Renata Russo Frasca Candido

ESTUDO DAS CARACTERÍSTICAS FÍSICO-QUÍMICAS E PROPRIEDADES MAGNÉTICAS DA SUPERFÍCIE DO OVO DE *Schistosoma mansoni* E *Schistosoma japonicum*

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Resumo

A esquistossomose é uma infecção crônica endêmica causada por parasitos do gênero *Schistosoma*, e ocorre em países 74 países na África, América do Sul e Ásia. Os três principais agentes desta infecção em humanos são: *Schistosoma mansoni* e *Schistosoma japonicum*, causadores da doença hepato-intestinal, e *Schistosoma haematobium*, responsável pela infecção genitourinária. Apesar de haver tratamento efetivo como o praziquantel, a esquistossomose permanece como a segunda infecção parasitária mais prevalente no mundo. O diagnóstico da esquistossomose intestinal é feito através da direta visualização dos ovos em amostras fecais. O método atualmente recomendado pela Organização Mundial de Saúde em estudos epidemiológicos é o método de Kato-Katz. Apesar de simples e barato, em áreas de baixa endemicidade esta técnica perde sensibilidade, levando à ocorrência de casos falso-negativos e subestimação da prevalência da área estudada. O Helmintex® é um método coproparasitológico altamente sensível que permite o isolamento de ovos de *Schistosoma* à partir de 30 gramas de fezes, baseado na interação entre os ovos e esferas paramagnéticas em um campo magnético. Entretanto, este método demanda tempo e equipamentos especializados, sendo de difícil manipulação em estudos de campo. O mecanismo que promove a interação das esferas paramagnéticas com os ovos de *Schistosoma* não é conhecido. Tendo em vista a necessidade de ferramentas diagnósticas sensíveis e de fácil aplicabilidade em estudos epidemiológicos em áreas de baixa transmissão, este trabalho tem por objetivo estudar características físico-químicas da superfície dos ovos de *S. mansoni* e *S. japonicum*, afim de aprimorar a eficiência do método Helmintex®. Ovos de *S. mansoni* e *S. japonicum* foram isolados de fígados de camundongos experimentalmente infectados. Os ovos foram submetidos à analise morfológica e estrutural utilizando Microscopia Eletrônica de Varredura e Transmissão e análise elementar utilizando Espectroscopia por Dispersão de Energia. A susceptibilidade magnética foi determinada utilizando-se o SQUID (Superconducting Quantum Interference Device) e a concentração dos elementos químicos foi determinada através de Espectroscopia por Emissão Atômica. Experimentos para elucidar as propriedades de interação dos ovos e das microesferas foram conduzidos incubando ovos de ambas as espécies com diferentes microesferas paramagnéticas. Os resultados mostram que a superfície do ovo de ambas as espécies é recoberta por uma camada densa de microespinhos, sendo estes mais curtos e menos espaçados em *S. mansoni*. Os ovos espontaneamente ligam-se às partículas, com maior preferência por material magnético. Os ovos de *S. japonicum* possuem maior afinidade pelas microsesferas paramagnéticas do que os ovos de *S. mansoni*. A presença de estreptavidina na superfície das microesferas aumenta a afinidade de ambas as espécies por microesferas não-magnéticas, porém diminui a afinidade por microesferas paramagnéticas. Apesar da presença de ferro na casca do ovo tanto de *S. mansoni* quanto de *S. japonicum*, a origem da interação não parece ser magnética, e sim, baseada na diferença de cargas eletrostáticas presentes na superfície dos ovos e das microesferas. A continuidade deste estudo é importante para determinar as características físico-químicas de ovos provenientes de fezes humanas, e pode levar ao aprimoramento e otimização do método Helmintex®. Estudos utilizando-se Microscopia de Força Atômica encontram-se em andamento.

Palavras-chave: *Schistosoma*; propriedades magnéticas; superfície do ovo.

Abstract

Schistososmiasis is a chronic endemic infection caused by parasites of the genus *Schistosoma,* and it occurs in 74 countries in Africa, South America and Asia. The three main agents of this infection in humans are: *Schistosoma mansoni* and *Schistosoma japonicum*, that cause the hepatic-intestinal disease, and *Schistosoma haematobium*, responsible for the genitourinary infection. Despite the effective treatment like praziquantel, schistososmiasis remains as the second most prevalent parasitic disease in the world. Diagnosis of the intestinal schistososmiasis is achieved through the direct visualization of the eggs in fecal samples. The current method recommended by the World Health Organization in epidemiological studies is the Kato-Katz method. Despite it being simple and cheap, in areas of low endemicity this technique loose sensibility, leading to the occurrence of false-negative cases and underestimation of the prevalence in the studied area. Helmintex™ is a coproparasitological method highly sensitive that allows the isolation of *Schistosoma* eggs from 30 grams of feces, based in the interaction between the eggs and paramagnetic microspheres in a magnetic field. However, this method demands time and specialized equipment, being of difficult manipulation in work field. The mechanism that promotes the interaction between the paramagnetic spheres with the *Schistosoma* eggs is not known. Considering the necessity of sensitive diagnostic tools of easy applicability in epidemiological studies in low endemicity areas, this work has the purpose to study the surface physical-chemical characteristics of *S. mansoni* and *S. japonicum* eggs, in order to enhance the efficiency of the Helmintex™ method. *S. mansoni* and *S. japonicum* eggs were isolated from livers of experimentally infected mice. The eggs were submitted to morphological and structural analysis using Scanning and Transmission Electron Microscopy and elemental analysis using Energy Disperssion Spectroscopy. The magnetic susceptibility was determined using SQUID (Superconducting Quantum Interference Device) and the concentration of the chemical elements was determined through Atomic Emission Spectroscopy. Experiments to elucidate the interaction properties of the eggs of the eggs and the microspheres were conducted incubating the eggs from both species with different paramagnetic microspheres. The results show that the egg surface of both species is recovered by a dense layer of microspines, being those shorter and less spaced in *S. mansoni*. The eggs spontaneously bind the particles, with a greater preference for magnetic material. *S. japonicum* eggs have a higher affinity for paramagnetic microspheres than *S. mansoni* eggs. The presence of streptavidin in the surface of the microspheres enhances the affinity of both species for non-magnetic material, however it decreases the affinity for paramagnetic microspheres. Despite the presence of iron in the eggshell of *S. mansoni* and *S. japonicum*, the origin of the interaction does not seem to be magnetic, but, based in the difference of electrostatic charges present in the surface of the eggs and the microspheres. The continuity of this study is important to determine the physical-chemical characteristics of eggs from human feces, and it can lead to the upgrading and optimization of the Helmintex™ method. Studies using Atomic Force Microscopy are in progress.

Keywords: *Schistosoma*; magnetic properties; egg surface.

Apresentação

A presente tese é composta por introdução, objetivos e justificativa, dois artigos científicos que compõem os resultados, discussão, considerações finais e perspectivas, referências bibliográficas e anexo.

A introdução aborda os principais aspectos da esquistossomose e sua importância na saúde pública. As dificuldades na obtenção de resultados diagnósticos precisos em áreas de baixa transmissão justificam este trabalho, e o objetivo se concentra na necessidade de otimização do método Helmintex®, técnica coproparasitológica altamente sensível recentemente desenvolvida pelo grupo de Parasitologia Biomédica da PUCRS, baseada na interação dos ovos de *Schistosoma* com microesferas paramagnéticas em um campo magnético.

O primeiro artigo foi publicado na revista Plos Neglected Tropical Diseases em maio de 2013, The Iron Distribution and Magnetic Properties of Schistosome Eggshells: Implications for Improved Diagnostics. Neste trabalho foram investigadas as propriedades magnéticas dos ovos de *Schistosoma mansoni* e *S. japonicum* e características responsáveis pela interação dos ovos com microesferas paramagnéticas.

O segundo artigo foi aceito em 9 de agosto de 2014 na revista International Journal for Parasitology, The Affinity of Magnetic Microspheres for *Schistosoma* Eggs. Neste trabalho foram estudadas as interações dos ovos de *Schistosoma mansoni* e *S. japonicum* por microesferas com diferentes coberturas e funcionalizações.

A discussão aborda os principais aspectos sobre as propriedades magnéticas e de interação dos ovos de *Schistosoma* estudados nos dois artigos apresentados, seguido das considerações finais e perspectivas futuras para novos estudos.

Por fim, o anexo apresenta um artigo em elaboração que investigou a afinidade dos ovos de *Schistosoma* pelas microesferas paramagnéticas a partir da interação de diferenças eletrostáticas nas superfícies, hipótese levantada com base nos resultados aqui apresentados.

Sumário

1. Introdução

1.1. Esquistossomose

A esquistossomose é uma infecção parasitária crônica endêmica causada por parasitos do gênero *Schistosoma*, e ocorre em países na África, América do Sul e Ásia (Chitsulo *et al.*, 2000), afetando de 200 a 300 milhões de pessoas no mundo todo (Liang & Spear, 2008), ocasionando 200.000 mortes a cada ano (Rutitzky *et al.*, 2005). Os três principais agentes desta infecção em humanos são: *Schistosoma mansoni* e *Schistosoma japonicum*, causadores da doença hepato-intestinal, e *Schistosoma haematobium*, responsável pela infecção genitourinária. Apesar dos esforços para o controle desta infecção (Favre *et al.*, 2001) e da possibilidade de erradicação desta parasitose através de medidas preventivas (Amauri *et al*., 2011) e tratamento efetivo adequado como o praziquantel (Savioli *et al.*, 1997), a esquistossomose permanece como a segunda infecção parasitária mais prevalente no mundo, perdendo somente para a malária (Chitsulo *et al*., 2000).

No Brasil, acredita-se que existam cerca de 6 milhões de indivíduos infectados com a esquistossomose mansônica, única espécie de interesse médico na região brasileira (Katz & Almeida, 2003), principalmente nos estados de Alagoas, Goiás, Sergipe, Pernambuco, Distrito Federal, Bahia, Espírito Santo, Maranhão, Paraíba, Rio Grande do Norte, Rondônia e Minas Gerais (Coura & Amaral, 2004), e cerca de 25 milhões vivem em áreas onde a transmissão desta helmintíase é possível (Vendrame *et al*, 2001).

No Sul do país, focos isolados foram detectados no Paraná (Curitiba, Uraí, Jacarezinho, Santo Antônio da Platina, Jataizinho e Porecatu) e em Santa Catarina (São Francisco do Sul, Araquari, Massaranduba, Jaguará do Sul, Joinville) (Rey, 1991; Schlemper Júnior *et al*., 1996). No Rio Grande do Sul o primeiro caso autóctone da doença foi descoberto em janeiro de 1997, quando foram encontrados ovos de *S. mansoni* em um paciente hospitalizado no município de Sapucaia e residente em Esteio (Graeff-Teixeira *et al.*, 1999). Desde então, o grupo de Parasitologia Biomédica da PUCRS vêm estudando este foco.

1.2. Ciclo biológico

O ciclo biológico do parasito é complexo e requer um hospedeiro definitivo e um hospedeiro intermediário. Fêmeas de *Schistosoma mansoni* colocam seus ovos nas ramificações mais finas das veias mesentéricas do seu hospedeiro definitivo, um vertebrado. Os ovos atravessam a mucosa intestinal e saem junto com as fezes, no meio líquido, liberando uma larva móvel e ciliada chamada miracídio (Xavier *et al*, 1998), que deve encontrar o hospedeiro intermediário, um caramujo do gênero *Biomphalaria*, em até 12 horas. Após penetração no caramujo, os miracídios alojam-se em diversos tecidos do molusco e transformam-se em esporocistos que, por poliembrionia, geram esporocistos filhos e depois, cercárias (Rey, 2001). As cercárias que abandonam o hospedeiro invertebrado ficam nadando na água, quase sempre em direção à superfície, até entrarem em contato com a pele de um hospedeiro vertebrado (Xavier *et al*, 1998), promovendo a penetração do corpo cercariano e a concomitante perda da cauda (Neves *et al*, 1997). O corpo transforma-se em esquistossômulo, que penetra nos vasos sanguíneos da pele e do tecido subcutâneo e atinge as câmaras cardíacas direitas, os capilares pulmonares e o coração esquerdo. Levados pela circulação sistêmica, os esquistossômulos disseminam-se para vários órgãos e tecidos. Somente os esquistossômulos que chegam ao sistema porta-hepático, cerca de três semanas após a penetração das cercárias, são capazes de amadurecer, transformando-se em machos e fêmeas trinta dias após a penetração (Rey, 2001). Nesses vasos, os adultos acasalam-se, migrando posteriormente aos pares e contra o fluxo sanguíneo, até as vênulas do plexo mesentérico inferior, onde se inicia a oviposição (Baptista & Andrade, 2005).

1.3. O ovo

O ovo do *S. mansoni* é muito típico, medindo, em média, 150 µm de comprimento por 65 µm de largura. Tem o pólo anterior mais delgado e o posterior mais volumoso, com um espinho lateral saliente e agudo em suas proximidades. Já o ovo do *S. japonicum* é menor, medindo cerca de 110 µm de comprimento por 50 µm de largura. Possui formato ovalado e um espinho rudimentar (Ford & Blankespoor, 1979).

A formação do ovo tem início na fêmea, quando um único oócito é produzido no ovário e liberado no oviduto. No oviduto, o oócito é fertilizado pelo esperma que vem do reservatório espermático. O ovo fertilizado segue pelo oviduto e une-se ao ducto

vitelínico, onde de 30-40 células vitelínicas irão envolver o ovo e seguir em direção ao oótipo. Uma vez no oótipo, este sofre contrações fazendo com que as células vitelínicas liberem grânulos contendo as proteínas precursoras da casca do ovo, dando início ao entrelaçamento de proteínas quinonas mediado pela atividade de enzimas tirosinases, que irão formar a casca endurecida e de coloração escura típica do ovo (Cordingley, 1987; deWalick *et al*., 2011).

A maturação do ovo tem início após a formação da casca e a passagem pelo útero da fêmea para a circulação sanguínea. Apenas um breve período de desenvolvimento ocorre dentro da fêmea, onde duas fases podem ser definidas: fase pré-zigótica, quando os oócitos deixam o ovário, e fase zigótica, quando o oócito é fertilizado (estágio 0). No estágio 1, os ovos recém depositados pela fêmea são levados pela corrente sanguínea até ficarem presos em pequenas veias do hospedeiro, onde terão início as primeiras clivagens, resultando em um primórdio embriônico que ocupará metade do comprimento transversal do ovo. No estágio 2, há a formação de uma massa sólida celular chamada de estereoblátula, ocupando todo o comprimento transversal do ovo. O estágio 3 é caracterizado por um aumento da estereoblástula, assumindo um formato alongado e ocupando 2/3 do comprimento longitudinal do ovo. Inicia-se a formação do envelope externo. No estágio 4, há a formação de um sincício ao redor de todo o embrião, iniciando a formação do envelope interno. Há também formação da massa neural primordial. No estágio 5, o embrião ocupa quase toda a área interna do ovo, e o envelope externo assumiu um aspecto fino, granular e anucleado. Na região anterior do embrião, duas células grandes e imaturas, que irão formar as glândulas laterais do miracídio, aparecem ao lado do primórdio da massa neural. O estágio 6 define o aparecimento de estruturas miracidiais, como o início da formação da glândula apical, e a formação do terebratório à partir da epiderme da região anterior do embrião. Na região posterior mediana do embrião ocorrerá a formação das células germinativas do miracídio. Precursores dos músculos começam a se diferenciar abaixo da epiderme. No estágio 7, o embrião alongado pré-formado ocupa toda a área do ovo, porém nenhum movimento é observado. O estágio 8 caracteriza o ovo maduro, quando o miracídio está totalmente formado, sendo possível observar movimentos rápidos no interior do ovo, contrações musculares, cílios e batimentos da célula-flama (Jurberg *et al.*, 2009).

O diagnóstico da esquistossomose intestinal é feito através da direta visualização dos ovos em amostras fecais. Métodos coproparasitológicos possuem a vantagem de serem altamente específicos devido à visualização dos ovos nas fezes, sendo quantitativos e de baixo custo, com a confirmação da espécie feita pela morfologia do ovo. Porém estes métodos demandam muito tempo e trabalho, principalmente em locais onde a endemicidade é muito baixa e os pacientes podem apresentar menos de 1 ovo por grama de fezes.

1.4. Diagnóstico

O método diagnóstico atualmente mais utilizado em serviços de saúde e recomendado pela Organização Mundial de Saúde (WHO, 2010) em estudos epidemiológicos é o método de Kato-Katz, que consiste na clarificação das fezes com uma mistura de água e glicerina contra-corada com verde de malaquita (Katz *et al*, 1972). Entretanto, apesar de ser um método simples e barato, em áreas de baixa endemicidade e de baixa carga parasitária, esta técnica perde sensibilidade, levando à ocorrência de casos falso-negativos e o não estabelecimento da real prevalência da área estudada (Enk *et al.*, 2008).

Como alternativa aos métodos parasitológicos, técnicas moleculares e testes imunológicos vêm sendo desenvolvidos para aumentar a sensibilidade no diagnóstico da esquistossomose. Métodos moleculares são baseados na amplificação de uma sequência altamente repetitiva do DNA do parasito (Pontes et al., 2002; Sandoval et al., 2006). Os métodos imunológicos indiretos detectam a presença de anticorpos específicos produzidos em resposta antígenos liberados pelo parasito, destacando-se a detecção de antígeno catódico circulante (CCA) e antígeno anódico circulante (CAA) através do ELISA (Van Lieshout *et al.*, 2000); imunoeletrotransferência utilizando antígeno bruto do parasito (Sulahian *et al.*, 2005), e a detecção por imunoblot de componentes de membrana de vermes adultos de *S. mansoni* (Cesari *et al.*, 2005). Ambos os métodos, apesar de apresentarem alta sensibilidade e especificidade principalmente em áreas de baixa prevalência da infecção, apresentam desvantagens por não estabelecerem a intensidade da infecção, e também por não servirem como diagnóstico da infecção ativa,

uma vez que os resultados das provas imunológicas podem permanecer positivos por muito tempo, mesmo após o tratamento da infecção (Sturrock, 2001).

O recém desenvolvido Helmintex® é um método coproparasitológico altamente sensível em áreas de baixa endemicidade. Sua técnica permite isolar os ovos de *Schistosoma* a partir de 30 gramas de fezes, baseada na interação entre os ovos e esferas paramagnéticas, em um campo magnético (Teixeira *et al.*, 2007). Estudos mostram que o Helmintex® é mais sensível que o método de Kato-Katz (Caldeira et al., 2012; Pinheiro *et al.*, 2012), chegando à 100% de sensibilidade até 1.3 ovos por grama de fezes (Teixeira *et al.*, 2007). Porém, apesar da alta sensibilidade, este método demanda tempo e equipamentos especializados, sendo de difícil manipulação em estudos de campo.

