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ORIGINAL ARTICLE

Longitudinal analysis of antigen specific response in individuals with *Schistosoma mansoni* infection in an endemic area of Minas Gerais, Brazil

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Background: Immunoepidemiologic studies have shown a relationship between IgE and IgG4 antibodies with age and with resistance and susceptibility to infection. It is believed that the IgE and IgG4 responses to soluble egg antigen (SEA) can be used for serological analysis of infection and post-treatment status. This study aimed to evaluate the association between *Schistosoma mansoni* infection and anti-SEA IgG4 and IgE reactivities, and determine whether these reactivities could be used as biomarkers of infection.

Methods: Between 2001 and 2009, a longitudinal study was performed in which parasitologic and blood specimens and socioeconomic and water-contact information were collected from 127 individuals. All patients positive for *S. mansoni* infection were treated.

Results: Schistosomiasis prevalence and the geometric mean of the egg count in 2001 were 59% and 61.05, respectively, decreasing to 26.8% and 8.78 in 2009. IgG4 anti-SEA reactivity in infected individuals was significantly higher than that in uninfected individuals at all time points. Analysis of receiver-operating characteristic (ROC) area showed that the IgG4 anti-SEA antibodies were able to predict infection by *S. mansoni* at each time point.

Conclusion: IgG4 anti-SEA reactivity can be used as a biomarker for immune monitoring of the presence of infection with *S. mansoni* in endemic areas.

Keywords: Schistosomiasis, Longitudinal study, IgG4, IgE, Immunoepidemiology, Biomarkers

Introduction

Water-contact behavior is an important determinant of infection and re-infection with *Schistosoma mansoni* in populations where the parasite is endemic.[1,2] However, behavioral factors alone cannot explain variations in infection and re-infection in such populations. It is increasingly apparent that other factors such as the immune response, age and genetic make-up of the host may contribute to variations in infection levels.[3–7] The WHO's recommend approach to schistosomiasis control integrates several actions and strategies, including treatment of infected individuals, promotion of health education, sanitation and treatment of the water supply.[8] In Brazil, studies that evaluated the impact of the national schistosomiasis control program showed that it was successful with regard to the control of morbidity and mortality but did not interrupt transmission and did not prevent new foci of infection.[9,10] Although disease control programs recognize that it is important to integrate all strategies, individual and mass treatment programs remain the major strategy for schistosomiasis control. The persistence of *S. mansoni* infections at a low level (<50–100 eggs per g of feces) makes it difficult to detect infection using the Kato–Katz method, which has low sensitivity.[11–15] Cure rates may be overestimated using this method,[16] and more sensitive diagnostic techniques such as immunological biomarkers need to be developed to monitor treatment effectiveness.

Biomarkers of immune responses may be useful as additional epidemiologic tools since they may be more sensitive and specific.

Studies of human immune responses to *S. mansoni* infection indicate that parasite-specific antibodies play an important part in susceptibility and resistance to infection and re-infection.[17–21] These studies identified a balance between IgE and IgG4 levels in the presence of *S. mansoni* infection, which suggests that high levels of IgE are related to parasite resistance and high levels of IgG4 to susceptibility. However, most studies have focused on one time point after treatment, preventing longitudinal evaluation of effective immune response to the infection.

Building on our previous published work, we carried out a longitudinal immunologic evaluation of the relationship between IgG4 and IgE to better evaluate whether determination of the reactivity of these antibodies could be used as a monitoring tool in endemic populations. The current study evaluates the association between *S. mansoni* infection and IgG4/IgE antibody reactivity to soluble egg antigens (SEA), controlling for socioeconomic and water-contact variables, to determine whether these antibody reactivities could be used in longitudinal studies as biomarkers of infection.

Materials and methods

Study area and population

The study population resides in the endemic area of Virgem das Graças, a rural community located in the Jequitinhonha Valley in northern Minas Gerais State, Brazil. This population lives in four dispersed hamlets (Cardoso 1, 2, 3, and Suçuarana) along the main Cardoso and Suçuarana streams and in a central village. The local population depends on subsistence farming of the staples corn and manioc, cattle husbandry and remittances from family members working in cities.

