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Overexpression of Schistosome-specific Antigens for Selection of scFv Antibody from Phage Display Libraries of Water Buffalo for Potential Use in Schistosomiasis Diagnosis

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Schistosomiasis is an acute and chronic parasitic disease caused by blood flukes of the genus *Schistosoma*. The Kato-Katz method is the standard for the diagnosis of intestinal schistosomiasis because of its specificity, simplicity, and low cost. However, Kato-Katz is less useful in light infections and other available detection methods are neither practical nor sensitive for routine or large-scale screening. Thus, improved tests are needed for the assessment of infection. The water buffalo is a natural host and reservoir of *Schistosoma* but is schistosomiasis-resistant, suggesting a robust immune repertoire, which can be tapped as a source of antibodies for diagnosis of schistosomiasis. Two schistosome antigens had been previously shown to be recognized by a phage display single-chain fragment of variation (scFv) library derived from a schistosome-infected water buffalo. However, specific antibodies have not been isolated using these antigens due to a lack of sufficient amounts and purified forms of the antigens. In this study, the schistosome antigens were cloned and overexpressed in vector with solubility tag, purified by immobilized metal affinity chromatography (IMAC), and used to interrogate the immune scFv library to isolate and identify specific schistosome antibody. An scFv specific to schistosome SAP domain-containing hypothetical protein was isolated but was only 105-bp long although certain regions align with the IgG V_L, tetraglycylserine (Gly_4Ser) linker, and IgG V_H sequences of synthetic scFv constructs. Despite its length, scFv A63 can be potentially designed as a ligand in direct ELISA (enzyme-linked immunosorbent assay) by fusion with enzyme conjugates such as alkaline phosphatase to detect *S. japonicum* schistosomule and adult worm. The results in this study also suggest the need to construct a bigger and more diverse scFv library and to test a wider range of schistosome antigens, specifically those that will be present on the surface of the schistosome at all stages of *S. japonicum* life cycle.

Keywords: *Schistosoma japonicum*, bovine scFv-phage library, recombinant antigen

INTRODUCTION

Schistosomiasis is an acute and chronic parasitic disease caused by blood flukes of the genus *Schistosoma* (Badr 1981). It is one of the neglected tropical diseases and the second most devastating of all human diseases caused by parasites. The World Health Organization recently estimates that more than 207 M people are infected worldwide, with over 700 M people at risk of infection – specifically in tropical and subtropical areas of South America, Africa, the Middle East, East Asia, and the

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Philippines. In China, zoonotic schistosomiasis japonica – also called "snail fever" – is associated with chronic liver and intestinal fibrosis (McManus *et al.* 2010).

Intestinal schistosomiasis is detected commonly by the Kato-Katz thick smear technique – which is rapid, simple, and inexpensive (Katz *et al.* 1972). However, Kato-Katz is less useful in light infections and other available detection techniques such as indirect, immunological, and polymerase chain reaction (PCR) based methods are neither practical nor sensitive enough for routine or large-scale screening (McManus *et al*. 2010). Thus, improved tests are demanded for assessment of infection. *S. japonicum* proteins such as thioredoxin peroxidase-1 and cathepsin B have been reported as potential diagnostic antigens for the Asian schistosomiasis (Macalanda *et al*. 2018, 2019) but specific antibodies must be generated to recognize these antigens.

The domestic water buffalo (*Bubalus bubalis*) can be tapped as a source of antibodies for the diagnosis of schistosomiasis because it naturally harbors *Schistosoma* but is often asymptomatic, suggesting a robust immune repertoire against the disease. In addition, *B. bubalis* belongs to the Bovidae family as with cows (*Bos taurus*), which feature ultralong complementarity-determining 3 region of the immunoglobulin heavy chains (CDR H3) that can range up to 70 amino acids and are encoded by a single variable, diversity, joining recombination that is extensively diversified prior to antigen exposure (Burke *et al*. 2020; Haakenson *et al*. 2018). The ultralong CDR H3 can generate a unique antigen-binding site that can bind to otherwise inaccessible epitope targets and neutralize several antigen variants (Burke *et al*. 2020). Since the water buffalo is related to cows, it is expected that its antibody repertoire will maintain the unique structural diversity found in cows. However, because the carabao is more exposed to harsher conditions and more parasites yet is more resistant to most of the diseases than domestic cattle (Deb *et al*. 2016), it should possess a more robust immune repertoire than the domestic cattle. We hypothesize that the carabao immune repertoire will offer a more diverse immune response and stable antibody frameworks, producing more potent antibodies than domestic cattle.

Bacteriophage libraries that display scFv fragments have proven to be useful reagents in the identification of antibodies against a specific target antigen. Hosking *et al*. (2015a) previously generated an scFv-phage library encoding B-cell heavy chain variable region (V_H) and light chain variable region (V_L) gene families from the local draining lymph node of *B. bubalis* in China following experimental infection with *S. japonicum* cercariae. A pool of selected scFv-phage recognized stage-specific schistosome antigens on protein microarray, including two hypothetical proteins with NCBI GenBank accession numbers AY811797 and AY814150. The former contains a DNA-binding SAP domain and is highly expressed in schistosomule and adult worm stages of *S. japonicum*. However, specific antibodies against these target antigens were not isolated from the selected pool of scFv phage because the antigens were not available in purified form and amounts sufficient for the selection of specific antibodies using the phage display libraries. In the present study, the genes encoding the schistosome antigens were each sub-cloned in an expression vector that carries maltose-binding solubility and His_6 purification tags, to allow soluble over-expression and purification of soluble antigens. The purified antigens were then used to screen the scFv libraries – to isolate specific antibodies against schistosome, which can be used as reagents for the diagnosis of schistosomiasis.

MATERIALS AND METHODS

PCR Amplification

The genes of CH2 (NCBI Accession No. AY811797) and CH4 (NCBI Accession No. AY814150) were amplified *via* PCR in 50 µL reaction that contained 1X PCR buffer, 1 mM $MgSO_4$, 0.3 µM of each primer (Appendix I), 0.3 mM dNTP mix, 1 U Platinum™ Pfx polymerase (Invitrogen, USA), and 100 ng DNA in Veriti™ 96-well thermal cycler (Applied Biosystems, USA) under the following conditions: 94 °C for 1 min, followed by 30 cycles of 94 °C for 0.25 min, 55 °C for 0.5 min, and 68 °C for 1 min, then 68 °C for 8 min. The amplicons were run in 0.8% agarose gel electrophoresis, stained with GelRed (Biotium, USA), for 1 h at 110 V and visualized under the Gel Doc™ XR+ documentation system (Bio-Rad, USA). The PCR fragment (621 bp for CH2 and 1065 bp for CH4) was manually excised and purified using GeneJet™ quick gel extraction kit (Thermo Scientific, USA). The purity and concentration of the amplicon were determined using the Epoch microplate spectrophotometer and Take3 software (Biotek Instruments, USA).