2. Objetivos e justificativa

O mecanismo que promove a afinidade das esferas paramagnéticas pelos ovos de *Schistosoma* não é conhecido. Apesar da interação aparentemente ser magnética devido à presença de ferro na casca de ovos de *S. japonicum* (Jones *et al.*, 2007), estudos anteriores mostraram que a superfície dos ovos de *S. mansoni* e *S. japonicum* é recoberta por uma matriz fibrosa denominada microespinhos, e que esta matriz pode ser responsável pela aderência dos ovos em vidros e formação de agregados (Ford & Blankespoor, 1979). Também foi observado, durante a padronização do método Helmintex®, que esferas paramagnéticas cobertas por estreptavidina possuíam um melhor desempenho de interação com os ovos do parasito (Teixeira et al., 2007). A estreptavidina é uma proteína tetramérica produzida pela actinobactéria *Streptomyces avidinii* (Chalet & Wolf, 1964), que possui alta afinidade à biotina (Weber *et al*., 1989). A biotina é um co-fator essencial presente em enzimas carboxilases que catalisam a transferência de CO_2 para os seus substratos nas células (Wood, 1977). A presença de biotina já foi descrita em *S. mansoni* em um estudo conduzido por Santos & Chaves (1997), que detectou biotina endógena em fibras musculares, mitocôndria e espinho de cercarias, e também nos tecidos de macho adulto. No entanto, não há relatos na literatura da presença de biotina na superfície dos ovos deste parasito.

Tendo em vista a necessidade de ferramentas diagnósticas sensíveis e de fácil aplicabilidade em estudos epidemiológicos em áreas de baixa transmissão, este trabalho tem por objetivo estudar a superfície do ovo de *Schistosoma mansoni* e *S. japonicum*, bem como suas propriedades magnéticas, características físico-químicas e possíveis alvos, a fim de desenvolver aplicações úteis e aprimorar a eficiência do método Helmintex® de forma que este se torne um método padrão diagnóstico em locais de baixa endemicidade para o controle da esquistossomose.

3. Resultados

3.1. Artigo científico publicado na revista Plos Neglected Tropical Diseases

The Iron distribution and magnetic properties of schistosome eggshells: Implications for improved diagnostics.

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The Iron Distribution and Magnetic Properties of Schistosome Eggshells: Implications for Improved Diagnostics

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Abstract

Background: *Schistosoma mansoni* and *Schistosoma japonicum* are the most frequent causative agents of human intestinal schistosomiasis. Approximately 200 million people in the world are infected with schistosomes. Diagnosis of schistosomiasis is often difficult. High percentages of low level infections are missed in routine fecal smear analysis and current diagnostic methodologies are inadequate to monitor the progress of parasite control, especially in areas with low transmission. Improved diagnostic methods are urgently needed to evaluate the success of elimination programs. Recently, a magnetic fractionation method for isolation of parasite eggs from feces was described, which uses magnetic microspheres to form parasite egg – magnetic microsphere conjugates. This approach enables screening of larger sample volumes and thus increased diagnostic sensitivity. The mechanism of formation of the conjugates remains unexplained and may either be related to specific surface characteristics of eggs and microspheres or to their magnetic properties.

Methods/Principal Findings: Here, we investigated iron localization in parasite eggs, specifically in the eggshells. We determined the magnetic properties of the eggs, studied the motion of eggs and egg-microsphere conjugates in magnetic fields and determined species specific affinity of parasite eggs to magnetic microspheres. Our study shows that iron is predominantly localized in pores in the eggshell. Parasite eggs showed distinct paramagnetic behaviour but they did not move in a magnetic field. Magnetic microspheres spontaneously bound to parasite eggs without the presence of a magnetic field. *S. japonicum* eggs had a significantly higher affinity to bind microspheres than *S. mansoni* eggs.

Conclusions/Significance: Our results suggest that the interaction of magnetic microspheres and parasite eggs is unlikely to be magnetic in origin. Instead, the filamentous surface of the eggshells may be important in facilitating the binding. Modification of microsphere surface properties may therefore be a way to optimize magnetic fractionation of parasite eggs.

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Introduction

Schistosomiasis is a helminth infection representing a major health burden for humans in tropical and developing nations. Some 200 million people are infected, and 600 million are currently estimated to be at risk of infection [1].

Recently, schistosomiasis control efforts have been increasingly focused on mass drug administration in endemic areas to alleviate morbidity in affected individuals [2]. Although it has been acknowledged that the goal to regularly administer chemotherapy to at least 75% of school-age children at risk of morbidity was not achieved by 2010, many countries are controlling schistosomiasis

Author Summary

In the present study, we investigated the mechanism underlying a novel diagnostic method for *Schistosoma* – one of the most widespread and frequently occurring parasites infecting humans in tropical countries. In recent years, the world has seen significant reduction in the burden of *Schistosoma* infections in many countries due to improved control and sanitation. However, it is becoming increasingly difficult to evaluate and monitor the progress of control towards elimination. At the moment it is extremely difficult to determine whether the parasite has been eliminated from a region. This is due to the absence of a sensitive and inexpensive method to detect the parasite. A series of recent studies describes a method with vastly improved diagnostic sensitivity based on the magnetic fractionation of parasite eggs from fecal samples. However, the mechanisms of action of this new diagnostic are not currently known. To further optimize and improve this method, we studied the magnetic properties of parasite eggshells and their binding characteristics to magnetic microspheres.

with increasing success using a combination of therapeutics and improved sanitation [3,4]. Sustained and effective drug therapy has the effect of pushing the disease into a state of low endemicity, where individuals carry low-level infections that are very difficult to detect. As a result, new efforts are required for parasite surveillance in regions of low endemicity [5].

It has become increasingly recognized that the evaluation and monitoring of control and elimination efforts for schistosomiasis is hindered by the lack of appropriate diagnostic techniques [5,6]. With a diagnostic limit of approximately 100 eggs per gram feces, the current WHO recommended test, the Kato-Katz method of fecal examination, is limited by poor sensitivity [7,8,9]. It is estimated that more than half of all infections with schistosomiasis are missed in cross sectional studies relying on the observation of only one fecal smear, necessitating the need to perform multiple smears [10]. Examining multiple fecal smears at different time points is logistically difficult, and time and labour intensive. There is, therefore, an urgent need to develop new diagnostic methodologies for intestinal schistosomiasis that are highly sensitive and applicable under field conditions [5]. There is also the need for a new gold standard method to which more sensitive, newly developed, simple and field applicable molecular and rapid diagnostic tests can be compared.

Recently, a novel method for *Schistosoma* egg detection based on magnetic fractionation of parasite eggs from fecal matter was developed [11]. For this method, termed Helmintex, magnetic microspheres are added to larger volumes of fecal samples (30 g). Parasite eggs and magnetic microspheres can then be co-purified from other fecal components through the application of a magnetic field and field gradient. The purified egg concentrates are more readily detectable by light microscopy. Teixeira and colleagues reported that magnetic microspheres coated with a variety of adsorbed molecules could be used to purify eggs of *Schistosoma mansoni* from fecal matter in a magnetic field [11]. The nature of the adsorbed molecules had no influence on the efficiency of the purification and even the use of uncoated microspheres resulted in the purification of *Schistosoma mansoni* eggs from feces in a magnetic field. The mechanism of interaction between parasite eggs and microspheres is unclear, yet it is important to characterize it further in order to optimize specificity and efficiency of the Helmintex method [12].

There are two possible explanations for the seemingly specific interaction of magnetic microspheres and *Schistosoma* eggs. Firstly, it could be that biochemical, chemical or physical surface properties of the eggs mediate the interaction. Secondly, the eggs could themselves be magnetic, leading to a magnetically mediated adhesion of the microspheres to the eggs. Since the interaction seems independent of the surface characteristics of the magnetic microspheres, the latter of these two possibilities seemed the more probable at the beginning of this investigation.

It has been shown that the eggshells of *S. japonicum* contain iron in concentrations detectable by energy dispersive X-ray spectroscopy in the transmission electron microscope [13]. The authors of that study suggested that iron assists in the formation of the biopolymer that makes up the eggshells. Eggshells are formed by polymerization of tyrosine-rich eggshell precursor proteins that are synthesised in the vitelline glands of the parasite. The tyrosine residues are oxidised by tyrosinases to *o*-quinones. Lysine and histidine residues on the same or adjacent eggshell precursors subject the *o*-quinones to nucleophilic attack, leading to a robust cross-linked polymer [14,15,16].

In other invertebrates, such as the bivalves *Mytilus*, DOPA-rich bonds in quinone-tanned protein polymers are stabilized by divalent metal-ions, including iron [17]. The vitelline glands of schistosomes are enriched with iron and the iron storage protein ferritin [18]. Thus, there is strong evidence for a role of iron in stabilizing the protein polymer that is the schistosome eggshell. Full verification of this hypothesis requires the refinement of methods for hydrolysis of the highly resistant shells [19].

Here we present results of experiments elucidating the elemental composition of the eggshell, with special focus on the organization of iron in the matrix and the resulting magnetic properties of the eggs. We performed a range of experiments to characterise the nature of magnetic microsphere interaction with parasite eggs and in order to investigate whether this interaction was a result of non-specific binding of the microspheres to the surface of the eggs, or whether it was the result of magnetically aided adhesion of eggs and microspheres.

Materials and Methods

All work using animals was approved by the Animal Ethics Committee of the Queensland Institute of Medical Research (Project P1289). This study was conducted according to guidelines of the National Health and Medical Research Council of Australia, as published in the *Australian Code of Practice for the Care and Use of Animals for Scientific Purposes*, 7th edition, 2004 (www.nhmrc.gov.au). *S. mansoni* was maintained in *Biomphalaria glabrata* snails and *S. japonicum* was sourced from infected *Oncomelania hupensis hupensis* snails collected in Anhui Province, China. Both, *S. mansoni* and *S. japonicum* worm stages were maintained in outbred Swiss mice.

Eggs of both species were purified from livers of mice by digestion of liver parenchymal tissues with collagenase B in phosphate buffered saline (PBS) in the presence of ethylenediaminetetraacetic acid (EDTA) as an iron chelator. The eggs were incubated in enzyme for 8 h at 37°C with agitation. For further purification eggs were centrifuged in Percoll gradients as described by Dalton et al. in 1997 [20].

Scanning electron microscopy (SEM)

Samples of *S. mansoni* and *S. japonicum* eggs were fixed in 2% (v/v) glutaraldehyde, 1% (w/v) paraformaldehyde in PBS for 60 min at 4°C and washed twice with PBS (pH = 7.4) in 1.5 mL Eppendorf tubes. The samples were then serially dehydrated in ascending

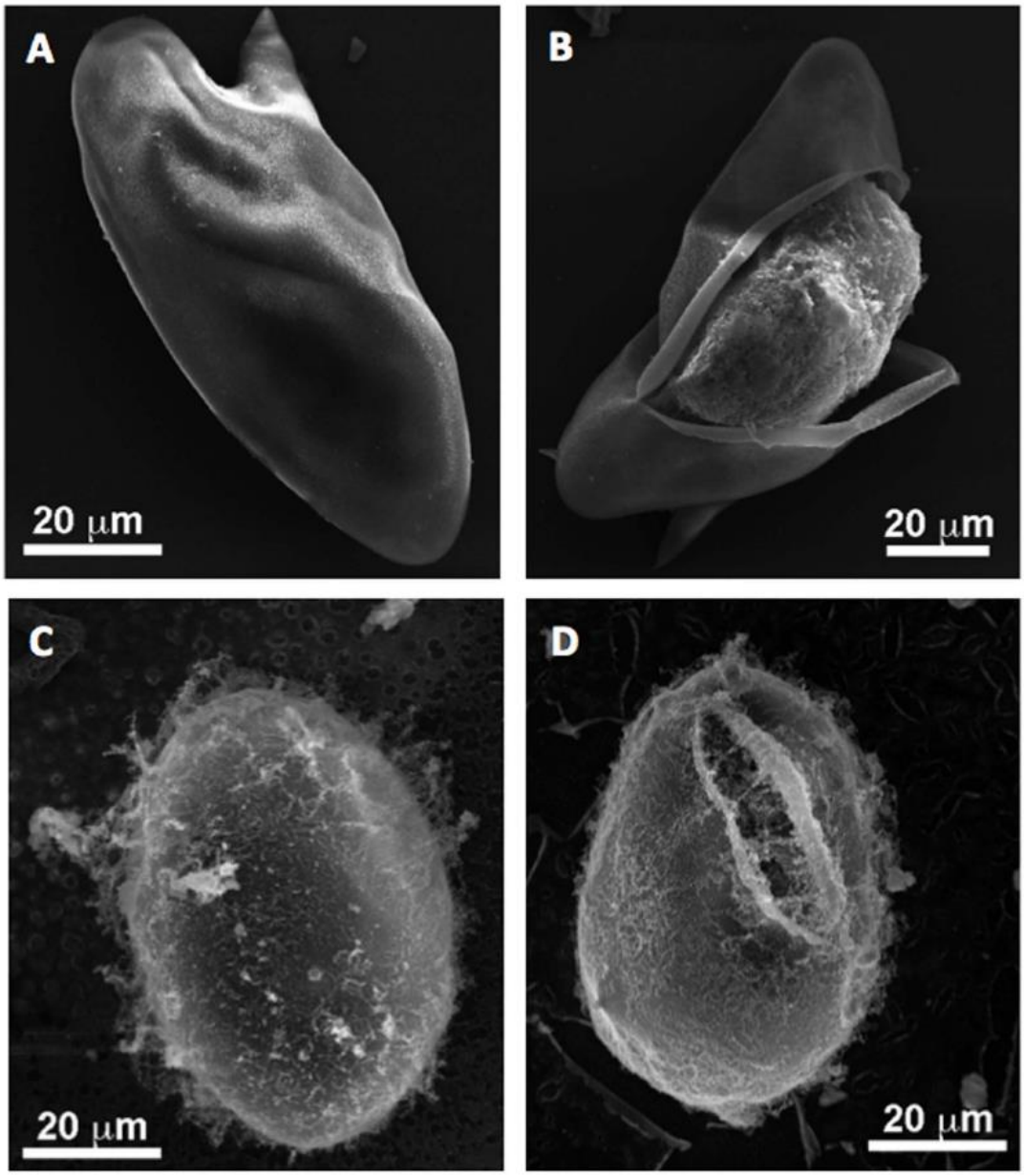

Figure 1. Morphology of *Schistosoma mansoni* and *Schistosoma japonicum* eggs. Panel A shows an intact egg of *S. mansoni*. Panel B shows an *S. mansoni* egg broken open with the miracidium still inside the egg. Panels C and D show similar images for *S. japonicum*.
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concentrations of ethanol (33%, 50%, 66%, and 100% (dry)) followed by two further washes in dry ethanol using a PELCO Biowave microwave processor (TedPella Inc., Redding, CA, USA). Dehydrated samples were transferred onto circular polylysine-coated glass coverslips and critically point dried (Emitech 850 Critical Point Drier, Quorum Technologies, Ashford, UK). The coverslips were then attached to aluminum sample holders and coated with a 5 nm thick platinum coating for morphological analyses. SEM was performed using the in-lens detector of a Zeiss 1555 VP field emission scanning electron microscope operating at 15 keV (Carl Zeiss, Überkochen, Germany).

Cryopreparation and High Angle Annular Dark Field Scanning Transmission Electron Microscopy (HAADF-STEM)

Eggs of both parasite species were fixed in 3% (v/v) glutaraldehyde in 0.1 M phosphate buffer. Eggs were trans-

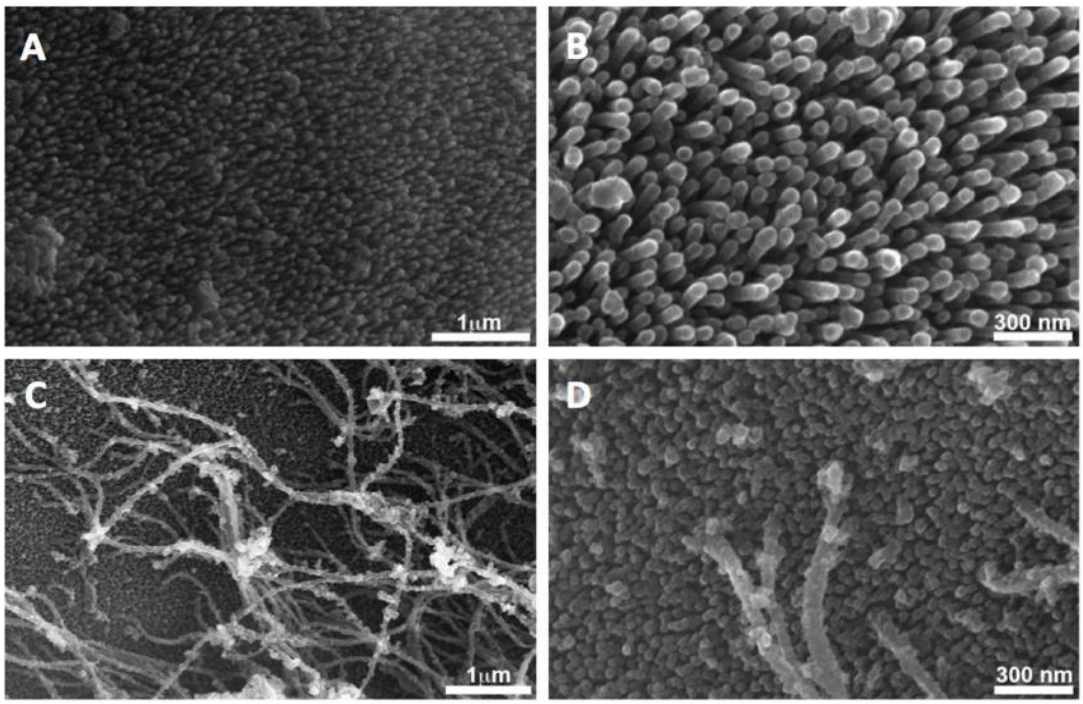

Figure 2. Surface characteristics of *Schistosoma mansoni* and *S. japonicum* eggshells. Panels A and B show the surface of a S. *mansoni* egg imaged with high resolution scanning electron microscopy illustrating that the surface is completely covered with filaments or microspines. Figures C and D show similar observations of *S. japonicum*. The microspines on the surface of *S. japonicum* are shorter and the surface is covered with an additional filamentous matrix.
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ferred to a solution of 20% (w/v) bovine serum albumin in PBS on a copper membrane and rapidly frozen in a Leica EM PACT2 High Pressure Freezer (Leica, Vienna, Austria). Subsequently, the membranes and samples were transferred in cryo-tubes under liquid nitrogen to a Leica EM AFS freeze substitution apparatus for fixation and dehydration in 2% (w/v) osmium tetroxide and 0.5% uranyl acetate (w/v) in 100% anhydrous acetone. The tissues were cryo-substituted for 3 days, according to the following protocol: The temperature of the substitution chamber was increased from −160°C to −85°C over 2 h, and maintained at −85°C for 48 h, after which the samples were brought to room temperature. After further washes in anhydrous acetone, the samples were infiltrated and embedded in Epon resin.

