

Multi-epitope protein production and its application in the diagnosis of opisthorchiasis



Abstract

Background Opisthorchiasis and cholangiocarcinoma (CCA) continue to be public health concerns in many Southeast Asian countries. Although the prevalence of opisthorchiasis is declining, reported cases tend to have a lightintensity infection. Therefore, early detection by using sensitive methods is necessary. Several sensitive methods have been developed to detect opisthorchiasis. The immunological detection of antigenic proteins has been proposed as a sensitive method for examining opisthorchiasis.

Methods The *Opisthorchis viverrini* antigenic proteins, including cathepsin B (OvCB), asparaginyl endopeptidase (OvAEP), and cathepsin F (OvCF), were used to construct multi-antigenic proteins. The protein sequences of OvCB, OvAEP, and OvCF, with a high probability of B cell epitopes, were selected using BepiPred 1.0 and the IEDB Analysis Resource. These protein fragments were combined to form OvCB_OvAEP_OvCF recombinant DNA, which was then used to produce a recombinant protein in *Escherichia coli* strain BL21(DE3). The potency of the recombinant protein as a diagnostic target for opisthorchiasis was assessed using immunoblotting and compared with that of the gold standard method, the modified formalin-ether concentration technique.

Results The recombinant OvCB_OvAEP_OvCF protein showed strong reactivity with total immunoglobulin G (IgG) antibodies against light-intensity *O. viverrini* infections in the endemic areas. Consequently, a high sensitivity (100%) for diagnosing opisthorchiasis was reported. However, cross-reactivity with sera from other helminth and protozoan infections (including taeniasis, strongyloidiasis, giardiasis, *E. coli* infection, enterobiasis, and mixed infection of *Echinostome* spp. and *Taenia* spp.) and no reactivity with sera from patients with non-parasitic infections led to a reduced specificity of 78.4%. In addition, the false negative rate (FNR), false positive rate (FPR), positive predictive value (PPV), negative predictive value (NPV), and diagnostic accuracy were 0%, 21.6%, 81.4%, 100%, and 88.9%, respectively.

Conclusions The high sensitivity of the recombinant OvCB_OvAEP_OvCF protein in detecting opisthorchiasis demonstrates its potential as an opisthorchiasis screening target. Nonetheless, research on reducing cross-reactivity should be undertaken by detecting other antibodies in other sample types, such as saliva, urine, and feces.

Keywords Multi-antigenic protein, B cell epitopes, Immunoblotting, Opisthorchiasis

*Correspondence:

Jittiyawadee Sripa

jittiyawadee.s@ubu.ac.th

¹ College of Medicine and Public Health, Ubon Ratchathani University, Warinchamrap 34190, Ubon Ratchathani, Thailand

² Research Group for Biomedical Research and Innovative Development (RG-BRID), College of Medicine and Public Health, Ubon Ratchathani University, Warinchamrap 34190, Ubon Ratchathani, Thailand

Background

Opisthorchiasis is a major public health concern in several Southeast Asian countries. The prevalence of opisthorchiasis has decreased in many regions because of intensive prevention and control programs, mass stool examinations, and drug treatments; however, most reported cases have shifted to light infections [1-4]. Thus, a sensitive and specific examination is required



© The Author(s) 2024. **Open Access** This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit http://creativecommons.org/licenses/by/4.0/. The Creative Commons Public Domain Dedication waiver (http://creativecommons.org/publicdomain/zero/1.0/) applies to the data made available in this article, unless otherwise stated in a credit line to the data.

for the early diagnosis of opisthorchiasis to ensure early chemotherapy and behavioral modifications in patients.

Molecular and immunological techniques have been investigated as alternative methods to overcome the limitations of traditional microscopic stool examinations, including low sensitivity [5], mistaking small intestinal flukes [6], and the inconvenience of fecal collection, which has prompted several residents to deny involvement in stool examinations [7-9].

Identifying strong reactivity between antigenic antigens and specific antibodies related to *Opisthorchis viverrini* infection is beneficial for diagnosing opisthorchiasis. Several antigenic antigens related to *O. viverrini*, such as snail antigens [10–13], *O. viverrini* excretory/secretory products (ES), crude antigens, and other secreted proteins, have been examined as diagnostic targets. Among the well-established antigens, secreted proteins such as cathepsin B (OvCB) [14], cathepsin F (OvCF) [15], and asparaginyl endopeptidase (OvAEP) [16] have been detected in ES and crude somatic extracts of *O. viverrini*. Thus, the host immune system can be exposed to OvCB, OvAEP, or OvCF to induce a humoral immune response.

