Serum Carbonic Anhydrase 1 is a Biomarker for Diagnosis of Human *Schistosoma mansoni* Infection

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Abstract. *Schistosoma mansoni* is a major public health threat in many parts of the world. The current diagnostic tests for schistosomiasis are suboptimal, particularly early in infection, when the parasite burden is low and with reinfection after treatment. We sought to identify novel biomarkers of active infection by studying serum proteins in a mouse model of schistosomiasis followed by confirmation in chronically infected patients. Acute (6 weeks) and chronic (12 weeks) sera from *S. mansoni*-infected C57BL/6 mice as well as sera from chronically infected patients were assessed using two proteomic platforms: surface-enhanced, laser desorption and ionization, time-of-flight mass spectrometry and Velos Orbitrap mass spectrometry. Several candidate biomarkers were further evaluated by Western blot and/or enzyme-linked immunosorbent assay (ELISA). Among the most promising was carbonic anhydrase 1 (CA1), a host protein found primarily in red blood cells and enterocytes that proved to be a negative biomarker for schistosomiasis in both mouse and human samples. Reduced serum CA-1 levels were confirmed by both Western blot (murine and human: both $P < 0.001$) and ELISA (human: $P < 0.01$). Western blots of serial mouse sera revealed a progressive reduction in serum CA1 levels over the 12-week infection period. CA1 is a promising negative serum biomarker for the diagnosis of *S. mansoni* infection.

INTRODUCTION

Schistosomiasis affects ~200 million people in more than 70 countries and causes at least 200,000 deaths/year. More than 600 million people are at risk of acquiring this disease. Diagnosis typically depends on the detection of eggs that first appear in stool or urine between 4 and 6 weeks postinfection. During the late stages of the infection, which are associated with liver fibrosis and other complications, eggs can be difficult to detect; therefore, greater emphasis is placed on serologic testing. Current immunoassays have major limitations, however, including the inability to distinguish active from resolved infection (i.e., posttreatment), poor specificity due to the use of crude antigens, and low sensitivity in patients with light infections.^{1,2} Recent and acute infections can also be missed due to a seronegative “window” that typically lasts 4–12 weeks postexposure.^{3–5} However, some patients can take up to 6 months to become seropositive.^{6–9}

Mouse models of *Schistosoma mansoni* infection vary widely with some inbred strains showing pathology similar to human disease (e.g., C57BL/6), whereas others develop more severe manifestations (e.g., CBA).^{10–13} Regardless of the model used, the acute stage of murine schistosomiasis typically starts within 6–8 weeks of infection when active granulomas begin to form around eggs deposited in the colonic wall or swept up the portal circulation into the liver. At 12–14 weeks postinfection, mice enter the chronic stage and granuloma size tends to decrease.^{14–16} Host granuloma formation and fibrosis associated with parasite eggs are the main pathologies associated with chronic schistosomiasis. These changes occur primarily in the liver and intestines in the case of *S. mansoni* infection.^{10–12}

Schistosoma mansoni infection in humans ranges from asymptomatic to severe hepatosplenic fibrosis with portal hypertension, gastrointestinal hemorrhage, and death.^{10–12} Because of the similarities between murine and human disease manifestations, we used the C57BL/6 model to study protein profiles after *S. mansoni* infection to look for candidate biomarkers.

We followed a structured approach to biomarker discovery beginning with high-throughput surface-enhanced, laser desorption and ionization, time-of-flight mass spectrometry (SELDI TOF MS) analysis of mouse sera followed by Orbitrap Velos mass spectrometry (MS) microsequencing and confirmatory immunologic testing (western blot and enzyme-linked immunosorbent assay [ELISA]). This approach revealed serum carbonic anhydrase 1 (CA-1) as a powerful biomarker in both acute and chronic infection in the mouse model and in chronically infected patients.

MATERIALS AND METHODS

Infection protocol. *Schistosoma mansoni*-infected *Biomphalaria glabrata* snails were obtained from the Biomedical Research Institute (Bethesda, MD). Cercariae were collected 35–45 days postinfection by exposing the snails to continuous light for 2 hours at room temperature (RT).¹⁷ Twenty-six, 6-week-old female C57BL/6 mice were purchased from Charles River (St. Constant, Canada). Twenty of the mice were infected by tail penetration (200 cercariae) as previously described,¹⁷ and six uninfected animals served as controls. All animal experiments were performed in accordance with the guidelines of the Canadian Council on Animal Care and with approval of the Animal Care Committee of McGill University.