For HAADF-STEM, resin sections were cut from blocks at a thickness of 150 nm using an EM UC6 ultramicrotome (Leica, Vienna, Austria) and mounted onto 200 µm mesh carbon filmed copper grids for analysis at 300 kV using a JEOL JEM 3000F FEGTEM transmission electron microscope (JEOL, Tokyo, Japan). A ~1 nm probe size was used to image the mass variation within the sections, with areas of high mass appearing bright. Energy Dispersive X-ray Spectroscopy (EDS) data was combined with STEM imaging using an Oxford Instruments INCA detector (Oxford Instruments NanoAnalysis, High Wycombe, UK) to map the composition of features of interest.

Superconducting quantum interference device (SQuID) magnetic susceptometry

SquiD magnetic susceptometry is a technique to determine the magnetic properties of any given solid material. Samples are exposed to a desired sequence of magnetic fields at constant temperature or a sequence of temperatures at a constant magnetic field. The magnetization of the sample material resulting from this exposure is recorded for each point in a sequence. Using standard sequences, basic magnetic properties (e.g., whether a material is ferromagnetic, paramagnetic or diamagnetic (non-magnetic)) can be determined.

Lyophilized *S. mansoni* and *S. japonicum* eggs were placed in gel capsules for magnetic characterization in a 7 Tesla (T) magnetic property measurement system SQuID magnetic susceptometer (Quantum Design, San Diego, CA, USA). Magnetic hysteresis loops were acquired between −7 T and 7 T at 5 K. Zero-field-cooled and field-cooled (ZFC-FC) magnetization versus temperature curves were obtained from 5 to 300 K, in a measurement field of 0.01 T.

Inductively coupled plasma atomic emission spectroscopy (ICP-AES)

The concentration of iron, copper and silicon was determined for both types of eggs using ICP-AES. Inductively coupled plasma atomic emission spectroscopy (ICP-AES) is an analytical technique

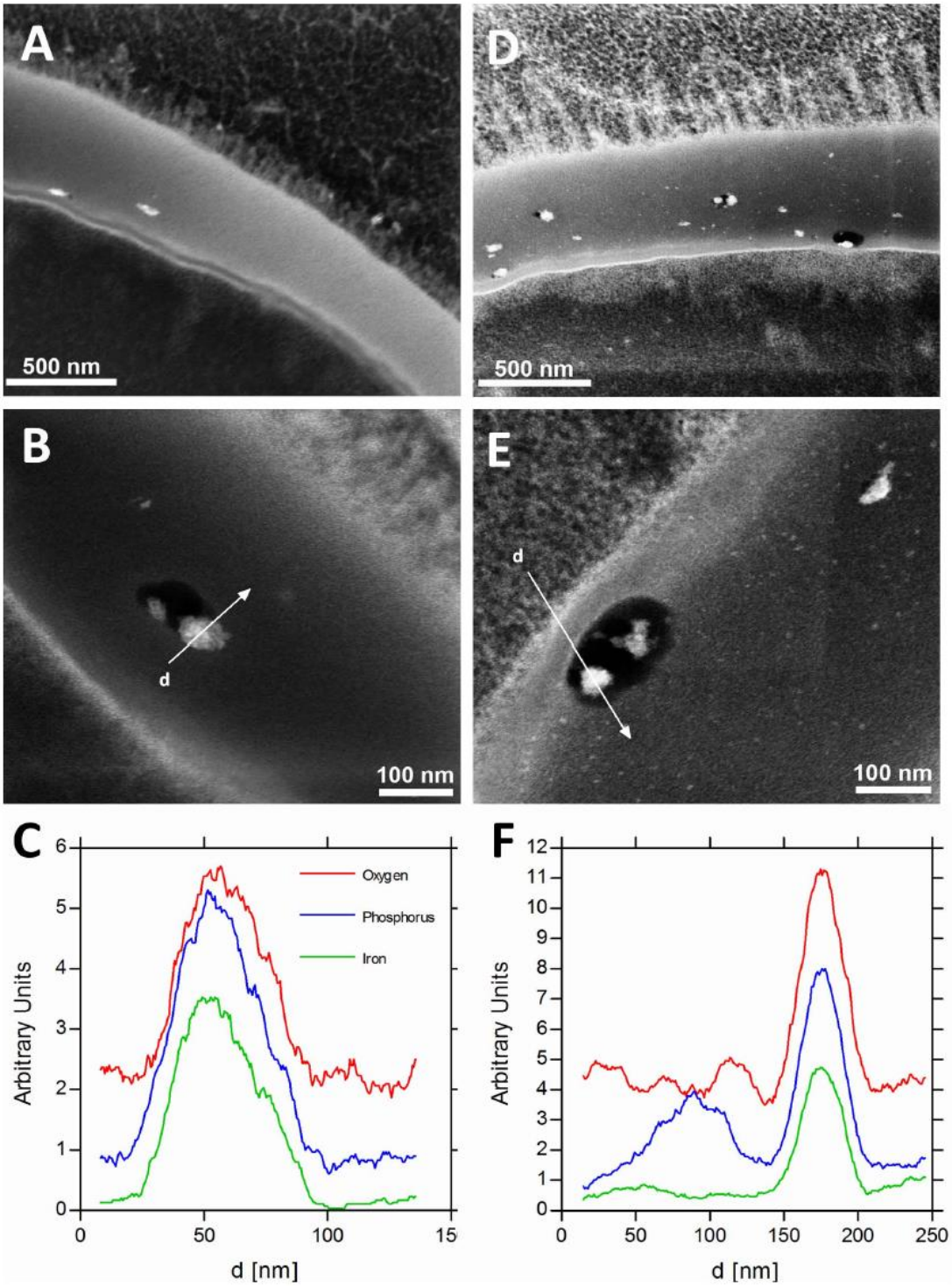
A
500 nm
D
500 nm
B
d
100 nm
E
d
100 nm
C
Oxygen
Phosphorus
Iron
Arbitrary Units
d [nm]
F
Arbitrary Units
d [nm]

Figure 3. Iron localization within the *Schistosoma* eggshell. Panel A shows inclusions of iron phosphate in the shell of *S. mansoni* at low resolution. Panel B shows similar inclusions in *S. mansoni* at a higher resolution. Panel C depicts the STEM-EDS spectra for iron, phosphorous and oxygen acquired when scanning across an inclusion, along the white line (d) shown in Panel B. Panels D, E and F show similar observations for *S. japonicum*.
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used to determine the elemental composition of a material. It uses inductively coupled plasma to produce excited atoms and ions that emit electromagnetic radiation at wavelengths characteristic of a particular element which are then detected by a detector.

The same samples as in the SQuID measurements were used. For ICP-AES analysis, three replicate samples were digested in 70% HNO_3 at 95°C. The analysis was performed at the Marine and Freshwater Research Laboratory at Murdoch University, Murdoch, WA, Australia.

Exposure of eggs to magnetic fields with and without magnetic microspheres

In order to assess the ability to manipulate parasite eggs using a magnetic field, approximately 100 glutaraldehyde-fixed eggs of *S. japonicum* were floated on a 100% Percoll/water interface in a 5 mL cell culture dish. No spontaneous hatching of eggs was observed in these fixed eggs. A cylindrical neodymium-iron-boron magnet was brought close to the eggs so that they were exposed to a magnetic field of approximately 0.1 T and a magnetic field gradient of approximately 35 T/m while being observed under an optical microscope.

To assess the capability of the different egg species to bind magnetic microspheres, eggs of the two species were incubated with microspheres at egg/microsphere ratios of 1:100 and 1:500. Unbound microspheres were washed out using custom made filters after an incubation time of 10 min with agitation. Images of the conjugated microsphere/egg suspension were taken at a 100-fold magnification and the distributions of the number of observed microspheres per egg were recorded and compared with the Poisson distribution.

Analysis of magnetic susceptometry data

The SQuID magnetometry data was fitted to two functions, the Brillouin function and Curie's law. These functions are used to determine the atomic iron specific moment in the samples [21]. The Brillouin function is specifically used to describe the response of an ideal paramagnet to an applied magnetic field. The spin state and thus the oxidation state of the iron atoms in a material can be deduced from the Brillouin fit using Equation 1.

$$M = Ng\mu_B J \left[\frac{2J+1}{2J}\coth\left(\frac{2J+1}{2J}\frac{g\mu_B JB}{k_B T}\right) - \frac{1}{2J}\coth\left(\frac{1}{2J}\frac{g\mu_B JB}{k_B T}\right)\right] + AB \quad (1)$$

The function describes the dependency of the magnetization (M) on the applied magnetic field (B) in an ideal paramagnet mixed with diamagnetic (non-magnetic) atoms and gives the total angular momentum quantum number J of the microscopic paramagnetic moments of the material. N is the number of paramagnetic atoms in the sample; g is the electron g-factor (−2.0023); μ_B is the Bohr Magneton (9.274×10^{-24} J T^{-1}); J is the total angular momentum quantum number for each paramagnetic atom; k_B is the Boltzmann constant (1.380×10^{-23} m^2 kg s^{-2} K^{-1}); T is the temperature and B is the magnetic flux density. The factor A is the diamagnetic susceptibility of the sample holder and the diamagnetic components of the eggs.

Curie's law is used to describe the temperature dependency of magnetic susceptibility (χ). Data for this analysis are often plotted as $1/\chi$ versus T. A linear relationship with line of best fit running through the coordinate origin indicates ideal paramagnetic behavior. In the present study, the magnetization versus temperature data were fitted with Curie's law shown in Equation 2.

$$\chi = \frac{M}{B} = \frac{Ng^2J(J+1)\mu_B{}^2}{3k_B T} + A \quad (2)$$

where χ is the magnetic susceptibility of the eggs and all other symbols correspond to those used in Equation 1.

Results

Scanning electron microscopy

SEM images of *S. mansoni* and *S. japonicum* eggs are shown in Figure 1. The eggs exhibit the typical features of *S. mansoni* (large spine) and *S. japonicum* (oval shape, small spine) [22]. The fibrous matrix surrounding the egg, also typical for *S. japonicum*, can clearly be seen in Figures 1C and 1D [22]. Figures 1B and 1D show examples of eggs of both species where the eggshell has broken open and the miracidium is still inside the egg. It can be seen that the eggshell curls outwards after initial rupture.

Figure 2 shows details of the surfaces of the eggs of both species at a higher magnification. The surface of *S. mansoni* is covered with evenly spaced structures previously termed microspines with a length of about 200–300 nm and a diameter of 60 nm [22,23]. The surface of *S. japonicum* is also covered with microspines, however they are considerably shorter. In addition a structure, previously termed the fibrous matrix covers the *S. japonicum* surface [22].

Transmission electron microscopy

The elemental composition and structure of the eggshells of both species was studied by HAADF-STEM and STEM-EDS (Figure 3). The TEM images show that the eggshells are about 700 nm thick. There are regions where the shell is interspersed with small holes of variable size (50–200 nm). These holes have been shown to be empty in other studies [23]. In the present study we show for the first time that these holes are partially filled with a material containing iron, phosphorous and oxygen and we hypothesise that this material is an iron-phosphate that is retained more readily by cryo-fixation and subsequent freeze-substitution processing compared to conventional TEM sample preparation methods (Figure 3).

Magnetic measurements

The results from the magnetometry measurements of the eggs are presented in Figure 4. Both, *S. mansoni* and *S. japonicum* eggs exhibited paramagnetic behaviour with no hysteresis at 5K (Figure 4A). The magnetic susceptibility versus temperature measurements were in nearly perfect agreement with Curie's Law (Figure 4B). The magnetic moment per iron atom (measured in Bohr magnetons - μ_B) obtained from fitting the Brillouin function with an additional diamagnetic contribution (Equation 1) to the magnetization versus magnetic field data (Figure 4A) at 5K was 4.8 and 4.1 $\times\mu_B$ for *S. mansoni* and *S. japonicum* respectively. It

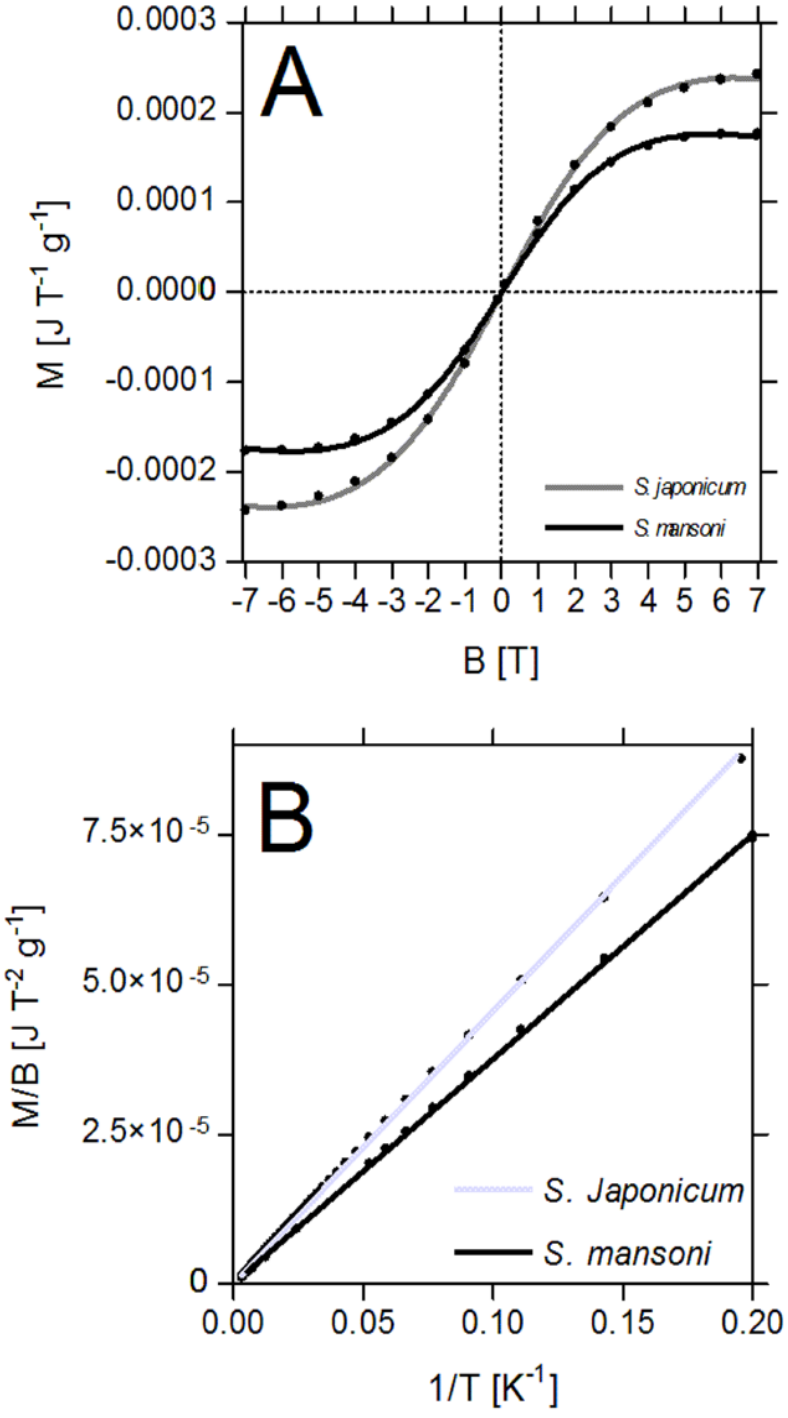

Figure 4. Results of magnetic susceptibility analysis. Figure 4A shows the curve fits of the Brillouin function with diamagnetic contribution (Equation 1) to the magnetization versus magnetic field data (for whole eggs at a temperature of 5 K). It can be seen that ideal paramagnetic behavior is approximated with good precision. (R^2>0.99) for both, *Schistosoma mansoni* and *Schistosoma japonicum* eggs. Figure 4B shows the curve

fits of Curie's law to the magnetization versus inverse temperature data. Again, ideal paramagnetic behavior can be observed. For *S. mansoni* the total magnetic moments were approximated to 4.84×μ_B using the Brillouin function and 4.76×μ_B using Curie's law. For *S. japonicum* the total magnetic moments were approximated to 4.12×μ_B using the Brillouin function and 3.71×μ_B using Curie's law.
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was assumed that iron was the only paramagnetic material in the eggs. Similar values were obtained from fits of Curies's law to the magnetization versus temperature data (4.76 and 3.7×μ_B for the *S. mansoni* and *S. japonicum* eggs respectively). These values agree but are slightly lower than what would be expected if all the iron in the sample were present as high spin Fe^{2+} ions (typically 5.4×μ_B) or high spin Fe^{3+} (typically 5.9×μ_B). We can therefore conclude that there is mix of high spin and low spin iron configurations present in the eggs. Further, more sophisticated measurements using, for example, Mössbauer spectroscopy, that can be used to detect the chemical environment around each iron atom, would be necessary to resolve the exact distribution of these configurations.

Elemental analysis using inductively coupled plasma atomic emission spectroscopy (ICP-AES)

ICP-AES data are summarized in Table 1. *S. mansoni* and *S. japonicum* eggs contained approximately 0.74 mg/g and 1.26 mg/g of iron respectively (dry weight). By comparison, 1 g of blood contains 3.39 mg iron and 1 g of normal human feces approximately 0.3 mg iron (dry weight) [24]. The concentrations of copper and silicon were also determined.

Exposure of eggs to magnetic fields with and without microspheres

No movement of parasite eggs suspended at the Percoll/water interface was observed when the eggs were exposed to a strong magnetic field and a high magnetic field gradient, and imaged with light microscopy. However, when magnetic microspheres were incubated with parasite eggs they readily bound to a fraction of the eggs even without the presence of a magnetic field. The microsphere-egg conjugates were very susceptible to magnetic fields and field gradients as shown in Figure 5 and the video file provided as supporting information (Video S1). Figure 6 illustrates the binding characteristics of the microspheres to the parasite eggs. For both tested parasite egg to microsphere ratios (1:100 and 1:500) the fraction of *S. japonicum* eggs that bound microspheres was statistically significantly higher than the fraction of *S. mansoni* eggs that bound microspheres (54% versus 41%, $p = 0.02$ for the 1:100 ratio and 76% versus 30%, $p<0.001$, for the 1:500 ratio, unpaired t-test). In addition the number of microspheres which bound to individual *S. japonicum* eggs was significantly higher than that for *S. mansoni* (Figure 6). The distribution of microspheres per egg was not well characterized by a single Poisson distribution, especially for the *S. japonicum* eggs but reasonable fits were obtained on the assumption that a fraction of the eggs had no binding capacity (for more details on the analyses using Poisson statistics please refer to the supporting Figures S1 and S2 as well as Text S1).