Vector Linearization

The cloning vector pET His_6 MBP TEV LIC (ligation independent cloning) (Addgene # 29708) was linearized using FastDigest™ *Ssp*I (Thermo Fisher Scientific, USA), then run in 0.8% agarose gel electrophoresis for 1 h at 110 V. The 6.0 kb fragment was excised and purified using GeneJet™ gel extraction kit (Thermo Fisher Scientific, USA).

T4 DNA Polymerase Treatment and LIC

To generate 5' overhangs, 50 ng of purified amplicon and 150 ng of purified, linearized vector were treated with 1 U of T4 DNA polymerase (Thermo Fisher Scientific, USA) in presence of 2.5 mM dCTP and 2.5 mM dGTP, respectively, at 22 °C for 30 min followed by 20 min at 75 °C. The T4 DNA polymerase-treated amplicon and vector were then co-incubated in 2:3 volume (µL) ratio at room temperature for 1 h.

Transformation into Competent *Escherichia coli* DH5α Cells

The LIC reaction mixture was aliquoted to 100 µL of competent *E. coli* DH5α. The reaction mixture was left on ice for 30 min, incubated at 42 °C for 45 s, then on ice for 5 min. Afterward, 950 µL of SOC was added to the tube, followed by 1 h incubation at 37 °C at 250 rpm. After mixing, 200 µL of the mixture was spread on Luria-Bertani (LB) agar plate containing ampicillin (100 µg/mL), which was then incubated at 37 °C for 18 h.

Gene Construct Isolation and Sequencing

A transformant colony was grown at 37 °C with 250 rpm shaking for 18 h in 5 mL of LB broth containing ampicillin (100 µg/mL). The plasmid was isolated using PureLink™ quick plasmid DNA MiniPrep kit (Invitrogen, USA) and sent to Apical Scientific Sdn. Bhd. for sequencing using primers LIC Fwd (5'- GTATTAACGCCGCCAGTCC -3') and LIC Rev (5'- GCTTTGTTAGCAGCCGGAT -3').

Overexpression, Extraction, Purification, and Sequencing of Schistosome Antigens

The recombinant schistosome antigens were each transformed into competent *E. coli* BL21(DE3), as previously described, then grown in Overnight Express™ Instant terrific broth medium (Novagen, Germany) for 36 h at 26 °C at 300 rpm. Cultures were harvested by centrifugation and treated with 500 µL B-PER™ bacterial protein extraction reagent (Thermo Fisher Scientific, USA) and 1.0 µL Lysonase™ bioprocessing reagent (Novagen, Germany). The recombinant antigens were purified by IMAC using HisPur™ Ni-NTA (Thermo Scientific, USA), followed by ultrafiltration using Amicon® Ultra-4 10K centrifugal filter devices (Merck Millipore, Germany) with 20 mM potassium phosphate (KH_2PO_4-K_2HPO_4) buffer (pH 7.5). The presence of purified antigens was confirmed by SDS-PAGE (sodium dodecyl sulfate – polyacrylamide gel electrophoresis) stained with Coomassie blue. The purified recombinant proteins were sent to Apical Scientific Sdn., Bhd. Mass for sequencing by MALDI-ToF MS/MS and spectral data were analyzed using Mascot sequence matching software (Matrix Science, USA) with MSPnr100 database and MS-Bridge protein prospector (http://prospector.ucsf.edu/prospector/cgi-bin/msform.cgi?form=msbridgestandard).

Screening of the Water Buffalo scFv-phage Libraries with the Overexpressed Schistosome Antigens

Four bacteriophage libraries displaying scFv (Appendix II; Hosking *et al*. 2015a, b) were utilized for the iterative selection process called biopanning to select for scFv that specifically bound to the antigens.

Rescue and Amplification of scFv Phage Libraries

One (1) mL of log phase *E. coli* TG1 cells was infected with 100 µL of scFv phage for 1 h at 37 °C. Infected cells were spread plated on 2xYT agar plates with chloramphenicol (25 µg/mL) and incubated overnight at 37 °C. Colony growth was scraped with 1 mL of 2xYT broth, inoculated in 50 mL of non-expressing medium (2xYT + 1% glucose + 25 µg/mL chloramphenicol) and incubated at 37 °C at 120 rpm until OD_{600} of 0.5 was attained. Subsequently, 1 x 10^{11} transducing units (tu) of M13K07 helper phage and 25 µL of 1.0 M IPTG (isopropyl β-D-1-thiogalactopyranoside) were added, followed by a 40-min incubation at 37 °C and centrifugation at 10,000 g at 4 °C for 15 min. The cell pellet was inoculated in 100 mL of low-expressing medium (2xYT + 1% glucose + 25 µg/mL chloramphenicol + 0.5 M IPTG) and incubated for 2 h, after which kanamycin was added (100 µg/mL). After overnight incubation at 26 °C at 120 rpm, the supernatant was collected after centrifugation for 15 min at 4,500 g, at 4 °C, and was co-incubated with 25 mL of 2.5 M NaCl/ 20% PEG 8000 for 2 h on ice. Phage pellet was recovered by 15-min centrifugation at 12,000 g and resuspended with 2 mL of 1X phosphate-buffered saline (PBS). Resuspended phages were precipitated using 0.5 mL of 2.5 M NaCl/ 20% PEG 8000 solution for 10 min on ice. Phages were recovered by centrifugation at 12,000 rpm for 15 min at 4 °C and resuspended with 0.5 mL of PBS. The titer of scFv phage libraries, expressed in tu/mL, was determined *via* serial dilution, transfection into 1 mL of mid-log phase *E. coli* TG1 cells, and plate count method (Hosking *et al.* 2015a, b).

Biopanning of scFv Phage Libraries

Each of the recombinant antigens and the control His_6-MBP-TEV N-terminal tag for background binding were diluted to a final concentration of 20 µg/mL with coating buffer (0.1 M bicarbonate-carbonate buffer, pH 9.6). Preliminarily, respective wells of the microplate were coated with 300 µL of the antigens and MBP overnight at 4 °C (or 2 h at 37 °C), as previously described (Hosking *et al*. 2015a). The coating solution was removed gently by pipetting and the wells were washed thrice using 0.01 M PBS with 0.05% (v/v) Tween 20 (PBST). The coated

and additional empty wells as null control were treated with 300 µL of blocking buffer (1% casein in PBS) for 1 h at ambient temperature, then washed with PBST.