The potential of OvCB, OvAEP, and OvCF to induce humoral immune responses and their high immunogenicity have been demonstrated by the strong reactivity between recombinant proteins and specific antibodies against *O. viverrini* infection. This strong immunological reaction indicated the presence of B-cell epitopes in the protein sequences of OvCB, OvAEP, and OvCF. Thus, modifying a single protein by combining it with OvCB, OvAEP, and OvCF protein fragments that represent a high epitope to B cells could increase the immunogenicity of the protein target and the sensitivity of immunological detection methods.

Thus, in this study, protein sequences from OvCB, OvAEP, and OvCF, which represent high-probability of B-cell epitopes, were selected to construct a multiepitope recombinant protein. Subsequently, the efficacy of the modified recombinant protein in the diagnosis of opisthorchiasis was tested. Human negative and positive parasitic infection sera, including opisthorchiasis and other helminth and protozoan infections, were used to test and establish immunoblotting. The sensitivity and specificity of the test were evaluated and compared with those of the gold standard method, the modified formalin–ether concentration technique (m-FECT).

Methods

Human sera

The parasitic infection sera used in this study were obtained from individuals with helminth eggs, larvae, and protozoan cysts detected in their feces using m-FECT. The individual parasitic infections were opisthorchiasis (35 cases), taeniasis (4 cases), strongyloidiasis (5 cases), hookworm infection (3 cases), giardiasis (3 cases), Entamoeba coli infection (2 cases), enterobiasis (1 case), trichuriasis (1 case), and mixed infections with Echinostome spp. and Taenia spp. (1 case). Individual negative sera (17 sera) obtained from subjects who lived in endemic areas and were negative for parasitic infection with m-FECT were included to examine the specificity and accuracy of the diagnosis. The intensity of helminth infections, including opisthorchiasis, hookworm infection, trichuriasis, and echinostomiasis, was low. All procedures involving human participants were performed in accordance with the Declaration of Helsinki. The procedures used in this study were reviewed and approved by the Human Ethics Committee of Ubon Ratchathani University (UBU-REC-46-2563).

B-cell epitopes prediction

The amino acid sequences of *O. viverrini* cathepsin B; OvCB (GenBank accession no. GQ303559.1), asparaginyl endopeptidase, or legumain; OvAEP (GenBank accession no. DQ402101.1) and cathepsin F or cysteine protease; and OvCF (GenBank accession no. AY821800.1), retrieved from the NCBI database, were used to predict linear B-cell epitopes using BepiPred 1.0 and the IEDB Analysis Resource. The peptide fragments of each protein showed epitope probability scores for B cells, and the peptide fragments with the highest scores were selected [17].

Construction of multi-epitopes recombinant DNA

The DNA sequences corresponding to the selected peptide fragments were used as templates for primer design. Primer pairs were designed to encompass the selected OvCB, OvAEP, and OvCF DNA sequences. Each primer was incorporated with restriction sites to facilitate fusion with other selected DNA sequences and ligation into pET32a+ (Table 1).

The selected DNA segments of OvCB, OvAEP, and OvCF amplified in 25 μ L of polymerase chain reaction (PCR) mixture containing 10 mM dNTP, 1X SuperFiTM II buffer, 0.5 μ M of each primer, 1 μ L of Platinum SuperFi II DNA Polymerase (Thermofisher Scientific), 200 ng of cDNA library of adult *O. viverrini*, and distilled water were added to bring the mixture's volume up to 25 μ L. The PCR reactions were subjected to amplification in Biometra T-Personal 48 Thermocycler (Analytik Jena GmbH, Germany) with the conditions as following: predenaturation at 95 °C for 5 min, followed by 35 cycles of denaturation at 72 °C for 1.5 min, and a final extension at 72 °C for 10 min.

