densitometric ELISA, with higher stability of the reagents. These advantages show that Dot-ELISA is a better choice for epidemiological field studies, providing good sensitivity and specificity. In this report, we standardized and compared a DOT-ELISA assay for IgM and IgG against *S. mansoni* antigens from eggs and worms with a routine IgG and IgM immunofluorescence assays using similar antigens, in sera from patients with quantified egg stool excretion.

MATERIAL AND METHODS

Patients

We used sera and stools collected from two groups of patients. The first one consisted of 27 patients suffering from intestinal schistosomiasis, with the presence of eggs in the stools. These patients lived in endemic area in Northeastern Brazil and had migrated to the São Paulo State in the past few years. None of them had clinically detectable hepatic involvement. The mean age of this group was 26.7 years (range 17 to 51 years), with 12 males. The mean egg count (eggs/gram of faeces egf) was 145 egf (range 8 to 570), as determined by the Kato-Katz quantitative stool test⁹.

The second group consisted of 50 samples from clinically normal nonschistosomotic patients (07), patients with intestinal helminthic diseases (20) whose stools were positive for *Ascaris lumbricoides* (02), *A. lumbricoides/Trichuris trichiura* (02), Ancylostomiasis (01) and *Giardia lamblia* and other intestinal

protozooses (15); and patients with other infectious or autoimmune diseases (23) such as syphilis (07), toxoplasmosis (04), autoimmune thyroiditis (04), systemic lupus erythematosus (03), streptococcal disease/Rheumatic disease (02), rubella (02) and *Mycoplasma* infection (01). All of them had at least two stool samples negative for *S. mansoni* eggs.

Antigens

The antigens of worms and eggs of *S. mansoni* for use in the indirect immunofluorescence assay were obtained from infected hamsters⁸. The mesenteric vessels were perfused with saline in order to remove adult worms, that were collected and stored frozen. After perfusion, livers were quick frozen and sliced on a cryostat to obtain sections with egg containing granulomas (IFI-E). The same procedure was used to obtain cryostat sections of the collected worms, after embedding in Tissue-Tek compound (IFI-W).

The *S. mansoni* antigens or immunoadsorption were prepared by disruption of worm and eggs in saline solution. Briefly, we used worms obtained from infected hamsters by mesenteric perfusion, or eggs purified by repeated sieving from intestinal walls of chronically infected hamsters. Both materials were washed and suspended in ten volumes of phosphate buffered saline (PBS). After ten cycles of freezing and thawing, they were submitted to sonication for one minute. After discarding the precipitated material by centrifugation at 800g/15 min, the supernatant protein was determined by the Folin-Lowry method¹¹ using bovine albumin as standard, and the extracts were aliquoted and stored at -20°C until use.

Immunofluorescence

The indirect immunofluorescence assay for antibodies against *S. mansoni* worm and egg antigen was performed as described elsewhere⁸. Briefly, appropriate serum dilutions were layered over cryostat preparations of eggs (IFI-E) or worms (IFI-W) and the bound antibody was visualized by subsequent incubation with anti-human IgG or anti-human IgM fluorescein conjugates. The reaction was contrasted with Evans Blue and observed by two independent observers using halogen lamp epifluorescence microscopy.

DOT-ELISA

The DOT-ELISA for quantitation of antibodies against extracts of *S. mansoni* was performed on nitrocellulose papers, with 0.22 µm pores cut as discs

with 6 mm in diameter. On these discs, antigens were preabsorbed in volumes of 1 μ containing worm antigen (Wag) or egg antigen (Eag). After drying, the free bindings sites on the paper were blocked by 2 h incubation in PBS containing 5% defatted dry milk. These adsorbed and blocked discs can be stored dry at -20°C until use. For antibody detection, the antigen discs were placed on 96 microwell plates, with the adsorption side exposed to PBS. After 1 h, they were carefully washed and appropriated sera dilution added to the wells. After another 1 h incubation at room temperature, the plates were washed with PBS, with care taken to avoid damage to the discs. The bound antibodies were visualized by subsequent incubation with anti-human IgG or anti-human IgM peroxidase conjugates, three washes in PBS and development with 4-chloro-1-naphthol (0.4mg/ml) and hydrogen peroxide (0.01%) in 0.01M citrate buffer, pH 5.0, for 20 minutes. The reaction was stopped by washing with PBS and by incubation with 0.01% Nonidet P-40 in PBS. The reactions were considered to be positive when the dot blue color intensity was higher than the appropriate controls in the same plate.