Murine and human serum samples. At 6 weeks postinfection (acute phase), 15 of the infected mice and three control animals were sacrificed. At 12 weeks postinfection (chronic phase), five infected and three control mice were killed. At sacrifice, blood was obtained by cardiac puncture,

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adult worms in the mesenteric veins were counted, and livers were collected for histology. Mouse sera were aliquoted and kept at -80°C until assays were performed. Human serum samples from a total of 25 patients (16 males and nine females; age range, 10–60 years) diagnosed with schistosomiasis were obtained from the National Reference Center for Parasitology (NRCP) serum bank (Montreal, Canada). These sera had been screened using an “in-house” *S. mansoni* and *Schistosoma haematobium* ELISA followed by species-specific testing (<https://www.mcgill.ca/tropmed/nrcp>). Human serum samples from a total of five patients (two males and three females; age range, 40–63 years) diagnosed with American trypanosomiasis (Chagas disease), human serum samples from a total of four patients (two males and two females; age range, 24–37 years) diagnosed with filariasis, human serum samples from a total of four patients (one male and three females; age range, 27–63 years) diagnosed with fascioliasis, and human serum samples from a total of 11 patients (three males and eight females; age range, 19–70 years) diagnosed with strongyloidiasis were also obtained from the NRCP. NRCP sera from an additional 28 subjects found to be negative for schistosomiasis (10 males and 18 females; age range, 19–66 years) were used as controls.

Histology. Mouse livers collected at 6 or 12 weeks after infection were fixed in 10% buffered formalin and embedded in paraffin (Histopathology Core, Montreal General Hospital, Montreal, Canada). Transverse sections (4 μm) were stained with hematoxylin and eosin (H and E) and examined by light microscopy for histopathological changes.

Initial proteomic analysis by SELDI. Individual murine and human serum samples were first analyzed by SELDI TOF MS. To detect as many candidate biomarkers as possible, fractionated serum samples were applied to Immobilized Metal Affinity Capture (IMAC30), Cation Exchange (CM10), and reverse-phase hydrophobic H50 Protein Chip arrays (Bio-Rad Laboratories, Hercules, CA) as previously described.¹⁸ Briefly, ProteinChip serum fractionation kit (Bio-Rad) was used to define six pH fractions prior to MS analysis using 96-well Q-Ceramic HyperD F resin filtration plates (Bio-Rad). To decrease complexity, 20 μL of each sample were first incubated with 30 μL of U9 buffer (9 M urea, 2% [w/v] 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate [CHAPS], and 50 mM Tris-HCl, pH 9) for 20 minutes. Then, samples were diluted with 50 μL of U1 buffer (1 M urea, 2% CHAPS, 50 mM Tris-HCl, pH 9) and added to preequilibrated filtration plates incubated on a MicroMix machine (MicroMix 5; Diagnostic Products Company, Los Angeles, CA) for 30 minutes. Five fractions were collected by serial elution in buffers of decreasing pH (pH 9, 7, 5, 4, 3). The wash buffers used were 1) 50 mM Tris-HCl, 0.1% octyl β -D-glucopyranoside (OGP), pH 9, 2) 50 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid 0.1% OGP, pH 7, 3) 100 mM Na acetate, 0.1% OGP, pH 5, 4) 100 mM Na acetate, 0.1% OGP, pH 4, and 5) 50 mM Na citrate, 0.1% OGP, pH 3. A final elution by vacuum using an organic buffer yielded fraction no. 6 (33.3% isopropanol, 16.7% acetonitrile (ACN), and 0.1% trifluoroacetic acid). All fractions were stored at -80°C until analysis.

