Supplemental Methods

Ethical Statement

Ethics approval for collection of samples from individuals from Thailand and Lao PDR was obtained from the Khon Kaen University Ethics Committee for Human Research (HE611507, HE631300, HE641114, HE641242 and HE664044). Clonorchiasis serum samples were approved by the Medical Ethics Committee of the National Institute of Infectious Diseases, Tokyo, Japan (Nos. 177 and 589). Collection and use of samples from individuals from Australia was approved by the James Cook University Human Research Ethics Committee under approval H8523. A de-identified panel of healthy control sera for protein microarray serology was collected at University of California Irvine General Clinical Research Center (GCRC), now Center for Clinical Research, under an approved protocol HS# 2007-5896. Excretory/secretory products of *O. viverrini* were obtained from flukes passaged through hamsters with approval from the Animal Ethics Committee of Khon Kaen University and according to the Ethics of Animal Experimentation of the National Research Council of Thailand (AEMDKKU 002/2018).

***O. viverrini* secreted proteomes and selection of proteins to construct the proteome microarray**

A total of 20 µg of *O. viverrini* soluble excretory/secretory (ES) products was dissolved in 50 mM ammonium bicarbonate (NH_4CO_3) and 20 mM dithiothreitol (DTT), followed by incubation at 65°C for 60 min. Alkylation was carried out by adding iodoacetamide (IAM) to a final concentration of 55 mM and incubating the mixture in the dark at room temperature for 40 minutes. Subsequently, a final incubation with 100 mM DTT was conducted at room temperature before adding 1 µg of trypsin and incubating the mixture at 37°C overnight. Thereafter, peptides were desalted using a Zip-Tip (Millipore Sigma, Burlington, MA, USA) and analysed by mass spectrometry using a Triple TOF 5600+ mass spectrometer (AB SCIEX) with a nano electrospray ion source coupled to a LC-MS/MS on a Shimadzu Prominance Nano HPLC as described.[1] Briefly, 15 µl of digested peptides was injected onto a 50 mm x 300 µm C18 trap column (Agilent Technologies, Santa Clara, CA, USA) at a constant flow rate (60 µl/min). Following desalting, the trap column was connected to a 150 mm x 100 µm 300SBC18, 3.5 µm nano HPLC analytical column (Agilent Technologies) and peptides were separated using a linear gradient of 2–40% solvent B (90% acetonitrile, 0.1% formic acid in water) over 80 minutes at a flow rate of 500 nL/min. This was followed by a 6-minute wash at 2% solvent B and a steeper gradient increasing from 40% to 80% solvent B over 10 minutes. Solvent B was maintained at 80% for 5 minutes to wash the column, and then reduced to 2% for re-equilibration before the next sample injection. The mass spectrometer settings included an ionspray voltage of 2,200V, a declustering potential of 100 V, a curtain gas flow of 25, a nebuliser gas 1 (GS1) of 12, and an interface heater temperature of 150°C. The mass spectrometer acquired 250 ms full scan TOF-MS data, followed by twenty 250 ms full scan product ion data in Information Dependent Acquisition (IDA) mode. Full scan TOF-MS data was collected over the mass range of 300–1600, and for product ion ms/ms, the range was 80–1600. Ions detected in the TOF-MS scan that exceeded a threshold of 150 counts and had a charge state of +2 to +5 triggered the acquisition of product ion ms/ms spectra of the 20 most intense ions. Data acquisition was managed using Analyst TF 1.6.1 (AB SCIEX).

Newly generated mass spectrometry data from *O. viverrini* ES products (PRIDE project PXD056031) and previously reported mass spectrometry data from *O. viverrini* extracellular vesicles (EVs) (PRIDE projects PXD020356 and PXD020345), including exosome-like vesicles (ELVs) and microvesicles (MVs)[2,3] were reanalysed using a proteomics database built based on newly generated genomic data (GenBank PRJNA230518) appended to the common Repository of adventitious proteins (cRAP) contaminants database. Other sequences including T265_16380, T265_07410 and T265_10096, and mucinase Ov-M60[4] were added to generate a final database. Analysis was performed using SearchGUI v 3.3.15[5] using X!Tandem v2015.12.15.2,[6] MS-GF+ (v2018.04.09)[7] and Tide[8] using the following parameters: trypsin (2 miss cleavages) as enzyme, carbamidomethylation of C as fixed modification, deamidation of N and Q and oxidation of M as variable modifications, 20 ppm as precursor mass tolerance and 0.2 Da as

fragment mass tolerance and precursor charges +2 to +4. Data were further processed with peptideShaker 1.16.40[9] to generate a report file. PSMs, peptides and proteins were validated at a 1.0% False Discovery Rate (FDR) estimated using the decoy hit distribution.