Pre-absorption was done by incubating the scFv-phage library (1 x 10^{11} tu/mL, diluted in 0.01 M PBS) in blocked wells for 1 h at ambient temperature at 100 rpm. The recovered scFv-phage library was then pre-absorbed in MBP-coated wells then into antigen-coated wells, both for 1 h at ambient temperature at 100 rpm. Bound scFv-phage was eluted using 100 µL of 50 mM glycine (pH 2.0) for 10 min, immediately followed by neutralization with 10 µL of 2.0 M Tris-HCl (pH 8.0). The neutralized phages were transfected into 1 mL of log phase *E. coli* TG1 cells, then rescued as described above. The biopanning was performed in three rounds, to improve the monoclonal character of the libraries' rounds (Hosking *et al.* 2015a, b). The relative enrichment was calculated based on the ratio between titer of the eluted scFv-phages and the titer of the input scFv-phages (1.0 x 10^{11} tu) as follows:

$$\text{Relative enrichment} = \frac{\text{Titer of the eluted scFv phage}}{\text{Titer of the input scFv phage}}$$

scFv-phage ELISA

scFv-phage ELISA was conducted, as previously described (Hosking *et al.* 2015a). The wells of microplates were coated with 100 µL of diluted purified antigen (0.01 µg/µL in 0.1 M bicarbonate-carbonate coating buffer, pH 9.6) for 1 h at 37 °C, washed with 200 µL of PBST, and blocked with 200 µL of casein in PBS (1% w/v) for 2 h at 37 °C. The blocker solution was discarded, then 100 µL of scFv-phage libraries (5 x 10^{11} tu) were added and incubated for 2 h at room temperature. After washing with PBST thrice, the wells were incubated with 100 µL of mouse anti-M13 pVIII-HRP antibody conjugate (GE Healthcare, USA) for 30 min at ambient temperature. Tetramethylbenzidine and 2.0 M HCl were added, to detect a signal and to stop the reaction, respectively. Absorbance at 450 nm was measured spectrophotometrically.

Selection of Schistosome Antigen-specific scFv

The library that exhibited the highest binding to each antigen was screened *via* colony PCR using Platinum® Taq DNA polymerase high fidelity (Invitrogen, USA) and primers, LacI Fwd (5'-GGAAAGCCGGCAGTGA -3') and scRev (5'- GGAATTCGGCCCCCGAG -3'). The scFv clones with 800 to 1000 bp amplicon were sequenced at Apical Scientific Sdn. Bhd, Malaysia.

Bioinformatics Analysis

The corresponding sequences of scFv clones were visualized and annotated *via* SnapGene® viewer (GSL Biotech LLC, USA), analyzed using Nucleotide BLAST (BLASTn), and translated *in silico* using Protein BLAST (BLASTp). The level of homology between the nucleotide and translated sequences of scFv clones was determined by multiple sequence alignment using MUSCLE with neighbor-joining as the clustering method in MEGA X (Kumar *et al*. 2018). Complementarity-determining regions (CDRs) and framework regions (FR) numbering of amino acid residues of the scFv clones was performed using abYsis (Swindells *et al.* 2017).

Specificity Testing of scFv

Amplified scFv clones (5 x 10^7 tu) were assessed for binding affinity towards the antigens *via* scFv-phage ELISA, as previously described. Cross-reactivity and specificity of the scFv clones were examined against MBP, bovine plasma proteins (bovine serum albumin and bovine hemoglobin), and bovine lactoferrin.

Statistical Analysis

All data were analyzed using Statistica 13 (TIBCO Software Inc.). The differences between groups were calculated with the one-way analysis of variance (ANOVA) test using Tukey's *post hoc* analysis where significant differences were validated at $p < 0.05$.

RESULTS AND DISCUSSION

Sequencing results and multiple sequence alignment *via* MEGA7 for the CH2 and CH4 gene constructs confirmed their correct insertion in the pET vector. BLASTn analysis suggests that CH2 is a portion of the *S. japonicum* CARP1 (*S. japonicum* Consortium 2009) with 99.52% sequence homology (NCBI Accession No. FN318530). Uniprot shows that *S. japonicum* CARP1 shares homology with cell-cycle and apoptosis regulator 1 (CCAR1; also known as CARP-1) that was originally identified as a regulator of apoptosis signaling and cell proliferation in human breast carcinoma cell lines (Rishi *et al*. 2003), and later shown to be a coactivator to multiple classes of transcription factors such as the tumor suppressor protein p53 (Kim *et al*. 2008) and members of the nuclear receptor superfamily (Kanno *et al*. 2019).

Antigen CH4 is 99% identical to *S. japonicum* SJCHGC06324 protein mRNA, the *S. japonicum* P40 major egg antigen (Sjp40; Hosking *et al.* 2015a, b). Sjp40 or *S. japonicum* heat shock protein 40 is abundantly present in soluble egg antigens and egg secretory proteins, as such is implicated in triggering the host immune response after secretion from eggs into host tissues (Xu *et al*. 2020). Zhou *et al*. (2010) also found that Sjp40 and its antibodies are detectable from the host at a relatively early phase (Day 21 post-infection with *S. japonicum*) and suggested that Sjp40 is a potential antigen

candidate for the early diagnosis of schistosomiasis.

Driguez *et al.* (2015) previously synthesized schistosome antigens CH2 and CH4 *in vitro* at a microarray-scale using a cell-free expression system. However, the antigens were not very pure and in small quantities; therefore, they did not allow isolation of specific antibodies. In the present study, cloning of CH2 and CH4 into a vector with solubility and His_6 purification tags allowed the production of sufficient amounts of purified antigens (Figure 1) at 69.99 mg/mL and 93.31 mg/mL, respectively,

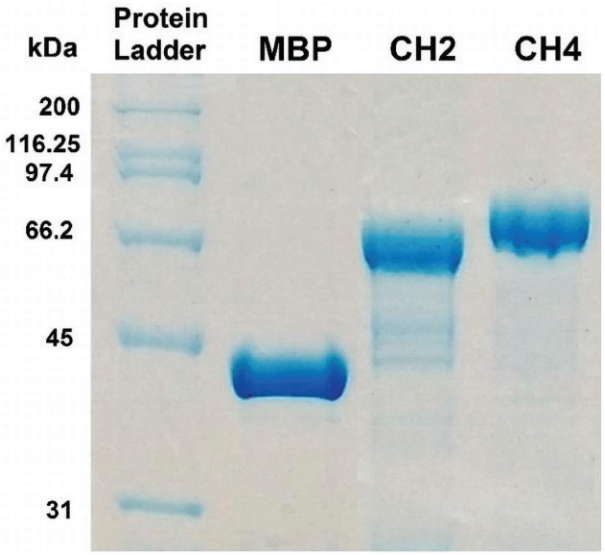

Figure 1. SDS-PAGE analysis of over-expressed and purified schistosome antigens. Proteins were resolved on a 12% polyacrylamide gel under reducing conditions.

in 50-mL expression medium. The yields were sufficient and highly purified for the screening of the scFv libraries.

The identity of the antigens was validated using MS Bridge Prospector and ExPASy's Peptide Mass tool. One hit of CH2 has a mass-to-charge ratio (m/z) of 1107.2814 with a corresponding peptide sequence of "KLAYENVLASKE" and is identical to a hypothetical protein containing DNA-binding SAP domain protein (SJCHGC09111). Two hits of CH4 (m/z = 833.2337 and 1150.6217) correspond to peptide sequence KEMERLR and RGMFALLPMDR, respectively, and were found to be identical to Sjp40 (SJCHGC06324). BLASTp reports also showed that CH4 is homologous to *S. mansoni* Smp40 (*S. mansoni* egg antigen p40), also known as heat shock protein 20, which is one of a number of previously identified soluble egg antigens and soluble worm antigen proteins (Hernandez and Stadecker 1999).