Table 1. Elemental concentration in *Schistosoma mansoni* and *Schistosoma japonicum* eggs.

	Fe (mg/g)	Si (mg/g)	Cu (mg/g)
S. mansoni	0.74	0.15	0.11
S. japonicum	1.26	0.03	0.03

doi:10.1371/journal.pntd.0002219.t001

Discussion

The present study investigated the magnetic properties as well as iron localization and content of *S. mansoni* and *S. japonicum* eggs and especially the eggshells. This investigation was conducted to elucidate the processes underlying the success of a previously developed magnetic fractionation approach for the detection of parasite eggs in fecal samples, namely the Helmintex method [11]. In recent years, this method has been used in a series of diagnostic studies and has consistently shown improved sensitivity when compared with the conventional Kato-Katz method of fecal evaluation and the saline gradient method [11,25,26,27].

The most important question for optimizing the existing Helmintex method was whether the magnetic properties of the *Schistosoma* eggs were the cause for the adhesion of the magnetic microspheres to the surface of the eggs or whether this binding was of another biochemical or physical nature.

We show that the eggshells of *S. mansoni* and *S. japonicum* eggs contain iron in concentrations measurable by STEM-EDS and ICP-AES, and that the eggs are distinctly paramagnetic, meaning they magnetize in an applied magnetic field and demagnetize when the magnetic field is taken away. These are the principal characteristics required for magnetic fractionation. Interestingly, most of the iron seems to be accumulated as iron-phosphate in pores in the eggshell.

However, the magnetization of the eggs was comparatively weak and the iron content was low. Furthermore we did not observe any movement of the eggs in magnetic fields and field gradients of a similar order of magnitude to those used in the Helmintex protocol. Therefore, the interaction between the eggs and the magnetic microspheres, which is the basis of the success of the Helmintex method, is unlikely to be magnetic in origin.

The original Helmintex studies have shown that magnetic microspheres coated with a wide variety of different ligands could be used to purify parasite eggs [11]. Here, we show for the first time that microspheres physically bind to the eggs. The high surface area of the filamentous outer structure of the eggs may be part of the explanation as this large surface area may provide strong overall adhesion from relatively weak interactions. This hypothesis is further supported by the observation that *S. japonicum* eggs with their additional fibrous matrix bound significantly more microspheres than *S. mansoni* eggs, which do not have this matrix. However, the distributions of microspheres per egg observed in the binding studies suggest that a fraction of the eggs have very little, if any, binding capacity. Furthermore, it should be noted that the fixation using glutaraldehyde may lead to modified surface characteristics of the eggs. Further studies are required to investigate the impact of fixation on particle binding.

The present study provides the first magnetic characterization of *S. mansoni* and *S. japonicum* eggs. We report the discovery of an iron-containing material, presumably iron phosphate located in pores within the eggshell. We provide evidence that *Schistosoma* eggs are not magnetic enough to move in an applied magnetic field of a similar order of magnitude as the one used in the Helmintex method. We show that magnetic microspheres spontaneously bind to eggs of *S. mansoni* and, to a greater degree, to *S. japonicum*. Based on these results we conclude that the conjugation of magnetic microspheres and parasite eggs is mediated not by magnetism but by the surface properties of eggs and microspheres. Systematic

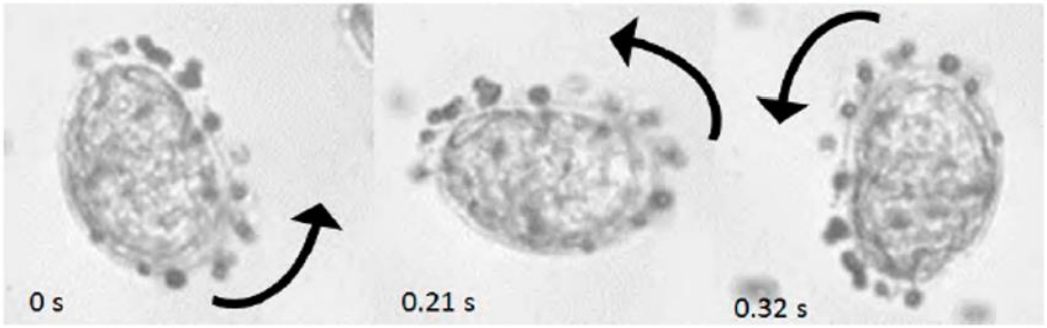

Figure 5. *Schistosoma mansoni* egg – paramagnetic microsphere conjugates. At least 15 microspheres can be seen bound to the surface of the egg. A magnet is rotated around the suspension by 180 degrees over approximately 0.5 seconds (black arrows indicate the movement of the magnet). The images represent frame captures from Video S1 available as supporting information.
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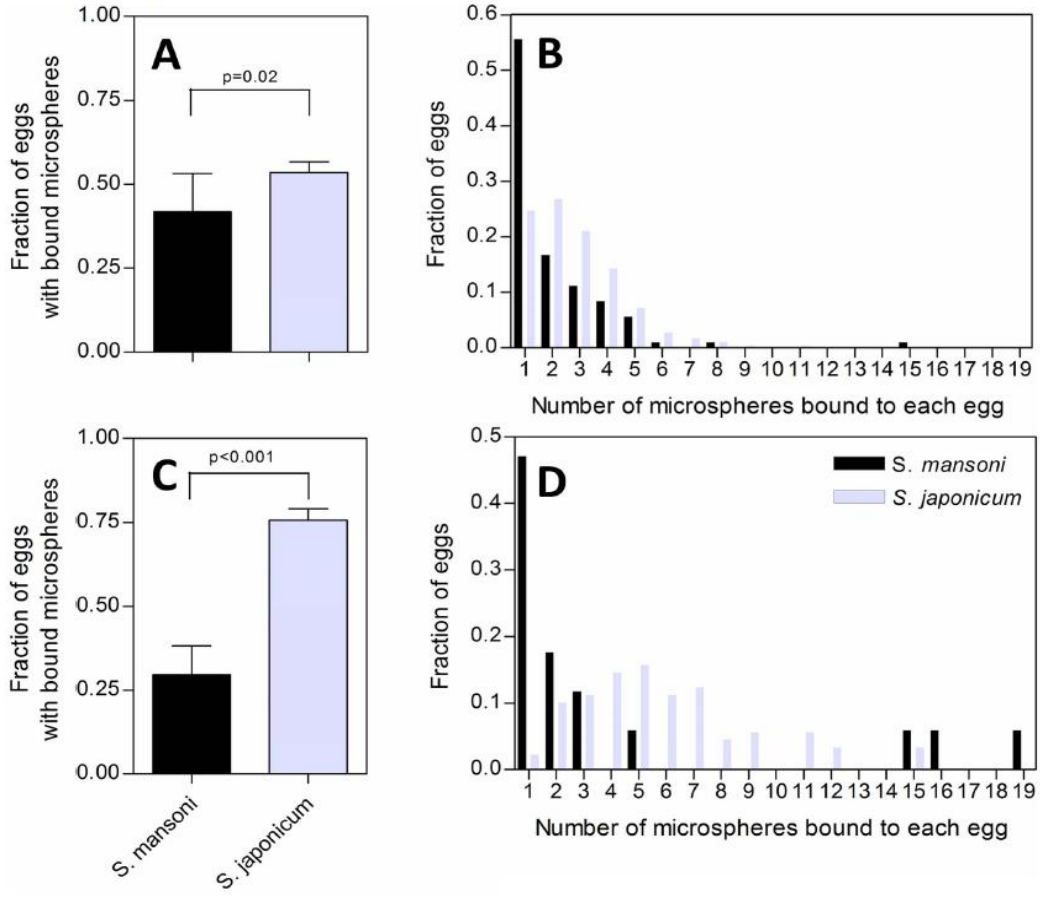

Figure 6. Microsphere binding characteristics to *S. mansoni* and *S. japonicum* eggs. Panel A shows the fraction of eggs that had at least one microsphere bound at an egg to microsphere ratio of 1:100. Panel B shows the distribution of the number of microspheres bound to eggs of the two parasite species at an egg to microsphere ratio of 1:100. Panels C and D show the same data for an egg to parasite ratio of 1:500. For both ratios *S. japonicum* eggs spontaneously conjugated with microspheres at a significantly higher frequency than *S. mansoni* eggs. Similarly, the average number of microspheres per individual egg was considerably higher for *S. japonicum* than for *S. mansoni* (Panels B and D).
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quantification of the binding of microspheres that have different surface functionalizations to parasite eggs is likely to represent an opportunity to optimize the Helmintex magnetic fractionation method. Previous field studies have indicated that such an optimized Helmintex method may be developed into a new gold standard to validate future rapid diagnostic and molecular methods for *Schistosoma* detection [25,26,27].

Supporting Information

Figure S1 Comparison of the calculated Poisson distribution (red) and measured distribution of microspheres per egg (black) at a microsphere to egg ratio of 100 microspheres per egg. Panel A shows the distribution of the number of microspheres bound to all *S. mansoni* eggs (including those eggs that had no microspheres bound). Panel B shows the distribution for *S. mansoni* when the eggs that had no microspheres bound to them were excluded. Panel C shows the distribution of the number of microspheres bound to all *S. japonicum* eggs (including those eggs that had no microspheres bound). Panel D shows the distribution for *S. japonicum* when the eggs that had no microspheres bound to them were excluded.
(TIF)

Figure S2 Comparison of the calculated Poisson distribution (red) and measured distribution of microspheres per egg (black) at a microsphere to egg ratio of 500 microspheres per egg. Panel A shows the distribution of the number of microspheres bound to all *S. mansoni* eggs (including those eggs that had no microspheres bound). Panel B shows the distribution for *S. mansoni* when the eggs that had no microspheres bound to them were excluded. Panel C shows the distribution of the number of microspheres bound to all *S. japonicum* eggs (including those eggs that had no microspheres bound). Panel D shows the distribution for *S. japonicum* when the eggs that had no microspheres bound to them were excluded.
(TIF)

Video S1 Movement of microsphere-egg conjugates when exposed to a magnetic field of approximately 0.1 T and a field gradient of approximately 35 T/m.
(WMV)

Text S1 Additional information on the statistical analysis of binding of magnetic microspheres to *S. mansoni* and *S. japonicum* eggs providing further explanations for figures S1 and S2.
(DOC)

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Author Contributions

Conceived and designed the experiments: SK LG RLM MJH MS JAS AS RCW AH CGT TGS MKJ. Performed the experiments: SK LG RLM RK RRFC SQT MS JAS AS RCW. Analyzed the data: SK LG MS RRFC SQT JAS AS AH MJH RCW CGT TGS MKJ. Contributed reagents/materials/analysis tools: MKJ SQT RLM CGT MS JAS AS RRFC SQT. Wrote the paper: SK LG RLM RRFC SQT RCW CGT TGS MKJ.

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3.2. Artigo científico aceito pela revista International Journal for Parasitology em 9 de agosto de 2014.

The Affinity of Magnetic Microspheres for *Schistosoma* Eggs.

De: **Int J Parasitol** (editor@IJP.org.au)

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Ms. Ref. No.: IJPara14_206R1
Title: The Affinity of Magnetic Microspheres for Schistosoma Eggs
International Journal for Parasitology

Dear Ms. Candido,

I am pleased to confirm that your paper "The Affinity of Magnetic Microspheres for Schistosoma Eggs" has been accepted for publication in the International Journal for Parasitology.

For every issue of the IJP the Editor will endeavour to feature an image on the cover from, or relating to, an article in that issue. We invite all authors to submit images that would be suitable. To maximize their aesthetic qualities, these images may be stylized/modified versions of pictures or diagrams from the author's article.

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Abstract: Schistosomiasis is a chronic parasitic disease of humans, with two species primarily causing the intestinal infection: Schistosoma mansoni and Schistosoma japonicum. Traditionally, diagnosis of schistosomiasis is achieved through direct visualization of eggs in faeces using techniques that lack the sensitivity required to detect all infections, especially in areas of low endemicity. A recently developed method termed Helmintex™, is a very sensitive technique for detection of Schistosoma eggs, exhibits 100 % sensitivity at 1.3 eggs per gram of faeces, enough to detect even low-level infections. The Helminthex™ method is based on the interaction of magnetic microspheres and schistosome eggs. Further understanding the underlying egg-microsphere interactions would enable a targeted optimization of egg-particle binding and may thus enable a significant improvement of the Helmintex™ method and diagnostic sensitivity in areas of low infection rates. We investigated the magnetic properties of S. mansoni and S. japonicum eggs and their interaction with microspheres with different magnetic properties and surface functionalization. Eggs of both species exhibited higher binding affinity to the magnetic microspheres than the non-magnetic microspheres. Binding efficiency was further enhanced if the particles were coated with streptavidin. S. japonicum eggs bound more microspheres as compared to S. mansoni. However, distinct differences within each species of eggs were also observed when the distribution of the numbers of microspheres bound per egg were modeled with double Poisson distributions. Using this approach, both S. japonicum and S. mansoni eggs fell into two groups, one having greater affinity for magnetic microspheres than the other indicating that not all eggs of a species exhibit the same binding affinity. Our observations suggest that interaction between the microspheres and eggs is more likely to be related to surface charge-based electrostatic interactions between eggs and magnetic iron oxide rather than through a direct magnetic interaction.

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Porto Alegre, 4th June 2014.

Dear Editor,

We would like submit the present manuscript entitled "The Affinity of Magnetic Microspheres for *Schistosoma* Eggs" to The International Journal for Parasitology.

In this study, we investigated the magnetic properties of *Schistosoma mansoni* and *S. japonicum* eggs and their interaction with microspheres with different surface functionalization. We believe that our work has provided important information underlying the binding properties between the eggs and the microspheres, which may lead to an improvement of the recently developed diagnostic technique Helmintex™ and increase sensitivity of such method in low endemicity areas.

Our observations indicate that the presence of magnetic iron oxide at the surface of the microspheres is the dominant factor that determines the affinity of the eggs for the microspheres. *S. japonicum* eggs have higher affinities for the magnetic microspheres than *S. mansoni*. Streptavidin-coating increases the affinity of both species of egg for non-magnetic microspheres but the effect is much smaller than that induced by magnetic iron oxide coating. Modelling the observed distribution of numbers of microspheres bound to the eggs with Poisson distributions indicates that there are two distinct categories of eggs in both *S. japonicum* and *S. mansoni* species, one having a higher affinity for magnetic material than the other category, indicating that not all eggs of a species exhibit the same binding affinity.

The results of our work imply that interaction between the microspheres and the eggs is more likely to be related to surface charge based electrostatic interactions. Further studies are necessary in order to elucidate the mechanisms underlying this newly observed phenomenon of the physical interactions between the *Schistosoma* eggs and the different microspheres.

All authors agree with the submission and are ready to take full responsability for the accuracy of the data. This study has not been submitted elsewhere. We appreciate your time and consideration.

Yours sincerely,

Renata Russo Frasca Candido

Vivian Favero

Mary Duke

Stephan Karl

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Robert C. Woodward

Carlos Graeff Teixeira

Malcolm K. Jones

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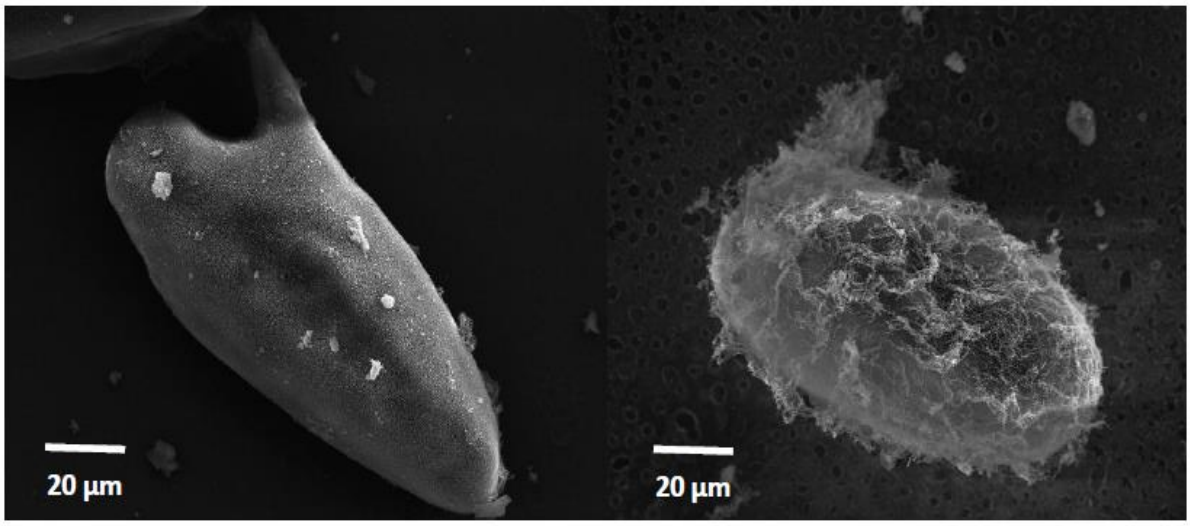
*Graphical Abstract (for review)

Graphical abstract



*Highlights (for review)

Highlights

- *Schistosoma* eggs physically bind magnetite-coated polystyrene microspheres.
- *S. japonicum* eggs have greater affinity for magnetic microspheres than *S. mansoni*.
- There are two groups of *Schistosoma* eggs, one binds weakly and the other, strongly.
- Source of binding could be differences of zeta potentials between eggs and spheres.