Proteins positively identified with ≥2 peptides in all three samples (ESP products, ELVs and MVs) were included in the final list of selected candidates. Additionally, proteins found in two of the samples and manually curated and selected based on homology with diagnostic candidates in other trematodes were also included. Proteins were searched for transmembrane domains and signal peptides using TMHMM - 2.0. and SignalP v4.0, respectively. Signal peptides were removed from the final sequences. For cloning, ORFs corresponding to proteins >120 kDa were split into ORFs of 1779 bp. The ES products and MV mass spectrometry proteomics data have been deposited in the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifiers PXD056031 and PXD02035, respectively.

Microarray assembly and printing

Open reading frames for all selected proteins (table S1) were codon optimised for expression in *Escherichia coli* and commercially synthesised and cloned in pXI vector (Twist Bioscience, San Francisco, CA, USA). DH5α competent cells were transformed using synthesised cloned genes for plasmid isolation. Proteins encoded by each purified plasmid were expressed *in vitro* (RTS 100 *E. coli* HY kit – Biotechrabbit, Berlin, Germany) according to the manufacturer's instructions and printed onto eight-pad nitrocellulose-coated AVID glass slides (Grace Biolabs, Bend, OR, USA) with an Omnigrid 100 microarray printer (Genomic Solutions, Ann Arbor, MI, USA). Vector containing no insert was similarly "expressed" and printed (in multiple locations) to serve as a negative control and multiple empty spots were left on each pad to serve as background controls. Purified human IgG and Ig-subclasses (IgG1 and IgG4), anti-human IgG and parasite extracts (*O. viverrini* adult stage ES products[10] and somatic antigen[11]) were also printed as positive controls. Expression quality control was assessed by detection of N-terminal HIS tags as previously described[12].

Recombinant proteins

The nine most reactive antigens from the proteome array were selected for progression to recombinant expression in *E. coli*. Open reading frames were cloned into the *Nde*I and *Xho*I sites of the pET41a *E. coli* expression vector, such that the vector's N-terminal GST tag was removed, to prevent the detection of non-specific immune responses upon ICT validation of recombinant proteins. Pilot expression assays showed P1-4 to be expressed in soluble form with P5-9 expressed in insoluble form. Expression and purification using immobilised metal ion affinity chromatography (IMAC) of soluble proteins was carried out in *E. coli* BL21(DE3) as described by us elsewhere.[13] Insoluble proteins were expressed as inclusion bodies and purified as described with the addition of 6M urea creating denaturing conditions. Fractions containing recombinant proteins (as determined by SDS-PAGE) were pooled and concentrated using Amicon Ultra-15 centrifugal devices with a 3 kDa MWCO and quantified using the Pierce BCA Protein Assay (ThermoFisher Scientific, Waltham, MA, USA. The final concentration of the proteins was adjusted to 2 mg/ml, and proteins were aliquoted and stored at -80°C.

Pilot development of PoC-ICTs

The recombinant proteins were successfully expressed and purified for printing on ICTs at a concentration of 2 mg/ml in PBS: P1 (OON19686.1), and 4 antigens at 2 mg/ml in 6M urea/PBS: P5 (Ov-M60-2); P6 (OON17288.1_C); P8 (OON14063.1); and P9 (OON23642.1). ICT components for P1, P5, P6, P8 and P9 antigens were laminated in five layers: (1) modified backing card (paper lower cassette), (2) nitrocellulose membrane (Sartorius Stedim Biotech SA, Goettingen, Germany) containing a recombinant antigen line (T-line) and anti-mouse IgG antibody (Lampire Biological Laboratories, Pipersville, PA, USA) line (C-line), (3) conjugated pad of antibody-labeled gold nanoparticle, (4) sample

