Ministério da Saúde

Fundação Oswaldo Cruz

Centro de Pesquisas René Rachou

Programa de Pós-graduação em Ciências da Saúde

Desenvolvimento e padronização de novas metodologias aplicadas ao diagnóstico e monitoração de cura da esquistossomose mansoni na fase inicial (aguda) e crônica

Por

Rafaella Fortini Grenfell e Queiroz

Belo Horizonte

Julho/2012

Ministério da Saúde

Fundação Oswaldo Cruz

Centro de Pesquisas René Rachou

Programa de Pós-graduação em Ciências da Saúde

Desenvolvimento e padronização de novas metodologias aplicadas ao diagnóstico e monitoração de cura da esquistossomose mansoni na fase inicial (aguda) e crônica

Por

Rafaella Fortini Grenfell e Queiroz

Tese apresentada com vistas à obtenção do título de Doutor em Ciências na área de Doenças Infecciosas e Parasitárias

Orientação: Dr. Paulo Marcos Zech Coelho

Dr. Donald A. Harn, Jr

Belo Horizonte

Julho/2012

Catalogação-na-fonte

Rede de Bibliotecas da FIOCRUZ

Biblioteca do CPqRR

Segemar Oliveira Magalhães CRB/6 1975

Q1d **Queiroz, Rafaella Fortini Grenfell e.**

2012

Desenvolvimento e padronização de novas metodologias aplicadas ao diagnóstico e monitoração de cura da esquistossomose mansoni na fase inicial (aguda) e crônica / Rafaella Fortini Grenfell e Queiroz. – Belo Horizonte, 2012.

XXII, 166 f: il.; 210 x 297mm.

Bibliografia: f. 170 - 185

Tese (doutorado) – Tese para obtenção do título de Doutor em Ciências pelo Programa de Pós-Graduação em Ciências da Saúde do Centro de Pesquisas René Rachou. Área de concentração: Doenças Infecciosas e Parasitárias.

1. Esquistossomose mansoni/diagnóstico 2. *Schistosoma mansoni*/parasitologia 3. Antígenos/imunologia I. Título. II. Coelho, Paulo Marcos Zech (Orientação). Iii. Harn Jr, Donald A (Orientação)

CDD – 22. ed. – 616.963

Ministério da Saúde

Fundação Oswaldo Cruz

Centro de Pesquisas René Rachou

Programa de Pós-graduação em Ciências da Saúde

Desenvolvimento e padronização de novas metodologias aplicadas ao diagnóstico e monitoração de cura da esquistossomose mansoni na fase inicial (aguda) e crônica

Por

Rafaella Fortini Grenfell e Queiroz

Foi avaliada pela banca examinadora composta pelos seguintes membros:

Prof. Dr. Paulo Marcos Zech Coelho (Presidente)

Prof. Dr. Ricardo Tostes Gazzinelli - CPqRR

Prof. Dr. José Mauro Peralta - UFRJ

Prof. Dr. José Augusto Nogueira Machado – Santa Casa de Misericórdia de Belo Horizonte

Prof. Dr. Olindo Assis Martins Filho CPqRR

Dissertação defendida e aprovada em: 06/07/2012

Dedico

Aos meus quatro pilares: meus pais, Silvio e Eliane; meu marido, Fabiano; meu mentor, Dr. Paulo Marcos. Por me mostrarem, cada um a seu modo, que o conhecimento é o nosso maior bem, que nenhum homem pode jamais tirar.

Ofereço

Aos indivíduos residentes de áreas endêmicas, nosso objetivo maior.

Sinto-me nascido a cada momento para a eterna novidade do Mundo.

Fernando Pessoa

SUPORTE FINANCEIRO

Conselho Nacional de Desenvolvimento Científico e Tecnológico (MCT/CNPq/CT-Saúde/MS/SCTIE/DECIT 576026/2008-5)

Conselho Nacional de Desenvolvimento Científico e Tecnológico (MCT/CNPq 143204/2008-4)

Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES/DRI/CGCI)

Fundação Oswaldo Cruz (Fiocruz/VDPR/PAPES V)

Fundação de Amparo à Pesquisa do Estado de Minas Gerais

National Institutes of Health AI R01 AI068109A to DAH

Research Program for SUS: shared management in health (PPSUS)

The Council of the International Educational Exchange of Scholars, The United States Department of State, Fulbright (IIE# 15104086)

AGRADECIMENTOS

"*Tudo o que sinto, vejo, escuto e toco, esbarra, quase sempre, em Deus*"
Adélia Prado

Eis que chegou o momento de expressar meus agradecimentos a muitos e tantos amigos que se revelaram indispensáveis ao longo desse tempo. Para maior percepção desse sentido, devo contar que esta não foi uma caminhada breve, mas uma travessia. Foi a quatro anos que comecei algo. De uma formal apresentação no escritório da Santa Casa de Misericórdia de Belo Horizonte fui levada a uma conversa particularmente informal e inspiradora de onde surgiu uma promissora ideia. Por aí começou. A ideia era audaciosa. Desenvolver novas alternativas para o diagnóstico de uma antiga doença, tão carente em projetos inovadores como ainda eram seus próprios pacientes. Inspirada pelas palavras empolgantes de meu mentor, vi-me desafiada a prosseguir. Parti-me, então, de uma certeza, que detinha a chave do sucesso deste projeto: a de me apoiar em grandes mestres.

Se o desafio era enorme, as motivações eram grandiosas. Somadas, ainda, às muitas generosidades vindas de pessoas muito amadas que compõem hoje o meu próprio elo com a pesquisa. Pessoas que transformaram momentos de anseios em uma estrada larga, margeada de frutos. Com estas pessoas aprendi a me apoiar na mais forte base existente, a busca de saberes, representada por uma plenitude de coisas possíveis.

Muitos percalços incidiram, mas longe de obscurecerem o trajeto, aumentaram-lhe o brilho. E, ao invés de me deterem, impulsionaram-me com mais força. Talvez esta tese seja o resultado mais visível desse processo de construção em meio a uma conjuração de afetos e amizades. Partindo então dessa premissa, faz-se necessário e bastante prazeroso dedicar algumas palavras àqueles que dela fazem parte diretamente.

Dr. Paulo Marcos Zech Coelho, quem cito primeiro. Poderia ter cumprido somente seu grandioso papel de orientador. Papel este, muito bem cumprido, necessário dizer. Mas ele não é mesmo um ser humano como outro qualquer. Serviu-me também como mentor, base de apoio e, mais, como um segundo pai. Deu-me conselhos profissionais nas muitas conversas que tivemos sobre meu futuro. Insistiu no meu fortalecimento pessoal e profissional. Tornou-me pesquisadora.

Dr. Naftale Katz, o grande precursor da pesquisa em diagnóstico. No início, uma pergunta em mim não se calava: como convencer o criador do mais respeitado método parasitológico de que precisávamos melhorar o diagnóstico de *Schisto*? Preocupação sem razão, como ele

mesmo disse certa vez. Foi com quem aprendi que é preciso fornecer as soluções práticas e aplicáveis que o mundo precisa.

Dr. Donald A. Harn, Jr, my supervisor, from whom I have learnt that good leadership, requires deep human qualities. You did your best to enrich our work and my graduation. You trusted in my skills and gave me the chance to be part of different projects, seminars and meetings. You helped me to improve my network. Our collaboration will be continually enriched by our new ideas, projects and products. So, I hope.

Cristina Toscano Fonseca pela co-orientação nos momentos cruciais que deram início ao projeto.

Élio Baba e Andréa Teixeira, escolhidos a dedo para minha qualificação. Os múltiplos resultados obtidos foram frutos do desafio, por vocês, lançados. Seria melhor uma glicoproteína purificada ou uma proteína recombinante? Numa decisão em conjunto, audaciosa devo dizer, optamos por ambas.

Pesquisadores componentes da banca: José Mauro Peralta, José Augusto Nogueira Machado, Carlos Alberto Pereira Tavares, Dirceu Grecco, Ricardo Gazzinelli, Olindo Assis Martins Filho, Andrea Teixeira, Luciana Gomes. Um agradecimento especial a estes que se fizeram presentes no momento decisivo da minha formação.

Todo o querido Laboratório de Esquistossomose, incluindo pessoas que lá não mais se encontram. Pelos momentos de partilha dos resultados experimentais e também da vida. Em especial, àqueles que fizeram parte da minha rotina de experimentos, alguns dos quais compõem hoje a equipe da Plataforma de Produção de Anticorpos. Vocês me deram o prazer de boas conversas e grandes ensinamentos sobre muito do que passou a texto: Elizandra Giani, Suedali Barata, Ana Carolina Mattos, Watson Martins. A Vanessa Silva-Moraes, um agradecimento mais que especial, por ter agregado tanta força ao nosso grupo.

Diana Taboada, responsável pela completa revisão da língua portuguesa. E Vera de Paula Ribeiro, pela conferência da língua inglesa.

Liliane Siqueira, Áureo Almeida e Martin Enk pelo bonito trabalho realizado em diferentes áreas endêmicas, por onde deixam esperança, cuidado e carinho para um grande número de indivíduos carentes de nosso país.

Jussara Miranda e Daniela Portela, as secretárias mais eficientes deste país! Vocês tornaram meu percurso mais leve e muito, muito mais alegre.

Queridíssima Vandinha, que em muitos dias nublados fez o sol aparecer. Uma destas pessoas que a vida não pode dar-se ao luxo de não ter.

Centro de Pesquisas René Rachou, Dra. Zélia Profeta, nossa atual diretora, e a Fundação Oswaldo Cruz, como um todo, no que inclui todos os doutores, técnicos, estagiários, colaboradores e amigos.

Biotério do CPqRR, especialmente Kátia, Fernanda, Jaci, Vera e Moisés pelo constante e eficiente esforço de ensinar, cuidar e aprimorar o cultivo dos animais experimentais.

Equipe do Moluscário do CPqRR, em especial à querida Suelene, pelo importante trabalho na manutenção do ciclo do *S. mansoni* e pela disponibilização das cercárias sempre que requisitadas.

Secretaria da pós-graduação do CPqRR, na representação de diferentes chefias ao longo destes 4 anos. Dedico um agradecimento especial a Cristiane e Andréa pelo interesse constante na solução de dúvidas e requisições.

Biblioteca do CPqRR em prover acesso gratuito local e remoto à informação técnico-científica com recursos públicos federais, integrante do rol de referências desta tese, também pela catalogação e normalização da mesma.

University of Georgia, my home for one year, extremely prepared to deal with international scholars.

Great friends and collaborators from the lab staff in UGA. People with the best expertise for handling complex scientific subjects: Smanla, Leena, Lisa, Cac, Bea, Hillary, Changlin, Farrah, Jennifer, Nathan.

My dear Ruth Davies from the Monoclonal Antibody facility. It was a great honor to work with you. You have inspired me and now it is overwhelming to have you as my collaborator on the Monoclonal Antibody Facility in Fiocruz.

Lavon Smith, my English teacher. Our great talks every Tuesday and Thursday became an inspiration for life.

Dan Colley who was just around the corner during my days in UGA. My first contact, responsible for my introduction to Don.

Colaboradores diretos deste projeto: Edward Oliveira e Neusa Araújo do CPqRR. Sandra Drummond e José Roberto Lambertucci, UFMG. Smanla Tundup e Ruth Davies, *UGA*.

Akram D'adara, *Harvard University*. Cito, especialmente, José Mauro Peralta da UFRJ, não somente pela colaboração, mas por ter me ensinado os passos-chaves que me levaram a instituição da Plataforma de Anticorpos Monoclonais.

Financiamentos concedidos por diferentes agências: CNPq, Capes, Fapemig, Fiocruz, PPSUS, *NIH*, *Fulbright*, *IIE*. Agradeço, especialmente, a Taís da CAPES que é um alento no auxílio dos pós-graduandos internacionais.

Agradecimentos especiais

Meus amados pais, Silvio e Eliane, pela real preparação para a vida. De fato, "um bom pai vale por cem mestres" (*Jean Jacques Rousseau*).

Minha amada avó Marieta Fortini que desde sempre foi uma segunda mãe, provendo-me com riquíssima educação.

Meus queridíssimos irmãos, Camilla e Silvio, que me ensinam a ter força diante das muitas adversidades da vida. Mais ainda, por nossa união que se faz mais forte à medida que crescemos.

D. Lena, Sr. Expedito, Júnior e Marcelo por me permitirem ter hoje minha família multiplicada por dois.

Todos os meus amigos, de toda a vida, que são parte indissolúvel da minha história.

Por fim, meu agradecimento final, ao maior incentivador deste projeto, meu querido Fabiano. Que sem dúvida transformou minha perspectiva sobre o que é ciência e como fazê-la. Especialmente sobre como somar ciência, gestão e inovação. Quem me presenteou com seus incontáveis valores e riquíssimo acervo de idéias, o que o tornou companheiro fiel deste projeto. Sempre incasável ao me doar coragem nas muitas vezes em que cheguei em casa querendo alçar vôos mais e mais altos. A fase foi longa, mas chega agora o seu fim. É a você que ofereço os frutos deste trabalho.

RESUMO

Neste projeto, foram testados diferentes antígenos candidatos a padronização de novos métodos diagnósticos a serem usados nas diferentes fases da infecção esquistossomótica. Inicialmente, foram analisados os antígenos brutos do parasito visto que seu baixo custo e simplicidade de obtenção merecem novas abordagens. Assim, antígenos de vermes adultos, ovos e tegumento de esquistossômulos foram analisados com soro de pacientes submetidos a rigoroso diagnóstico parasitológico para determinação de infecção e/ou diagnóstico clínico e imunológico. Ao serem aplicados no método indireto de ELISA, estes antígenos apresentaram alta sensibilidade e especificidade em fases distintas da infecção murina. Através desses resultados foi possível confirmar que o uso de antígenos obtidos de diferentes formas evolutivas do parasito serve como ferramenta potencial para análise da evolução cronológica da infecção. Quando aplicados em amostras humanas, o uso de antígenos de vermes adultos mostrou-se promissor para o diagnóstico de pacientes residentes de áreas endêmicas que apresentavam baixa carga parasitária, com índices de sensibilidade e especificidade de 95%. Tendo sido, nestas condições, superior aos antígenos de ovos. O estudo de pacientes em fase aguda da infecção permitiu a validação da técnica indireta de ELISA com antígenos de tegumento de esquistossômulos. Esta técnica apresentou uma significativa sensibilidade na identificação de grande parte dos pacientes recentemente infectados. A outra abordagem adotada por este tarbalho envolveu o uso do Antígeno Catódico Circulante (CCA), antígeno este secretado/excretado por vermes jovens e adultos, que foram direcionados para o desenvolvimento de novas e promissoras metodologias diagnósticas. Para este propósito, o CCA foi utilizado em diferentes formas antigênicas: como glicoproteína purificada a partir de vermes, como proteína recombinante e como peptídeos imunogênicos de 20 aminoácidos. Estes antígenos foram usados na padronização do Método de Separação Imunomagnética, denominado IMS. O uso de CCA recombinante no método de IMS indireto levou aos índices mais significativos de sensibilidade e especificidade, sem que qualquer resultado falso-negativo fosse detectado. Por outro lado, o uso da glicoproteína CCA purificada demonstrou ser superior no diagnóstico para monitorização de cura. A partir destes resultados, mais promissores que a ELISA convencional, partimos para a padronização final desta técnica para a detecção direta do CCA nas mesmas amostras, de forma a permitir somente a identificação de infecções ativas. Para isto, anticorpos monoclonais específicos para a glicoproteína CCA foram produzidos e conjugados a marcadores. A escolha do clone foi baseada na reduzida especificidade deste pela porção responsável pelas reações cruzadas do CCA, a porção

glicídica *Lewis x*. O novo método IMS para detecção direta demonstrou alta sensibilidade de 94% e especificidade de 100%, apresentando correlação direta com a carga parasitária destes pacientes determinada pela contagem de ovos nas fezes. Os excelentes resultados na detecção de antígeno circulante obtidos no presente trabalho, que contrapõem os obtidos em outros trabalhos publicados, se devem a nova metodologia empregada que utiliza a concentração destes antígenos ao invés da diluição de amostras. Por fim, idealizamos um último método, denominado FluoIMS, destinado a identificação qualitativa da presença de CCA através da microscopia de fluorescência. Este método, de detecção direta e de execução bastante simples, apresentou significativos índices de sensibilidade quando três lâminas individuais para cada amostra foram analisadas. Nossos resultados trazem grandes expectativas para a melhoria do diagnóstico dos muitos pacientes infectados por baixas cargas do *Schistosoma mansoni*, em diferentes fases da infecção, e apontam novas perspectivas para aplicação destes métodos no controle de cura pós-tratamento.

ABSTRACT

In this project, various candidate antigens for standardization of new diagnostic methods were tested for use at different phases of schistosome infection. Initially, the crude antigens of the parasite were analyzed, since their low cost and ease of obtaining deserve new approaches. Thus, antigens of adult worms, egg antigens, and tegument antigens of schistosomula were analyzed by means of sera of patients submitted to a rigorous parasitological diagnosis for determination of infection. When applied to the indirect method of ELISA, these antigens presented high sensitivity and specificity levels at different phases of murine infection. Based on these results, it was possible to confirm that the use of antigens obtained at different evolutive phases of the parasite acts as a potential tool for analysis of the chronological evolution of infection. When applied to human samples, the use of adult worm antigens was promising for diagnosis of patients living in endemic areas, and presenting low worm burden, with sensitivity and specificity levels of 95%. Under these conditions, they were considered superior than the egg antigens. The study related to tourists presenting acute phase of infection allowed the validation of the indirect technique of ELISA, with tegument antigens of schistosomula. This technique showed a significant sensitivity for identification of a large part of recently infected patients. Another approach used in this study involved the use of Circulating Cathodic Antigen (CCA), which was secreted/excreted by juvenile and adult worms, that were directed to development of new and promissing diagnostic methodologies. For this purpose, the CCA was used at different forms of antigens: as purified glycoprotein obtained from worms, as recombinant protein and as immunogenic peptides of 20 aminoacids. These antigens were used for standardization of the Immunomagnetic Separation Method, named IMS. The use of recombinant CCA in the indirect method of IMS showed the most significant levels of sensitivity and specificity, and no false-negative results could be detected. On the other hand, the use of purified glycoproteinCCA demonstrated to be superior for diagnosis of cure control. Based on these results, which were more promissing than the conventional ELISA, we started the final standardization of this technique for the direct detection of CCA in the same samples, in order to allow only the identification of active infections. For this purpose, specific monoclonal antibodies for glycoprotein CCA were produced and conjugated to merchandises. The choice of the clone was based on the lack of its specificity by the portion responsible for the cross-reactions of CCA, the glicidic portion *Lewis x*, and in order that a low level of cross-reactivity could be detected by this method, in future analyses. The new method IMS demonstrated high levels of sensitivity (94%) and

specificity (100%), reaching superior levels than the ones showed by the current immunological methods, as well as presenting a direct correlation with those patients´worm burdens, which were obtained by fecal egg counts. The excellent results obtained in the present study regarding the detection of circulating antigen, that did not corroborate the results obtained in other published papers, are due to the new methodology used, that utilizes the concentration of these antigens and not the dilution of samples. Finally, we planned another method, named FluoIMS, for the qualitative identification of the presence of CCA by means of fluorescence microscopy. This method, offering direct detection and ease of execution, showed significant levels of sensitivity, when three individual glass-plates for each sample were analyzed. Our results offer great expectancy for the improvement of diagnosis of infected patients with low *Schistosoma mansoni* burdens, at different phases of infection, and indicate new perspectives for application of these methods in the post-treatment cure control.

SUMÁRIO

LISTA DE FIGURAS

LISTA DE TABELAS

LISTA DE ABREVIATURAS E SÍMBOLOS

WHO	*World Health Organization*
DALY	*Disability adjusted to life years*
TAL	*Tegumnetal-Allergen-Like*
SmStoLP-2	*Stomatin-like 2*
Le^x	*Lewis x*
CCA	Antígeno Catódico Circulante
CAA	Antígeno Anódico Circulante
SEA	Antígeno solúvel de ovos
IPSE/α-1	*IL-4 inducing principle*
LDN-DF	GalNAcb1-4(Fuca1-2Fuca1-3)GlcNAcb1
HPJ	Técnica de Hoffmann, Pons & Janer
PCR	Reação em Cadeia da Polimerase
ELISA	*Enzyme-linked Immunosorbent Assay*
SWAP	Antígeno solúvel de vermes adultos
MAS	*Major Serological Antigen*
Mama	*Adult Microssomal Antigen*
IMS	Método de Separação Imunomagnética
FluoIMS	Método de Separação Imunomagnética para análise em microscopia de fluorescência

ELISA-SWAP *Enzyme-linked Immunosorbent Assay* sensibilizada com Antígeno Solúvel de Vermes Adultos

ELISA-SEA *Enzyme-linked Immunosorbent Assay* sensibilizada com Antígeno Solúvel de Ovos

SmTeg Antígeno de Tegumento de Esquistossômulos

ELAC *Earl's salts plus lactalbumin hydrolyzate*

LB Meio Luria Bertani

IPTG Isopropil β-D-tiogalactose

TMB Substrato 3,3',5,5-tetrametilbenzidina

HAT Meio hipoxantina-aminopterina-timidina

mAbCCA Anticorpos monoclonais específicos para glicoproteína CCA purificada

BSA Albumina Sérica Bovina

ELISA-SmTeg *Enzyme-linked Immunosorbent Assay* sensibilizada com Antígeno de Tegumento de Esquistossômulos

ELISA-CCA *Enzyme-linked Immunosorbent Assay* sensibilizada com Antígeno Catódico Circulante purificado

ELISA-CCAr *Enzyme-linked Immunosorbent Assay* sensibilizada com Antígeno Catódico Circulante recombinante

ELISA-CCApep1 *Enzyme-linked Immunosorbent Assay* sensibilizada com peptídeo 1 do Antígeno Catódico Circulante

ELISA-CCApep2 *Enzyme-linked Immunosorbent Assay* sensibilizada com peptídeo 2 do Antígeno Catódico Circulante

ELISA-mAbCCA *Enzyme-linked Immunosorbent Assay* sensibilizada com anticorpos monoclonais específicos para Antígeno Catódico Circulante purificado

IMS-CCA Método de Separação Imunomagnético sensibilizado com Antígeno Catódico Circulante purificado

IMS-CCAr Método de Separação Imunomagnético sensibilizado com Antígeno Catódico Circulante recombinante

IMS-CCApep1 Método de Separação Imunomagnético sensibilizado com peptídeo 1 de Antígeno Catódico Circulante

IMS-CCApep2 Método de Separação Imunomagnético sensibilizado com peptídeo 2 de Antígeno Catódico Circulante

IMS-mAbCCA Método de Separação Imunomagnético sensibilizado com anticorpos monoclonais específicos para Antígeno Catódico Circulante purificado

1 INTRODUÇÃO

1 INTRODUÇÃO

1.1 Aspectos epidemiológicos da esquistossomose

A esquistossomose é uma doença causada por espécies do gênero *Schistosoma* que afeta 200 milhões de indivíduos em 74 países do mundo segundo a *World Health Organization* (WHO) (2004). Estima-se que 207 milhões de pessoas estão acometidas, sendo que 20 milhões apresentam a forma grave da doença e 779 milhões vivem em áreas de risco (Chitsulo et al., 2000; Van der Werf et al., 2003; Steimann et al., 2006) (Figura 1). Além disso, aproximadamente 280 mil mortes por ano são atribuídas às esquistossomoses.

Nos últimos 50 anos, houve uma mudança em sua distribuição geográfica e mesmo com programas de controle bem sucedidos, o número de pessoas infectadas ou sob risco de contrair a doença não foi reduzido (Van der Werf et al., 2003). O acometimento é mais frequente em localidades com deficiência de saneamento básico, o que obriga indivíduos de baixo poder aquisitivo à uma vida insalubre pela falta de escolha e disponibilidade de fontes de águas seguras para fins recreativos, domésticos ou profissionais (Hagan, Ndhlovu & Dunne, 1998).

Entre as helmintoses, a esquistossomose representa a principal doença em termos de morbidade e mortalidade causando perdas anuais de até 4,5 milhões em *Disability adjusted to life year* (DALY) (WHO, 2004).

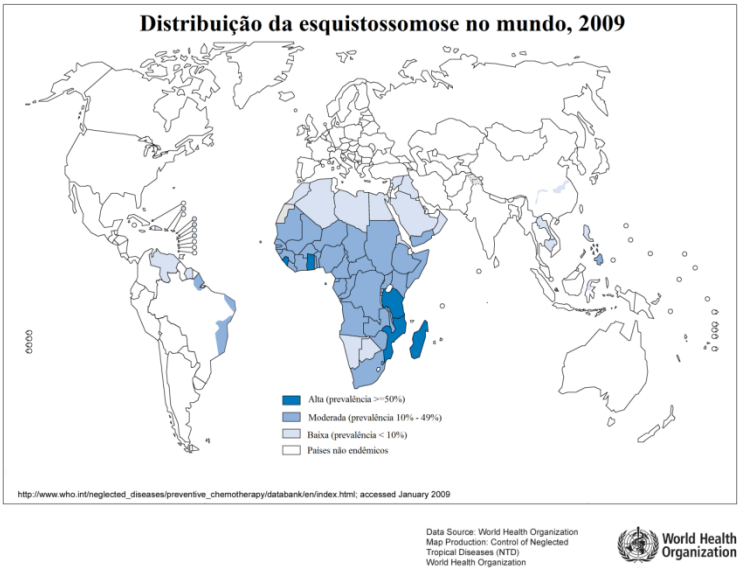


Figura 1 Distribuição da esquistossomose no mundo. Extraído: WHO, 2009

No Brasil, estima-se uma prevalência de 5,4% de indivíduos infectados pelo *Schistosoma mansoni* (Coura & Amaral, 2004), com cerca de 30 milhões vivendo em regiões onde há transmissão da doença atingindo quase todos os estados brasileiros, principalmente as regiões Nordeste, Sudeste e Centro-oeste (Katz & Peixoto, 2000; Oliveira et al., 2004). A transmissão contínua é observada nas regiões Sudeste e Nordeste, desde o Maranhão até o Espírito Santo e Minas Gerais, enquanto nas regiões norte e sul, existem apenas áreas de média extensão e focos isolados (Coura & Amaral, 2004) (Figura 2). Segundo Katz & Peixoto (2000), mais de 8 milhões de pessoas estão infectadas, enquanto outras 30 milhões estão expostas ao risco de infecção.

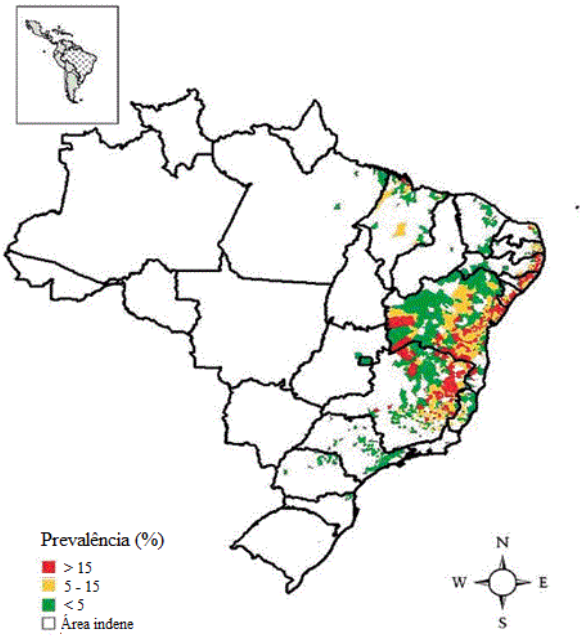


Figura 2 Áreas endêmicas da esquistossomose mansoni no Brasil. Extraído: Amaral RS, Taiuil, Lima DD, Engels D 2006. Memórias do Instituto Oswaldo Cruz 101 (Suppl. I): 79-85

O programa de controle da esquistossomose, implantado no Brasil entre 1976 e 1993, resultou em significativa redução da prevalência da doença e também da incidência de formas graves, no entanto, foi observado o surgimento de novos focos. Investigações mais aprofundadas são necessárias para que se possa verificar se a redução na prevalência da esquistossomose em áreas endêmicas representa realmente uma redução no número de indivíduos infectados. Ou, por outro lado, se simplesmente reflete uma diminuição na carga parasitária dos indivíduos das áreas tratadas, dificultando sua identificação pelos métodos diagnósticos disponíveis atualmente. Resultados recentes obtidos por nosso grupo (Enk et al., 2008) reforçam a

segunda hipótese ao sugerirem que a prevalência da esquistossomose no Brasil está sendo subestimada devido à dificuldade de se diagnosticar indivíduos com baixa carga parasitária.

1.2 Patologia da esquistossomose mansoni

A transmissão da doença para o homem ocorre pelo contato deste com águas onde existam moluscos infectados do gênero *Biomphalaria* que liberam cercárias em contato com a água. A cercária infecta o homem por penetração ativa na pele ou mucosa e, num longo percurso inicialmente extravascular e, depois, intravascular, o parasito perfaz vários ciclos na circulação sistêmica e mudanças marcantes ocorrem na sua morfologia (Lenzi et al., 2008). O parasito passa de cercária para esquistossômulo, verme imaturo ou jovem e verme adulto macho ou fêmea. Os esquistossômulos migram para os pulmões a partir do 4º dia após a penetração e, posteriormente, a partir do 8º dia, atingem o sistema porta hepático. O desenvolvimento dos vermes jovens completa-se nos vasos intra-hepáticos, onde se acumulam lentamente, havendo um evidente assincronismo no desenvolvimento dos vários exemplares, podendo ser encontrados vermes jovens ao lado de espécimes adultos durante vários dias entre o quarto e o 23º dias (Faust, Jones & Hoffman, 1934; Barbosa et al., 1978). Já com 168 horas, alguns exemplares jovens apresentam sangue ingerido, bem como aumento de seu volume corporal (Pinto & Almeida, 1948). Novas papilas sensoriais são formadas, enquanto a abertura da boca se desenvolve com a formação da ventosa oral (Crabtree & Wilson, 1980).

Após a maturação, aproximadamente 35 dias após a infecção, vermes adultos se alojam no plexo mesentérico onde podem permanecer por vários anos (Coelho, 1970). As fêmeas maduras passam a depositar ovos, aproximadamente 350 ovos por dia (Pellegrino & Coelho, 1978; Valadares et al., 1981), que ao saírem do hospedeiro vertebrado, através das fezes, liberam miracídios, que infectam o hospedeiro invertebrado. Grande parte dos ovos é eliminada junto às fezes. Contudo, ovos ainda ficam retidos na mucosa intestinal e nos capilares do sistema porta do hospedeiro, onde desencadeiam uma reação inflamatória granulomatosa. Esta reação, que se forma ao redor dos ovos, é a principal causa da patogenia da esquistossomose. A reação granulomatosa resulta em fibrose de tecidos que pode evoluir para a obstrução do plexo venoso podendo levar à hipertensão portal, hepatomegalia, esplenomegalia, aumento do volume abdominal representado por ascite e formação de varizes esofagianas e hemorroidais.

1.2.1 Patologia da fase aguda

A esquistossomose se apresenta no homem sob uma forma transitória chamada de aguda e três formas crônicas, a intestinal, a hepatoesplênica e a hepatointestinal. O quadro agudo reflete uma infecção de instalação recente, com forte resposta imunológica, geralmente vista em pacientes infectados pela primeira vez. Assim, tem sido visto mais comumente em indivíduos que visitam áreas endêmicas vindos de áreas endenes. Em moradores de áreas endêmicas raramente são verificados casos agudos, o que sugere que estes últimos podem ter desenvolvido regulação imunológica e tolerância a antígenos do parasito (Andrade, 2008). Estes indivíduos adquirem a doença após entrarem em contato com águas de riachos, lagoas, poços, piscinas ou outros locais contaminados por cercárias. Por outro lado, várias pessoas se infectam ao mesmo tempo durante excursões ou períodos de férias (Prata & Coura, 2008). O surgimento de vários casos da forma aguda num pequeno intervalo de tempo serve inclusive para denunciar focos de instalação recente de transmissão, numa população sem contato prévio com a parasitose (Andrade, 2008).

Durante a penetração da cercária na pele, ocorre uma reação inflamatória em dois estágios. No primeiro estágio, caracterizado por uma resposta imediata, com liberação de substâncias *histamina-like*, formam-se em poucos minutos manchas avermelhadas, por dilatação de arteríolas e capilares, e prurido, caracterizando-se por uma reação de hipersensibilidade imediata. O segundo estágio ocorre de 16 a 24 horas após a penetração das cercárias na pele, com o desenvolvimento de pápulas decorrente de uma hipersensibilidade tardia, que caracteriza a dermatite cercariana. Importante consideração foi feita por Neves (1965) de que a referida dermatite cercariana não é indício seguro da instalação da forma aguda e nem sua ausência afasta a possibilidade do diagnóstico desta.

Os principais sinais e sintomas agudos desta fase são febre, cefaléia, apatia, dores abdominais, diarréia, anorexia, tosse seca e eosinofilia (Neves, Martins & Tonelli, 1966). Algumas destas manifestações podem surgir alguns dias após a exposição cercariana ou, mais frequentemente, cerca de um mês depois, coincidindo com o começo da eliminação de ovos nas fezes (Van der Werf et al., 2003). Daí se dizer que a forma aguda pode ser pré ou pós-postural (Hyatt et al., 1979). Dados experimentais obtidos com camundongos infectados, sacrificados em sequência, apresentaram o fígado e o baço histologicamente sem nenhuma alteração até o momento em que os ovos maduros se fizeram presentes (Andrade & Azevedo, 1987; Pearce et al., 1991).

A sintomatologia da fase aguda raramente se apresenta como mencionado, podendo também ser discreta, apenas com febrícula, cefaléia, astenia e anorexia, ou mesmo assintomática. Em outros casos, com grande carga parasitária, a infecção pode se tornar invasiva, com a disseminação sistêmica dos ovos, principalmente nos pulmões, apresentando intensa reação inflamatória (forma toxêmica) pela formação de granulomas. A febre e os sintomas da fase aguda desaparecem paulatinamente, com a fase crônica se instalando após 4 meses de infecção. A quase totalidade dos pacientes de fase crônica não refere história de fase aguda (Prata & Coura, 2008).

1.2.2 Patologia da fase crônica

Apesar da importância da fase aguda da infecção, a esquistossomose é de fato uma doença crônica, onde cerca de 90% dos pacientes desenvolvem a forma leve, sendo muitas vezes assintomáticos, e de 4 a 10% apresentam formas graves (Andrade, 2008). A forma leve é comumente encontrada em residentes de áreas endêmicas, que geralmente possuem baixa carga parasitária (Cheever, 1968) e apresentam granulomas periovulares isolados no fígado em várias fases de evolução para a cicatrização. Além de aparecerem isoladamente, os granulomas na forma leve se formam nas mais finas ramificações terminais da veia porta, diferentemente da forma grave onde a deposição de ovos ocorre no espaço periportal (Andrade & Prata, 1963).

Já a forma grave hepatoesplênica é morfologicamente caracterizada pela fibrose hepática periportal descrita por Symmers (1904), que pode vir acompanhada de lesões destrutivas e obstrutivas da veia porta (Andrade, 2004). A fibrose portal é o resultado da deposição de numerosos ovos, com consequente reação inflamatória crônica granulomatosa e destruição vascular. A repercussão clínica se traduz nos sinais de hipertensão porta, com esplenomegalia e varizes de esôfago, o que atribui a esta forma a designação de hepatoesplênica, comumente associada a altas cargas parasitárias. A forma hepatoesplênica é considerada importante pela sua gravidade, embora o paciente possa sofrer regressão após o tratamento curativo da esquistossomose (Bina & Prata, 1983; Mohamed-Ali et al., 1991; Richter, 2003). O baço aumentado de volume é o outro componente morfológico destacado na forma hepatoesplênica. Este aumento resulta de uma combinação de proliferação dos seus elementos celulares, como consequência da hipertensão portal. O exame de elementos do sangue periférico revela leucopenia, anemia e plaquetopenia (Prata & Coura, 2008).

As manifestações clínicas da chamada forma hepatointestinal são imprecisas visto que muitos pacientes são assintomáticos. Cerca da metade apresenta episódios de diarréia ocasional e de curta duração, intercalados com períodos de obstipação. Outros pacientes referem dores abdominais, intolerâncias alimentares, sonolência pós-prandial, náusea, insônia, emagrecimento, mialgia e, mais raramente, impotência e sudorese. Tanto no intestino como no fígado pode haver lesões mais avançadas que levam ao aumento da espessura da parede intestinal, nódulos hepáticos e fibrose periportal (Andrade & Prata, 1963). Tais alterações acabam por serem idênticas às encontradas na forma hepatoesplênica, desta se diferenciando por ter baço impalpável ou palpável, mas sem atingir o rebordo costal (Prata & Coura, 2008).

O envolvimento do sistema nervoso central na infecção por *S. mansoni* pode ocorrer como resultado da migração de vermes adultos para tecidos próximos ao sistema nervoso, seguido da deposição pontual de ovos (Lambertucci, 2010). A mielorradiculopatia esquistossomótica é a forma ectópica mais grave da esquistossomose. Sua prevalência em centros médicos no Brasil e na África encontra-se em torno de 5%. Os sinais e sintomas iniciais incluem: dor lombar e/ou dor em membros inferiores, paraparesia, disfunções urinária e intestinal e impotência no homem (Lambertucci, Silva & Amaral, 2007).

As perspectivas de redução da prevalência da infecção esquistossomótica e de sua morbidade e mortalidade, assim como o bloqueio de sua expansão para áreas ainda não endêmicas dependem de ações dos programas de controle da doença nos seus principais níveis de atenção. O saneamento básico com o suprimento de água tratada e rede de esgoto com tratamento, a educação individual e comunitária, o tratamento precoce e a busca ativa e segura de casos, constituindo assim os quatro pilares de um programa completo de controle.

A busca ativa de casos, sobretudo considerada neste trabalho, é dificultada em função das plurais manifestações clínicas da esquistossomose mansoni que podem em muito se assemelhar a inúmeras outras doenças. Este fato dificulta o diagnóstico, retarda o tratamento e sua devida notificação. Por esta perspectiva, torna-se imprescindível que o diagnóstico diferencial seja feito para cada fase da infecção, uma vez que o diagnóstico de certeza só é estabelecido através de exames parasitológicos, segundo relatado pela Fundação Nacional de Saúde (FUNASA) (1998).

O diagnóstico de certeza permite o tratamento quimioterápico seguro dos pacientes infectados. A importância deste tratamento consiste em curar a doença, impedir a evolução

para as formas graves, e também minimizar a eliminação dos ovos do helminto de forma a prevenir sua transmissão (Centro de Vigilância Epidemiológica, 2007).

1.3 Características biológicas relevantes para uso em diagnóstico de esquistossômulos, vermes jovens, vermes adultos e ovos

1.3.1 Esquistossômulos

A cercária do *S. mansoni*, após penetração no hospedeiro definitivo, sofre alterações bioquímicas e morfológicas, especialmente devido a mudanças do ambiente. Além da perda da cauda, o trematódeo forma rapidamente uma camada de microvilosidade sobre todo o tegumento, modifica a respiração para anaeróbica, perde o glicocálice, e a membrana inicial trilaminar passa a ser heptalaminar (Stirewalt, 1974). Toda essa adaptação estrutural dá origem a um novo estágio denominado esquistossômulo.

Diversos autores procuraram estabelecer os fatores envolvidos na morfogênese do tegumento durante a transição de cercária para esquistossômulo (Gilbert et al., 1972; Stirewalt et al., 1974; Ramalho-Pinto et al., 1975; Haas, 1984), assim como fatores de migração dos esquistossômulos pela pele de seu hospedeiro (Grabe & Haas, 2004a). Este esclarecimento precede a aplicação de agentes do tegumento do parasito para fins vacinais e de diagnóstico.

Os esquistossômulos migram por vasos sanguíneos, passam pela pequena circulação e chegam aos pulmões, ao que parece, por volta de quatro a cinco dias após a infecção. Nesta fase, são mais finos, com cerca de 400 µm de comprimento e, portanto, mais estreitos do que os encontrados na pele. Possuem as extremidades cobertas por espículos e as principais mudanças no tegumento são a superfície pregueada e escavada, ao passo que a membrana externa continua heptalaminada (McLaren, 1980; Hockley & McLaren, 1973).

Os espécimes coletados no pulmão já apresentam o ceco preenchido por pigmento, resultante da digestão de hemoglobina (Hockley & McLaren, 1973). Os primeiros esquistossômulos chegam ao fígado em torno de uma semana após a infecção (Barbosa et al., 1978), quando novas mudanças fisiológicas e morfológicas ocorrem, especialmente no tubo digestivo. Clegg (1965) descreveu que a ingestão de hemácias começa nos espécimes com 15 dias quando eles são recuperados do fígado.

Componentes de superfície ou próximos da superfície de esquistossômulos podem ter um alto padrão imunogênico quando expostos, o que pode favorecer seu uso como agentes de diagnóstico, apesar de pouca informação disponibilizada sobre a identidade destes componentes. Sabe-se que o tegumento é delimitado, na sua superfície basal, por uma membrana plasmática (Hockley & McLaren, 1973) e que componentes protéicos permanecem expostos sendo reconhecidos pelo sistema imunológico do hospedeiro. Proteínas como a Sm22,6, pertencente ao grupo *Tegumental-Allergen-Like* (TAL), apresentam importantes epitopos para IgE (Fitzsimmons et al., 2012). Sua aplicação no diagnóstico, no entanto, é desestimulada por ser igualmente encontrada em espécies de *Fasciola*.

Com o avanço da proteômica, outros componentes protéicos do tegumento de esquistossômulos foram identificados como pertencentes a uma variedade de classes, incluindo transportadores de nutrientes, receptores, enzimas e outros sem função definida (Braschi et al., 2006a,b; Castro-Borges et al., 2011). Dentre elas, podemos citar algumas de maior relevância quanto a imunogenicidade, como fosfodiesterase SmNPP-5 (Bhardwaj et al., 2011), enzimas glicolíticas como gliceraldeído-3P-desidrogenase e triose-fosfato desidrogenase (Mansour et al., 2000), anexina 2 (Tararam et al., 2010), *Stomatin-like* 2 (SmStoLP-2) (Farias et al., 2010) e Sm20,8 (Mohamed et al., 1998) que são especialmente capazes de serem reconhecidas por agentes da resposta humoral. Ainda, a Sm-p80 que foi demonstrada como antígeno secretado/excretado por esquistossômulos de pulmão em métodos *ex vivo* e, igualmente, demonstrou ser reconhecida por IgG (El Ridi et al., 2009; Zhang et al., 2011).

O caráter imunogênico do tegumento do parasito não se limita somente às proteínas. Há muito se sabe que a reposta imune humoral na esquistossomose é prioritariamente direcionada contra glicoconjugados, localizados na superfície do parasito ou secretados para a circulação sanguínea (Nash et al., 1981; Aronstein et al., 1983; Omer Ali et al., 1988). Mais recentemente, muitos desses carboidratos foram identificados e corroboram com os primeiros achados de que estão diretamente envolvidos na resposta imunogênica (Omer-Ali et al., 1986; Cummings & Nyam, 1996; 1999; Eberl et al., 2001).

A marcação da superfície do esquistossômulo por auto-radiografia mostra, pelo menos, 12 glicoproteínas que não são identificadas em cercárias. Apesar do aumento no interesse sobre a biologia e a imunologia associadas às glicanas, elas ainda permanecem enigmáticas quanto a suas funções ou efeitos no hospedeiro. Publicações de três décadas têm reportado glicanas

inespecíficas do *Schistosoma* que induzem respostas imunes (adaptativa e inata) ou que são detectadas como circulantes no homem (Hokke et al., 2007). Conforme foi demonstrado por diversos estudos que utilizaram anticorpos específicos, muitas glicanas do *Schistosoma* apresentam mudanças em sua expressão durante a evolução de seu ciclo de vida enquanto outras permanecem iguais nos diferentes estágios (Van Remoortere et al., 2000; Robijn et al., 2005). Exemplo desta é a Gal(β1-4)[Fuc(α1-3)]GlcNAc ou *Lewis x* (Le^x), altamente expresso em glicoproteínas do ovo, cercária e vermes adultos (Wuhrer et al., 2006).

1.3.2 Vermes jovens e adultos

Após chegarem ao fígado, as formas imaturas rapidamente dão início a organogênese, que é bem caracterizada a partir do terceiro estágio, em 21 dias. A morfologia dos vermes foi estudada há quase cem anos no Brasil por Pirajá da Silva (1908). A superfície mais externa do tegumento que interage com o hospedeiro é formada por duas camadas lipídicas justapostas, enquanto a superfície basal interna é limitada por uma única membrana de camada dupla. A membrana externa ainda possui aspecto heptalaminar e, agora, apresenta também invaginações. O tegumento conecta-se às células subtegumentares por conexões citoplasmáticas ligadas por microtúbulos. A superfície dorsal é coberta por numerosos tubérculos com espinhos, enquanto a superfície entre os tubérculos é composta por estrias rasas e planas.

Assim como os esquistossômulos, vermes jovens e adultos também apresentam importantes proteínas e glicanas em sua superfície que assumem relação de reconhecimento pelo sistema imunológico. A Sm13 é uma das mais importantes proteínas do tegumento dos vermes, reconhecida por anticorpos, cuja confirmação foi feita por anticorpos purificados após imunização murina com proteínas do tegumento de vermes (Abath et al., 2000). Da mesma forma, a Sm60 é uma proteína de ligação à manose, com propriedades inflamatórias, presente não somente nos vermes, mas também em ovos (Coelho-Castelo et al., 2002) e, ainda, a Sm29, um antígeno de ligação com a membrana de vermes adultos fortemente relacionado com reconhecimento por IgG1 e IgG3 (Cardoso et al., 2008).

À semelhança de outros trematódeos, o sistema digestivo dos vermes adultos se inicia com a boca localizada no fundo da ventosa oral, seguida por um esôfago curto, que se bifurca a altura do acetábulo. A ventosa oral é, portanto, utilizada para a ingestão de alimentos e, de

maneira bastante importante, para a eliminação de materiais residuais do metabolismo e da própria alimentação (Hockley, 1973), por processo de regurgitação.

O tubo digestivo é o sítio dos principais antígenos circulantes produzidos pelos vermes. Em vermes marcados com anticorpos monoclonais específicos contra os antígenos circulantes se verificou intensa marcação no ceco ramificado, por microscopia confocal (Water et al., 1987; Borges et al., 1994).

Machos e, principalmente, fêmeas adultas, ingerem uma grande quantidade de hemácias dos vasos onde estão alojados. O sangue digerido é rapidamente hemolisado em seus intestinos: nas fêmeas adultas, cerca de 330 mil hemácias por hora, enquanto nos machos adultos cerca de 30 mil hemácias por hora (Lawrence, 1973). A hemolisina, presente no esôfago, lisa as células vermelhas, liberando a hemoglobina para o tubo digestivo, onde é catalisada em peptídeos ou aminoácidos livres, essenciais para o desenvolvimento, o crescimento e a reprodução dos parasitos. Estes peptídeos difundem-se para ou são incorporados nas células gastrointestinais (Bogitsh, 1989). Da hemoglobina então, a porção globina é utilizada, e o produto final da oxidação do grupo heme, caracterizado como hemozoína (Oliveira et al., 2000) é regurgitado. A regurgitação da hemozoína pelo parasito se dá em decorrência de movimentos peristálticos e acaba por induzir a excreção/secreção de antígenos isolados pertencentes ao próprio sistema digestivo do parasito.