The Affinity of Magnetic Microspheres for *Schistosoma* Eggs

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Abstract

Schistosomiasis is a chronic parasitic disease of humans, with two species primarily causing the intestinal infection: *Schistosoma mansoni* and *Schistosoma japonicum*. Traditionally, diagnosis of schistosomiasis is achieved through direct visualization of eggs in faeces using techniques that lack the sensitivity required to detect all infections, especially in areas of low endemicity. A recently developed method termed Helmintex™, is a very sensitive technique for detection of *Schistosoma* eggs, exhibits 100 % sensitivity at 1.3 eggs per gram of faeces, enough to detect even low-level infections. The Helminthex™ method is based on the interaction of magnetic microspheres and schistosome eggs. Further understanding the underlying egg-microsphere interactions would enable a targeted optimization of egg-particle binding and may thus enable a significant improvement of the Helmintex™ method and diagnostic sensitivity in areas of low infection rates. We investigated the magnetic properties of *S. mansoni* and *S. japonicum* eggs and their interaction with microspheres with different magnetic properties and surface functionalization. Eggs of both species exhibited higher binding affinity to the magnetic microspheres than the non-magnetic microspheres. Binding efficiency was further enhanced if the particles were coated with streptavidin. *S. japonicum* eggs bound more microspheres as compared to *S. mansoni*. However, distinct differences within each species of eggs were also observed when the distribution of the numbers of microspheres bound per egg were modeled with double Poisson distributions. Using this approach, both *S. japonicum* and *S. mansoni* eggs fell into two groups, one having greater affinity for magnetic microspheres than the other indicating that not all eggs of a species exhibit the same binding affinity. Our observations suggest that interaction between the microspheres and eggs is more likely to be related to surface charge-based electrostatic

50 interactions between eggs and magnetic iron oxide rather than through a direct magnetic

51 interaction.

52 Keywords: Schistosomiasis; diagnosis; low endemicity; Helmintex™; magnetic properties.

1. Introduction

Schistosomiasis is a public health problem that affects more than 200 million people in 74 countries in Africa, South America and Asia, with 10 % of the affected persons presenting the severe form of the disease, and up to 60 % presenting clinical manifestations (WHO, 1993). The disease is caused by helminths of the genus *Schistosoma*, with three species causing most of the infection in humans: *Schistosoma mansoni* and *Schistosoma japonicum*, responsible for the hepato-intestinal manifestations, and *S. haematobium*, that causes genitourinary infection. Despite the efforts to control this infection, which are based on treatment of infected people with appropriate and effective chemotherapies like praziquantel (Davis, 1993; Savioli et al., 1997), schistosomiasis remains the second-most widespread parasitic infection in the world, after malaria (Chitsulo et al., 2000).

Currently, schistosomiasis infections are diagnosed through direct visualization of eggs of characteristic shape in faecal samples. The eggs are formed by female schistosomes in the ootype (egg mould), a bottle-neck in the reproductive tract that forms eggs one by one (DeWalick et al., 2012), with a production of 100-300 eggs per day for *S. mansoni* and 500-3500 for *S. japonicum* (Pittella, 1997). To continue the life cycle, the egg must migrate from the mesenteric vasculature across the endothelial and mucosal barriers to the lumen of the intestine with subsequent excretion. When the eggs are not detected in the coproparasitological examination in areas of low transmission or after mass drug administration, the true prevalence of the infection of the studied population cannot be established. Cases of misdiagnosis in areas of low or very low transmission intensities and low worm burden currently occur worldwide because the available diagnostic techniques are too expensive to be used on greater scale or are not sensitive enough to detect low burdens of

77 eggs in the stool, leading to a number of false-negative diagnoses (Engels et al., 1996; Enk et
78 al., 2008), which can result from daily variations in egg excretion within an individual (Van
79 Etten et al., 1997).

80 Several molecular methods (Pontes et al., 2002; Sandoval et al., 2006; tenHove et al.,
81 2008) and immunological methods (Deelder et al., 1989; De Jonge et al., 1990; Deelder et al.,
82 2000; Doenhoff et al., 2004) and have been developed in an attempt to improve diagnostic
83 sensitivity. Molecular methods are based on the amplification of a highly repeated parasite
84 DNA sequence, using the polymerase chain reaction (PCR) in human samples, but those
85 techniques require a well-equipped laboratory and proper skills to perform it. Antibody
86 detection tests provide information about whether an individual has been exposed to the
87 parasite. However, their specificity for detection of active infections is limited since specific
88 antibodies in the host, once developed against *Schistosoma* species, are long-lived and
89 therefore could be often present in individuals who have already cleared the infection
90 (Sturrock, 2001). The most common immunologic method used is the ELISA (Enzyme-
91 Linked Immunosorbent Assay) which consists of detecting host antibodies to parasite´s
92 antigens. However, ELISA also demands well-trained people, and some authors have shown
93 cross-reactivity between diagnostic antigens for schistosomiasis and antigens of other
94 parasites (Correa-Oliveira et al., 1988; Valli et al., 1997).

95 Errors in assessing prevalence and intensity of infection are exaggerated due to an
96 inherent lack of sensitivity and accuracy in common diagnostic techniques, particularly in
97 areas of low prevalence and in individuals in the earliest or latest stages of schistosome
98 infection. The problem will thus be further exacerbated when the prevalence and intensity of
99 schistosomiasis is being reduced through the introduction of effective control measures
100 (Hamilton et al., 1998). The importance of diagnosing individuals with undetected infections
101 due to low-parasitic burden can be summarized in the following points: i. the degree of

pathology and egg count are not always correlated; ii. undetected and untreated infections may be responsible for persistence of transmission; iii. the proportion of missed infections increases after chemotherapy, which overestimates cure rates and, iv. persistent light infections may maintain concomitant immunity leading to acquired resistance, which interferes with vaccine trials and with conventional treatment (DeVlas and Gryseels, 1992).

The World Health Organization (WHO) currently recommends the Kato-Katz thick smear technique for diagnosing intestinal schistosomiasis infection in epidemiological studies. Kato Katz technique has the advantage of being a simple, low-cost procedure, and also allows for quantification of egg loads (Katz et al., 1972). However, due to the relatively small amount of fecal matter observed with this method, it lacks sensitivity. This leads to an underestimated number of positive cases and thus, an inaccurate measurement of the prevalence of the disease especially in areas of low endemicity (Ebrahim et al., 1997; Zhanga et al., 2009).

The Helmintex™ technique is a very sensitive method for detection of *Schistosoma* eggs by isolating the eggs from a larger volume of faeces. Helmintex™ is based on the interaction of the eggs with magnetic particles, and this novel method has been shown to exhibit 100 % sensitivity for egg intensities of 1.3 eggs per gram of faeces (Teixeira et al., 2007).

Studies have shown that eggshells of *S. japonicum* and *S. mansoni* contain iron (Jones et al., 2007; Karl et al, 2013). The iron is believed to help the stabilization of the proteins that form the eggshells (Jones et al., 2007). Recently, we provided the first magnetic characterization of eggshells of *Schistosoma* species, showing that, despite the shells containing paramagnetic iron compounds, the interaction between magnetic particles and the eggs is unlikely to be purely magnetic in origin (Karl et al., 2013). Mediators of the

126 interactions were postulated to be surface elaborations of the shells, notably the microspines,
127 demonstrated in earlier studies (Ford and Blankespoor, 1979).

128 In order to clarify the properties responsible for the interaction between the eggs and
129 the microspheres and to optimize the Helmintex™ method, we characterize here the affinity
130 of *S. mansoni* and *S. japonicum* eggs for a variety of polystyrene microspheres using direct
131 microscopic observations and Poisson analysis.

2. Materials and Methods

2.1. Maintenance of the parasite life cycles

Schistosoma mansoni and *S. japonicum* were maintained at the QIMR Berghofer Medical Research Institute by passage in *Swiss* mice and *Biomphalaria glabrata* snails for *S. mansoni*, and *Oncomelania hupensis hupensis* snails collected in Anhui Province (China) for *S. japonicum*. The use of animals was approved by the Animal Ethics Committee of the Queensland Institute of Medical Research (Project P1289). The experiments were conducted in the School of Physics, The University of Western Australia, Perth, Australia, and in the Queensland Institute of Medical Research, Brisbane, Australia.

2.2. Acquisition of parasite's eggs

Mice infected with either *S. mansoni* and *S. japonicum* were euthanased at approximately 42 days post-infection and the livers were removed for digestion with Collagenase B in phosphate buffered saline (PBS) overnight at 37 °C. On the following day, samples were sieved for isolation of the eggs and purified using Percoll density gradient centrifugation as described by Dalton and colleagues (Dalton et al., 1997). The eggs were stored in PBS at -80 °C until use.

2.3. Incubation of eggs with microspheres

We used four types of commercial polystyrene microspheres with diameters of ~4 µm, schematically represented in Figure 1 (Spherotec Inc, USA) as follows:

a) Uncoated polystyrene microspheres (PP-40);

b) Magnetite-coated polystyrene microspheres (PM-40);

c) Streptavidin-coated polystyrene microspheres (SVP-40) and,

d) Magnetite-streptavidin-coated polystyrene microspheres (SVM-40).

Streptavidin was chosen as ligand in both magnetic and non-magnetic particles due to its capability to bind schistosome eggs (Teixeira et al., 2007). The eggs and microspheres were incubated in 1.5 mL microtubes (Eppendorf, USA) and mixed in agitation using a Rotary Suspension Mixer (Ratek Lab, Australia) in pH 7 for 30 minutes. A custom-made filter of 42 µm pore size was cut and glued to the end tip of the microtubes in an attempt to try to remove as many unbound particles as possible. The mixture of microspheres and eggs was placed inside the microtubes with the filter and centrifuged at 800 x *g* for 5 minutes. The egg-microsphere conjugates were collected from the filter using a pipette and transferred to a glass slide for further analysis using optical microscopy.

2.4. Determining egg/microspheres ratio

To determine a suitable egg to microsphere ratio for subsequent experiments, four different incubations with 1:100, 1:200, 1:500 and 1:1000 eggs/microsphere ratios respectively were tested and the best egg/microsphere ratio was chosen through visual inspection using optical microscopy. Only magnetite-coated microspheres (Figure 1B) were used in these experiments due to their relatively high affinity for *Schistosoma* eggs (Teixeira et al., 2007). Egg/microsphere conjugates were placed onto a glass slide and examined using optical microscopy and the concentrations were determined using a haemocytometer (Bright-line, Sigma-Aldrich).

177

178 *2.5. Determining the incubation time of the egg/microsphere conjugates*

179 After determining the best egg/microsphere concentration as previously indicated,
180 magnetite-coated microspheres were also used to determine the optimal time to incubate the
181 eggs with the microspheres. The microspheres were incubated under agitation with *S.*
182 *mansoni* and *S. japonicum* eggs for 3 different durations: 10 minutes, 30 minutes and 60
183 minutes. The conjugates were transferred onto a glass slide and analyzed using optical
184 microscopy.

185

186 *2.6. Image acquisition*

187 Each condition was prepared and repeated four times. From each incubation
188 experiment, around 30 fields of view with around 30 eggs per field of view were collected at
189 random using an Optiphot-2 Optical Microscope (Nikon, Japan) with a ProgRes C10 Plus 3
190 MP Digital Camera (Jenoptik, Germany). In each digital image, the number of microspheres
191 bound to the visible edge of each egg was recorded using the objective lens magnification x10
192 of the optical microscope. Only microspheres bound at the visible (lateral) edge of focused
193 eggs were counted, in order to minimize errors caused by microspheres bound at the upper
194 and lower surfaces of the egg where microsphere identification was significantly more
195 difficult because of occlusion by the mottled appearance of the internal structure of the egg.

196

197 *2.7. Data Analysis and Statistics*

198 *2.7.1. Analysis of proportions*

For each of the investigated microsphere-type/egg-species combination, the proportions of eggs that had visibly bound to microspheres to their edges was compared to the proportion of eggs that had no microspheres visibly bound to the edge using Fisher's Exact Test. Specifically, the influence of *i)* magnetic coating, *ii)* streptavidin coating and *iii)* parasite species on the number of eggs that had bound microspheres was investigated. These three data analysis tests were carried out on the same experimental data set.

2.7.2. *Poisson distribution modelling*

The number, n, of microspheres bound to the visible edge of an egg was used as a measure of the relative affinity of the egg for microspheres rather than as an absolute measurement of the number of microspheres bound. Given the discrete nature of n, the distribution of n for a given species of egg and type of microsphere could be expected to be a single Poisson distribution (based on a random chance of binding a population of eggs with a constant affinity for the microspheres). However, in general, a single Poisson distribution was a poor fit to the data while a double Poisson distribution modelled the observed data well. The double Poisson distribution is given by

$$p(n) = f\frac{\lambda_1^n}{n!}e_1^{-\lambda} + (1-f)\frac{\lambda_2^n}{n!}e_2^{-\lambda}$$

where $p\ (n)$ is the probability of finding n microspheres attached to the edge of an egg when the egg is randomly taken from a population of eggs comprising two groups: group 1 comprising a fraction, f, of the population of eggs and having a mean number, λ_1, microspheres bound per egg, and group 2 comprising a fraction (1 - f) of the population of eggs and having a mean number, λ_2, of microspheres bound per egg. The double Poisson distribution was fitted to the data using a sum of squares minimization routine yielding three

parameters (f, λ_1, and λ_2) to describe the observed distribution. In order to assess the standard errors on f, λ_1, and λ_2 for a given distribution, the raw data for the distribution were resampled to generate 300 new distributions using the bootstrap method (Efron and Gong, 1983). Each of the 300 resampled distributions were fitted with the double Poisson distribution model, enabling the standard deviations of f, λ_1, and λ_2 to be estimated.

3. Results

3.1. Influence of incubation time on the formation of egg/microsphere conjugates

The numbers of magnetic microspheres bound to eggs did not vary for incubation times of 30 and 60 minutes (Figure 2). However, after only 10 minutes of incubation, many unbound microspheres were found free in suspension, indicating that, either the particles separated from the eggs after washing procedures, or it was not enough time to allow the binding of eggs and microspheres (Figure 3). The results are summarized in Table 1.

3.2. Analysis of proportions using Fisher's Exact Test

3.2.1. Influence of streptavidin coating on the binding

Figure 4 shows the results from the comparative analysis between incubation experiments using streptavidin-coated microspheres versus experiments using microspheres that were not coated with streptavidin. It is shown that with the exception of *S. japonicum* binding to magnetite-coated microspheres, the presence of streptavidin generated a statistically significant ($p<0.05$) increase in the proportion of eggs that had bound to microspheres. However, the increase in the proportion of eggs that had bound to microspheres was relatively small (~1.2-2 fold).

3.2.2. Influence of magnetic coating on the binding

Figure 5 shows the results from the comparative analysis between incubation experiments using magnetite-coated microspheres versus experiments using microspheres that were not coated with magnetite. Generally, in the absence of magnetic iron oxide coating, there was very little affinity of the eggs for the microspheres. Magnetite coating led to a statistically significant increase in the proportion of eggs that had bound to microspheres in all cases. The increase in the proportions was much larger than that seen for streptavidin (>3 fold).

3.2.3. Influence of parasite species on microsphere binding

Figure 6 shows the results from the comparative analysis between incubation experiments with *S. mansoni* versus *S. japonicum* eggs. Generally a larger proportion of *S. japonicum* eggs bound microspheres ($p<0.05$) presumably due to the presence of the longer filamentous matrix on the surface of these eggs. However, the difference in the proportions between the two species was relatively small (1-2 fold).

3.3. Poisson distribution

The observed distributions of the number of microspheres bound to the eggs generally fitted poorly with single Poisson distributions. Double Poisson distributions gave good fits to the data indicating that both *S. japonicum* and *S. mansoni* eggs appeared to fall into two distinct categories, one having a greater affinity for microspheres than the other (Figure 7 and Figure 8). At pH 7, *S. japonicum* eggs had a significantly higher affinity for magnetite-coated microspheres than the *S. mansoni*

eggs. Streptavidin coating increased the affinity for both species. In the absence of magnetite coating, there was very little affinity of the eggs for the microspheres.

4. Discussion

The Helmintex™ method, which is based on the interaction between schistosome eggs and magnetite-coated microspheres, has proven to be more sensitive for detection of schistosome eggs in faeces than the Kato Katz technique (Caldeira et al., 2012; Pinheiro et al., 2012). Magnetic microspheres have been used for several years in serological diagnosis techniques, such as MEIA (Magnetic Microbead-Based Enzyme Linked Immunosorbent Assay) used in the detection of the parasite's circulating anodic antigens (Gundersen et al., 1992) and detection of the parasites antibody in human serum (Liu et al., 2010), but never in a coproparasitological test.

The eggshells of schistosomes are composed of a cross-linked polymerized protein matrix (Deelder et al., 2000). The exterior of the eggshells contains a filamentous structure, microspines, that are especially abundant and elongate on the surface of *S. japonicum* eggs, while the *S. mansoni* eggs have a dense matt of very short fibers (Karl et al., 2013). These structures may promote the adhesive properties of eggs to different surfaces and could be important in the binding of magnetic particles to eggs observed here and previously (Teixeira et al., 2007; Karl et al., 2013). However, this complex surface on the eggs is unlikely to be the only source of binding as *S. mansoni* eggs which have less microspines, still have substantial binding capacity (Teixeira et al., 2007).

The results of the experiments described in this study indicate that, for both parasite species, the presence of a magnetic coating on the surface of the polystyrene microspheres results in an increase in the number of eggs that are observed to bind the microspheres (Figure 5). The results also indicate that there are at least two distinct categories of egg in each species characterized by the affinity of the egg for magnetite-

296 coated microspheres (Figure 7). For the category of eggs that show an enhanced affinity
297 for magnetite-coated microspheres, the underlying mechanism for the affinity is not
298 fully understood. Our initial hypothesis as to the success of the Helmintex™ approach
299 was that the eggs were magnetic and thus attracted magnetic microspheres. However,
300 while schistosome eggs have paramagnetic properties, that is they are weakly magnetic
301 in a magnetic field, this explanation now seems unlikely. The eggs and the microspheres
302 physically bind without the application of any magnetic field, and the low iron content
303 coupled with the form of iron in the eggshells is unlikely to promote a magnetic
304 interaction (Karl et al., 2013). Another possibility that could explain the binding is that
305 different electrostatic interactions between the oppositely charged surfaces of the eggs
306 and the microspheres could be promoting the adhesion, but further research is required
307 to test this hypothesis and could involve binding tests such as demonstrated here using
308 polymer microspheres with different surface coatings to modify the zeta potential of the
309 microspheres.

310 It is known that specific molecules are often required to promote cell to cell or
311 cell to surface adhesion. The fact that cells do not stick to each other or to surfaces
312 unless a number of specific cell-cell bridges can form, argues for the existence of a
313 repulsive barrier that must be overcome by bridging molecules (Bell et al., 1984). Some
314 studies measuring electrophoretic mobility and hydrophobicity of bacteria have shown
315 the relationship between physicochemical surface parameters and the adhesion of
316 bacterial cells to negatively charged polystyrene structures (Van Loosdrecht et al.,
317 1990). Most natural solids, as well as bacteria, are negatively-charged (Loder and Liss,
318 1985). In aquatic environments, these surface charges are counterbalanced by oppositely
319 charged ions, some of which are bound to the surface. The adhesion of microorganisms
320 to surfaces is influenced by long-range, short-range and hydrodynamic forces,

composed by two additive terms: electrostatic repulsion and van der Walls attraction (Ruter and Vincent, 1984).