Sample preparation for LTQ-Orbitrap Velos. The LTQ-Orbitrap Velos (Thermo Scientific, Bremen, Germany) analysis was performed in the Clinical Proteomics Platform,

McGill University. Pooled serum samples (1 $\mu\text{L}/\text{lane}$) from control (12 weeks) as well as acutely (6 weeks) and chronically infected mice (12 weeks) were loaded onto precast 10% mini-protean TGX precast gels (BioRad, Mississauga, Canada) and run for 45 minutes at 200 V. Gels were then stained and destained using standard Coomassie procedures.¹⁹ Each lane was sliced into 20 horizontal bands that were processed for proteomic analysis as previously described.²⁰ Briefly, bands were placed in 1.5-mL tubes and exposed to 40 μL of 100 mM NH_4HCO_3 for 5 minutes followed by centrifugation at $14,000 \times g$ for 10 minutes. After aspiration of the liquid, the gel pieces were vortexed with 40 μL of 100 mM NH_4HCO_3 /50% ACN (Sigma-Aldrich, St. Louis, MO) for 10 minutes to shrink the gel. The gel pieces were dried in a microplate at 60°C in a vacuum concentrator for 30 minutes to remove excess ACN and then placed in 40 μL of 10 mM dithiothreitol/100 mM NH_4HCO_3 (Sigma-Aldrich) at 56°C in a closed water bath for 60 minutes. Gel pieces were then alkylated in 40 μL of 55 mM iodoacetamide + 100 mM NH_4HCO_3 for 45 minutes in 1.5-mL tubes in the dark at RT. After centrifugation at $14,000 \times g$ for 15 minutes, the fluid was aspirated, and gel pieces were washed in the same 1.5-mL tube in 100 μL of 100 mM NH_4HCO_3 . Tubes were then centrifuged at $14,000 \times g$ for 10 minutes, followed by removal of the fluid and washing of the gel in 40 μL of 100 mM NH_4HCO_3 for 5 minutes at RT after which 40 μL of ACN was added to make a 1:1 solution and incubation was continued for 15 minutes at RT. This last wash step with 100 μL of 25 mM NH_4HCO_3 in 50% ACN was repeated once, then the gel pieces were dried (60°C as above) and digested with 10–20 μL (enough to cover pieces) of trypsin (Roche, Mannheim, Germany) overnight at 37°C (12.5 ng/ μL). After digestion, peptides were extracted with 100 μL 1% formic acid and vortexing at 37°C for 15 minutes. Tubes were then centrifuged at $14,000 \times g$ for 15 minutes, and the supernatants were transferred to new 1.5-mL tubes (first extraction). A second extraction was performed with 100 μL 5% formic acid/50% ACN (Sigma-Aldrich), and after centrifugation ($14,000 \times g$ for 15 minutes), the two extraction supernatants were pooled. The pooled supernatants were then dried in a vacuum centrifuge (Hermle Labortechnik, Wehingen, Germany) for ~1–2 hours at 50°C . Dried samples were resuspended in 40 μL 0.05% formic acid and stored at -20°C until used for MS analysis.

Velos parameters. The samples were analyzed using a Thermo Easy-nano LC (Thermo Scientific, Bremen, Germany) connected to LTQ-Velos Orbitrap. Tryptic peptides were eluted at a flow rate of 300 nL/minute: taking 60 minutes to go from 8% to 45% ACN containing 0.1% (v/v) formic acid; 5 minutes at 80%, and finally 15 minutes back to 0%. The total run time for each sample was 80 minutes. MS spectra were acquired with a resolution of 60,000 (scan range: 380–1,400 m/z). The top 5–7 peptide signals (positive polarity) from each MS scan were submitted to MS/MS. The peptides were fragmented in the linear ion-trap by the collision-induced dissociation cell (1.0-m/z isolation width, 10-ms activation time, 35% normalized activation energy, Q value of 0.25). Dynamic exclusion was applied with a repeat count of 1 and an exclusion time of 15 seconds.^{21,22}

Protein identification. All MS/MS data were analyzed using Mascot (version 2.3.01; Matrix Science, London,

FIGURE 1. Liver histology at 6 and 12 weeks after *Schistosoma mansoni* infection of C57Bl/6 mice with 200 cercariae by tail penetration (hematoxylin and eosin stain: 10 $\times$ magnification). (A) Hepatic granulomas at 6 weeks postinfection, and (B) hepatic granulomas 12 weeks postinfection.