When CH2 was used to interrogate BpLn+A library, the library attained high titer on its two biopanning rounds, inferring that a huge portion of the population of scFv from the adult worm-pre-selected antibody repertoire recognized epitopes of the schistosome protein. On the other hand, significant enrichment was obtained after the

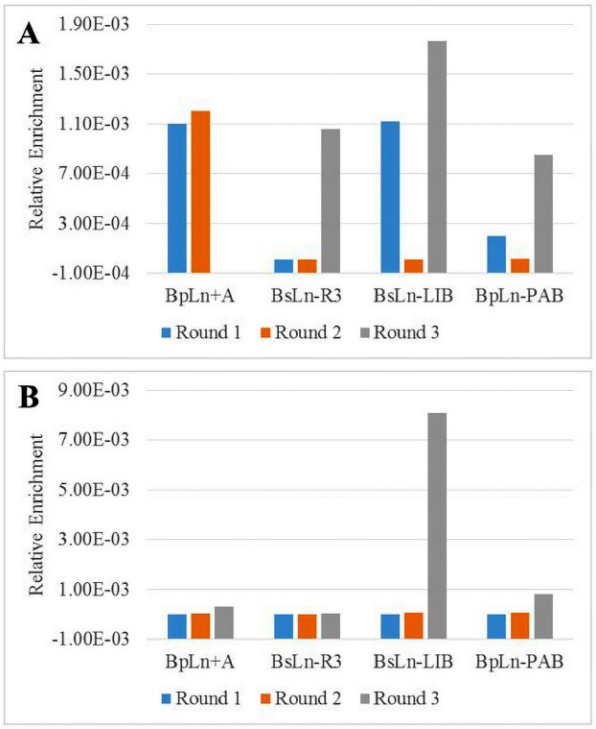

Figure 2. Relative enrichment of immune bovine scFv-phage libraries against recombinant *S. japonicum* DNA-binding SAP domain-containing protein (**A**) and recombinant *S. japonicum* P40 major egg antigen (**B**).

third round of biopanning the BsLn-LIB library using CH4 (Figure 2).

Figure 3 suggests that specific antibodies towards CH2 and CH4 may be obtained from BpLn+A (second panning) and BsLn-LIB (third panning), respectively. To identify the scFv that was bound to the antigens, more than 300 and 500 colonies from BpLn+A and BsLn-LIB, respectively, were screened for the presence of scFv by PCR. Colonies that produced approximately 1-kb amplicon were selected and sequenced for characterization (Hosking *et al.* 2015a, b). For CH4, one candidate scFv (E6; Appendix III) shows significant alignment to the immunoglobulin gamma heavy chain variable region, partial (*Bos taurus*) (ABF48133.1; Kaushik *et al*. 2009) with E-value of 1e–26. Several putative conserved immunoglobulin domains have also been detected in E6, including specific hits IgV and V-set, and a non-specific hit for IgVH. However, when scFv-phage ELISA was performed on this scFv clone, no specific binding was observed as shown by very low absorbance at 450 nm (Appendix IV). Although not cross-reactive with non-schistosome proteins, CH4 binds with E6 as well as

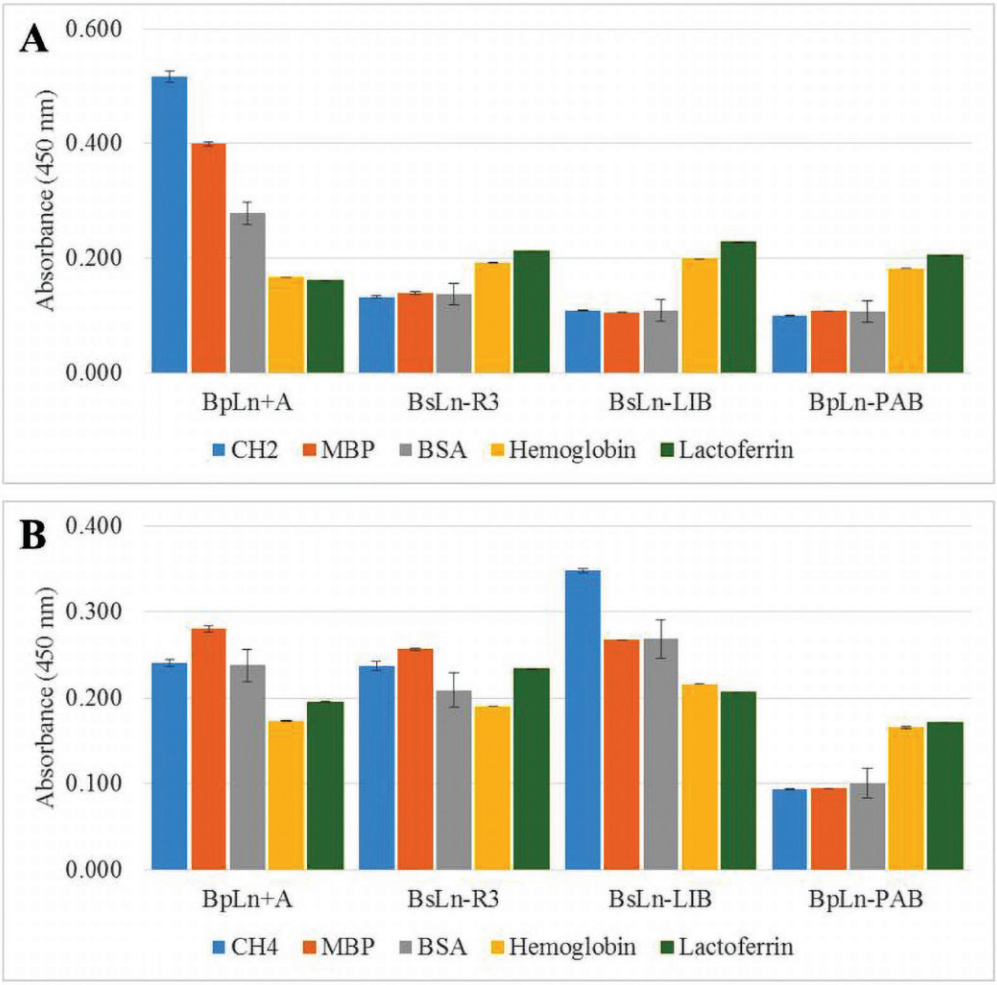

Figure 3. scFv-phage ELISA of libraries enriched with *S. japonicum* DNA-binding SAP domain-containing protein (CH2; **A**) and recombinant *S. japonicum* P40 major egg antigen (CH4; **B**). Results are presented as mean ± standard deviation of three independent trials performed in triplicate.

with the negative controls M13KO7 helper phage and F12, which is a clone with aberrant sequence and with no known V_H and/or V_L fragment, suggesting that the recombinant antigen CH4 lacks specificity. CH4 appears to be a sticky antigen that tends to bind non-specifically.

