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Research Paper

A Parallel Comparison of Antigen Candidates for Development of an Optimized Serological Diagnosis of Schistosomiasis Japonica in the Philippines

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ABSTRACT

Schistosoma japonicum is stubbornly persistent in China and the Philippines. Fast and accurate diagnostic tools are required to monitor effective control measures against schistosomiasis japonica. Promising antigen candidates for the serological diagnosis of schistosomiasis japonica have generally been identified from the Chinese strain of *S. japonicum*. However, the Chinese (SjC) and Philippine (SjP) strains of *S. japonicum* express a number of clear phenotypic differences, including aspects of host immune responses. This feature thereby emphasized the requirement to determine whether antigens identified as having diagnostic value for SjC infection are also suitable for the diagnosis of SjP infection. In the current study, 10 antigens were selected for comparison of diagnostic performance of the SjP infection using ELISA. On testing of sera from 180 subjects in the Philippines, SjSAP4 exhibited the best diagnostic performance with 94.03% sensitivity and 98.33% specificity using an optimized serum dilution. In another large scale testing with 412 serum samples, a combination (SjSAP4 + Sj23-LHD (large hydrophilic domain)) provided the best diagnostic outcome with 87.04% sensitivity and 96.67% specificity. This combination could be used in future for serological diagnosis of schistosomiasis in the Philippines, thereby representing an important component for monitoring integrated control measures.

1. Introduction

Schistosomiasis japonica is a disease of poverty and remains as a public health issue in China, the Philippines and Indonesia. In China, the epidemiology of schistosomiasis is changing due to extensive integrated control efforts (Collins et al., 2012; Li et al., 2014b). The estimated number of infected people dropped from ~840,000 in 2004 to 185,000 in 2013 (Xu et al., 2016). A number of endemic areas are nearing schistosomiasis transmission interruption (Li et al., 2014b; Xu et al., 2016). In contrast, as of 2010, an estimated 580,000 individuals were reported infected in the Philippines (Rollinson et al., 2013), with very high prevalences recently reported in a number of endemic provinces (Olveda et al., 2016). For China, the need for improved diagnostic tools is urgently required for effective surveillance and determination of elimination; for the Philippines, it is imperative to develop affordable and accurate field diagnostic tools for schistosomiasis control.

There are four major types of methods available for the diagnosis of schistosomiasis: parasitological detection (e.g. the Kato-Katz (KK) method), antibody-detection (AbD), antigen-detection (AgD) and circulating nucleic acids (DNA and RNA) detection (CNAD) (Cai et al., 2016a; Weerakoon et al., 2015). The KK method shows low sensitivity, while AbD based on crude extracted antigens, such as soluble egg antigen (SEA), exhibits cross-reactivity with other helminth infections, which is particularly relevant in many schistosomiasis-endemic areas. There has been considerable focus on the detection of the presence of schistosome ova, or SEA-specific antibodies but these approaches are limited for early diagnosis. More sensitive methods based on PCR technology, including qPCR (Gordon et al., 2015), droplet digital PCR (Weerakoon et al., 2016), and LAMP (Wang et al., 2011a; Xu et al., 2015) have been developed as diagnostic procedures for schistosomiasis. However, the cost of these CNAD tools may limit their practical application for large-scale surveillance of schistosomiasis. Of the available methods, ELISA-based AgD detection has several advantages, such as being affordable, easy-to-operate, there are no requirements for advanced equipment, there is less chance for cross-contamination as reported with PCR methods, such as nested-PCR (Li et al., 2014a) and

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LAMP (Karthik et al., 2014), and provides a balance of sensitivity and specificity once an appropriate antigen is identified and employed.

In this post-genomics era, an increasing number of high-throughput immunological studies have been carried out on schistosomes. These reports have identified a panel of tegumental and excretory-secretory antigens as potential diagnostic targets with high levels of sensitivity and specificity (Chen et al., 2014; Lu et al., 2012; McWilliam et al., 2014; Sangfuang et al., 2016; Xu et al., 2014). For example, Xu et al. identified a saposin-like protein, SjSP-13, as a potential diagnostic candidate based on a genome-wide screening of secretory proteins (Xu et al., 2014), whereas Liu et al. identified other saposin members showing better diagnostic performance than SjSP-13 (Liu et al., 2016). Since different groups employ different human cohorts with variable infection intensity and use different experimental assay conditions, a comparative study was required to compare the clinical diagnostic performance of different candidate *S. japonicum* antigens.

The majority of studies undertaken on the serological diagnosis of schistosomiasis japonica have been carried out using the Chinese strain of *S. japonicum*. However, the Chinese (SjC) and Philippine (SjP) strains of *S. japonicum* show clearly different phenotypes in terms of virulence, fecundity, pathology, drug sensitivity and immunology (Hope et al., 1996; Moertel et al., 2006; Weerakoon et al., 2017). Here, we carried out a comparative study with ten antigens to validate the most promising candidate for diagnosis of schistosomiasis in the Philippines. An optimized formula for serological diagnosis of SjP infection was further suggested based on this parallel comparative study.