Dentre os antígenos excretados/secretados pelo verme na corrente sanguínea do hospedeiro, destacam-se dois que foram mais extensamente estudados, o Antígeno Catódico Circulante (CCA) e o Antígeno Anódico Circulante (CAA), glicoproteínas carregadas eletronicamente em pH neutro. Os estudos mais detalhados feitos até hoje datam de 1980 a 1985 e se baseiam em análises de fluorescência. Estes estudos foram feitos com camundongos infectados por cercárias de *S. mansoni* que demonstraram a presença destes antígenos circulantes em macrófagos do fígado tão cedo quanto três semanas após a infecção, sendo ainda reconhecidos após sete semanas (Van Marck et al., 1980; El-dosoky et al., 1984; Deelder et al., 1985; Agnew et al., 1995), estando assim presentes em fase bastante inicial da infecção. Water et al. 1987 demonstraram ainda que da terceira a sétima semana de infecção, a quantidade de CCA e CAA aumenta gradativamente. Em testes *in vitro* os resultados foram ainda além, com CCA sendo excretado/secretado por esquistossômulos imediatamente no início da cultura. Comparativamente, fêmeas jovens e adultas produziram mais antígenos que machos, sendo a concentração de CCA superior a de CAA (Van Dam et al., 1996).

Apesar de não existir nenhuma descrição detalhada sobre a distribuição intracelular destes antígenos, já foi demonstrado que ambos são encontrados em grânulos ou corpos inclusos, similares morfologicamente a lisossomos, e em vesículas endocíticas nos macrófagos. Geralmente encontrados como imunocomplexos, possuem significativa imunogenicidade com forte capacidade de ligação com anticorpos IgM e IgG (Water et al., 1987). Esta característica atribui aos antígenos circulantes a capacidade de serem detectados em amostras sanguíneas de pacientes infectados em fase aguda e em fase crônica.

Deelder et al. (1985) sugerem ainda que ambos antígenos circulantes se depositam no glomérulo renal. O CCA, com seu peso molecular em torno de 30 kDa, pode ser facilmente detectado na urina de pacientes infectados pelo *S. mansoni* (De Clercq et al., 1997). Em amostras de urina de camundongos infectados obtidas em sete semanas, o CCA novamente se mostrou o antígeno mais predominante dentre os circulantes (Van Dam et al., 1996).

1.3.3 Ovos

A migração dos vermes da região hepática em direção aos vasos mesentéricos começa em quatro e cinco semanas de infecção (Clegg, 1965). Ainda hoje, não se tem completo conhecimento sobre os fatores determinantes dessa migração para o sítio de oviposição. No entanto, sabe-se bem que os ovos são os elementos de maior responsabilidade pela patologia do hospedeiro vertebrado. Ainda imaturos, são eliminados pelas fêmeas do parasito e podem permanecer vivos nos tecidos do hospedeiro, por um período de mais de 12 dias, após maturação que demanda 6 dias (Prata, 1957; Cançado et al., 1965). Aproximadamente 50% dos ovos liberados pelas fêmeas são carregados pela circulação portal e ficam nos tecidos do hospedeiro (Warren, 1978), induzindo a formação de granulomas hepáticos e intestinais.

O próprio ovo libera uma grande variedade de imunógenos, conhecidos como Antígenos Solúveis do Ovo (SEA) que desencadeiam respostas imunológicas (Lenzi et al., 1998; Loeffler et al., 2002). Prova disso é a imunização com SEA sendo capaz de induzir imunidade em camundongos, levando a ativação de células T CD8+, chegando a protegê-los parcialmente do desafio com cercárias de *S. mansoni* (El-Ahwany et al., 2012).

Muitos estudos estão atualmente em andamento na tentativa de identificar os antígenos mais imunogênicos presentes no SEA. Estes estudos são direcionados especialmente para a identificação de fatores de morbidade, componentes isolados para servirem de adjuvantes

vacinais, extratos protéicos para uso como agentes vacinais ou, ainda, antígenos a serem utilizados no diagnóstico da fase crônica da infecção. Exemplos são a ω-1 e a *IL-4 inducing principle* (IPSE/α-1), glicoproteínas secretadas por ovos viáveis e algumas das principais desencadeadoras da resposta imune (Zaccone et al., 2011). Adicionalmente, ovos são repletos de glicanas, como a GalNAcb1-4(Fuca1-2Fuca1-3)GlcNAcb1 (LDN-DF) contra a qual alto título de anticorpos é observado (Robijn et al., 2004), além do próprio Le^x já anteriormente descrito.

Há, portanto, uma grande diversidade de antígenos do *S. mansoni*, no que se atribui especial referência às proteínas ou glicoproteínas de tegumento, proteínas solúveis liberadas pelo parasito, glicoproteínas excretadas/secretadas em seus diferentes estágios de vida. Muitos componentes do parasito são atualmente conhecidos e estão sendo bem descritos pelo uso de novos métodos de identificação e classificação. Várias áreas de desenvolvimento, que até pouco tempo se encontravam deficientes, têm se beneficiado a cada novo achado.

Desta forma, torna-se passível de ser aprimorado o desenvolvimento de novas metodologias diagnósticas ou o aperfeiçoamento de métodos usuais. Principalmente, quando este diagnóstico pode e deve ser validado para aplicação em qualquer das fases da infecção, de maneira a prevenir precocemente a gravidade da patologia e sua cronicidade e permitir o tratamento eficaz e seguro, mesmo em casos difíceis de serem detectados.

1.4 Diagnóstico da esquistossomose mansoni

O diagnóstico preciso da esquistossomose consiste em um instrumento-chave para aspectos importantes da infecção, como determinantes epidemiológicos, fatores relacionados à morbidade, avaliações de intervenções terapêuticas e acompanhamento de medidas de controle.

Durante o século XX, muito se alcançou em termos de métodos diagnósticos para esquistossomose, embora tenham sido limitados os investimentos em métodos nesta área. Entretanto, ainda hoje, métodos ideais que associem elevada eficácia, baixo custo e simplicidade operacional, ainda não estão disponíveis (Rabello et al., 2008). A busca atual se volta para o desenvolvimento de metodologias que certamente contemplem estes requisitos, mas que, além disto, sejam eficientes para atender características peculiares de cada fase da

infecção, aguda ou crônica, em período pré-patente ou posterior a oviposição, mesmo em pacientes com baixa carga parasitária.

Quando em 1852, Theodor Bilharz, médico alemão que trabalhava no Egito, descreveu pela primeira vez a doença parasitária que mais tarde se chamaria esquistossomose, apresentou também a primeira contribuição às técnicas diagnósticas através de desenhos esquemáticos do ovo espiculado. Desde então, a utilização de técnicas de diagnóstico tem acompanhado passo a passo o desenvolvimento tecnológico científico mundial (Rabello et al., 2008). Hoje, o desenvolvimento de novas abordagens é possível não somente devido ao crescimento biotecnológico, mas, principalmente, ao melhor entendimento da resposta imunológica e da interação entre o parasito e o hospedeiro.

Os métodos de diagnóstico disponíveis hoje podem ser agrupados em categorias distintas. Inicialmente, podem ser divididos em duas categorias: métodos de detecção direta e métodos de detecção indireta. São considerados diretos os métodos que detectam o parasito, ou componentes deste, como ovos, antígenos, moléculas ou fragmentos destas. Os métodos indiretos identificam evidências indiretas da presença do parasito e dependem de marcadores clínicos, bioquímicos ou, especialmente, imunológicos associados à infecção.

Uma segunda divisão permite classificar os métodos como qualitativos ou quantitativos. Métodos qualitativos são comumente mais fáceis e rápidos de serem feitos, porém não geram projeções sobre a dinâmica de uma infecção, informando somente a presença do parasito. Já métodos quantitativos refletem a carga parasitária e/ou a resposta imunológica de um indivíduo ou grupo populacional e permite o estabelecimento de indicadores epidemiológicos em programas de controle.

Um exemplo desta importância é a avaliação dos resultados alcançados com as medidas de controle implementadas em determinada região endêmica que podem se mostrar reduzidos se baseados exclusivamente na determinação de prevalência, mas podem revelar importantes repercussões ocorridas na intensidade da infecção na população tratada (WHO, 1994). Ao considerar indicadores de intensidade de infecção e morbidade, métodos quantitativos eficientes permitem a definição de estratégia de tratamento e controle da área ou foco endêmico. Igualmente, podem servir como indicadores de cura para a verificação do impacto da terapêutica, quando de detecção direta, permitindo avaliar se a eficácia de uma droga foi parcial ou absoluta (Rabello et al., 2008).

1.4.1 Métodos parasitológicos

Inúmeras técnicas parasitológicas encontram-se descritas na literatura científica, muitas das quais já estão em desuso por não serem consideradas adequadas e apresentarem baixa sensibilidade. São exemplos destas, exames parasitológicos utilizados no passado com o uso de lâminas sem qualquer preparação (Martins, 1937), técnicas de flutuação (Willis, 1921; Faust et al., 1939) e métodos de centrifugação (Faust, Ingalls & See, 1946; Sapero & Lawless, 1953).

Já técnicas recentes, como o método de eclosão de miracídios e de gradiente salínico estão sendo validados. Por outro lado, a técnica de sedimentação espontânea das fezes (HPJ) (Hoffmann, Pons & Janer, 1934), de centrifugação com formol-acetato de etil (TF-Test®) modificado (Gomes et al., 2004; Siqueira et al., 2011) e o método de Kato-Katz (Katz et al., 1972) são amplamente utilizados, pela simplicidade de execução e por apresentaram considerável índice de sensibilidade para pacientes com alta carga parasitária.

O método de eclosão de miracídios é baseado no forte comportamento fototrópico dos miracídios. Amostras fecais são colocadas em frasco próprio que é completado com água desclorada até o orifício localizado no ápice do funil. O frasco permanece dentro de uma caixa de madeira permitindo que somente o ápice do funil fique exposto a luz. Desta forma, os miracídios são atraídos pela luz artificial, quando a partir daí, podem ser coletados e contados. Testes com 1,5 g de fezes para determinação de sua eficiência demonstraram que a sensibilidade de um único teste é equivalente a 36 lâminas de Kato-Katz (Jurberg et al., 2008). Ensaios em trabalhos de campo mostraram dificuldades logísticas em seu uso.

O método de gradiente salínico foi criado com o objetivo de desenvolver uma ferramenta simples e sensível para o diagnóstico da esquistossomose no campo. Baseia-se em um método de baixo custo, de execução fácil e rápida e que traz como grande vantagem a redução no número final de análises ao microscópio. O fluxo de solução salina a 3% provoca a suspensão e a retirada de sedimentos de baixa densidade da amostra fecal que está diluída em solução salina a 0,9%. Os ovos de *S. mansoni* possuem maior densidade e permanecem na superfície de uma placa porosa, quando são analisados ao microscópio ótico. Resultados obtidos em laboratório demonstraram que o método de gradiente salínico apresenta maior sensibilidade do que 12 lâminas de Kato-Katz de uma única amostra fecal de 500 mg (Coelho et al., 2009).

Esta técnica está sendo validada em condições de campo e tem demonstrado resultados promissores.

O método de sedimentação foi descrito por Lutz (1919) e padronizado por Hoffmann, Pons & Janer (1934), tornando-se bastante conhecido e utilizado. Neste procedimento, as fezes são homogeneizadas em água e filtradas em tela metálica ou gaze cirúrgica, para retenção de resíduos fecais de maiores dimensões. Após isso, são deixadas em sedimentação espontânea. Este é o método qualitativo mais difundido entre os laboratórios de análises clínicas, por ser de fácil execução e de baixo custo, não exigir aparelhagem especial e permitir o diagnóstico simultâneo de outras parasitoses (Rabello et al., 2008).

O TF-Test® foi primeiramente feito para servir como método qualitativo (Immunoassay Ind & Com SA, Brasil). Modificações foram propostas por Gomes et al. (2004) e Siqueira et al. (2011) de forma a aumentar a sensibilidade e permitir a padronização de método quantitativo a partir da metodologia inicial. Amostras fecais são filtradas e transferidas para solução de formol e acetato de etil e o material é centrifugado. O sedimento é analisado em microscópio ótico. O TF-Test® modificado demonstrou resultados similares aos do método de Kato-Katz quando amostras de pacientes com alta carga parasitária foram analisadas. No entanto, para amostras de pacientes de baixa carga, os resultados foram discordantes quanto a sua sensibilidade. O TF-Test® modificado foi superior ao Kato-Katz para Gomes et al. (2004), mas Siqueira et al. (2011) mostraram o inverso.

Já a técnica de Kato-Katz compreende os requisitos necessários de um bom método diagnóstico o que faz com que seja considerado o método de escolha pela Organização Mundial de Saúde (1994). Vantagens como simplicidade de execução, baixo custo, possibilidade de armazenamento e transporte de lâminas em temperatura ambiente por meses, sem prejuízo dos resultados, fizeram deste método o mais utilizado em estudos epidemiológicos realizados em diversos países há 40 anos. Em 1972, Katz, Chaves e Pellegrino modificaram o método descrito por Kato & Miura (1954), simplificando a realização da técnica quantitativa ao substituir a pesagem prévia da amostra fecal por uma pequena placa com orifício de 6 mm de diâmetro em seu centro que permitia a medição da quantidade para análise (42,7 $\pm$ 2,0 mg). Assim sendo, após determinação da quantidade de fezes, a contagem de ovos é feita em microscópio ótico, utilizando-se lamínula de celofane tratada com glicerina, água e verde malaquita. Admitindo-se que toda lâmina tem 45 mg de

fezes, multiplica-se o número de ovos por 24 para se calcular o número de ovos em 1 g de fezes.

Fatores limitantes da técnica de Kato-Katz foram descritos por diversos autores. É impossível realizar o teste com amostras diarréicas, apesar de ser este um quadro comum na infecção por *S. mansoni*, possui baixa reprodutibilidade em diferentes lâminas do mesmo indivíduo e leituras desiguais são obtidas por examinadores diferentes (Kongs et al., 2001; Berhe et al., 2004; Gentile et al., 2011). Outra limitação é a necessidade de diferentes amostras do mesmo paciente para que um resultado mais sensível seja alcançado, através da análise de múltiplas lâminas. Para ensaios epidemiológicos, exames repetidos se tornam pouco práticos e economicamente inviáveis. E, ainda, em áreas onde a prevalência é baixa, menor que 10%, ou no diagnóstico de indivíduos com baixa carga, se faz necessário o uso de metodologias complementares para que o nível de sensibilidade desse diagnóstico seja significativamente próximo da real prevalência (Barreto et al., 1990; Engels et al., 1996; Kongs et al., 2001; Enk et al., 2008; Siqueira et al., 2011).

1.4.2 Métodos moleculares

A reação em cadeia da polimerase (PCR) é, atualmente, uma das técnicas mais destacadas da ciência, após seu surgimento em 1985 (Saiki et al.), quando rapidamente se tornou uma nova ferramenta a ser considerada para o diagnóstico de doenças infecciosas. O método permite a amplificação de sequências de DNA ou RNA do agente infeccioso, copiando-as em larga escala de maneira bastante específica. A sua importância se faz sentir principalmente em infecções leves, nas quais um método extremamente sensível é necessário para o diagnóstico preciso.

Alguns métodos de PCR foram descritos como ferramentas diagnósticas para a esquistossomose mansoni (Pontes, Dias Neto & Rabello, 2002; Pontes et al., 2003; Gomes et al., 2004; Oliveira et al., 2010). Estes métodos se baseiam em sequências de DNA abundantes no material genético do *S. mansoni*. Todos se mostraram eficientes na identificação dos casos positivos, mesmo em pacientes de baixa carga parasitária, e no correto diagnóstico dos casos negativos. Foram relatados pelos autores níveis de sensibilidade próximos a 90% e níveis de especificidade próximos a 100%.

Hussein et al. (2012) identificaram, ainda, DNA do parasito no soro de camundongos com apenas 3 dias de infecção, demonstrando que a sensibilidade da PCR pode ser também aplicada para o diagnóstico precoce na fase pré-patente da infecção.

Uma modificação da PCR, denominada PCR-ELISA, desenvolvida pela primeira vez por Musiani et al. (2007), foi proposta para a detecção de DNA do parasito em amostras fecais como um alternativa ao diagnóstico de pacientes com baixa carga (Gomes et al., 2010). O sistema permite a amplificação de uma região específica do DNA em uma primeira etapa e a aplicação do produto da PCR em sistema baseado na coloração enzimática por peroxidase como etapa final. Gomes et al. (2010) mostraram que 30% de uma população endêmica no Brasil foi encontrada positiva para a infecção com a PCR-ELISA, em comparação com os 18% determinados por exame parasitológico, comprovando a eficiência desse método, apesar da complexidade de execução.

Oliveira et al. (2010) desenvolveram e validaram uma técnica de PCR, especialmente destinada a pacientes de baixa carga parasitária provenientes de áreas endêmicas. A técnica molecular foi capaz de identificar 91% destes pacientes, tendo sido ineficaz somente para três pacientes com carga inferior a 10 ovos por grama de fezes. A especificidade encontrada foi de 100%.

A PCR em tempo real desenvolvida por Gentile et al. (2011) demonstrou ser superior no diagnóstico experimental da esquistossomose mansoni em comparação com métodos parasitológicos e imunológicos. No entanto, demonstrou desempenho diagnóstico inferior que métodos imunológicos na detecção de infecção recente.

Desvantagens associadas ao uso da PCR são semelhantes às encontradas em relação ao diagnóstico molecular de outras doenças. Tecnicamente, o maior problema é a possibilidade de contaminação das amostras analisadas, o que pode ser minimizado pela utilização de ambientes, equipamentos e reagentes exclusivos para a realização de cada etapa envolvida no processo. Além disso, a ocorrência da contaminação deve ser monitorada pela inclusão de controles negativos (não contendo DNA extraído) por cada reação de amplificação (Rabello et al., 2008).

A sofisticação e as grandes exigências infra-estruturais da tecnologia da PCR limitam, hoje, sua utilização em algumas situações específicas, como o diagnóstico no campo e/ou o

acompanhamento em massa de uma população. Certamente, à medida que o processo tecnológico caminha, tais limites serão progressivamente diminuídos. Mesmo não sendo o método diagnóstico de escolha, a PCR já se faz importante em análises individuais e na validação de novas metodologias onde é necessário um procedimento confirmatório adicional de alto desempenho.

1.4.3 Métodos imunológicos

Existe uma ampla variedade de métodos imunológicos descritos, entretanto eles têm demonstrado valor limitado no diagnóstico da esquistossomose. A maior parte dos métodos imunológicos descritos se aplica à detecção de anticorpos, sendo, portanto indiretos e a sua positividade não define a presença de infecção esquistossomótica ativa, indicando somente a resposta do sistema imune do hospedeiro a determinados antígenos do parasito. Além disso, comumente apresentam reações cruzadas com outras helmintoses e podem permanecer positivos durante anos após a cura quimioterápica (Smithers & Doenhoff, 1982; Mott & Dixon, 1982; Montenegro, 1992; Rabello, 1997; Rabello et al., 2008).

Apesar dessas limitações, no entanto, vários autores (Coelho e Tavares, 1991; Rey, 2001; Da Frota et al., 2011) salientam que métodos imunológicos de detecção indireta são justificados em situações em que estão sendo estudadas áreas endêmicas de baixa intensidade de infecção, onde também é baixa a eficiência dos métodos parasitológicos. Sendo assim, há um importante direcionamento das técnicas indiretas como métodos auxiliares em levantamentos epidemiológicos (Berghist, 1992).

A elevada sensibilidade que pode ser atingida por estas técnicas estimula sua utilização não somente para o diagnóstico de indivíduos de áreas endêmicas, mas em especial turistas que retornam infectados para suas cidades (Doenhoff, Chiodini & Hamilton, 2004). Atualmente, a técnica mais utilizada é o método imunoenzimático *Enzyme-linked Immunosorbent Assay* (ELISA), que foi introduzido em 1971 por um grupo sueco (Engvall, Jonsson &Perlmann, 1971) e outro holandês (Van Weemen & Schuurs, 1971).

Uma das dificuldades no desenvolvimento destes testes é a escolha dos antígenos apropriados. Existem diversas dificuldades que influenciam a escolha de um antígeno ideal como: produtividade e facilidade de obtenção, elevada estabilidade em condições simples de estocagem e capacidade antigênica (Rabello et al., 2008).

Os antígenos podem ser obtidos de diversos estágios evolutivos do parasito. Os mais utilizados são os extratos brutos, preparados mediante ruptura de vermes, cercárias ou ovos. O antígeno solúvel de vermes adultos (SWAP) é a fonte mais fácil e abundante de material antigênico (Doenhoff, Chiodini & Hamilton, 2004). Antígenos de cercárias são menos frequentemente empregados devido a sua baixa sensibilidade e especificidade (Lunde & Ottensen, 1980). O homogeneizado de ovos, conhecido como SEA, contém igualmente grande número de frações antigênicas, apesar de somente uma minoria desses constituintes ser liberada por ovos viáveis, como demonstrado por Ashton et al. (2001). Sendo assim, os extratos brutos apresentam a grande vantagem de serem facilmente preparados, entretanto a utilização de preparações purificadas é uma possibilidade cada vez mais considerada.

A ocorrência de reações cruzadas é um dos grandes problemas da pesquisa de anticorpos e é especialmente observada com a utilização de extratos antigênicos brutos que possuem frações antigênicas compartilhadas por diversos parasitos, protozoários e até bactérias. Por esta razão, as pesquisas se tornam cada vez mais refinadas na utilização de antígenos individuais purificados que induzam a formação de ligações antígeno-anticorpo mais específicas. São exemplos de antígenos purificados já estudados o *Major Serological Antigen* (MSA) (Stek et al., 1983), o antígeno CEF6 que envolve frações antigênicas de ovos (Doenhoff et al., 2003; Turner et al., 2004), os antígenos *Adult Microsomal Antigen* (Mama) (Hancock & Tsang, 1986; Torres et al., 2001) e um antígeno larval de 37 kDa que demonstrou ser um bom marcador de susceptibilidade (Wu, 2002).

Sejam extratos brutos ou antígenos purificados, a utilização de técnicas indiretas leva indiscutivelmente à análise detalhada dos níveis de anticorpos como marcadores de imunidade assim como fornecem importantes dados em estudos soro-epidemiológicos que posteriormente servirão como base para estudos vacinais (Deelder, 1992).

Os ensaios imunológicos mais promissores são, no entanto, os métodos considerados diretos por detectar antígenos do parasito ou moléculas de ácidos nucléicos, discutidos anteriormente, em amostras de soro ou urina. Os antígenos excretados/secretados pelo *S. mansoni* na circulação do hospedeiro estão presentes exclusivamente em infecções ativas, e os níveis detectados podem ser correlacionados com a intensidade da infecção. Foi por meio da detecção de antígenos circulantes do parasito depositados nos tecidos de múmias egípcias, que hoje sabemos que a humanidade convive com a esquistossomose mansoni desde 3000 anos

A.C. (Miller et al., 1972). Adicionalmente, este achado demonstrou a grande estabilidade dos antígenos circulantes.

A grande maioria da pesquisa de antígenos circulantes é direcionada para dois antígenos principais como já descrito, o CCA e o CAA. Os métodos para detectá-los envolvem a captura dos mesmos através de técnicas como ELISA, utilizando-se de amostras sorológicas (Deelder et al., 1989; Polman et al., 1998; Al-Sherbiny et al., 1999) e, mais recentemente, o *dipstick* que usa um anticorpo monoclonal específico para uma das porções glicídicas do CCA para promover a detecção do antígeno em urina (Vam Dam et al., 2004).

Os primeiros métodos para detecção de CCA foram baseados na técnica de ELISA, apresentando bons resultados quanto à sensibilidade de detecção em amostras murinas. No entanto, logo demonstraram níveis muito baixos de sensibilidade quando passou a ser padronizado com amostras humanas, o que se justificou pela baixa quantidade de antígenos circulantes considerando a biomassa do ser humano. Este fato poderia ser revertido ao se utilizar um maior volume de soro humano na realização do teste. Contrariamente a isto, os autores trabalharam com amostras diluídas em até 100 vezes (Deelder et al. 1989; Polman et al. 1998, Al-Sherbiny et al., 1999).

Já o uso do *dipstick* demonstrou uma grande oscilação nos níveis de sensibilidade em estudos realizados em áreas endêmicas africanas, atingindo 56%, 89% e 94% de sensibilidade, respectivamente (Ashton et al., 2011; Coulibaly et al., 2011; Shane et al., 2011). Adicionalmente, por utilizar anticorpos monoclonais específicos para uma das porções glicídicas do CCA que, como já descrito, divide epitopos com diversos outros microrganismos e células humanas, o método vem apresentando um número significativo de resultados falso-positivos, em muito relacionado com reações cruzadas, levando a níveis baixos de especificidade (43% e 59%, respectivamente) (Legesse & Erko, 2008; Shane et al., 2011).

Erros conceituais cometidos na padronização dos métodos convencionais de detecção direta não justificam o fim dos estudos de desenvolvimento de metodologias mais apropriadas para a determinação de níveis de infecção por detecção de antígenos circulantes. Vantagens diversas estão vinculadas a esta prática, visto que a presença dos antígenos se correlaciona diretamente com o número de vermes vivos no hospedeiro (Deelder et al., 1976; 1994; Polman et al., 1998), sua detecção está relacionada com infecção ativa, podendo ainda ser

utilizada em infecções recentes, devido a presençca de vermes jovens nesta fase, e para o controle de cura pós-quimioterapia. Finalmente, sua especificidade se torna alta se padronizada com anticorpos monoclonais direcionados para epitopos não compartilhados com outros parasitos.

Para o controle da esquistossomose em áreas endêmicas ou em pequenos grupos populacionais, a prevalência da infecção, o tamanho populacional, a disponibilidade de infra-estrutura e recursos humanos e financeiros pesam tanto na escolha do método como suas características intrínsecas, como sensibilidade e especificidade. Assim, métodos simples podem se mostrar suficientes para o controle da morbidade, especialmente importante em áreas de elevada prevalência, mas também necessário em áreas de baixa prevalência. A utilização de um exame mais sensível representa um meio eficaz de diagnosticar indivíduos com baixas cargas parasitárias que são de difícil detecção através dos métodos atualmente disponíveis. Indivíduos estes que não estão sendo diagnosticados e, logo, não serão tratados e continuarão a manter o processo de transmissão.

2 OBJETIVOS

2 OBJETIVOS

2.1 Objetivo geral

Desenvolver e avaliar o desempenho de novas alternativas para o diagnóstico sorológico da esquistossomose mansoni, em fase aguda e crônica da infecção.

2.2 Objetivos específicos

Padronizar técnicas de ELISA para o diagnóstico diferencial de fase crônica, utilizando antígenos de vermes adultos e antígenos de ovos de *S. mansoni* com uso de camundongos experimentalmente infectados

Padronizar técnica de ELISA com antígenos de tegumento de esquistossômulos de *S. mansoni* para o diagnóstico precoce, em fase pré-patente, de camundongos experimentalmente infectados

Avaliar o desempenho das técnicas padronizadas de ELISA com antígenos de vermes adultos e antígenos de ovos para o diagnóstico de fase crônica de pacientes de áreas endêmicas com baixa carga parasiotária

Avaliar o desempenho da técnica de ELISA com antígenos de esquistossômulos para o diagnóstico de fase aguda de indivíduos não residentes de áreas endêmicas, recentemente infectados em foco endêmico da esquistossomose mansoni

Obter diferentes formas do antígeno circulante CCA, incluindo a glicoproteína purificada, a proteína recombinante e peptídeos individuais, para uso na padronização de métodos diagnósticos

Desenvolver e avaliar o desempenho de novos métodos para o diagnóstico indireto de pacientesde áreas endêmicas com baixa carga parasitária por metodologia quantitativa, denominada IMS pelo uso das diferentes formas de CCA

Produzir anticorpos monoclonais específicos para a glicoproteína CCA purificada através de metodologia *in vitro*

Desenvolver e avaliar o desempenho do método quantitativo IMS e do método qualitativo FluoIMS para detecção direta de CCA em amostras sorológicas de pacientes de áreas endêmicas com baixa carga parasitária, antes e após o tratamento quimioterápico.

3 MATERIAIS E MÉTODOS

3 MATERIAIS E MÉTODOS

3.1 Autorização dos Comitês de Ética para manuseio de animais e amostras humanas

Este projeto foi aprovado pela Comissão de Ética no Uso de Animais da Fundação Oswaldo Cruz (CEUA L-023/08) para manuseio dos animais experimentais em todas as metodologias aplicadas. Este projeto foi igualmente aprovado para utilização de amostras humanas pelo Comitê de Ética em Pesquisa com Seres Humanos (CEPSH/03/2008) e pelo Comitê de Ética Nacional (784/2008, CONEP 14886) para utilização das amostras obtidas na região de Pedra Preta, Minas Gerais. Este projeto foi, ainda, aprovado pelo Comitê de Ética da FUNASA, em Minas Gerais, para utilização das amostras obtidas na região de São João Del Rei. Os objetivos do estudo foram apresentados e explicados a todos os participantes, e os termos de consentimento foram individualmente assinados e armazenados.

3.2 Amostras sorológicas murinas

Camundongos *swiss* fêmeas (4 a 6 semanas) foram mantidos no Biotério de Experimentação do Centro de Pesquisas René Rachou (CPqRR). Cada animal foi exposto a 40 cercárias (desvio padrão de $\pm$ 10) de *S. mansoni* (cepa LE) por via subcutânea (Peters & Warren, 1969). Estas cercárias foram obtidas no Laboratório de Malacologia do CPqRR. Amostras sanguíneas foram coletadas pelo plexo retro-orbital em 30, 60 e 140 dias após a infecção, quando os animais foram sacrificados por deslocamento cervical e submetido à perfusão do sistema porta-hepático com solução salina 0,85% e 50 U/L heparina (Pellegrino & Siqueira, 1986). As amostras de sangue foram centrifugadas a 3000 g por 5 minutos e armazenadas a -20ºC. Vermes adultos obtidos da perfusão foram contados em lupa (Zeiss Stemi DV4). Camundongos sem infecção foram também selecionados para coleta sanguínea de forma a serem usados como controle negativo de infecção.

3.3 Estudos em áreas e focos endêmicos para esquistossomose mansoni

3.3.1 Área endêmica de baixa prevalência: Pedra Preta, Minas Gerais

Este estudo foi realizado nas comunidades de Buriti Seco e Morro Grande em Pedra Preta, um pequeno município, localizado na região rural de Montes Claros, no estado de Minas Gerais, onde a esquistossomose mansoni é endêmica. As amostras coletadas para este estudo fizeram parte de um projeto mais abrangente realizado pela equipe do Laboratório de

Esquistossomose do CPqRR (Siqueira et al., 2011). Esta área endêmica foi selecionada por conter uma população com baixo índice de migração e por nunca ter sido submetida ao tratamento quimioterápico da esquistossomose. Adicionalmente, baseado nos dados fornecidos pelo Centro de Controle de Zoonosis de Montes Claros, o índice de prevalência de 12% foi registrado em 2005. No total, 201 indivíduos participaram do estudo (93 mulheres/108 homens) e estes indivíduos continham entre 1 e 88 anos de idade.

Para a obtenção de amostras fecais, quatro amostras de cada indivíduo foram coletadas em quatro dias consecutivos, com tubos coletores plásticos de 100 ml. Cada amostra foi devidamente identificada pelo nome e número de cada participante e, ainda, pelo número de suas residências. Dezoito lâminas, contendo 45 mg cada, foram analisadas para a presença de ovos de *S. mansoni* e outros helmintos pela técnica de Kato-Katz (Katz et al., 1972), preparadas da seguinte maneira: 12 lâminas da primeira amostra e duas lâminas da segunda, terceira e quarta amostras, em um total de 750 mg de amostra analisada. Estas mesmas amostras foram também submetidas ao TF-Test® quantitativo (Siqueira et al., 2011). Para este teste, as amostras foram filtradas em malha de nylon e quantificadas em placas de metal. Cada porção de 500 mg foi transferida para tubo contendo solução preservativa de formol a 10% e acetato de etil e centrifugada a 500 g por 2 minutos. O sedimento foi ressuspendido em solução salina 0,85% para análise no microscópio ótico. A intensidade da infecção foi expressa em opg, usando a média aritmética da contagem dos ovos obtidos das 18 lâminas analisadas multiplicadas por 24. Amostras de sangue foram coletadas no primeiro dia de coleta fecal e centrifugadas a 3000 g por 5 minutos para obtenção do soro, que foi armazenado a - 20^{o}C.

Todos os participantes que apresentaram ovos de *S. mansoni* nas fezes foram tratados com praziquantel em dose única de 50 mg/kg quando adultos e de 60 mg/kg quando crianças. Infecções por outros helmintos foram tratadas com dose única de 400 mg de albendazol, como recomendado pelo Ministério da Saúde. Os pacientes positivos foram ressubmetidos ao exame parasitológico em 30 e 90 dias após a quimioterapia com praziquantel. Pacientes com resultados positivos foram novamente submetidos ao tratamento.

3.3.2 Foco endêmico com turistas: Colônia do Teodoro

Oitenta indivíduos fizeram parte de um grupo hospedado em um sítio localizado em Colônia do Teodoro, próximo a cidade de São João Del Rei, estado de Minas Gerais. O grupo esteve

frequentando o sítio durante o período de Dezembro de 2009 a Março de 2010 e os indivíduos tiveram contato com uma piscina de água natural que continha, como posteriormente comprovado, exemplares do hospedeiro invertebrado *Biomphalaria glabrata*. A identificação da área foi feita pela Fundação Nacional de Saúde em Março de 2010. Para verificar os casos sintomáticos, entrevistas com cada indivíduo, identificação de sinais e sintomas relacionados com a esquistossomose aguda e exames clínicos foram feitos. Desta forma, a infecção por *S. mansoni* foi definida para indivíduos que tiveram contato com a água contaminada e, adicionalmente, apresentaram um dos seguintes critérios: presença de ovos em amostras fecais, título de IgG detectado por ELISA sensibilizada com SWAP (ELISA-SWAP), mieloradiculopatia detectada por ultrasonografia, e/ou sintomas compatíveis com a esquistossomose aguda. Os sintomas que definiram a infecção foram febre, tosse, dermatite cercariana e angioedema. Sintomas não específicos, como fadiga, alterações gastrointestinais e cefaléia, foram também reportados.

Amostras de sangue foram coletadas de todos os pacientes por punção venosa, centrifugadas a 3000 g por 5 minutos e armazenadas a - 20^{o}C. Vinte e quatro indivíduos concordaram em doar amostras fecais e estas amostras foram analisadas pelo método de Kato-Katz (Katz et al., 1972), sendo feitas três lâminas de cada amostra. Cinco pacientes foram hospitalizados com formas graves da infecção, onde o primeiro desenvolveu uma infecção pulmonar grave, o segundo com mieloradiculopatia esquistossomótica e, três com desidratação grave causada pela forma hepatointestinal.

Todos os pacientes positivos foram igualmente tratados seguindo a recomendação do Ministério da Saúde.

3.4 Doadores voluntários de amostras sorológicas para grupo controle

Cinquenta e três voluntários (35 mulheres/18 homens) com idades entre 22 e 65 anos foram doadores de amostras de sangue para que compusessem o grupo de controle negativo da infecção. Nenhum dos voluntários era residente ou visitante de áreas endêmicas ou apresentavam qualquer histórico médico relacionado com uma infecção prévia de esquistossomose. As amostras sorológicas foram obtidas e armazenadas da mesma forma descrita no item 3.3.1. Além da seleção dos doadores com base no histórico individual, cada um foi submetido a duas análises confirmatórias realizadas por ELISA-SWAP e ELISA sensibilizada com SEA (ELISA-SEA) para detecção de possíveis títulos de IgG para os

antígenos de *S. mansoni* utilizados. Pacientes reativos para os ensaios de ELISA foram removidos do grupo controle.

3.5 Preparação de antígenos de *S. mansoni*

3.5.1 Antígeno solúvel de vermes adultos

Após 45 dias da infecção de camundongos *swiss* com 100 cercárias (desvio padrão $\pm$ 10), os animais foram submetidos à perfusão do sistema porta-hepático para obtenção dos vermes adultos, como já descrito no item 3.2. Os vermes adultos obtidos foram lavados três vezes com tampão fosfato 0,15M pH 7,2 (PBS), submetidos a trituração por 15 minutos (Virtiz Precisa), e centrifugados a 9500 g por 1 hora a 4ºC (Eppendorf AG). Foi realizada a diálise do sobrenadante obtido em membrana de celulose (Sigma-Aldrich) contra solução salina 0,9% por 48 horas a 4ºC. O extrato antigênico foi centrifugado a 1250 g por 15 minutos a 4ºC e o sobrenadante foi separado em alíquotas e estocado a - 20ºC. Uma alíquota foi submetida à dosagem protéica pelo método de Bradford (Bradford, 1976).

3.5.2 Antígeno solúvel de ovos

Após realizar a perfusão do sistema porta-hepático, o fígado de cada camundongo infectado por 100 cercárias foi obtido e a obtenção dos ovos foi feita segundo protocolo de Carter & Colley (1978) com modificações. Os fígados foram incubados *overnight* a 4ºC e, em seguida, a 37ºC por 2 horas, quando foram macerados em liquidificador. O extrato obtido foi filtrado em quatro tamises de porosidade gradual (425 µm, 180 µm, 106 µm e 45 µm). Os ovos retidos no tamis de menor porosidade foram triturados (Virtiz Precisa) por 40 minutos. O extrato obtido foi centrifugado a 21000 g por 1 hora a 4ºC e filtrado em filtro de 0,45 µm. Ao fim do processo, foi feita a diálise de 48 horas contra solução salina a 0,9% e a dosagem protéica por método de Bradford (1976).

3.5.3 Antígeno de tegumento de esquistossômulos (SmTeg)

Cercárias foram mecanicamente transformadas em esquistossômulos pela técnica de Ramalho-Pinto et al. (1974), com algumas modificações. Após serem acondicionadas em tubo cônico e deixadas em banho de gelo por 30 minutos, foram centrifugadas a 200 g por 3 minutos a 4ºC (Eppendorf 5820R). O sedimento foi ressuspendido em meio *Earl's salts plus lactalbumin hydrolyzate* (ELAC) gelado. As cercárias tiveram as caudas separadas em vórtex

(Scientific Industries Genie-2) na velocidade máxima por 2 minutos. Para retirada completa das caudas, lavagens sucessivas foram realizadas com meio ELAC a 37^{o}C. Os esquistossômulos recém-transformados foram incubados por 90 minutos a 37^{o}C, lavados com solução salina a 0,9% e centrifugados a 200 g por 1 minuto. Para remoção do tegumento, 2 ml de cloreto de cálcio 0,3M foram adicionados e a suspensão foi novamente submetida ao vórtex por 7 minutos e centrifugada a 200 g por 1 minuto. O sobrenadante obtido foi, então, centrifugado a 50000 g por 1 hora em ultracentrífuga (Sorvall) e o sedimento contendo o tegumento foi ressuspendido em solução salina a 0,9%. A diálise do material obtido foi feita contra solução salina a 1,7% por 72 horas e, a determinação da dosagem protéica, pelo método de Bradford (1976).

3.5.4 Antígeno Catódico Circulante: Glicoproteína purificada

Vermes adultos foram triturados (Virtiz Precisa) por 15 minutos e centrifugados a 25000 g por 1 hora a 4ºC em ultracentrífuga (Sorvall). O sobrenadante foi aquecido a 100ºC por 30 minutos, como descrito por Deelder et al. (1976), e filtrado em filtro de 50 kDa com capacidade de retenção de proteínas com $\geq$ 45 kDa (Millipore Amicon, Sigma-Aldrich) através de etapas de centrifugação a 2700 g por 30 a 45 minutos. Foi realizada a diálise do produto purificado final contra solução salina a 0,9% por 48 horas a 4ºC e mantidos a - 20ºC. Dosagens protéicas destas amostras foram feitas em Nanodrop (Thermo Scientific 2000).

3.5.5 Antígeno Catódico Circulante: Proteína recombinante

Vermes adultos foram macerados em tubo de vidro com 1 ml de Trizol (Invitrogen) e incubados por 10 minutos a 25ºC. Posteriormente, 200 µl de clorofórmio foram adicionados e a suspensão foi incubada por 5 minutos e centrifugada a 15000 g por 15 minutos a 4ºC para reserva da camada superior contendo RNA. O RNA foi precipitado por adição de 500 µl de isopropanol, incubado por 10 minutos e, novamente, centrifugado por 10 minutos. Etanol 75% gelado foi adicionado ao sedimento, que foi centrifugado por 5 minutos. Após a remoção do etanol, o sedimento final seco foi ressuspendido em 50 µl de água livre de RNase e mantido a - 20ºC. cDNA foi obtido por kit de acordo com o protocolo sugerido pelo fabricante (SuperScript® II Reverse Transcriptase, Invitrogen).

A sequência do gene da CCA foi obtida no banco de dados do *National Center for Biotechnology Information* (NCBI, 2012) sob o número de referência AAB53003. Para a

produção da CCA recombinante, *primers* específicos foram desenhados da seguinte forma: *sense* 5'- CCC*GGATCC*ATGACGTTTGATTTCATGTTAAAG - 3' and *antisense* 5'- GGG*CTCGAG*TAGGGAGTTAATCATTTGATTCATAGC - 3' de forma a conter os sítios de restrição de *Bam*HI e *XHO*I (em itálico na sequência), respectivamente. A PCR foi realizada com 32 ciclos a 95°C por 45 segundos como etapa desnaturante, 60°C por 45 segundos como etapa de anelamento e 72°C por 1 minuto como etapa de alongamento. O produto obtido da PCR foi primeiramente subclonado em plasmídio pCR-*Blunt* II TOPO (Invitrogen) e transformado em bactérias *Escherichia coli* competentes TOP10 (Invitrogen). O DNA recombinante foi isolado e digerido com as enzimas de restrição. Os fragmentos resultantes desta digestão foram subclonados em plasmídeos pET21a igualmente clivados por *Bam*HI e *XHO*I. O plasmídeo de expressão obtido ao final do processo (CCA-pET21a) foi sequenciado para confirmação de que o inserto estava de acordo com a sequência de genes original da proteína e, então, foi inserido em bactérias *E. coli* competentes BL21 Gold (Agilent Technologies). As bactérias transformadas tiveram o crescimento estimulado por incubação *overnight* a 37°C em meio Luria Bertani (LB) com 75 μg/ml de ampicilina para, em seguida, ser diluída 100 vezes em meio LB e ser novamente incubada até que valores de absorbância a 600 nm alcançassem o mínimo de 0,5. Para indução da expressão da proteína recombinante, isopropil β-D-tiogalactose (IPTG) 1 mM foi adicionado e as bactérias foram estimuladas por 3 horas, centrifugadas por 3200 g por 10 minutos e sonicadas por 30 segundos com intervalos de 1 minuto com 10 repetições (Biologics Inc 3000). A purificação da proteína foi feita em coluna de afinidade *His-Trap* (Amersham/Pharmacia). A determinação da concentração protéica foi feita em Nanodrop (Thermo Scientific 2000).

3.5.6 Antígeno Catódico Circulante: Peptídeos de 20 aminoácidos

A sequência completa de 347 aminoácidos da CCA foi obtida no banco de dados da NCBI/*protein* (NCBI, 2012). Para a predição de epitopos para células B, a sequência inteira de aminoácidos foi analisada por *software BCPreds: B-cell epitope prediction server 1.0*. As duas melhores sequências de 20 aminoácidos com regiões expostas a células B foram consideradas, tendo como referência valores de *cut off* > 0,9. Desta forma, os peptídeos contidos na tabela 1 foram sintetizados por Mimotopes (San Diego, EUA).

Tabela 1 Peptídeos de 20 aminoácidos da CCA sintetizados a partir da predição de epitopos para células B.

Identificação	Sequência de aminoácidos	Posição dos aminoácidos	*Cut off* BCPred
1	Pro-Asn-Pro-Ser-Asp-Asp-Ser-Ser-Asn-Ser-Gly-Thr-Ile-Ser-Gly-Asn-His-Ser-Asp-Glu	307	1
2	Lys-Gln-Leu-Glu-Gln-Leu-Lys-Ile-Glu-Asn-Lys-Thr-Leu-Arg-Asn-Ser-Leu-Asp-Glu-His	83	0.926

3.6 Análise de especificidade para antígenos de CCA

Cada um dos antígenos de CCA produzidos e purificados foi analisado por método de afinidade, seguindo a técnica de Harlow & Lane (1988). Placas MaxiSorpTM (NUNC) foram sensibilizadas com 1 μg/ml de cada antígeno, tendo-se utilizado SWAP como controle positivo para a presença de CCA e albumina sérica bovina (BSA) como controle negativo. Após bloqueio das placas, 100 μl/poço de anticorpo monoclonal IgG1 anti-CCA conjugado à peroxidase (5F4.B4, University of Georgia, Monoclonal Antibody Facility) foi adicionado (1:8000). A revelação foi feita com substrato 3,3',5,5-tetrametilbenzidina (TMB) (Invitrogen) por 10 minutos até ser parada com ácido sulfúrico 2N. Os resultados foram obtidos em leitor de microplaca (BioRad 3550) a 450 nm em valores de absorbância.

3.7 Produção de anticorpos monoclonais específicos para glicoproteína CCA purificada (mAbCCA)

Camundongos BALB/c fêmeas (9 semanas de idade) foram imunizados, por via subcutânea, com 0,1 mg da glicoproteína CCA purificada em associação com novo adjuvante patenteado por Donald Harn e Rafaella Queiroz (*US patent n*. 61/476,431). Em duas semanas, os camundongos receberam reforço. Amostras sorológicas foram obtidas antes e após cada imunização e testadas por ELISA para determinação do título de anticorpos específicos para CCA durante cada etapa. Camundongos com o maior título de anticorpos após as imunizações foram selecionados e receberam novo reforço 15 dias após a segunda inoculação, por via intraperitoneal. Após três dias, os animais foram submetidos à esplenectomia e células do

baço foram obtidas por centrifugação a 210 g por 3 minutos. Estas células foram fundidas a mielomas (Sp2/O-Ag14) por adição de polietilenoglicol (Sigma-Aldrich). Os hibridomas obtidos foram incubados em placas de 96 poços e selecionados com meio hipoxantina-aminopterina-timidina (HAT). Cada poço foi monitorado por microscopia ótica para verificação do crescimento e da viabilidade celular.

Para seleção de clones produtores de anticorpos anti-CCA, foram feitos ensaios de ELISA (Harlow & Lane, 1988), com placas sensibilizadas com 1µg/ml de CCA, adição de 100 µl de sobrenadante de cultura e anticorpos conjugados à peroxidase na diluição de 1:5000 (Southern Biotech). Um segundo ensaio de ELISA foi realizado para que hibridomas produtores de anticorpos específicos para epitopos do Le^x fossem identificados. Este ensaio foi realizado da mesma forma, no entanto, com placas sensibilizadas com tetrassacarídio Le^x (Sigma-Aldrich). Hibridomas não reativos para epitopos Le^x foram selecionados e, novamente por ELISA, tiveram o subtipo de imunoglobulina determinado, de acordo com kit do fabricante (Sigma-Aldrich).

3.8 Purificação e conjugação de mAbCCA

O clone selecionado para este estudo (16D7.C10 IgM) foi crescido em meio DMEM suplementado com soro fetal bovino a 20% (Invitrogen) e penicilina (100 U/ml) e estreptomicina (100 mg/ml) (Invitrogen). Sobrenadantes destas culturas foram submetidos à precipitação por solução saturada de sulfato de amônio (Chick & Martin, 1913) e incubadas por 30 minutos a 4ºC. As proteínas precipitadas foram separadas por centrifugação a 1000 g por 15 minutos a 4ºC, ressuspendidas em PBS e dialisadas. Posteriormente, foram submetidas a segunda etapa de purificação por coluna de proteína G (Sigma-Aldrich) seguindo a instrução do fabricante. Após determinar a dosagem protéica de cada fração por Nanodrop a 280 nm, as soluções de mAbCCA foram armazenadas a - 20ºC. Alíquotas foram conjugadas à peroxidase ou à Alexa Flúor 647, com kit de conjugação a peroxidase ou a fluorocromos (Invitrogen), seguindo procedimento explicitado pelo fabricante.