Examination of F12 and its clones for false positive, target-unrelated peptides (TUPs) using SAROTUP (http://immunet.cn/sarotup/index.html) (Huang *et al*. 2010) revealed similarity of their sequence to MTQAPSV, which is found in a lectin-like oxidized LDL receptor-binding peptide (White *et al.* 2001) and to another sequence, MQNPTQAPSVGL, which binds palladium powder (Frascione *et al*. 2013; Kriplani and Kay 2005). These sequence similarities suggest that the peptide MTQAPS in F12 and its clones is a TUP without any actual affinity towards the target antigen. F12 and its clones may be TUPs that bind to other components of the selection system rather than the desired target (Menendez and Scott 2005), or TUPs that are enriched by proliferation-related pressures (Thomas *et al*. 2010).

For CH2, two distinct scFv clones (A63 and A73) were detected in the BpLn+A library and were tested for affinity and specificity (Figure 4). During scFv-phage ELISA, clone A63 exhibited high significant binding to the antigen when compared with clone A73, M13KO7 helper phage, MBP, bovine serum albumin, hemoglobin, and lactoferrin, suggesting CH2 antigen-specificity of clone A63. When analyzed by SnapGene viewer, clone A63 showed the vector features such as the upstream regulatory elements, PelB signal sequence, FLAG and myc tags, short version of gene III, as well as the Ipp terminator (Figure 5). The Lac promoter, ribosome-binding site, PelB signal sequence, and Ipp terminator were also in place. The scFv insert was in frame with the N-terminal FLAG tag, C-terminal myc tag, and the super short version of gene III of the f1 phage, indicating the scFv that can be displayed by the phage.

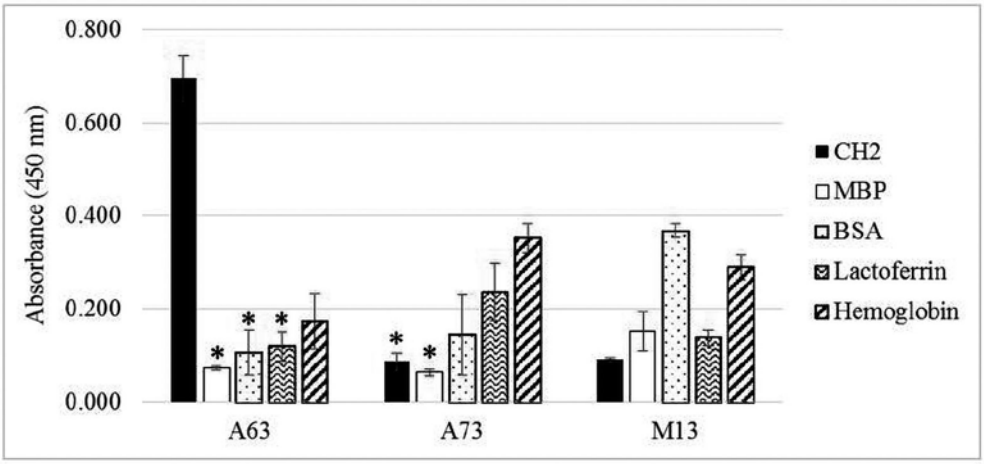

Figure 4. scFv phage ELISA of selected clones against antigens. Asterisk (*) indicates no significant difference between the binding specificity of the antibody (A63 and A73) or M13K07 to a certain antigen at $p > 0.05$. Error bar: mean ± standard deviation. Mean: product of two independent trials done in triplicate. A63 and A73 – candidate antibodies; M13K07 helper phage – negative control.

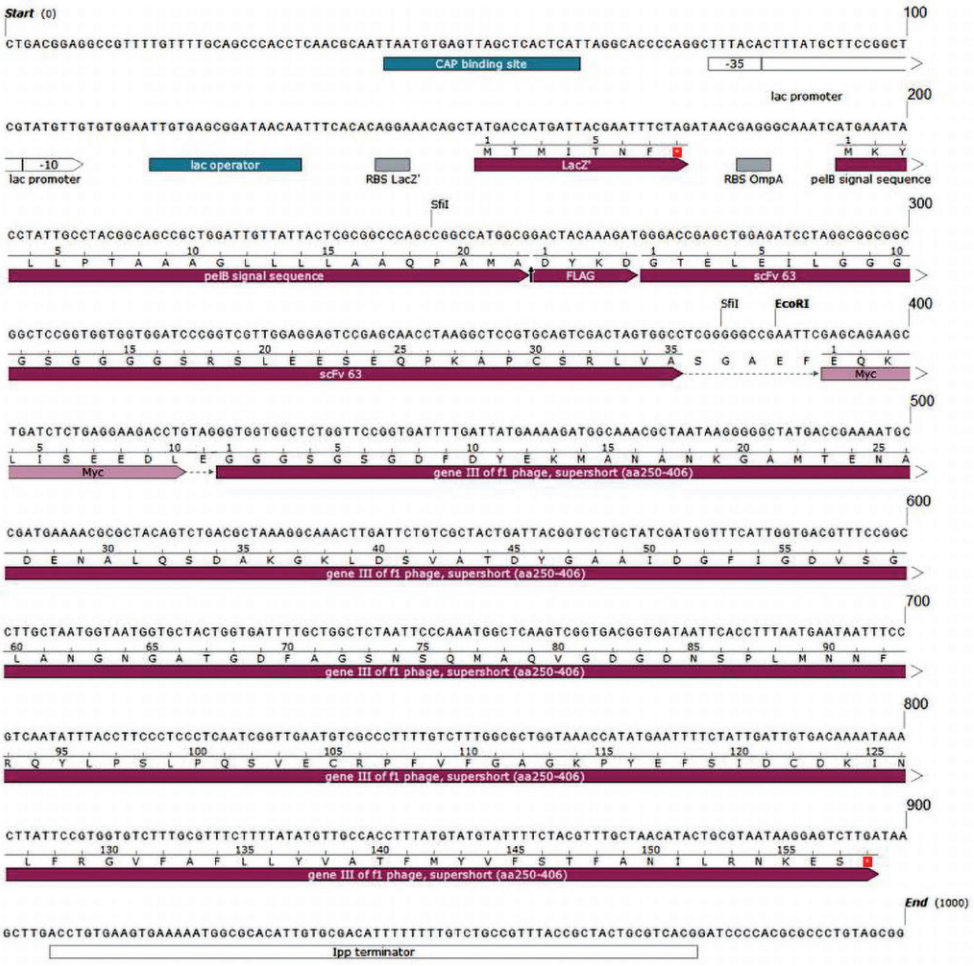

Figure 5. Analysis of A63 sequence by SnapGene® Viewer.

The amber codon (UAG) was also present in the 5' end of myc tag, which is read-through as glutamic acid due to the amber suppressor gene (SupE) in *E. coli* TG1.