Statistical analysis

Qualitative data, such as frequencies, were analyzed by the χ^2 test, with Yates correction. Quantitative data, such as serum titers, were analyzed by the Kruskal Wallis test. The correlation between tests was calculated by Pearson linear correlation or by Spearman ranks correlation. Differences were considered significant when the probability of equality was less than 0.05 ($p < 0.05$). Concordance tests and kappa index were also performed in the comparison of serological tests^{4,15}. The diagnostic performance of the IgM and IgG Dot-ELISA was evaluated in terms of sensitivity, specificity, efficiency and positive and negative predictive values as elsewhere described⁵.

RESULTS

Antigen preparation

One thousand worms of both sexes were obtained from perfused livers of chronically infected hamsters, washed and processed as described in Methods, resulting in a saline worm extract with 3.6 mg protein/ml and a yield of 36 mg of protein. Eggs were purified from intestinal walls of chronically infected hamsters and one gram of washed eggs was extracted as described in Methods, resulting in a saline egg extract with 1.0 mg protein/ml and 10mg of total protein yield.

These extracts were aliquoted and stored at -20°C until use.

Dot-Elisa standardization

In order to standardize this reaction, we performed several tests to define the best antigen concentration per disc and the appropriate conjugates dilution. These tests, using standard positive and negative sera, revealed an optimum of 700 ng per dot form worm antigen and 750 ng per dot for egg antigen. These concentrations, allied to the conjugate dilution (1/500), provide a clear discrimination of positive and negative reactions. Less antigen or higher conjugate dilution provide less clear results, but probably these values should be corrected for each conjugate or antigen preparation.

Antibody detection

We tested the four antigenic preparations both for IgG and IgM detection in sera from non schistosomotic or schistosomotic patients. The titers of schistosomotic patients in the DOT-ELISA using the two antigenic preparations and conjugates directed against IgG and IgM can be seen in Figure 1. These tests showed that the best cut-off for all reactions was correspondent to 1/40 dilution.

Once the cut-off was established, we calculated the sensitivity, specificity, efficiency, and positive and negative predictive values, which could be seen in the table 1.

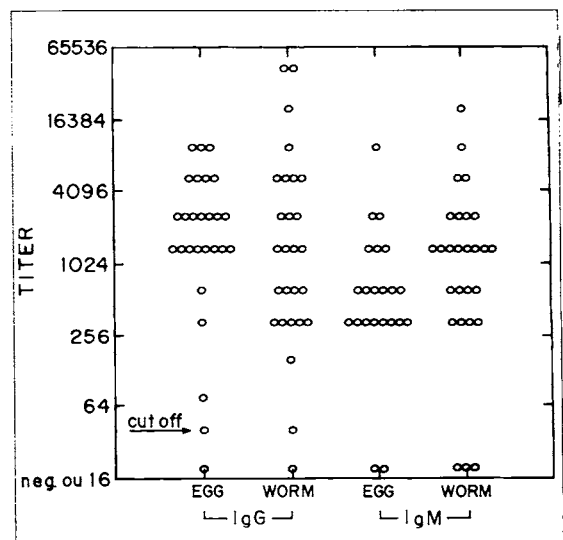


Fig. 1 - Distribution of Dot-ELISA titers in serological analysis.

TABLE 1
Sensitivity, specificity, efficiency, positive predictive value and negative predictive value of the four antibody detection tests, for anti-*S. mansoni* IgG and IgM.

Antibody detection test	Antibody class	No. positive (tested) Patients/Other diseases	Sensitivity	Specificity	Efficiency	Positive predictive value	Negative predictive value
DOT-ELISA (Worm)	IgG	26(27)/3(50)	0.963	0.940	0.948	0.896	0.978
	IgM	24(27)/0(45)	0.889	1.000	0.958	1.000	0.937
DOT-ELISA (Egg)	IgG	25(27)/6(50)	0.926	0.880	0.896	0.806	0.956
	IgM	20(22)/0(45)	0.909	1.000	0.970	1.000	0.957
IFI (Worm)	IgG	25(27)/3(50)	0.926	0.940	0.935	0.892	0.959
	IgM	26(27)/5(50)	0.963	0.900	0.922	0.839	0.978
IFI (Egg)	IgG	26(27)/2(50)	0.778	0.960	0.896	0.913	0.889
	IgM	21(27)/4(50)	0.778	0.920	0.870	0.840	0.885

When the antigens were compared in the same detection system, high agreement was observed between tests, as shown by the kappa index. These data are detailed in Table 2.

TABLE 2
Concordance analysis between antigens in the same serological test.