3.9 *Enzyme-linked Immunosorbent Assay* (ELISA)

Os ensaios de ELISA foram baseados na técnica de Harlow & Lane (1988). Placas MaxiSorpTM foram sensibilizadas com 100 µl/poço da solução antigênica diluída em tampão carbonato-bicarbonato 0,05M pH 9,6 por 16 horas a 4ºC. As placas foram lavadas três vezes

com tampão de lavagem e, os sítios não específicos foram bloqueados com 300 µl de tampão de lavagem contendo 10% de soro fetal bovino ou 2,5% de proteínas do leite a 37ºC por 1 hora. Após novas lavagens, 100 µl de cada amostra sorológica testada foi diluída em PBS e adicionada em triplicata e as placas incubadas a temperatura ambiente por 1 hora. Em seguida, as placas foram submetidas a novas lavagens e incubadas a temperatura ambiente por 1 hora com anticorpos anti-IgG conjugados à peroxidase diluídos em tampão de lavagem (Southern Biotech). Após novas lavagens, 100 µl de substrato TMB foram adicionados a cada poço. A reação foi parada após 20 minutos de incubação pela adição de 50 µl/poço de ácido sulfúrico 2N. Os resultados foram obtidos em valores de absorbância a 450 nm por leitor de microplaca (BioRad 3550).

Amostras sorológicas murinas ou humanas, sabidamente negativas ou positivas, foram adicionadas como controle em todas as placas utilizadas para cada ELISA. A quantidade de antígeno para sensibilização das placas, assim como as diluições das amostras sorológicas e de anticorpos conjugados, foram padronizados por curvas de diluição limitante. Cada ensaio foi repetido duas vezes, tendo sido feitas triplicatas para cada amostra em cada um destes ensaios.

3.9.1 ELISA-SWAP

Para os ensaios de ELISA-SWAP foram utilizados 1 µg/ml de SWAP para sensibilização das placas e a diluição de 1:100 para amostras murinas e de 1:50 para amostras humanas. Para análise de amostras murinas, foi utilizada a diluição de 1:5000 de anticorpos conjugados à peroxidase e, para amostras humanas, a diluição de 1:60000. O *cut off* determinado foi de 0,250 para ELISA-SWAP murina e de 0,150 para ELISA humana.

3.9.2 ELISA-SEA

Para este ensaio, foram utilizados 3 µg/ml de antígeno SEA para sensibilização das placas. Amostras murinas foram diluídas 1:100 e amostras humanas 1:150, para adição de 1:15000 e 1:40000 de anticorpos conjugados, respectivamente. Os valores de *cut off* foram 0,544 para os ensaios ELISA-SEA murina e de 0,250 para os ensaios de ELISA-SEA humana.

3.9.3 ELISA-SmTeg

A sensibilização das placas foi feita com 1 μg/ml de antígeno SmTeg e a mesma diluição de 1:100 foi utilizada para ambas as amostras murinas e humanas. A adição de anticorpos conjugados foi feita na diluição de 1:15000 para amostras murinas e 1:60000 para amostras humanas. O *cut off* de 0,150 foi usado para ELISA-SmTeg murina e de 0,110 para análise com amostras humanas.

3.9.4 ELISA-antígenos de CCA

Para os ensaios que envolveram os antígenos de CCA, as placas foram sensibilizadas com 1 μl/ml de cada um dos antígenos utilizados: glicoproteína CCA purificada, CCA recombinante, CCA peptídeos 1 e 2. Amostras de soro humano foram adicionadas 1:100 e, os anticorpos conjugados, 1:60000. Valores de *cut off* foram determinados como 0,250 para ELISA-CCA, 0,103 para ELISA-CCAr, 0,117 para ELISA-CCApep1 e, 0,166 para ELISA-CCApep2.

3.9.5 ELISA-mAbCCA

O ensaio de ELISA para detecção direta de CCA em amostras sorológicas foi realizado com 1 μg/ml de mAbCCA (16D7.C10) e a diluição de 1:100 de amostra de soro. mAbCCA conjugados à peroxidase foram utilizados na diluição de 1:400. O *cut off* determinado foi de 0,031.

3.10 Método de Separação Imunomagnética: Método indireto

Microesferas paramagnéticas de 0,4 μm (Estapor, Merck) foram sensibilizadas com cada antígeno de CCA (10^6 microesferas com 1 μg/ml de antígeno/ensaio). Todas as etapas de incubação foram feitas com agitação de forma a intensificar a ligação entre antígeno-anticorpo. Para cada etapa de sensibilização, os antígenos foram diluídos em tampão carbonato-bicarbonato 0,05M pH 9,6 e incubados por 16 horas a 4ºC. As microesferas foram lavadas quatro vezes com tampão de lavagem, utilizando-se uma base magnética (Invitrogen) para aderência das mesmas. O bloqueio dos sítios não específicos foi feito com solução de proteínas do leite a 20% em tampão de lavagem a 4ºC por 16 horas. As microesferas foram lavadas novamente e mantidas a 4ºC até o uso.

No dia da análise, microesferas já sensibilizadas e bloqueadas foram lavadas por três vezes e a cada tubo de 1,5 ml contendo 10^6 microesferas foram adicionados 100 µl de soro não diluído em duplicata. Os tubos foram incubados a 37ºC por 1 hora. Após nova lavagem, as microesferas foram incubadas a 37ºC por 1 hora com 100 µl de anticorpos anti-IgG humano conjugados à peroxidase (Sigma-Aldrich) na diluição de 1:60000 em tampão de lavagem. Cada tubo foi lavado por mais cinco vezes e 100 µl de substrato TMB foi adicionado, seguido da adição de 100 µl de ácido sulfúrico 2N após 10 minutos de revelação (figura 3). Com o auxílio da base magnética, o sobrenadante foi transferido para placa de 96 poços para obtenção dos resultados, como valores de absorbância, a 450 nm em leitor de microplaca. Valores de *cut off* foram 0,197 para IMS-CCA, 0,063 para IMS-CCAr, 0,164 para IMS-CCApep1 e 0,133 para IMS-CCApep2.

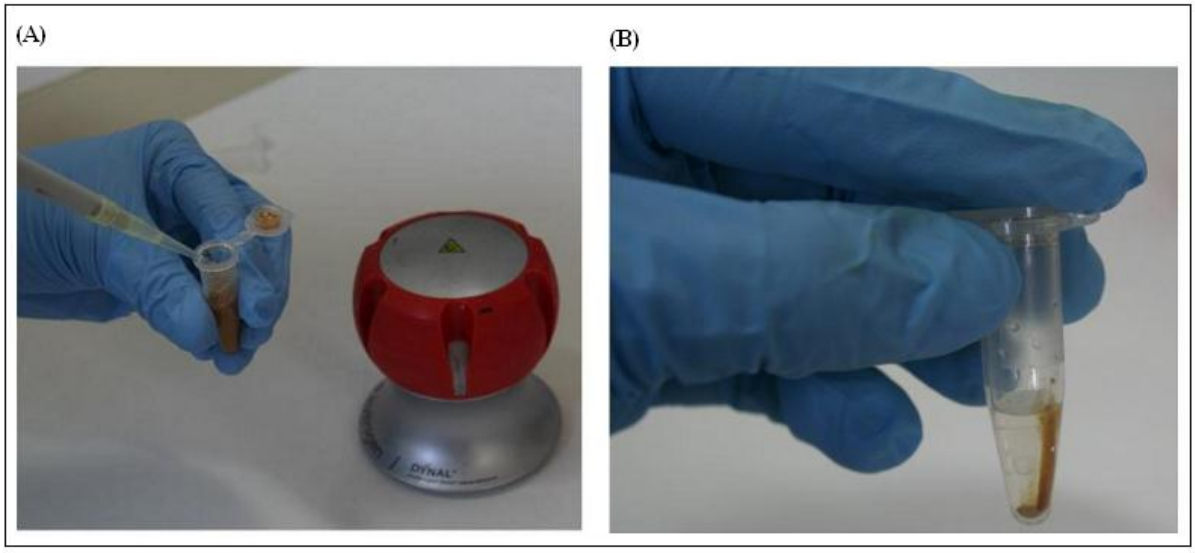


Figura 3 Demonstração do método de IMS. Em (A), adição de amostra sorológica em tubos de 1,5 ml para incubação, (B) lavagem do tubo de 1,5 ml após etapa de incubação e separação das microesferas paramagnéticas em base magnética.

3.11 Método de Separação Imunomagnética: Método direto

O ensaio IMS-mAbCCA foi padronizado como descrito no item 3.5, com algumas modificações. Às microesferas paramagnéticas sensibilizadas com 1 µg/ml de mAbCCA (16D7.C10) e devidamente bloqueadas, adicionou-se 200 µl de soro não diluído e a incubação foi feita a 37ºC por 2 horas, sob agitação. Por fim, foram utilizados 100 µl de mAbCCA

conjugados à peroxidase diluídos 1:400 em tampão de lavagem. O valor de *cut off* padronizado foi de 0,036.

3.12 Método de Separação Imunonagética por fluorescência: Método direto

O mesmo procedimento adotado para IMS-mAbCCA foi utilizado para este ensaio, com a substituição do anticorpo conjugado. Neste caso, utilizou-se 100 µl de mAbCCA conjugado à Alexa Flúor 647 na diluição de 1:400 em tampão de lavagem. A análise qualitativa foi realizada em estudo duplo cego pela análise em microscopia de fluorescência (Karl Zeiss Axiostar) de 5 µl da suspensão de microesferas sobre lâminas de vidro (642 nm, filtro LP 590). A visualização de microesferas fluorescentes revelou amostras positivas para a infecção. Registros foram feitos com máquina fotográfica digital (Canon EOS Digital Rebel XT).

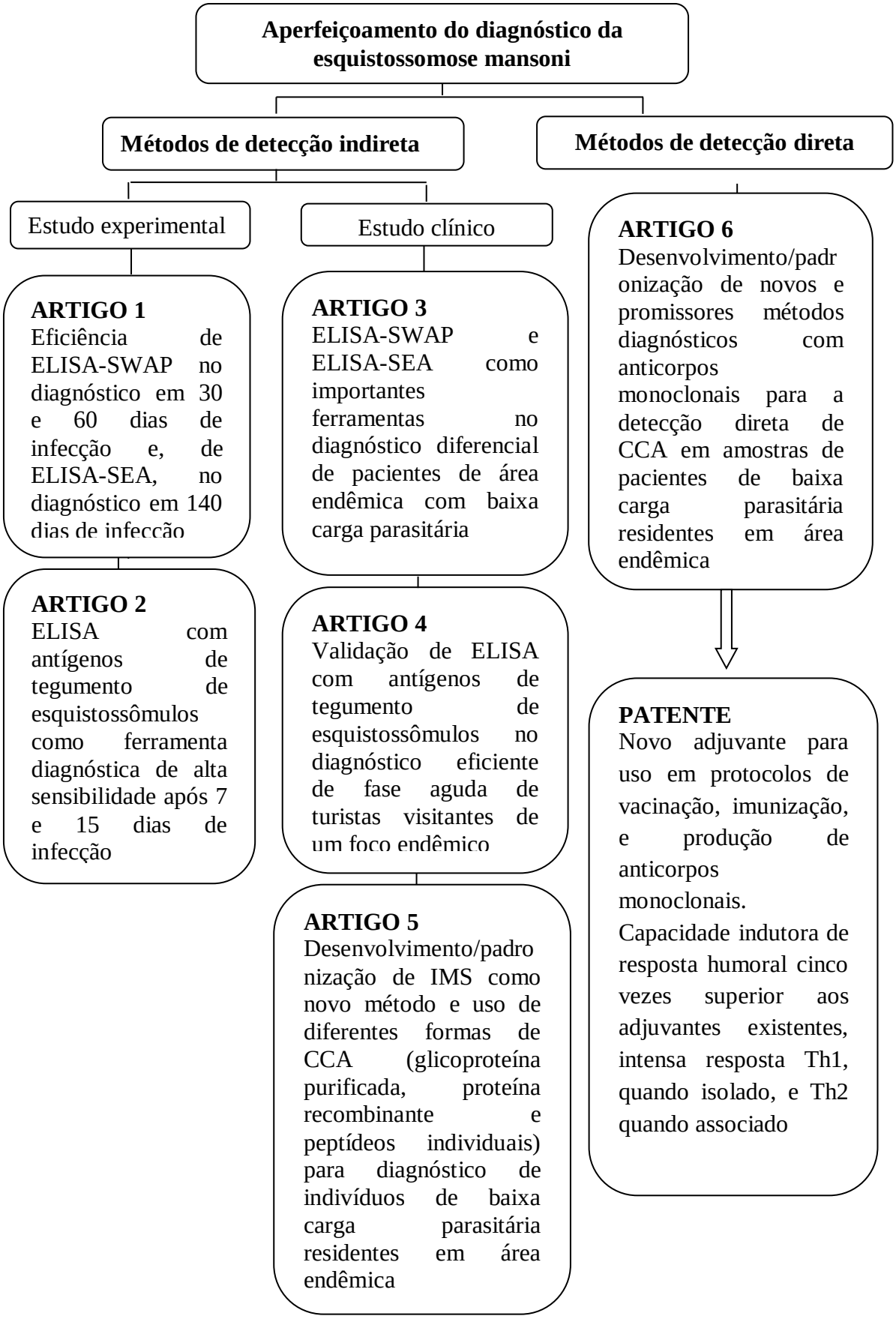
3.13 Análise estatística

Resultados obtidos dos métodos padronizados em valores de absorbância foram analisados pelo *software* Minitab por teste de normalidade de Kolmogorov-Smirnov. Dados com distribuição normal foram analisados por teste *t* de Student, enquanto os dados restantes foram analisados por teste de Mann-Whitney, ambos para níveis de significância com $p \leq 0,05$. Comparações entre porcentagens foram determinados por teste de Qui-Quadrado (χ^2) ou por teste de duas proporções de Fisher ($p \leq 0,05$). Valores de sensibilidade, especificidade e *cut off* foram determinados por curva Roc em *software* Prism. Concordância entre os métodos foram descritas por coeficiente de Cohen (Cohen, 1968) e analisadas de acordo com a definição de Landis & Koch (1977), com *software* ComKappa 2.0: 1.00 - 0,81 *almost perfect*; 0,80 - 0,61 *substantial*; 0,60 - 0,41 *moderate*; 0,40 - 0,21 *fair*; 0,20 - 0 slight; < 0 *poor*.

4 ARTIGOS E PATENTE

4 ARTIGOS E PATENTE

O delineamento deste projeto foi feito com base no seu principal objetivo de aperfeiçoar o diagnóstico da esquistossomose mansoni através do desenvolvimento e da validação de diferentes metodologias sorológicas com alta eficiência. A escolha por diferentes métodos que incluiu desde o aperfeiçoamento de métodos conhecidos, como a ELISA convencional, até o desenvolvimento e a padronização de novos métodos, como o IMS e o FluoIMS, foi realizada tendo-se como referência o conceito de que dificilmente um único método terá alta sensibilidade e especificidade de forma simultânea para o diagnóstico das diferentes fases da infecção. Para isto, foram padronizadas técnicas de detecção indireta, por determinação de títulos de imunoglobulinas IgG, e de detecção direta, por determinação dos níveis de CCA circulante no soro de pacientes infectados. Adicionalmente, diferentes antígenos foram utilizados, incluindo antígenos brutos e antígenos altamente purificados, assim como anticorpos monoclonais com alta especificidade, para avaliação do desempenho individual de cada método no diagnóstico da esquistossomose mansoni em suas diferentes fases de infecção, no que especialmente abrangeu fases aguda e crônica, fase pré-patente e fase pós-quimioterapia, como descrito pelo seguinte delineamento.


Aperfeiçoamento do diagnóstico da esquistossomose mansoni
Métodos de detecção indireta
Métodos de detecção direta
Estudo experimental
Estudo clínico
ARTIGO 1
Eficiência de ELISA-SWAP no diagnóstico em 30 e 60 dias de infecção e, de ELISA-SEA, no diagnóstico em 140 dias de infecção
ARTIGO 2
ELISA com antígenos de tegumento de esquistossômulos como ferramenta diagnóstica de alta sensibilidade após 7 e 15 dias de infecção
ARTIGO 3
ELISA-SWAP e ELISA-SEA como importantes ferramentas no diagnóstico diferencial de pacientes de área endêmica com baixa carga parasitária
ARTIGO 4
Validação de ELISA com antígenos de tegumento de esquistossômulos no diagnóstico eficiente de fase aguda de turistas visitantes de um foco endêmico
ARTIGO 5
Desenvolvimento/padronização de IMS como novo método e uso de diferentes formas de CCA (glicoproteína purificada, proteína recombinante e peptídeos individuais) para diagnóstico de indivíduos de baixa carga parasitária residentes em área endêmica
ARTIGO 6
Desenvolvimento/padronização de novos e promissores métodos diagnósticos com anticorpos monoclonais para a detecção direta de CCA em amostras de pacientes de baixa carga parasitária residentes em área endêmica
PATENTE
Novo adjuvante para uso em protocolos de vacinação, imunização, e produção de anticorpos monoclonais.
Capacidade indutora de resposta humoral cinco vezes superior aos adjuvantes existentes, intensa resposta Th1, quando isolado, e Th2 quando associado

4.1 ARTIGO 1

Revista da Sociedade Brasileira de Medicina Tropical 45(4):, Jul-Aug, 2012

Article/Artigo

Antigens of worms and eggs showed a differentiated detection of specific IgG according to the time of *Schistosoma mansoni* infection in mice

Antígenos de vermes e ovos demonstraram detecção diferenciada de IgG baseado no tempo de infecção pelo *Schistosoma mansoni* em camundongos

Rafaella Fortini Queiroz Grenfell[1], Watson Hermann Martins[1], Vanessa Silva-Moraes[1], Suedali Villas-Boas Barata[2], Elizandra Giani Ribeiro[1], Edward Oliveira[3] and Paulo Marcos Zech Coelho[1]

ABSTRACT

Introduction: The correlation between the immunological assay and the antibody titer can offer a tool for the experimental analysis of different phases of the disease. **Methods:** Two simple immunological assays for *Schistosoma mansoni* in mice sera samples based on specific IgG detection for worms soluble antigens and eggs soluble antigens were standardized and evaluated in our laboratory. Fifty mice were used in negative and positive groups and the results obtained by enzyme-linked immunosorbent assays (ELISA) assays were compared with the number of worms counted and the IgG titers at different times of infection. **Results:** Data showed that ELISA using adult worm antigens (ELISA-SWAP) presented a satisfactory correlation between the absorbance value of IgG titers and the individual number of worms counted after perfusion technique (R^2=0.62). In addition, ELISA-SWAP differentially detected positive samples with 30 and 60 days post infection (p=0.011 and 0.003, respectively), whereas ELISA using egg antigens (ELISA-SEA) detected samples after 140 days (p=0.03). **Conclusions:** These data show that the use of different antigens in immunological methods can be used as potential tools for the analysis of the chronological evolution of *S. mansoni* infection in murine schistosomiasis. Correlations with human schistosomiasis are discussed.

Keywords: Schistosomiasis mansoni. IgG. Worm/egg antigens. Acute/chronic phases.

RESUMO

Introdução: A correlação entre o ensaio imunológico e o título de anticorpos serve como ferramenta para a determinação das diferentes fases da doença. **Métodos:** Dois ensaios imunológicos simples para detecção de IgG específico para antígenos de vermes adultos e ovos do *Schistosoma mansoni* com amostras de soro murino foram padronizados e avaliados em nosso laboratório. Cinquenta camundongos negativos e positivos foram avaliados e os resultados obtidos por *enzyme-linked immunosorbent assays* (ELISA) foram comparados com o número de vermes adultos contados em tempos diferentes de infecção. **Resultados:** Os dados mostraram que a ELISA com antígenos de vermes adultos (ELISA-SWAP) apresentou uma correlação satisfatória entre a absorbância obtida para os títulos de IgG e o número individual de vermes contados por perfusão do sistema porta hepático (R2=0,62). Adicionalmente, a ELISA-SWAP foi capaz de detectar diferencialmente amostras positivas com 30 e 60 dias de infecção (p=0,011 e 0,003, respectivamente), enquanto a ELISA com antígenos de ovos (ELISA-SEA) detectou amostras positivas com 140 dias de infecção (p=0,03). **Conclusões:** Estes dados mostram que o uso de antígenos diferentes em métodos imunológicos pode ser usado como ferramentas potenciais para a análise da evolução cronológica da infecção por *S. mansoni* na esquistossomose murina. Correlações com a esquistossomose humana devem ser discutidas.

Palavras-chaves: Esquistossomose mansonica. IgG. Antígenos de vermes/ovos. Fases aguda/crônica.

1. Laboratório de Esquistossomose, Centro de Pesquisas René Rachou, Fundação Oswaldo Cruz, Belo Horizonte, MG. 2. Departamento de Ciências Básicas, Laboratório de Bioquímica, Universidade Federal dos Vales do Jequitinhonha e Mucuri, Diamantina, MG 3. Laboratório de Pesquisas Clínicas, Centro de Pesquisas René Rachou, Belo Horizonte, MG.

Address to: Dr. Paulo Marcos Zech Coelho. Lab. Esquistossomose/CPqRR/FIOCRUZ. Av. Augusto de Lima 1715/sala 205, 30190-002 Belo Horizonte, MG, Brasil.

Phone: 55 31 3349-7740; Fax: 55 31 3295-3115

e-mail: coelhopm@cpqrr.fiocruz.br

Received in 31/10/2011

Accepted in 13/02/2012

INTRODUCTION

Schistosomiasis is the most important of the human helminthiases in terms of morbidity and mortality associated with subtle but persistent morbidities[1-4]. During schistosomiasis progression, schistosomes mature to adults in the hepatic circulation and then in pairs migrate to inhabit in the mesenteric veins, where they mate and lay a large number of eggs in the vessels of the intestinal wall[5] and other tissues, mainly in the liver. The major pathological consequences of chronic schistosomiasis are associated with soluble egg antigens secreted from schistosome egg that produces a vigorous inflammatory response resulting in granuloma formation[6].

Understanding the dynamic responses of the hosts with schistosomiasis related to the time of infection is important to provide insights into the mechanisms underlying disease progression and thus could be potentially useful for the differential diagnosis in schistosomiasis evolution. Patent schistosome infections are highly immunogenic and there is not difficulty to demonstrate the presence of anti-schistosome antibodies[7]. Together, different levels of immunoglobulin G (IgG) isotypes against *Schistosoma mansoni* antigens have been demonstrated before and after chemotherapy in experimental schistosomiasis[8-10], but there have been no studies that evaluated the IgG levels against different antigens at different times after infection.

In this work, we standardized and evaluated enzyme-linked immunosorbent assays (ELISA) using soluble worm or egg antigens for detection of IgG levels in different times after mice infection, including acute and chronic phases. The use of different antigens was previously tested in ELISA for human sera showing that the use of soluble antigens is justified by the high degree of sensitivity, specificity and correlation between the quantitative seroreactivity and the faecal eggs count, irrespective of their purity[11]. We also investigated the correlation between the IgG titers and the parasite burden.

METHODS

Mice sera

Thirty *swiss* female mice (4-6 weeks), purchased at the Animal Facility of René Rachou Research Center, Oswaldo Cruz Foundation (FIOCRUZ), were exposed to an average of 40 cercariae of *S. mansoni* (LE strain) per animal, by subcutaneous route[12]. Serum samples were collected by retro orbital sinus puncture at 30, 60 and 140 days after infection, when mice were sacrificed by cervical dislocation and submitted to perfusion of the hepatic portal system using saline solution (0.85% NaCl) plus 50U/L heparin[13]. Adult worms were counted in a stereoscopic microscope (Zeiss Stemi DV4, New Jersey, USA). Twenty swiss female mice were used as negative control of infection. The use of animals was approved by the Ethical Commission in the Use of Animals, FIOCRUZ, Brazil (CEUA No. L-0023/08).

Preparation of *Schistosoma mansoni* adult worm soluble antigen

Swiss female mice (4-6 weeks) were subcutaneously infected by syringe injection, with 100 cercariae[12]. Aiming to recover the adult worms, after 45 days the animals were sacrificed by cervical dislocation, and underwent perfusion of the hepatic portal system using saline solution (0.85% NaCl) plus 50U/L heparin[13]. Adult worms were washed three times with 0.15M phosphate buffer saline pH7.2, submitted to mechanical grinding (Virtiz Precisa, Dietikon, Switzerland), and centrifuged at 9,500g for 1h at 4ºC (Eppendorf AG, Hamburg, Germany). The supernatant obtained was dialyzed in cellulose membrane (Sigma-Aldrich D9777, St Louis, USA) against saline solution (0.9% NaCl) for 48h at 4ºC. The antigen was centrifuged at 1,250g for 15min at 4ºC and the supernatant was aliquoted and stored in freezer at -20ºC. An aliquot was submitted to protein assessment following Bradford method[14] and the obtained concentration was used as parameter in the standardization of the immunoassay for detection of murine antibody, called here as enzyme-linked immunosorbent assay-soluble adult worm antigens (ELISA-SWAP).

Preparation of *Schistosoma mansoni* eggs soluble antigen

After performing the perfusion of hepatic portal system of infected mice, the livers of these animals were removed to provide eggs recovery. The antigen used in this study was prepared by the method described by Colley et al.[15]. Eggs of *S. mansoni* were homogenized and ground in Virtiz (Virtiz Precisa, Dietikon, Switzerland) with 0.85% saline solution for 40 minutes. The homogenate was centrifuged at 9,500g for an hour at 4ºC. After 48h of dialysis in cellulose membrane (Sigma-Aldrich D9777, St Louis, USA) against 0.9% saline solution, the supernatant was submitted to protein assessment[14]. The final concentration was used as parameter in the standardization of the immunoassay for detection of human and murine antibody, named here as enzyme-linked immunosorbent assay-soluble egg antigens (ELISA-SEA).

Enzyme-linked immunosorbent assays

ELISA-SWAP: microtiter plates MaxiSorpTM Surface (NUNC Brand Products, Roskilde, Denmark) were sensitized with 100µl/well of 1µg/mL of SWAP diluted in buffer 0.05M carbonate-bicarbonate pH 9.6 for 16h at 4ºC. The plates were washed three times with 0.15M phosphate buffer saline pH 7.2 with 0.05% of Tween 20 (PBS-T) (LGC Biotecnologia São Paulo, SP, BR) and, the non specific sites were blocked with 10% fetal bovine serum in PBS-T 0.05% at 37ºC for an hour. After new washing steps, 100µl of serum samples diluted 1:100 in PBS were added in triplicate into each well and the plates were incubated at room temperature for one hour. Then, the plates were submitted to washing steps and incubated at room temperature for one hour with conjugated anti-IgG mouse-peroxidase (Southern Biotech, Birmingham, USA) diluted 1:5,000 in PBS-T 0.05%. The plates were again washed and 100µl of substrate 3,3',5,5-tetramethylbenzidine solution (TMB (Invitrogen, Carlsbad, USA) were added to each well. The reaction was stopped after 20 minutes of incubation in the dark by addition of 50µl/well of 2N sulfuric acid. The results were obtained as absorbance values at 450nm in microplate reader (Model 3550, Bio-Rad Laboratories, Tokyo, JA). The cut off value was determinate as 0.250 of absorbance, using Roc curve (area=0.997). Positive and negative sera were added to the plates, and wells without antigen and antibodies were kept as controls of the assay. The standard dilution used in each technique was determined by a dilution curve, performed with the same reagents and equipment[16].

ELISA-SEA: Microtiter plates MaxiSorpTM Surface (NUNC Brand Products, Roskilde, Denmark) were sensitized with 100µL/well of 3µg/mL of SEA antigen diluted in buffer 0.05M carbonate-bicarbonate buffer pH 9.6 for 16h at 4ºC. The plates were washed three times with 0.15M phosphate buffer saline pH 7.2 with PBS-T 0.05% of Tween 20 and the non specific sites were blocked with 10% fetal bovine serum in PBS-T 0.05% at 37ºC for 1h. After new washing steps, 100µL of serum samples diluted 1:100 in PBS were added in triplicate into each well and the plates were incubated at room temperature for one hour. Following, the plates were submitted to washing steps and incubated at room temperature for one hour with conjugated anti-IgG mouse-peroxidase (Southern Biotech, Birmingham, USA) diluted 1:15,000 in PBS-T 0.05%. The plates were washed and the results were obtained as described for ELISA-SWAP. The cut off value was determined as 0.544, using Roc curve (Area=0.828). Positive and negative sera were added to the plates, and wells without antigen and antibodies were used as controls of the assay. The standard dilution used in each technique was determined by a dilution curve, performed with the same reagents and equipment[16].

Statistical analysis

Data deriving from absorbance values were analyzed with Minitab software by Kolmogorov-Smirnov normality test. Normal distributed data were analyzed by Student's *t* test and non-normal distributed data were analyzed by Mann-Whitney test, $p \leq 0.05$ as significance level. Significance levels for percentages were determined by Chi-square test (x^2). The cut off values and likelihood ratio were determined with Prism 4.0 software.

Ethical considerations

All the experiments on animals reported were performed after the Oswaldo Cruz Foundation ethics committee approval (L-0023/08).

RESULTS

Serum samples from 30 mice infected with *S. mansoni* were tested by the ELISA methods (ELISA-SWAP and ELISA-SEA). Together, 20 mice were used as negative control. Comparison of the results obtained by the ELISA-SWAP and ELISA-SEA systems was firstly

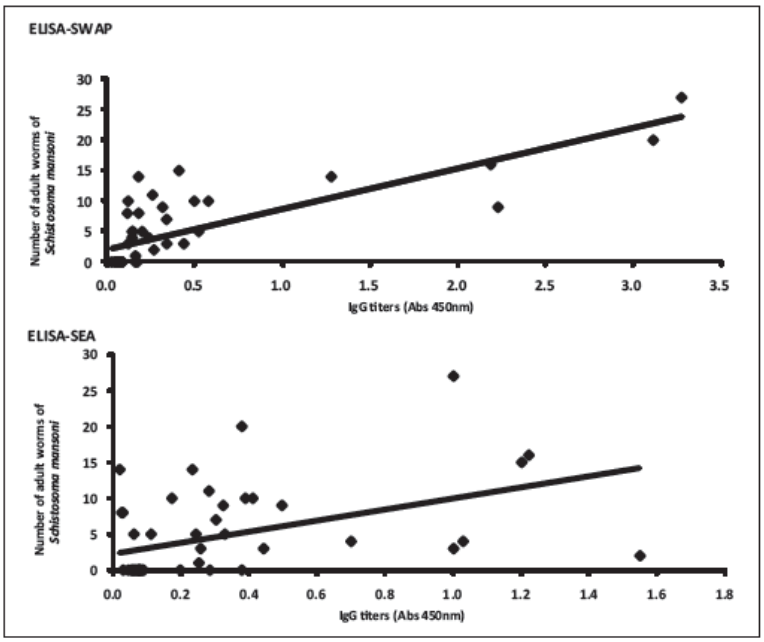


FIGURE 1 - Relationship between individual values of the number of worms after perfusion of hepatic portal system and IgG levels in mice serum detected by ELISA assays at 140 days after infection. ELISA-SWAP with a cut off value of 0.250 showed a linear regression equation of y = 6.683x + 1.923 (R2=0.62). ELISA-SEA with a cut off value of 0.544 showed a linear regression equation of y = 7.755x + 2.266 (R2=0.21).

ELISA-SWAP: enzyme-linked immunosorbent assay-soluble adult worm antigens; **ELISA-SEA:** enzyme-linked immunosorbent assay-soluble egg antigens.

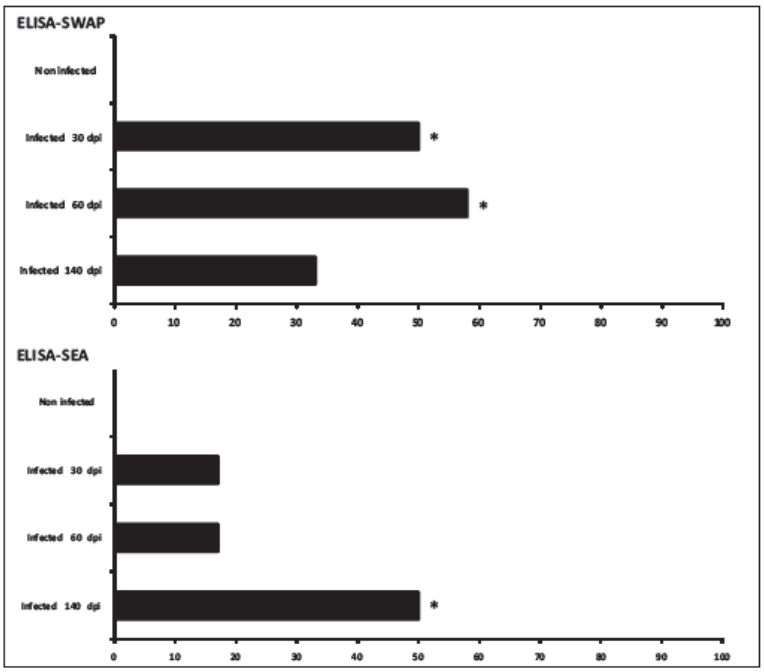


FIGURE 2 - Percentage of positive mice sera for the detection of IgG levels by ELISA-SWAP and ELISA-SEA. Mice were exposed to 40 cercariae of S. mansoni and serum samples were collected at 30, 60 and 140 days after infection. Each column represents the percentage of mice sera that were positive for the presence of specific IgG anti-SWAP (*p=0.011, 0.003 respectively for 30 and 60 days after infection) and anti-SEA (*p=0.029 for 140 dpi) for Chi-square test. The Cut off values were 0.250 and 0.544, respectively.
ELISA-SWAP: enzyme-linked immunosorbent assay-soluble adult worm antigens; **ELISA-SEA:** enzyme-linked immunosorbent assay-soluble egg antigens.

performed using cut off values determined by the ROC curve, and compared with worms recovered by perfusion technique. The cut off values of 0.250 and 0.544 were determined for ELISA-SWAP and ELISA-SEA, respectively. The geometric mean of the number of worms per mouse previously infected with 40 cercariae, estimated by the perfusion technique, was 8.

Analysis of results showed that 7 samples of infected mice were negative for both ELISA-SWAP and ELISA-SEA assays. Four of these samples were from infected mice with low parasite burden (3-5 worms recovered) (p=0.001) while 1 sample was from an infected mice with absence of worms. Two other samples were from mice with 10 and 14 worms recovered after perfusion technique.

The relationship between individual values of the number of worms after perfusion and specific IgG levels in mice sera after both ELISA assays was evaluated. As showed in **Figure 1**, ELISA-SWAP showed a more confident association with a R^2 of 0.62 in comparison with ELISA-SEA with a R^2 of 0.21.

To analyze the capability of both assays in detecting IgG titers in different times of infection by *S. mansoni*, replicates of the same samples were assessed on three different days post infection (30, 60 and 140 dpi). As shown in **Figure 2**, ELISA-SWAP was capable of detecting specific IgG antibodies in 50% of serum samples from mice with 30 days of infection (p=0.01) and in 58% of samples with 60 days (p=0.003). On the other hand, only 33% of the positive serum from mice after 140 days of infection was detected.

Whereas, ELISA-SEA was sensible for detecting specific IgG titers in only 17% of serum samples from mice with 30 or 60 days of infection, but showed a much better result for 140 dpi samples with 50% of positive detection (p=0.03). All negative samples were also negative for both ELISA assays.

DISCUSSION

Patent schistosome infection is highly immunogenic and it is easy to demonstrate the presence of anti-*Schistosoma* antibodies response in infected patients and experimental schistosomiasis. Many different assays have been used to display such immunological reactivity, including skin hypersensitivity reactions against

injected antigens, complement fixation, indirect immunofluorescence, indirect haemagglutination, radioimmunoassay, and various flocculation and precipitation tests[17-20]. But thus far, all these methods revealed low sensitivity, demonstrating the remaining inefficiency correlation between results from direct and indirect methods.

In this study, we evaluated the efficiency of two different ELISA assays based on the detection of IgG antibodies (against worm soluble antigens and egg soluble antigens) as a tool to confirm the results obtained by the portal system perfusion technique considering the number of worms from mice infected with 40 cercariae. Worm antigens are most abundant and easily obtained source of antigenic material. Crude extracts of worms work well in ELISA[11,21], and worm antigens generally give higher sensitivity and specificity than those from larvae[16]. Collectively, antigens from schistosome eggs are highly immunogenic. Their exit from the host after all depends on it[21] and, in consequence, anti-*Schistosoma* antibody titers rise after the onset of infection patency, as defined by the detection of eggs in clinical specimens.

For that propose, infected mice were submitted to perfusion technique after 140 days after infection and the number of worms were counted. We notice that ELISA-SWAP showed a better correlation between the number of adult worms and the titer of IgG for each individual mouse with R^2=0.62. All the non infected mice samples analyzed were diagnosed as real negative reaching a 100% of specificity. Two samples showed incoherent results with 10 and 14 worms and low absorbance for IgG specific for SWAP antigens.

The same analysis was performed for ELISA-SEA that showed a very low correlation with the individual number of adult worms (R^2=0.21). Seven serum samples from mice with 10-20 worms recovered from perfusion technique presented disjointed correlation with IgG titers for soluble egg antigens.

Pathologic lesions of schistosomiasis are mainly caused by the eggs deposited in various tissues[22-24]. Host immune response to antigens excreted from the embryonated mature eggs results in the formation of granulomas that in chronic infections lead to fibrotic changes. The female adult worms start laying eggs 35 days postinfection, and about 5 weeks postinfection, the eggs may be seen in host liver tissues, and 6 weeks postinfection, egg granulomas appear in infected liver. If *S. mansoni* infections could be detected in the early stage the chemotherapy could abort the pathology[8,25].

In order to determine the ability of both assays in detecting IgG titers in different times of the infection, we assessed the titers of IgG on three different days post-infection (30, 60 and 140dpi). Data showed that IgG against adult worms antigens were easily detected in most of the positive samples 30 and 60 days post-infection. In this case, 50% of serum samples from mice infected with 40 cercariae after 30 days of infection (p=0.01) and 58% of samples with 60 days (p = 0.003) were properly detected. In contrast, ELISA-SWAP assay was able to detect positive serum samples collected after 140 days of infection in only 33% of the cases. When we checked the IgG levels against eggs antigens by ELISA-SEA, the assay showed low detection capability for serum after 30 and 60 days of infection since only 17% of serum samples were detected as positive samples. On the other hand, the ELISA-SEA showed a much better result for 140 dpi samples with 50% of positive detection (p=0.03). Accordingly, all negative samples were also negative for both ELISA assays.

Results from individual laboratories and, from multicentre trials, suggest that egg antigens provide greater diagnostic sensitivity and specificity than worm antigens for the detection of an infection[11,16,26]. In the present study, we show that diagnostic tests based on egg antigens should be postponed until egg laying is started. To obtain a positive result, the parasite cycle must be completed within the definitive host with the development of males and female adult worms, which reproduce and produce the oviposition[27]. Also, although extracts prepared by homogenizing *Schistosoma* eggs contain a large number of molecules, only a minority of the constituents of SEA might be released by viable eggs *in vivo*, as demonstrated *in vitro*[28] which can explain the low detection capability of ELISA-SEA for the detection of IgG from positive mice sera. On the contrary, others have shown that during the acute phase of the disease there is an increase in anti-worm antibody titers and this fact may be due to the production (at least initially) of antibodies specific for glycanic epitopes which schistosome larvae and worms, and probably also other parasites[29], have in common.

Briefly, the use of ELISA-SWAP or SEA as a possible tool for the diagnosis of *S. mansoni* infection has been reaffirmed. Importantly, although both methods showed limitations, a higher ratio of anti-*Schistosoma* antibodies could be useful in discriminating between acute and chronic phases of the infection using antigens from different stage forms of the helminth in murine model. It is worth to note that these findings are not seen in the diagnosis of human schistosomiasis and further analysis is recommended. Grenfell et al. (unpublished results) found that immunological assays using SWAP antigens showed high sensitivity even in chronic phase of human infection.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

FINANCIAL SUPPORT

The present work was financially supported by The National Council for Scientific and Technological Development (CNPq-DECIT 576026/2008-5), Research Support Foundation of the State of Minas Gerais (FAPEMIG), Oswaldo Cruz Foundation (FIOCRUZ), Rene Rachou Research Center (CPqRR), Research Program for SUS: shared management in health (PPSUS). CNPq also supported Coelho PMZ as a Senior Fellow and Grenfell RFQ as a Doctoral Fellow.

REFERENCES

1. King CH, Sturrock RF, Kariuki HC, Hamburger J. Transmission control for schistosomiasis - why it matters now. Trends Parasitol *2006;* 22:575-582.
2. Utzinger J, N'Goran EK, N'Dri A, Lengeler C, Xiao S, Tanner M. Oral artemether for prevention of *Schistosoma mansoni* infection: randomized controlled trial. Lancet 2000; 355:1320-1325.
3. Prata A, Ruiz-Guevara R, Antunes CM, Marinho CC, Queiroz LC, Voieta I, et al. Comparison between clinical and ultrasonographic findings in cases of periportal fibrosis in an endemic area for schistosomiasis mansoni in Brazil. Rev Soc Bras Med Trop 2010; 43:129-134.
4. Lambertucci JR, Silva LC, Amaral RS. Guidelines for the diagnosis and treatment of schistosomal myeloradiculopathy. Rev Soc Bras Med Trop 2007; 40:574-581.
5. Wu J, Xu W, Ming Z, Dong H, Tang H, Wang Y. Metabolic changes reveal the development of schistosomiasis in mice. PLoS Negl Trop Dis 2010; 4:e807.
6. Kasinathan RS, Greenberg RM. *Schistosoma mansoni* soluble egg antigens trigger erythrocyte cell Death. Cell Physiol Biochem 2010; 26:767-774.

7. Beltran S, Gourbal B, Boissier J, Duval D, Kieffer-Jaquinod S, Pierce RJ, et al. Vertebrate host protective immunity drives genetic diversity and antigenic polymorphism in *Schistosoma mansoni*. J Evol Biol 2011; 24:554-572.
8. Coelho PM, Tavares CA. ELISA detection of specific circulating antibodies against *Schistosoma mansoni* in mice after treatment with oxamniquine. Braz J Med Biol Res 1991; 24:485-493.
9. Ribeiro F, Mello RT, Tavares CA, Kusel JR, Coelho PM. Synergistic action of praziquantel and host specific immune response against *Schistosoma mansoni* at different phases of infection. Rev Inst Med Trop Sao Paulo 2004; 46:231-233.
10. Ruppel A, Xing Y, Dell R, Numrich P, Shi YE. *Schistosoma mansoni* and *S. japonicum*: decline of antibodies against diagnostic adult worm antigens (Sm31/32) following praziquantel treatment of mice. Trop Med Parasitol 1991; 42:325-331.
11. Mott KE, Dixon H. Collaborative study on antigens for immunodiagnosis of schistosomiasis. Bull World Health Organ 1982; 60:729-753.
12. Peters PA, Warren K. A rapid method of infecting mice and other laboratory animals with *Schistosoma mansoni*: subcutaneous injection. J Parasitol 1969; 55:558.
13. Pellegrino J, Siqueira A. Técnica de perfusão para colheita de *Schistosoma mansoni* em cobaias experimentalmente infestadas. Rev Bras Malar Doenças Trop 1956; 8:589-597.
14. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976; 7:248-254.
15. Colley DG, Lewis FA, Goodgame RW. Immune Responses During Human Schistosomiasis. IV. Induction Of Suppressor Cell Activity By *Schistosome* Antigen Preparations And Concanavalin. A J Immunol 1978; 120:1225-1232.
16. McLaren M, Draper CC, Roberts JM, Minter-Goedbloed E, Ligthart GS, Teesdale CH, Amin et al. Studies on the enzyme linked immunosorbent assay (ELISA) test for *Schistosoma mansoni* infections. Ann Trop Med Parasitol 1978; 72:243-253.
17. Bergquist NR. Present aspects of immunodiagnosis of schistosomiasis. Mem Inst Oswaldo Cruz 1992; 87 (supl IV):29-38.
18. Hamilton JV, Klinkert M, Doenhoff MJ. Diagnosis of schistosomiasis: antibody detection, with notes on parasitological and antigen detection methods. Parasitology 1998; 117:S41-S57.
19. Kagan IG, Cahill KM. Parasitic serologic studies in Somaliland. Am J Trop Med Hyg 1968; 17:392-396.
20. Maddison SE. Serodiagnosis of parasitic infections. Clin Microbiol Rev 1991; 4:457-469.
21. Maddison SE, Slemenda SB, Tsang VC, Pollard RA. Serodiagnosis of *Schistosoma mansoni* with adult worm antigen in an enzyme-linked immunosorbent assay using a standard curve developed with a reference serum pool. Am J Trop Med Hyg 1985; 34:484-494.
22. Doenhoff MJ. Granulomatous inflammation and the transmission of infection: schistosomiasis - and TB too? Immunol Today 1998; 19:462-467.
23. Asahi H, Stadecker MJ. Analysis of egg antigens inducing hepatic lesions in schistosome infection. Parasitol Int 2003; 52:361-367.
24. Ross AG, Bartley PB, Sleigh AC, Olds GR, Li Y, Williams GM, et al. Schistosomiasis. N Engl J Med 2002; 346:1212-1220.
25. Helmy MM. Touchdown PCR, ELISA and stool examination for early diagnosing of *Schistosoma mansoni* in mice. J Egypt Soc Parasitol 2007; 37:903-913.
26. Mott KE, Dixon H, Carter CE, Garcia E, Ishii A, Matsuda H, et al. Collaborative study on antigens for immunodiagnosis of *Schistosoma japonicum* infection. Bull World Health Organ 1987; 65:233-244.
27. Chaiworaporn R, Maneerat Y, Rojekittikhun W, Ramasoota P, Janecharut T, Matsuda H, et al. Therapeutic effect of subcurative dose praziquantel on *Schistosoma mansoni* infected mice and resistance to challenge infection after treatment. Southeast Asian J Trop Med Public Health 2005; 36:846-852.
28. Ashton PD, Harrop R, Shah B, Wilson RA. The schistosome egg: development and secretions. Parasitology 2001; 122:329-338.
29. Noya BA, Cesari IM, Losada S, Colmenares C, Balzan C, Hoebeke J, et al. Evaluation of alkaline phosphatase immunoassay and comparison with other diagnostic methods in areas of low transmission of schistosomiasis. Acta Trop 1997; 66:69-78.

4.2 ARTIGO 2

Schistosomula tegument antigen as potential candidate for the early serological diagnosis of schistosomiasis mansoni

Rafaella Grenfell[1], Watson Martins[1], Vanessa Silva-Moraes[1], Neusa Araujo[1], Edward Oliveira[2], Cristina Fonseca[1], Paulo Marcos Z. Coelho[1]

[1]Laboratório de Esquistossomose, Centro de Pesquisas René Rachou, Fundação Oswaldo Cruz (Fiocruz), Belo Horizonte, Minas Gerais, Brazil, 30.190-002.

[2]Laboratório de Pesquisas Clínicas, Centro de Pesquisas René Rachou, Fundação Oswaldo Cruz (Fiocruz), Belo Horizonte, Minas Gerais, Brazil, 30.190-002.

Corresponding Author. Mailing address: Fundação Oswaldo Cruz, Centro de Pesquisas René Rachou, Av. Augusto de Lima, 1715, Belo Horizonte, MG, Brazil. 30.190-002. Phone: 55 31 3349 7740, e-mail: coelhopm@cpqrr.fiocruz.br

ABSTRACT

If *Schistosoma mansoni* infection could be detected in the early stages, especially before the egg deposition in the host tissues, the development of severe pathologic lesions might be prevented efficiently. So, we developed an indirect enzyme-linked immunosorbent assay based on the detection of specific IgG against schistosomula antigens (ELISA-SmTeg). The assay was applied in sera samples from non-infected and infected mice collected after 7 and 15 days post-infection. The results were compared to the number of adult worms obtained by perfusion of the murine hepatic system after 50 days post-infection. The sensitivity and specificity of the ELISA-SmTeg were 100% (p = 0.0032, 0.0048, respectively for 7 and 15 days of infection) with a cut off value of 0.15 (p = 0.0002). Our findings show a novel low cost serological assay using easy to obtain antigens that was capable of detecting all the infected mice as soon as 7 days post-infection.