However, the scFv region was only 105 bp long (Figure 5), in contrast with those expected in the library where it originated from with scFv sizes ranging from 800–850 bp (Hosking *et al.* 2015a, b). The Gly_4Ser repeat linker was only 30 bp while the candidate V_L and V_H chains were 21 bp and 54 bp, respectively (Figure 5). BLASTn analysis shows certain regions of scFv A63 (Figure 6) aligning with the IgG V_L, Gly_4Ser repeat linker, and IgG V_H sequences of synthetic scFv constructs such as that of AutoH1 scFv fragment (NCBI Accession No. DQ862468), an ovine auto-antibody fragment with a binding affinity towards the ovine small intestinal tissue and *Mycobacterium avium* subsp. *paratuberculosis* (Berger *et al.* 2007). *In silico* analysis suggests that the scFv in A63 is novel. However, its N-terminal sequence aligned with accessions of the rabbit immunoglobulin kappa light chain (Figure 7A) while the C-terminal sequence aligned with both prokaryotic and eukaryotic hypothetical proteins (Figure 7B). Neither FR nor CDR was identified. Similar studies generated single clones of antigen-specific scFv that are not full length. For instance, the G2/clone 15 scFv derived from a human patient suffering from metastatic breast cancer was composed of a full-length human variable heavy chain, a Gly_4Ser repeat linker, and a truncated human variable light Kappa chain yet the clone manifested high binding affinity and specificity towards GD2/GD3 gangliosides (Kotlan *et al.* 2005). Mishra *et al.* (2010) obtained a human antibody-based monoclonal scFv with a 1-mer of Gly_4Ser linker that showed high binding affinity and specificity to murine anti-PSA IgG2A clone 735. Phipps *et al.* (2016) found that the presence of antibiotics as selective agents for bacteria infected with both the scFv-phage library and the helper phage were responsible for the shortening of the scFv sequences at the DNA level as biopanning proceeds from one round to another. However, moderation of the selection pressure did not mitigate such a phenomenon. Even if it does, reducing the concentration of antibiotics in the culture may result in loss of plasmid, thereby decreasing antibody fragment diversity. Currently, such a phenomenon is not completely understood.

Despite its length, it is worthwhile to perform structural studies on scFv A63, which may reveal strategies in designing scFv A63 as a reporter ligand in direct ELISA. The scFv A63 can be cloned and overexpressed in a vector with enzyme conjugates such as alkaline phosphatase or horseradish peroxidase to detect *S. japonicum* schistosomule and adult worm. The ELISA

Score	Expect	Identities	Gaps	Strand
64.4 bits(70)	8e-07	59/71(83%)	3/71(4%)	Plus/Plus

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CDS:                1    G  T  E  L  E  I  L  G  G  G  G  S  G  G  G  G  S   R   S   L  E  E  S
Query: scFv A63     1    GGGACCGAGCTGGAGATCCTAGGCGGCGGCGGCTCCGGTGGTGGTGGATCCC-GGT-CG-TTGGAGGAGTC  68
                         || ||| |||| ||||||  ||| || ||||||||||||||||||||||| ||| ||  || |||||||
Sbjct: DQ862468    391  GGAACCAAGCTAGAGATCAAAGGTGGTGGCGGCTCCGGTGGTGGTGGATCCCAGGTGCGACTGCAGGAGTC  461
CDS                 131  G  T  K  L  E  I  K  G  G  G  G  S  G  G  G  G  S  Q  V  R  L  Q  E  S
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Figure 6. BLASTn analysis of scFv A63. Pairwise alignment with the synthetic scFv construct AutoH1 scFv fragment (NCBI accession no. DQ862468), which shows the highest percentage identity with scFv A63 and E-value nearest to 1.

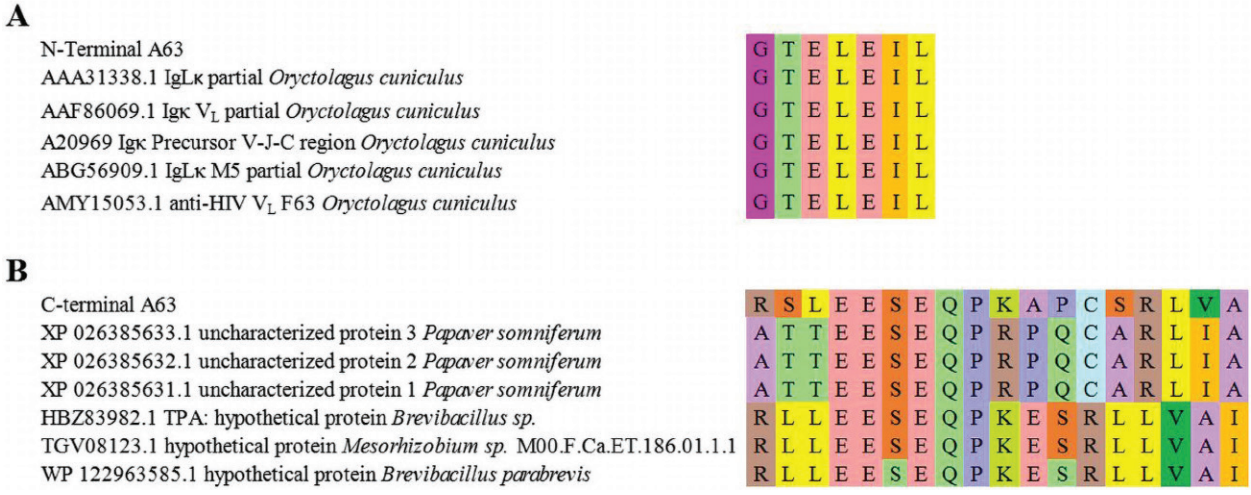

Figure 7. MUSCLE alignment of the N- (**A**) and C- (**B**) termini of the scFv in A63 excluding the Gly_4Ser linker.

can be used to diagnose schistosomiasis in water buffalo and later humanized for applications in humans. Several approaches have been described for such purpose, which include rational and empirical methods (Almagro and Fransson 2008). In rational methods such as CDR grafting, resurfacing, superhumanization, and human string content optimization, a small set of variants of antibody structure and/or sequence is generated and then assessed on their binding or any other characteristic of interest. In empirical methods such as framework libraries, guided selection, framework shuffling, and humaneering, large combinatorial libraries are generated that are then used in selecting the desired variants by enrichment technologies such as phage, ribosome, or yeast display, or by high throughput screening techniques (Almagro and Fransson 2008).

Results in this study also suggest the need to construct a bigger library with higher diversity to increase the probability of finding an antibody against a schistosome antigen with sufficiently high affinity.

CONCLUSION

Schistosome-specific antigens were overexpressed and purified by employing a vector with solubility and polyhistidine purification tags, and used to isolate and characterize schistosome-specific scFv from the immune bovine antibody library. The specific scFv may be designed as a reporter ligand in direct ELISA for specific, simple, and inexpensive diagnosis of schistosomiasis.

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NOTE ON APPENDICES

The complete appendices section of the study is accessible at http://philjournalsci.dost.gov.ph

STATEMENT ON CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

REFERENCES

ALMAGRO JC, FRANSSON J. 2008. Humanization of antibodies. Front Biosci 13(1): 1619–1633.