Individuals eligible for inclusion in the study were males and females aged 6 years and over who had provided stool and blood samples in 2001, 2002, 2005 and 2009 and answered all questions in the socioeconomic and water-contact questionnaires. Pregnant and lactating women were excluded and did not receive treatment as determined by Brazilian health regulations. According to our census in 2001, 658 people lived in 146 households in the Virgem das Graças study area. One hundred and four individuals were under 6 years of age and 40 women were pregnant or lactating during the study periods. This left 514 individuals eligible for the study, 387 of whom were lost to follow-up. The final sample for this study therefore consisted of 127 individuals who participated throughout the 8-year longitudinal study.

Parasitological and blood collection survey

Longitudinal parasitologic examinations and blood collection surveys were carried out in 2001, 2002, 2005 and 2009. Stool specimens were examined for *S. mansoni*, *Ascaris lumbricoides*, hookworm and *Trichuris trichiura* eggs using the Kato–Katz method.[22] All study participants received three name-coded, 80-ml plastic tubes for the collection of fecal samples. The participants were instructed to deposit one fecal sample per day in a fresh tube for 3 consecutive days and to return each sample immediately to the collection point, where the tubes were stored at 4°C. Two slides for each stool sample (a total of six slides per individual) were prepared within 24 h of collection, as described by Gazzinelli et al. (2006).[23] In all four phases of the trial (2001, 2002, 2005 and 2009), 10 ml of blood were collected from each patient. Serum samples were obtained from peripheral blood samples by centrifugation (10 min, 3000 rpm at room temperature) and these samples transferred to the Cellular and Molecular Immunology Laboratory at the Centro de Pesquisas René Rachou/FIOCRUZ in Belo Horizonte, Minas Gerais, where they were stored at −70°C until needed.

Preparation of crude *S. mansoni* antigens

Soluble egg antigens (SEA) were prepared according to methods previously described.[24]

Enzyme-linked immunosorbent assay

IgE and IgG4 reactivity (anti-SEA) were determined in the patients' sera by ELISA. All collected and stored specimens were run at the same time at the end of the study. One hundred microliters of soluble antigens of *S. mansoni* SEA in 0.05 M carbonate–bicarbonate buffer, pH 9.6, at a concentration of 5 μg/ml was added to each well of a polystyrene 96-well plate (Maxisorb; Nunc, Roskild, Denmark). The plates were incubated overnight at 4°C. The following day the plates were washed five times, using an automatic washer, with a solution containing 0.15 M phosphate-buffered saline (PBS, pH 7.2) containing 0.05% Tween 20 (PBS-T) (Sigma, St Louis, MO, USA). The plates were blocked by adding 200 μl of blocking solution (3% fetal bovine serum diluted in PBS-T) to each well and incubated for 1 h. After blockage the plates were again washed as previously and 100 μl of sera, diluted 1:50, added in duplicate for IgE analysis and 1:100 for IgG4 analysis. The plates were incubated for an additional hour. Measurements of the dilutions of sera were obtained from a curve with different concentrations of a pool of sera. The plates were again washed with PBS-T and then 100 μl per well of anti-human immunoglobulin IgG4 or IgE conjugated with biotin (Zymed, San Francisco, CA, USA) diluted 1:1000 was added to each well. After incubation the plates were washed with PBS-T and 100 μl of streptavidin horseradish peroxidase (Amersham, Piscataway, NJ, USA) at 1:1000 dilution added to each well. After incubation for 90 min at room temperature (25–27°C) the plates were washed with PBS-T and 100 μl of ortho-phenylenediamine (OPD) (Sigma) containing 0.03% hydrogen peroxidase added to each well. Optical density was measured at 492 nm. Reactions were carried out using the same batch of reagents, antibodies and equipment for measurement.

Water-contact survey and exposure determination

A questionnaire was used to obtain information on water-contact behavior for all individuals over the age of 6 years. Parents answered all questions for children below 10 years of age. All participants were asked about the frequency and type of contact with local water sources for domestic, recreational and occupational purposes. Contact with all water bodies, including streams, canals, springs, wells, ponds and swamps, was included because of the widespread distribution in the study area of the snail *Biomphalaria glabrata,* a major intermediate host of *S. mansoni*.[25] In 2001, direct observation of water contact was carried out at 14 contact sites in three of the five study communities to

determine the duration and intensity (percent body exposure) of each contact; these parameters, together with frequency of contacts, are used to determine the total body minutes (TBM) of exposure for each individual.[26] Additional observations during household and snail surveys established that the water-contact pattern observed at the 14 observation sites was representative of that at the remaining 57 contact sites in the study area.