Keywords: Acute schistosomiasis, Diagnosis, Immunological assay, Schistosomula antigens.

Sponsorship: Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundação de Amparo à Pesquisa do Estado de Minas Gerais (Fapemig), Fundação Oswaldo

Cruz (Fiocruz)/Centro de Pesquisas Rene Rachou (CPqRR), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (Capes), The Council of the International Educational Exchange of Scholars (Fulbright, U.S. Department of State).

SUMÁRIO

A detecção da infecção pelo helminto *Schistosoma mansoni* quando realizada nas fases iniciais, especialmente antes da oviposição nos tecidos do hospedeiro, pode impedir de forma eficiente o desenvolvimento de graves lesões patológicas. Baseado nisto, foi desenvolvido um ensaio imunoenzimático indireto para detecção de anticorpos IgG específicos contra antígenos de esquistossômulos (ELISA-SmTeg). Este ensaio foi aplicado em amostras sorológicas de camundongos não infectados, da mesma forma que de camundongos recentemente infectados, após 7 e 15 dias de infecção. Os resultados foram comparados com o número de vermes adultos obtidos por perfusão do sistema hepático murino 50 dias pós-infecção. A sensibilidade e a especificidade do novo método, denominado ELISA-SmTeg, foram de 100% (p = 0,0032, 0,0048, respectivamente, durante 7 e 15 dias de infecção) com um valor de corte de 0,15 (p = 0,0002). Nossos resultados mostraram que um ensaio de baixo custo, que utiliza antígenos de fácil obtenção, é capaz de discriminar a esquistossomose mansoni em modelo experimental de forma precoce, incluindo 7 dias pós-infecção.

INTRODUCTION

As an important zoonosis, schistosomiasis remains a significant global public health problem. Approximately 200 million humans and a significant number of livestock are infected with schistosomes, and approximately 20 million patients suffer from severe consequences (17). Severe complications of schistosomiasis after only a few weeks of exposure have been reported (13-15) and those patients are at high risk of developing chronic manifestations with irreversible fibrosis (16). Despite the public health importance of schistosomiasis and the risk that the disease might further spread and intensify in the absence of wide-ranging improvement measures, schistosomiasis has been neglected for decades, due to many consistent factors (18). The most imperative factor regarding the difficult of controlling the spread of the disease is the absence of diagnostic methods capable of detecting the disease, especially in the pre-patent phase.

Host immune response to antigens excreted from the embryonated mature eggs results in the formation of granulomas that in chronic infections lead to fibrotic changes (19). In the majority of schistosome infections, the female adult worms start to discharge eggs 30 days post-infection, and the eggs could be seen in host liver tissues about 5 weeks post-infection. After 6 weeks, egg granulomas appear in infected liver (2). If schistosome infections could be detected before the egg deposition in the host tissues, the chemotherapy could be effective and the development of severe pathologic lesions in host tissues might be prevented (6, 7). Therefore, it is imperative to have an easy to do and low cost assay expressing the capability to detect the initial phase of the infection that can be used in the field and/or for increasing travelers and floating population.

The objective of the present study was to develop and test the capability of a novel assay which would serve as a potential methodology candidate for the early diagnosis of schistosomiasis mansoni. For that matter, we used schistosomula tegument antigens in an enzyme-linked immunosorbent assay for the detection of specific IgG (ELISA-SmTeg). The performance of the assay was evaluated for murine schistosomiasis, especially for 7 to 15 days of infection. Nevertheless, it is still essential to evaluate the effectiveness of this assay for acute human schistosomiasis.

MATERIALS AND METHODS

Mice sera

Twenty six swiss female mice (4-6 weeks) purchased at the Animal Facility of René Rachou Research Center/FIOCRUZ were infected with 40 cercariae (standard deviation of $\pm$ 10) of *S. mansoni* (LE strain) per mouse by subcutaneous route (11). Fifteen swiss female mice were used as negative control of infection. The serum samples were collected by retro orbital sinus puncture at days 7 and 15 after infection. After 50 days, mice were sacrificed by cervical dislocation and submitted to perfusion of the hepatic portal system using saline solution (1.17% NaCl) plus 50 U/L heparin (10). Adult worms were counted in a stereoscopic microscope (Zeiss Stemi DV4, Nettetal, GE). The use of animals was approved by the Commission for Ethics in the Use of Animals (CEUA FIOCRUZ), Brazil (L-02308).

Preparation of *S. mansoni* schistosomula tegument antigen (SmTeg)

Cercariae of the LE strain were obtained at the Laboratory of Malacology of the René Rachou Research Center/Fiocruz and were mechanically transformed in to schistosomula through the previously described technique, with some modifications (12). Briefly, cercariae were placed into conical tubes and left in an ice bath for 30 minutes before the centrifugation (Eppendorf Centrifuge 5820R, Hamburg, GR) at 200g for 3 minutes at 4°C. The pellet was ressuspended in cold Earl's salts plus lactalbumin hydrolyzate medium (ELAC). The cercarial tails were broken in vortex (Scientific Industries Genie-2) at maximum speed for 2 minutes. Later the tails were removed from the medium through repeated washing steps with ELAC at 37°C, and schistosomula were incubated for 90 minutes at 37°C and washed with 0.9% saline solution. This step was followed by centrifugation at 200g for 1 minute. For tegument removal, 2 ml of 0.3M calcium chloride were added to the schistosomula that was stirred in vortex for 7 minutes and centrifuged at 200g for 1 minute. The supernatant was centrifuged at 50000g for an hour and the pellet enriched of membrane was ressuspended in 0.9% saline and dialyzed against 1.7% saline solution for 72 hours. Protein concentration was determined by Bradford method (3). The final concentration used in standardized tests was 0.52 μg/μl.

Indirect Enzyme-Linked Immunosorbent Assay

Based on the technique previously described (9) with some modifications. In brief, microtiter plates MaxiSorpTM Surface (NUNC Brand Products, Roskilde, DK) were coated with 100 μl per well of SmTeg diluted at 1 μl/ml in buffer 0.05M carbonate-bicarbonate pH 9.6 for 16 hours at 4°C. Next, the plates were washed three times with 0.15M phosphate buffer saline pH 7.2 containing 0.05% of polysorbate sodium (LGC Biotecnologia, São Paulo, BR) (washing buffer). In follow, the free sites were saturated with 300 μl per well of 2.5% skim milk diluted in washing buffer, incubating at 37°C for 1 hour. After further washing steps, 100 μl of individual mice sera (diluted 1:100) in 0.15M phosphate buffer saline pH 7.2 were added to the plates and were incubated at room temperature for 1 hour. The plates were submitted to washing steps and incubated at room temperature for 1 hour with anti-IgG conjugated to peroxidase diluted in washing buffer (anti-mouse IgG Fc specific-Southern Biotech, Birmingham, USA) at the dilution of 1:15000. Plates were washed again and 100 μl of substrate solution (3,3',5,5-tetramethylbenzidine) (Invitrogen, Grand Island, USA) were added to each well. The enzymatic reaction was stopped after 8 minutes of incubation in the dark by

adding 50 µl per well of 2N sulfuric acid. The results were obtained as absorbance values at 450 nm in microplate reader (BIORAD 3550, Tokio, JA). The cut off value of the ELISA-SmTeg for murine sera samples was 0.15 (0.66 ± 0.09), determined by ROC curve (A = 1.0).

Positive and negative controls were assayed properly, also wells without antigen and sera samples as control of nonspecific adsorption of conjugate. The standard dilution was determined by a dilution curve, performed with the same reagents and equipment based on six different dilutions.

Statistical analysis

Data deriving from absorbance values were analyzed with Minitab software (Minitab Inc, College, USA) by Kolmogorov-Smirnov normality test. Normal distributed data were analyzed by Student's *t* test and non-normal distributed data were analyzes by Mann-Whitney test ($p < 0.05$ as significance level). The sensitivity, specificity, cut off values and likelihood ratios were determined with Prism 4.0 software.

RESULTS

The performance of ELISA-SmTeg was firstly evaluated with 34 mice sera samples of which 26 mice were infected with 40 cercariae (standard deviation of ± 10) of *S. mansoni* by subcutaneous route. The results obtained in absorbance values were initially compared to the cut off value of 0.15 which was determined after a screening of all positive and negative samples and it was based on 100% of sensitivity and specificity. Afterward, the results were compared to the number of worms obtained after perfusion technique. The geometric mean of the number of adult worms per mouse previously infected with 40 cercariae estimated by the perfusion technique was 19.6 (standard deviation of ± 5.7) and it corroborated to the absorbance values founded after ELISA-SmTeg assay.

The results are shown on figure 1 and demonstrates that ELISA-SmTeg was capable of detecting specific IgG antibodies in all of the 26 sera samples from mice recently infected with *S. mansoni* (7 and 15 days after infection) with a cut off value of 0.15 ($p = 0.0002$). Accordingly, all negative samples firstly diagnosed by immunological assays using soluble egg antigens or soluble adult worm antigens (unpublished observations) were also negative for the ELISA assay performed.

From table 1, compared with the control at each time interval by *t* test, statistically significant increase appeared in the infected group on both days 7 and 15 post-infection (p = 0.032 and 0.048, respectively). The level of circulatory anti-SmTeg IgG in the infected mice group didn't show an increasing trend within the infection time analyzed, whereas no obvious change was found in the control group, and significant difference was found between the infected mice group and the control group.

DISCUSSION

A good serological test for the diagnosis of schistosomiasis should be capable of detecting *S. mansoni* very early after infection. A tool is needed to solve the problem of differential diagnosis due to the non-specific symptoms of the early stages of the disease (8) and doubly due to the possibility of treatment before the elimination of eggs in pre-patent phase. In experimental hosts, anti-schistosome antibody reactivity remains low for worm antigens, until the infections become patent (1, 5). This could be a result of the early stages of infection being poorly immunogenic, which, in turn, helps explain why a good test to diagnose pre-patent infections has not yet been devised.

This work addresses the development of an indirect immunological assay for the early diagnosis of human infection using schistosomula tegument antigens. The scope was particularly based on the exposure time to the schistosomula tegument in infected individuals before the onset of eggs by the parasite.

We evaluated the sensitivity/specificity of ELISA-SmTeg for the detection of specific titers of IgG using mice sera. Therefore, sera samples were collected from mice (40 cercariae) at day 7 and 15 after the subcutaneous infection. All mice groups were submitted to perfusion technique for attainment of worms after 50 days of infection, when the parasite burden for each sample was established. We could noticed that the ELISA-SmTeg was properly standardized and it was efficient to detect serum antibodies in mice, since there was a noticeable statistically difference between the antibodies titers from infected and non-infected groups with a cut off value of 0.15 (p = 0.0002). It was possible to achieve total efficiency on the diagnosis of all samples with 100% of sensitivity and specificity in view of the fact that all the negative and positive mice presented the accurate result, as shown by the ROC curve (figure 1).

Our data indicates that antigens from the schistosomula tegument may be a potential candidate antigen for early diagnosis of infection with *S. mansoni*, which could be further developed toward early serological diagnosis of human schistosomiasis mansoni. The levels of circulatory anti-SmTeg IgG in infected mice sera showed a statistically significant increase from day 7 and 15 post-infection compared with the mice sera prior to infection as the control group (table 1). These results suggest that anti-SmTeg IgG could be detectable from the host at a very early phase, at least on day 7 after the infection (figure 1) and this forms a basis for further studies.

From table 1, no fluctuation in circulatory anti-SmTeg IgG level in infected mice sera was observed from days 7 to 15 post-infection, showing that anti-SmTeg IgG level in maintained during the time of schistosomula exposure. As a tegument extract of proteins, the extent of SmTeg antigens exposure might be enough to stimulate the host to produce abundant specific IgG for keeping the continual rise until the eggs began to be trapped in tissues and developed to miracidium on day 35 of the infection.

It was anticipated that the difficulties associated with parasitological diagnosis might be overcome by adoption of immunological methods (4), especially when this diagnosis can be performed in pre-patent phase and that may provide evidence that an infection is present. Thus, measuring assay for antibody activity before the eggs laying could therefore provide useful information on the programmes' effectiveness (4). Therefore, SmTeg is a potential antigen for early diagnosis of schistosomiasis. These findings provide foundation for further studies to make this crude antigen an especially attractive target for profound utilization in prevention and control of schistosomiasis.

REFERENCES

1. Ambroise-Thomas P, Andrews P. Development of fluorescent antibodies directed against larval stages, eggs, and adults of *Schistosoma mansoni* in mice harbouring unisexual or bisexal infections. Tropenmed Parasitol. 1976; 27(4): 483-488.

2. Barbosa MA, Pellegrino J, Coelho PM, Sampaio IB. Quantitative aspects of the migration and evolutive asynchronism of *Schistosoma mansoni* in mice. Rev Inst Med Trop Sao Paulo. 1978; 20 (3): 121-132.

3. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 1976; 72: 248-254.

4. Doenhoff MJ, Chiodini PL, Hamilton JV. Specific and sensitive diagnosis of schistosome infection: can it be done with antibodies? TRENDS in Parasitology. 2004; 20(1): 35-39.

5. Dunne DW, Bain J, Lillywhite J, Doenhoff MJ. The stage-, strain- and species-specificity of a Schistosoma mansoni egg antigen fraction (CEF6) with serodiagnostic potential. Trans R Soc Trop Med Hyg. 1984; 78: 460-470.

6. Enk MJ, Lima AC, Drummond SC, Schall VT, Coelho PM. The effect of the number of stool samples on the observed prevalence and the infection intensity with *Schistosoma mansoni* among a population in an area of low transmission. Acta Trop. 2008; 108 (2-3): 222-228.

7. Enk MJ, Amaral GL, Costa e Silva MF, Silveira-Lemos D, Teixeira-Carvalho A, Martins-Filho OA, *et al*. Rural tourism: a risk factor for schistosomiasis transmission in Brazil. Mem Inst Oswaldo Cruz. 2010; 105 (4): 537-540.

8. Evengard B, Hammarstromi L, Smitht CIE, Linder E. Early antibody responses in human schistosomiasis. Clin Exp Immunol. 1990; 80: 69-76.

9. Harlow E, Lane D. Antibodies: A Laboratory Manual. Imperial Cancer Research Fund Laboratories. 1988; 726 pp.

10. Pellegrino J, Siqueira A. Técnica de perfusão para colheita de *Schistosoma mansoni* em cobaias experimentalmente infestadas. Rev Bras Malar Doenças Trop. 1956; 8: 589-597.

11. Peters PA, Warren K. A rapid method of infecting mice and other laboratory animals with *Schistosoma mansoni*: subcutaneous injection. J Parasitol. 1969; 55: 558.

12. Ramalho-Pinto FJ, Grazzinelli G, Howells RE, Mota-Santos TA, Figueiredo EA, Pelegrino J. *Schistosoma mansoni*: defined system for stepwise transformation of cercariae to schistossomule in vitro. Exp Parasitol. 1974; 36(3): 360-372.

13. Scully RE, Mark EJ, McNeely BU. Weekly clinico-pathological exercises. Case 21. New Engl J Med. 1985; 312: 1376-1383.

14. Shi GF, Xu ZY, Fu Q, Weng XH, Ma QY, Zhang JS. Dynamic study on relationship between serum SEAIC level and hepatic pathological changes in mice infected with *Schistosoma japonicum*. Chin J Parasitol Parasit Dis. 1994; 12: 205-209.

15. Tarp B, Black FT, Petersen E. The immunofluorescence antibody test (IFAT) for the diagnosis of schistosomiasis used in a non-endemic area. Trop Med Int Health. 2000; 5(3): 185-191.

16. Warren KS, Boros DL, Hang LM, Mahmoud AA. The *Schistosoma japonicum* egg granuloma. Am J Pathol. 1975; 80: 279-294.

17. World Health Organization. The social context of schistosomiasis and its control: an introduction and annotated bibliography. 2008; Available from: http://dosei.who.int/uhtbin/cgisirsi/tLEqou8myB/26990009/5/0.

18. Utzinger J, Raso G, Brooker S, De Savigny D, Tanner M, Ornbjerg N, *et al.* Schistosomiasis and neglected tropical diseases: towards integrated and sustainable control and a word of caution. Parasitology. 2009; 136(13): 1859-1874.

19. Zhou X, Wu J, Huang X, Kunnon SP, Zhu X, Chen X. Identification and characterization of *Schistosoma japonicum* Sjp40, a potential antigen candidate for the early diagnosis of schistosomiasis. Diagnostic Microbiology and Infectious Disease. 2010; 67: 337-345.

FIGURE, TABLE AND LEGENDS

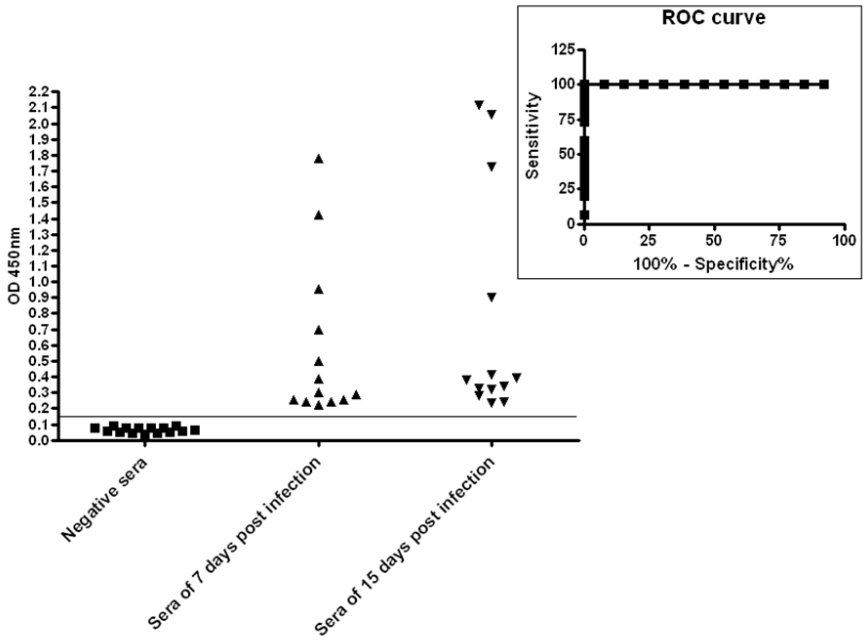


Figure 1. IgG levels detected in mice sera by ELISA-SmTeg. Mice were exposed to 40 cercariae of *S. mansoni* and serum samples were collected at 7 and 15 days after infection. Each sample is represented by the mean of four absorbance values for two independent experiments. Cut off value are represented by bar. The box graphic indicates the ROC curve for 100% of sensitivity and specificity for mice sera.

Table 1. The amount of circulatory IgG anti-SmTeg in mice sera at days 0, 7 and 15 post-infection.

Groups	*n*	The amount of circulatory IgG anti-SmTeg in mice sera (OD at 450 nm, mean ± SD)		
		0 dpi	7 dpi	15 dpi
Infection	13		0.580 ± 0.096	0.749 ± 0.100
Control	15	0.080 ± 0.014		
p value			0.032	0.0048

p value. Control group versus infected group.

4.3 ARTIGO 3

Schistosoma mansoni in a low prevalence area in Brazil: the importance of additional methods for the diagnosis of hard to detect individual carriers by low cost immunological assays

Rafaella Grenfell[a], Watson Martins[a], Martin Enk[a], Áureo Oliveira[a], Liliane Siqueira[a], Vanessa Silva-Moraes[a], Edward Oliveira[b], Paulo Marcos Z. Coelho[a*]

[a]Laboratório de Esquistossomose, Centro de Pesquisas René Rachou, Fundação Oswaldo Cruz (FIOCRUZ). Avenida Augusto de Lima 1715, Belo Horizonte, Minas Gerais, Brazil. 30.190-002.

[b]Laboratório de Pesquisas Clínicas, Centro de Pesquisas René Rachou, Fundação Oswaldo Cruz (FIOCRUZ). Avenida Augusto de Lima 1715, Belo Horizonte, Minas Gerais, Brazil. 30.190-002.

***Corresponding Author**: Fundação Oswaldo Cruz, Centro de Pesquisas René Rachou, Av. Augusto de Lima, 1715, Belo Horizonte, MG, Brazil. 30.190-002. Phone: 55 31 3349 7740. Fax number: 55 31 3295 3115. E-mail: coelhopm@cpqrr.fiocruz.br

Running title: Diagnosis of low burden schistosomiasis patients

Summary

The diagnosis of schistosomiasis is currently based on stool parasitological examinations for egg detection, which is laborious and lacks sensitivity, especially for patients with low parasite burden. There are assays that detect the anti-schistosomal antibodies in patient sera but they usually demonstrate low sensitivity and specificity, especially for patients with low parasite burden which is common in endemic areas. Two simple and well known immunological assays for *Schistosoma mansoni* detection based on specific IgG detection for worms (SWAP) and eggs (SEA) antigens were evaluated in our laboratory with sera samples of individuals from an endemic area with very low parasite burden. Data showed that ELISA-SWAP presented a significant result for human diagnosis with 95% of sensitivity and specificity willing to confirm Kato-Katz diagnosis (18 slides from 4 samples of faeces) with an almost perfect agreement by Kappa index (0.85). Although ELISA-SEA presented the same sensitivity, it showed 85% of specificity, a Kappa index of 0.75 and it seemed to be

more suitable to cross-reaction. These immunological assays were shown to be potential tools for the additional diagnosis of *S. mansoni* infection, jointly to the parasitological method, including low burden individuals that are hardly well diagnosed by current methods.

Keywords: Schistosomiasis mansoni, Immunodiagnosis, Low parasite burden, Endemic area individuals, additional diagnosis.

1 Introduction

Endemic to many countries in the developing world, schistosomiasis continues to be a serious public health problem and the most important of the human helminthiases in terms of morbidity and mortality associated with subtle but persistent morbidities.[1] It is a chronic and debilitating disease with an active transmission not only in highly endemic areas, but in previously non-endemic areas, despite major advances in its control.[2]

Estimation of the intensity of schistosomal infection is currently based on quantitative egg counts by Kato-Katz technique,[3] which can be highly variable.[4] Therefore, patient management, based solely on the presence of ova, is overly conservative and may result in patients with low egg count being undiagnosed.[5] Innovative and useful methods have been developed by our group as saline gradient system for egg counts,[6] miracidia hatching device for miracidia visualization,[7] and eggs DNA detection.[8] However, the sensitivity of parasitological methods decreases in areas of low endemicity.[4] Together, those methods do not allow the detection of the infection stage once the elimination of eggs in faeces is required prior to diagnosis.

Antibody-detecting assays with the proper standardization can be highly specific and sensitive.[9] These tests are promising for the diagnosis of patients living in low endemicity areas, especially if used concurrently with the coproscopy.[10, 11] The simultaneous use of different diagnostic methods has been applied to monitor the human population, to identify the small number of infected people once morbidity control is achieved with higher sensitivity and to diagnose early stages of the infection.[4, 6, 12]

The main target of this work is to properly standardize and evaluate Enzyme-Linked Immunosorbent Assays (ELISA) using easy to obtain worm or egg antigens as an improvement over the serological tests previously studied. In order to make this an innovative work, the performance of the assays was evaluated with sera from Brazilian individuals living

in low endemicity area for *S. mansoni* infection, well diagnosed by eighteen slides of faeces obtained on four different days by Kato-Katz for the estimation of the real intensity of infection.

2 Materials and methods

2.1 Community survey

This study was performed in the communities of Buriti Seco and Morro Grande in Pedra Preta, a little village in a schistosomiasis endemic area in the rural region of Montes Claros, state of Minas Gerais at the southeast region of Brazil, as published.[5] This area was chosen due to the fact that the population was not treated for schistosomiasis and it also had a low migration index with a fixed resident population. Additionally, according to data provided by Montes Claros Control Centre of Zoonosis, an infection rate of 12% was found in 2005. Forty people from Pedra Preta aged 28-64 participated in this study (female/male: 22/18). Jointly, 20 healthy donors aged 22-65 participated as negative controls throughout the standardization and evaluation of the assay (female/male adults: 14/06).

Sera and stool samples - Four stool samples per individual were collected on four consecutive days using 100 ml plastic together with one serological sample. The samples were identified using the name and number of the participant and, in case of endemic area individuals, together with the identification of the residence. Written informed consent was obtained from all participants. Eighteen glass slides (41.7 mg/smear each) were evaluated for the presence of *S. mansoni* and other helminth eggs by the Kato-Katz technique,[3] prepared as follows for each participant: 12 slides of the first sample and two slides each of the second, third and fourth sample in a total of 750 mg of faeces. The intensity of infection was expressed in eggs per gram of faeces (epg), using the arithmetic mean of egg counts obtained from the 18 slides multiplied by 24.

Treatment of positive cases - All participants who were positive for schistosomiasis were treated with praziquantel in a single dose of 50 mg/kg. Infections with other helminthes were treated with a single dose of 400 mg albendazole, as recommended by the Brazilian Ministry of Health. Positive patients were resubmitted to stool examination by Kato-Katz assay thirty days post chemotherapy and retreated as needed.

2.2 Preparation of antigens

S. mansoni adult worm soluble antigen (SWAP) - Swiss female mice (4-6 weeks) were infected subcutaneously, with 100 cercariae. After 45 days the animals were sacrificed by cervical dislocation, and underwent perfusion of the hepatic portal system using saline solution 0.85% plus 50 U/l heparin.[13] Adult worms were washed three times with 0.15M phosphate buffer saline pH 7.2, submitted to mechanical grinding (Virtiz Precisa, Switzerland), and centrifuged at 9500g for 1 hour at 4ºC (Eppendorf AG, Germany). The supernatant obtained was dialyzed in cellulose membrane (Sigma-Aldrich, USA) against saline solution 0.9% for 48 hours at 4ºC. Antigen was centrifuged at 1250g for 15 minutes at 4ºC and supernatant was stored at -20ºC. An aliquot was submitted to protein assessment (Nanodrop, Thermo Scientific 2000, USA) and the obtained concentration was used as parameter in the standardization of the immunoassay for detection of human and murine antibodies, called here as ELISA-SWAP.

S. mansoni eggs soluble antigen (SEA) - After performing the perfusion of hepatic portal system of infected mice, the liver of these animals was removed to provide egg recovery. The antigen used in this study was prepared as previously described.[14] Eggs of *S. mansoni* were homogenized and ground in Virtiz (Virtiz Precisa) with 0.85% saline solution for 40 minutes. The homogenate was centrifuged at 9500g for an hour at 4ºC. After 48 hours of dialysis in cellulose membrane (Sigma-Aldrich) against 0.9% saline solution, the supernatant was submitted to protein assessment (Nanodrop, Thermo Scientific 2000). The final concentration was used as parameter in the standardization of the immunoassay for detection of human and murine antibody, named here as ELISA-SEA.

2.3 Evaluation of Enzyme-linked Immunosorbent Assays

ELISA-SWAP and -SEA were first standardized with sequentially incubation of diluted sera (1:50, 1:150, 1:300 in PBS), diluted conjugate (1:40000, 1:60000, 1:80000, 1:100000 in PBS-T 0.05%), and finally, with substrate 3,3',5,5-tetramethylbenzidine solution (Invitrogen, USA) (TMB:H2O2) to determine sera and anti-IgG conjugated to peroxidase units of reactivity using a dilution curve. Two patient sera were selected for the construction of the standard curve.

ELISA-SWAP - Microtiter plates MaxiSorpTM Surface (NUNC, Denmark) were sensitized with 100 µl/well of 1 µg/ml of SWAP diluted in buffer 0.05M carbonate-bicarbonate pH 9.6 for 16 hours at 4ºC. The plates were washed three times with 0.15M phosphate buffer saline pH 7.2 with 0.05% of Tween 20 (LGC Biotecnologia, BR) (washing buffer) and, the non specific sites were blocked with 10% fetal bovine serum in washing buffer at 37ºC for an hour. After new washing steps, 100 µl of sera samples diluted 1:50 in PBS were added in triplicate into each well and the plates were incubated at room temperature for an hour. Following, the plates were submitted to washing steps and incubated at room temperature for an hour with conjugated anti-IgG human peroxidase (Southern Biotech, USA) diluted 1:60000 in washing buffer. The plates were washed again and 100 µl of substrate TMB/H2O2 were added to each well. The reaction was stopped after 20 minutes of incubation in the dark by addition of 50 µl/well of 2N sulfuric acid. The results were obtained as absorbance values at 450 nm in microplate reader (Bio-Rad Laboratories 3550, JA).

ELISA-SEA - Microtiter plates MaxiSorpTM Surface (NUNC) were sensitized with 100 µl/well of 3 µg/ml of SEA antigen diluted in buffer 0.05M carbonate-bicarbonate buffer pH 9.6 for 16 hours at 4ºC. The plates were washed three times with 0.15M phosphate buffer saline pH 7.2 with washing buffer and the non specific sites were blocked with 10% fetal bovine serum in washing buffer at 37ºC for an hour. After new washing steps, 100 µL of sera samples diluted 1:150 in PBS were added in triplicate into each well and the plates were incubated at room temperature for an hour. Following, the plates were submitted to washing steps and incubated at room temperature for an hour with conjugated anti-IgG human-peroxidase (Southern Biotech) diluted 1:40000 in washing buffer. The plates were washed and the results were obtained as described for ELISA-SWAP.

2.4 Statistical analysis

Data deriving from absorbance values were analyzed with Minitab software by Kolmogorov-Smirnov normality test. Normal distributed data were analyzed by Student's *t* test and non-normal distributed data were analyzed by Mann-Whitney test, $p \leq 0.05$ as significance level. Significance levels for percentages were determined by Chi-square (χ^2) analysis ($p \leq 0.05$ as significance level). The sensitivity, specificity, cut off values, likelihood ratio and positive predictive values were determined by Prism 5 software. The agreement between the methods was measured using the Cohen coefficient[15] and analyzed according Landis & Koch

definition,[16] with software ComKappa 2.0: 1.00 - 0,81 almost perfect; 0,80 - 0,61 substantial; 0,60 - 0,41 moderate; 0,40 - 0,21 fair; 0,20 - 0 slight; < 0 poor.

3 Results

ELISA-SWAP and –SEA standardization was first performed in order to determine sera and anti-IgG conjugated to peroxidase (IgG-HRP) units of reactivity using a dilution curve. Selected patients samples showed high reactivity in both ELISA tests and high parasite burden in Kato-Katz. The figure 1 depicts the titration patterns of each selected sera.

Human diagnostic characteristics of the ELISA methods were first determined separately in order to evaluate the sensitivity and specificity of both assays. For this purpose, faecal samples from 40 inhabitants from the low endemic area selected were diagnosed by Kato-Katz method as the gold standard according to the World Health Organization and divided into negative and positive patients. All the positive patients presented low parasite burden (1-39 epg; $\mu = 12 \pm 11$), but two showed 156 and 555 epg of faeces. Plus, 20 negative samples obtained from non endemic area individuals were used as negative controls.

ELISA-SWAP and ELISA-SEA were both capable of detecting specific IgG antibodies in 19 (95%) of 20 positive samples from patients from an endemic area for *S. mansoni*, including all the low parasite burden samples, but one. ELISA-SWAP was capable to detect 20 negative samples from the non-endemic residents (100%) (Figure 2), whereas ELISA-SEA was capable to diagnose 17 negative samples (85%) (Figure 3). The cut-off values were determined based in the ROC curve as 0.15 for ELISA-SWAP and 0.25 for ELISA-SEA.

To verify the diagnostic concordance between the egg counts by Kato-Katz and the IgG absorbance values demonstrated by ELISA, a correlation was done between the three methods according to the parasite burden, as shown on table 1.

Although ELISA-SWAP and ELISA-SEA presented a Kappa index correlation of 0.81 ($\pm$ 0.15) based on Landis & Koch definition, the first method presented a better correlation with Kato-Katz technique with a Kappa index of 0.85 ($\pm$ 0.16), indicating almost perfect agreement. Whereas, ELISA-SEA presented a substantial agreement in comparison with Kato-Katz egg counts, with a Kappa index of 0.75 ($\pm$ 0.16).

The analysis of discordant results revealed that 11 negative samples from endemic area patients were positive for IgG detection in both ELISA. Plus, three Kato-Katz negative samples were positive for ELISA-SEA.

Since cross-reactivity might occur with indirect immunological assays, final analysis were performed for both ELISA techniques using sera samples from 9 patients that were uniquely positive for *Trichuris trichiura*, hookworms or *Enterobius vermicularis*. Among these 9 patients, 5 showed high titers of IgG in ELISA-SWAP and, 7 in ELISA-SEA (table 2).

Finally, the sensitivity values of ELISA assays were determined by comparison of the absorbance values and the number of eggs estimated by Kato-Katz technique. An important finding showed that only 14 patients showed eggs in faeces when 12 slides of the first sample were analyzed. Differently, eggs were found in faeces of more 6 patients when 18 slides of 4 different samples were analyzed. So, using the complete analysis that diagnosed 20 patients as positive for schistosomiasis, both ELISA assays presented 90% of sensitivity, whereas ELISA-SWAP presented 95% and ELISA-SEA presented 85% of specificity.

4 Discussion

Diagnosis of heavily infected patients with *S. mansoni* (high worm burden) can be easily done with field-applicable parasitological methods.[8] However, it is increasingly noticeable the number of patients with low parasite burden that are unlikely to be correctly diagnosed by stool examinations. Together, mass treatment of individuals from endemic areas performed with single-dose oral can lead to persistence of low parasite burden infections. In an attempt to attain the accurate diagnosis for these patients, there is need for sensitive diagnostic methods that can be used to confirm parasitological methods that may show very low sensitivity for those patients.[17]

Patent schistosome infection is highly immunogenic and there is no difficulty in demonstrating the presence of anti-*Schistosoma* antibodies or cell-mediated immune responsiveness in infected subjects. Many different assays have been used to display such immunological reactivity, including skin hypersensitivity reactions against injected antigens, complement fixation, indirect immunofluorescence, indirect haemagglutination, radioimmunoassay, and various flocculation and precipitation tests.[18, 19] But thus far all of these methods showed low sensitivity demonstrating a lack of correlation between results

from direct and indirect methods. In this study, we evaluated the efficiency of two different ELISA assays based on the detection of IgG antibodies for easy to obtain crude antigens (adult worm soluble antigens and egg soluble antigens) in reproducing results obtained by an intensive and extensive search of positive cases by Kato-Katz technique, considered by the World Health Organization as the standard method for schistosomiasis mansoni.

Worm antigens are a most abundant and easily obtained source of antigenic material. Crude extracts of worms work well in ELISA assay and, generally gives higher sensitivity and specificity than those from larvae.[20] Jointly, antigens from schistosome eggs are highly immunogenic. Their exit from the host after all depends on it and, in consequence, anti-*Schistosoma* antibody titers rise after the onset of infection patency, as defined by the detection of eggs in clinical specimens.[21] It is noticeable that both antigens may lead to a low cost method.

A method should be both sensitive and specific for the human diagnosis, each of which is defined mathematically. This applies not only to those living in endemic areas, but also to tourists and other travelers to the region who return home infected. Based on that, forty samples were obtained from individuals in an endemic area for *S. mansoni* and them examined by eighteen glass slides of the parasitological method. From the 40 samples, 18 were from positive patients with low parasite burden (01 to 200 epg/faeces) and 2 were from positive patients with high parasite burden (201-600 epg/faeces). Other 20 samples were negative for *S. mansoni* eggs by Kato-Katz technique. Jointly, sera samples from 20 non-endemic properly diagnosed individuals were also obtained.

Due to the difficulty of diagnosing patients with low parasite burden with the gold standard method, the inclusion of individuals in this study was done with an intensive search for eggs in faeces that was done with 18 slides from 4 different samples by Kato–Katz thick smears. We showed that an important difference was seen when analyzing 12 slides from a single sample when only 14 patients were properly diagnosed, while 6 more patients were diagnosed when 18 slides were used. As found by others, [22, 23] absence of infection cannot be deduced from a reduced number of Kato–Katz thick smears, and examination of multiple stool samples is recommended in order to estimate prevalence more accurately.

We could find consistent results for ELISA-SWAP after the analysis of human samples reaching a Kappa index of 0.85 as an almost perfect agreement in comparison to Kato-Katz

technique. Nineteen of all 20 negative non-endemic human samples were seen as negative for this immunological method. The same result was found for positive endemic individuals, including 17 samples from patients with very low parasite burden (1-40 epg/faeces) and two samples from patients with high parasite burden, according to the World Heath Organization. Discordant results were found for eleven patients that were negative for *S. mansoni* eggs but jointly presented significant IgG titers. Not surprisingly, all of these patients were from endemic area and could have been previously infected. Jointly, nine of these individuals presented eggs of other helminthes in faeces, as hookworms, *Trichuris trichiura* and *Enterobius vermicularis*.

Finally, 95% of all low parasite burden positive cases and non-endemic negative cases were properly detected by ELISA-SWAP. The sensitivity and specificity calculated for the cut off value of 0.137 were then 95%. These data show that the methodology assumed in this work lead to a higher sensitivity and specificity than immunological methods standardized by others.[17, 24]

Analysis with ELISA-SEA for non-endemic individuals showed that also 17 sera samples of all the 20 presented likelihood for the negative diagnosis. For the positive patients instead, one sample was diagnosed as false negative with no significant IgG titers. The kappa index of 0.75 showed a moderate agreement with Kato-Katz technique and, 0.81 in comparison to ELISA-SWAP. ELISA-SEA showed a poorer confidence with 85% of specificity, although it reached 95% of sensibility for a cut off of 0.223. The ELISA-SEA data were found to be very similar to data found by other authors,[25] differently from data found for ELISA-SWAP that were shown to be accurate for the confirmation diagnosis allied to Kato-Katz technique.

Lack of sensitivity is a common problem to both parasitological and antigen/antibody-detection methods and usually immunological methods have poor specificity (i.e. a high proportion of egg-negative, antibody-positive results).[26] Solutions are available as we had shown. All the data obtained in this work demonstrated that a simple technique as ELISA using SWAP can reach a suitable moderate Kappa index agreement and can be perfectly capable of performing the diagnosis of schistosomiasis mansoni even in individuals living in endemic areas showing very low egg output. As an additional tool, these indirect immunological methods can be used in association with a small amount of slides for the Kato-Katz examination as recommended by the World Health Organization, or with multiple slides and samples as recommended by our work.

Results from individual laboratories and from multicentre trials suggest that egg antigens provide greater diagnostic sensitivity and specificity than worm antigens for the detection of infection. [20, 27, 28] In disagreement, we had shown that the use of egg antigens as a tool for diagnosing schistosomiasis can be controversial due to false positive results and the cross-reactivity. The fact that after patency there is an increase in anti-worm, in addition to anti-egg antibody titers is perhaps best explained by the production (at least initially) of antibodies specific for glycanic epitopes which schistosome larvae and worms, and probably also other parasites, have in common.[29] Extracts prepared by homogenizing *Schistosoma* eggs contain a large number of molecules, although only a minority of the constituents of SEA might be released by viable eggs in vivo, as demonstrated in vitro.[30]

5 Conclusions

Briefly, the high sensitivity and specificity of a single ELISA-SWAP examination has been confirmed. Otherwise, ELISA-SEA presented consistently lower results than ELISA-SWAP when compared to Kato-Katz results and, a significant number of false positive cases when compared to ELISA-SWAP. This warrants additional studies, especially for researches directly related to individuals from schistosomiasis endemic areas that usually present low parasite burden and are hardly well diagnosed. We showed interesting results with two simple and well known tests as indispensable and additional tools for patients diagnosis and analysis on rigorous monitoring of community-based helminthes control programs.

Declarations

This work was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq-Decit 34/2008), Research Suport Foundation of the State of Minas Gerais (FAPEMIG), Fundação Oswaldo Cruz (FIOCRUZ), Centro de Pesquisas René Rachou (CPqRR), Research Program for SUS: shared management in health (PPSUS). CNPq also supported Coelho PMZ as a Senior Fellow and Grenfell RFQ as a Doctoral Fellow.

Ethical approval

This project was approved by the Ethical Research Committee of the René Rachou Research Institute-Fiocruz (CEPSH/CPqRR 03/2008) and the National Brazilian Ethical Board (784/2008, CONEP 14886). The study objectives were presented and explained to all

participants and written consent was obtained through signing a form before admission to this study.

References

1. Utzinger J, N'Goran EK, N'Dri A, et al. Oral artemether for prevention of *Schistosoma mansoni* infection: randomized controlled trial. *Lancet* 2000; **355**:1320-5.

2. King CH, Dangerfield-Cha M. The unacknowledged impact of chronic schistosomiasis. *Chronic Illn* 2008; **4**(1):65-79.

3. Katz N, Chaves A, Pellegrino J. A simple device for quantitative stool thick-smear technique in schistosomiasis mansoni. *Rev Inst Med Trop Sao Paulo* 1972; **14**:397–400.

4. Enk MJ, Katz N, Zech Coelho PM. A case of *Schistosoma mansoni* infection treated during the prepatent period. *Nat Clin Pract Gastroenterol Hepatol* 2008; **5**(2):112-5.

5. Siqueira LMV, Coelho PMZ, Oliveira AA, et al. Evaluation of two coprospic techniques for the diagnosis of schistosomiasis in a low-transmission area in the state of Minas Gerais, Brazil. *Mem Inst Oswaldo Cruz* 2011; **106**(7):844-50.

6. Coelho PMZ, Jurberg AD, Oliveira AA, Katz N. Use of a saline gradient for the diagnosis of Schistosomiasis. *Mem Inst Oswaldo Cruz* 2009; **104**(5):720-3.

7. Jurberg AD, Oliveira AA, Lenzi HL, Coelho PMZ. A new miracidia hatching device for diagnosing Schistosomiasis. *Mem Inst Oswaldo Cruz* 2008; **103**(1):112-4.

8. Gomes LI, Marques LHS, Enk MJ, Oliveira MC, Coelho PMZ, Rabello A. Development and Evaluation of a Sensitive PCR-ELISA System for Detection of *Schistosoma* Infection in Feces. *PLoS Negl Trop Dis* 2010; **4**(4):e664.

9. Abdel-Fattah M, Al-Sherbiny M, Osman A, Charmy R, Tsang V. Improving the detection limit of quantitative diagnosis of anti-*S. haematobium* antibodies using Falcon Assay Screening Test (FAST) ELISA by developing a new standard curve. *Parasitol Res* 2011; **108**:1457–63.

10. Gonçalves MM, Barreto MG, Peralta RH, Gargioni C, Gonçalves T, Igreja RP, Soares MS, Peralta JM. Immunoassays as an auxiliary tool for the serodiagnosis of Schistosoma mansoni infection in individuals with low intensity of egg elimination. Acta Trop 2006; **100**(1-2):24-30.

11. Igreja RP, Matos JA, Gonçalves MM, Barreto MM, Peralta JM. Schistosoma mansoni-related morbidity in a low-prevalence area of Brazil: a comparison between egg excretors and seropositive non-excretors. Ann Trop Med Parasitol 2007; **101**(7):575-84.

12. World Health Organization. Report of the Scientific Working Group Meeting on Schistosomiasis. *Geneva* 2008;14-16 november.

13. Pellegrino J, Siqueira A. Técnica de perfusão para colheita de *Schistosoma mansoni* em cobaias experimentalmente infestadas. *Rev Bras Malar Doenças Trop* 1956; **8**:589-97.

14. Colley DG, Lewis FA, Goodgame RW. Immune Responses During Human Schistosomiasis. Iv. Induction Of Suppressor Cell Activity By Schistosome Antigen Preparations And Concanavalin A. *J Immunol* 1978; **120**:1225.

15. Cohen J. Weighted kappa: nominal scale agreement with provisions for scales disagreement of partial credit. *Psychol Bull* 1968; **70**:213-20.

16. Landis JR, Koch GG. The measurement of observer agreement for categorical data. *Biometrics* 1977; **33**(1):159–74.

17. Sorgho H, Bahgat M, Poda JN, et al. Serodiagnosis of *Schistosoma mansoni* infections in an endemic area of Burkina Faso: performance of several immunological tests with different parasite antigens. *Acta Tropica* 2005; **93**:169–80.

18. Maddison SE. Serodiagnosis of parasitic infections. *Clin Microbiol* 1991; **4**:457–69.

19. Hamilton JV, Klinkert M, Doenhoff MJ. Diagnosis of schistosomiasis: antibody detection, with notes on parasitological and antigen detection methods. *Parasitology* 1998; **117**:S41–S57.

20. Mott KE, Dixon H. Collaborative study on antigens for immunodiagnosis of schistosomiasis. *Bull World Health Organ* 1982; **60**:729–53.

21. Doenhoff MJ. Granulomatous inflammation and the transmission of infection: schistosomiasis - and TB too? *Immunol Today* 1998; **19**:462–7.

22. De Vlas SJ, Gryseels B. Underestimation of Schistosoma mansoni prevalences. *Parasitol Today* 1992; **8**(8):274-7.

23. Kongs A, Marks G, Verlé P, Van der Stuyft P. The unreliability of the Kato-Katz technique limits its usefulness for evaluating *S. mansoni* infections. *Trop Med Int Health* 2001; **6**(3):163-9.

24. Nassr AKh, Hassan M.M., Abdel Salam F.M., Lashin A.H., Shahin W.A., Amin H. IgG isotypes in schistosomiasis patients before and after praziquantel. *J Egypt Soc Parasitol* 2002; **32**(3):931-52.

25. Stothard JR, Sousa-Figueiredo JC, Standley C, et al. An evaluation of urine-CCA strip test and fingerprick blood SEA-ELISA for detection of urinary schistosomiasis in schoolchildren in Zanzibar. *Acta Trop* 2009; **111**(1):64-70.

26. Doenhoff MJ, Chiodini PL, Hamilton JV. Specific and sensitive diagnosis of schistosome infection: can it be done with antibodies? *TRENDS in Parasitology* 2004; **20**(1):35-3.

27. McLaren M, Draper CC, Roberts JM, et al. Studies on the enzyme linked immunosorbent assay (ELISA) test for *Schistosoma mansoni* infections. *Ann Trop Med Parasitol* 1978; **72**:243–53.

28. Mott KE, Dixon H, Carter CE, et al. Collaborative study on antigens for immunodiagnosis of *Schistosoma japonicum* infection. *Bull World Health Organ* 1987; **65**:233–44.

29. Alarcón de Noya B, Colmenares C, Lanz H, Caracciolo MA, Losada S, Noya O. *Schistosoma mansoni*: immunodiagnosis is improved by sodium metaperiodate which reduces cross-reactivity due to glycosylated epitopes of soluble egg antigen. *Exp Parasitol* 2000; **95**:106-12.

30. Ashton PD, Harrop R, Shah B, Wilson RA. The schistosome egg: development and secretions. *Parasitology* 200; **122**:329-38.

Tables, Figures and legends

Table 1. Correlation between ELISA-SWAP and ELISA-SEA with Kato-Katz results obtained after the analysis of 18 slides from 4 faeces samples from endemic area positive patients.

Eggs/g of faeces	*ELISA-SWAP*			*ELISA-SEA*			*Number of individuals*
	Absorbance	*%*	*PPV*[1]	*Absorbance*	*%*	*PPV*[1]	
1-10	0.316	100	1	0.570	100	1	11
11-20	0.247	100	1	0.740	100	1	4
21-30	0.116	50	0.5	0.541	50	0.5	2
31-40	0.373	100	1	0.435	100	1	1
100-200	0.216	100	1	0.634	100	1	1
500-600	0.138	0	0	0.237	0	0	1

[1]*Positive Predictive Value*

Table 2. Cross-reactivity results from ELISA-SWAP and ELISA-SEA using sera samples from patients previously diagnosed by schistosomiasis mansoni and other helminthiasis by Kato-Katz technique.