pad (Sigma Millipore) and (5) absorbent pad (Whatman Schleicher & Schuell, Dassel, Germany). The laminate was cut into strips of 0.4 cm in length using a guillotine. The test strip laminate was inserted into a plastic cassette cartridge, and the cassette was closed with a cover (Adtec Inc, Oita, Japan). The nitrocellulose membrane was coated with each of the recombinant proteins at the T-line (at each concentration of 0.8 µg protein/line) and anti-mouse IgG antibody as C-line, using the XYZ3000 Dispensing Platform (BioDot Inc., Irvine, CA, USA), at a flow rate of 0.1 µL/mm. Monoclonal anti-human IgG or IgG4 antibody-labelled gold nanoparticles (Kestrel BioSciences Co., Pathumthani, Thailand) were sprayed onto a glass microfiber filter (GF33; Whatman Schleicher & Schuell, Germany) at a flow rate of 1 µL/mm to produce the conjugated pad.

ICT optimization and screening

The cassettes were optimized for antibody (IgG and IgG4) detection of P1-, P5-, P6-, P8- and P9-containing ICTs. Briefly, 5 µL of each diluted serum sample in running buffer was applied onto a sample well (S). Next, 60 µL of running buffer was applied into the buffer well (B). ICT results were read at 15 min with a naked eye and judged as positive or negative by reference to a color card, with the cutoff for positivity defined. The appearance of red bands at the T-line and the C-line were judged as positive, whereas only the C-line appeared in negative cases. In the absence of the red band at the C-line, the test was determined to be invalid. Optimal conditions for ICT detection are shown in table S3 and a schematic outlining the methodology is shown in figure S1. The overall study design is depicted in figure 1.

1. Sotillo J, Pearson MS, Becker L, et al. In-depth proteomic characterization of *Schistosoma haematobium*: Towards the development of new tools for elimination. *PLoS Negl Trop Dis* 2019; **13**(5): e0007362.
2. Chaiyadet S, Sotillo J, Krueajampa W, et al. Silencing of *Opisthorchis viverrini* tetraspanin gene expression results in reduced secretion of extracellular vesicles. *Front Cell Infect Microbiol* 2022; **12**: 827521.
3. Mulvenna J, Sripa B, Brindley PJ, et al. The secreted and surface proteomes of the adult stage of the carcinogenic human liver fluke *Opisthorchis viverrini*. *Proteomics* 2010; **10**(5): 1063-78.
4. Ta BTT, Nguyen DL, Jala I, et al. Identification, recombinant protein production, and functional analysis of a M60-like metallopeptidase, secreted by the liver fluke *Opisthorchis viverrini*. *Parasitol Int* 2020; **75**: 102050.
5. Vaudel M, Barsnes H, Berven FS, Sickmann A, Martens L. SearchGUI: An open-source graphical user interface for simultaneous OMSSA and X!Tandem searches. *Proteomics* 2011; **11**(5): 996-9.
6. Craig R, Beavis RC. TANDEM: matching proteins with tandem mass spectra. *Bioinformatics* 2004; **20**(9): 1466-7.
7. Kim S, Pevzner PA. MS-GF+ makes progress towards a universal database search tool for proteomics. *Nat Commun* 2014; **5**: 5277.
8. Diament BJ, Noble WS. Faster SEQUEST searching for peptide identification from tandem mass spectra. *J Proteome Res* 2011; **10**(9): 3871-9.
9. Vaudel M, Burkhart JM, Zahedi RP, et al. PeptideShaker enables reanalysis of MS-derived proteomics data sets. *Nat Biotechnol* 2015; **33**(1): 22-4.
10. Sripa B, Kaewkes S. Localisation of parasite antigens and inflammatory responses in experimental opisthorchiasis. *Int J Parasitol* 2000; **30**(6): 735-40.
11. Intapan PM, Maleewong W. *Opisthorchis viverrini*: influence of maternal infection in hamsters on offspring infected with homologous parasite and their IgG antibody response. *Exp Parasitol* 2006; **113**(2): 67-74.

12. Gaze S, Driguez P, Pearson MS, et al. An immunomics approach to schistosome antigen discovery: antibody signatures of naturally resistant and chronically infected individuals from endemic areas. *PLoS Pathog* 2014; **10**(3): e1004033.
13. Pearson MS, Tedla BA, Mekonnen GG, et al. Immunomics-guided discovery of serum and urine antibodies for diagnosing urogenital schistosomiasis: a biomarker identification study. *Lancet Microbe* 2021; **2**(11): e617-e26.