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Proteomic and immunomic analysis of *Schistosoma mekongi* egg proteins

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ABSTRACT

Schistosomiasis remains a global health problem. In the Mekong river basin, approximately 80,000 people are at risk of infection by *Schistosoma mekongi*. The parasite's eggs become entrapped in the host's organs and induce massive inflammation, contributing to the pathogenesis of schistosomiasis. In addition, egg antigens are important in circumoval precipitin tests (COPTs) and other diagnostic techniques. Little is known regarding the egg proteins of *S. mekongi*, and so we applied immunoblotting and mass spectrometry-based proteomic approaches to study these proteins and their antigenicity. A total of 360 unique proteins were identified in *S. mekongi* eggs using proteomic analyses. The major protein components of *S. mekongi* eggs were classified into several groups by functions, including proteins of unknown function, structural proteins, and regulators of transcription and translation. The most abundant proteins in *S. mekongi* eggs were antioxidant proteins, potentially reflecting the need to neutralize reactive oxidative species released from host immune cells. Immunomic analyses revealed that only DNA replication factor Cdt1 and heat shock protein 70 overlap between the proteins recognized by sera of infected mice and humans, illustrating the challenges of knowledge transfer from animal models to human patients. Forty-one immunoreactive protein bands were recognized by either mouse or patient sera. Phosphoglycerate kinase, fructose-1,6-bisphosphate aldolase and elongation factor 1 appeared to be interesting immunogens of *S. mekongi* eggs as these proteins were recognized by polyclonal IgMs and IgGs in patient sera. Our findings provide new information on the protein composition of *S. mekongi* eggs as well as the beginnings of a *S. mekongi* immunogen dataset. These data may help us better understand the pathology of schistosomiasis as well as natural antibody responses against *S. mekongi* egg proteins, both of which may be useful in including *S. mekongi* to other schistosoma diagnostic, vaccine and immunotherapy development.

1. Introduction

Schistosomiasis is a tropical disease resulting from infection by trematodes of the genus *Schistosoma*. Schistosomiasis is prevalent and an important public health and societal concern in developing countries. Transmission of human schistosome parasites (major species: *S. mansoni*, *S. japonicum* and *S. haematobium)* has been reported in at least 78 countries (Vic-Dupont et al., 1957). *S. mekongi* was first identified in 1978 and shares many features with its close relative *S. japonicum* (Voge et al., 1978). *S. mekongi* is endemic to one province of the Lao People's Democratic Republic (Lao PDR) and two provinces of Cambodia (Sayasone et al., 2012). In Lao PDR, co-infection by *Opisthorchis viverrini* and *S. mekongi* was found to contribute to hepatobiliary morbidity (Sayasone et al., 2011). The life cycle of *S. mekongi* involves humans and other animals, including dogs, pigs and possibly rats (Kitikoon et al., 1973). The *Neotricula aperta* snail serves as an intermediate host, and its habitat is found in rocky bank areas of the Mekong River. Parasite cercariae emerge from infected snails during the daytime and live under the water surface. Humans and animals are infected via skin penetration after contact with contaminated waters (Urbani et al., 2002). In Lao PDR, the national WHO control program is implemented through mass drug administration and through information, education and communication campaigns in high-risk provinces (WHO Expert Committee, 2002; Phongluxa et al., 2015). However, the prevalence of *S. mekongi* infection remains high in many regions. The disease affects travelers as well as locals: in 2011, *S. mekongi* was

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discovered unexpectedly from colon biopsies of a traveler five years after a visit to Lao PDR (Campa et al., 2014).

All available evidence suggests that schistosome eggs, not adult worms, are responsible for the morbidity of schistosomiasis. Most parasite eggs are not excreted and become permanently trapped in the intestines or liver (Burke et al., 2009), directly mediating the symptoms of schistosomiasis. The eggs induce granulomas, causing chronic inflammation that leads to the disease manifestations of schistosomiasis. Although one *S. mekongi* worm produces ~3000 eggs per day, schistosome eggs are only sporadically observed in stools. The Kato-Katz technique is considered as the most widely used method for diagnosis of schistosomiasis but lacks sensitivity in low- and mid-intensity infections (Bergquist et al., 2009). Immunodiagnostics are considered to be more sensitive than conventional methods (Alarcón de Noya et al., 2007). However, immunoassays using crude antigens from *S. japonicum* or *S. mansoni* are of limited utility given their inability to discriminate between schistosome parasites and between active and past infections (Alarcon de Noya et al., 1996). Currently, no *S. mekongi* antigen dataset is available.

In this study, the proteome of *S. mekongi* eggs was analyzed using mass spectrometry (LC-MS/MS). In addition, two-dimensional gel electrophoresis (2DE) and immunoblotting were used to identify immunogens of *S. mekongi* eggs using sera from *S. mekongi*-infected mice and humans as probes. These results deepen our understanding of the complete schistosome proteome and provide a foundation for further investigations into the molecular basis of schistosome modulation of host immunity. Moreover, our results provide an antigen dataset, which may be useful in including *S. mekongi* with schistosoma diagnostic, vaccine and immunotherapy development. This increases the possibility to control Mekong schistosomiasis.

2. Material and methods

2.1. Preparation of mouse and human sera

Protocols for mouse experiments were approved by the Animal Care and Use Committee, Faculty of Tropical Medicine, Mahidol University (No. 004–2016). Six ICR mice were infected with cercariae following abdomen exposure using a hairloop. Sera were collected pre- and post-infection at 2nd, 4th and 8th weeks.