Endemic area individuals	*Positive (+) or Negative (-) results*					
	Kato-Katz	*ELISA-SWAP*	*ELISA-SEA*	*Hookworms*	*Trichuris trichiura*	*Enterobius vermicularis*
1	-	+	+	+	-	-
2	-	-	+	+	-	-
3	-	+	+	+	-	-
4	-	-	+	+	-	-
5	-	-	-	+	-	-
6	-	+	+	+	-	-
7	-	+	+	+	-	-
8	-	+	-	+	-	+
9	-	-	+	-	+	-

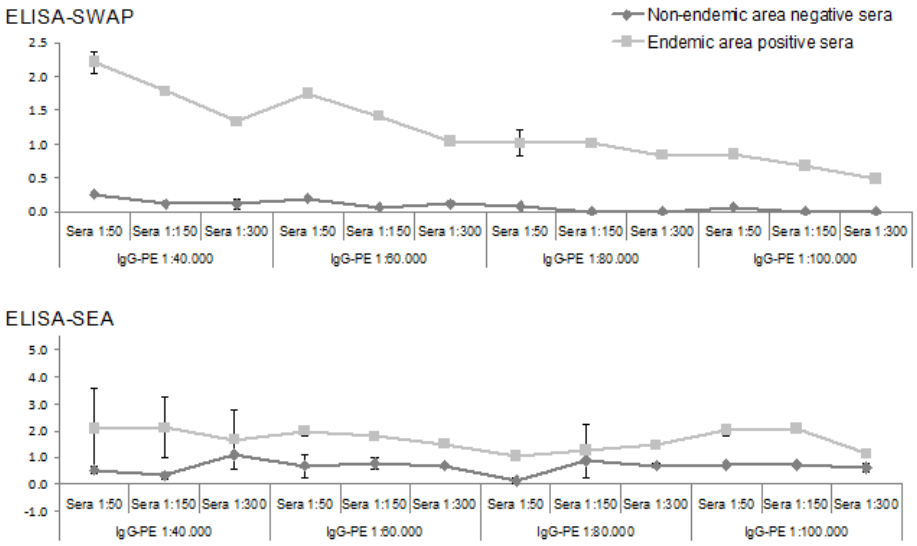


Figure 1. Human sera titration in ELISA-SWAP and ELISA-SEA. Antigen: SWAP or SEA (1µg/ml, 3µg/ml, respectively); Sera: endemic area positive sera and non-endemic area negative sera, diluted (1:50-1:300) in PBS; Conjugate: peroxidase labeled anti-human IgG diluted (1:40000-1:100000) in PBS-T; substrate: TMB/H2O2.

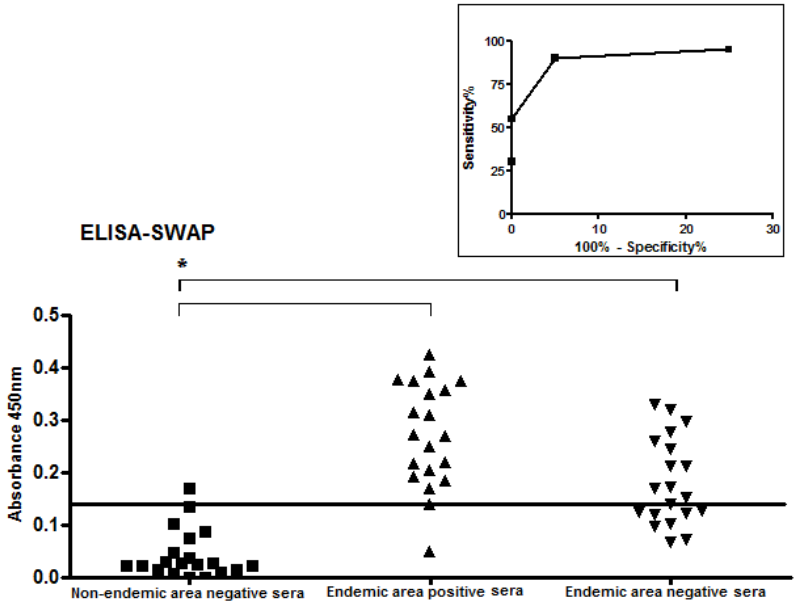


Figure 2. Reactivity of 60 serological samples from non-endemic and endemic individuals in ELISA-SWAP. Antigen: SWAP (1µg/ml); sera: non-endemic negative samples, endemic

Kato-Katz positive and negative samples diluted 1:50 PBS-T; conjugate: anti-IgG PE diluted 1:60000 PBS-T; substrate: TMB/H2O2. Black line indicates the cut off value of 0.15. The box graphic indicates the Roc Curve for 90% of sensitivity and 95% of specificity. Statistical results are represented by * for *p* value < 0.001.

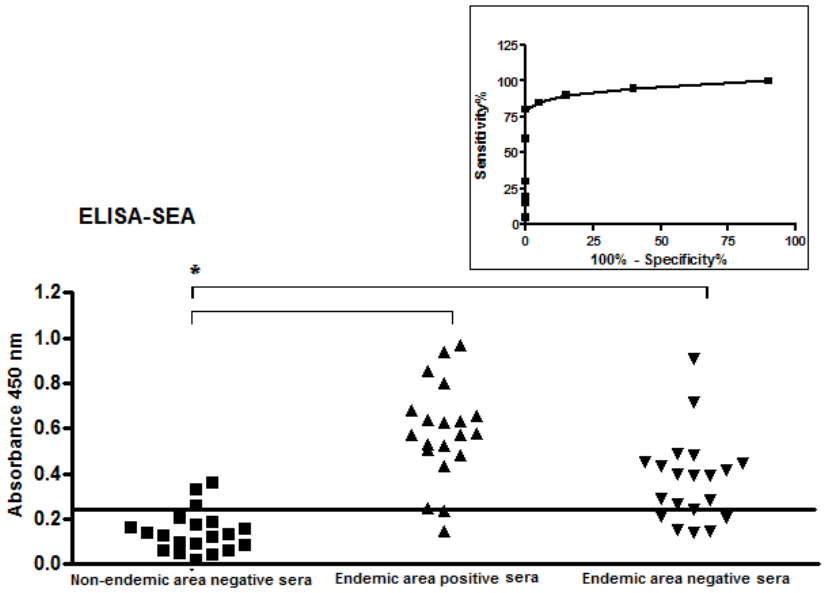


Figure 3. Reactivity of 60 serological samples from non-endemic and endemic individuals in ELISA-SEA. Antigen: SEA (3µg/ml); sera: non-endemic negative samples, endemic Kato-Katz positive and negative samples diluted 1:150 PBS-T; conjugate: anti-IgG PE diluted 1:40000 PBS-T; substrate: TMB/H2O2. Black line indicates the cut off value of 0.25. The box graphic indicates the Roc Curve for 90% of sensitivity and 85% of specificity. Statistical results are represented by * for *p* value < 0.003.

4.4 ARTIGO 4

Acute schistosomiasis: An innovative method for the diagnosis of travelers recently infected in a new focus of *Schistosoma mansoni*

Rafaella Grenfell[1], Watson Martins[1], Sandra Drummond[3], Carlos Maurício Antunes[5], Izabela Voieta[4], Alba Otoni[4], Áureo Oliveira[1], Vanessa Silva-Moraes[1], Eduardo Ribeiro[1], Edward Oliveira[2], Cristina Fonseca[1], José Roberto Lambertucci[4], Paulo Marcos Z. Coelho[1]

[1]Laboratório de Esquistossomose, Centro de Pesquisas René Rachou, Fundação Oswaldo Cruz (Fiocruz), Belo Horizonte, Minas Gerais, Brazil.

[2]Laboratório de Pesquisas Clínicas, Centro de Pesquisas René Rachou, Fundação Oswaldo Cruz (Fiocruz), Belo Horizonte, Minas Gerais, Brazil.

[3]Fundação Nacional de Saúde, Minas Gerais, Brazil.

[4]Serviço de Doenças Infecciosas e Parasitárias, Faculdade de Medicina, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil.

[5]Santa Casa de Misericórdia de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil.

Corresponding Author. Mailing address: Fundação Oswaldo Cruz, Centro de Pesquisas René Rachou, Av. Augusto de Lima, 1715, Belo Horizonte, MG, Brazil. 30.190-002. Phone: 55 31 3349 7740, e-mail: coelhopm@cpqrr.fiocruz.br

ABSTRACT

Background: The diagnosis of schistosomiasis mansoni on early stages of infection is important to prevent late morbidity. A simple, cheap, sensitive and specific assay for routine diagnosis of schistosome infection based on the detection of specific IgG for schistosomula tegument antigen was developed by our group (ELISA-SmTeg). Methodology/Principal Findings: We describe here an acute outbreak involving a travel group of 80 individuals from a non-endemic area of the State of Minas Gerais, Brazil. These individuals were in contact with a freshwater pool where *Biomphalaria glabrata* was found. Results obtained from our new methodology were compared to IgG antibody titers against adult worm antigens (SWAP) by ELISA and, also to parasitological examination, ultrasonography and clinical findings.

ELISA-SmTeg was capable of detecting 64 positive cases among the 80 individuals participating at the survey with a positivity ratio of 80% and a higher sensitivity than ELISA-SWAP that was only sensitive for 56% of positive cases. Besides, a significant correlation was found for the severity of the infection and the specific IgG titers against SmTeg. Conclusions/significance: Our data showed that ELISA-SmTeg might serve as the initial diagnostic tool for acute stages of the infection in community-based helminth control programs or for non-endemic patients' surveillance.

Keywords: Schistosomiasis mansoni, Immunological assay, Schistosomula tegument antigen and Acute outbreak.

AUTHOR SUMMARY

Schistosomiasis is a neglected disease caused by helminthes of the genus *Schistosoma*. The transmission cycle requires the contact with contaminated water where specific snails are found as intermediate hosts. The diagnosis of schistosomiasis mansoni in individuals from non-endemic countries is challenging and few data are available on the accuracy of serological diagnosis in those patients. This study aimed to evaluate a new immunological assay called ELISA-SmTeg for the early detection of IgG antibodies against schistosomula antigens in serum from a group of travelers hosted in a new focus of schistosomiasis mansoni in Brazil. Data revealed a significant sensitivity of our new assay in comparison to ELISA-SWAP immunological assay. Results were also compared to Kato-Katz parasitological technique, ultrasonography and individual clinical findings. In conclusion, ELISA-SmTeg showed a good result for the initial diagnosis of patients with the acute form of the infection.

INTRODUCTION

Schistosomiasis continues to be a major worldwide public health problem that affects 200 million people and about 779 million people live in endemic areas in the Middle East, South America, Caribbean, Southeast Asia and particularly sub-Saharan Africa (1). Nonetheless, the disease is seen in growing numbers in recently detected foci of transmission due to increased immigration from endemic areas and tourism. Severe sequelae due to schistosomiasis are rare in travelers from non-endemic areas because the risk of developing significant pathological manifestations after short-term exposure that frequently result in high parasite burden is limited by the life span of the adult worm. The most important factor regarding the difficulty

of controlling the spread of the disease is the absence of diagnostic methods capable of detecting the disease, especially in the early patent phase.

Clinical findings make possible the division of the disease in two phases. First is the pre-patent phase, which includes cercarian dermatitis caused by the penetration of cercariae into the skin and the local presence of schistosomula. Together, the Katayama fever that may appear 3 to 7 weeks after an exposure and is characterized by fever, anorexia, abdominal pain and headaches. In this phase, the inflammatory reaction is well established and involves predominantly Th1 bias, when immunoglobulin levels are elevated in the serum (2). The second phase that occurs after 40 to 60 days post-infection is the acute phase, characterized by the egg laying and a predominantly Th2 inflammatory response. Its clinical manifestations vary depending on the parasite load and host immune response. Patients may present hepatointestinal, hepatosplenic, schistosomal myeloradiculopathy and acute forms with the latter being a main indicator of schistosomiasis severity (3, 4).

Although the treatment with oxamniquine on the first days after the infection is effective in obtaining a coprological cure (5, 6, 7), the lack of methods to detect the pre-patent phase precludes the patients from a successful therapy. The diagnosis is first guided by the patient history of water contact in an endemic area. This evidence is further confirmed by the presence of the *S. mansoni* eggs in stool samples after egg laying, on post-postural period (8). Even during early patent phase, the absence of *S. mansoni* eggs in the faeces does not rule out the diagnosis due to the low specificity this methodology may present. There is usually a miliary distribution of eggs in the organs of the host and laparoscopy frequently reveals whitish nodules, as granulomas, on the surface of liver, intestines and visceral peritoneus. Hence, it is expected that the parasitological methods do not have properly efficiency for the diagnosis, predominantly for patients in acute phase once the female has not yet laid eggs (2).

Serological techniques have proven advantages in diagnosis of schistosomiasis (9,10,11) since the humoral response especially related to IgG antibodies in acute patients to egg and worm antigens does not differ from the chronic phase as a high level is detected, but they are not yet part of the routine of neither private nor the public health laboratories (12).

Regarding the urgent necessity of having a reliable diagnostic tool to determine pre-patent positive cases, this work focus on the evaluation of an Indirect Enzyme Linked Immunosorbent Assay using schistosomula tegument antigen (ELISA-SmTeg). This

methodology was first standardized using 7 to 10 days post-infection mice sera when it showed to be a promising diagnostic tool for early stages of the disease. In this report, we describe a case involving a group of travelers in Brazil that was exposed to a contaminated freshwater pool. Early detection of the clinical symptoms or signs of schistosomiasis mansoni combined with rapid investigation of the entire group enabled us to observe this group prospectively.

METHODS

Ethics Statement

The Ethical Committee of Fundação Nacional de Saúde in Minas Gerais, Brazil granted ethical approval for this study. Written informed consent was obtained from each individual.

Travelers group survey

Eighty individuals were part of a group that was hosted on a country house in Colônia do Teodoro, a recently endemic focus for schistosomiasis mansoni, next to the city of São João Del Rei in the State of Minas Gerais, in southeast Brazil, from December/2009 to March/2010. The individuals were exposed to a freshwater pool where *Biomphalaria glabrata* snails were found. To identify symptomatic cases, we initiated the interviews with exposed travelers on March/2010. Follow-up focused on the detection of symptoms or signs of chronic schistosomiasis previously described in travelers, together with current symptoms and signs, including pulmonary symptoms, gastrointestinal symptoms (e.g. abdominal pain or diarrhea), constitutional symptoms (e.g. fatigue or weight loss), and myeloradiculopathy involvement.

Schistosoma mansoni infection was defined as exposure to the freshwater pool plus one of the following criteria: presence of eggs in faecal samples, IgG antibody titers by ELISA using adult worm antigens, myeloradiculopathy detected by ultrasonography, and/or symptoms compatible with acute schistosomiasis. Symptoms defining infection in this group were fever, cough, cercarial dermatitis, and angioedema. Nonspecific symptoms (e.g. fatigue, gastrointestinal complaints, and headache) were reported as well. Blood samples were collected by venipuncture of all those patients. Individual serum samples were obtained after centrifugation of blood samples at 3000g for 5 minutes. These samples were maintained at –20^{o}C. Twenty four out of 80 individuals agreed to submit faeces for examination. These samples were firstly analyzed by Kato-Katz parasitological assay (13). Three glass slides

(41.7 mg/smear) of a single fecal sample were examined. Five patients were hospitalized, one with advanced pulmonary stage, one with schistosomal myeloradiculopathy and, three with severe dehydration caused by the hepatointestinal form.

Positive individuals for one of the described criteria were treated with a single oral dose of praziquantel (50 mg/kg), according to the recommendation of the Brazilian Ministry of Health.

ELISA-SWAP was performed in microtiter plates MaxiSorpTM Surface (NUNC, Denmark) sensitized with 100µl/well of 1g/ml of SWAP diluted in buffer 0.05M carbonate-bicarbonate pH 9.6. The incubation was done for 16 hours at 4ºC. The plates were washed three times with 0.15M phosphate buffer saline pH 7.2 with 0.05% of Tween 20 (LGC Biotecnologia, BR) (washing buffer) and, the non specific sites were blocked with 10% fetal bovine serum in washing buffer at 37ºC for an hour. After new washing steps, 100µl of human serum samples diluted 1:50 in PBS were added in triplicate into each well and the plates were incubated at room temperature for an hour. Following, the plates were submitted to washing steps and incubated at room temperature for an hour with conjugated anti-IgG human peroxidase (Southern Biotech, USA) diluted 1:60000 in washing buffer. The plates were washed again and 100µl of substrate 3,3',5,5-tetramethylbenzidine solution (TMB/H2O2) (Invitrogen, USA) were added to each well. The reaction was stopped after 20 minutes of incubation in the dark by addition of 50µl/well of 2N sulfuric acid. The results were obtained as absorbance values at 450 nm in microplate reader (Bio-Rad Laboratories 3550, JA). The cut off value of the ELISA-SWAP was 0.188 (standard deviation of 0.08), determined by ROC curve (A = 0.70, $p < 0.0001$).

Evaluation of the Indirect Enzyme-linked Immunosorbent Assay using schistosomula tegument antigen (ELISA-SmTeg)

Serologic studies were conducted at the Brazilian Excellence Center for the Diagnosis of Schistosomiasis mansoni, Oswaldo Cruz Foundation (Brazilian Ministry of Health) in Belo Horizonte. The serologic test performed is based on the detection of specific IgG against schistosomula tegument antigen by ELISA methodology.

Preparation of S. mansoni schistosomula tegument antigen - Cercariae of the LE strain were obtained at the Laboratory of Malacology of the René Rachou Research Center, Oswaldo

Cruz Foundation and were mechanically transformed into schistosomula (14). Briefly, cercariae were placed into conical tubes and left in ice bath for 30 minutes before centrifugation (Eppendorf Centrifuge 5820R, Hamburg, GR) at 200g for 3 minutes at 4°C. The pellet was ressuspended in cold Earl's salts plus lactalbumin hydrolyzate medium (ELAC). The cercarial tails were broken in vortex (Scientific Industries Genie-2, New York, USA) at maximum speed for 2 minutes. Later the tails were removed from the medium through repeated washing steps with ELAC at 37°C, and schistosomula were incubated for 90 minutes at 37°C and washed with 0.9% saline solution. This step was followed by centrifugation at 200g for 1 minute. For tegument removal, 2 ml of 0.3M calcium chloride were added to the schistosomula that was stirred in vortex for 7 minutes and centrifuged at 200g for 1 minute. The supernatant was centrifuged at 50000g for an hour and the pellet enriched of membrane was ressuspended in 0.9% saline and dialyzed against 1.7% saline solution for 72 hours. Protein concentration was determined by Bradford method (15). The final concentration used in standardized tests was 0.52 mg/ml.

Indirect Enzyme-Linked Immunosorbent Assay - Microtiter plates MaxiSorpTM Surface were coated with 100µl per well of SmTeg diluted at 1µl/ml in buffer 0.05M carbonate-bicarbonate pH 9.6 for 16 hours at 4°C. Next, the plates were washed three times with washing buffer. After that, the free sites were saturated with 300µl per well of 2.5% skim milk diluted in washing buffer, incubating at 37°C for 1 hour. After further washing steps, 100µl of individual serum sample (diluted 1:100) in 0.15M phosphate buffer saline pH 7.2 were added to the plates and were incubated at room temperature for 1 hour. The plates were submitted to washing steps and incubated at room temperature for 1 hour with anti-IgG conjugated to peroxidase diluted in washing buffer (anti-human IgG Fc specific peroxidase) at the dilution of 1:60000. Plates were washed again and 100µl of substrate solution were added to each well. The enzymatic reaction was stopped after 10 minutes of incubation in the dark by adding 50µl per well of 2N sulfuric acid. The results were obtained as absorbance values at 450 nm in microplate reader (BIORAD 3550, Tokio, JA). The cut off value of the ELISA-SmTeg was 0.110 (standard deviation of 0.02), determined by ROC curve (A = 0.92, $p < 0.0001$).

Positive and negative controls were properly assayed, also wells without antigen and serum samples as control of nonspecific adsorption of conjugate. The standard dilution was

determined by a dilution curve, performed with the same reagents and equipment based on six different dilutions.

Statistical analysis - Data deriving from absorbance values were analyzed with Minitab software (Minitab Inc, College, USA) by Kolmogorov-Smirnov normality test. Normal distributed data were analyzed by Student's t test and comparisons between methods were done by Chi-square analysis ($p< 0.05$ as significance level). The sensitivity, specificity, cut off values and positivity ratios were determined with Prism 5.0 software.

RESULTS

All the 80 individuals hosted on the country house that had contact with the freshwater pool participated on this survey as serum sample donors after they had been interviewed and the clinical criteria had been identified. Serum samples were submitted to a well established indirect ELISA-SWAP assay that allowed us to identify the levels of specific IgG against *S. mansoni* adult worm antigens of each individual patient. After the *S. mansoni* infection was defined by one of the defined criteria, a correlation was done with a new indirect immunological assay, called ELISA-SmTeg, which has been previously standardized in our laboratory. This assay allows the determination of specific IgG antibody levels against schistosomula tegument antigen as a promising tool for the indirect and confirmatory diagnosis of recently infected individuals with the acute stage of the infection.

The cut off value for this new assay had been previously determined as 0.110 using fifty three volunteers' serum samples as our control group by the ROC curve ($A = 0.92$, p value < 0.0001), as shown on figure 1. It is important to notice that the high sensitivity described was determined with clinical acute phase patients and the same effectiveness was not seen with the chronic form patients (data not shown).

Using the defined cut off value, samples from the 80 individuals were applied on ELISA-SmTeg assay and a first comparison was done with data obtained from another indirect immunoassay usually used as the routine method, the ELISA-SWAP. Figure 2 shows the Optical Density (OD) values of serum samples from each individual patient determined by each assay.

Among the 80 positive patients, only 45 individuals were properly diagnosed by ELISA-SWAP. On the other hand, ELISA-SmTeg showed a higher sensitivity by being capable of

identifying 64 positive individuals (p = 0.001). The positivity ratio of ELISA-SmTeg assay of 80% was superior to 56% obtained by ELISA-SWAP. The Cohen's Kappa Index of 0.385 (standard deviation of + 0.094) indicates fair agreement between both immunological assays, again showing a superior fulfillment of ELISA-SmTeg when diagnosing patients with clinical acute form.

A second analysis was done based on the clinical investigation. *S. mansoni* infection was defined as positive when individuals exposed to the freshwater pool were also positive for at least one of the pre-defined criteria: presence of eggs in stools, IgG antibody titers by ELISA-SWAP (superior to the cut off value of 0.188, schistosomal myeloradiculopathy detected by ultrasonography, and/or symptoms compatible with acute schistosomiasis. Twenty four individuals agreed to be donors of faecal samples that were analyzed by Kato-Katz methodology. Results revealed that these 24 individuals showed eggs in stools and, additionally, demonstrated high levels of specific IgG antibodies for ELISA-SmTeg with an average of 0.401 (standard deviation of + 0.016). Three positive patients with eggs in stools were not detected by ELISA-SWAP showing an OD average of 0.157 (+ 0.026).

Patients with clinical symptoms and/or signs that were properly diagnosed by both methods, Kato-Katz and ELISA-SWAP, showed also high levels of IgG against SmTeg. The IgG levels for these patients were significantly higher than from patients whom were positive only for ELISA-SWAP, presenting no eggs in stools (p = 0.002). Figure 3 shows this relation after patients were divided in four individual groups. Group 1 were limited to patients with symptoms and/or signs of acute schistosomiasis but negative for any other diagnostic method used (total of 28 individuals) whereas, group 2, with 26 individuals, involved patients that were positive for clinical examination plus ELISA-SWAP. Patients with positive results for these two last criteria that, additionally, presented eggs in stools were represented by group 3, with a total of 21 individuals. Accordingly, patients diagnosed as negative ELISA-SWAP, but had all the clinical symptoms compatible with acute schistosomiasis also presented lower IgG titers for SmTeg in comparison to group 2 (p = 0.002). Lastly, all the 5 hospitalized patients with severe schistosomiasis were represented by group 4. This last group allows us to reaffirm that ELISA-SmTeg presented once more a high positivity ratio by being capable of diagnosing all the severe cases and, additionally presented a correlation between specific IgG levels and the severity of the infection. Statistical differences were found when comparisons

were made for the antibody level of patients on groups 1 and 2 in comparison to group 4 (p = 0.003 and 0.001, respectively).

DISCUSSION

Acute schistosomiasis is one of the clinical manifestations of infection with *Schistosoma* sp. (16). The pre-postural phase occurs after cercarial penetration in skin and, clinical symptoms are due to the migration of young parasite to portal system followed by the maturation of male and female worms (7 to 8 weeks). Oviposition, which defines the postural phase of the disease, typically occurs 45 to 60 days later. This symptomatic acute phase is mostly severe in non-endemic (non-immune tolerant) individuals (i.e. tourists, travelers) exposed to fresh water in endemic areas (16, 17, 18). It is considered to be a toxemic and allergic reaction to the migrating and maturing schistosomula (19). The severity of the clinical presentation varies according to the cercarial burden and the immune response to the released parasite antigens (6).

Circulating immune complexes are found in 55–93% of patients with acute schistosomiasis, and their presence and amount are correlated to the intensity and severity of symptoms (20, 21). These symptoms are typically seen before oviposition, egg-laying, and the appearance of granulomatous reactions around eggs. Oviposition begins at the end of adult maturation and migration to the vesical plexus or mesenteric veins (22). In the early stages of the acute phase, a search for eggs in stools is typically negative, and will remain so until the end of the entire life cycle. Nonetheless, eggs may still be detected in stools of patients complaining of symptoms compatible with clinical acute form (17).

At any rate, the diagnosis of acute schistosomiasis relies on serological testing. Still, it is crucial to select an antigenic portion of the helminth in order to solve the problem of variability on the sensitivity that may occur according to the antigen used. Even so, the primary investigation must be done by a physician in order to determine the medical history of each individual case and then, serological findings ought to be used as a confirmatory methodology. Among the immunological assays available, ELISA is the most commonly used, especially with egg or worm antigens. Its methodology sensitivity can be high for patients with chronic form, reaching values of 89-96% for egg antigens and 90-94% for worm antigens, which is related to the cut off titre defining positivity (23, 24). Nonetheless, early

treatment with praziquantel or oxamniquine shows promise and should be ready for exposed travelers (6, 7, 25).

Reports of outbreaks in travelers with a single and recent exposure have contributed to a better description of the natural history of schistosomiasis and, particularly in those cases, diagnosis relies mainly on positive serological testing. We report here a recent (December/2009 – March/2010) focus of schistosomiasis mansoni where 80 individuals were hosted in Colônia do Teodoro, an endemic focus, in State of Minas Gerais, southeast Brazil. All the 80 individuals had contact with a freshwater pool where *B. glabrata* snails were detected. Those individuals were immediately interviewed and symptoms and/or signs were registered as the primary diagnosis. As a consequence, all the individuals exposed to contaminated water presented clinical findings compatible with acute schistosomiasis and were further investigated for IgG antibody levels by ELISA-SWAP. Data revealed 45 patients with high levels of IgG against adult worm antigens, but 35of them were below the cut off of 0.188 determined for this assays. Among those 35 patients, 3 presented eggs in stools determined by Kato-Katz assay after 50 days of the infection. Although the Kato-Katz analysis was not performed for all the 80 patients, the sensitivity of ELISA-SWAP is low when it is being used as a tool for the immunodiagnosis of patients with acute form.

Thus, based on the statement that difficulties associated with clinical or parasitological diagnosis in early patent phase might be overcome by adoption of immunological methods (26), we developed a new indirect ELISA methodology using schistosomula antigen based on the helminth life-cycle. Data from ELISA-SmTeg showed a significant positivity ratio when 64 patients presented high levels of specific IgG (p = 0.001), demonstrating a significantly more reliable result than ELISA-SWAP, the most used immunological method for that purpose, especially when 19 individuals were differentially diagnosed by ELISA-SmTeg. Positivity ratio was 56% and 80%, respectively for ELISA-SWAP and ELISA-SmTeg. When a comparison between the two methods was done by Cohen´s Kappa Index, only a fair agreement (0.385 + 0.094) was seen between methods. This final analysis corroborates with our first findings showing that ELISA-SmTeg is more sensitive for the differential diagnosis of patients with acute schistosomiasis. There is generally a trade-off between sensitivity and specificity, and the relative performance of a new diagnostic test is therefore calculated as an index in which both these parameters are accounted for. Sensitivity, however, has more

relevance in a public health context, especially in situations where the goal is the complete elimination of a new focus (26).

After performing the Kato-Katz analysis of faecal samples from the 24 donors, it was revealed that all these patients were positive for the presence of eggs in stools. When comparing this parasitological result of each individual patient to the specific IgG antibody level for ELISA-SmTeg, once again, the effectiveness of our new immunoassay was confirmed when the assay had properly diagnosed all these 24 positive patients. On the other hand, ELISA-SWAP did not achieve the same effectiveness after 3 patients with eggs in stools were not differently diagnosed showing low IgG titers (0.157 + 0.026).

When patients were divided into groups based on the results obtained by different diagnostic methodologies and on the severity of infection, it could be seen that the IgG titers against SmTeg antigen significantly increase as the positivity ratio became higher. Individuals that were positive only for the clinical examination (group 1) showed significant lower levels of IgG antibody than those that were also positive for ELISA-SWAP assay (group 2) (p = 0.002). Accordingly, patients that were positive for Kato-Katz other than clinical examination and ELISA-SWAP (group 3) demonstrated significant higher IgG titers in sera than group 1 and group 2 (p = 0.002). No doubt that including clinical findings as a key to look closely for evidence of infection is important in patient management. But correlating immunodiagnostic results and antibody levels with clinical data should allow us to improve our practicing in rural endemic areas, which in turn would significantly enhance the efficiency of the anti-schistosomiasis control program (27).

Finally, five patients were hospitalized with the severe form of the infection, one with advanced pulmonary stage, one with schistosomal myeloradiculopathy and, three with severe intestinal form. These five patients (group 4) were positive for the presence of eggs in stools and, once more, presented high levels of specific IgG determined by ELISA-SmTeg corroborating with the initial findings that shows a significant sensitivity for this new immunoassay. A positivity ratio of 80% was observed for ELISA-SmTeg and a correlation was confirmed when comparing IgG levels against SmTeg and the severity of the infection (p = 0.003 and 0.001, groups 1 and 2, respectively). Furthermore, the low sensitivity for ELISA-SWAP was again reported when the assay missed the diagnosis of two patients with the severe form of the infection.

While antibody-based methods suffer from low sensitivity, especially for the diagnosis of acute schistosomiasis (2), the ELISA-SmTeg was noticed here as an important tool to confirm real positive samples plus as a tool for measuring the severity of the infection. Hence, an immunoassay capable of diagnosing individuals with acute form, especially before the egg laying will allow the increase on the control programme's effectiveness (26).

ACKNOWLEDGMENTS

We thank the financial support provided by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundação de Amparo à Pesquisa do Estado de Minas Gerais (Fapemig), Fundação Oswaldo Cruz (Fiocruz)/Centro de Pesquisas René Rachou (CPqRR), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (Capes), The Council of the International Educational Exchange of Scholars (Fulbright, U.S. Department of State). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

REFERENCES

1. World Health Organization (2008) The social context of schistosomiasis and its control: an introduction and annotated bibliography. Available: http://dosei.who.int/uhtbin/cgisirsi/tLEqou8myB/26990009/5/0. Accessed 10 April 2012.

2. Lambertucci J (2010) Acute schistosomiasis mansoni: revisited and reconsidered. Mem Inst Oswaldo Cruz 105 (4): 422-435.

3. Lambertucci JR, Serufo JC, Gerspacher-Lara R, Rayes AA, Teixeira R, et al. (2000) *Schistosoma mansoni*: assessment of morbidity before and after control. Acta Trop 77 (1): 101-109.

4. Pereira LF, Gazzaneo AL, de Melo RM, Tenório HC, de Oliveira DS, et al. (2010) Clinical and laboratory evaluation of schistosomiasis mansoni patients in Brazilian endemic areas. Mem Inst Oswaldo Cruz 105 (4): 449-453.

5. García Goyco C, Nazario-López H, Ruiz-Tiben E, Vázquez G, Borges T, et al. (1997) The efficacy of oxamniquine in acute schistosomiasis: a clinical analysis of 28 treated patients. Bol Asoc Med P R 89 (4-6): 63-69.

6. Coelho PM, Enk MJ, Katz N (2009) Treatment of clinical schistosomiasis at the prepatent phase: an option? Trends Parasitol 25: 299-300.

7. Enk MJ, Katz N, Coelho PMZ (2008) A case of *Schistosoma mansoni* infection treated during the prepatent period. Nature Clin Pract Gastroenterol & Hepatol 5 (2): 112-115.

8. Berhe N, Medhin G, Erko B, Smith T, Gedamu S, et al. (2004) Variations in helminth faecal egg counts in Kato-Katz thick smears and their implications in assessing infection status with *Schistosoma mansoni*. Acta Trop 92: 205-212.

9. Turner P, Lalloo K, Bligh J, Armstrong M, Whitty CJ, et al. (2004) Serological speciation of human schistosome infections by ELISA with a panel of three antigens. J Clin Pathol 57 (11): 1193-1196.

10. Gonçalves MM, Barreto MG, Peralta RH, Gargioni C, Gonçalves T, et al. (2006) Immunoassays as an auxiliary tool for the serodiagnosis of *Schistosoma mansoni* infection in individuals with low intensity of egg elimination. Acta Trop 100 (1-2): 24-30.

11. Oliveira EJ, Kanamura HY, Takei K, Hirata RD, Valli LC, et al. (2008) Synthetic peptides as an antigenic base in an ELISA for laboratory diagnosis of schistosomiasis mansoni. Trans R Soc Trop Med Hyg 102 (4): 360-366.

12. Caldas IR, Campi-Azevedo AC, Oliveira LF, Silveira AM, Oliveira RC, et al. (2008) Human schistosomiasis mansoni: immune responses during acute and chronic phases of the infection. Acta Trop 108 (2-3): 109-117.

13. Katz N, Chaves A, Pellegrino J (1972) A simple device for quantitative stool thick-smear technique in schistosomiasis mansoni. Rev Inst Med Trop Sao Paulo 14: 397-400.

14. Ramalho-Pinto FJ, Gazzinelli G, Howells RE, Mota-Santos TA, Figueiredo EA, et al. (1974) *Schistosoma mansoni*: defined system for stepwise transformation of cercariae to schistossomule in vitro. Exp Parasitol 36 (3): 360-372.

15. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 7 72: 248-254.

16. Ross AG, Vickers D, Olds GR, Shah SM, McManus DP (2007) Katayama syndrome. Lancet Infect Dis 7: 218-224.

17. Jauréguiberry S, Paris L, Caumes E (2010) Acute schistosomiasis, a diagnostic and therapeutic challenge. Clin Microbiol Infect 16 (3): 225-231.

18. Gryseels B, Polman K, Clerinx J, Kestens L (2006) Human schistosomiasis. Lancet 368: 1106-1118.

19. Corachan M (2002) Schistosomiasis and international travel. Clin Infect Dis 35: 446-450.

20. Hiatt RA, Sotomayor ZR, Sanchez G, Zambrana M, Knight WB (1979) Factors in the pathogenesis of acute schistosomiasis mansoni. J Infect Dis 139: 659-666.

21. Hiatt RA, Ottesen EA, Sotomayor ZR, Lawley TJ (1980) Serial observations of circulating immune complexes in patients with acute schistosomiasis. J Infect Dis 142: 665-670.

22. Southgate VR, Bray RA (2003) Medical helminthology. In: Cook GC, Zumla A, eds. Manson's tropical diseases. Saunders Elsevier, London.

23. Van Gool T, Vetter H, Vervoort T, Doenhoff MJ, Wetsteyn J, et al. (2002) Serodiagnosis of imported schistosomiasis by a combination of a commercial indirect hemagglutination test with *Schistosoma mansoni* adult worm antigens and an enzyme-linked immunosorbent assay with *S. mansoni* egg antigens. J Clin Microbiol 40: 3432-3437.

24. Sorgho H, Bahgat M, Poda JN, Song W, Kirsten C, et al. (2005) Serodiagnosis of *Schistosoma mansoni* infections in an endemic area of Burkina Faso: performance of several immunological tests with different parasite antigens. Acta Trop 93 (2): 169-180.

25. Enk MJ, Lima AC, Drummond SC, Schall VT, Coelho PM (2008) The effect of the number of stool samples on the observed prevalence and the infection intensity with *Schistosoma mansoni* among a population in an area of low transmission. Acta Trop 108 (2-3): 222-228.

26. Doenhoff MJ, Chiodini PL, Hamilton JV (2004) Specific and sensitive diagnosis of schistosome infection: can it be done with antibodies? TRENDS in Parasitology 20 (1): 35-39.

27. Al-Sherbiny MM, Osman AM, Hancock K, Deelder AM, Tsang VC (1999) Application of immunodiagnostic assays: detection of antibodies and circulating antigens in human schistosomiasis and correlation with clinical findings. Am J Trop Med Hyg 60 (6): 960-966.

FIGURES AND FIGURE LEGENDS

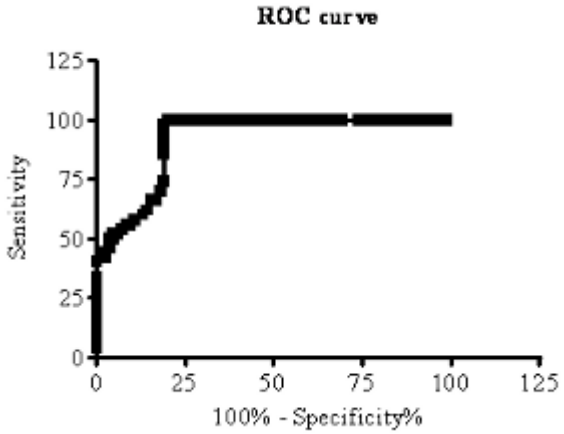


Fig 1 ROC curve of ELISA-SmTeg assay. The assay defines levels of specific IgG antibodies for schistosomula antigens as a diagnostic tool for clinical acute form patients. Artwork created by Prism 5.0 software

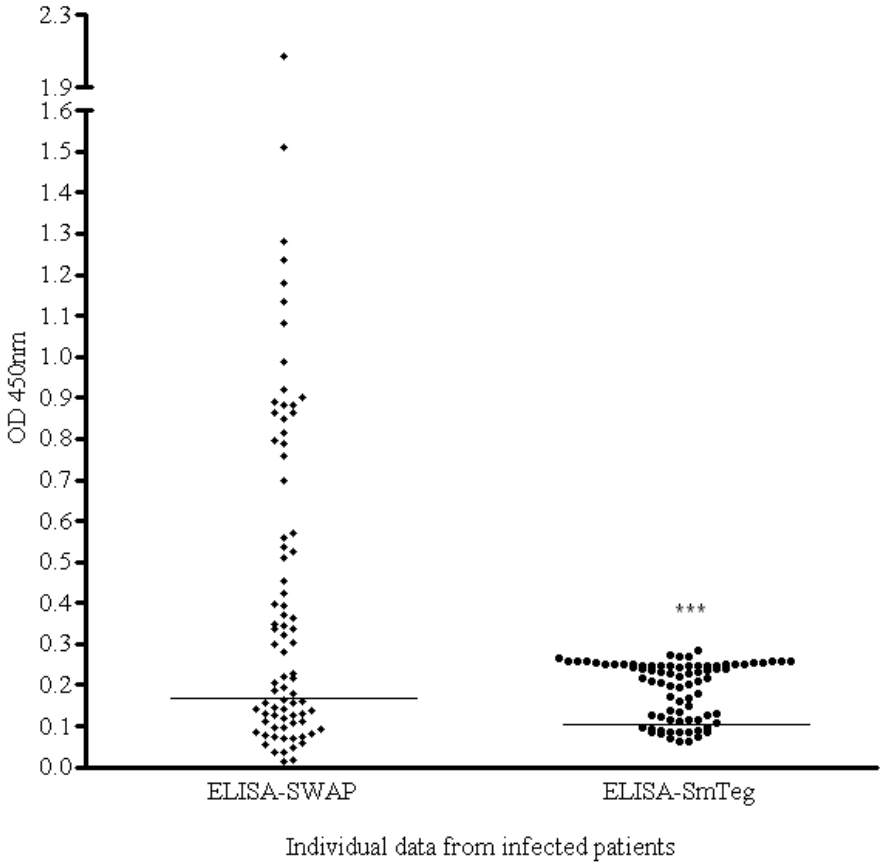


Fig 2 Individual analysis of the 80 serum samples by ELISA-SWAP and ELISA-SmTeg. Each OD value is representative for the mean of four absorbance values. Cut off values are represented by bars (0.188 for ELISA-SWAP and 0.110 for ELISA-SmTeg). Statistical differences between the number of positive individuals by both assays are represented by *** (p = 0.001) after Chi-square analysis. Artwork created by Prism 5.0 software

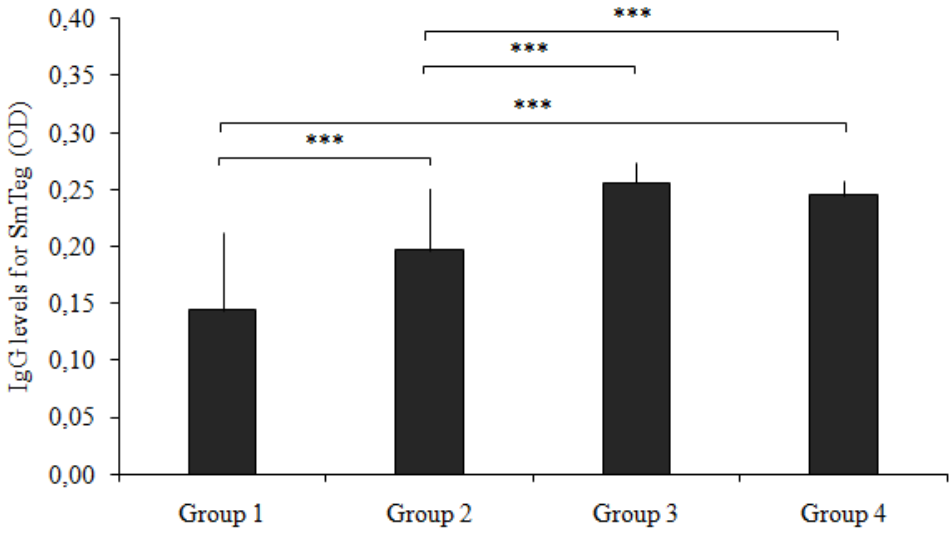


Fig 3 Relation between IgG titers detected by ELISA-SmTeg and groups divided according to the diagnostic results and severity of infection. Patients were divided in four groups: (Group 1) 28 patients positive only for clinical examination; (group 2) 26 patients positive for clinical examination plus ELISA-SWAP; (group 3) 21 patients positive for clinical examination, ELISA-SWAP and Kato-Katz; (group 4) 5 hospitalized patients with severe schistosomiasis. IgG levels for SmTeg are representative by the mean of four absorbance values at 450 nm. Statistical differences between groups are represented by *** after Student *t* test, for p = 0.002 and 0.003, respectively for groups 1 and 2 and, groups 1 and 4; p = 0.002 and 0.001, for groups 2 and 3 and, groups 2 and 4. Artwork created by Prism 5.0 software

4.5 ARTIGO 5

New approaches with different types of CCA for the diagnosis of patients with low *Schistosoma mansoni* load after intensive parasitological trial

Target antigens for schistosomiasis diagnosis

Rafaella Grenfell[a,b], Donald A. Harn[b], Smanla Tundup[b], Akram Da'dara[c], Liliane Siqueira[a], Paulo Marcos Z. Coelho[a]

[a] Schistosomiasis Laboratory, Rene Rachou Research Center, Oswaldo Cruz Foundation (Fiocruz), Belo Horizonte, Minas Gerais, Brazil.

[b] Department of Infectious Diseases, College of Veterinary Medicine and the Center for Tropical and Emerging Diseases, University of Georgia, Athens, Georgia, United States of America.

[c] Tufts University, Grafton, Massachusetts, United States of America.

Corresponding Author. Mailing address: Rene Rachou Research Center, Oswaldo Cruz Foundation. Av. Augusto de Lima 1715, Belo Horizonte, MG, Brazil. 30.190-002. Phone: 55 31 3349 7740, e-mail: coelhopm@cpqrr.fiocruz.br

ABSTRACT

Schistosomiasis mansoni is a serious debilitating and sometimes fatal disease. Accurate diagnosis plays a key role in patient management and infection control. However, currently available diagnostic methods are not ideal. Therefore, the selection of target diagnostic antigen candidates has turned out to be a promising tool for the development of new, more sensitive diagnostic methods. In previous investigations, crude antigens were tested and presented some advantages, though false-positive results were frequent. Recently, we turned our focus to developing innovative methodologies that employ highly purified *Schistosoma mansoni* antigens. Specifically, we focused on purified Circulating Cathodic Antigen (CCA) glycoprotein, a recombinant CCA protein and two individual CCA peptides. These schistosome proteins/peptides were tested in a new diagnostic method employing Immunomagnetic separation based on the improvement of antigen-antibody binding. Use of

recombinant CCA as a diagnostic antigen allowed us to develop a diagnostic assay with high sensitivity and specificity with no false-negative results. Interestingly, purified CCA worked as a better diagnostic antigen to demonstrate cure after praziquantel treatment to eliminate schistosomes. Lastly, our new diagnostic method was superior to Enzyme-linked Immunosorbent Assay (ELISA) in discriminating positive and negative cases, even for low endemicity patients.

Keywords: *Schistosomiasis mansoni*, Immunodiagnosis, Antigens candidates, Circulating Cathodic Antigen, recombinant protein, peptides.

Sponsorship: National Counsel of Technological and Scientific Development (CNPq), Oswaldo Cruz Foundation (Fiocruz)/ Rene Rachou Research Center (CPqRR), Coordination for the Improvement of Higher Level Education Personnel (Capes), The Council of the International Educational Exchange of Scholars (Fulbright, U.S. Department of State). National Institutes of Health AI R01 AI068109A to DAH. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

AUTHOR SUMMARY

Currently available diagnostic methods for *schistosomiasis mansoni* are not sensitive for patients with low parasite load. The selection of target diagnostic antigen candidates is a promising tool for the development of a new and more sensitive assay. In this study, we focused on purified Circulating Cathodic Antigen (CCA) glycoprotein, a recombinant CCA protein and two individual CCA peptides for development of an innovative assay. Best results were seen for the recombinant CCA that showed high sensitivity and specificity with no false-negative results, while purified CCA glycoprotein was a good antigen for the control of cure. Our new assay was superior to Enzyme-linked Immunosorbent Assay (ELISA) in discriminating positive and negative cases, especially related to low endemicity patients.

INTRODUCTION

Schistosomiasis is a disease caused by infection with *Schistosoma mansoni*, *S. haematobium*, *S. japonicum*, and less frequently, *S. mekongi* and *S. intercalatum*. Schistosomiasis occurs in the tropics and subtropics and is among the most important parasitic diseases worldwide, with a significant socio-economic impact (1). Approximately 74 countries are endemic, with roughly 120 million individuals being symptomatically infected and 20 million being severely

affected (2,3). Schistosomiasis control programs are largely based on treatment of infected populations, therefore adequate case-finding is important for effective implementation of chemotherapy control programs (4). Moreover, schistosomiasis represents an increasing problem in non-endemic areas, due to the growing number of immigrants and tourists (5-8). Herein, diagnosis plays a crucial role in the monitoring of early infection as well as efficacy of treatment. Currently, the gold-standard for diagnosis in much of the world remains the detection of schistosome eggs in stools or urine (9). However, because of low and sporadic egg production, the risk of having a large percentage of individuals go as undiagnosed is tremendous. Undiagnosed individuals remain infected and contribute to transmission of the disease (10,11).