United Kingdom) set up to search both schistosome and rodent protein databases (n_sch201202 and n_rodents20120120, respectively—all entries). Trypsin was used as the digestion enzyme. Mascot was searched with a fragment ion mass tolerance of 0.50 Da and a parent ion tolerance of 2.5 ppm. Carbamidomethylation of cysteine was specified as a fixed modification, and oxidation of methionine was specified as a variable modification. After searching, Mascot files were imported into Scaffold (version 3.6.3; Proteome Software Inc., Portland, OR) to validate the peptide/protein identifications. Identifications were accepted if the protein achieved $\geq 95.0\%$ probability using the PeptideProphet algorithm^{23,24} and contained at least one unique peptide. Proteins that contained similar peptides and could not be differentiated based on MS/MS alone are not included in the displayed results.

Western blots. Mouse and human sera were separated by one-dimensional electrophoresis on 4–12% Bis-Tris NuPAGE gels (Invitrogen, Burlington, Canada). Separated proteins were transferred to nitrocellulose membranes (Invitrogen, Kiryat Shmona, Israel) at 100 V for 7 minutes, which were then stained with Ponceau S to verify transfer and as an initial loading control.^{25,26} Membranes were then blocked with 5% skim milk in 0.05% Tween 20 in phosphate-buffered saline (PBS) (PBST) buffer for 1 hour at RT, followed by three washes with PBST for 5 minutes each. Membranes were incubated with a rabbit monoclonal antibody that recognizes human and mouse CA1 (78% identity between mouse and human CA1) (Abcam, Toronto, Canada) at a 1:150 dilution overnight at 4°C. Membranes were then washed three times with PBST for 5 minutes each. Incubations with horseradish peroxidase (HRP)-conjugated anti-rabbit IgG were performed at 1:20,000 at RT for 1 hour (Amersham Biosciences Co., Piscataway, NJ). Membranes were washed once in PBST for 15 minutes followed by two PBST washes of 5 minutes each, then incubated in Super Signal West Pico detection solution for 1 minute at RT (Pierce, Rockford, IL) and exposed to X-ray film. Glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) was used as an internal loading control. Membranes were stripped with 100 mL of stripping solution: 2% sodium dodecyl sulfate (SDS), 62.5 mM Tris HCL (pH 6.7), 100 mM mercaptoethanol for 30 minutes at 55°C, followed by

reprobing with a mouse anti-GAPDH monoclonal (1:5,000, Abcam). Blots were incubated with HRP-conjugated anti-mouse IgG at 1:10,000 at RT for 1 hour (Amersham Biosciences Co.) and exposed to X-ray film. Image J software (National Institutes of Health) was used to analyze densities of selected bands.

CA1 enzyme-linked immunosorbent assay. A human CA1 ELISA (USCN, Life Science Inc., Wuhan, China) was used according to the manufacturer's instructions. Briefly, 100 μ L of standards or heat-inactivated serum samples (56°C for 30 minutes) were analyzed in duplicate wells, and CA1 concentrations were calculated based on the internal standard curve. Samples that initially yielded results above the highest standard were diluted 2-fold with PBS and rerun. CA1 values are reported in pg/mL.

Statistical analysis. The Statistical Package for the GraphPad Prism 6 (GraphPad Software, San Diego, CA) was used for statistical analysis. All results are presented as means $\pm$ SEM. Levels of CA1 in the serum were evaluated

FIGURE 2. Example of a ~2,794.38-Da candidate biomarker in infected mice that gradually increases from 6 to 12 weeks after infection (6W and 12W, respectively; $P < 0.01$) vs. uninfected control animals (CTL). Serum SELDI TOF MS mass spectra obtained for F6CSL (Fraction 6, CM10 chip, low laser intensity) from infected mice (top two spectra) vs. noninfected mice (bottom spectrum). The x axis indicates the mass-to-charge ratio (m/z). The y axis indicates the protein abundance expressed as absolute intensity (μ A/laser pulse). SELDI TOF MS = surface-enhanced, laser desorption and ionization, time-of-flight mass spectrometry.