BADR M. 1981. Detection of bladder cancer. In: Bladder Cancer. El-Bolkany C ed. Cairo, Egypt: Al-Ahram Press. 1p.

BERGER S, BANNANTINE JP, GRIFFIN FT. 2007. Autoreactive antibodies are present in sheep with Johne's disease and cross-react with *Mycobacterium avium* subsp. *paratuberculosis* antigens. Microbes Infect 9(8): 963–970.

BURKE MJ, STOCKLEY PG, BOYES J. 2020. Broadly neutralizing bovine antibodies: highly effective new tools against evasive pathogens? Viruses 12(4): 473.

DEB GK, NAHAR TN, DURAN PG, PRESICCE GA. 2016. Safe and sustainable traditional production: the water buffalo in Asia. Front Environ Sci 4(38): 1–7.

DRIGUEZ P, DOOLAN D, MOLINA D, LOUKAS A, TRIEU A, FELGNER P *et al*. 2015. Protein microarrays for parasite antigen discovery. In: Parasite Genomics Protocols. Peacock C ed. New York: Springer. p. 221–233.

FRASCIONE N, CODINA-BARRIOS A, BASSINDALE AR, TAYLOR PG. 2013. Enhancing *in vitro* selection techniques to assist the discovery, understanding and use of inorganic binding peptides. Dalton T 42(28): 10337–10346.

HAAKENSON JK, HUANG R, SMIDER VV. 2018. Diversity in the cow ultralong CDR H3 antibody repertoire. Front Immunol 9(1262): 1–7.

HERNANDEZ HJ, STADECKER MJ. 1999. Elucidation and role of critical residues of immunodominant peptide associated with T cell-mediated parasitic disease. J Immunol 163(7): 3877–3882.

HOSKING CG, MCWILLIAM HEG, DRIGUEZ P, PIEDRAFITA D, LI Y, MCMANUS DP, ILAG LL, MEEUSEN ENT, DE VEER MJ. 2015a. Generation of a novel bacteriophage library displaying scFv antibody fragments from the natural buffalo host to identify antigens from adult *Schistosoma japonicum* for diagnostic development. PLoS Negl Trop Dis 9(12): e0004280.

HOSKING CG, DRIGUEZ P, MCWILLIAM HEG, ILAG LL, GLADMAN S, LI Y, PIEDRAFITA D, MCMANUS DP, MEEUSEN ENT, DE VEER MJ. 2015b. Using the local immune response from the natural buffalo host to generate an antibody fragment library that binds the early larval stages of *Schistosoma japonicum*. Int J Paras 45(11): 729–740.

HUANG J, RU B, LI S, LIN H, GUO FB. 2010. SAROTUP: scanner and reporter of target-unrelated peptides. J Biomed Biotechnol 2010: 101932.

KANNO Y, ZHAO S, YAMASHITA N, SAITO N, UJIIE A, IIJIMA R, KIKAWA N, NEMOTO K, INOUYE Y. 2019. Cell cycle and apoptosis regulator 1, CCAR1, regulates enhancer-dependent nuclear receptor CAR transactivation. Mol Pharmacol 95(1): 120–126.

KATZ N, CHAVES A, PELLEGRINO J. 1972. A simple device for quantitative stool thick smear technique in *Schistosomiasis mansoni*. Rev Inst Med Trop Sao Paulo 14(6): 397–400.

KAUSHIK AK, KEHRLI JR ME, KURTZ A, NG S, KOTI M, SHOJAEI F, SAINI SS. 2009. Somatic hypermutations and isotype restricted exceptionally long CDR3H contribute to antibody diversification in cattle. Vet Immunol Immunop 127(1–2): 106–113.

KIM JH, YANG ROEDER RG, WOOJIN A, STALLCUP MR. 2008. CCAR1, a key regulator of mediator complex recruitment to nuclear transcription complexes. Mol Cell 31(4): 510–519.

KOTLAN B, SIMSA P, TEILLAUD J-L, FRIDMAN WH, TOTH J, MCKNIGHT M, GLASSY MC. 2005. Novel ganglioside antigen identified by B cells in human medullary breast carcinomas: the proof of principle concerning the tumor-infiltrating B lymphocytes. J Immunol 175(4): 2278–2285.

KRIPLANI U, KAY BK. 2005. Selecting peptides for use in nanoscale materials using phage-displayed combinatorial peptide libraries. Current Opin Biotech 16(4): 470–475.

KUMAR S, STECHER G, LI M, KNYAZ C, TAMURA K. 2018. MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms. Mol Biol Evol 35(6): 1547–1549.

MACALANDA AMC, ANGELES JMM, MOENDEG KJ, DANG ATM, HIGUCHI L, INOUE N, XUAN X, KIRINOKI M, CHIGUSA Y, LEONARDO LR, VILLACORTE EA, RIVERA PT, GOTO Y, KAWAZU S-I. 2018. Evaluation of *Schistosoma japonicum* thioredoxin peroxidase-1 as a potential circulating antigen target for the diagnosis of Asian schistosomiasis. J Vet Med Sci 80(1): 156–163.

MACALANDA AMC, ANGELES JMM, MOENDEG KJ, DANG-TRINH M-A, HIGUCHI L, KIRINOKI M, CHIGUSA Y, LEONARDO LR, VILLACORTE EA, RIVERA PT, GOTO Y KAWAZU S-I. 2019. *Schistosoma japonicum* cathepsin B as potential diagnostic antigen for Asian zoonotic schistosomiasis. Parasitol Res 118(9): 2601–2608.

MCMANUS DP, GRAY DJ, LI Y, FENG Z, WILLIAMS GM, STEWART D, REY-LADINO J, ROSS AG. 2010. Schistosomiasis in the People's Republic of China: the era of the Three Gorges Dam. Clin Microbiol Rev 23(2): 442–466.

MENENDEZ A, SCOTT JK. 2005. The nature of target-unrelated peptides recovered in the screening of phage-displayed random peptide libraries with antibodies. Anal Biochem 336(2): 145–157.

MISHRA R, VON DER OHE M, SCHULZE C, BIAN S, MAKHINA T, LOERS G, KLEENE R, SCHACHNER M. 2010. Functional role of the interaction between polysialic acid and extracellular histone H1. J Neuro Sci 30(37): 12400–12413.

PHIPPS ML, LILLO AM, SHOU YM, SCHMIDT EN, PAAVOLA CD, NARANJO L, BEMDICH S, SWANSON BI, BRADBURY ARM, MARTINEZ JS. 2016. Beyond helper phage: using "helper cells" to select peptide affinity ligands. PLoS ONE 11(9): e0160940.

RISHI AK, ZHANG L, BOYANAPALLI M, WALI A, MOHAMMAD RM, YU Y, FONTANA JA, HATFIELD JS, DAWSON MI, MAJUMDAR APN, REICHERT U. 2003. Identification and characterization of a cell cycle and apoptosis regulatory protein-1 as a novel mediator of apoptosis signaling by retinoid CD437. J Biol Chem 278(35): 33422–33435.