Immunodiagnostic techniques are rapid, sensitive, convenient, and easily applied and have been used to estimate infection rates with the goal of improving diagnosis in epidemiological surveys and identifying individuals to target for treatment (12-15). Nonetheless, low specificity is frequently a problem in immunodiagnostic assays, largely because of the use of crude antigens that are either intact material from the parasite or a soluble extract of the parasite or eggs, both of which contain many antigens that might be shared with unrelated pathogens (unpublished data). The systematic purification of antigens from *Schistosoma* sp should allow for the development of new anti-schistosome antibodies that will be valuable diagnostic tools (16-18). Antigens excreted by adult worms into the circulation of the host, "circulating antigens", have repeatedly been shown to be potent diagnostic target molecules (19-22). Research on circulating antigens has focused on two genus-specific proteoglycan antigens derived from the schistosome gut: circulating anodic antigen (CAA) and circulating cathodic antigen (CCA). The diagnostic methods available that use circulating antigens unfortunately have low efficiency, especially for diagnosis of low intensity parasite burdens. Thus antibody levels against schistosome circulating antigens can currently only be used as a marker for infection for patient populations with moderate to high levels of parasite burden, or perhaps as assays to determine efficacy of future vaccination trials (23).

For this reason, defined diagnostic antigen(s) that increase sensitivity and specificity of serological assays and that can detect patients with low parasite loads patients would be of tremendous benefit to schistosome control programs. In this regard, a new immunological assay, called Immunomagnetic separation (IMS), was developed and refined by our group using paramagnetic beads. A benefit of this approach is to effectively concentrate, rather than

dilute, patient serum during incubation. We compared IMS to enzyme-linked immunosorbent assay (ELISA) using the same antigens in order to evaluate the effectiveness of this new approach. Standardization of IMS included tests to optimize the schistosome diagnostic antigens. Therefore we assessed the sensitivity of different forms of CCA for their diagnostic potential for clinical schistosomiasis. The antigens we focused on were: 1) CCA purified glycoprotein, 2) CCA recombinant protein (CCAr) and 3) two individual CCA peptides (CCApep1 and CCApep2). A longitudinal survey was performed with individuals from a low endemicity area for *schistosomiasis mansoni* according to the Brazilian law. Final analyses were done by comparing IMS results to data obtained during this longitudinal survey using Kato-Katz and TF-Test as parasitological assays.

Here, we demonstrate that a well standardized immunological assay is sensitive and specific for the discrimination of low parasite load cases, by demonstrating that (1) the levels of parasite-specific immunoglobulin G (IgG) are significantly different from positive and negative individuals when IMS is performed with CCAr; (2) IMS-CCAr achieved the most significant positivity ratio for diagnosis with no false-negative results; (3) specific IgG antibody levels drop significantly 30 days post-chemotherapy for all CCA antigens; (4) IMS-CCA was shown to be a reliable assay for monitoring cure post-chemotherapy; and finally (5) IMS methodology was superior to ELISA in detecting the presence of schistosome infection in patients with low parasite loads.

METHODS

Community survey

A longitudinal study was performed in the communities of Buriti Seco and Morro Grande in Pedra Preta, a little village in a schistosomiasis endemic area in the rural region of Montes Claros, state of Minas Gerais at the southeast region of Brazil, as published (24). This area was chosen based on the fact that the population had not being treated for schistosomiasis and, additionally has a low population migration index. A prevalence rate of 12% was reported in 2005 according to data provided by the Montes Claros Zoonosis Control Centre. The total amount of residents participating in the survey was 201 individuals (93 women/108 men).

Stool samples analysis – Each survey participant provided four separate stool samples on each of four consecutive days for Kato-Katz analysis (9). This method was performed using 18 slides, which were prepared as follows for each participant: 12 slides for the first sample and two slides each for the second, third and fourth samples in a total of 750 mg of faeces (18 x 41.7 mg). The same samples were analyzed by quantitative TF-Test as previously described (24). Briefly, samples was passed through a nylon mesh and quantified in metal plates. Each 500 mg portion was transferred to a tube containing preservative solution (10% formalin) and processed using ethyl acetate. Samples were centrifuged at 500g for 2 min. Sediment was ressuspended in 0.85% saline solution and analyzed using optical microscopy (25).

Serum samples processing – Among the 201 individuals participating on the survey, fifty patients with parasite loads varying between 1 and 555 eggs per gram of feces (epg) were selected to provide serum samples (24 women/26 men, between 8 and 88 years old). Individual serum samples were obtained after centrifugation of blood samples at 3000g for 5 minutes. These samples were maintained at -20^{o}C.

Treatment of positive cases – All participants who were positive for schistosomiasis were treated with praziquantel in a single dose of 60 mg/kg for children and 50 mg/kg for adults. Infections with other helminthes were treated with a single dose of 400 mg albendazole, as recommended by the Brazilian Ministry of Health. Positive patients were submitted new stool samples 30 days post-chemotherapy for examination by Kato-Katz assay. Individuals testing positive were retreated as needed. Serum samples were also obtained 30 days after treatment.

Healthy volunteers

Fifty three healthy volunteers (35 women/18 men, between 22 and 65 years old) were selected as donors to be used as our negative control group of individuals. The volunteers were non-endemic area residents or visitors with no medical history of previous schistosomiasis. Serum samples were processed as described.

Confirmatory diagnosis of healthy donors – In addition to patient history, a confirmatory diagnosis was performed using two individual ELISA assays for the detection of IgG antibody against soluble adult worm antigens (ELISA-SWAP) and against soluble egg antigens (ELISA-SEA). Both assays were done as previously described (in press). Patients reactive for both ELISA assays were removed from the "Healthy" cohort.

CCA antigens preparation

Purification of S. mansoni CCA glycoprotein – Adult worms from *S. mansoni* (LE strain) were obtained by perfusion of hepatic portal system of *swiss* female mice (4-6 weeks) 45 days post-infection with 100 cercariae (26). Adult worms were washed three times with 0.15M phosphate buffer saline pH 7.2, submitted to mechanical grinding (Virtiz Precisa, Switzerland) and ultracentrifugation at 25000g for 1 hour at 4ºC (Sorvall, Buckinghamshire, UK). Supernatant was collected then heated to 100ºC for 30 minutes, as previously described (27) then filtered through a 50kDA exclusion filter (Millipore Amicon, Sigma-Aldrich, St. Louis, USA) by centrifuging the solution through the filter at 2700g. Final purified product was dialyzed in cellulose membrane (Sigma-Aldrich, USA) against saline solution 0.9% for 48 hours at 4ºC and maintained at - 20ºC prior to use. An aliquot was analyzed for protein concentration (Nanodrop, Thermo Scientific 2000, USA). A silver stained Tris-glycine SDS-PAGE (12% gel) was performed to assess purity (28).

Preparation of CCA recombinant protein - Adult worms were homogenized by glass homogenizer with 1 ml of Trizol (Invitrogen, Grand Island, USA) and incubated for 10 minutes at 25ºC. Further, 200 μL of chloroform was added and the suspension was incubated for 5 minutes. This suspension was centrifuged at 15000g for 15 minutes at 4ºC and the upper layer was reserved. RNA was precipitated by addition of 500 μl of isopropanol then incubated for 10 minutes, then the solution centrifuged at 4ºC for 10 minutes. Cold 75% ethanol was added to resuspend the pellet then centrifugation for 5 minutes. The ethanol was removed and the final pellet dried, ressuspended in 50 μl of RNase-free water and kept at -20ºC until use. cDNA was obtained according to the manufacturers instructions (SuperScript® II Reverse Transcriptase, Invitrogen).

CCA gene reference sequence was obtained from the database of the National Center for Biotechnology Information (NCBI) (29) under reference number AAB53003. To express the CCA domain, gene-specific primers were designed as follows: sense 5'-CCC*GGATCC*ATGACGTTTGATTTCATGTTAAAG - 3' and antisense 5'-GGG*CTCGAG*TAGGGAGTTAATCATTTGATTCATAGC - 3' which contain *Bam*HI and *XHO*I restriction sites (italicized), respectively. The PCR conditions through 32 cycles were 95°C for 45 seconds as denaturing step, 60°C for 45 seconds as an annealing step and 72°C for 1 minute as an elongation step. The PCR product was first subcloned into the pCR-Blunt

II TOPO plasmid (Invitrogen) and transformed into TOP10 competent cells. Recombinant plasmid DNA was isolated and digested with the restriction enzymes. The resulting fragment was purified and subcloned into *Bam*HI-*Xho*I-cleaved pET21a. The final recombinant expression plasmid (CCA-pET21a) was sequenced to ensure that the insert was in the correct reading frame and subsequently introduced into *E. coli* strain BL21 Gold competent cell (Agilent Technologies, La Jolla, USA), which lacks Ompt, Lon proteases. Cells were grown overnight at 37°C in Luria Bertani (LB)-medium containing 75 μg/ml ampicillin. The overnight culture was diluted 100-fold in LB-medium and grown until an O.D.600 for 0.5 was reached. To induce protein expression, isopropyl β-D- thiogalactoside (IPTG) was added to 1.0 mM and the cells were grown for another 3 hours. Cells were harvested by centrifugation and sonicated. Purification was done by affinity chromatography on His-Trap columns (Amersham/Pharmacia). The homogeneity of the recombinant protein was analyzed by silver stained Tris-glycine SDS-PAGE (12% gel) (28).

Selection and production of CCA peptides - The complete 347 amino acid sequence of CCA was retrieved from NCBI/protein. For prediction of B cell epitopes, the full length protein sequence was subjected to B cell prediction at BCPreds: B-cell epitope prediction server 1.0 (30-32). Two best conformations of surface exposed B cell epitope sequences having the cut off value for BCPreds (> 0.9) were taken into consideration. Peptides (Table 1) were synthesized in a stepwise manner on a Fmoc solid-phase synthesis strategy to obtain C-terminally amidated peptides (Mimotopes, San Diego, USA).

Purification evidence for CCA antigens – A confirmation method was used for each of the four CCA antigens obtained. Microtiter plates MaxiSorpTM Surface (NUNC, Denmark) were coated with 100 μl/well of 1 μg/ml of each antigen (purified CCA glycoprotein, CCA recombinant protein and each individual CCA peptide) diluted in 0.05M carbonate-bicarbonate buffer pH 9.6 for 16 hours at 4ºC. As positive controls we included soluble adult worm antigen (SWAP). Plates were washed three times with 0.15M phosphate buffered saline pH 7.2 with 0.05% of Tween 20 (LGC Biotecnologia, BR) (PBS-T 0.05%) then plates were blocked by incubation with 2.5% skim milk diluted in PBS-T 0.05% at 37ºC for an hour. Plates were washed with PBS-T 0.05% then 100 μl/well of an peroxidase conjugated IgG1 monoclonal antibody against CCA conjugated was added diluted in PBS-T 0.05% (1:8000) (Lot 5F4.B4, University of Georgia, Monoclonal Antibody Facility, USA) then incubated at RT for 1 hr. Plates were washed in PBS-T 0.05%, then 100 μl of substrate 3,3',5,5-

tetramethylbenzidine solution (Invitrogen) were added and the reaction stopped after 15 minutes of incubation in the dark by addition of 50 µl/well of 2N sulfuric acid. Results were obtained as optical density (O.D.) at 450 nm using a microplate reader (Model 3550, Bio-Rad Laboratories, Tokyo, JA).

Immunomagnetic Separation technique (IMS) with CCA antigens

Paramagnetic microspheres (0.4µm) (Estapor microspheres, Merck, Lyon, FR) were sensitized with CCA antigens (10^6 microspheres with 1µg/ml of antigen/assay) for each individual CCA antigen preparation: 1) with purified CCA, 2) CCA recombinant protein, 3) CCA peptide 1, and 4) CCA peptide 2. All incubation steps were performed under rotation to improve antigen-antibody binding. For each sensitization step, antigens were diluted in 0.05M carbonate-bicarbonate buffer pH 9.6 for 16 hours at 4ºC. Microspheres were washed four times with 0.15M phosphate buffer saline pH 7.2 with 0.05% of Tween 20 (LGC Biotecnologia, São Paulo, BR) (washing buffer) using a 1.5ml tube magnetic base (Invitrogen, Grand Island, USA). Non specific-binding was blocked using 20% skim milk proteins in washing buffer at 4ºC for 16 hours. The microspheres were washed and maintained at 4ºC until use. Prior to use, microspheres were washed then 100µl of a non-diluted serum sample were added in duplicate, followed by incubation at 37ºC for an hour. Microspheres were next washed then incubated at 37ºC for an hour with 100µl of peroxidase conjugated anti-human IgG Fc specific (Sigma-Aldrich, St. Louis, USA) diluted 1:60000 in washing buffer. Tubes were washed again and 100µl of substrate 3,3',5,5-tetramethylbenzidine solution (TMB/H_2O_2) (Invitrogen, Grand Island, USA) were added to each well. The reaction was stopped after 10 minutes of incubation in the dark by addition of 50µl/tube of 2N sulfuric acid. Using the magnetic base, supernatant were transferred to a microtiter plate and results were obtained as absorbance values at 450nm in a microplate reader (Bio-Rad Laboratories 3550, Tokyo, JA).

Indirect Enzyme-Linked Immunosorbent Assay (ELISA) with CCA antigens

ELISA using CCA antigens were standardized based on the technique described (33) with some modifications. Microtiter plates MaxiSorpTM Surface (NUNC Brand Products, Roskilde, DK) were coated with 100µl per well of CCA antigens diluted at 1µl/ml in 0.05M carbonate-bicarbonate buffer pH 9.6 for 16 hours at 4ºC. Next, the plates were washed three times with washing buffer, then blocked by addition of 300µl per well of 2.5% skim milk

diluted in washing buffer, incubating at 37ºC for 1 hour. Plates were washed then 100µl of individual serum sample diluted 1:100 in 0.15M phosphate buffer saline pH 7.2 were added to the plates in duplicate and incubated at room temperature for 1 hour. The plates were then washed and incubated at room temperature for 1 hour with peroxidase conjugated anti-human IgG Fc specific (Sigma-Aldrich, St. Louis, USA) diluted in washing buffer at 1:60000. Plates were washed again and 100µl of substrate solution were added to each well. The enzymatic reaction was stopped after 10 minutes of incubation in the dark and O.D. at 450 nm determined by microplate reader. The cut off value of each ELISA method was determined by ROC curve and they were defined as 0.250 for ELISA-CCA, 0.103 for ELISA-CCAr, 0.117 for ELISA-CCApep1 and, 0.166 for ELISA-CCApep2 (A = 0.765; 0.924; 0.954; 0.824, respectively). Positive and negative controls were assayed for both techniques.

Statistical analysis

Data derived from absorbance values were analyzed with Minitab software (Minitab Inc, College, USA) by Kolmogorov-Smirnov normality test. Normal distributed data were analyzed by Student's *t* test and non-normal distributed data were analyzed by Mann-Whitney test. Comparisons between methods were done by Chi-square (χ^2) analysis ($p \leq 0.05$ as significance level). The sensitivity, specificity, cut off values and likelihood ratios were determined with Prism 4.0 software. Agreement between methods was measured using the Cohen coefficient (34) and analyzed according the Landis & Koch definition (35), with software ComKappa 2.0: 1.00 - 0,81 almost perfect; 0,80 - 0,61 substantial; 0,60 - 0,41 moderate; 0,40 - 0,21 fair; 0,20 - 0 slight; < 0 poor.

Ethics

This project was approved by the Ethical Research Committee of the Rene Rachou Research Center, Oswaldo Cruz Foundation (CEPSH/CPqRR 03/2008) and the National Brazilian Ethical Board (784/2008, CONEP 14886). The study objectives were presented and explained to all participants and written consent was obtained through signing a form before admission to this study.

RESULTS

CCA antigen preparations

To define a more efficient methodology for *schistosomiasis mansoni* diagnosis, especially for areas of low endemicity, four different forms of CCA antigens were obtained and tested in the Immunomagnetic Separation assay, a new immunological methodology designed for use with non-diluted serum. Initially, the purified CCA glycoprotein was obtained from adult worm extracts and the CCA recombinant protein were induced in *E. coli*. Final products are shown on Figure 1 and both demonstrated a 30 kDa protein, correlating with previously reported characteristics of CCA (27).

Afterwards, two CCA peptides were synthesized based on predicted B cell epitopes. These four CCA antigens were then tested as diagnostic assay candidates using a monoclonal IgG1 antibody against *S. mansoni* CCA (Lot 5F4.B4, University of Georgia, Monoclonal Antibody Facility, USA). Results are shown on Figure 2 where a significant reaction was seen for all four CCA antigens tested in comparison to Bovine Serum Albumin (BSA) as our negative control.

IMS validation for low endemicity area residents

The longitudinal study involved the communities of Buriti Seco and Morro Grande in Pedra Preta, southeast Brazil. These communities are areas of low endemicity for schistosomiasis mansoni, and with low migration index and no history of previous treatment. Among the 201 individuals participating on the survey, 50 patients including adults and children were selected to provide serum samples (24 women/26 men). These patients were first diagnosed by Kato-Katz and TF-Test and results showed a parasite load range between 1 and 555 epg among the group. All patients were treated as recommended and they resubmitted stools for Kato-Katz testing 30 days post chemotherapy when serum samples were obtained. Retreatment was done in all reinfection cases.

The 50 serum samples selected from people of Pedra Preta, together with the healthy donors' serum samples were screened by IMS using the four different antigens described: sensitized with purified CCA glycoprotein (IMS-CCA), CCA recombinant protein (IMS-CCAr), CCA peptides 1 and 2 (IMS-CCApep1 and IMS-CCApep2, respectively). The 53 healthy donors, s were initially tested for antibodies to schistosomes by ELISA-SWAP and -SEA. Only one

individual was reactive for both antigens and was not therefore removed from the healthy (negative) control group. Further, cut off value, positivity ratio, sensitivity and specificity of each IMS methodology was determined by ROC curves, which are represented on Figure 3.

IMS-CCA presented a sensitivity of 90% and a specificity of 92% for a cut off value of 0.197, which showed that the purified CCA glycoprotein might be considered a good marker for schistosome infection with 5 missing positive patients and 4 missing negative individuals. Moreover, IMS using CCA recombinant protein showed an excellent result providing an excellent sensitivity of 100% and specificity of 96% for a cut off of 0.063, where only two negative individuals presented false positive results. Finally, IMS using CCA peptides 1 and 2 showed similar effectiveness with the same sensitivity (80%) plus a specificity of 90% and 92%, respectively for cut off values of 0.164 and 0.133. When analyzing false-positive and -negative results, we could see that the use of these 20 amino acids peptides decreased the diagnostic effectiveness with 10 false-negative results for both peptides, 5 for IMS-CCApep1 and 4 for IMS-CCApep2. The positivity ratios achieved by each IMS method were 91% (93/102), 98% (100/102), 85% (87/102) and 86% (88/102), for IMS-CCA, IMS-CCAr, IMS-CCApep1 and IMS-CCApep2. The positivity ratio achieved by IMS-CCAr was significantly higher than the other three IMS assays ($\chi 2 = 0.74$, $p < 0.001$). Figure 4 shows the individual O.D. for each positive and negative patient as determined by each IMS protocol.

Not all the infected patients showed an adequate post-treatment follow-up, since no eggs were found in any patient stools 30 days post-chemotherapy. Forty two of the 50 PZQ treated patients agreed to donate serum samples once more. Diagnostic results obtained by the four IMS protocols from both time points were compared with the purpose of detecting any differences in IgG antibody titers. All four CCA antigens showed statistical differences when the mean of patients' O.D. were compared on the timeline, as shown in Figure 5. From the observations in each period, 98% of the patients became negative via IMS-CCA (41/42), whereas 81% became negative via IMS-CCApep1 (34/42) and 93% via IMS-CCApep2 (39/42). IMS using CCA recombinant protein identified 55% of the patients as negative for the disease 30 days after treatment (23/42).

Comparative analysis of the effectiveness of IMS with ELISA

In addition to the fact that IMS was standardized with non-diluted serum, the incubation steps were performed under rotation with the purpose of improving antigen-antibody binding and

thus, diagnostic sensitivity. To test this hypothesis, purified CCA glycoprotein, the CCA recombinant protein and the CCA peptides 1 and 2 were used in ELISA (ELISA-CCAr, ELISA-CCApep1 and ELISA-CCApep2) and the results were compared to data obtained using IMS analysis. Significant differences were observed in the positivity ratios. Forty five positive patients were correctly diagnosed by IMS-CCA and only 35 were diagnosed by ELISA-CCA ($\chi 2 = 0.21$, $p < 0.001$). All the patients were positive for IMS-CCAr in comparison to 48 patients diagnosed by ELISA-CCAr ($\chi 2 = 0.48$, $p < 0.001$). On the other hand, IMS- and ELISA-CCApep1 presented no difference with 40 positive patients. However, comparing CCApep2, statistical differences were detected with 40 patients diagnosed by IMS-CCApep2 and, 37 by ELISA-CCApep2 ($\chi 2 = 0.21$, $p < 0.001$). Analysis of Cohen's Kappa Index showed a moderate agreement of 0.467 ($\pm$ 0.103) (69/102) between IMS-CCA and ELISA-CCA. The same agreement was found for IMS-CCApep2 and ELISA-CCApep2 that showed an agreement of 0.479 ($\pm$ 0.106) (66/102). A better agreement was found for IMS-CCAr versus ELISA-CCAr and, IMS-CCApep1 versus ELISA-CCApep1 which indicated a substantial agreement of 0.664 ($\pm$ 0.096) with a positivity of 84/102 and 0.699 ($\pm$ 0.106) with 75/102, respectively.

Data obtained from a prospective parasitological diagnosis with 18 slides of Kato-Katz plus TF-Test confirmed the low parasite load of residents of Pedra Preta that were infected by *S. mansoni* (1 to 555 epg). Based on individual parasite load, patients were divided into three groups according to the range of 1 to 10 epg, 11 to 30 epg and greater than 31 epg. Groups were examined by IMS and ELISA methods. Results are shown on Table 2.

DISCUSSION

Population and treatment-based control programs have been successful in reducing the intensity of infection and severe morbidities associated with schistosomiasis; however, transmission remains active in highly endemic areas, and recurring low-level reinfection is likely to be associated with subtle but persistent morbidities (36-38). Adequate case-finding is essential for the effective execution of control programs. Diagnosis has mainly depended upon finding eggs in patients' faecal samples. However, fluctuation in egg output and the chance of missing light infections necessitate repeated examinations. Serologic testing has been used to enhance our ability to detect the disease and try to be more sensitive in demonstrating light infections (13,23,39). Extensive research on the development of antibody-assays has result in promising methods that cannot easily be applied as follow-up quality

control for chemotherapy and present little potential for discriminating positive from negative patients. Therefore, the diagnosis of schistosomiasis is challenging and few data are available on the accuracy of serological diagnosis in prospective studies. This study applied a multievaluation approach combining specific antibody detection for four different antigens with an investigation performed on parasitological data in efforts to produce a more field-applicable assay format.

The identification and description of CCA as a constitutional glycoprotein from adult worms gut (40) has allowed the development of assays for detecting antibodies or circulating antigens in urine and serum samples of infected individuals (21,41-43). When CCA was used in those assays, sensitivity was lower than expected which was partly explained as a consequence of low levels of circulating antigens being regurgitated by adult worms (44), especially in patients with low parasite loads. To solve this problem, we standardized an innovative method called IMS that uses paramagnetic beads in contact with non-diluted serum and is based on incubation steps performed under rotation, allowing for increased antigen-antibody binding. We chose CCA was as the adult worm antigen to focus on for our IMS assay (45). The IMS method was evaluated with four different CCA antigens, including the CCA purified glycoprotein, the CCA recombinant protein and, also, two individual peptides of 20 amino acids, with the intention of selecting the ideal candidate for the indirect schistosomiasis serological diagnosis. Since schistosomiasis epidemiological profiles show an increase in the number of low endemicity areas, the sensitivity of each IMS was validated with patients' samples from an endemic area in southeast Brazil where most of them showed low parasite load after 18 slides of Kato-Katz plus TF-Test determination. Patients in that area had never been treated for schistosomiasis and have been evaluated in a longitudinal study based on reevaluations and treatment schemes every time a reinfection case is detected.

The purification of CCA glycoprotein from adult worms of *S. mansoni* and the production of the CCA recombinant protein were confirmed by SDS-PAGE (Figure 1) and via binding of a CCA-specific monoclonal antibody (Figure 2). Although both antigens functioned well in IMS, IMS using the recombinant protein presented more significant results when all the positive cases were properly detected with a sensitivity of 100% and, only two false-positive results, giving rise to a specificity of 96% ($\chi 2 = 0.74$, $p < 0.001$). Whereas IMS-CCA achieved 90% of sensitivity and 92% of specificity, with 5 false-negative and 4 false-positive results.

Comparison between the positivity ratios revealed that IMS-CCAr was significantly superior to IMS-CCA on diagnosing low endemicity patients ($\chi 2 = 0.74$, $p < 0.001$). The prior structural difference between CCA and CCAr that justifies their specificity lays on the fact that CCA was purified in its native form as a whole glycoprotein, while the recombinant CCA was expressed in *E. coli* and contains the protein portion of the native CCA, which was not glycosylated. Native CCA glycoprotein contains 0-linked poly (Le^x) carbohydrate chains with approximately 25 repeating units. Carbohydrate chains containing multiple Le^x determinants have been identified on several glycolipids not only from schistosome but from other parasites (46,47), from human adenocarcinomas (48) and also, circulating granulocytes carry relatively high abundance of branched N-linked polysaccharides having Le^x repeating units (49). Additionally, Le^x sequence is particularly immunogenic, playing an important role during inflammatory processes, especially in granulocyte and monocyte adhesion processes and recruiting granulocytes to sites of inflammation (50). It is conceivable that the use of the CCA glycoprotein in schistosome diagnosis leads to false-positive results, when IgG antibodies against its most immunogenic portion (the Le^x units) can be mistakenly detected. In contrast, the CCA protein sequence of 347 amino acids, obtained by recombinant expression, is exclusively found in genus *Schistosoma* with no description in any other parasite or human proteins, which can be confirmed by Blast search.

The use of synthetic peptides corresponding to a single continuous epitope may increase the specificity of an immunoassay in the same way that monoclonal antibodies recognizing a single epitope do compared to polyclonal antiserum. Thus, with the complete 347 amino acid sequence of CCA, prediction of B cell epitopes was performed and the two best conformations were considered (cut off value for BCPreds > 0.9). Same identity analysis was done and both peptides were recognized by the CCA-specific monoclonal antibody (Figure 2). When evaluating each peptide for the diagnosis of *S. mansoni* using IMS methodology, data showed similar results for these two methods, as showed by the ROC curves (Figure 3). IMS-CCApep1 presented 80% of sensitivity and 90% of specificity with 10 missing positive cases and 5 missing negative individuals. IMS-CCApep2 showed the same sensitivity and 4 missing negative cases, leading to a specificity of 80%. Despite the possible advantage of increasing diagnosis specificity by using individual peptides, our data did not show any disparity in specificity between IMS-CCA and -CCApep1 or -CCApep2 when a similar same amount of false-positive cases were detected by the three methods (Figure 4).

In a final comparison of the four antigens used in IMS methodology, CCAr continued to yield a higher positivity ratio of 98% compared to CCA (91%), CCApep1 (85%) and CCApep2 (86%) ($\chi 2 = 0.74$, $p < 0.001$). Recombinant protein based diagnosis offers important advantages because higher antigen concentrations can be used, and nonspecific moieties are not present in those proteins, as they may be in crude antigens or in native proteins. Nevertheless, due to the restricted amino acids sequence (epitope) of a single peptide, the use of each sequence has been deemed impractical. This suggests that for peptides to be used, a large pool of epitopes would be required to achieve wide population coverage and the cost would increase significantly.

All the individuals that presented eggs in stools were treated, as recommended by the Brazilian Ministry of Health. These positive patients were resubmitted to stool examination by Kato-Katz after 30 days of chemotherapy and none of them presented eggs in stool at that time. Among those 50 patients, 42 were followed up by IMS methodology with the aim of determining specific IgG titer against each CCA antigen. A significant decrease was detected on the mean of IgG antibody levels for all the four IMS tested ($p < 0.001$) (Figure 5). When individual O.D. was analyzed, IMS-CCA yielded the best results with only 1 missing negative patient (41/42), whereas IMS-CCAr missed 19 negative patients and, IMS-CCApep1 and -CCApep2 missed 8 and 3, respectively. The distinctive patient who was positive for IMS-CCA was also positive for the other IMS methodologies showing no inconsistency on the diagnosis performed for IMS. The possibility that this patient may have been reinfected or presented immature worms at the moment of treatment cannot be eliminated. No data involving the detection of CCA in serum of patients infected for less than 6 weeks have been published to date. However, CCA can be detected in mice 3 weeks post-infection plus, plus freshly transformed schistosomula, or isolated adult worms excrete CCA *in vitro* immediately after transformation (51). Nevertheless, even though IMS-CCAr presented the most significant positivity ratio and a high capability of distinguishing positive and negative individuals, it did not show a reliable performance as a cure control assay, as was in the case for IMS-CCA.

Since other investigators have reported low sensitivity values for the detection of antibodies against CCA or the circulating antigen itself in urine and serum samples using immunological assays as ELISA (21,41-43), we compared our IMS data with results obtained by ELISA assays that were standardized with the same CCA antigens. The main differences of these two

methodologies were two: (1) IMS was performed with non-diluted serum while ELISA used only diluted samples (1:100) and, (2) incubation steps were done under rotation for IMS, different from the ELISA. The main goal of this comparison was to confirm that the sensitivity of IMS is superior to ELISA based on an accurate capture of antibodies by CCA antigens. This theory was confirmed for CCA, CCAr and CCApep2 when IMS presented a significant improved sensitivity than ELISA. IMS-CCA were capable of detecting 10 extra positive patients than ELISA-CCA ($\chi 2 = 0.21$, $p < 0.001$), whereas IMS-CCAr and -CCApep2 detected 2 and 3 extras positive patients, respectively than each corresponded ELISA ($\chi 2 = 0.48$ and 0.21, $p < 0.001$). The superiority of IMS in detecting positive cases was evident when low egg burden patients were divided into three groups based on parasite load. Interesting data showed that IMS-CCA, -CCAr and -CCApep2 continued to show a higher sensitivity than ELISA even for patients with parasite loads as low as 1 to 10 epg, and this observation was especially demonstrated by IMS-CCAr. Cohen's Kappa Index confirmed a moderate agreement between IMS and ELISA for CCA (0.467 ± 0.103) and CCApep2 (0.479 ± 0.106) and a substantial agreement for CCAr (0.664 ± 0.096) and CCApep1 (0.699 ± 0.106).

The present study was undertaken to develop an assay that is more field applicable than the ELISA for testing of serum samples of low endemicity areas. IMS methodology was standardized and accurately validated in our study and demonstrated to be superior to ELISA that is usually used in routine diagnosis despite the low sensitivity and specificity it may present. The comparison between different CCA antigens in IMS diagnosis allowed the evaluation of the specific capability of each assay in diagnosing positive and negative individuals and the occurrence of false-positive and/or -negative results. IMS-CCAr presented the most significant positivity ratio for the primary diagnosis, while IMS-CCA was the most reliable assay for cure control. Due to the restricted single epitopes of individual peptides, the 20 amino acids sequence used here showed no advantages in comparison to the native glycoprotein or the recombinant protein. In conclusion, results revealed that the detection of specific IgG antibody against CCA in serum may be used as a definitive diagnosis tool for *S. mansoni* infection, even for patients presenting low parasite load.

ACKNOWLEDGEMENTS

We thank Áureo Almeida for the help provided in parasitological analysis and Leena Srivastava for the assistance offered for the recombinant protein constructs. We also thank the

Monoclonal Antibody Facility of University of Georgia, especially Ms. Ruth Davies and, Estapor, Merck for supplying the microspheres.

REFERENCES

1. King CH, Dickman K, Tisch DJ (2005) Reassessment of the cost of chronic helmintic infection: a meta-analysis of disability-related outcomes in endemic schistosomiasis. Lancet 365: 1561–1569.

2. Croft SL, Vivas L, Brooker S (2003) Recent advances in research and control of malaria, leishmaniasis, trypanosomiasis and schistosomiasis. East Mediterr Health J 9: 518–533.

3. Chitsulo L, Engels D, Montresor A, Savioli L (2000) The global status of schistosomiasis and its control. Acta Trop 77: 41–51.

4. Guo JJ, Zheng HJ, Xu J, Zhu XQ, Wang SY, et al (2012) Sensitive and Specific Target Sequences Selected from Retrotransposons of *Schistosoma japonicum* for the Diagnosis of Schistosomiasis. PLoS Negl Trop Dis 6 (3): e1579.

5. Patz JA, Graczyk TK, Geller N, Vittor AY (2000) Effects of environmental change on emerging parasitic diseases. Int J Parasitol 30: 1395–1405.

6. Ross AG, Bartley PB, Sleigh AC, Olds GR, Li Y, et al (2002) Schistosomiasis. N Engl J Med 346: 1212–1220.

7. Enk MJ, Amaral GL, Costa e Silva MF, Silveira-Lemos D, Teixeira-Carvalho A, Martins-Filho OA, et AL (2010) Rural tourism: a risk factor for schistosomiasis transmission in Brazil. Mem Inst Oswaldo Cruz 105 (4):37-540.

8. Carvalho OS, Scholte RG, Guimarães RJ, Freitas CC, Drummond SC, et al (2010) The Estrada Real project and endemic diseases: the case of schistosomiasis, geoprocessing and tourism. Mem Inst Oswaldo Cruz 105 (4): 532-536.

9. Katz N, Chaves A, Pellegrino J (1972) A simple device for quantitative stool thick-smear technique in *schistosomiasis mansoni*. Rev Inst Med Trop Sao Paulo 14: 397–400.

10. Rabello A, Pontes LA, Dias-Neto E (2002) Recent advances in the diagnosis of *Schistosoma* infection: the detection of parasite DNA. Mem Inst Oswaldo Cruz 97: Suppl. 1171–1172.

11. Enk MJ, Lima AC, Drummond SC, Schall VT, Coelho PM (2008) The effect of the number of stool samples on the observed prevalence and the infection intensity with *Schistosoma mansoni* among a population in an area of low transmission. Acta Trop 108 (2-3): 222-228.

12. Turner P, Lalloo K, Bligh J, Armstrong M, Whitty CJ, et al (2004) Serological speciation of human schistosome infections by ELISA with a panel of three antigens. J Clin Pathol 57 (11): 1193-1196.

13. Gonçalves MM, Barreto MG, Peralta RH, Gargioni C, Gonçalves T, et al (2006) Immunoassays as an auxiliary tool for the serodiagnosis of *Schistosoma mansoni* infection in individuals with low intensity of egg elimination. Acta Trop 100 (1-2): 24-30.

14. Oliveira EJ, Kanamura HY, Takei K, Hirata RD, Valli LC, et al (2008) Synthetic peptides as an antigenic base in an ELISA for laboratory diagnosis of *schistosomiasis mansoni*. Trans R Soc Trop Med Hyg 102 (4): 360-366.

15. Jin YM, Lu K, Zhou WF, Fu ZQ, Liu JM, et al (2010) Comparison of Recombinant Proteins from *Schistosoma japonicum* for Schistosomiasis Diagnosis. Clin Vaccine Immunol 17 (3): 476-480.

16. Elhag SM, Abdelkareem EA, Yousif AS, Frah EA, Mohamed AB (2011) Detection of schistosomiasis antibodies in urine patients as a promising diagnostic maker. Asian Pac J Trop Med 4 (10): 773-777.

17. Wang J, Yu CX, Yin XR, Qian CY, Song LJ, et al (2011) Evaluation of early diagnostic value of 6 antigens from *Schistosoma japonicum* in mice. Zhongguo Xue Xi Chong Bing Fang Zhi Za Zhi 23 (3): 273-278.

18. Kinkel HF, Dittrich S, Bäumer B, Weitzel T (2012) Diagnosis of imported schistosomiasis: Evaluation of eight serological tests. Clin Vaccine Immunol.

19. Qian ZL, Deelder AM (1982) Circulating antigens in *Schistosoma*-infections. Acta Leiden 49: 71-80.

20. Qian ZL, Wen HC (1983) Schistosome circulating antigens (CSA) as a possible diagnostic parameter for active infections. Acta Leiden 51: 37-52.

21. De Jonge N, Kremsner PG, Krijger FW, Schommer G, Fillié YE, et al (1990) Detection of the schistosome circulating cathodic antigen by enzyme immunoassay using biotinylated monoclonal antibodies. Trans Roy Soc Trop Med Hyg 84: 815-818.

22. Deelder AM, Qian ZL, Kremsner PG, Acosta L, Rabello AL, et al (1994) Quantitative diagnosis of *Schistosoma* infections by measurement of circulating antigens in serum and urine. Trop Geograph Med 46: 233-238.

23. Deelder AM (1992) Diagnostic markers in schistosomiasis. Mem Inst Oswaldo Cruz 87 (V): 125-127.

24. Siqueira LM, Coelho PM, de Oliveira AA, Massara CL, Carneiro NF, et al (2011) Evaluation of two coproscopic techniques for the diagnosis of schistosomiasis in a low-transmission area in the state of Minas Gerais, Brazil. Mem Inst Oswaldo Cruz 106 (7): 844-850.

25. Gomes JF, Hoshino-Shimizu S, Dias LCS, Araújo AJUS, Castilho VLP, et al (2004) Evaluation of a novel kit (TF-Test) for the diagnosis of intestinal parasitic infections. J Clin Lab Anal 18: 132-138.

26. Pellegrino J, Siqueira A (1956) Técnica de perfusão para colheita de *Schistosoma mansoni* em cobaias experimentalmente infestadas. Rev Bras Malar Doenças Trop 8: 589-597.

27. Deelder AM, Klappe HT, van den Aardweg GJ, van Meerbeke EH (1976) *Schistosoma mansoni*: demonstration of two circulating antigens in infected hamsters. Exp Parasitol 40 (2): 189-197.

28. Schagger H, von Jagow G (1987) Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. Anal Biochem 166 (2): 368–379.

29. National Center for Biotechnology Information (NCBI) 2012; Available: http://ncbi.nlm.nih.gov/protein. Accessed 20 May 2012.

30. Chen J, Liu H, Yang J, Chou K (2007) Prediction of linear B-cell epitopes using amino acid pair antigenicity scale. Amino Acids 33: 423-428.

31. EL-Manzalawy Y, Dobbs D, Honavar V (2008) Predicting linear B-cell epitopes using string kernels. J Mol Recognit 21: 243-255.

32. EL-Manzalawy Y, Dobbs D, Honavar V (2008) Predicting flexible length linear B-cell epitopes. 7th International Conference on Computational Systems Bioinformatics, Stanford, CA pp.121-131.

33. Harlow E, Lane D (1988) Antibodies: A Laboratory Manual. New York: Cold Spring Harbor Laboratory Press. 495 p.

34. Cohen J (1968) Weighted kappa: nominal scale agreement with provisions for scales disagreement of partial credit. Psychol Bull 70: 213-220.

35. Landis JR, Koch GG (1977) The measurement of observer agreement for categorical data. Biometrics 33 (1): 159–174.

36. King CH, Sturrock RF, Kariuki HC, Hamburger J (2006) Transmission control for schistosomiasis - why it matters now. Trends Parasitol 22: 575–582.

37. King CH (2009) Toward the elimination of schistosomiasis. N Engl J Med 360: 106–109.

38. Lammie PJ, Fenwick A, Utzinger J (2006) A blueprint for success: integration of neglected tropical disease control programmes. Trends Parasitol 22: 313–321.

39. Lei JH, Su BT, Xu H, Shen JL, Guan XH et al (2011) Evaluation of an IgY-based immunomagnetic enzyme-linked immunosorbent assay system for detection of circulating *Schistosoma japonicum* antigen in serum samples from patients in China. Am J Trop Med Hyg 85 (6): 1054-1059

40. Deelder AM, el-Dosoky I, Van Marck EA, Qian ZL (1985) Immunofluorescent localization of *Schistosoma mansoni* circulating cathodic antigen in tissues of infected mice using monoclonal antibody. Z Parasitenkd 71 (3): 317-323.

41. Deelder AM, De Jonge N, Fillié YE, Kornelis D, Helaha D, et al (1989) Quantitative determination of circulating antigens in human *schistosomiasis mansoni* using an indirect hemagglutination assay. Am J Trop Med Hyg 40 (1): 50-54.

42. Van Dam GJ, Qian ZL, Fillié YE, Rotmans JP, Deelder AM (1993) Detection of IgM antibodies directed against the gut-associated circulating cathodic antigen in sera from *Schistosoma mansoni* infected patients. Development and comparison of three enzyme-linked immunoassays. Trop Geogr Med 45 (2): 59-65.

43. Fillié YE, Van Lieshout L, Kornelis D, Deelder AM (1994) Evaluation of an ELISA for combined measurement of CAA and CCA in *schistosomiasis mansoni*. Acta Trop 57 (4): 279-287.

44. Disch J, Garcia MM, Krijger GW, Amorim MN, Katz N, et al (1997) Daily fluctuation of levels of circulating cathodic antigen in urine of children infected with *Schistosoma mansoni* in Brazil. Trans R Soc Trop Med Hyg 91 (2): 222-225.

45. Van Dam GJ, Claas FH, Yazdanbakhsh M, Kruize YC, van Keulen AC, et al (1996) *Schistosoma mansoni* excretory circulating cathodic antigen shares Lewis-x epitopes with a human granulocyte surface antigen and evokes host antibodies mediating complement-dependent lysis of granulocytes. Blood 88 (11): 4246-4251.

46. Dissanayake S, Shahin A (2007) Induction of interferon-gamma by Taenia crassiceps glycans and Lewis sugars in naive BALB/c spleen and peritoneal exudate cells. Mol Immunol 44 (7): 1623-1630.

47. Haslam SM, Coles GC, Morris HR, Dell A (2000) Structural characterization of the N-glycans of Dictyocaulus viviparus: discovery of the Lewis(x) structure in a nematode. Glycobiology 10 (2): 223-229.

48. Hakomori S, Nudelman E, Levery SB, Kannagi R (1984) Novel fucolipids accumulating in human adenocarcinoma. I. Glycolipids with di- or trifucosylated type 2 chains. J Biol Chem 259: 4672-4680.

49. Spooncer E, Fukuda M, Klock JC, Oates JE, Dell A (1984) Isolation and characterization of polyfucosylated lactosaminoglycan from human granulocytes. J Biol Chem 259: 4792-4801.

50. Van Dam GJ, Bergwerff AA, Thomas-Oates JE, Rotmans JP, Kamerling JP, et al (1994) The immunologically reactive O-linked polysaccharide chains derived from circulating cathodic antigen isolated from the human blood fluke *Schistosoma mansoni* have Lewis x as repeating unit. Eur J Biochem 225 (1): 467-482.

51. Van Dam GJ, Bogitsh BJ, van Zeyl RJ, Rotmans JP, Deelder AM (1996) *Schistosoma mansoni*: in vitro and in vivo excretion of CAA and CCA by developing schistosomula and adult worms. J Parasitol 82 (4): 557-564.

TABLES

Table 1.Predicted B cell epitopes for *Schistosoma mansoni* CCA.

Reference Nr	Peptide Sequence	Amino acid positions	BCPred Score
1	Pro-Asn-Pro-Ser-Asp-Asp-Ser-Ser-Asn-Ser-Gly-Thr-Ile-Ser-Gly-Asn-His-Ser-Asp-Glu	307	1
2	Lys-Gln-Leu-Glu-Gln-Leu-Lys-Ile-Glu-Asn-Lys-Thr-Leu-Arg-Asn-Ser-Leu-Asp-Glu-His	83	0.926

Table 2 Relation between the number of eggs determined by Kato-Katz and TF-Test and the diagnosis determined by IMS and ELISA assays.

Number of eggs (epg)	n	Positive results detected by each method							
		IMS-CCA	IMS-CCAr	IMS-CCApep1	IMS-CCApep2	ELISA-CCA	ELISA-CCAr	ELISA-CCApep1	ELISA-CCApep2
< 10	30	26	30	23	24	18	29	24	19
11 – 20	11	11	11	11	8	8	10	8	9
> 31	9	8	9	6	8	9	9	8	9

FIGURES AND FIGURE LEGENDS

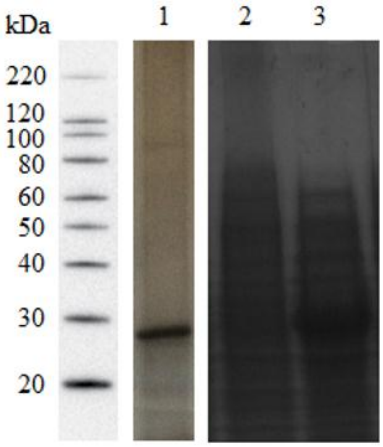


Fig 1 SDS-PAGE analysis of purified CCA glycoprotein and CCA recombinant protein. Aliquots of samples corresponding to the final product of adult worm extract submitted to purification steps (2) and, the CCA recombinant protein expressed in *E. coli* before (3) and after (4) induction with IPTG were subjected to silver stained SDS-PAGE analysis. Electrophoresis was done using 12% Tris-glycine gels. Molecular weight standards are shown in (1).