Although the addition of streptavidin to the microspheres generally resulted in an increase in the proportion of eggs observed to bind microspheres (Figure 4), when the two categories of eggs within each species were considered separately, there was some evidence that the addition of streptavidin to the magnetite-coated microspheres may have reduced the proportion of eggs in the high affinity category (Figure 7). The explanation for this could be that although the streptavidin provides a weak affinity mechanism between eggs and microspheres and increases slightly the affinity of eggs in the low affinity group, the streptavidin coating may increase the distance of closest approach of egg surfaces with the magnetic iron oxide coating and hence reduce the interaction force (magnetic or electrostatic) between the eggs and microspheres in the high affinity group (Teixeira et al., 2007).

A significantly larger proportion of *S. japonicum* eggs bound microspheres than *S. mansoni* eggs (Figure 6). In addition, the percentage of eggs with a high binding affinity was larger for *S. japonicum* than *S. mansoni* (Figure 7). There is little difference in the affinity (Lambda) of binding for eggs with a high binding affinity in the two groups. One possible explanation for the enhanced binding of the *S. japonicum* may be related to the difference in the specialized filamentous structures that have been observed on the outer edges of the two types of eggs (Karl et al., 2013).

The observation that two distinct categories of eggs can be identified in each species raises the question of the origin of the difference. One possibility is that the two categories represent two stages of maturity of the eggs. Since the eggs in this study were purified from infected livers, the ratios of eggs in the two categories may not be

345 the same as those found in eggs located in faeces. As such, further studies will be
346 required to determine whether (1) eggs in faeces have similar affinities for magnetite-
347 coated microspheres and (2) eggs in faeces have similar population fractions in the
348 affinity categories. Answers to these questions will provide valuable information for
349 strategies to improve the efficiency of the Helmintex™ method.

350 The fact that two distinct categories of eggs have been identified also raises a
351 caveat for any studies on small numbers of eggs such as is typically the case for
352 transmission electron microscopy studies, for example. Care needs to be taken in any
353 conclusions drawn regarding the nature of eggs from such studies since currently there
354 may be some uncertainty regarding the category within which the egg falls.

355 In this work we have examined variations in the binding of different
356 microspheres to two different species of *Schistosoma* eggs, *S. japonicum* and *S.*
357 *mansoni*. Our observations showed that the binding between *Schistosoma* eggs and the
358 microspheres are non specific, happening to different types of particles, with a high
359 preference for particles coated with magnetic material. The source of the binding is
360 unclear but an electrostatic force based on differences of zeta potentials between the
361 eggs and the microspheres seems the most likely. An unexpected observation was that
362 the number of microspheres bound per egg is not the same for all eggs. We found that
363 the binding of microspheres to eggs could be best described in terms of two populations
364 of eggs, one group that binds weakly and a second group that binds strongly.

365 A critical question for future work is whether these two groups of low and high
366 affinity exist within eggs that make their way into faeces. In order to improve the
367 Helmintex™ method one needs to firstly increase the proportion of eggs with high
368 affinity for the microspheres and secondly increase the magnitude of the affinity of eggs

369 for microspheres in this group. Far from limiting the effectiveness of the Helmintex™,
370 this work opens the way to further investigations to elucidate the nature of the egg and
371 microsphere binding, which would be used in a further development of the Helmintex™
372 method for enhanced diagnostics in areas of low transmission and low parasite burdens.

373

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375

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537

538 **Figure Legends**

539

540 Figure 1. Schematically representation of the microspheres (Spherotec, USA). 1 A.
541 Polystyrene particle (Cat. Nº PP-40-10); 1 B. Polystyrene magnetic particle (Cat. Nº
542 PM-40-10); 1 C. Streptavidin polystyrene particle (Cat. Nº SVP-40-5); 1 D. Streptavidin
543 magnetic particle (Cat. Nº SMV-40-10).

544

545 Figure 2. *Schistosoma japonicum*. Eggs and microspheres incubation times. A. 10
546 minutes. B. 30 minutes. The arrow indicates a microsphere.

547

548 Figure 3. *Schistosoma mansoni*. Eggs and microspheres incubation times. A. 10
549 minutes. B. 30 minutes. The arrow indicates a microsphere.

550

551 Figure 4. Comparative analysis between incubation with streptavidin-coated
552 microspheres versus microspheres that were not coated with streptavidin.

553

554 Figure 5. Comparative analysis between incubation with magnetite-coated microspheres
555 versus microspheres that were not coated with magnetite.

556

557 Figure 6. Comparative analysis between incubation with *S. mansoni* versus *S.*
558 *japonicum* eggs.

559

560 Figure 7. Results from a Poisson distribution analysis. A: Magnetic microspheres group;
561 B: Non magnetic microspheres group. The figure schematically illustrates the results of
562 the analyses. The two different categories of eggs are represented as the blue and the red

563 boxes. The height of the box indicates the magnitude of "Lambda", the measure of the
564 likelihood of the egg binding a microsphere. The width of the box indicates the fraction
565 of eggs of that category in the sample being studied. The bars are the standard
566 uncertainties on the widths and heights of the boxes.
567
568 Figure 8. Example fit of a double Poisson distribution (red) to the observed distribution
569 (blue) of the number (k) of microspheres bound to the edge of eggs (*S. japonicum* with
570 magnetite-coated polystyrene microspheres). The fit yields the fraction of eggs
571 described by each Poisson distribution together with the parameter lambda (λ) that
572 describes the magnitude of the affinity for microspheres of each of the two categories of
573 egg.

1 **Tables**

2

3 Table 1. Incubation times experiment. The results show the percentage of eggs with magnetic

4 microspheres bound after each time.

5

Time (minutes)	*S. mansoni* eggs (%)	*S. japonicum* eggs (%)
10	25	43
30	37	48
60	35	49

6

Figure

1 Figure 1.

2

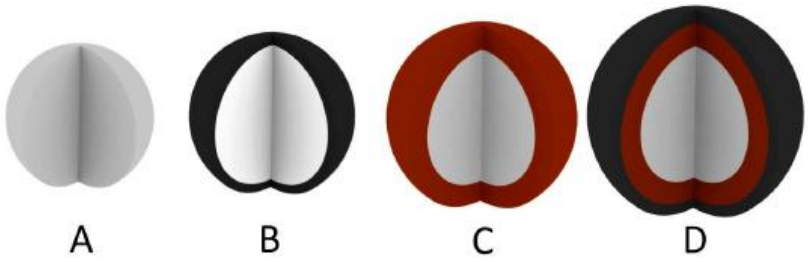
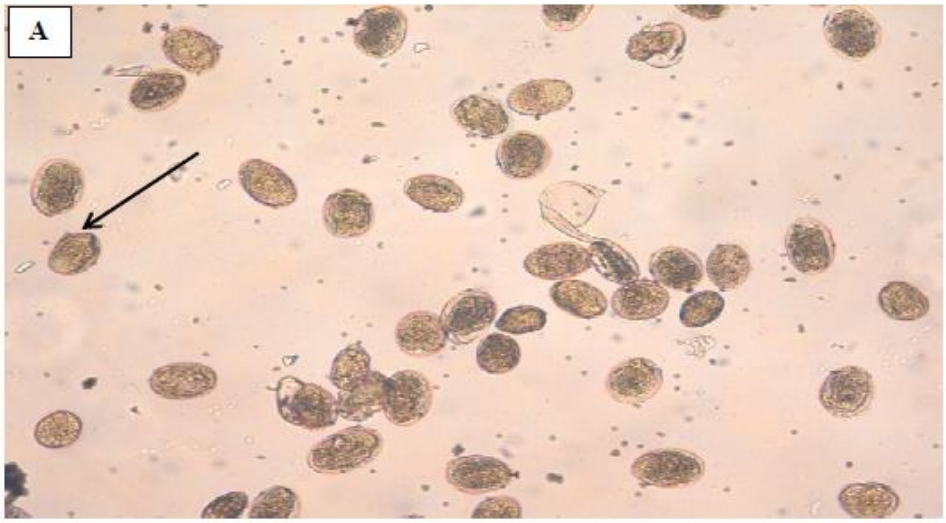


Figure 2.



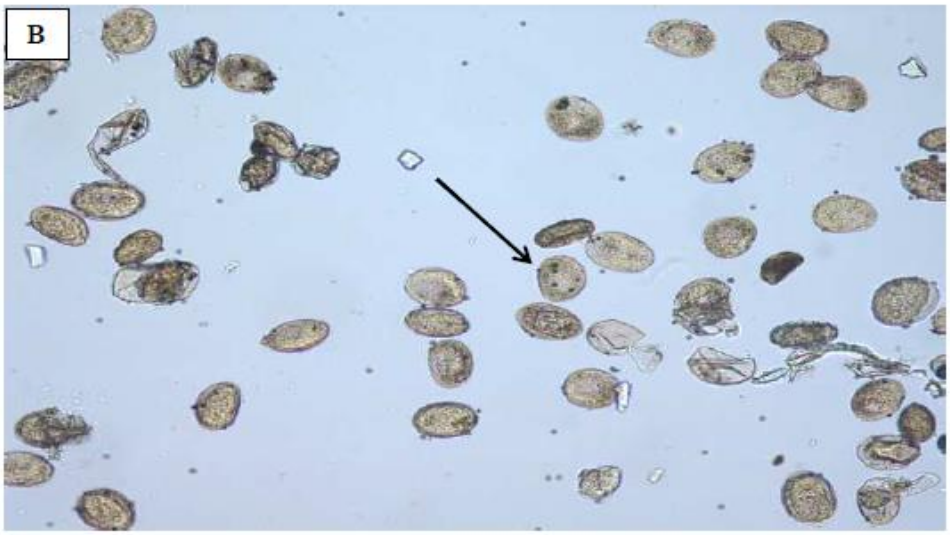
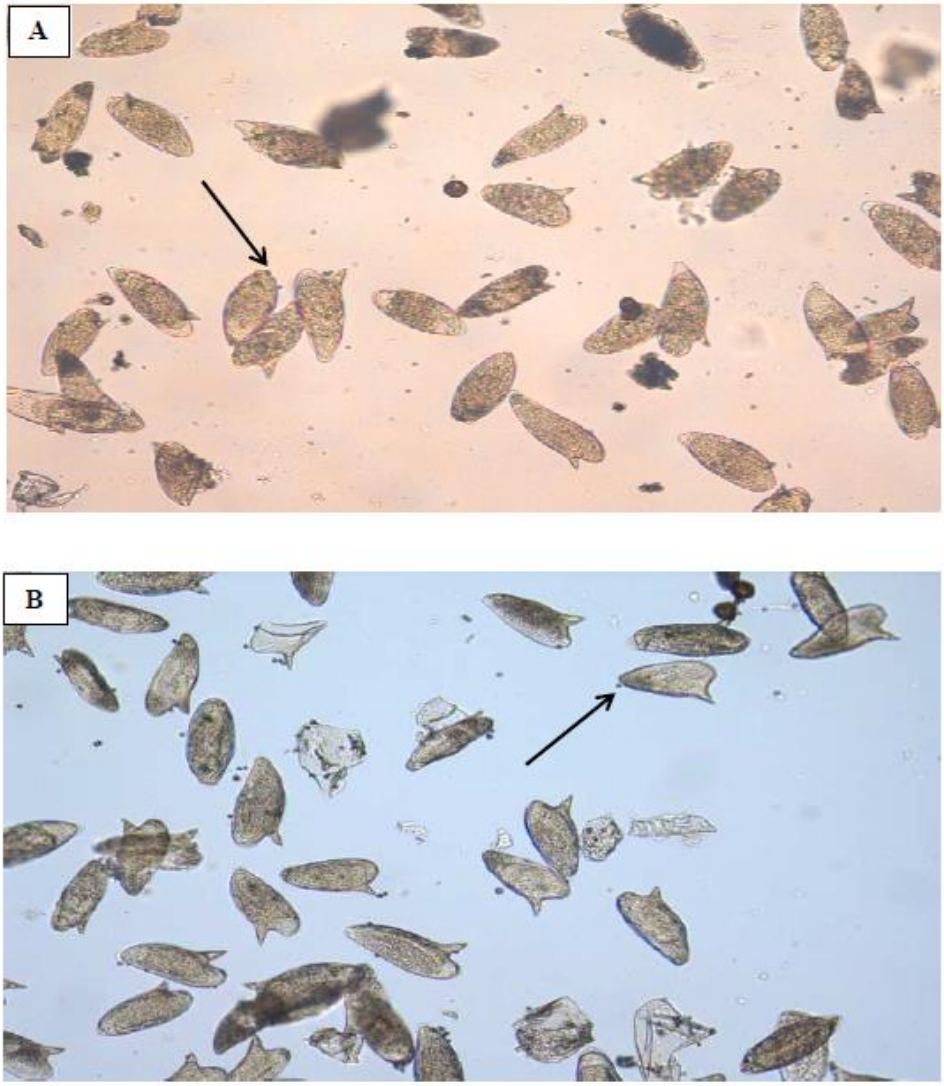
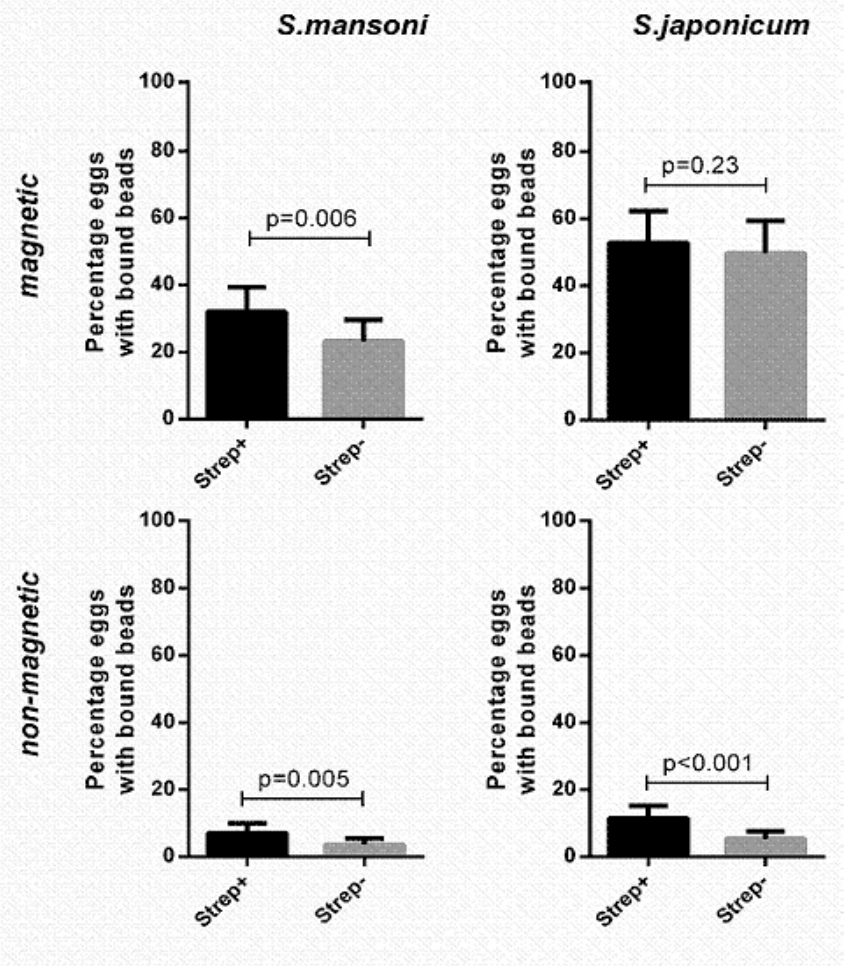


Figure 3.



14 Figure 4.



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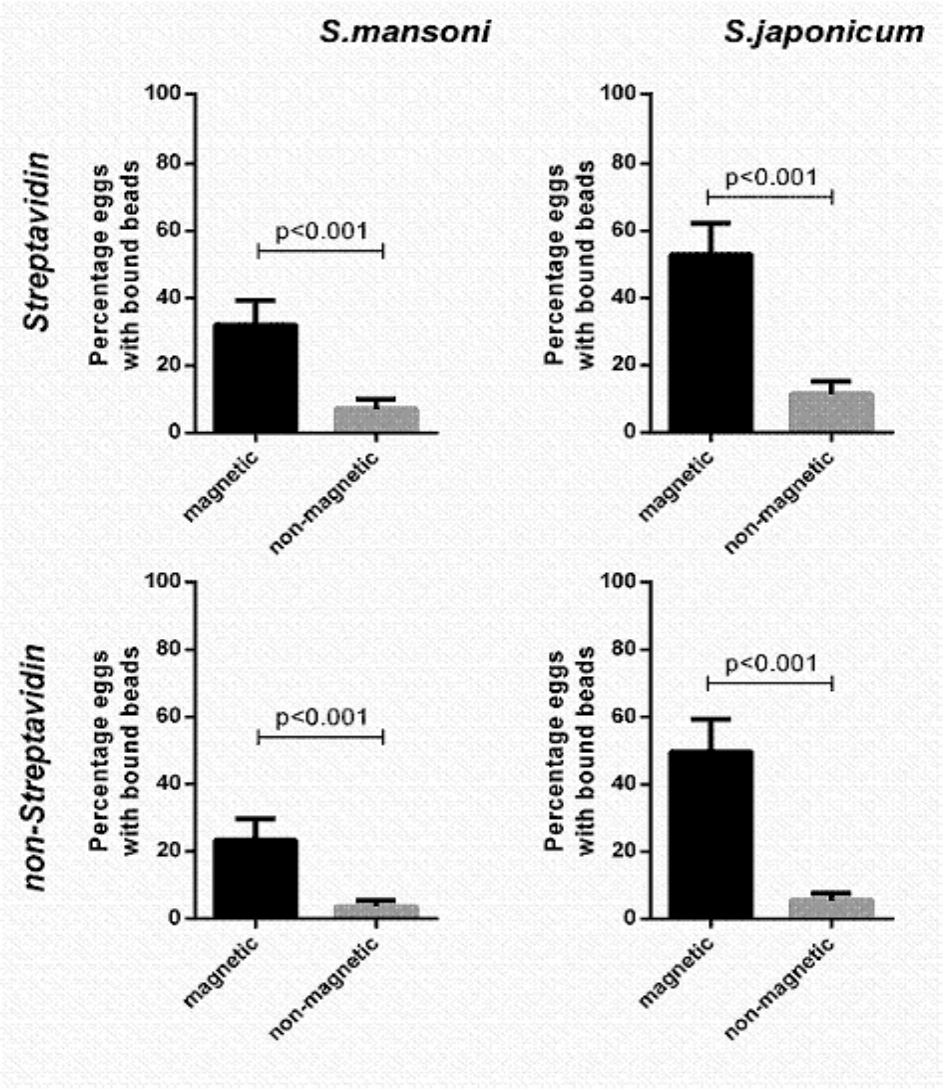
16 Figure 5.