[*S. japonicum* Consortium] *Schistosoma japonicum* Genome Sequencing and Functional Analysis Consortium. 2009. The *Schistosoma japonicum* genome reveals features of host-parasite interplay. Nature 460(7253): 345–351.

SWINDELLS MB, PORTER CT, COUCH M, HURST J, ABHINANDAN KR, NIELSEN JH, MACINDOE G, HETHERINGTON J, MARTIN AC. 2017. abYsis: integrated antibody sequence and structure-management, analysis, and prediction. J Mol Bio 429(3): 356–364.

THOMAS WD, GOLOMB M, SMITH GP. 2010. Corruption of phage display libraries by target-unrelated clones: diagnosis and countermeasures. Anal Biochem 407(2): 237–240.

WHITE SJ, NICKLIN SA, SAWAMURA T, BAKER AH. 2001. Identification of peptides that target the endothelial cell—specific LOX-1 receptor. Hypertension 37(2): 449–455.

XU Z, JI M, LI C, DU X, HU W, MCMANUS DP, YOU H. 2020. A biological and immunological characterization of *Schistosoma japonicum* Heat Shock Proteins 40 and 90α. Int J Mol Sci 21(11): 4034.

ZHOU XH, WU JY, HUANG XQ, KUNNON SP, ZHU XQ, CHEN XG. 2010. Identification and characterization of *Schistosoma japonicum* Sjp40, a potential antigen candidate for the early diagnosis of schistosomiasis. Diagn Microbiol Infect Dis 67(4): 337–345.

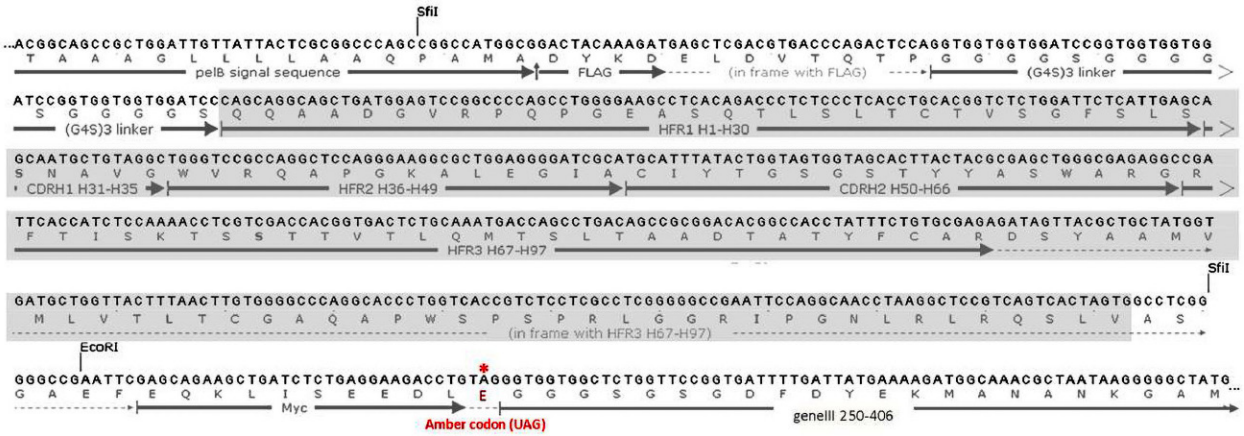
APPENDICES

Appendix I. Primers used to amplify the schistosome antigens CH2 and CH4.

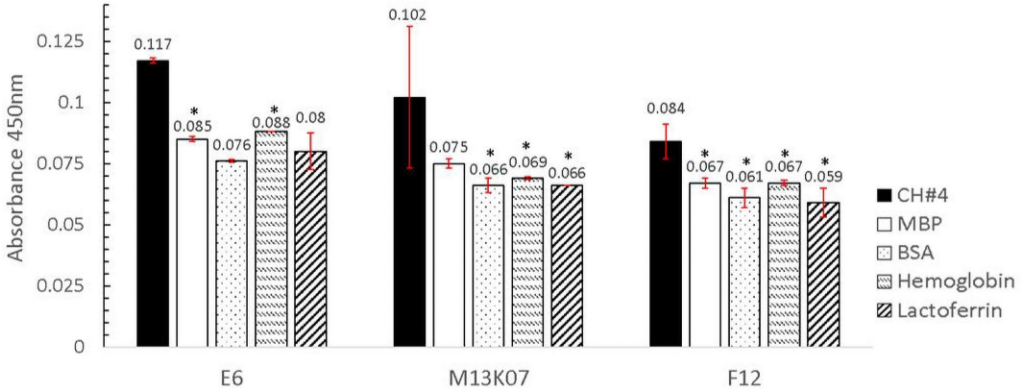
Target antigen	Primer name	Primer sequence (5' → 3')
CH2	CH2F	**TACTTCCAATCCAATGCA**ATGAGCAAACCGTTTCATC
	CH2R	**TTATCCACTTCCAATGTTA**TTACTCCTCTTCTTGAATTGCC
CH4	CH4F	**TACTTCCAATCCAATGCA**ATGTCAGGTACACATCAAAACC
	CH4R	**TTATCCACTTCCAATGTTA**CTAATGAGCGATTTCGTGTTG

Appendix II. List of water buffalo scFv-phage libraries.

Library name	Description
BpLn+A	Buffalo portal lymph node scFv-phage library selected against *S. japonicum* adult worms (Hosking *et al*. 2015a)
BsLn-R3	Buffalo skin lymph node scFv-phage library selected against *S. japonicum* schistosomula (Hosking *et al*. 2015b)
BsLn-LIB	Buffalo portal lymph node scFv-phage library unpanned (Hosking e*t al*. 2015b)
BpLn-PAB	Buffalo portal lymph node scFv-phage library selected against mouse infected sera (Hosking *et al*. 2015b)



Appendix III. Sequence analysis of the scFv clone E6. CDR numbering and regions of E6 (501 bp) displaying 1–97 (highlighted in grey) of 138 heavy chain amino acid residues using Kabat numbering scheme. Upstream: pelB signal sequence, N-terminal *Sfi*I site, and FLAG tag. In-frame with FLAG tag is the glycine-serine linker or G4S)3. Downstream: *Sfi*I and *Eco*RI restriction sites, Myc tag, and the start of genelll (250–406). The amber codon (UAG) represented by asterisk (*) is read-through as glutamic acid. Amino acids are auto-translation of standard genetic code by SnapGene® viewer (GSL Biotech LLC, USA).



Appendix IV. Binding affinity and specificity of CH4-enriched scFv clone E6 via ELISA. Asterisk (*) indicates no significant difference between the binding specificity of the antibody (E6, M13K07, or F12) to a certain antigen, at $p > 0.05$. Error bar: mean ± standard deviation. Mean: product of two independent trials done in triplicate. E6 – candidate antibody (Ab); M13K07 helper phage – negative control; F12 – candidate Ab; representative of the Ab with the highest number of clones isolated from the enriched library.