TBM were calculated from the direct observation and questionnaire data using the method described by Gazzinelli et al.[27] We used the means of activity-specific TBM based on the water-contact surveys carried out during the study.

Treatment

In 2001 all individuals, infected and non-infected with *S. mansoni*, were treated (baseline) with a single oral dose of praziquantel (60 mg/kg for those aged 15 years or below and 50 mg/kg for those aged more than 15 years. Individuals infected with *A. lumbricoides, T. trichura* or hookworm were treated with a single oral dose of 400 mg of albendazole. In 2002, 2005 and 2009, only patients stool-positive for parasites were treated. Treatment of all individuals in year 1 (2001) was performed following guidelines from the Brazilian Ministry of Health, which call for mass treatment for schistosomiasis when the prevalence is higher than 50%.

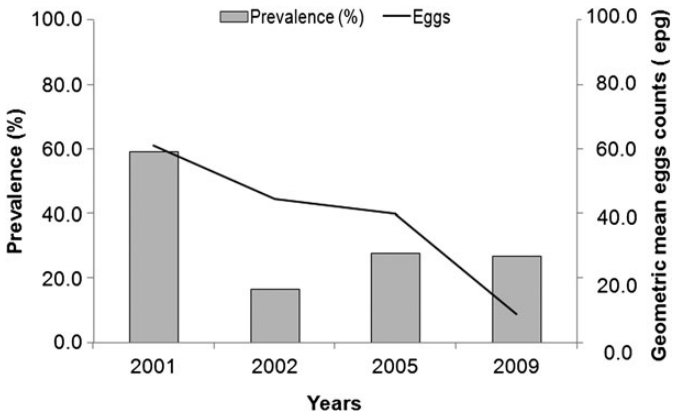

Figure 1. Prevalence and intensity of *Schistosoma mansoni* infection between 2001 and 2009 in Virgem das Graças, Brazil (n=127).

Statistical analysis

The Mann–Whitney *U* test was used to compare data between uninfected and infected individuals for each year. To analyze the predictive ability of the immune biomarker a receiver-operating characteristic (ROC) curve was built. The ROC plot displayed sensitivity versus 1–specificity, such that areas under the curve (AUC) varied from 0.5 to 1.0, with higher values indicating increased discriminatory ability. When the variable under study cannot distinguish between uninfected and infected individuals, the area is equal to 0.5 (the ROC curve will coincide with the diagonal).

Poisson multivariate logistic regression with the generalized estimating equations (GEE) method was used to examine the relationship between age, water contact and antibody reactivity to infection status.[28] This method allowed inclusion of the time variable. The relative risk and 95% CI were estimated. We present three models with different immunologic variables because of the presence of multicollinearity. One of these models refers to the IgG4/IgE ratio. We used SPSS V.19.0 statistical software for ROC analysis and STATA V.10.0 for Poisson logistic analysis.[29,30] The differences were considered significant at $p<0.05$.

Results

Study population and infection parameters

The prevalence of *S. mansoni* at baseline (2001) was 59.0% (95% CI=50.38–67.72) and the geometric mean of eggs per gram of feces (epg) was 61.05 (95% CI=58.70–63.40). In 2001 all individuals received treatment. One year after treatment (2002), both *S. mansoni* prevalence and epg were significantly reduced to 16.5% (95% CI=9.98–23.08) and 40.6 epg (95% CI=37.80–43.42), respectively. By 2005, the prevalence had increased to 27.6% (95% CI=19.68–5.43), but the parasite burden remained similar to that in 2002, with an epg of 39.81 (95% CI=37.27–42.35). The 2009 data showed prevalence nearly unchanged from 2005, at 26.8% (95% CI=18.96–34.57), but epg significantly lower at 8.78 (95% CI=6.45–11.11) compared to previous years (Figure 1).