10
0
. <u> </u>
ò
5
ě
\mathcal{O}
~
\sim
.±
0
10
\Box
0
2
0
~
(1)
×
\circ
0
Ť.
$\overline{\Omega}$
Φ
-
-
Ψ
U
ī
\cap
_
5
<u> </u>
2
Φ
$\overline{\mathbf{O}}$
ă
0
0
<u>a</u>
Ψ
<u> </u>
\cup
Š
Ž
ð
QVO
0v0 b
DVO br
and Ov(
and Ov(
, and Ov(
EP, and Ov(
vEP, and Ov(
AEP, and Ov(
vAEP, and Ov(
DvAEP, and Ov(
OvAEP, and Ov(
, OvAEP, and Ov(
B, OvAEP, and Ov(
CB, OVAEP, and Ovo
CB, OvAEP, and OvC
vCB, OvAEP, and Ov(
DvCB, OvAEP, and Ov(
OvCB, OvAEP, and Ov(
f OvCB, OvAEP, and Ov(
of OvCB, OvAEP, and Ov(
of OvCB, OvAEP, and Ov(
s of OvCB, OvAEP, and Ov(
es of OvCB, OvAEP, and Ov(
ces of OvCB, OvAEP, and Ov(
nces of OvCB, OvAEP, and Ov(
ences of OvCB, OvAEP, and Ov
ences of OvCB, OvAEP, and Ov(
uences of OvCB, OvAEP, and Ov(
quences of OvCB, OvAEP, and OvC
equences of OvCB, OvAEP, and OvC
equences of OvCB, OvAEP, and OvC
sequences of OvCB, OvAEP, and Ov(
A sequences of OvCB, OvAEP, and OvC
A sequences of OvCB, OvAEP, and Ov(
VA sequences of OvCB, OvAEP, and OvC
JNA sequences of OvCB, OvAEP, and OvC
DNA sequences of OvCB, OvAEP, and OvC
DNA sequences of OvCB, OvAEP, and OvC
d DNA sequences of OvCB, OvAEP, and OvC
ed DNA sequences of OvCB, OvAEP, and OvC
ted DNA sequences of OvCB, OvAEP, and OvC
cted DNA sequences of OvCB, OvAEP, and OvC
ected DNA sequences of OvCB, OvAEP, and OvC
lected DNA sequences of OvCB, OvAEP, and OvC
elected DNA sequences of OvCB, OvAEP, and OvC
selected DNA sequences of OvCB, OvAEP, and OvC
Selected DNA sequences of OvCB, OvAEP, and OvC
Selected DNA sequences of OvCB, OvAEP, and OvC
1 Selected DNA sequences of OvCB, OvAEP, and OvC
1 Selected DNA sequences of OvCB, OvAEP, and OvC
e 1 Selected DNA sequences of OvCB, OvAEP, and OvC
Ie 1 Selected DNA sequences of OvCB, OvAEP, and OvC