2. Materials and Methods

2.1. Ethical Statement

All animal work was conducted according to the Australian Code for the Care and Use of Animals for Scientific Purposes (8th edition) and with the approval of the QIMR Berghofer Medical Research Institute Animal Ethics Committee (Ethics Approval: Project P288). Serum samples from the study participants in the Philippines were collected with informed written consent, and ethical approval was provided by the Institutional Review Board of the Research Institute for Tropical Medicine, Department of Health, Manila, the Philippines (Institutional Review Board Numbers 2012-13-0 and 2015-12) and the Human Research Ethics Committee, QIMR Berghofer Medical Research Institute, Brisbane, Australia (Ethics Approval: Project P524). All serum samples from healthy humans were obtained with informed written consent, and the protocol was approved by the ethics committee of the Institute of Pathogen Biology, Chinese Academy of Medical Sciences (Beijing, China).

2.2. Mice and Parasites

Eight-week-old female BALB/c mice were percutaneously infected with 14 *S. japonicum* cercariae (Chinese mainland strain, Anhui population) or 25 *S. japonicum* cercariae (Philippine strain, Sorsogon population). Blood samples were taken from animals at 4, 6, 7, 9 and 11 weeks post infection (p.i.). Blood samples from five naive mice were used as controls. The liver tissues were collected at 11 weeks post infection. Eggs per gram of liver were calculated as a measure of hepatic egg burden and general infection level, as described (Cai et al., 2015). Six Swiss mice were infected with approximately 25 *S. japonicum* cercariae (Philippine strain). After 6 weeks, these mice were orally administered 150, 200, 250, 300 and 350 mg/kg praziquantel prepared in 2.5% (v/v) Cremophor EL (Sigma, USA) for 5 consecutive days (Chuah et al., 2016). Serum samples were collected before infection and at 2, 4 and 6 weeks post-infection as well as at 1, 2 3, 4, 5, 6 and 7 months after chemotherapy.

2.3. Gene Expression Analysis

A next-generation oligonucleotide microarray was used to determine the expression patterns of the obtained selected target proteins in four developmental stages of *S. japonicum* (eggs, cercariae, hepatic schistosomula and adult worms). The design and construction of the microarray, as well as the hybridization procedures and feature extraction, have been reported previously (Cai et al., 2016b; Cai et al., 2017). Raw data and normalized gene level data from the array have been deposited in the public database Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) under accession numbers for the platform GPL18617, and series GSE57143. The expression pattern of the genes encoding target antigen candidates in the four developmental stages was extracted from the above dataset. A heatmap was generated based on the relative signal intensities of forward probes against the egg stage using HemI 1.0 software.

2.4. Cloning, Expression and Purification of Recombinant Proteins

Primers were designed to amplify a specific region of the target proteins (Supplementary Table 1). The DNA fragments were amplified by PCR from cDNA isolated from adult worms of *S. japonicum* (Philippine strain). After digestion with restriction enzymes, DNA fragments were cloned into the pET-28a vector. Recombinant plasmids were confirmed by sequencing and transformed into *E. coli* BL21 (DE3). Expression of the recombinant proteins was induced by 0.5 mM IPTG. Recombinant proteins were purified under native or denaturing conditions using Ni-NTA agarose (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Purified recombinant proteins were analyzed by 12% (w/v) SDS-PAGE.

2.5. Western Blotting

Purified recombinant protein samples were loaded onto 12% (w/v) SDS-PAGE and transferred to 0.2-μm PVDF membranes at 100 V for 10 min and then at 100 mA for 1 h using a wet western blotting system. After blocking with 5% (v/v) non-fat milk in PBST for 90 min, the membrane was incubated with a mouse anti-His monoclonal antibody (Sigma-Aldrich Co, MO, USA) at 4 °C overnight. After washing, the membrane was incubated with an HRP-conjugated goat anti-mouse IgG (H + L) antibody (ThermoFisher Scientific, MA, USA) for 1 h at room temperature. SuperSignal West Pico Chemiluminescent Substrate (ThermoFisher Scientific, MA, USA) was used to detect signals.

2.6. Human Serum Samples

Serum samples from schistosomiasis patients were obtained from Northern Samar, the Philippines. For the first serum cohort ($n = 180$), 67 and 113 patients were confirmed as positively and negatively infected, respectively, in 2012 by examining eggs in stool samples using the Kato-Katz method. The second serum batch ($n = 412$) were obtained from the same endemic area in 2015, two years after a program of mass drug (i.e., 40 mg/kg praziquantel) administration. Of these, 108 and 304 patients were confirmed as positive and negative infection, respectively, using the Kato-Katz method (Olveda et al., 2017). Serum samples of healthy individuals were obtained from Heilongjiang Province, a non-endemic area for schistosomiasis in China.

2.7. Evaluation of Diagnostic Candidates for Schistosomiasis Japonica by ELISA

Recombinant proteins were quantified by the bicinchoninic acid assay (BCA assay) (ThermoFisher Scientific, MA, USA). For determining IgG levels induced in SjC or SjP-infected BALB/c mice, all recombinant proteins were diluted to a final concentration of 1 μg/mL with coating buffer overnight at 4 °C with 100 μL added per well. All wells were

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