TABLE 1

Eight proteins were increased in the sera of mice during both the acute and chronic stages of schistosomiasis compared with controls

Proteins identified by LTQ-Orbitrap Velos	Accession no.	Molecular weight	Taxonomy
Myoglobin	21359820	17.07 kDa	Unknown
L chain L, crystal structure of the complex Fab M75-peptide	160285546	24.12 kDa	LK3 transgenic mice
Unnamed protein product	12843046	26.41 kDa	LK3 transgenic mice
Proteasome (prosome, macropain) subunit, beta type 1	37231712	26.40 kDa	Unknown
Apolipoprotein F precursor	19527216	35.43 kDa	Unknown
Leucine-rich alpha-2-glycoprotein precursor	16418335	37.43 kDa	Unknown
Extracellular matrix protein 1 isoform 1 precursor	170295832	62.83 kDa	Unknown
Maltase-glucoamylase, intestinal-like	354489684	403.52 kDa	Unknown

by comparing the results in the control group with those in the other parasitic infection groups. To do this, the means of the control group and the means of the other parasitic infection groups were calculated and compared using one-way analysis of variance (ANOVA) with Brown–Forsythe test and Bartlett’s test for equal variances. $P \leq 0.01$ was considered to be statistically significant. We further performed Tukey’s post hoc analysis adjusted for multiple comparisons.

RESULTS

Worm burden and histopathologic examination. The number of worm pairs identified was similar in groups killed at either 6 or 12 weeks postinfection (mean, 15 ± 10 pair/mouse). H and E-stained liver sections at 6 weeks after the infection showed an early granulomatous response surrounding the entrapped eggs. Consistent with previous reports,²⁷ these granulomas appeared to consist primarily of macrophages and eosinophils surrounded by lymphocytes. At 12 weeks postinfection, liver granulomas were surrounded by large numbers of epithelioid cells and lymphocytes with occasional vacuolated giant cells (Figures 1 and 2).

Strategy for selecting the most promising biomarkers. This study used two proteomic approaches to identify novel biomarkers for schistosomiasis. Since proteomic studies typically yield large numbers of candidate biomarkers, we moved back and forth between platforms and between murine and human samples to select candidates with the greatest potential to be clinically useful. Our first experiments used SELDI and prefractionated sera from the mice at 6 and 12 weeks postinfection to ask the simple question “Are there differences in serum protein profiles following schistosomiasis infection?” This “first look” revealed marked

differences in the SELDI spectra between the uninfected and infected serum proteomes with at least 74 possible biomarkers based simply on m/z ratios. A representative example of discriminatory peaks which are presented in Figure 2 were able to prove that the signal intensity of the peak increased with the prolonged time of infection.

Identification of differential proteins by LTQ-Velos Orbitrap. LTQ-Velos Orbitrap was used for brute-force MS analysis of 1-dimensional SDS–polyacrylamide gel electrophoresis gel slices of pooled sera from uninfected and infected mice. This approach also generated large numbers of potential biomarkers (e.g., $> 1,122$ between uninfected and infected mice) tentatively identified based on MS microsequencing. This platform also permitted us to use protein identification probability (PIP) algorithms based on percentage of total spectra, number of assigned spectra, number of unique spectra, peak intensities, and percent coverage both to assess the level of confidence in the identifications and to generate semiquantitative results. The latter capacity is based on the assumption that high-abundance proteins will produce more MS/MS spectra than low-abundance proteins^{28,29} so that comparing the number of spectra from the same protein between any two samples can serve as a crude relative quantitation of this protein.³⁰

Use of the two proteomic platforms. Of the 1,122 proteins tentatively identified by the Scaffold software, application of a 95% PIP filter brought the number down to 239. From these 239 differentially expressed proteins, serum levels of 22 were higher than control levels in both acute or chronic infection mouse serum, and only eight were upregulated in both the acute and chronic stages (Table 1). Nineteen were higher in the control serum than during acute or chronic infection with nine downregulated in both acute and chronic stages (Table 2). We then used the predicted molecular weights of the 17 proteins to query the mouse

TABLE 2

Nine proteins were reduced in the serum of mice during both acute and chronic stages of schistosomiasis compared with controls