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18 Figure 6.

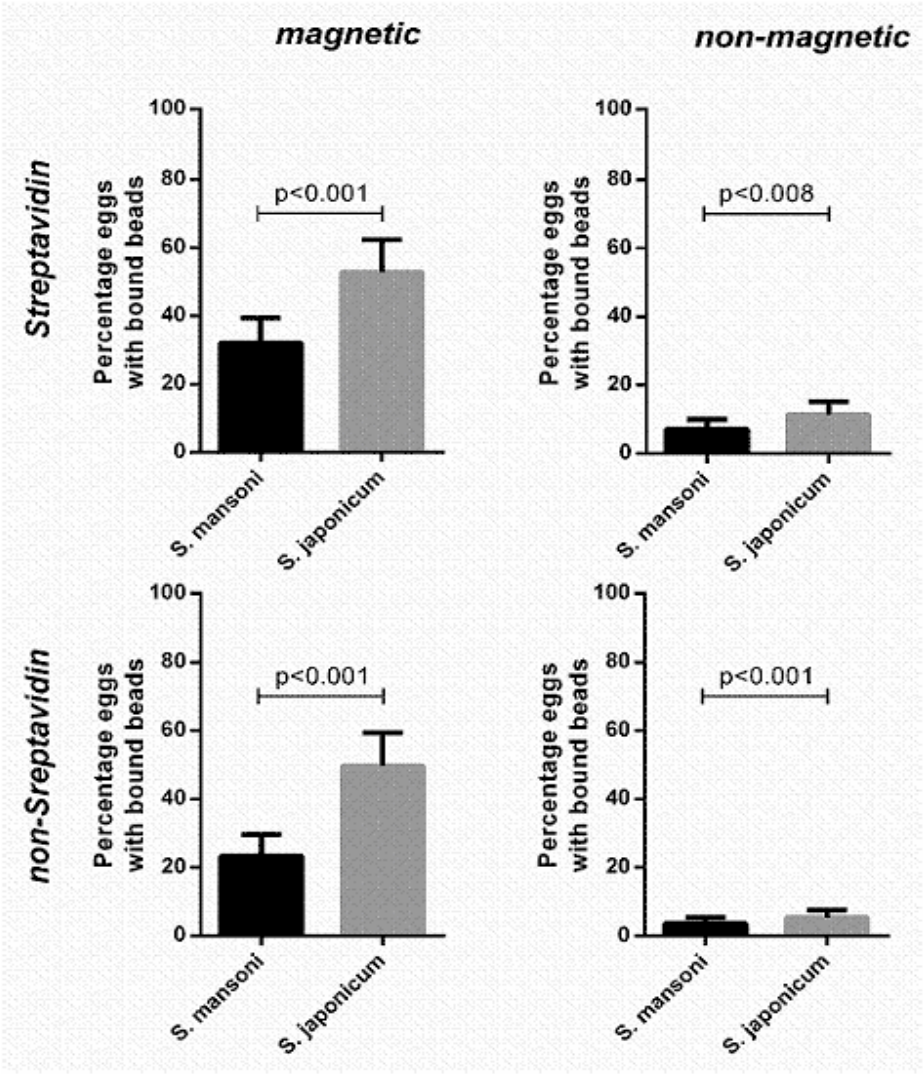

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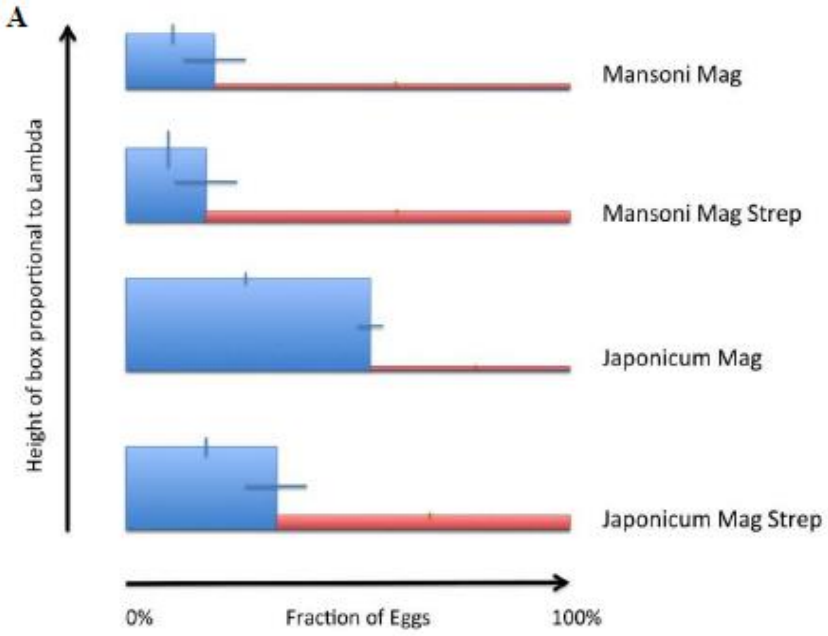
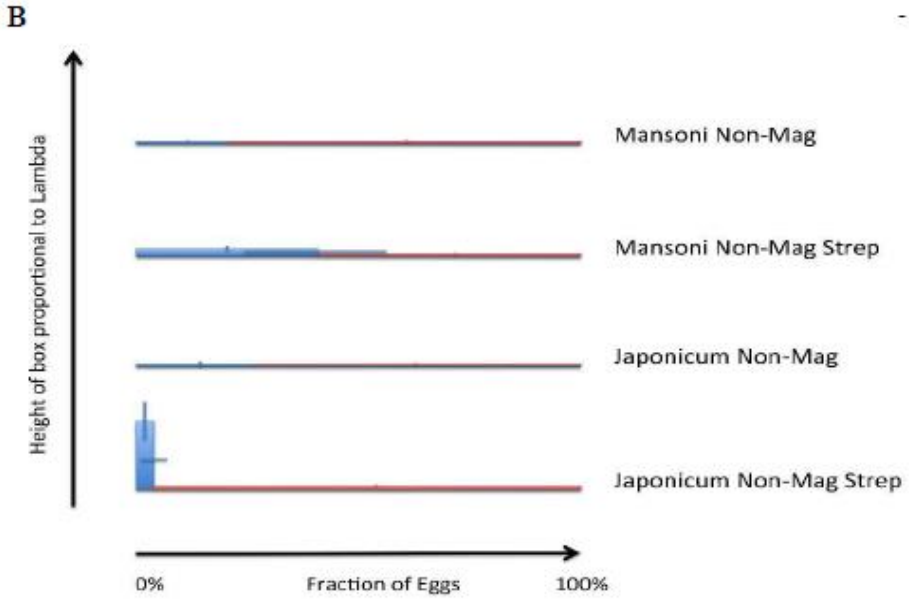
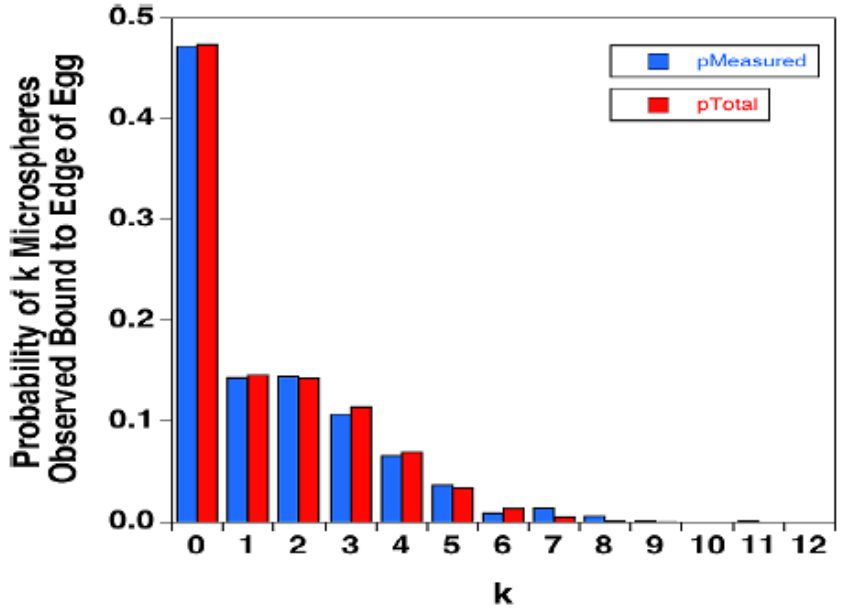
Figure 7.


A
Height of box proportional to Lambda
Mansoni Mag
Mansoni Mag Strep
Japonicum Mag
Japonicum Mag Strep
0%
Fraction of Eggs
100%


B
Height of box proportional to Lambda
Mansoni Non-Mag
Mansoni Non-Mag Strep
Japonicum Non-Mag
Japonicum Non-Mag Strep
0%
Fraction of Eggs
100%

27 Figure 8.

28



4. Discussão

Pouco se sabe sobre estruturas, moléculas e propriedades físicas presentes na casca do ovo de *Schistosoma* sp. que possam mediar sua interação com diferentes superfícies. Análises proteômicas identificaram proteínas na casca do ovo que auxiliam em sua aderência ao endotélio, com conseqüente extravasamento para a corrente sanguínea (Mathieson & Wilson, 2010). Um estudo recente mostrou que o ovo de *Schistosoma* adere-se ao fator de Von Willebrand, ao fibrinogênio e à fibronectina, proteínas do plasma essenciais na formação do coágulo (deWallick et al., 2014). Porém os mecanismos que contribuem para estas e outras interações ainda não foram totalmente explorados.

Os resultados dos trabalhos aqui apresentados mostram estruturas típicas já observadas na superfície dos ovos de *S. mansoni* e *S. japonicum*, porém com alta resolução, evidenciando diferentes características para cada espécie. Na superfície do ovo de *S. mansoni*, estes microespinhos são mais curtos e menos espaçados, apresentando morfologia semelhante entre eles. Já na superfície de *S. japonicum*, estes microfilamentos apresentam-se mais finos e longos, envolvendo todo o ovo. Nos experimentos de incubação dos ovos com as microesferas, foi observado que os ovos de *S. japonicum* possuem uma maior afinidade pelas microesferas do que os ovos de *S. mansoni*, especialmente quando as microesferas são cobertas por material magnético. Esta diferença estrutural e morfológica poderia explicar o porquê dos ovos de *S. japonicum* aderirem mais microesferas do que os ovos de *S. mansoni*, porém não explica o porquê da afinidade pelas microesferas aumentar quando estas são envoltas por material magnético.

Outra questão que permanece não compreendida é o fato de, após cada experimento de incubação, haver microesferas livres no sedimento e ovos sem partículas aderidas, principalmente nos experimentos envolvendo microesferas magnéticas. Estas observações sugerem que há dois grupos distintos dentro de uma mesma espécie de *Schistosoma*, um grupo possuindo grande afinidade por material magnético, e outro grupo sem esta mesma capacidade de interação.

Uma das questões que poderia ajudar na compreensão desta diferença de comportamento seria a presença de estreptavidina na superfície das microesferas. A

estreptavidina é uma proteína que se liga à biotina através de pontes de hidrogênio (Grubmuller et al., 1996). No grupo de ovos que aparentemente não possui afinidade por material magnético, a adição de estreptavidina na superfície das microesferas evidenciou um aumento na afinidade destas pelos ovos. Porém, no grupo de ovos com capacidade de interação com as microesferas paramagnéticas, a adição de estreptavidina ocasionou uma diminuição nesta interação, sugerindo que, se a interação dos ovos pelas microesferas paramagnéticas fosse de origem magnética, a adição de moléculas de estreptavidina aumentaria a distância do material magnético pelo ferro presente na casca do ovo, deixando menos sítios disponíveis para a interação magnética acontecer.

A presença do ferro na casca do ovo de *S. japonicum* já havia sido demonstrada por Jones e colaboradores (2007), porém esta é a primeira caracterização magnética do ovo de *S. mansoni*. As análises por microscopia eletrônica de transmissão revelaram partículas de ferro em poros presentes nas cascas dos ovos. Análises por espectroscopia de emissão atômica revelaram que os ovos de *S. mansoni* e *S. japonicum* contêm, respectivamente, 0.74 mg/g e 1.26 mg/g de ferro. Estes resultados poderiam explicar a afinidade dos ovos pelas esferas paramagnéticas, hipótese que deu origem à este trabalho. Porém, a análise elementar deste material através de espectroscopia por dispersão de energia revelou se tratar de fosfato de ferro, uma forma pobre de ferro (Thomas & George, 2010), sugerindo que o ferro presente não poderia ser o fator determinante da interação.

Uma hipótese levantada ao longo deste estudo foi de que as interações aqui evidenciadas poderiam ser explicadas com base na diferença de forças eletrostáticas na superfície dos ovos e das esferas. Uma ferramenta atualmente muito utilizada para determinar forças de interação entre superfícies é a microscopia de força atômica. Esta técnica consiste na varredura da superfície de uma amostra com a ajuda de uma sonda, para obtenção de informações da topografia estrutural, distribuição de cargas, e forças de van der Waals, eletrostáticas e magnéticas (Meyer, 1992).

Aikawa e colegas (1996), em um estudo sobre malária cerebral utilizando microscopia de força atômica, investigaram o processo de aderência de eritrócitos infectados com o protozoário *Plasmodium falciparum* a células endoteliais, o que ocasiona a obstrução de microvasos cerebrais. Os resultados revelaram que a estrutura responsável pela adesão é positivamente carregada, enquanto que a membrana

plasmática do eritrócito é negativamente carregada, resultando em diferenças de cargas que podem desempenhar um papel importante na citoaderência destes dois tipos de células. Em outro estudo, a microscopia de força atômica foi utilizada para examinar e compreender a ultraestrutura do flagelo do parasito *Tripanossoma cruzi* (Rocha *et al*., 2010), órgão envolvido na aderência do parasito à células epiteliais do sistema gastrointestinal no inseto vetor (Schmidt *et al*, 1998).

O estudo de parasitos através de microscopia eletrônica é um campo em crescimento e deve ser incentivado para a investigação de alvos físicos e moleculares que possam contribuir para a otimização de ferramentas diagnósticas utilizadas na saúde humana. Análises por Microscopia de Força Atômica da superfície dos ovos de *Schistosoma mansoni* e *S. japonicum* para verificação da presença de biotina e investigação das forças de interação, adesão e retração por microesferas com diferentes coberturas e funcionalizações encontram-se em andamento. Resultados preliminares mostram que a amplitude da força de repulsão depende diretamente do comprimento e da largura dos microfilamentos presentes na superfície externa dos ovos de *Schistosoma mansoni* e *S. japonicum*, sendo menos intensa em ovos de *S. mansoni*.

5. Considerações Finais e Perspectivas

O presente estudo foi conduzido com o objetivo de investigar a superfície dos ovos de *Schistosoma mansoni* e *S. japonicum*, e suas propriedades físicas, químicas e magnéticas, e otimizar o método Helmintex®. Após realização de inúmeros experimentos, algumas conclusões relevantes devem ser destacadas:

- As superfícies dos ovos de ambas as espécies de *Schistosoma* são cobertas por uma matriz fibrosa, sendo a de *S. mansoni* coberta por microespinhos mais curtos e menos espaçados que a superfície do ovo de *S. japonicum*;

- As cascas dos ovos de ambas as espécies de *Schistosoma* possuem cerca de 700 nm de espessura com pequenos poros de 50-200 nm de largura, e são parcialmente preenchidas por um material contendo ferro, fósforo e oxigênio;

- Ovos de *S. mansoni* e *S. japonicum* interagem e ligam-se espontaneamente sem a aplicação de um campo magnético;

- A interação entre os ovos e as microesferas paramagnéticas não é específica, ocorrendo com diferentes tipos de microesferas. Além disso, ovos de *S. japonicum* aderem mais microesferas do que os ovos de *S. mansoni*, com ambas as espécies possuindo preferência por material magnético;

- Ovos de ambas as espécies de *Schistosoma* dividem-se em dois grupos distintos, um grupo possuindo alta capacidade de interação com as microesferas, e o outro, baixa força de interação, evidenciando que nem todos os ovos de uma mesma espécie possui capacidade de ligação às microesferas;

- Microesferas não-magnéticas cobertas por estreptavidina aumentam a capacidade de interação pelos ovos tanto de *S. japonicum* quanto de *S. mansoni*. Já a presença da proteína na superfície de microesferas magnéticas diminui essa afinidade aos ovos de ambas as espécies;

- A interação entre os ovos de *S. mansoni* e *S. japonicum* não parece ser de origem magnética, sugerindo ser de origem eletrostática, baseada nas diferenças de cargas existentes nas superfícies dos ovos e das microesferas.

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7. Anexo

7.1.Artigo em elaboração

Investigation of the Surface Schistosome Eggshells Using Atomic Force Microscopy

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Keywords: *Schistosoma mansoni*; *Schistosoma japonicum*; diagnosis; electron microscopy.

Abstract

Background: Schistosomiasis is a public health problem affecting more than 200 million people in Asia, Africa and America, with two main species causing the intestinal infection in humans: *Schistosoma mansoni* and *S. japonicum*. The Helmintex™ diagnostic technique is a very sensitive method for detecting *Schistosoma* eggs based on the interaction of eggs and magnetic particles in a magnetic field. The mechanisms underlying this interaction are unknown. Previous studies show the interaction is unlikely to be purely magnetic. The goal of this work is to investigate the strength of the interaction between schistosomes egg surface and different particles to assess whether the interactions between magnetic beads and eggs are made trough electrostatic forces.

Methods / Principal Findings: Eggs from both species were washed with pure water and dried onto a positively charged slide, followed by a period of rehydration for 3 h. Four different particles (non-magnetic, magnetic, non-magnetic streptavidin-coated and magnetic streptavidin-coated) of around 4 μm diameter were attached at the end of a tipless cantilever using epoxy resin. Cantilevers had a nominal spring constant of 0.32 N/m. The interactions between particles and eggs were investigated using an Asylum Research MFP-3D AFM in force spectroscopy mode. For all experiments, the loading force was constant at 10 nN and the velocity was 500nm/s. Eggs of both schistosomes presented long range repulsive interactions with all four different particles, without adhesion upon retraction for uncoated particles. For magnetic microspheres, the repulsion is less intense, which indicates stronger interaction between the microspheres and the eggs. Once the streptavidin is added to the surface of a microsphere, the interaction greatly increases.

Conclusion / Significance: The result of our work implies that the amplitude of repulsion strongly depends on the length/thickness of the microfilaments present at the external surface of the eggshells, which in. *S. mansoni* eggs is thinner. Our observations also indicate that, the presence of streptavidin increases the interaction between the microspheres and the eggs of both species, especially for non-magnetic particles. The differences of charges present in the surface of the microspheres and the eggs may act as a determinant factor in the binding. Further studies are required in order to understand the properties behind this phenomenon.