Table 1. Median specific antibody reactivities IgE and IgG4 (OD 492 nm) against *Schistosoma mansoni* soluble egg antigen (SEA) in uninfected and infected individuals, Minas Gerais, Brazil

	IgE anti-SEA: median (P25–P75)			IgG4 anti-SEA: median (P25–P75)		
Year	Negative	Positive	p[a]	Negative	Positive	p[a]
2001	0.004 (0.00–0.04)	0.013 (0.00–0.04)	NS	1.196 (0.24–2.15)	2.320 (2.28–2.35)	0.000
2002	0.024 (0.00–0.05)	0.019 (0.00–0.05)	NS	1.429 (0.32–2.25)	2.298 (1.99–2.31)	0.000
2005	0.461 (0.25–0.64)	1.010 (0.57–1.21)	0.000	0.835 (0.17–2.07)	2.228 (1.50–2.31)	0.000
2009	1.156 (0.68–1.64)	1.314 (0.86–2.12)	NS	1.209 (0.38–2.05)	2.000 (1.03–3.27)	0.007

P: percentile; NS: non-signficant.
[a]Mann–Whitney test.

Table 2. Multivariate model of the association between demographic, behavioral and immunologic variables and schistosomiasis infection, controlling for sex. Study period=4 years; no. of individuals=127; total observations=508

	Model 1			Model 2			Model 3		
Variables	RR (95% CI)	z test	p	RR (95% CI)	z test	p	RR (95% CI)	z test	p
Age group (years)									
6–14	1.933 (1.354–2.761)	3.63	<0.001	2.526 (1.851–3.447)	5.84	<0.001	1.938 (1.413–2.658)	4.11	<0.001
15–29	1.882 (1.342–2.637)	3.67	<0.001	1.586 (1.177–2.137)	3.03	0.002	1.888 (1.385–2.574)	4.02	<0.001
30–49	1.349 (0.947–1.921)	1.66	NS	1.222 (0.891–1.675)	1.25	NS	1.355 (0.981–1.872)	1.85	NS
50+	1.00	–	–	1.00	–	–	1.00	–	–
Water contact									
Crossing streams	2.128 (1.694–2.673)	6.49	<0.001	1.836 (1.500–2.248)	5.89	<0.001	2.105 (1.717–2.581)	7.16	<0.001
Fishing	1.132 (1.083–1.184)	5.51	<0.001	1.053 (1.019–1.088)	3.13	0.002	1.115 (1.078–1.153)	6.38	<0.001
Immunologic characteristics									
IgE anti-SEA	0.963 (0.814–1.139)	–0.44	NS						
IgG4 anti-SEA				1.754 (1.535–2.003)	8.28	<0.001			
Log ratio IgG4/IgE anti-SEA							1.118 (1.079–1.158)	6.25	<0.001
	ic =–0.039			ic =–0.066			ic =–0.066		
	Wald χ^2=97.28			Wald χ^2=194.22			Wald χ^2=165.48		

RR: relative risk; Log: natural logarithm of the ratio IgG4/IgE anti-SEA; z test: test to assess the significance of each variable in the model; ic: intraclass correlation; NS: non-significant.

IgE and IgG4 reactivity to SEA in infected and uninfected individuals

The anti-SEA IgE reactivity was not different between infected and uninfected individuals in the study years. Only in 2005 was higher IgE anti-SEA reactivity observed in infected patients when compared to egg-negative individuals. The IgE longitudinal analysis against SEA antigen showed an increase over time. However, the levels of antibody in 2005 were higher for both infected and uninfected individuals. The anti-SEA IgG4 levels were significantly higher in infected patients when compared to egg-negative individuals in all study years. This result suggests that anti-SEA IgG4 antibody may be useful as a biomarker for follow-up studies of susceptibility to infection in *S. mansoni* endemic areas (Table 1).

Table 3. Performace of IgG4 anti-SEA antibodies to discriminate infected patients from patients without schistosomiasis, Minas Gerais, Brazil

	IgG4 anti-SEA		Ratio IgG4/IgE anti-SEA[a]	
Year	AUC (%) (95% CI)	p	AUC (%) (95% CI)	p
2001	84 (76.6–90.9)	0.000	60 (50.7–70.6)	0.042
2002	74 (61.3–81.3)	0.003	65 (53.4–77.3)	0.030
2005	74 (64.3–85.1)	0.000	54 (43.9–64.3)	NS
2009	66 (54.8–77.9)	0.010	59 (49.2–70.0)	NS

SEA: soluble egg antigen; AUC: area under the ROC curve; ROC: receiver-operating characteristic; NS: non-significant.
[a]natural logarithm of the ratio.