Proteins identified by LTQ-Orbitrap Velos	Accession no.	Molecular weight (kDa)	Taxonomy
Proteasome subunit beta type-2	227116345	22.90	LK3 transgenic mice
Carbonic anhydrase 1	116063531	28.33	Unknown
Bisphosphoglycerate mutase	6680806	29.98	LK3 transgenic mice
Pancreatic alpha-amylase-like isoform 1	354486764	57.21	Unknown
BC026782 protein	20071242	58.07	LK3 transgenic mice
Peptidoglycan recognition protein 2	124245041	57.59	Unknown
Complement C2 precursor	157951694	84.74	Unknown
Rat xanthine oxidoreductase mutant	158428238	146.10	Buffalo rat
Murineoglobulin-2 precursor	50657404	161.59	Buffalo rat

TABLE 3
Predicted molecular weights of the 17 serum proteins identified by Orbitrap

Proteins identified by Orbitrap™	Accession no.	Molecular weight (kDa)	Mouse SELDI	Human SELDI
Myoglobin	21359820	17.07	N/A	N/A
Proteasome subunit beta type-2	227116345	22.90	23.17	N/A
L chain L, crystal structure of the complex Fab M75-peptide	160285546	24.12	N/A	N/A
Unnamed protein product	12843046	26.41	N/A	N/A
Proteasome (prosome, macropain) subunit, beta type 1	37231712	26.40	N/A	N/A
Carbonic anhydrase 1	116063531	28.33	28.85	28.97
Bisphosphoglycerate mutase	6680806	29.98	N/A	N/A
Apolipoprotein F precursor	19527216	35.43	N/A	N/A
Leucine-rich alpha-2-glycoprotein precursor	16418335	37.43	N/A	N/A
Pancreatic alpha-amylase-like isoform 1	354486764	57.21	59.83	N/A
BC026782 protein	20071242	58.07	59.83	N/A
Peptidoglycan recognition protein 2	124245041	57.59	59.83	N/A
Extracellular matrix protein 1 isoform 1 precursor	170295832	62.83	N/A	N/A
Complement C2 precursor	157951694	84.74	N/A	N/A
Rat xanthine oxidoreductase mutant	158428238	146.10	145.32	N/A
Murinoglobulin-2 precursor	50657404	161.59	N/A	N/A
Maltase-glucoamylase, intestinal-like	354489684	403.52	N/A	N/A

SELDI TOF MS = surface-enhanced, laser desorption and ionization, time-of-flight mass spectrometry. The 17 serum proteins were consistently increased or decreased in murine schistosomiasis and SELDI TOF MS peaks in both mouse and human serum that distinguished between infected and control samples.

FIGURE 3. Immunologic validation of CA1 as a candidate biomarker. (A) Representative Western blot of CA1 in pooled sera from *Schistosoma mansoni*-infected mice (6 and 12 weeks postinfection: 6W and 12W, respectively) and controls (C). Ponceau-stained gel and Western blot of GAPDH served as loading controls. (B) Relative density of CA1 levels normalized to GAPDH ($P < 0.001$). CA1 = carbonic anhydrase-1; GAPDH = glyceraldehyde-3-phosphate-dehydrogenase.

SELDI TOF MS database for the corresponding peaks ($\pm 5\%$ mass range). Distinct SELDI peaks that differed substantially between infected and control sera were detected at m/z values of 28.85 ($P = 0.007$), 59.83 ($P = 0.02$), and 145.32 ($P = 0.01$) kDa on the IMAC30 ProteinChips and at 23.17 ($P = 0.02$) kDa on the CM10 ProteinChips. Together, these data suggested that murine CA1 (mCA1: 28.85 kDa), proteasome subunit beta type-2 (59.83 kDa) and rat xanthine oxidoreductase mutant (145.32 kDa) were among the most promising biomarkers. The last SELDI peak of interest at 59.83 kDa was more complicated since this molecular weight corresponded to three proteins of similar molecular weight in the Orbitrap database: pancreatic alpha-amylase-like isoform 1, BC026782 protein, and peptidoglycan recognition protein 2. When we used these tentative molecular weights to query our human serum SELDI database (data not shown), we found only one peak at 28.97 kDa on the H50 ProteinChip arrays that potentially corresponded to human homologue of CA1 (hCA1 28 kDa, Table 3).

Immunologic confirmation by Western blot and ELISA.