Experiments using human sera were approved by the Ethics Committee of the Faculty of Tropical Medicine, Mahidol University (MUTM 2017-067-01). Two patient sera of *S. mekongi* COPT-positive but negative infection with *Trichinella spiralis*, *Gnathostoma spinigerum*, *Angiostrongylus cantonensis*, *Echinostoma spp.*, *Toxocara spp.*, *Wuchereria bancrofti*, *Brugia malayi, Paragonimus spp.* and sparganum infections were used in immunomic experiments. In addition, five healthy sera were served as controlled.

2.2. Preparation of egg samples

S. mekongi cercariae emerged from *Neotricula aperta* snails 4 weeks after exposure to miracidia. ICR mice (N = 6) were infected with cercariae following abdomen exposure using a hairloop. Sera were collected pre- and post-infection (weeks 2, 4 and 8). After 6–8 weeks, infected mice were sacrificed, dissected, and the livers and intestines were homogenized in normal saline solution (NSS). Homogenates were sequentially passed through 80, 120, 160, and 260 mesh stainless steel sieves to separate *S. mekongi* eggs from liver and intestinal tissue. The eggs were washed three times with NSS and stored in liquid nitrogen until used.

2.3. Protein extraction

S. mekongi eggs were washed with phosphate-buffered saline (PBS) and centrifuged at 13,000 × g for 2 min. After discarding the supernatant, liquid nitrogen was added and the eggs were crushed manually with a mortar and pestle on ice. After adding 0.5 mL of lysis buffer containing 1% (w/v) sodium dodecyl sulfate (SDS; Sigma Aldrich, USA), 1% (v/v) Triton X-100 (OmniPur, USA), 0.5% (w/v) NaCl (Merck, USA) and 0.1% protease inhibitor cocktail (Sigma Aldrich, USA), egg samples were sonicated on ice for 50 s with an interval of 2 min and centrifuged at 10,000 × g for 10 min at 4 °C to remove cell debris. The protein concentration of the egg lysate was measured using Bradford reagent (Bio-Rad, USA).

2.4. Gel electrophoresis

For SDS polyacrylamide gel electrophoresis (SDS-PAGE), egg lysate (30 μg) was mixed with Laemmli buffer (Bio-Rad Laboratories, USA) and denatured by heating at 65 °C for 10 min. The protein sample was loaded onto a 5% (w/v) acrylamide stacking gel and separated on a 12% acrylamide resolving gel (Bio-Rad Laboratories, USA). The separation was performed at 120 V until the bromophenol blue dye front reached the bottom of the gel. The gel was stained using Coomassie Brilliant Blue G-250. Three biological replications were performed. Whole gel lanes were excised (15 slices per gel) and subjected to tryptic digestion prior to mass spectrometry (MS) analyses.

For 2DE, a non-linear immobilized pH gradient (IPG) strip (pH 3–10; Amersham Bioscience, USA) was rehydrated overnight in IPG sample buffer containing 8 M urea, 2% (w/v) 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), 15 mM dithiothreitol (DTT), and 0.5% IPG sample buffer. Isoelectric focusing was performed using an Ettan IPGphorII instrument (Amersham Bioscience, USA) using the following parameters: 30 V for 14 h, 200 V for 1 h, 500 V for 1 h, 1000 V for 1 h, 3500 V for 1 h, and 8000 V for 18 h. After focusing, the strips were equilibrated with DTT for 15 min and with iodoacetamine for 15 min, then loaded onto a 12% SDS-PAGE gel. Second dimension separation was performed at 120 V until the bromophenol blue dye front reached the bottom of the gel. All three 2DE gels were stained with silver and the immunoreactive spots in these gels were excised and pooled for mass spectrometric analysis. Other eight 2DE were used for immunoblotting.

2.5. Immunoblotting

Proteins from eight 2DE were transferred onto a nitrocellulose membrane, individually. The membranes were blocked using 5% (w/v) non-fat milk in PBS for 2 h at room temperature, then rinsed with PBS containing 0.05% (v/v) Tween-20. Pooled sera from *S. mekongi*-infected and uninfected mice, diluted 1:200 in PBS containing 1% non-fat milk, were added to the membranes and incubated overnight at 4 °C (2 membranes for uninfected mouse serum and another two membranes for infected mouse serum). On the other hands, pooled sera from *S. mekongi*-infected and uninfected human with the same dilution were added to the membranes (2 membranes for uninfected human serum and another two membranes for infected human serum). After washing, horseradish peroxidase-conjugated goat anti-mouse IgM and IgG secondary antibodies were added and incubated for 1 h. Immunogen spots were visualized by detection of peroxidase activity using the Ultra TMB-Blotting Solution (ThermoFisher Scientific, UK). Immunoreactive protein spots were excised from silver-stained 2DE gels and subjected to in-gel digestion.

2.6. In-gel tryptic digestion

Gel slices from SDS-PAGE were destained using 50% acetonitrile in 50 mM ammonium bicarbonate and gel slices from 2DE were destained using 30 mM potassium ferricyanide and 100 mM sodium thiosulfate until until colorless. Gel pieces were incubated in 4 mM DTT at 60 °C for 15 min. Proteins were alkylated by adding 250 mM iodoacetamide and incubating at room temperature in the dark for 30 min. The reaction