Multivariate model

The multivariate analysis, controlled for demographic, immunological and water-contact variables, showed that a higher risk of infection by *S. mansoni* was associated with younger age (6–14 and 15–29 years) and with fishing and crossing streams (Table 2). In regard to the immunologic characteristics, no association was observed between anti-SEA IgE reactivity and *S. mansoni* infection. However, there was a significant association between *S. mansoni* infection and both anti-SEA IgG4 and the anti-SEA reactivity IgG4/IgE ratio (Table 2). These results show that there is a relationship between infection and IgG4 levels and between infection and the IgG4/IgE ratio. This association suggests that the IgG4 antibody response to SEA can be useful as an infection biomarker.

Anti-SEA IgG4 areas, observed under the ROC curve, were significantly different from 50%, indicating that analysis of this antibody reactivity can be used to predict infection in every study year. The IgG4 presented a high predictive power in 2001, as observed by a curve area of 84%, and a small predictive power in 2009 (curve area 66%) (Table 3 and Figure 2). A similar analysis

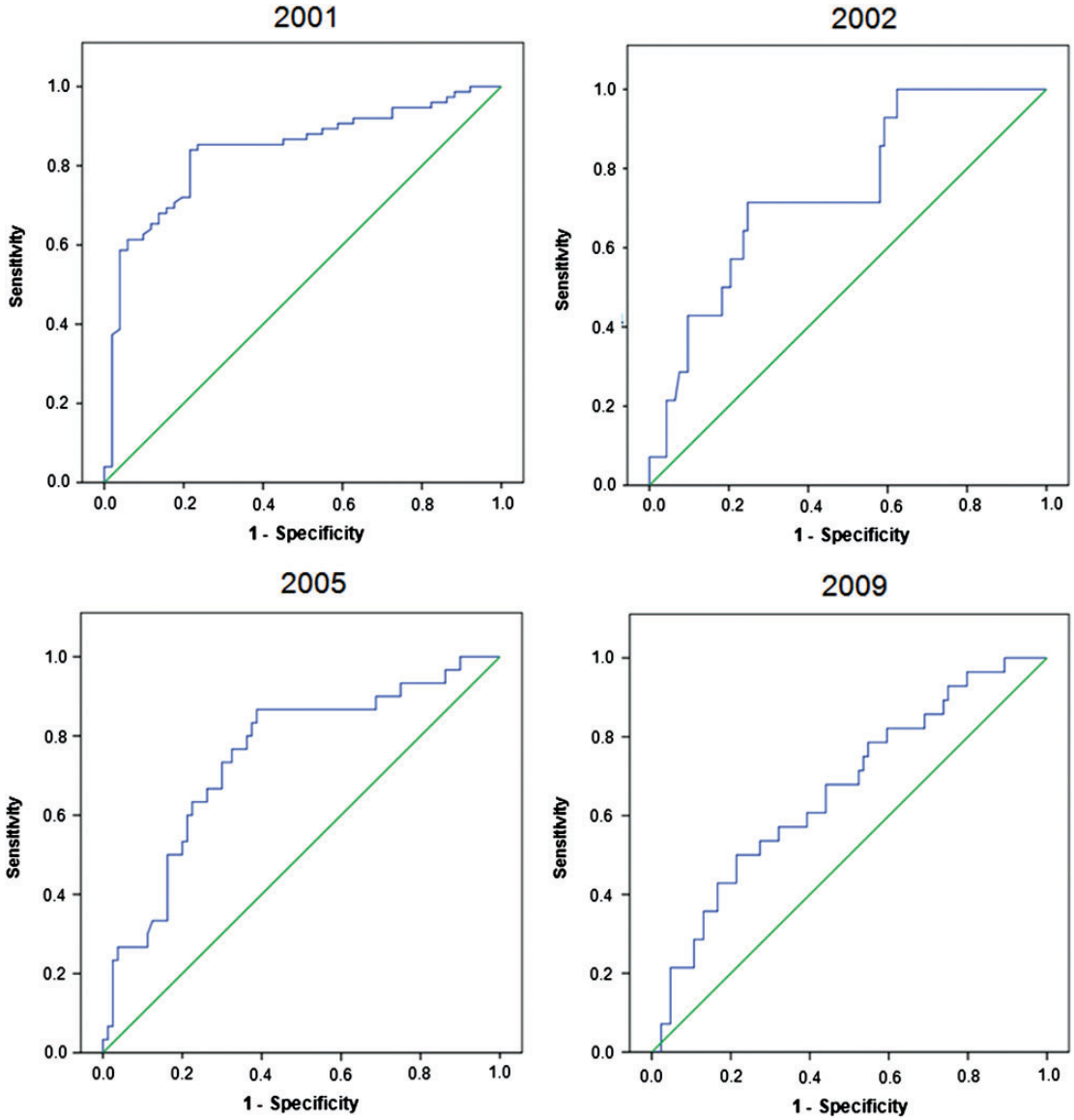

Figure 2. A receiver operating characteristic (ROC) plot, illustrating the ability of IgG anti-SEA antibodies to discriminate infected from uninfected individuals. Higher areas under the curve indicate better discrimination.