Western blot analysis of individual murine (Figure 3) and human serum samples (Figure 4) revealed striking reductions in serum CA1 levels in infected animals and patients compared with uninfected control sera. In mice, when the timing of infection was known, there was a progressive decline in serum CA1 levels between 6 and 12 weeks after infection (Figure 3B). The mean values, standard deviations, and results of comparisons among different groups (control, schistosomiasis, Chagas disease, filariasis, fascioliasis, and strongyloidiasis) are presented in Table 4. One-way ANOVA revealed significant differences among the groups ($P < 0.0001$) graphically represented in Figure 5. Then the Tukey's post hoc test was applied to identify whether these differences occur. Significant differences between different groups were found only when the comparisons were among schistosomiasis and all other groups. CA1 concentration was consistently lower in schistosomiasis than all other groups; however, statistical significance ($P < 0.001$) was also found when the comparison was between control and filariasis, and between schistosomiasis and filariasis (CA1 concentration was higher in filariasis than

FIGURE 4. Validation of CA1 as a candidate biomarker in human serum. (A) Representative Western blot of CA1 in patients chronically infected with *Schistosoma mansoni* (lanes I₁–I₈) vs. controls (C₁–C₂). Ponceau-stained gel and Western blot of GAPDH served as loading controls. (B) Relative densities of CA1 bands in infected patients (I₁–I₈) vs. controls (C₁–C₂) ($P < 0.001$). CA1 = carbonic anhydrase-1; GAPDH = glyceraldehyde-3-phosphate-dehydrogenase.

FIGURE 5. Serum carbonic anhydrase-1 (CA1) levels were significantly lower in patients chronically infected with *Schistosoma mansoni* compared with healthy controls and patients with Chagas disease, filariasis, fascioliasis, and strongyloidiasis ($P < 0.0001$). The data are presented as box-and-whisker graphs. The boxes indicate 25th to the 75th percentile; the line in the box indicates the median. The error bars indicate the highest and the lowest values. Serum CA1 concentration was significantly higher in the filariasis group compared with the control group ($P < 0.001$).

control and schistosomiasis). There was no significant difference among the other groups.

DISCUSSION

Schistosomiasis remains a serious health threat in many low- and middle-income countries. Despite the wide availability and relatively low cost of praziquantel, treatment is often delayed because the diagnostic tests available have low sensitivity both early and late in disease and because reinfection is common. There are currently no reliable biomarkers for active infection other than detection of living eggs in stool or urine, and egg shedding can persist for years after treatment. There is an urgent need to develop new tests that can address some of these diagnostic “gaps.” Long considered to be a research tool, MS has recently entered the clinical microbiology laboratory for the rapid identification of pathogens.³¹ In this work, we used two proteomic platforms and both murine and human serum samples to pursue novel biomarkers for schistosomiasis.

TABLE 4

Statistical evaluation on the serum levels (expressed in pg/mL) of significant CA1 in the healthy controls and in five patient groups

	Control	Schistosomiasis	Chagas disease	Filariasis	Fascioliasis	Strongyloidiasis
Minimum	48.97	45.87	76.28	150.8	73.51	75.17
25% Percentile	84.68	65.04	79.38	156	73.6	97.81
Median	103	71.13	137.9	176.8	90.05	103.4
75% Percentile	130	84.72	177.2	186	118.8	119.2
Maximum	198.7	99.4	193.3	187.3	122.9	162
Mean	106.9	72.87	130.2	172.9	94.14	109.5
Standard deviation	35.68	15.19	50.46	16.1	24.57	21.88
Standard error of mean	6.743	3.921	22.57	8.049	12.29	6.598
Lower 95% CI of mean	93.08	64.46	67.57	147.3	55.04	94.81
Upper 95% CI of mean	120.7	81.28	192.9	198.5	133.2	124.2
Sum	2,994	1,093	651.1	691.7	376.6	1,205

CA = carbonic anhydrase; CI = confidence interval. Minimum CA1 serum level, 25% percentile, median, 75% percentile, maximum, mean, standard deviation, standard error of mean, lower 95% CI of mean, and upper 95% CI of mean are reported.