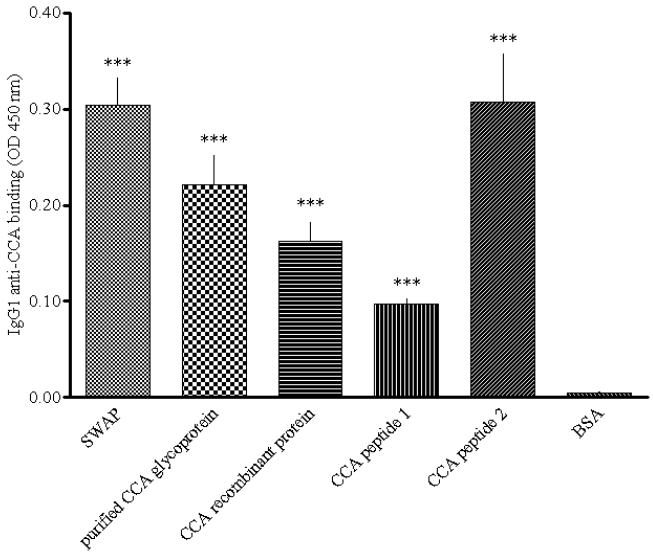


Fig 2 Binding of CCA-specific monoclonal IgG1 antibody to the four CCA antigens. Antigens represented by bars are: SWAP – soluble adult worm antigen extract, as the positive control; purified CCA glycoprotein; CCA recombinant protein; CCA peptides 1 (BCPred Score = 1) and 2 (BCPred Score = 0.926). Each O.D. value is representative for the mean of four absorbance values. Statistical differences for comparisons done to BSA are represented by *** ($p < 0.05$) after Student *t* test. Artwork created by Prism 5.0 software

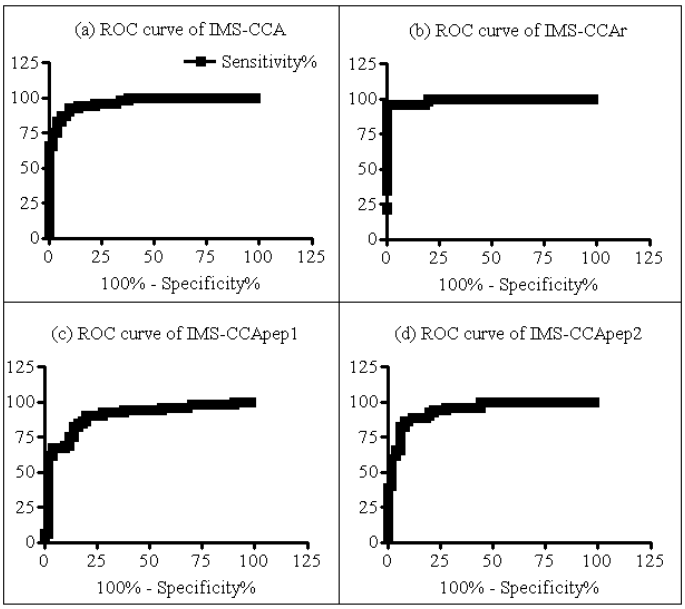


Fig 3 ROC curves of each IMS protocol. In (a) IMS-CCA (A = 0.967, p < 0.0001); (b) IMS-CCAr (A = 0.993, p < 0.0001); (c) IMS-CCApep1 (A = 0.899, p < 0.0001); and (d) IMS-CCApep2 (A = 0.944, p < 0.0001). Artwork created by Prism 5.0 software

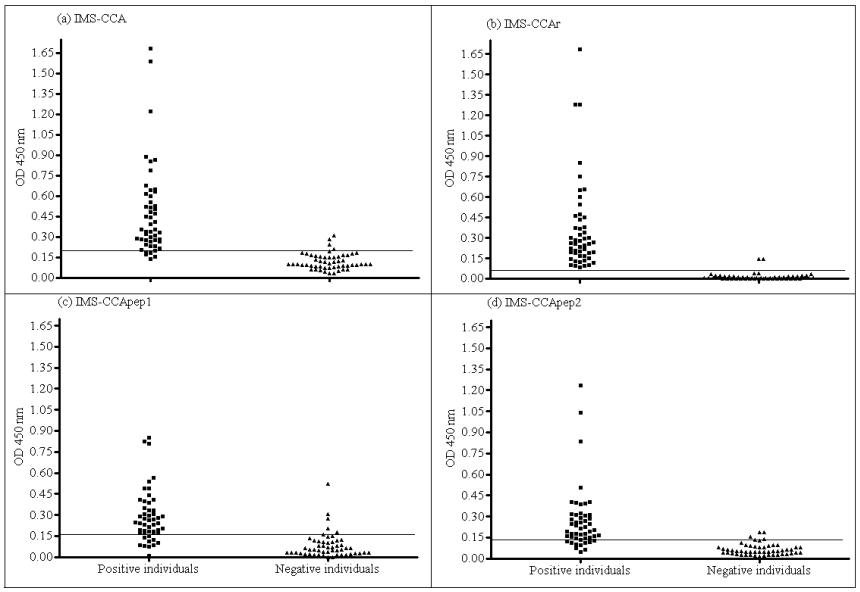


Fig 4 Individual analysis of 102 serum samples by the IMS protocols. Each O.D. value is representative for the mean of four absorbance values. Cut off values are represented by bars. In (a) IMS-CCA (cut off = 0.197); (b) IMS-CCAr (cut off = 0.063); (c) IMS-CCApep1 (cut off = 0.164); and (d) IMS-CCApep2 (cut off = 0.133). Artwork created by Prism 5.0 software

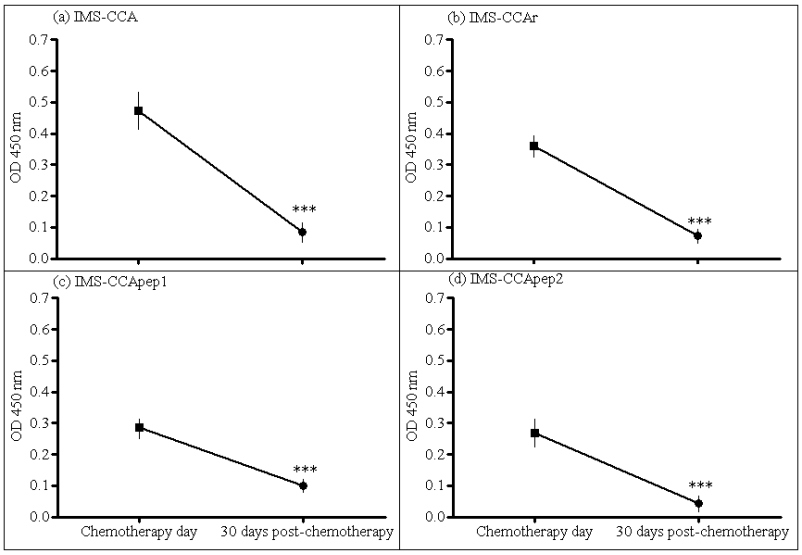


Fig 5 Timeline of positive individuals prior to and 30 days after chemotherapy diagnosed by IMS protocols. Each O.D. value is representative for the mean of specific IgG titers of all the 42 patients after chemotherapy. Boxes represents: (a) IMS-CCA; (b) IMS-CCAr; (c) IMS-CCApep1; and (d) IMS-CCApep2. Statistical differences between the absorbance value of pre- and post-treated individuals by each assay are represented by *** ($p < 0.001$) after Mann-Whitney analysis. Artwork created by Prism 5.0 software

4.6 ARTIGO 6

Newly established monoclonal antibody diagnostic assays for *Schistosoma mansoni* CCA detection in areas of low endemicity

New method for the direct diagnosis of schistosomiasis

Rafaella FQ Grenfell[a,b], Paulo Marcos Z Coelho*[a], Diana Taboada[a], Ana Carolina A Mattos[a], Ruth Davies[c], Donald A Harn[b]

[a] Schistosomiasis Laboratory, Rene Rachou Research Center, Oswaldo Cruz Foundation (Fiocruz). Avenida Augusto de Lima 1715 / 201 Belo Horizonte, Minas Gerais, Brazil 30190-002. Phone: 55 31 33497740, Fax: 55 31 32953115

[b] Department of Infectious Diseases, College of Veterinary Medicine, and Center for Tropical and Emerging Global Diseases, University of Georgia (UGA). 501 D.W. Brooks Drive, Athens, Georgia, 30602, United States of America. Phone: 1 706 5424569, Fax: 1 706 5830297

[c] Monoclonal Antibody Facility, College of Veterinary Medicine, UGA. 501 D.W. Brooks Drive, Athens, Georgia, 30602, United States of America. Phone: 1 706 5421848, Fax: 1 706 5830297

***Corresponding Author.** Mailing address: Schistosomiasis Laboratory, Rene Rachou Research Center, Oswaldo Cruz Foundation (Fiocruz), Av. Augusto de Lima, 1715, Belo Horizonte, MG, Brazil. 30.190-002. Phone: 55 31 3349 7740, e-mail: coelhopm@cpqrr.fiocruz.br

ABSTRACT

We developed three, novel diagnostic methodologies for the directly detection of schistosome infection in sera samples. These three new methods were evaluated with positive patients from a low endemicity area in southeast Brazil. The basis for these new assays was the production of a monoclonal antibody to the protein portion of highly purified CCA glycoprotein. This anti-CCA mAb was selected having no specificity for the *Lewis x* epitope. Three diagnostic methodologies were developed and validated, (1) Immunomagnetic Separation based on improved incubation steps of non-diluted sera, (2) Direct Enzyme-linked Immunosorbent Assay and (3) Fluorescent Microscopy Analysis as a qualitative assay. The two first quantitative methods presented a high sensitivity (94% and 92%, respectively) and specificity (100%) showing a significant correlation for determination of cure. The Immunomagnetic Separation technique showed excellent correlation with parasite burden. The third method was significant when a single sera sample was analyzed with 3 separate slides via an easy-to-do method capable of discriminating positive from negative cases, even for patients with low parasite burdens.

Keywords: *Schistosomiasis mansoni*, Direct diagnosis, Monoclonal antibody, Quantitative diagnosis, Fluorescent detection.

Sponsors: National Counsel of Technological and Scientific Development (CNPq), Oswaldo Cruz Foundation (Fiocruz)/ Rene Rachou Research Center (CPqRR), Coordination for the Improvement of Higher Level Education Personnel (Capes), The Council of the International Educational Exchange of Scholars (Fulbright, U.S. Department of State). National Institutes of Health AI R01 AI068109A to DAH.

INTRODUCTION

Schistosomiasis mansoni is a major parasitic disease associated with considerable morbidity and mortality in the developing world that can lead to sequelae of acute and chronic infection, including hepatointestinal disease, portal and pulmonary hypertension, liver fibrosis, and less common conditions such as myelo-radiculitis.[1] The gold standard for the diagnosis of *schistosomiasis mansoni* is the detection of parasite characteristic eggs in stools. The direct detection of eggs is difficult and not always possible in patients with low parasite burdens, thus low egg-shedding rates. Serological tests such as enzyme-linked immunosorbent assays (ELISA), immunofluorescence assays and indirect haemagglutination assays are used widely to detect antibodies against worm or soluble egg antigens. However, these assays are unable to differentiate between persistent and inactive infection.[2] While polymerase chain reaction methods can detect schistosomal egg DNA in stool, and parasite DNA in serum samples, none of the published PCR methods has been evaluated for utilization in routine diagnosis.[3,4]

Measurement of circulating antigens, as Circulating Cathodic Antigen (CCA) that are genus-specific glycoconjugates associated with the gut of the worm, appear promising as an alternative to egg counts for the detection of active infection for the diagnosis of *S. mansoni*, *S. haematobium*, and *S. japonicum* infections.[5-8] CCA is regurgitated from female and male worms into the circulatory system and levels of CCA are related to the presence and intensity of schistosome infection.[9,10] However, the techniques for this method are cumbersome and still have a low rate of sensitivity. The most recent advancement of the CCA test is a CCA dipstick test, a simple method used in endemic areas in Africa that unfortunately showed poor accuracy for diagnosis implying that the test was not suitable for rapid mapping of schistosomiasis.[11,12] Fluorescence imaging has also become a valuable approach for antigen detection.[13,14] The visualization of the parasite in whole or in part in fluorescence microscopy,

especially for an antigen that is a marker of active infection, should be considered. The first step in improving diagnostic tests for parasite-based diagnosis of schistosomiasis is of paramount importance. Misdiagnosis of schistosomiasis is costly and results in considerable morbidity and mortality, due to the fact that it contributes to both a delay in treatment for the correct diagnosis and to increasing drug pressure and thus resistance, thereby speeding up the obsolescence of affordable drugs. For utilization under field conditions, an assay should be rapid, specific and, most importantly, sensitive enough to discriminate between active infections, and those of recently treated patients.

This study evaluates the potential of three new diagnostic methods for schistosome infection; two quantitative and one qualitative, each based on the direct detection of CCA for determining active schistosome infection or to monitor the effectiveness of chemotherapy. Special characteristics allow these new methods to achieve a higher sensitivity compared to existing diagnostic assays. The new methods takes advantage of 1) paramagnetic microspheres that are then coated with the anti-CCA monoclonal antibody for the specific detection of protein epitopes of CCA avoiding potential cross-reactivity with carbohydrate epitopes; 2) incubation steps under rotation allowing for increased binding of antigen-antibody; (3) use of non-diluted sera permitting the detection of CCA even in patients with low parasite burdens. To test these concepts, individuals from an endemic area of low endemicity were selected and intensively monitored by parasitological assays for the first time before and after chemotherapy prior to the analysis of the new methods.

RESULTS

Generation of CCA-specific mAbs

The purified CCA glycoprotein obtained from *S. mansoni* adult worm extract was analyzed by SDS-PAGE and ELISA. Results are shown in Figure 1. In (a), SDS-PAGE results show a purified protein of 30 kDa, corroborating previous findings[19] and, in (b), the positive binding of the purified glycoprotein and the specific mAb compared to Bovine Serum Albumin (BSA) as negative control and SWAP as positive control.

The fusion of splenocytes from CCA-immunized mice with Sp2/0-Ag14 myeloma cells yielded a total of 186 HAT-resistant hybridoma clones. Thirteen clones with high CCA binding as determined by ELISA were selected. Among these thirteen, 5 clones were eliminated as they bound to *Lewis x*. Clones secreting mAbs against the protein portion of CCA were then isotyped, expanded and stored in liquid nitrogen. The characterization of the mAbs reacting specifically with CCA antigens is summarized in Table 1.

Purified mAbs (16D7.C10) were conjugated to peroxidase and also to Alexa fluor 647 and used for further experiments.

Validation of IMS-mAbCCA and ELISA-mAbCCA as quantitative methods for the diagnosis of schistosomiasis mansoni

An area of low endemicity for *schistosomiasis mansoni* in Brazil called Pedra Preta was selected for our survey. Among the 201 individuals participating on the survey, 50 patients including adults and children were selected to provide serum samples (24 women/26 men). These patients were first diagnosed by Kato-Katz and TF-Test and results showed a parasite load range between 1 and 555 epg among the group. All positive patients were treated as

recommended by the Brazilian Ministry of Health and resubmitted stools for Kato-Katz exam 30 and 90 days post chemotherapy. Thirty six of these patients agreed to donate sera samples. Retreatment was done in all reinfection cases. Fifty two sera samples from schistosome negative healthy donors were selected to be the negative group after these samples were properly diagnosed as negative by ELISA-SEA and ELISA-SWAP.

The 50 positive patients together with the 52 negative individuals were initially screened by two new quantitative assays, the IMS- and ELISA-mAbCCA, for the evaluation of the capability of each methodology to directly detect CCA in sera. The first analysis allowed the determination of the cut off values, positivity ratios, sensitivity and specificity of both methodologies by using a ROC curve analysis, represented in Figure 2.

The IMS methodology demonstrated 94% sensitivity and 100% specificity (cut off = 0.036), whereas ELISA showed similar effectiveness with 92% and 100% sensitivity and specificity, respectively (cut off = 0.031). No false-positives were seen, but IMS-mAbCCA presented 3 false-negative while ELISA-mAbCCA presented 4 false-negative results. Analysis of the positivity ratios were 97% (99/102) for IMS and 96% (98/102) for ELISA. Thirty six of the selected positive patients from Pedra Preta donated sera and faecal samples 30 and 90 days after chemotherapy. Faecal samples were intensively revaluated by Kato-Katz and TF-Test as described and no eggs were found in any samples 30 days after chemotherapy. On the other hand, two patients presented 1 epg after 90 days of chemotherapy. Individual OD results determined by each quantitative methodology for each patient are shown in Figure 3.

Analysis of Cohen's Kappa Index showed an almost perfect agreement of 0.941 ($\pm$ 0.099) between parasitological results and IMS-mAbCCA. Similar result was found for ELISA-mAbCCA in comparison to the gold standard with an agreement of 0.921 ($\pm$ 0.099). Same agreement was found for IMS- and ELISA-mAbCCA comparison (kappa index = 0.921 $\pm$

0.099). The correlation between IMS-mAbCCA results and fecal egg output for the 50 positive patients is shown on Figure 4 (R^2 = 0.99). ELISA-mAbCCA did not show the same positive correction between absorbance values and egg counts (data not shown) than IMS (p = 0.005). Three exceptions for this correlation were found for patients with 4, 7 and 39 epg that were not detected by IMS-mAbCCA.

Validation of FluoIMS-mAbCCA as a qualitative method for the diagnosis of *schistosomiasis mansoni*

The identical 102 sera samples from positive and negative individuals were used for standardization and validation of a qualitative method called FluoIMS-mAbCCA where microspheres were sensitized with mAbCCA to promote the binding of schistosome CCA antigen present in sera. By using Alexa fluor 647 attached to a second specific mAb, microspheres can be visualized using fluorescent microscopy. Double blind analysis showed that the microsphere's small size of 0.4 μm decreased the visibility creating a limitation for this assay. Identification of positive microspheres can be seen on Figure 5.

Nonetheless, the positivity ratio for FluoIMS was 74% (75/102) when one slide was performed. Thirty two positive individuals out of 50 and 43 negative individuals out of 52 were properly detected. Moreover, six false-positive results were seen for the thirty six samples obtained after 30 days of chemotherapy, decreasing the specificity of the assay. The accuracy of FluoIMS was increased when two extra slides were performed for each false-negative result for the 3 patients mistakenly diagnosed ($p < 0.001$). Completed data are shown on Table 2.

DISCUSSION

Definitive and accurate diagnosis is increasingly required for monitoring locality prevalence and severity of *schistosomiasis mansoni*. Presently, selective chemotherapy with praziquantel is being widely used, including by national schistosomiasis programs.[25] Identification of populations to be targeted for individual treatment and broad-spectrum chemotherapy in schistosomiasis-endemic areas, assessment of chemotherapy efficacy, morbidity, and evaluation of control strategies need to be based on reliable and available diagnostic tools. Faecal detection lacks sufficient sensitivity and patient compliance,[26-28] especially in areas where the endemicity is low and poor sensitivity limits application in large-scale and individual diagnosis.[25,29]

Indirect serologic tests, although well-accepted, cannot differentiate active from treated infections in surveillance and thus cannot identify reinfection.[30] Detection of circulating antigens secreted by living parasites has been considered as an alternative diagnostic method to distinguish active infections,[31-33] including Circulating Cathodic Antigen. Therefore, our group has been working on the development and validation of new methodologies in which the direct detection of CCA glycoprotein in sera samples is provided by concentration steps and the use of mAbs that lead to enhanced sensitivity and specificity. Initially, we validated the IMS methodology, a quantitative method based on the use of a specific IgM monoclonal antibody with the results compared to those obtained by direct ELISA. The use of similar microsphere immunoassays has become a popular approach for the diagnosis of many food-borne and infectious diseases.[34,35] This innovative technique involves immobilizing antibodies on micro-sized paramagnetic beads and uses antibody-coated beads to trap antigens from liquid samples. Furthermore, the small size and shape of the microspheres enables them to be

evenly dispersed in non-diluted samples improving the effectiveness of the antigen-antibody conjugation, and consequently enhancing the sensitivity of antigen detection.

The purification of CCA glycoprotein from adult worms of *S. mansoni* was confirmed by SDS-PAGE and by ELISA (Figure 1). Having purified CCA allowed for the production and purification of CCA-specific monoclonal antibodies (Table 1). We chose to select for anti-CCA monoclonal antibodies that did not bind to the *Lewis x* portion of CCA to increase the specificity of the assays, reducing false-positive data associated with cross-reactivity, mainly to other helminthes. Native CCA glycoprotein was described in terms of an O-linked poly or *Lewis x* carbohydrate chain with approximately 25 repeating units. Carbohydrate chains containing multiple *Lewis x* determinants have been identified on several glycolipids not only from schistosomes but from other parasites,[36,37] from human adenocarcinomas[38] and also, circulating granulocytes carry branched N-linked polysaccharides having *Lewis x* repeating units.[39] Thus, the use of mAbs against the glycan epitopes of schistosome CCA might lead to false-positives, especially when *Lewis x* from other sources is detected, as found by others.[5-8,12,40]

Therefore, our new assays were standardized and validated with the selected mAb for the sensitization of paramagnetic microspheres for IMS methodology and, 96-well microtiter plates for the direct ELISA. Since schistosomiasis epidemiological profile has shown an increase in the number of low endemicity areas and the direct diagnosis of those patients becomes more challenging, the confiability of each methodology was validated with 50 patients' samples from an endemic area in southeast Brazil where a low parasite burden was confirmed after an intensive and extensive analysis of 18 slides of faecal samples by Kato-Katz plus additional TF-Test analysis. Patients in that area had never been treated for schistosomiasis and had not been evaluated in a survey based on reevaluations and treatment

schemes every time a reinfection case is detected. Together, negative samples were obtained from volunteers with no medical history of previous schistosomiasis, who were also submitted to indirect ELISA-SWAP and ELISA-SEA diagnosis confirmation.

The IMS-mAbCCA based on quantitative peroxidase analysis demonstrated 94% of sensitivity and 100% of specificity for a cut off value of 0.036. Similarly, ELISA-mAbCCA presented 92% and 100% of sensitivity and specificity, respectively for a cut off value of 0.031 (Figure 2). The use of the new methods utilizing the CCA-specific IgM mAb, not related to *Lewis x* epitope, yielded three false-negative results via IMS-mAbCCA and four via the ELISA-mAbCCA method (Figure 3). These false-negative patients each had low egg burdens, from 4 to 39 epg. It is relevant that eggs were only detected in the stools of these individuals after a minimum of 10 Kato-Katz slides. Although IMS-mAbCCA was validated with non-diluted sera, differently from the ELISA, positivity ratios were similar with 97% for IMS when 99 out of 102 patients were correctly diagnosed in comparison to a positivity ratio of 96% for ELISA when 98 individuals were diagnosed.

Thirty six of the endemic positive patients were followed up 30 and 90 days after praziquantel chemotherapy. Once again, 18 slides of Kato-Katz together with TF-Test results were used as the gold standard. No eggs were found in faecal samples for any of these patients 30 days post-treatment and only 2 patients presented eggs in stools after 90 days. Analysis of the control and drug treated patients demonstrated that both IMS- and ELISA-mAbCCA reproduced parasitological results showing no OD reaction for those patients. These data corroborate with previous findings that showed that the level of circulating antigens in sera drops rapidly after three to six weeks of successful chemotherapy[10,41] and the direct detection of CCA is a considerable marker for the cure determination. Discordant results for the 2 reinfected or not cured patients may be explained by the low egg output determined by the

intensive analysis of parasitological assays that revealed only 1 epg for each patient. Nonetheless, additional investigations are suggested. Complementary data showed that IMS, but not ELISA, presented a positive correlation between the light absorbency intensity and egg output ($R^2 = 0.99$; $p = 0.005$) (Figure 4). Others[42] showed that the amount of circulating schistosome antigen was closely related to worm burden in infected mice. These observations were confirmed[43] when evaluating human samples. The use of microspheres for the antigen-antibody detection presents some advantages over the usual microtiter plates used for most diagnostic assays. The shape of the micro-beads enables them to be dispersed in the patient samples allowing for increased contact with antigens. Further, our analysis validated IMS with non-diluted sera, while a 100x dilution was used for ELISA.

Cohen's Kappa Index analysis revealed an almost perfect agreement between Kato-Katz and TF-Test and IMS- or ELISA-mAbCCA. Even with the established similarity of IMS- and ELISA-mAbCCA confiability for detecting active schistosomiasis infection, the superiority of IMS needs to be considered for use in field trials examining the impact of mass drug administration. The total time required for IMS was 2.3 hours in comparison to 4 hours for ELISA examination. It is possible to reduce this time with a diagnostic kit, when microspheres may be provided ready-to-use for the sera application. The standardization of an IMS kit may lead to a feasible production of an easy-to-do diagnostic assay.

We showed once more that quantitative methods are important tools for the direct diagnosis of active infections, especially when the individual parasite burden can be determined. To date, qualitative methods have not been demonstrated for the detection of circulating antigens, despite the advantages this approach has in reducing assay time associated with the simplicity of execution. Taken together with the simple training for technicians these methods may be more cost-effective as well as more accurate. We developed and validated the FluoIMS

methodology that is based on the same procedure described for IMS-mAbCCA except for the addition of the second mAb labeled here with Alexa fluor 647 for fluorescence microscopy analysis. Final data of a double blind analysis showed that an enhanced sensitivity of 94% was seen after the evaluation of 3 slides of each positive patient ($p < 0.001$). Same result was not seen when one single slide was performed and the assay lost on sensitivity (64%). On the other hand, the analysis of a single slide was enough to achieve 92% specificity for negative cases and 83% sensitivity for patients evaluated after chemotherapy. In conclusion, the confiability and positivity ratio of FluoIMS was significantly increased by two extra slides performed for each false-negative result ($p < 0.001$) when 15 positive patients become detectable presenting fluorescent microspheres (Table 2). Although promising, FluoIMS should be improved by extra validation, especially in order to make easier the small microspheres visualization under fluorescent microscopy (Figure 5) which will improve the sensitivity. Plus, false-positive results may be reduced by increasing washing steps after incubation to reduce the background. Finally, a quantification of the fluorescent microspheres could be further standardized.

The present study was undertaken to validate promising new schistosome diagnostic assays that might be more sensitive and specific than the currently employed assays. A special concern was to validate these new methodologies for their capability of discriminate active infections from previous contact using samples from low endemicity areas. The difficulty of diagnosing patients with low parasite burden needs urgent intervention since low endemicity areas will increase in number as control programs continue to use a small number of parasitological slides or large scale treatment, decreasing the infection intensities and underreporting reinfection rates. Overall, our results revealed that these new diagnostic methods, especially IMS-mAbCCA, showed high sensitivity, compliance, practicability even for the diagnosis of patients with low parasite burden and can be a potential alternative for the

individual and/or field diagnosis and finally, for the control of cure of *schistosomiasis mansoni* with a high degree of precision.

ACKNOWLEDGEMENTS

We thank Áureo Almeida and Liliane Siqueira for the help provided in Kato-Katz and TF-Test analysis. We also thank the Monoclonal Antibody Facility of University of Georgia for the collaboration and Merck Estapor for the donation of microspheres.

FIGURE LEGENDS

Fig 1 Confirmation analysis of CCA glycoprotein purification. Aliquots of *S. mansoni* adult worm extract purification products were analyzed by silver stained SDS-PAGE (a). Electrophoresis was performed as described. Molecular weight standards are shown in lane 1. Products were also analyzed by ELISA (b). Antigens represented by bars are: SWAP – soluble adult worm antigen extract, as the positive control; purified CCA glycoprotein and BSA – Bovine Serum Albumin. Each OD value is representative for the mean of four absorbance values. Statistical differences are represented by *** ($p < 0.05$) after Student *t* test. Artwork created by Prism 5.0 software

Fig 2 ROC curves for the quantitative methodologies based on the direct detection of CCA in sera. In (a) IMS-mAbCCA (A = 0.957, p < 0.0001) and (b) ELISA-mAbCCA (A = 0.982, p < 0.0001). Artwork created by Prism 5.0 software

Fig 3 Individual analysis of sera samples by IMS- and ELISA-mAbCCA protocols. Each OD value is representative for the mean of four absorbance values. Groups are represented by 50 positive individuals from Pedra Preta, 52 negative individuals and 36 patients after 30 and 90

days of chemotherapy. Cut off values are represented by bars. In (a) IMS-mAbCCA (cut off = 0.036) and (b) ELISA (cut off = 0.031). Artwork created by Prism 5.0 software

Figura 4 Correlation between optical densities (OD) obtained in IMS-mAbCCA and *S. mansoni* egg counts (egg per gram of faces - epg) in sera samples from positive patients. The epg ranged from 1-555 epg, which is represented by the logarithmic transformation along the x-axis. A high correlation was found between the OD values and egg counts (R^2 = 0.99). Artwork created by Prism 5.0 software

Fig 5 Representative images of FluoIMS-mAbCCA. In (a) negative sera sample, (b) microspheres visualization under white light, (c) and (d) positive sera samples with fluorescent microspheres under 642 nm, emission filter LP 590.

TABLES

Table 1 Characterization of mAbs produced against antigens of purified CCA glycoprotein.

		ELISA	
Clones	Ig-subclass	CCA specificity	*Lewis x* specificity
1.3C2b	IgG1	+++	-
1.2C6	IgG1	++	-
4.4C3	IgG1	+++	-
5.1B4	IgG1	+++	-
5.1B1	ND	++	+++
5.2A3	IgG1	++	-
5.1D3	IgG1	++	-
5F4.E4	ND	++	++
16D7.C10	IgM	+++	-
16D7.C4	ND	+++	++
16D7.B9	ND	+++	++
12D3.F2	IgM	+++	-

12D3.G8	ND	++	++

+ = positive reaction; - = negative reaction; ND = not determined.

Table 2 Performance of FluoIMS-mAbCCA on the detection of CCA in patients' sera.

	Results with 1 slide				Results with 3 slides			
	n		%		*N*		%	
Individuals	+	-	+	-	+	-	+	-
Positive (*n* = 50)	32***	18	64	36	47***	3	94	6
Positive post chemotherapy (*n* = 36)	3	33	8	92	ND[2]	ND	ND	ND
Negative (*n* = 52)	9	43	17	83	ND	ND	ND	ND

[1] Comparison analysis performed using Kato-Katz and TF-Test results as gold standards. [2] ND = not determined. *** represents statistical differences between 1 and 3 slides of FluoIMS ($p < 0.001$).

ONLINE METHODS

Community survey-We performed a study in Buriti Seco and Morro Grande in Pedra Preta, a schistosomiasis endemic area in the rural region of Montes Claros, state of Minas Gerais, southeast Brazil.[15] This area was chosen based on the fact that the population had not being treated for schistosomiasis and, additionally, it has a low population migration index. The residents participating in the survey was 201 individuals aged 1-88 (93 women/108 men).

Four stool samples per individual were collected on four consecutive days for Kato-Katz analysis.[16] This method was performed using 18 slides, which were prepared as follows for each participant: 12 slides of the first sample and two slides each for the second, third and fourth sample in a total of 750mg of faeces (18x42.7mg). Samples were also analyzed by quantitative TF-Test[15] and sediment was analyzed using optical microscopy.[17]

Among the individuals participating in the survey, fifty patients aged 8-88 were selected to provide serum samples (24 women/26 men). Among these individuals, parasite burdens ranged from 1-555 eggs per gram of faeces (epg). Individual serum samples were obtained after centrifugation of blood samples at 3000g for 5 minutes. These samples were maintained at –20^{o}C.

All positive participants for schistosomiasis were treated with praziquantel in a single dose of 60mg/kg for children and 50mg/kg for adults. Infections with other helminthes were treated with 400mg albendazole, as recommended by the Brazilian Ministry of Health. Thirty six positive patients were re-examined for schistosome infection by Kato-Katz and TF-Test 30 and 90 days post chemotherapy and retreated as needed, when serum samples were also obtained.

Healthy donors-Fifty two schistosome infection negative volunteers aged 22-65 (34 women/18 men) were selected to be part of the negative control group. These volunteers were non-endemic area residents or visitors with no medical history of previous schistosome infection. Serum samples of these donors were processed as described earlier.

Besides the historical criteria used to select healthy donors, we also performed ELISA assays for the detection of IgG antibodies against soluble adult worm antigens (ELISA-SWAP) and soluble egg antigens (ELISA-SEA).

Production of monoclonal antibody specific for CCA (mAbCCA)-*S. mansoni* (LE strain) adult worms were obtained by hepatic portal perfusion of *swiss* female mice 45 days post-infection with 100 cercariae.[18] Adult worms were washed with PBS, mechanically ground (Virtiz Precisa) then centrifuged at 25000g for 1h at 4ºC (Sorvall). Supernatant was collected and incubated at 100ºC for 30 minutes[19], then passed through a 50kDa filter (Millipore

Amicon, Sigma-Aldrich) by centrifuging the solution at 2700g. Finally, the product was dialyzed in cellulose membrane (Sigma-Aldrich) against saline solution 0.9% for 48h at 4ºC. An aliquot was submitted for protein assessment (Nanodrop, Thermo Scientific 2000). The final product was analyzed via silver stained Tris-glycine SDS-PAGE (12% gel)[20] and by an immunoassay when microtiter plates MaxiSorpTM Surface (NUNC) were sensitized with 1µg/ml of the purified CCA glycoprotein in 0.05M carbonate-bicarbonate pH9.6 (coating-buffer) for 16h at 4ºC. Positive and negative controls were SWAP and Bovine Serum Albumin (BSA). Plates were washed with PBS 0.05% Tween 20 (washing buffer) and, the non specific sites were blocked with 2.5% skim milk at 37ºC for an hour. After washing, 100µl/well of a specific IgG1 monoclonal antibody conjugated to peroxidase (5F4.B4, Monoclonal Antibody Facility, UGA) was added (1:8000) for 1h. Plates were washed again, then 100µl of substrate 3,3',5,5-tetramethylbenzidine solution (TMB) (Invitrogen) were added followed by 15 minutes incubation and the addition of 50µl/well of 2N sulfuric acid. Results were obtained as optical density (OD) at 450nm in microplate reader (Model 3550, Bio-Rad Laboratories).

Nine-week-old female BALB/c mice were immunized subcutaneously with 0.1mg of purified *S. mansoni* CCA using a new vaccine delivery method (US patent n.61/476,431) as adjuvant. Two weeks later, mice were boosted. Sera from mice were tested by ELISA to determine the antibody titer against CCA. Mice with the highest antibody titers were given an additional boost 15 days after the first boost by intraperitoneal injection of 0.1mg of CCA. Three days later, spleen cells were fused with Sp2/O-Ag14 myeloma cells using polyethyleneglycol. The fused cells were cultured on 96-well plates and selected with hypoxanthine-aminopterin-thymidine (HAT) medium. The initial screen of positive growth wells was by ELISA for antibodies to CCA. CCA was diluted 1µg/ml in coating buffer and microtiter plates were coated at 4ºC for 16h. After blocking with 2.5% of skim milk for 1h, 100µl of culture

supernatants of the HAT-selected hybridomas were added and incubated for 1h. After washing steps, the bound antibodies were detected using peroxidase-conjugated antimouse IgG (1:5000) (Southern Biotech) and TMB. ELISA positive hybridomas were selected. A second ELISA was performed to differentiate the hybridomas producing mAbs against the carbohydrate portion of CCA glycoprotein versus hybridomas producing mAbs to the protein portion of CCA. Plates were coated with *Lewis x* tetrasacharide (Sigma-Aldrich, Saint Louis, US) and the ELISA performed as described. Hybridomas non-reactive for *Lewis x* were selected and Ig-subclasses were determined by a kit for monoclonal isotyping (Sigma-Aldrich).

The selected clone (16D7.C10 IgM) was grown in hybridoma medium (Invitrogen) supplemented with penicillin (100U/mL) and streptomycin (100mg/mL). Culture supernatants were harvested and used for ammonium sulfate precipitation.[21] Precipitated proteins were dissolved in PBS, dialyzed against PBS and then the mAb purified by protein G purification column (Sigma-Aldrich) according to the manufacturers instructions. After measuring the OD at 280nm of the fractions, the protein-containing fractions were stored at -20ºC. Aliquots of the mAb were conjugated to peroxidase with Zenon Mouse Labeling Kit (Invitrogen) and, also to Alexa Fluor 647 with Fluorochrome Protein Labeling Kit (Invitrogen), according to the manufacturer's protocol.

Immunological assay evaluation for the direct detection of CCA in individual sera- *Immunomagnetic Separation technique with mAbCCA (IMS-mAbCCA)*-Paramagnetic microspheres (0.4µm; 10^6 microspheres/assay) (Merck) were sensitized with 1µg/ml of mAbCCA diluted in coating-buffer. The following steps were performed with a rotating suspension system to improve antigen-antibody binding. Microspheres were incubated for 16h at 4ºC and then, washed four times with washing buffer using a 1.5ml tube magnetic base

(Invitrogen). Non specific sites were blocked with 20% skim milk at 4ºC for 16h. The microspheres were then maintained at 4ºC until use. On the day of the analysis, microspheres were washed then 200µl of undiluted serum samples were added into duplicate tubes, followed by incubation at 37ºC for 2h. Microspheres were washed, then incubated at 37ºC for an hour with 100µl of peroxidase conjugated mAbCCA (16D7.C10) diluted 1:400. Each tube was washed again and 100µl of TMB were added to each well. The reaction was stopped after 10 minutes incubation by addition of 100µl/tube of 2N sulfuric acid. Using the magnetic base, supernatant was transferred to a microtiter plate and results were obtained at 450nm in a microplate reader.

ELISA with mAbCCA (ELISA-mAbCCA)-ELISA was standardized based on the technique previously described[22] with some modifications. Microtiter plates were coated with mAbCCA diluted at 1µg/ml in coating buffer for 16h at 4ºC. Next, plates were washed with washing buffer then blocked by addition of 300µl per well of 2.5% skim milk, incubating at 37ºC for 1h. After additional washing, 100µl of individual sera diluted 1:100 were added to the plates in duplicate wells and incubated for 1h. Plates were then washed and incubated for 1h with peroxidase conjugated mAbCCA diluted 1:400. Plates were washed, then 100µl of TMB added to each well and the enzymatic reaction stopped after 10 minutes of incubation in the dark. Results were obtained at 450nm in microplate reader.

Fluorescent microscopy analysis of IMS products using mAbCCA (FluoIMS-mAbCCA)-The same procedure adopted for IMS-mAbCCA was used here for a double blind analysis. After microspheres were sensitized and blocked, 200µl of sera samples were added to 1.5ml tubes and incubated under rotation for 2h at 37ºC. Microspheres were then washed and 100µl of Alexa Fluor 647 conjugated mAbCCA diluted 1:400 in washing buffer were added. Qualitative analysis of 5µl of microspheres suspension was performed by examination on a

glass slide with a fluorescent microscope (Karl Zeiss) to visualize fluorescent microspheres (642nm, emission filter LP590). Photographic records were taken with a digital camera (Canon EOS).

Positive and negative controls were assayed for each technique as control of nonspecific adsorption of conjugate.

Statistical analysis-Absorbance value data were analyzed with Minitab Inc. by Kolmogorov-Smirnov normality test. Normal distributed data were analyzed by Student's *t* test and non-normal data by Mann-Whitney test. Comparisons between methods were done by 2 proportions' Fisher analysis ($p \leq 0.05$ as significance level). Sensitivity, specificity and cut off values were determined with Prism4.0. Agreement between methods was measured using Cohen coefficient[23] and analyzed by Landis & Koch definition[24].

Ethics-This project was approved by the Ethical Research Committee of Fiocruz for animal use (CEUA-L.0023/08) according to the International Guiding Principles for Biomedical Research Involving Animals developed by the Council for International Organizations of Medical Sciences. The Ethical Research Committee of Fiocruz (CEPSH-03/2008) and the National Brazilian Ethical Board (784/2008,CONEP-14886) approved the human study. The study objectives were explained to all participants and written consent was obtained through signing a form before admission to this study. Parents/guardians provided written consent on behalf of child participants.

REFERENCES

1. Edungbola, L.D., Asaolu, S.O., Omonisi, M.K., Aiyedun, B.A. *Schistosoma haematobium* infection among schoolchildren in the Babana district, Kwara State, Nigeria. *Afr. J. Med. Sci.* **17**:187-193 (1988).

2. Doenhoff, M.J., Chiodini, P.L., Hamilton, J.V. Specific and sensitive diagnosis of schistosome infection: can it be done with antibodies? *Trends Parasitol.* **20**:35-39 (2004).

3. Allam, A.F., Kader, O., Zaki, A., Shehab, A.Y., Farag, H.F. Assessing the marginal error in diagnosis and cure of *Schistosoma mansoni* in areas of low endemicity using Percoll and PCR techniques. *Trop. Med. Int. Health.* **14**(3):316-321 (2009).

4. Gomes, L.I., et al. Development and evaluation of a sensitive PCR-ELISA system for detection of *Schistosoma* infection in feces. *PLoS Negl. Trop. Dis.* **4**(4):e664 (2010).

5. Deelder, A.M., et al. Sensitive determination of circulating anodic antigen in *Schistosoma mansoni* infected individuals by an enzyme-linked immunosorbent assay using monoclonal antibodies. *Am. J. Trop. Med. Hyg.* **40**:268-272 (1989).

6. De Jonge, N., et al. Detection of the schistosome circulating cathodic antigen by enzyme immunoassay using biotinylated monoclonal antibodies. *Trans. R. Soc. Trop. Med. Hyg.* **84**:815-818 (1990).

7. Van't Wout, A.B., et al. Schistosome circulating anodic antigen in serum of individuals infected with Schistosoma japonicum from The Philippines before and after chemotherapy with praziquantel. *Trans. R. Soc. Trop. Med. Hyg*. **86**:410- 413 (1992).

8. Kremsner, P.G., et al. Circulating anodic and cathodic antigen in serum and urine from Schistosoma haematobium infected Cameroonian children receiving praziquantel: a longitudinal study. *Clin. Infect. Dis.* **18**:408-413 (1994).

9. Van Lieshout, L., et al. Improved diagnostic performance of the circulating antigen assay in human schistosomiasis by parallel testing for circulating anodic and cathodic antigens in serum and urine. *Am. J. Trop. Med. Hyg.* **47***:* 463–469 (1992).

10. Deelder, A.M., et al. Quantitative diagnosis of Schistosoma infections by measurement of circulating antigens in serum and urine. *Trop. Geogr. Med.* **46**(4 Spec No):233-8 (1994).

11. Ashton, R.A., et al. Accuracy of circulating cathodic antigen tests for rapid mapping of Schistosoma mansoni and S. haematobium infections in Southern Sudan. *Trop. Med. Int. Health*. **16**(9):1099-103 (2011).

12. Coulibaly, J.T., et al. Accuracy of urine circulating cathodic antigen (CCA) test for Schistosoma mansoni diagnosis in different settings of Côte d'Ivoire. *PLoS Negl. Trop. Dis.* **5**(11):e1384 (2011).

13. Hassan Sel, D., Haggaz, A.E., Mohammed-Elhassan, E.B., Malik, E.M., Adam, I. Fluorescence microscope (Cyscope) for malaria diagnosis in pregnant women in Medani Hospital, Sudan. *Diagn. Pathol.* **6**:88 (2011).

14. Tarp, B., Black, F.T., Petersen, E. The immunofluorescence antibody test (IFAT) for the diagnosis of schistosomiasis used in a non-endemic area. *Trop. Med. Int. Health.* **5**(3):185-91 (2000).

15. Siqueira, L.M., et al. Evaluation of two coproscopic techniques for the diagnosis of schistosomiasis in a low-transmission area in the state of Minas Gerais, Brazil. *Mem. Inst. Oswaldo Cruz.* **106** (7): 844-850 (2011).

16. Katz, N., Chaves, A., Pellegrino, J. A simple device for quantitative stool thick-smear technique in schistosomiasis mansoni. *Rev. Inst. Med. Trop.* Sao Paulo. **14**:397–400 (1972).

17. Gomes, J.F., et al. Evaluation of a novel kit (TF-Test) for the diagnosis of intestinal parasitic infections. *J. Clin. Lab. Anal.* **18**: 132-138 (2004).

18. Pellegrino, J., Siqueira, A. Técnica de perfusão para colheita de *Schistosoma mansoni* em cobaias experimentalmente infestadas. *Rev. Bras. Malar. Doenças Trop.* **8**: 589-597 (1956).

19. Deelder, A.M., Klappe, H.T., van den Aardweg, G.J., van Meerbeke, E.H. *Schistosoma mansoni*: demonstration of two circulating antigens in infected hamsters. *Exp. Parasitol.* **40** (2): 189-197 (1976).

20. Schagger, H., von Jagow, G. Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Anal. Biochem.* **166**(2):368–79 (1987).

21. Chick, H., Martin, C.J. The precipitation of egg-albumin by ammonium sulphate. A contribution to the theory of the "salting out" of proteins. *Biochem. J.* **7**:380-398 (1913).

22. Harlow, E., Lane, D. *Antibodies: A Laboratory Manual.* Cold Spring Harbor Laboratory Press, New York, NY (1988).

23. Cohen, J. Weighted kappa: nominal scale agreement with provisions for scales disagreement of partial credit. *Psychol. Bull.* **70**:213-20 (1968).

24. Landis, J.R., Koch, G.G. The measurement of observer agreement for categorical data. *Biometrics* **33**(1):159–74 (1977).

25. Bergquist, N.R. Schistosomiasis: from risk assessment to control. *Trends Parasitol.* **18**: 309–314 (2002).

26. Kongs, A., Marks, G., Verlé, P., Van der Stuyft, P. The unreliability of the Kato-Katz technique limits its usefulness for evaluating S. mansoni infections. *Trop. Med. Int. Health* **6**: 163–169 (2001).

27. Berhe, N., et al. Variations in helminth faecal egg counts in Kato-Katz thick smears and their implications in assessing infection status with Schistosoma mansoni. *Acta Trop.* **92**: 205–212 (2004).

28. Enk, M.J., Lima, A.C., Drummond, S.C., Schall, V.T., Coelho, P.M. The effect of the number of stool samples on the observed prevalence and the infection intensity with Schistosoma mansoni among a population in an area of low transmission. *Acta Trop.* **108**(2-3):222-228 (2008).

29. de Vlas, S.J., Gryseels, B. Underestimation of Schistosoma mansoni prevalences. *Parasitol. Today* **8**: 274–277 (1992).

30. Rabello, A.L., Garcia, M.M., Pinto da Silva, R.A., Rocha, R.S., Katz, N. Humoral immune responses in patients with acute Schistosoma mansoni infection who were followed up for two years after treatment. *Clin. Infect. Dis.* **24**: 304–308 (1997).

31. de Jonge, N., Boerman, O.C., Deelder, A.M. Time-resolved immunofluorometric assay (TR-IFMA) for the detection of the schistosome circulating anodic antigen. *Trans. R. Soc. Trop. Med. Hyg*. **83**: 659–663 (1989).

32. Kremsner, P.G., et al. Quantitative determination of circulating anodic and cathodic antigens in serum and urine of individuals infected with Schistosoma intercalatum. *Trans. R. Soc. Trop. Med. Hyg.* **87**: 167–169 (1993).

33. Ndhlovu, P., et al. Circulating anodic antigen (CAA) levels in different age groups in a zimbabwean rural community endemic for Schistosoma haematobium determined using the magnetic beads antigen-capture enzyme-linked immunoassay. *Am. J. Trop. Med. Hyg.* **54**: 537–542 (1996).

34. Nagasaki, Y., Kobayashi, H., Katsuyama, Y., Jomura, T., Sakura, T. Enhanced immunoresponse of antibody/mixed-PEG co-immobilized surface construction of high-performance immunomagnetic ELISA system. *J. Colloid. Interface Sci.* **309**: 524–530 (2007).

35. Chen, L., Deng, L., Liu, L., Peng, Z. Immunomagnetic separation and MS/SPR end-detection combined procedure for rapid detection of Staphylococcus aureus and protein A. *Biosens. Bioelectron*. **22**: 1487–1492 (2007).

36. Dissanayake, S., Shahin, A. Induction of interferon-gamma by Taenia crassiceps glycans and Lewis sugars in naive BALB/c spleen and peritoneal exudate cells. *Mol. Immunol.* **44**(7):1623-30 (2007).

37. Haslam, S.M., Coles, G.C., Morris, H.R., Dell, A. Structural characterization of the N-glycans of Dictyocaulus viviparus: discovery of the Lewis(x) structure in a nematode. *Glycobiology.***10**(2):223-9 (2000).

38. Hakomori, S., Nudelman, E., Levery, S.B., Kannagi, R. Novel fucolipids accumulating in human adenocarcinoma. I. Glycolipids with di- or trifucosylated type 2 chains, *J. Biol. Chem.* **259**,4672-4680 (1984).

39. Spooncer, E., Fukuda, M., Klock, J.C., Oates, J.E., Dell, A. Isolation and characterization of polyfucosylated lactosaminoglycan from human granulocytes, *J. Biol. Chem.* **259**,4792-4801 (1984).

40. Shane, H.L., et al. Evaluation of urine CCA assays for detection of Schistosoma mansoni infection in Western Kenya. *PLoS Negl. Trop. Dis.* **5**(1):e951 (2011).

41. Van Lieshout, L., et al. Monitoring the efficacy of different doses of praziquantel by quantification of circulating antigens in serum and urine of schistosomiasis patients. *Parasitology*. **108** (Pt 5): 519-526 (1994).

42. Feldmeier, H., et al. Detection and quantification of circulating antigen in schistosomiasis by monoclonal antibody. II. The quantification of circulating antigens in human schistosomiasis mansoni and haematobium: relationship to intensity of infection and disease status. *Clin. Exp. Immunol*. **65**: 232–243 (1986).

43. Van Etten, L., et al. Fluctuation of schistosome circulating antigen levels in urine of individuals with Schistosoma mansoni infection in Burundi. *Am. J. Trop. Med. Hyg.* **54**(4):348-51 (1996).