was performed to evaluate whether the anti-SEA IgG4/IgE ratio would improve the predictive power of infection. The results showed that the anti-SEA IgG4/IgE ratio presented a low area under the curve. This area was significantly different only in 2001 and 2002 (Table 3 and Figure 3).

Discussion

In our study population *S. mansoni* prevalence was observed to peak in the second decade of life, with lower prevalence among individuals up to 50 years of age, a pattern found in all endemic areas.[31–36] It has been argued that this characteristic infection curve reflects the inability of the immature immune system of children and teenagers to eliminate the parasite.[18,19,21] Evaluation of the IgE and IgG4 anti-SEA reactivity over time showed that after 2005 anti-SEA IgE reactivity was significantly increased for both infected and uninfected individuals. Webster et al.[37] showed that higher IgE serum levels among individuals living in endemic areas are a consequence of persistent antigen exposure, which is consistent with our observation. An explanation for the persistent higher IgE reactivity in uninfected individuals may be that they develop distinct responses to different parasite antigens. This has been previously suggested by Harn et al.[38] and Silveira et al.[19] In addition, the higher IgE reactivity observed in uninfected individuals may also result from the occurrence of common antigens in both eggs and schistosomula.[38] Other authors argue that resistance may also be attributable to the release of dying worms, since the life span of an adult worm is estimated to be around 5–10 years. These hypotheses may be applicable simultaneously in situations where the population is treated.