We used SELDI TOF MS to generate protein expression profiles from the serum of individual mice and patients (versus controls). Although these spectral data suggested the presence of major differences between infected and noninfected mice/patients, the translation of SELDI "peaks" into identified candidate biomarkers can be very difficult due to the relatively low mass accuracy of this platform.³² Not unexpectedly, there were also large differences between the SELDI spectra in infected mice and patients. To address these issues, we turned to the Orbitrap platform that can provide much higher mass accuracy and resolution in addition to the identification of the potential biomarkers. The Orbitrap data were then used to query both the murine and human SELDI spectral database for highly discriminatory biomarkers. This multistep process yielded CA1 as a candidate "negative" biomarker for both murine and human schistosomiasis that was subsequently confirmed by both western blot and ELISA.

Carbonic anhydrases (CAs) are metalloenzymes that mediate the reverse hydration of CO₂ to H⁺ and HCO₃⁻ and subsequent exchange of the H⁺ and HCO₃⁻ ions for Na⁺ and Cl⁻ across cell membranes.³³ These enzymes are present in all living things where they participate in a wide range of physiologic reactions including respiration, photosynthesis, pH regulation, electrolyte secretion, and many biosynthetic reactions.³⁴ CAs can be grouped into five distinct genetic families or classes: α , β , γ , δ and ζ . These families differ primarily in the metal ion used for catalysis; α -, β -, and δ -CAs use zinc (Zn) ions at the active site, the γ -CAs may use iron (Fe),³⁵ but they can also bind Zn or cobalt (Co), and the ζ -class uses cadmium (Cd).^{36,37} α -CAs are the dominant form in humans and other animals (CA1 is one of α -CA isoforms). β -CAs are expressed in most species of bacteria, yeast, algae, plants, and some invertebrates such as nematodes and insects. γ -CAs have been reported in plants, archaea, and some bacteria.^{38,39} The last two CA classes: δ - and ζ -CAs are structurally similar to the β -CAs.

CAs have long been recognized as important drug targets, and both activators and inhibitors are currently used as therapeutic agents for a wide range of conditions including infections, cancer, obesity, and glaucoma.^{34,40} Consistent with their central role in diverse biological processes, deficiencies in CA activity can lead to many different symptoms. In mice and humans, CA1 is found primarily in the red blood cells,⁴¹ the colonic epithelium,⁴² and neutrophils.⁴³ Abnormalities in CA1 isoenzymes are thought to contribute to several types of anemia,⁴⁴ and lower levels of CA1 mRNA and protein as well as total CA activity are found in the inflamed mucosa of patients with ulcerative colitis compared with controls despite the heavy infiltration of these tissues by neutrophils.^{45,46} Deposition of *S. mansoni* eggs also causes acute and chronic inflammation of the colorectal mucosa.⁴⁷ If reduction of CA1 expression and activity is a common feature of colonic inflammation, this could explain the reduction of CA1 levels we observed in the serum of *S. mansoni*-infected mice and humans. Another possible explanation for the low serum CA1 concentration we observed in schistosomiasis is zinc deficiency. Serum zinc levels are low in subjects infected with *S. mansoni* and recover after treatment.⁴⁸ Zinc is essential for the structure and activity of human CAs.⁴⁹ Although zinc levels were not measured in either the mouse

or human sera used in this study, lower zinc levels in *S. mansoni*-infected mice and patients may have contributed to the reduced levels of CA that we observed.

In conclusion, serum CA1 appears to be a promising negative biomarker for human infection with *S. mansoni* at least during the chronic stage. Studies are planned to determine whether serum CA1 levels return to normal after praziquantel therapy and the kinetics of such normalization. We are curious to know whether serum CA1 is reduced in subjects with other *Schistosoma* species as well as other noninfectious conditions that cause bowel inflammation. It will also be important to determine the possible contribution of zinc deficiency to lower CA1 levels in schistosomiasis patients.

Received January 7, 2016. Accepted for publication October 31, 2016.

Published online February 27, 2017.

Acknowledgments: We are grateful to the Clinical Proteomics Platform, McGill University (<http://www.clinprot.org/>) for LTQ-Orbitrap Velos mass spectrometry analysis. We also thank Nathalie Martel for providing technical assistance.

Financial support: Manal Ibrahim Kardoush is the recipient of a scholarship from the Government of Egypt.

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