Introduction

Schistososmiasis remains endemic in 74 countries and territories, and it is considered the second most spread parasitic disease in the world, affecting more than 200 million, of whom 120 million are symptomatic and 20 million have severe disease (Chitsulo, 2000). While the distribution of schistosomiasis has changed over the last 50 years, and there have been successful control projects, the number of people infected or at risk of infection has not been reduced (Engels et al., 2002).

While the blood feeding activity of schistosome worms and translocation of the eggs through the intestines may be associated with anemia (Friedman *et al*., 2005; Koukounari *et al*., 2006), the main cause of morbidity in chronic schistosomiasis is caused by parasite eggs and the immune responses they evoke. While many eggs successful migrate to the gut lumen and are evacuated with the feces, a small proportion becomes trapped in host tissues especially in the liver. In some cases eggs may also migrate to the lungs or the brain (Wynn et al., 2004). Liver-entrapped eggs will mature and die, inducing a potent granulomatous immune response after embolism, causing intestinal obstruction, liver fibrosis and portal hypertension, which are the primary causes of morbidity, and in some cases, mortality, in infected individuals (Wilson et al., 2007).

Diagnosing schistosomiasis is difficult, especially in areas of low transmission. The gold standard diagnostic method is trough direct visualization of eggs in the faecal sample or in biopsies. The Helmintex™ method, a sensitive technique based on the interaction of *Schistosoma* eggs and magnetic microspheres when applied in a magnetic field has the limit of detection of 1.3 eggs per gram of feces, making this method the most sensitive until today. Preview works have shown that, despite *Schistosoma* eggs contain iron in the eggshells, this iron, a phosphate type of iron, is not strong enough to bind the magnetic microspheres, making this interaction of another nature (Karl et al., 2013).

The Atomic Force Microscopy (AFM) or scanning force microscopy (SFM) is included in a wider group of techniques named scanning probe microscopies (SPM). In all these techniques, the surface of the sample is scanned by a probe, following parallel lines, measuring a local interaction in the near-field region, and registering its value for each position. Thus, the probe is always the basic component of SPM, conditioning the resolution of each microscope (Santos e Castanho, 2004). It has become a powerful tool

in biology that can provide three-dimensional images of surface topography of biological specimens in ambient liquid or gas environments. Unlike other techniques, atomic force microscopy can use samples with just minor preparation, e.g. staining, coating etc., over a large range of temperatures and in repetitive studies (Alonso & Goldmann, 2003). It has been demonstrated its use in several works in the biological field, including the visualization of the flagellar surface of protists (Rocha et al., 2010).

In this work we present evidences of electrostatic interactions between *Schistosoma* eggs and four groups of 4 *micron* microspheres (magnetite-coated; magnetite- and streptavidin-coated; uncoated and streptavidin-coated), which could explain the nature of the interaction of the Helmintex™ method.

Materials and Methods

Maintenance of the parasites life cycles

Schistosoma mansoni and *S. japonicum* were maintained at the QIMR Berghofer Medical Research Institute, trough passages in *Swiss* mice and *Biomphalaria glabrata* snails for *S. mansoni*, and *Oncomelania hupensis hupensis* snails collected in Anhui Province (China) for *S. japonicum*. The use of animals was approved by the Animal Ethics Committee of the Queensland Institute of Medical Research (Project P1289). The experiments were conducted in the Australian National Fabrication Facility (ANFF) of the Australian Institute for Bioengineering and Nanotechnology, Brisbane, Australia.

Acquisition of S. mansoni and S. japonicum eggs

Mice infected with either *S. mansoni* and *S. japonicum* were euthanased at approximately 42 days post-infection and the livers were removed for digestion with Collagenase B in phosphate buffered saline (PBS) overnight at 37 °C. On the following day, samples were sieved for isolation of the eggs and purified using Percoll density gradient centrifugation as described by Dalton et al., in 1997. The eggs were stored in PBS at -80 °C until use.

Preparation of the eggs

Eggs from *S. mansoni* and *S. japonicum* were washed with distilled water and left to dry onto a positively charged slide for 24 hours, followed by a period of rehydration for 3 h. Four different 4 µm size microspheres (magnetite-coated; magnetite- and streptavidin-coated; uncoated and streptavidin-coated) were attached, separately, at the end of a tipless cantilever using epoxy resin. The atomic force microscope was conducted in the tapping mode, and a graphic was created for each approach/retraction measurement.

Investigation of biotin molecules on the surface of Schistosoma eggs

A partir das hipóteses levantadas nos artigos anteriores, de que a superfície do ovo de *Schistosoma* possui biotina ou molécula similar, e pela tecnologia hoje disponível no laboratório de Microscopia Eletrônica da PUCRS, iremos realizar o mesmo ensaio de atração e retração, porém utilizando moléculas de estreptavidina acopladas à sonda, conforme Lee e colaboradores (1994).

Results

(Texto em preparação. Algumas figuras já estão sendo apresentadas logo após a bibliografia.)

Discussion

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Figures:

Figure 1. *Schistosoma japonicum* egg. Force versus piezo movement in approach. Red line. Non-magnetic microspheres. Blue line. Magnetic microspheres. Green line. Non-magnetic streptavidin-coated microspheres. Black line. Magnetic streptavidin-coated microspheres. Applied force of 10nN and velocity of 500 nm/s.

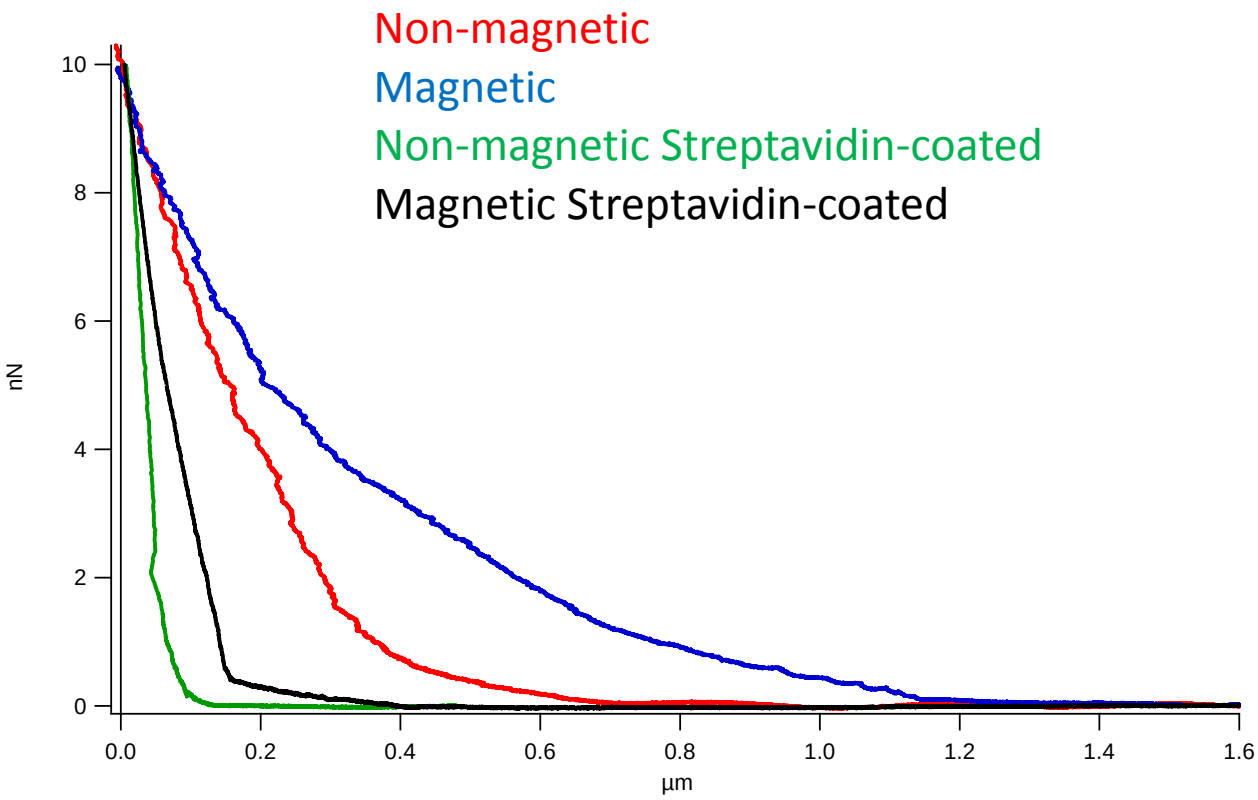

Figure 2. *Schistosoma japonicum* egg. Force versus piezo movement in retraction. Red line. Non-magnetic microspheres. Blue line. Magnetic microspheres. Green line. Non-magnetic streptavidin-coated microspheres. Black line. Magnetic streptavidin-coated microspheres. Applied force of 10nN and velocity of 500 nm/s.

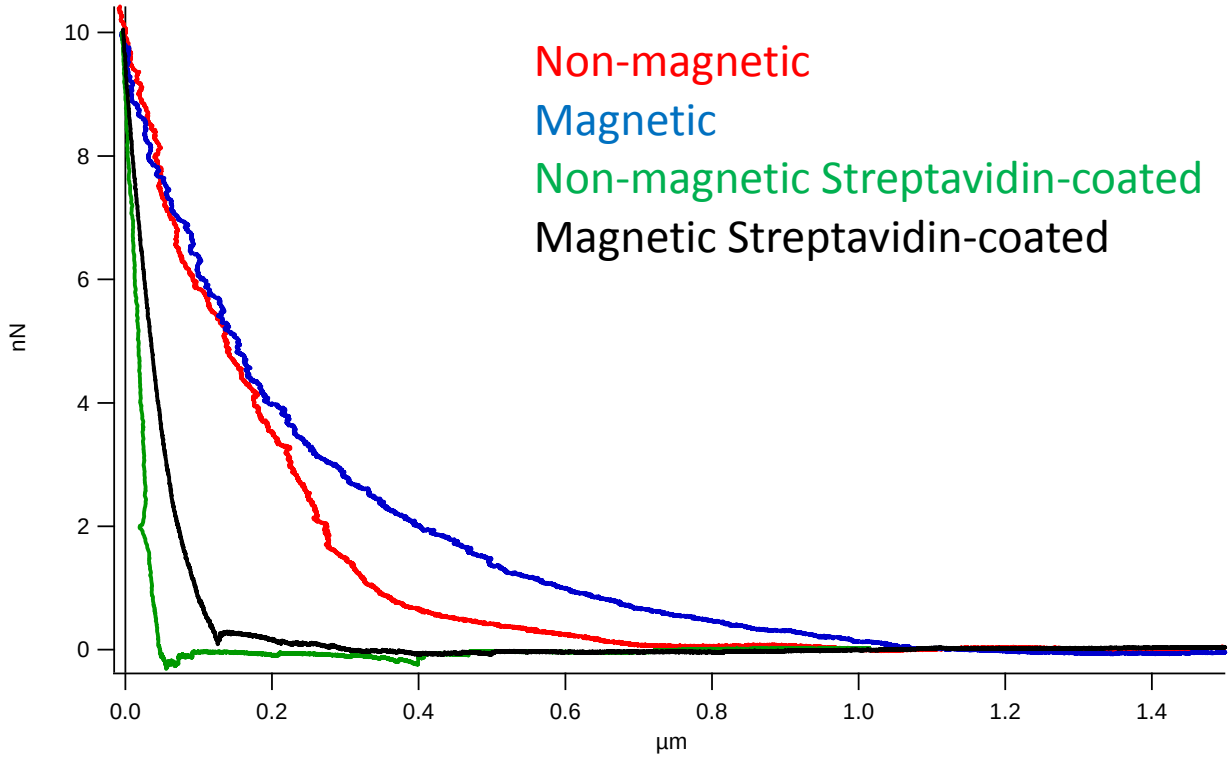

Figure 3. *Schistosoma mansoni* egg. Force versus piezo movement in approach. Red line. Non-magnetic microspheres. Blue line. Magnetic microspheres. Green line. Non-magnetic streptavidin-coated microspheres. Black line. Magnetic streptavidin-coated microspheres. Applied force of 10nN and velocity of 500 nm/s.

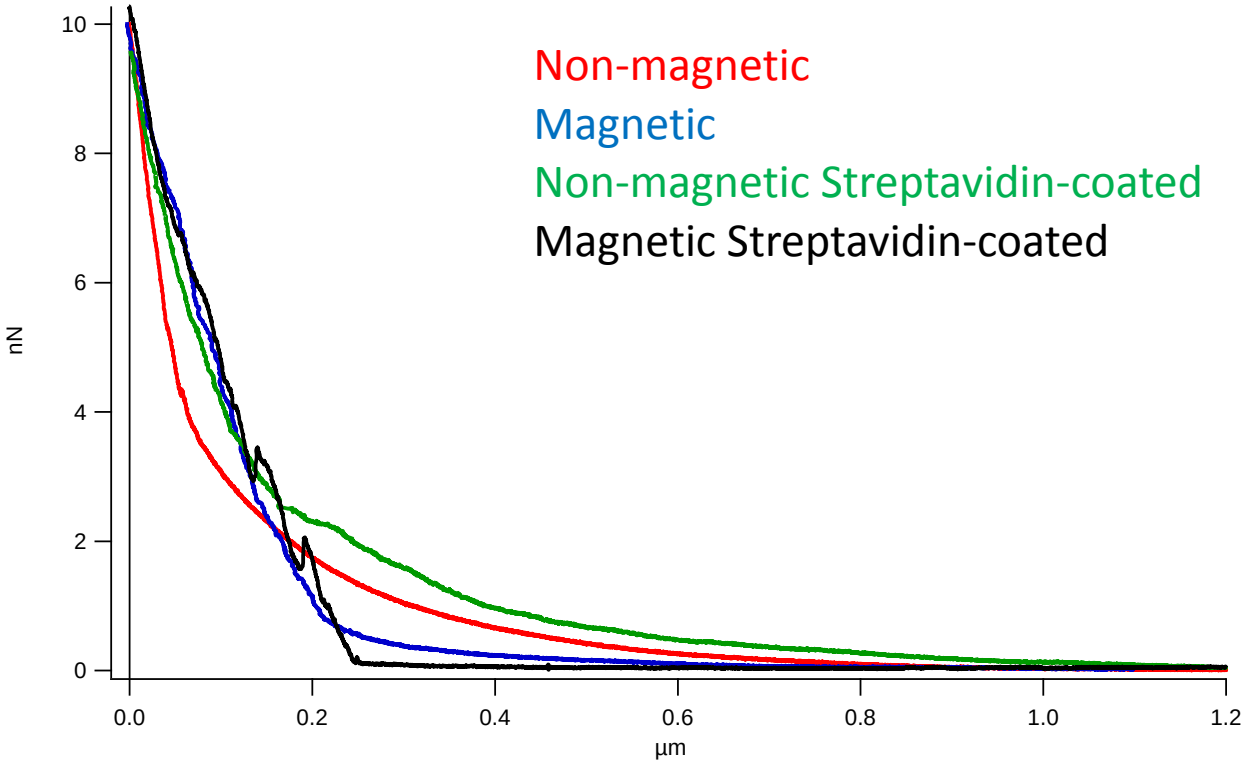

Figure 4. *Schistosoma mansoni* egg. Force versus piezo movement in retraction. Red line. Non-magnetic microspheres. Blue line. Magnetic microspheres. Green line. Non-magnetic streptavidin-coated microspheres. Black line. Magnetic streptavidin-coated microspheres. Applied force of 10nN and velocity of 500 nm/s.

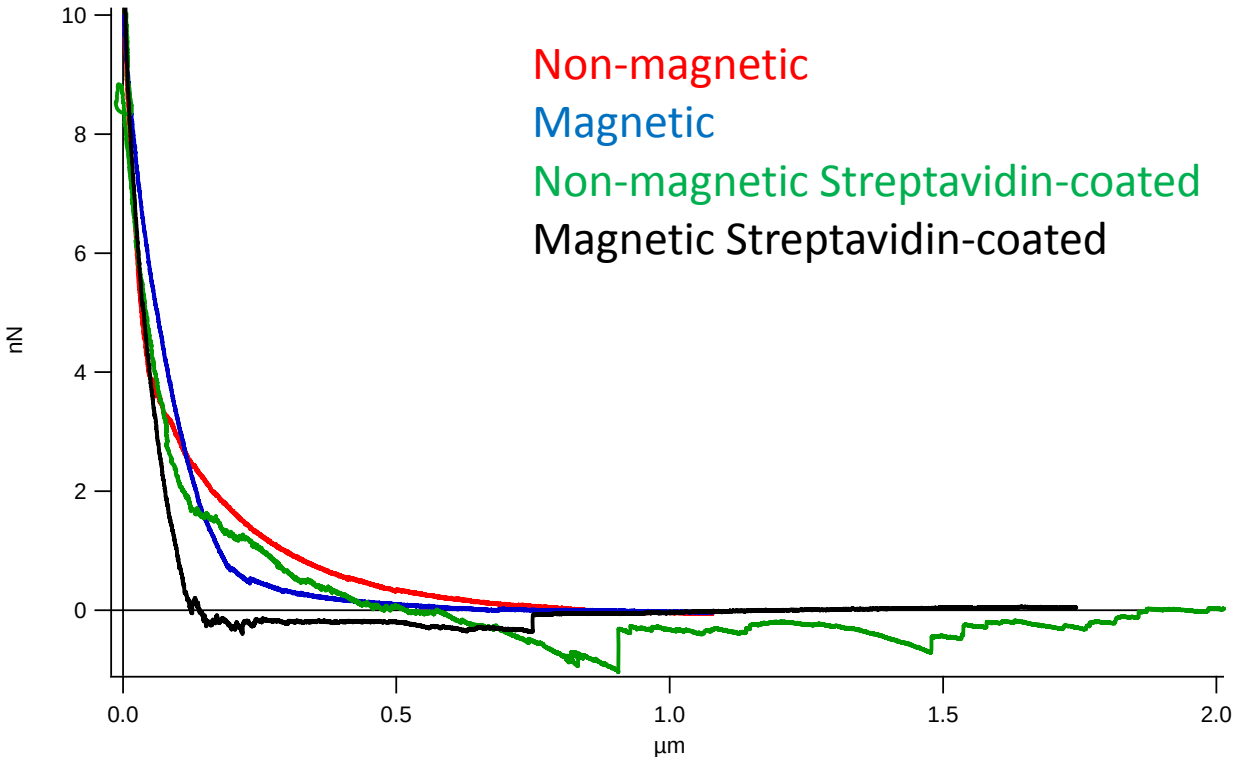

Figure 5. Example of a microsphere added to the cantilever.