Serological Test	Kappa index (K)	Zo
DOT-ELISA - IgG	0.918	11.7
DOT-ELISA - IgM	1.000	12.6
IFI - IgG	0.853	8.6
IFI-IgM	0.776	10.0

We also analyzed the positivity of all tests correlated with the status of egg excretion of the patients at the same time. According to egg excretion,

detected by quantitative stool analysis (egf), the patients presented irregular distribution, mainly bimodal, probably with two populations: one consisting of heavily infected, high excretors with more than 50 egf and the other consisting of low excretors with a low parasitic burden, less than 50 egf. The distribution of these patients is illustrated in Figure 2. The distribution of positivity for these two populations in the serological tests performed, could be seen at Table 3.

When we searched for correlations between serum titers and quantitative egg excretion, the only correlation found was with specific IgM antibodies as detected by DOT-ELISA using egg antigen, which presents a correlation coefficient of 0.552 ($p=0.0127$). The others antibody classes and tests presented no correlation with egg excretion.

TABLE 3
Positivity found in serological assays according to egg excretion rates by the schistosomiasis patients at blood sampling.

Serological test	Antigenic Preparation	% Positivity (no. positive/total tested)		
		Total	>50 egf	<50 egf
DOT-ELISA (Worm)	IgG	96.3%(26/27)	100%(15/15)	91.7%(11/12)
	IgM	88.9%(24/27)	93.3%(14/15)	83.3%(10/12)
DOT-ELISA (Egg)	IgG	92.6%(25/27)	100%(15/15)	83.3%(10/12)
	IgM	90.0%(20/22)	92.9%(13/14)	87.5%(7/8)
IFI (Worm)	IgG	92.6%(25/27)	100%(15/15)	83.3%(10/12)
	IgM	96.3%(26/27)	100%(15/15)	91.7%(11/12)
IFI (Egg)	IgG	77.8%(21/27)	100%(15/15)	50.0%(6/12)
	IgM	77.8%(21/27)	86.7%(13/15)	66.7%(8/12)

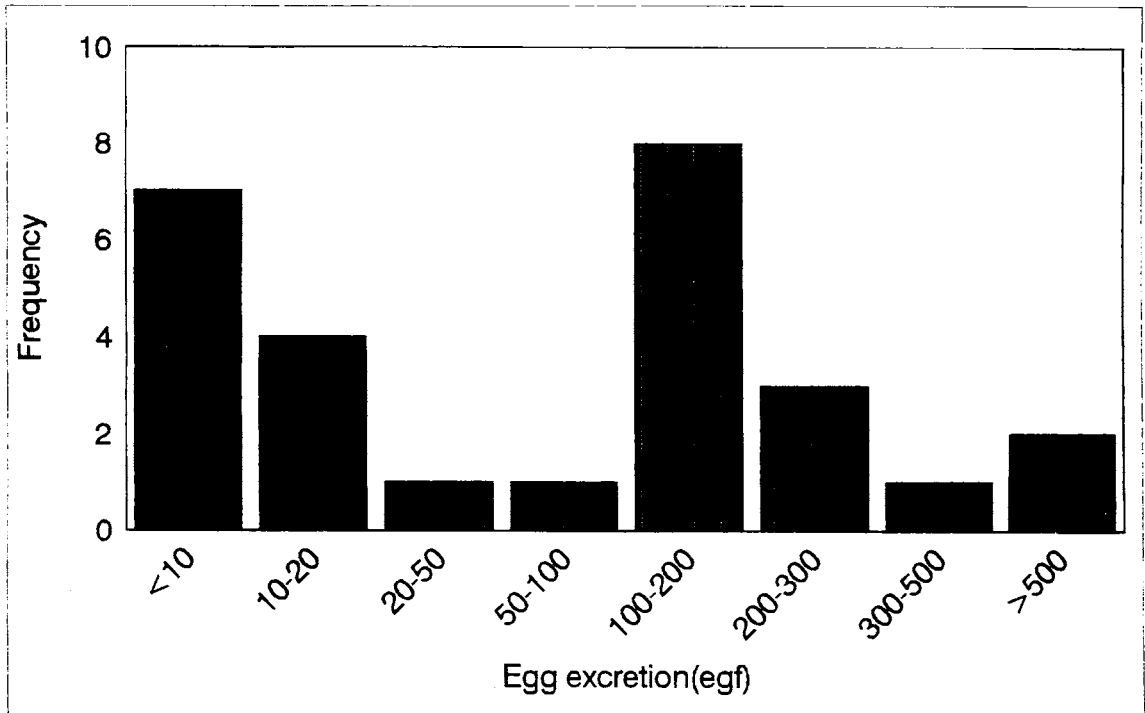


Fig. 2 - Distribution of egg excretion in schistosomiasis patients.