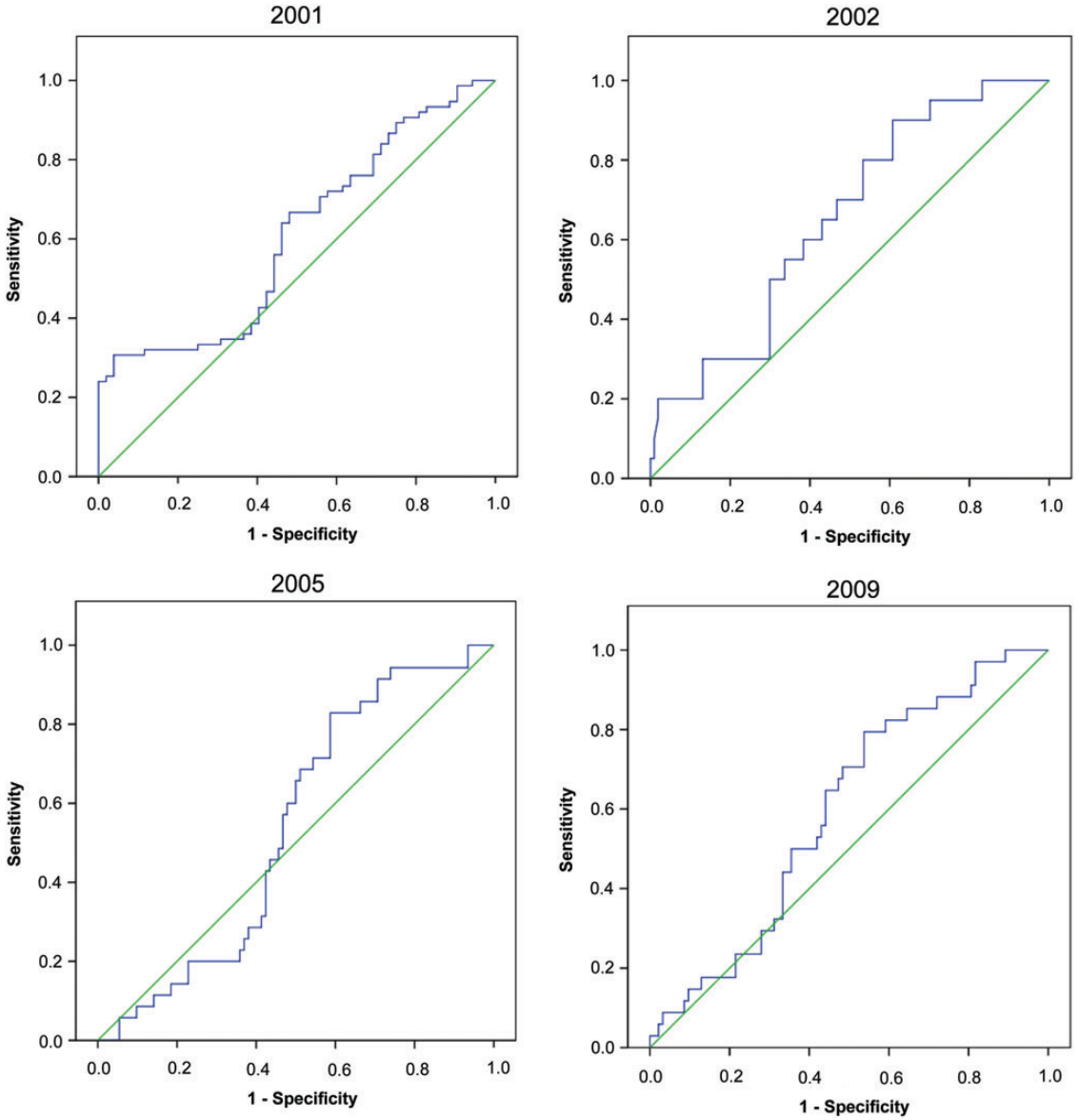

Figure 3. A receiver operating characteristic (ROC) plot, illustrating the ability of IgG4/IgE anti-SEA antibodies to discriminate infected from uninfected individuals. Higher areas under the curve indicate better discrimination.

Additionally, there are significant differences between our studies and others with respect to exposure to infection and treatment regimen.[39] In studies in Africa, the treatment dose is generally significantly lower than that given in Brazil, and studies performed in Brazil comparing the two doses have shown the cure rate to be significantly lower with lower dose used in Africa.[40] It is possible, therefore, that a combination of factors, related to faster killing of worms with the higher dose and the turnover of dying worms, induce concomitantly antigen exposure that affects the outcome of the immune response to infection or re-infection or both.

Murine assays have also suggested that there are common antigens associated with cercariae, schistosomula and miracidia.[41] As a consequence, when uninfected individuals have contact with cercariae, schistosomula and eggs a common set of antigens maintains the stimulation of the immune response that is necessary to induce elevated IgE secretion.[42] Our study showed that the anti-SEA IgG4 reactivity in infected individuals was significantly higher when compared to that in uninfected individuals. In contrast, Grogan et al.[43] observed no significant differences between this specific isotype against SEA and soluble worm antigen preparation (SWAP) over a period of 2 years after treatment in the infected population. However, it was observed that uninfected individuals had lower IgG4 anti-SEA levels, corroborating our findings. Using the recombinant antigen Sm 22.6, a member of the tegumental-allergen-like (TAL) family (TAL-1),[37] showed a relationship between resistance and reactivity to this antigen. However, this antigen is not present in eggs and therefore

cannot be directly compared with the data presented here. An additional analysis may be necessary to evaluate whether this recombinant antigen can indeed be used as a biomarker of infection at different time points in a longitudinal study.