FIGURES AND FIGURE LEGENDS

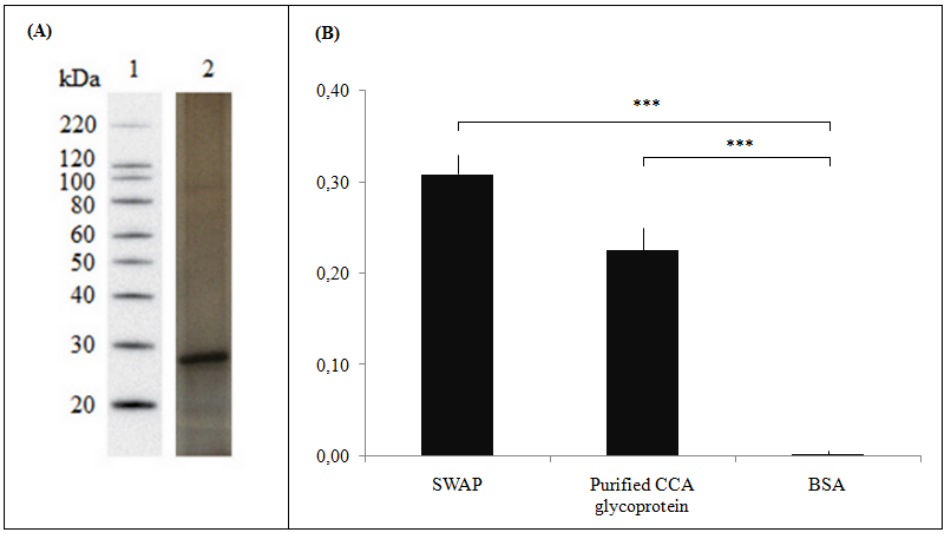


Fig 1 Confirmation analysis of CCA glycoprotein purification. Aliquots of *S. mansoni* adult worm extract purification products were analyzed by silver stained SDS-PAGE (A). Electrophoresis was performed as described. Molecular weight standards are shown in lane 1. Products were also analyzed by ELISA (B). Antigens represented by bars are: SWAP – soluble adult worm antigen extract, as the positive control; purified CCA glycoprotein and BSA – Bovine Serum Albumin. Each OD value is representative for the mean of four

absorbance values. Statistical differences are represented by *** ($p < 0.05$) after Student *t* test. Artwork created by Prism 5.0 software

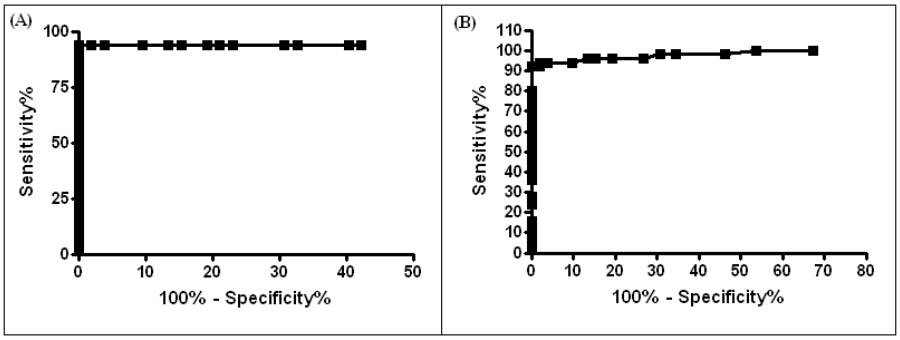


Fig 2 ROC curves for the quantitative methodologies based on the direct detection of CCA in sera. In (A) IMS-mAbCCA (A = 0.957, p < 0.0001) and (B) ELISA-mAbCCA (A = 0.982, p < 0.0001). Artwork created by Prism 5.0 software

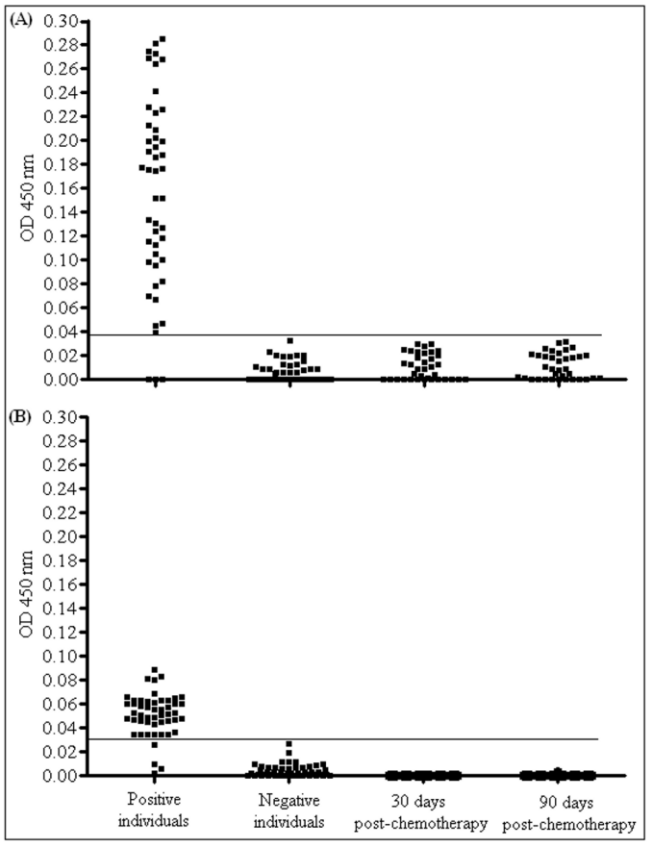


Fig 3 Individual analysis of sera samples by IMS- and ELISA-mAbCCA protocols. Each OD value is representative for the mean of four absorbance values. Groups are represented by 50 positive individuals from Pedra Preta, 52 negative individuals and 36 patients after 30 and 90

days of chemotherapy. Cut off values are represented by bars. In (A) IMS-mAbCCA (cut off = 0.036) and (B) ELISA (cut off = 0.031). Artwork created by Prism 5.0 software

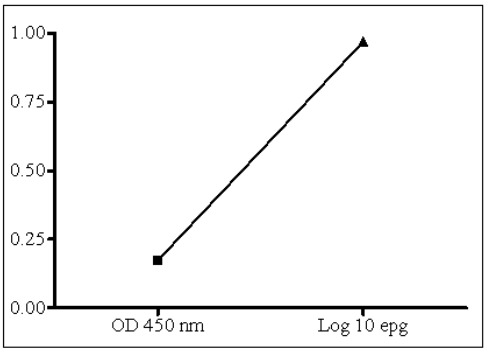


Figura 4 Correlation between optical densities (OD) obtained in IMS-mAbCCA and *S. mansoni* egg counts (egg per gram of faces - epg) in sera samples from positive patients. The epg ranged from 1-555 epg, which is represented by the logarithmic transformation along the x-axis. A high correlation was found between the OD values and egg counts (R^2 = 0.99). Artwork created by Prism 5.0 software

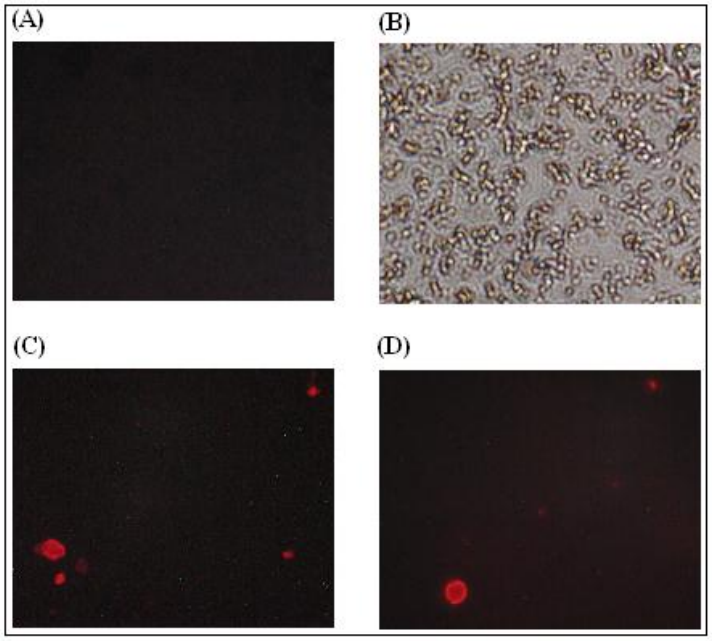


Fig 5 Representative images of FluoIMS-mAbCCA. In (A) negative sera sample, (B) microspheres visualization under white light, (C) and (D) positive sera samples with fluorescent microspheres under 642 nm, emission filter LP 590.

4.7 PATENTE

Registrada: ***New Vaccine Delivery Method*** **no** ***The United States Patent and Trademark Office*** **em 18.04.2011**

5 PERSPECTIVAS

5 PERSPECTIVAS

1 Para os métodos de diagnóstico que utilizam SmTeg, fazer avaliação experimental, em ordem cronológica, iniciando-se no terceiro dia da infecção e finalizando na fase crônica, para deteminar se a metodologia detecta a trasiência entre fase aguda e crônica.

2 Determinar a ocorrência de reações cruzadas com outros helmintos nas técnicas desenvolvidas no presente estudo.

3 Produzir anticorpos monoclonais específicos para a proteína CCA recombinante e para seus peptídeos de 20 aminoácidos para futura padronização dos métodos de IMS e FluoIMS para detecção de direta do CCA em amostras sorológicas.

4 Testar os métodos de detecção direta de CCA em amostras de urina, uma vez que esta glicoproteína é largamente eliminada por esta via. Desta forma, padronizaremos métodos não invasivos com promissora capacidade diagnóstica.

5 Testar os métodos de IMS e FluoIMS em amostras de pacientes com infecções por *Schistosoma* de outras espécies humanas, baseando-se na comprovação de que CCA é excretado/secretado por outras espécies do parasito conforme bibliografia publicada. Desta forma, será possível confirmar a efetividade dos novos métodos no diagnóstico das espécies de *Schistosoma*. Este projeto será feito em parceria com pesquisadores que traalham com outras espécies do parasito.

6 Desenvolver kits de diagnóstico utilizando os anticorpos monoclonais anti-CCA aderidos nas microesferas com base no baixo custo e na simplicidade de execução. Etapa atualmente negociada com a Merck.

7 Padronizar estas técnicas para estudos epidemiológicos e individuais.

8 Ampliar a validação dos métodos de IMS e FluoIMS em outros estudos epidemiológicos para controle de cura.

9 O adjuvante patenteado descrito neste trabalho já foi testado em algumas vacinas experimentais, envolvendo vírus recombinante da hepatite B, *Influenza* e outras, com resultados superiores aos adjuvantes utilizados em modelos experimentais (adjuvante de Freund) e humano (Alum). Novos estudos, que envolvem diferentes infecções, estão sendo iniciados.

10 Este trabalho gerou a criação de infraestrutura de produção de anticorpos monoclonais que resultou na consolidação de uma Plataforma institucional voltada para a produção de anticorpos em geral.

6 REFERÊNCIAS BIBLIOGRÁFICAS

6 REFERÊNCIAS BIBLIOGRÁFICAS

Abath FG, Xavier EM, Allen R, Gomes YM, Lucena-Silva N, Baliza M, Simpson AJ. Characterization of Sm13, a tegumental antigen of *Schistosoma mansoni*. Parasitol Res 2000; 86 (9): 745-52.

Agnew AM, Fulford AJC, De Jonge N, Krijger FW, Rodriguez-Chacon M, Gutsmann V, Deelder AM. The relationship between worm burden and levels of a circulating antigen (CAA) of five species of *Schistosoma* in mice. Parasitol 1995; 111: 67-76.

Al-Sherbiny MM, Osman AM, Hancock K, Deelder AM, Tsang VC. Application of immunodiagnostic assays: detection of antibodies and circulating antigens in human schistosomiasis and correlation with clinical findings. Am J Trop Med Hyg 1999; 60 (6): 960-6.

Andrade ZA, Azevedo TM. A contribution to the study of acute schistosomiasis (an experimental trial). Mem Inst Oswaldo Cruz 1987; 82: 311-17.

Andrade ZA, Prata A. Asymptomatic schistosomiasis studied by needle biopsy of the liver. The Am J Trop Med & Hyg 1963; 5: 236-42.

Andrade ZA. A patologia da esquistossomose humana. In: Carvalho OS, Coelho PMZ, Lenzi H. *Schistosoma mansoni* & Esquistossomose: uma visão multidisciplinar. Rio de Janeiro: Fiocruz; 2008. p. 547.

Andrade ZA. Schistosomal hepatopathology. Mem Inst Oswaldo Cruz 2004. 99 (Suppl. 1): 51-7.

Aronstein WS, Strand M. Identification of species-specific and gender-specific proteins and glycoproteins of three human schistosomes. J Parasitol. 1983; 69 (6): 1006-17.

Ashton PD et al. The schistosome eggs: development and secretions. Parasitology 2001; 122: 329-38.

Ashton PD, Harrop R, Shah B, Wilson RA. The schistosome egg: development and secretions. Parasitology 2001; 122 (Pt 3): 329-38.

Ashton RA, Stewart BT, Petty N, Lado M, Finn T, Brooker S, Kolaczinski JH. Accuracy of circulating cathodic antigen tests for rapid mapping of *Schistosoma mansoni* and *S. haematobium* infections in Southern Sudan. Trop Med Int Health 2011; 16 (9): 1099-103.

Barbosa MA. Quantitative aspects of the migration and evolutive asynchronism of *Schistosoma mansoni* in mice. Rev Inst Med Trop São Paulo 1978; 20: 121-132.

Bergquist NR. Present aspects of immunodiagnosis of schistosomiasis. Mem Inst Oswaldo Cruz 1992; 87: 29-38.

Berhe N, Medhin G, Erko B, Smith T, Gedamu S, Bereded D, Moore R, et al. Variations in helminth faecal egg counts in Kato-Katz thick smears and their implications in assessing infection status with *Schistosoma mansoni*. Acta Trop 2004; 92 (3): 205-12.

Bhardwaj R, Krautz-Peterson G, Da'dara A, Tzipori S, Skelly PJ. Tegumental Phosphodiesterase SmNPP-5 Is a Virulence Factor for Schistosomes. Infetion and Immunity 2011; 4276–4284.

Bina JC, Prata A. regressão da hepatoesplenomegalia pelo tratamento específico da esquistossomose. Ver Soc Bras Med Trop 1983; 16: 213-18.

Bogitsh BJ. Observation on digestion in schistosomes or 'blood and guts'. Trans Am Microscopical Soc 1989; 108: 1-5.

Borges JJ et al. Immunofluorescent visualization of the excretory and gut system of *Schistosoma mansoni* by confocal laser scanning microscopy. The Am J Trop Med & Hyg 1994; 50: 612-19.

Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976; 7 (72): 248-254.

Braschi, S, Borges WC, Wilson RA. Proteomic analysis of the schistosome tegument and its surface membranes. Mem Inst Oswaldo Cruz 2006a; 101 (Suppl. 1): 205–212.

Braschi, S, Curwen RS, Ashton PD, Verjovski-Almeida S, Wilson A. The tegument surface membranes of the human blood parasite *Schistosoma mansoni*: a proteomic analysis after differential extraction. Proteomics 2006b; 6: 1471–1482.

Cançado JR et al. Evaluation of the treatment of human *Schistosoma mansoni* infection by the quantitative oogram technique. Bull WHO 1965; 33: 557-66.

Cardoso FC, Macedo GC, Gava E, Kitten GT, Mati VL, de Melo AL, Caliari MV, et al. *Schistosoma mansoni* tegument protein Sm29 is able to induce a Th1-type of immune response and protection against parasite infection. PLoS Negl Trop Dis 2008; 2 (10): e308.

Carter CE, Colley DG. An electrophoretic analysis of *Schistosoma mansoni* soluble egg antigen preparation. J Parasitol 1978; 64 (3): 285-90.

Castro-Borges, W, Dowle A, Curwen RS, Thomas-Oates J, Wilson RA. Enzymatic shaving of the tegument surface of live schistosomes for proteomic analysis: a rational approach to select vaccine candidates. PLoS Negl Trop Dis 2011; 5: e993.

Centro de Vigilância Epidemiológica (CVE), Coordenadoria do Controle de Doenças. Vigilância Epidemiológica e Controle da Esquistossomose: Normas e Instruções Controle da Esquistossomose do Estado de São Paulo/PCE-SP, Brasil, versão 2007.

Cheever AW. A quantitative post mortem study of schistosomiasis mansoni in man. The Am J Trop Med & Hyg 1968; 17: 38-64.

Chick H, Martin CJ. The precipitation of egg-albumin by ammonium sulphate. A contribution to the theory of the "salting out" of proteins. Biochem J 1913; 7: 380-398.

Chitsulo L, Engels D, Montresor A, Savioli L. The global status of schistosomiasis and its control. Acta Trop 2000; 77 (1): 41-51.

Clegg JA. In vitro cultivation of *Schistosoma mansoni*. Experim Paarsitol 1965; 16: 133-47.

Coelho MV. O parasito *Schistosoma mansoni*. In: Cunha, AS. Esquistossomose mansoni. São Paulo: Editora Savier; 1970. p. 1-12.

Coelho PM, Jurberg AD, Oliveira AA, Katz N.Use of a saline gradient for the diagnosis of schistosomiasis. Mem Inst Oswaldo Cruz 2009; 104 (5): 720-3.

Coelho PMZ, Tavares CAP. Diagnostico imunológico. In: Castro LP, RochaPRS, Cunha AS. Topicos em gastroenterologia. Rio de Janeiro: Medsi; 1991.

Coelho-Castelo AA, Panunto-Castelo A, Moreno AN, Dias-Baruffi M, Jamur MC, Oliver C, Roque-Barreira MC, et al. Sm60, a mannose-binding protein from *Schistosoma mansoni* with inflammatory property. Int J Parasitol 2002; 32 (14): 1747-54.

Cohen J. Weighted kappa: nominal scale agreement with provisions for scales disagreement of partial credit. Psychol Bull 1968; 70: 213-20.

Coulibaly JT, Knopp S, N'Guessan NA, Silué KD, Fürst T, Lohourignon LK, Brou JK, et al. Accuracy of urine circulating cathodic antigen (CCA) test for *Schistosoma mansoni* diagnosis in different settings of Côte d'Ivoire. PLoS Negl Trop Dis 2011; 5 (11): e1384.

Coura JR, Amaral RS. Epidemiological and control aspects of schistosomiasis in Brazilian endemic areas. Mem Inst Oswaldo Cruz 2004; 99 (5 Suppl 1): 13-9.

Crabtree JE, Wilson RA. *Schistosoma mansoni:* a scanning electron microscope study on the developing schistosomulum. Parasitology 1980; 81: 553-564.

Cummings RD, Nyame AK. Glycobiology of schistosomiasis. FASEB J 1996; 10 (8): 838-48.

Cummings RD, Nyame AK. Schistosome glycoconjugates. Biochim Biophys Acta 1999; 1455 (2-3): 363-74.

Cummings RD, Nyame AK. Schistosome glycoconjugates. Biochim Biophys Acta 1999; 1455: 363–374.

Da Frota SM, Carneiro TR, Queiroz JA, Alencar LM, Heukelbach J, Bezerra FS. Combination of Kato-Katz faecal examinations and ELISA to improve accuracy of diagnosis of intestinal schistosomiasis in a low-endemic setting in Brazil. Acta Trop 2011; 120 Suppl 1: S138-41.

De Clercq D, Sacko M, Vercruysse J, vanden Bussche V, Landouré A, Diarra A, Gryseels B, et al. Circulating anodic and cathodic antigen in serum and urine of mixed *Schistosoma haematobium* and *S. mansoni* infections in Office du Niger, Mali. Trop Med Int Health 1997; 2 (7): 680-5.

Deelder AM, De Jonge N, Boerman OC, Fillie´ YE, Hilberath GW, Rotmans JP, Gerritse MJ, et al. Sensitive determination of circulating anodic antigen in *Schistosoma mansoni* infected

individuals by an enzyme-linked immunosorbent assay using monoclonal antibodies. Am J Trop Med Hyg 1989; 40: 268–272.

Deelder AM, El-Dosoky I, Van Marck EAE, Qian ZL. Immunofluorescent localization of *Schistosoma mansoni* circulating cathodic antigen in tissues of infected mice using monoclonal antibody. Zeitschrift fur Parasitekunde 1985; 71: 317-23.

Deelder AM, Klappe HT, van den Aardweg GJ, van Meerbeke EH. *Schistosoma mansoni*: demonstration of two circulating antigens in infected hamsters. Exp Parasitol 1976; 40 (2): 189-197.

Deelder AM, Qian ZL, Kremsner PG, Acosta L, Rabello AL, Enyong P, Simarro PP, van Etten EC, Krijger FW, Rotmans JP, et al. Quantitative diagnosis of *Schistosoma* infections by measurement of circulating antigens in serum and urine. Trop Geogr Med 1994; 46 (4 Spec No): 233-8.

Deelder AM. Diagnostic markers in schistosomiasis. Mem Inst Oswaldo Cruz 1992; 87 Suppl 5: 125-7.

Doenhoff MJ, Chiodini PL, Hamilton JV. Specific and sensitive diagnosis of schistosome infection: can it be done with antibodies? Trends in Parasitol 204; 20 (1): 35-9.

Doenhoff MJ, Wheeler JG, Tricker K, Hamilton JV, Sturrock RF, Butterworth AE, Ouma JH, et al. The detection of antibodies against *Schistosoma mansoni* soluble egg antigens (SEA) and CEF6 in ELISA, before and after chemotherapy. Ann Trop Med Parasitol 2003; 97 (7): 697-709.

Eberl M, Langermans JA, Vervenne RA, Nyame AK, Cummings RD, Thomas AW, Coulson PS, et al. Antibodies to glycans dominate the host response to schistosome larvae and eggs: is their role protective or subversive? J Infect Dis 2001; 183: 1238–1247.

El Ridi R, Tallima H. *Schistosoma mansoni* ex vivo lung-stage larvae excretory-secretory antigens as vaccine candidates against schistosomiasis. Vaccine 2009; 27: 666–673.

El-Ahwany E, Bauiomy IR, Nagy F, Zalat R, Mahmoud O, Zada O. T Regulatory Cell Responses to Immunization with a Soluble Egg Antigen in *Schistosoma mansoni*-Infected Mice. Korean J Parasitol 2012; 50 (1): 29–35.

El-Dosoky I, Van Marck EAE, Deelder AM. Presence of *Schistosoma mansoni* antigens ion liver, spleen and kidnet of infected mice: a sequential study. Zeitschrift fur Parasitekunde 1984; 70: 491-7.

Engels D, Sinzinkayo E, Gryseels B. Day-to-day egg count fluctuation in *Schistosoma mansoni* infection and its operational implications. Am J Trop Med Hyg 1996; 54: 319-324.

Engvall E, Jonsson K, Perlmann P. Enzyme-lonked immunosorbent assay. II. Qauntitative assay of protein antigen, immunoglobulin G, by means of enzyme-labelled antigen and antibody-coated tubes. Biochimica et Biophysica Acta 1971; 251: 424-34.

Enk MJ, Lima AC, Drummond SC, Schall VT, Coelho PM. The effect of the number of stool samples on the observed prevalence and the infection intensity with *Schistosoma mansoni* among a population in an area of low transmission. Acta Trop 2008; 108 (2-3): 222-8.

Farias LP, Cardoso FC, Miyasato PA, Montoya BO, Tararam CA, Roffato HK, Kawano T, et al. *Schistosoma mansoni* Stomatin like protein-2 is located in the tegument and induces partial protection against challenge infection. PLoS Negl Trop Dis 2010; 4 (2): e597.

Faust EC et al. Comparative efficiency of various techniques for the diagnosis of protozoa and helminthes in fezes. The J Parasitol 1939; 26: 241-62.

Faust EC, Ingalls JW, See JK. The diagnosis of schistosomiasis japonicum. II. Technics for the recovery of the eggs of *Schistosoma japonicum.* The Am J Trop Med & Hyg 1946; 26: 559-84.

Faust EC, Jones CA, Hoffman WA. Estudios sobre la esquistosomiasis mansoni em Puerto Rico. III. Estudio biológico. 2. La etapa mamífera del ciclo vital. Puerto Rico public H & Trop Med 1934; 10: 197-254.

Fitzsimmons CM, Jones FM, Stearn A, Chalmers IW, Hoffmann KF, et al. The *Schistosoma mansoni* Tegumental-Allergen-Like (TAL) Protein Family: Influence of Developmental Expression on Human IgE Responses. PLoS Negl Trop Dis 2012; 6 (4): e1593.

Fitzsimmons CM, McBeath R, Joseph S, Jones FM, Walter K, et al. Factors affecting human IgE and IgG responses to allergen-like Schistosoma mansoni antigens: Molecular structure and patterns of in vivo exposure. Int Arch Allergy Immunol 2007; 142: 40–50.

Fundação Nacional de Saúde. Controle da esquistossomose: manual de diretrizes técnicas. Brasília: Ministério da Saúde, Brasil; 1998.

Gentile R, Gonçalves MM, da Costa Neto SF, da Costa MM, Peralta RH, Peralta JM. Evaluation of immunological, parasitological and molecular methods for the diagnosis of *Schistosoma mansoni* infection before and after chemotherapy treatment with praziquantel in experimentally infected Nectomys squamipes. Vet Parasitol 2011; 180 (3-4): 243-9.

Gilbert B et al. *Schistosoma mansoni: in vitro* transformation of cercaria into schistosomula. Parasitology 1072; 64: 333-39.

Gomes JF, Hoshino-Shimizu S, Dias LCS, Araújo AJUS, Castilho VLP, Neves FAMA. Evaluation of a novel kit (TF-Test) for the diagnosis of intestinal parasitic infections. J Clin Lab Anal 2004; 18: 132-138.

Gomes LI, Dos Santos Marques LH, Enk MJ, de Oliveira MC, Coelho PM, Rabello A. Development and evaluation of a sensitive PCR-ELISA system for detection of *schistosoma* infection in feces. PLoS Negl Trop Dis 2010; 4 (4): e664.

Grabe K, Haas W. Navigation within host tissues: cercariae orientate towards dark after penetration. Parasitol Res 2004a; 93: 111-13.

Haas W. Cercaricida effect of 2-tetradecenoic acid, a penetration stimulant. Experim Parasitol 1984; 58: 215-22.

Hagan P, Ndhlovu P, Dunne D. Schistosome Immunology: more questions than answers. Parasitol Today 1998; 14 (10): 407-12.

Hancock K, Tsang VC. Development and optimization of the FAST-ELISA for detecting antibodies to *Schistosoma mansoni*. J Immunol Methods 1986; 92 (2): 167-76.

Harlow E, Lane D. Antibodies: A Laboratory Manual. Imperial Cancer Research Fund Laboratories. 1988; 726 pp.

Hockley DJ, McLaren DJ. *Schistosoma mansoni:* changes in the outer membrane of the tegument during development from cercariae to adult worm. International J Parasitol 1973; 3 (1): 13-25.

Hockley DJ. Ultrastrutucure of teh tegumento f *Schistosoma mansoni*. Advances in Parasitol 1973; 11: 233-305.

Hoffmann WA, Pons JÁ, Janer JL. Sedimentation concnetration method in schistosomiasis mansoni. Puerto Rico J Public Health and Trop 1934; 9: 283-98.

Hokke CH, Deelder AM, Hoffmann KF, Wuhrer M. Glycomics-driven discoveries in schistosome research. Exp Parasitol 2007; 117: 275–283.

Hokke CH, Deelder AM. Schistosome glycoconjugates in host-parasite interplay. Glycoconjugate J 2001; 18: 573–587.

Hokke CH, Deelder AM. Schistosome glycoconjugates in host-parasite interplay. Glycoconj J 2005; 18: 573–587.

Hussein HM, El-Tonsy MM, Tawfik RA, Ahmed SA. Experimental study for early diagnosis of prepatent schistosomiasis mansoni by detection of free circulating DNA in serum. Parasitol Res 2012.

Hyatt RA et al. Factors in the pathogenics of acute schistosomiasis mansoni. The J Infect Dis 1979; 139: 659-666.

Jurberg AD, Oliveira AA, Lenzi HL, Coelho PM. A new miracidia hatching device for diagnosing schistosomiasis. Mem Inst Oswaldo Cruz 2008; 103 (1): 112-4.

Katja Polman, Dirk Engels, Lorraine Fathers, Andre Deelder, Bruno Gryseels. Day-to-day fluctuation of schistosome circulating antigen levels in serum and urine of humans infected with *Schistoosma mansoni* in Burundi. Am J Trop Med Hyg1998; 59 (1): 150–154.

Katz N, Chaves A, Pellegrino J. A simple device for quantitative stool thick-smear technique in schistosomiasis mansoni. Rev Inst Med Trop Sao Paulo 1972; 14: 397–400.

Katz N, Peixoto SV. Critical analysis of the estimated number of Schistosomiasis mansoni carriers in Brazil. Rev Soc Bras Med Trop 2000; 33 (3): 303-8.

Khoo KH. Structural variations in schistosomal glycans. Trends Glycosci. Glycotechnol 2001; 31: 493–506.

Kongs A, Marks G, Verlé P, Van der Stuyft P. Limitations of Kato-Katz technique for evaluating *S. mansoni* infections. Trop Med Int Health 2001; 6: 163-169.

Lambertucci JR, Silva LC, do Amaral RS. Guidelines for the diagnosis and treatment of schistosomal myeloradiculopathy. Rev Soc Bras Med Trop 2007; 40 (5): 574-81.

Lambertucci JR. Acute *Schistosomiasis mansoni*: revisited and reconsidered. Mem Inst Oswaldo Cruz 2010; 105 (4): 422-435

Landis JR, Koch GG. The measurement of observer agreement for categorical data. Biometrics 1977; 33 (1): 159–74.

Lawrence JD. The ingestion of red blood cells by *Schistosoma mansoni*. The J Parasitol 1973; 59: 60-3.

Legesse M, Erko B. Field-based evaluation of a reagent strip test for diagnosis of schistosomiasis mansoni by detecting circulating cathodic antigen (CCA) in urine in low endemic area in Ethiopia. Parasite 2008; 15 (2): 151-5.

Lenzi HJ et al. Histoarchitecture of schistossomal granulomas development and involution: morphogenetic and biomechanical approaches. Mem Inst Oswaldo Cruz 1998; 93 (Suppl. 1): 141-51.

Lenzi HL, Jurberg AD, Coelho PM, Lenzi JA. Migração e desenvolvimento do *Schistosoma mansoni* no hospedeiro definitivo. In: Carvalho OS, Coelho PMZ, Lenzi H. *Schistosoma mansoni* & Esquistossomose: uma visão multidisciplinar. Rio de Janeiro: Fiocruz; 2008. p. 85.

Loeffler DA et al. Soluble eggs antigens from *Schistosoma mansoni* induce angiogenesis-related processes by up-regulating vascular endothelial growth factor in human endothelial cells. The J Infec Dis 2002; 185: 1650-1656.

Lunde MN, Ottensen EA. Enzyme-linked immunosorbent assay (ELISA) for detecting IgM and IgE antibodies in human schistosomiasis. The A J Trop Med & Hyg 1980; 29: 82-5.

Lutz A. *Schistosoma mansoni* and schistosomiasis observed in Brazil. Mem Inst Oswaldo Cruz 1919; 11: 109-140.

MacLaren DJ. *Schistosoma mansoni:* the parasite surface in relation to the host immunity. Letchworth: Research Studies Press 1980.

Malagueño E, Santana JV. Etiologia da esquistossomose. In: Malta J. Esquistossomose mansônica. Recife: Editora Universitária. 1994.

Mansour JM, McCrossan MV, Bickle QD, Mansour TE. *Schistosoma mansoni* phosphofructokinase: immunolocalization in the tegument and immunogenicity. Parasitology 2000; 120 (Pt 5): 501-11.

Martins AV. Sobre a pesquisa de ovos de *Schistosoma mansoni* pelo método de sedimentação-concentração. Brazil-médico 1937; 5: 319-21.

Mckerrow J, Salter J. Invasion of skin by *Schistosoma* cercariae. Trends Parasitol 2002; 18 (5): 193-5.

Miller FH, Tulloch GS, Kuntz RE. Scanning electron microscopy of integumental surface of *Schistosoma mansoni*. The Journal of Parasitology 1972; 58: 693-698.

Miller RL et al. Paraeoepidemiology of *Schistosoma* infection in mummies. BMJ 1992; 304: 555-6.

Mohamed MM, Shalaby KA, LoVerde PT, Karim AM. Characterization of Sm20.8, a member of a family of schistosome tegumental antigens. Mol Biochem Parasitol 1998; 96 (1-2): 15-25.

Mohamed-Ali Q et al. Ultrasonography investigation of periportal fibrosis in children with *Schistosoma mansoni* infection: reversibility of morbidity seven months after treatment with praziquantel. The Am J Trop Med & Hyg 1991; 44: 444-51.

Montenegro SML. Immunodiagnosis. Mem Inst Oswaldo Cruz 1992; 87: 333-35.

Mott D, Dixon H. Collaborative study on antigens for Immunodiagnosis of schistosomiasis. Bull WHO 1982; 60: 729-53.

Musiani M, Venturoli S, Gallinella G, Zerbini M. Qualitative PCR-ELISA protocol for the detection and typing of viral genomes. Nat Protoc 2007; 2 (10): 2502-10.

Nash TE, Lunde MN, Cheever AW. Analysis and antigenic activity of a carbohydrate fraction derived from adult *Schistosoma mansoni*. J Immunol 1981; 126 (2): 805-10.

National Center for Biotechnology Information (NCBI) 2012. Desponível em: http://ncbi.nlm.nih.gov/protein. Acesso em 10/janeiro/2012.

Neves J, Martins NR, Tonelli E. Forma toxêmica da esquistossomose mansônica. Considerações diagnósticas em torno de 50 casos identificados em Belo Horizonte. O Hospital 1966; 70: 1583-1603.

Neves J. Estudo clinic da fse pré-postural da esquistosomose mansônica. Ver Assoc Med Minas Gerais 1965; 16: 1-4.

Nyame AK, Kawar ZS, Cummings RD. Antigenic glycans in parasitic infections: implications for vaccines and diagnostics. Arch Biochem Biophys 2004; 426: 182–200.

Oliveira GC, Rodrigues NB, Romanha A, Bahia D . Genome and genomics of Schistosomes. Canadian J Zool 2004; 82: 375-390.

Oliveira LM, Santos HL, Gonçalves MM, Barreto MG, Peralta JM. Evaluation of polymerase chain reaction as an additional tool for the diagnosis of low-intensity *Schistosoma mansoni* infection. Diagn Microbiol Infect Dis 2010; 68 (4): 416-21.

Oliveira MF et al. haemozoin in *Schistosoma mansoni*. Molecul Bioch Parasitol 2000; 111: 217-21.

Omer Ali P, Smithers SR, Bickle Q, Phillips SM, Harn D, Simpson AJ. Analysis of the anti-*Schistosoma mansoni* surface antibody response during murine infection and its potential contribution to protective immunity. J Immunol 1988; 140 (9): 3273-9.

Omer-Ali P, Magee AI, Kelly C, Simpson AJG. A major role for carbohydrate epitopes preferentially recognized by chronically infected mice in the determination of *Schistosoma mansoni* schistosomulum surface antigenicity. J Immunol 1986; 137: 3601–3607.

Pearcce EJ et al. Downregulation of Th1 cytokine production accompanies induction of Th2 responses by a parasitic helminth, *Schistosoma mansoni*. The J Exp Med 1991; 173: 159-166.

Pellegrino J, Coelho PMZ. *Schistosoma mansoni*, wandering capacity of a worm couple. J Parasitol 1978; 64: 181.

Pellegrino J, Siqueira A. Técnica de perfusão para colheita de *Schistosoma mansoni* em cobaias experimentalmente infestadas. Rev Bras Malar Doenças Trop 1956; 8: 589-597.

Peters PA, Warren K. A rapid method of infecting mice and other laboratory animals with *Schistosoma mansoni*: subcutaneous injection. J Parasitol 1969; 55: 558.

Pinto C, Almeida A. Schistosomiasis mansoni no Brasil. Mem Inst Oswaldo Cruz 1948; 5: 287.

Pirajá da Silva MA. Contribuição para o estudo da esquistossomose na Bahia. Brazil Medico 1908; 22: 281-83.

Polman K, Engels D, Fathers L, Deelder AM, Gryseels B. Day-to-day fluctuation of schistosome circulating antigen levels in serum and urine of humans infected with *Schistosoma mansoni* in Burundi. Am J Trop Med Hyg 1998; 59 (1): 150-4.

Pontes LA et al. Comparison of a PCR assay and the Kato-Katz technique for diagnosing *Schistosoma mansoni* infection. The Am J Trop Med & Hyg 2003; 68: 652-6.

Pontes LA, Dias Neto E, Rabello A. Detection by polymerase chain reaction of *Schistosoma mansoni* DNA in human serum and feces. The Am J Trop Med & Hyg 2002; 66: 157-62.

Prata A. Biópsia na esquistossomose mansônica. Bases e aplicações no diagnostico e tratamento. Tese de doutorado 1957; Rio de Janeiro: Serviço nacional de educação sanitária, Ministério da Saúde.

Prata AR, Coura JR. Fases e formas clínicas da esquistossomose mansoni. In: Carvalho OS, Coelho PMZ, Lenzi H. *Schistosoma mansoni* & Esquistossomose: uma visão multidisciplinar. Rio de Janeiro: Fiocruz; 2008. p. 739.

Rabello A, Pontes LA, Enk M, Montenegro SM, Morais CNL. Diagnostico parasitológico, imunológico e molecular da esquistossomose mansoni. In: Carvalho OS, Coelho PMZ, Lenzi H. *Schistosoma mansoni* & Esquistossomose: uma visão multidisciplinar. Rio de Janeiro: Fiocruz; 2008. p. 895.

Rabello A. diagnosing schistosomiasis. Mem Inst Oswaldo Cruz 1997; 92 (5): 669-76.

Ramalho-Pinto FJ, Grazzinelli G, Howells RE, Mota-Santos TA, Figueiredo EA, Pelegrino J. *Schistosoma mansoni*: defined system for stepwise transformation of cercariae to schistossomule in vitro. Exp Parasitol 1974; 36 (3): 360-372.

Ramalho-Pinto RJ et al. Factors affecting surface changes in intact cercariae bodies of *Schistosoma mansoni*. Parasitology 1975; 71: 12-25.

Rey L. Parasitologia. 4ª ed. Rio de Janeiro: Ed. Guanabara-Koogan; 2008.

Richter J. The impact of chemotherapy on morbidity due to schistosomiasis. Acta Trop 2003; 86: 161-83.

Robijn MLM, Wuhrer M, Kornelis D, Deelder AM, Geyer R, Hokke CH. Mapping fucosylated epitopes on glycoproteins and glycolipids of *Schistosoma mansoni* cercariae, adult worms and eggs. Parasitology 2005; 130: 67–77.

Robjin M, Wuhrer M, Kornelis D, Deelder AM, Geyer R, Hokke CH. Mapping fucosylated epitopes on glycoproteins and glycolipids of *Schistosoma mansoni* cercariae, adult worms and eggs. Parasitology 2005; 130: 67–77.

Saiki RK et al. Enzymatic amplification of beta-globulin genomic sequences and restriction site analysis for diagnosis os sickle cell anemia. Science 1985; 230: 1350-54.

Sapero JJ, Lawless DK. The MIF satin-preservation technic for identification of intestinal protozoa. The Am J Trop Med & Hyg 1953; 2: 613-19.

Shane HL, Verani JR, Abudho B, Montgomery SP, Blackstock AJ, Mwinzi PN, Butler SE, et al. Evaluation of urine CCA assays for detection of *Schistosoma mansoni* infection in Western Kenya. PLoS Negl Trop Dis 2011; 5 (1): e951.

Siqueira LM, Coelho PM, de Oliveira ÁA, Massara CL, Carneiro NF, Lima AC, Enk MJ. Evaluation of two coproscopic techniques for the diagnosis of schistosomiasis in a low-transmission area in the state of Minas Gerais, Brazil. Mem Inst Oswaldo Cruz 2011; 106 (7): 844-50.

Smithers SR, Doenhoff MJ. Schistosomiasis. In: Cohen S, Warren KS. Immunology of Parsitic Infections. Londres: Year Book Medical Pub; 1982.

Steinmann P, Keiser J, Bos R, Tanner M, Utzinger J. Schistosomiasis and water resources development: systematic review, meta analysis, and estimates of people at risk. Lancet infect Dis 2006; 6: 411-426.

Stek M Jr, Coulis PA, Boctor FN, Pelley RP. Reactivity of anti-MSA-1 monoclonal antibody with schistosomal and non-schistosomal antigenic extracts. Lancet 1983; 2 (8348): 522-3.

Stirewalt MA, Dorsey CH. *Schistosoma mansoni*: cercarial penetration of host epidermis at the ultrastructural level. Exp Parasitol 1974; 35 (1): 1-15.

Symmers WSTC. Note on a new form of liver cirrhosis due to the presence of the ova of *Bilharzia haematobia.* J Pathol Bact 1904; 9: 237-239.

Tararam CA, Farias LP, Wilson RA, Leite LC. *Schistosoma mansoni* Annexin 2: molecular characterization and immunolocalization. Exp Parasitol 2010; 126 (2): 146-55.

Torres EA, Acosta H, Cruz M, Weinstock J, Hillyer GV. Seroprevalence of *Schistosoma mansoni* in Puerto Ricans with inflammatory bowel disease. P R Health Sci J 2001; 20 (3): 211-4.

Turner P, Lalloo K, Bligh J, Armstrong M, Whitty CJ, Doenhoff MJ, Chiodini PL. Serological speciation of human schistosome infections by ELISA with a panel of three antigens. J Clin Pathol 2004; 57 (11): 1193-6.

Valadares TE, Coelho PMZ, Pellegrino J, Sampaio IBM. *S. mansoni*: Aspectos da oviposição da cepa LE em camundongos infectados com um casal de vermes. Rev Inst Med Trop São Paulo 1981; 23: 6.

Van Dam GJ, Bogitsh BJ, van Zeyl RJ, Rotmans JP, Deelder AM. *Schistosoma mansoni*: in vitro and in vivo excretion of CAA and CCA by developing schistosomula and adult worms. J Parasitol 1996; 82 (4): 557-64.

Van Dam GJ, Wichers JH, Ferreira TM, Ghati D, van Amerongen A, Deelder AM. Diagnosis of schistosomiasis by reagent strip test for detection of circulating cathodic antigen. J Clin Microbiol 2004; 42 (12): 5458-61.

Van Der Werf MJ, Borsboom GJ, De Vilas SJ. No effect of recall period length on prevalence of self-reported haematuria in *Schistosoma haematobium*-endemic areas. Trans R Soc Trop Med & Hyg 2003; 97: 373-374.

Van Marck EA, Deelder AM, Gilgase PLJ. Schistosomal glomerulopathy: Role of the circulating anodic pollyssacharide antigen. In: Van Den Bossche H. The host-invader interplay. Amsterdam: Elsevier; 1980.

Van Remoortere A, Hokke CH, van Dam GJ, van Die I, Deelder AM, van den Eijnden DH. Various stages of schistosoma express Lewis(x), LacdiNAc, GalNAcbeta1-4 (Fucalpha1-3)GlcNAc and GalNAcbeta1-4(Fucalpha1-2Fucalpha1-3)GlcNAc carbohydrate epitopes: detection with monoclonal antibodies that are characterized by enzymatically synthesized neoglycoproteins. Glycobiology 2000; 10 (6): 601-9.

Van Weemen BK, Schuurs AHM. Immunoassay using antigen-enzyme conjugates. Febs Letters 1971; 15: 232-6.

Warren KS. The pathology, pathobiology and pathogenesis of schistosomiasis. Nature 1978; 273: 609-12.

Willis HH. A simple levitation method for the detcetion of hookeworm ova. The Med J Australia 1921; 6: 375-76.

World Health Organization (WHO) 2004. Schistosomiasis disease information. Disponível em: http://www.who.int/tdr/diseases/schisto/diseaseinfo.htm. Acesso em: 20/Maio/2012.

World Health Organization (WHO). O controle da esquistossomose. (Relatório Técnico, 830). Genebra: OMS 1994.

Wu G. A historical perspective on the Immunodiagnosis of schistosomiasis in China. Acta Tropica 2002; 82: 193-8.

Wuhrer M, Koeleman C, Fitzpatrick J, Hoffmann K, Deelder AM, Hokke CH. Gender-specific expression of complex-type N-glycans in schistosomes. Glycobiology 2006; 16 (10): 991–1006.

Zaccone P, Burton OT, Gibbs SE, Miller N, Jones FM, Schramm G, Haas H, et al. The *S. mansoni* glycoprotein ω-1 induces Foxp3 expression in NOD mouse $CD4^+$ T cells. Eur J Immunol 2011; 41 (9): 2709-18.

Zhang W, Ahmad G, Torben W, Siddiqui AA. *Schistosoma mansoni* antigen Sm-p80: Prophylactic efficacy of a vaccine formulated in human approved plasmid vector and adjuvant (VR 1020 and alum). Acta Trop 2011; 118 (2): 142–151.

7 ANEXOS

7 ANEXOS

7.1 Confirmação da autorização do CEUA

MINISTÉRIO DA SAÚDE / FUNDAÇÃO OSWALDO CRUZ
VICE-PRESIDÊNCIA DE PESQUISA E DESENVOLVIMENTO TECNOLÓGICO
Comissão de Ética no Uso de Animais
CEUA-FIOCRUZ

CERTIFICADO

Certificamos que o protocolo intitulado "**Desenvolvimento de técnicas diagnósticas para esquitossomose com microesferas Estapor por separação magnética e aglutinação**" sob a responsabilidade do **Dr. Paulo Marcos Zech Coelho,** foi licenciado pelo nº **L-0023/08** e está de acordo com os Princípios Éticos na Experimentação Animal adotado pelo Colégio Brasileiro de Experimentação Animal (COBEA) e foi APROVADO pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA - FIOCRUZ) em reunião ordinária de 24/03/2008. Na presente formatação, este programa está licenciado e tem validade até 24 de março de 2012.

Rio de Janeiro, 24 de março de 2008.

Octavio Augusto França Presgrave
Coordenador da CEUA
FIOCRUZ

Avenida Brasil nº 4365 Manguinhos – sala 110 - Manguinhos - CEP 21045-900 - Rio de Janeiro - Brasil
Tel: (+55) 21.3885-1698 - FAX: (+55) 21. 2590-9490 E. mail: ceua@fiocruz.br

7.2 Confirmação da autorização do CEPSH

Ministério da Saúde

FIOCRUZ
Fundação Oswaldo Cruz

Centro de Pesquisas René Rachou
Comitê de Ética em Pesquisa em Seres Humanos

PARECER Nº 11/2004 – CEPSH- CPqRR

Protocolo CEPSH/CPqRR nº: 08/2003

Projeto de Pesquisa: "Desenvolvimento de nova técnica quantitativa de exame de fezes e aperfeiçoamento da técnica de ELISA para detecção de antígenos circulantes e anticorpos para o diagnóstico antes e após tratamento quimioterápico em área de baixa endemicidade para esquistossomose mansoni"

Pesquisador Responsável: Paulo Marcos Zech Coelho

Instituição: Centro de Pesquisas René Rachou / FIOCRUZ

Data de entrada no CEP: 7 de março de 2003.

Ao se proceder à análise do protocolo em questão, após consideradas as respostas as pendências anteriores, constatou-se que as informações enviadas atendem aos aspectos fundamentais da Resolução CNS 196/96, sobre Diretrizes e Normas Regulamentadoras de Pesquisa envolvendo Seres Humanos.

Diante do exposto, o Comitê de Ética em Pesquisa em Seres Humanos do Centro de Pesquisas René Rachou / FIOCRUZ, de acordo com as atribuições definidas na Resolução 196/96, manifesta-se pela aprovação do projeto de pesquisa proposto.

Situação: **PROJETO APROVADO**

Belo Horizonte, 26 de junho de 2004.

Álvaro José Romanha
Coordenador do CEPSH- CPqRR

Centro de Pesquisa René Rachou / FIOCRUZ
Av. Augusto de Lima, 1715, 30190-002 Belo Horizonte - MG - Brasil
Tel: (0055) (31) 3295-3566 (ramal 180) - Fax: (0055) (31) 3295-3115
e-mail: romanha@cpqrr.fiocruz.br