DISCUSSION

In this study, we standardized a simple and feasible method for preparing antigenic extracts of *S. mansoni*, that provided good and reproducible antigens to be dotted on nitrocellulose membranes and could be used in moderately equipped laboratories. This advantage is important because described methods usually involve sophisticated technology to purify antigens or equipment that are not available to most laboratories in underdeveloped endemic countries. The use of adsorption on nitrocellulose membranes also provided an higher stability of antigenic fractions in a dried, ready to use, form, that could be transported at room temperature without loss of sensitivity at least for two weeks (data not shown).

Our results, using sera from other pathologies and parasitic infections, showed that no cross reaction could be observed with the IgM DOT-ELISA using egg or worm extracts as antigen, which occur with other serological tests³. These false positive results obtained by IgG DOT-ELISA occurred mainly with autoimmune diseases, such as autoimmune thyroiditis (2/4), rheumatic disease (1/2) and less frequently with parasitic

infections, such as toxoplasmosis (1/4), but the IFI assay gave more cross-reactions, also with helminthic infections, as described elsewhere, using ELISA¹⁴. Unfortunately, we did not have tested sera from patients suffering teniasis or neurocysticercosis that could present same cross-reactions¹³. The high specificity observed in some of our tests was obtained under conditions of strict laboratory standards, using selected groups of patients and trained personnel which is rarely the case for field conditions.

Our study population clearly presents two subgroups, one composed of low egg excreters (less than 50 egf) and the other of high excreters (higher than 50 egf). We did not find any significant difference for positivity for any of our tests between those groups, but larger studies are needed. The analysis of any other characteristics of these subgroups was considered to be beyond the scope of this report.

Despite its higher specificity, the IgM DOT-ELISA using egg antigen had lower sensitivity, but enough to be used as a positive predictive test, mainly for epidemiological studies, avoiding problems that occur with the other serological methods, which

produce a significant number of false negative results, specially in low prevalence areas. These findings were also observed in similar egg antigen ELISA procedures, but with lower specificity ¹⁴.

The diagnosis of light infections or low parasitic burden in isolated schistosomiasis patients can be partially solved by this test, by virtue of its positive prediction, eliminating the need for multiple stool sampling for a correct diagnosis in most patients ¹⁰. The correlation with its titer and quantitative egg stool excretion also allows a rough estimation of parasitic burden, as described by others, specially in children, with an ELISA using purified egg antigen, but without antibody class analysis ³. The other serological methods tested in this report do not provide any correlation with this parameter, similarly to the findings reported in similar analysis ¹⁴. These data show that the DOT-ELISA assay can be used for the detection of specific antibodies against *S. mansoni* in sera from suspected patients or in epidemiological studies and, with further purification of egg antigen and larger samples, as a possible tool for rough estimation of parasite burden in epidemiological studies.

RESUMO

DOT-ELISA para a detecção de anticorpos IgG e IgM para antígenos de ovos e vermes de *Schistosoma mansoni*, em associação com excreção de ovos por pacientes

A esquistossomose humana, causada pelo *Schistosoma mansoni*, é altamente prevalente no Brasil e é usualmente diagnosticada através de exames de fezes, que são demorados. Os testes sorológicos nesta doença limitam-se aos estudos epidemiológicos, mostrando apenas o contato prévio com o parasita e não a infecção realmente ativa. Neste trabalho, nós padronizamos e comparamos um novo ensaio de Dot-ELISA, para detecção de IgM e IgG específicas contra antígenos de ovo e vermes de *S. mansoni*, comparando seus resultados com a técnica clássica de imunofluorescência (IFI), tanto para IgG ou IgM, utilizando antígenos similares, no estudo de uma população de 27 pacientes com quantificação de ovos nas fezes. A positividade do teste de Dot-ELISA foi de 96,3% para IgG e 88,9% para IgM, com antígeno de verme, e 92,6% para IgG e 90,9% para IgM com antígeno de ovo, que foi comparado com a IFI que apresentou resultados similares com antígeno de verme, 92,6% (IgG) e 96,3%

(IgM) e menores com antígeno de ovo, 77,8% (IgG e IgM). Nosso grupo de pacientes claramente apresentava dois subgrupos, quanto à excreção de ovos nas fezes, com maior positividade dos testes sorológicos para o grupo composto por pacientes com maior excreção fecal de ovos. Quando comparamos a excreção quantitativa de ovos nas fezes com os títulos sorológicos, detectamos apenas correlação entre estes valores e o Dot-ELISA IgM ($r=0,552$ $p=0.0127$).