We evaluated whether specific IgE and IgG4 levels can be used as indicators of infection by *S. mansoni,* taking into account an individual's age, sex and water contact. We observed that specific IgG4 reactivity, IgG4/IgE ratio, age and exposure to water activities such as crossing streams and fishing were associated with *S. mansoni* infection in Virgem das Graças. This was observed when we applied a multivariate model for the analysis. In contrast, IgE reactivity was not related with infection in this model. Our results showed a clear association between IgG4 anti-SEA reactivity and *S. mansoni* infection that is not as evident with the IgG4/IgE ratio and *S. mansoni* infection. These results suggest that the anti-SEA IgG4 reactivity may be an important tool to predict infection in endemic areas.

Demeure et al.[44] showed, using a multivariate model adjusted by water contact, age and sex, that high IgG4 production was strongly associated with increased susceptibility to re-infection. In contrast low levels of IgG4 production in patients resistant to infection and re-infection was found. Our data which shows a decrease in IgG4 is in agreement with Hagan et al. and our previous studies.[17,19,20]

This study aimed to identify a putative immune biomarker of *S. mansoni* infection that could be easily applied to longitudinal studies. With large-scale use of chemotherapy in several countries in recent years, and its now extensive application in some African countries following the WHO World Health Assembly resolutions 54.19 and 65.21, sensitive and faster methods for monitoring infection and for the identification of individuals targeted for treatment are needed.[46,47] Therefore, it is important to monitor infection and to evaluate the treatment impact on populations covered by schistosomiasis control programs.[48–50] We showed IgG4 anti-SEA reactivity to be a significantly powerful predictor of infection in all four study periods (2001, 2002, 2005 and 2009). The predictive power as measured by the ROC area ranged from 84% at baseline to 66% for 2009.

One reason for this reduction in predictive power could be a reduction in parasite burden, with consequent reduced exposure to the antigen that stimulates secretion of this antibody isotype. That is, a decline in infection intensity over time may have influenced this biomarker's predictive power. Correlation between parasite burden and increased anti-SEA IgG4 reactivity has been shown by others in longitudinal studies.[20,43,51] We hypothesized that an increase in IgE isotype over time could contribute to the reduction of the predictive power of anti-SEA IgG4 reactivity.

The increase in anti-SEA IgE reactivity over time observed in our study supported our hypothesis. Conversely, other studies have suggested that the immune response to *S. mansoni* re-infection depends on the balance between IgE and IgG4 antibodies.[17–19,52,53] In our study we performed a longitudinal analysis of the anti-SEA IgG4/IgE ratio to test this hypothesis and observed that it did not improve the predictive power of infection when compared with IgG4 anti-SEA alone. Therefore, our results are not in agreement with these previous studies, since the anti-SEA IgG4 reactivity was always higher over the years in both infected and uninfected individuals. This result is important because this balance may not be maintained when individuals are followed for several years.[17–19,21,44,52–55] Studies like ours have intrinsic limitations related to the biological system being studied, such as intrahost and interhost variations, sensitivity of the method of diagnosis and our limited capacity to control for all variables related to infection. Longitudinal immunological studies related to the analysis of serologic factors such as antibodies have additional variability in the instability of serum factors such as IgE and of frozen samples, and these limitations apply to other previously published studies. To further validate our studies, we are performing comparative analysis between sera collected from different endemic areas that we have studied over the years. To date we have not observed any significant variability that would question the results presented in this manuscript.

In conclusion, our data suggest that IgG4 reactivity to egg antigens can be used as an immune biomarker for the monitoring of infection with *S. mansoni* in endemic areas and may be an important high-throughput tool for large-scale population monitoring. However, the anti-SEA IgG4/IgE ratio did not have significant predictive power at any of the four study points.

Authors' contributions: LFM carried out the entire data collection, analysis, and interpretation of the data; ROP, PTL and HK drafted the manuscript; MNSA performed the statistical analyzes; RTF performed the immunoassays; AG and HK supervised data collection; AG and RCO conceived and designed the study. All authors critically revised the manuscript for intellectual content and read and approved the final version. LFM and AG are the guarantors of the paper.

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Competing interests: None declared.

Ethical approval: Ethical clearance was provided by the ethics committee at the Universidade Federal de Minas Gerais (no. ETIC194/06-EX01/09) and the Brazilian National Committee for Ethics in Research (CONEP no. 612/2007). Consent was obtained from all participants prior to the commencement of the study.

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