Estes dados mostram que o teste de Dot-ELISA pode ser usado para a detecção de anticorpos específicos contra o *S. mansoni* em soros de pacientes suspeitos, para estudos epidemiológicos de prevalência; e, com maior purificação dos antígenos de ovos e amostragem ampliada, poderá tornar-se um teste útil para uma estimativa grosseira da carga parasitária em estudos epidemiológicos.

ACKNOWLEDGEMENT

We wish to thank Almir Robson Ferreira for graphs composition. This work was partially supported by FAPESP (92/2142-9) and LIMHCFMUSP 46 and 49.

REFERENCES

1. BOCTOR, F.N.; STEK, M.J.; PETER, J.B. & KAMAL, R. - Simplification and standardization of DOT-ELISA for human schistosomiasis mansoni. *J. Parasit.*, 73: 589-592, 1987.
2. DEELDER, A.M.; KORNELIS, D.; MAKBIN, M. et al. - Applicability of different antigen preparations in the enzyme linked immunosorbent assay for *schistosomiasis mansoni*. *Amer. J. trop. Med. Hyg.*, 29: 401-410, 1980.
3. DOENHOFF, M.J.; BUTTERWORTH, A.E.; HAYES, R.J. et al. - Seroepidemiology and serodiagnosis of schistosomiasis in Kenya using crude and purified egg antigens of *Schistosoma mansoni* in ELISA. *Trans. roy. Soc. trop. Med. Hyg.*, 87: 42-48, 1993.
4. FLEISS, A.R. - Clinical epidemiology. In: FLEISS, A.R. - The architecture of clinical research. 2. ed. Philadelphia, W.B. Saunders, 1985.p.185-186.
5. GALEN, R.S. & GAMBINO, S.R. - Beyond normality: the predictive value and efficiency of medical diagnosis. New York, John Wiley & Sons, 1975.
6. HOSHINO-SHIMIZU, S.; CAMARGO, M.E.; KANAMURA-KAWADA, H.Y.; SILVA, L.C. & DIAS, L.C.S. - Aspectos sorológicos e soropidemiológicos da esquistossomose mansônica. In: Modernos conhecimentos na esquistossomose mansônica. Belo Horizonte, Academia Mineira de Medicina, 1986. p.67-87. (Biblioteca da Academia Mineira de Medicina, v.14)
7. JANISTSCHKE, K.; REINHOLD, A. & BODE, L. - Nitrocellulose DOT-ELISA for serodiagnosis of schistosomiasis. *Trans. roy. Soc. trop. Med. Hyg.*, 81: 956-958, 1987.

8. KANAMURA-KAWADA, H.Y.; HOSHINO-SHIMIZU, S.; CAMARGO, M.E. & SILVA L.C. - Class specific antibodies and fluorescent staining patterns in acute and chronic form of schistosomiasis mansoni. **Amer. J. trop. Med. Hyg.**, 28: 242-248, 1979.
 9. KATZ, N.; CHAVES, A. & PELLEGRINO, J. - A simple device for quantitative stool thick smear technique in schistosomiasis mansoni. **Rev. Inst. Med. trop. S. Paulo**, 14: 397-400, 1972.
 10. LIMA, J.N.; PATTUCI, R.M.J.; YOSHIMI, J. et al. - Avaliação parasitológica da esquistossomose mansônica em área de baixa endemicidade. **Rev. Soc. bras. Med. trop.**, 24 (suppl. 2): 69, 1991.
 11. LOWRY, O.H.; ROSEBROUGH, N.J.; FARR, A.L. & RANDALL, R.J. - Protein measurement with the folin phenol method. **J. biol. Chem.**, 193: 265-275, 1951.
 12. MOTT, K.E. & DIXON, H. - Collaborative study on antigen for immunodiagnosis of schistosomiasis. **Bull. Wld. Hlth. Org.**, 60: 729-753, 1982.
 13. PAMMENTER, M.D.; EPSTEIN, S.R. & REES, R.T. - Cross reactions in the immunodiagnosis of schistosomiasis and cysticercosis by a cerebrospinal fluid enzyme linked immunosorbent assay. **Trans. roy. Soc. trop. Med. Hyg.**, 86: 51-52, 1992.
 14. TANABE, M.; AKAZAKI, M.; KOBAYASHI, S. et al. - Serological studies on schistosomiasis mansoni in the northeast Brazil. **Rev. Inst. Med. trop. S. Paulo**, 32: 121-131, 1990.
 15. WOOLSON, R.F. - Statistical methods for the analysis of biomedical data. New York, John Wiley and Sons, 1987.
- Recebido para publicação em 24/08/1994.
Aceito para publicação em 23/01/1995.