Table 1 Selected DNA sequences o	of OvCB, OvAEP, and OvCF encoded high	B-cell epitope probability peptides			
Genes	The B-cell epitope-rich peptides	The selected cDNA encoded B-cell epitope	B-cell epitope probability	Primers	Total length of DNA (bp)
Cathepsin B (GenBank accession no. GQ303559.1)	21-40, 48-87, 89-108, 132-139, 150-157, 168-227, 238-240, 242, 263-279, 307-310	GANTIC ACT GGA GCA CGA TGG ATA TCT GGA AGA CAT TCG ANA GGA TTC GAA TCT GAC CAC CTG ATT CAC ACG TTT GGA GCC AAG ATG GAA ACT GCA GCA CAA AAA GCG CAG GGA ACT GCA GCA CAA AAA GCG CAG AGG CAA ACG GTC AAG CAC GTG GGA TTT GAT GCC GGT CTC AAA AGG CAC TTT GAT GCC GGA TCT AAA TGG CG CAT TGC TCT TCC GTC AGT GAG ACT TGA GCC GGA TCT AAA TGG CG CAT TGC TCT TCC GTC AGT GAG AGT CAG GCG TTC GGG GCA GTG GGA TCG ATC GGG GCA GTG GGA TCC ATG GCG TTC GGG GCA GTG GGA TCCA AGT GGA TGT CAAA AGC CTC AGT GCG GTA GGG TGG GGG TGG GGA GGA TTTC AAA AGG CTC AGT GGC GTG GGA TTC CAT TCA AAT GGC GTG GGA CTG GGG TGG GGA GGA TAT CCT GCT GTG TGG GGA GGA TAT CCT GCT GTG TGG GGA GGA TAT CCT GCT GTG TGG GGA GGA TAT CCT GGC TTAT CCA TTT CCA ACT GGG AGG ACT CAT GGT CAA GGC TCT GGC TCT TAT CCA TTT CCA ACT GGG AGG ACT CCT GGT TAT CCA ACT GGG AGG ACT CCT GGT TAT CCA ACT GGG AGG ACT CCT GGT TAT CCA ACT GGG AGG TCT TAT CCA TTT CCA ACT TGC GGA AGG TCT TAT CCA TTT CCA ACT TGG GGA GGA TCT TAT CCA TTT CCA ACT TGC GGA AGG TCT TAT CCA TTT CCA ACT TCC CCA CCG CCA TT TAT CCA TTT CCA ACT TCC CCA TGT CCA CGA AGG TCC CGA AGA TCC ACA TTT CCA CCA CG CCA CGA TTG TCA ACT TT CCA CCA CG CCA CGA TTT CCA ACT TT CCA CCA CG CCA CGA TT CCA TT CCA ACT TT CCA CCA CG CCA CGA TT CCA TT CCA ACT TCCA CCA CG CCA CGA TT CCA TT CCA ACT TT CCA CCA CG CCA CGA TT CCA CGA CGA TT CCA ACT TT CCA CCA CG CCA CGA TT CCA CGA CGA TT CCA ACT TT CCA CCA CG CCA CGA TT CCA CGA TT CCA ACT TT CCA CCA CCA CGA CGA CGA TT CCA CGA TT CCA ACT TT CCA CCA CCA CGA CGA TT CCA CGA TT CCA ACT TT CCA CCA CCA CGA CGA TT CCA CGA TT CCA CGA TT CCA ACT TT CCA CCA CCA CGA CGA CGA TT CCA CGA CGA TT CCA CGA TT CCA CGA TT CCA CGA TT CCA CGA CGA TT CCA CG	0.493	Fwd: 5'- gCG CGC GA TTC ACT GGA GCA CGA TGG ATA TCT GGA -3' Rev: 5'- GCG CGC GAG CTC GGA GAT GTT AGC TCT CGT CTT ATC -3'	603
Asparaginyl endopeptidase, also known as legumain (GenBank accession no. DQ402101.1)	24–34, 68–70, 85–91, 97–119, 127–141, 202–206, 220–231, 249–260, 265–294, 304–317, 321, 323, 339–357, 370–378, 393–401	GAG CTC GAG CAT CAC GAT CTG TCG CAT CGC ACA CTG GAT GAG TTC CAA TCG GTG AAA CAG AAT ACC AAG CAA AGT CAC GTA TCG AGA TTC GGG GAA CTG CCT CAG GTA CTT CAT AGC CAT CCG TCA GGT GTG ACAT TTG GAA CTG CCA CGG GGA CAT TG GAA CTG CG TCG CGA GAA GAA GAA AAA GCC GAA ACC GAG AGA GAA CAT GAA TTG GCA TCC CGA AAA CTA TAT CGT GAA TTG GCA TCC CGA AAA CTA TAT CGT GAA TTG GCA TGC CGA AAA CTA TAT CGT AAA GAC GAA ACC GAG AAA CTA TAT CGT AAA GAA ACA TTC GAA GAA ATC GTC ACG GAT GTA ACC TTC CAT CAG CCA ACC ATG CGC AGA GAC GTC GAG CCA ACC ATG CGC GAG AAA CGGTC ACG GAT GTA ACC TTC CAT CAG CCA ACC ATG CGC AGA GTC GAC CCA ACC ATG CGC GAG GTC GAC CCA ACC ATG CGC GAG GTC GAC	0.469	Fwd: 5'- gcg cgc gag ctc gag cat cac gat ctg tgg cat cgc -3' Rev: 5'- gcg cgc gtc gag ctg gag TTC ctc cga ctttga caa -3'	33.6

Genes	The B-cell epitope-rich peptides	The selected cDNA encoded B-cell epitope	B-cell epitope probability	Primers	Total length of DNA (bp)
Cathepsin F (GenBank accession no. AY821800.1)	22–31 , 37–53, 60–75, 78–133, 155–163, 174–177, 192–219, 225–234, 254–269, 283–286, 298–300	GTC GAC CAA TITTICC GAC CTG ACC AGT GAG GAG TTC AAG ACG CGG TAT TTG AGG ATG CAA TTC GAT GAG CGG ATT GTC AAT GAG GAT CAC CCA CAA GAA GAT GAG GAT CAC ACC CAA GAA GAT GTG ACG ATG GAT AAC AGC AAT TTT GAT TGG CGA GAT CAT GGT GCA GTC GGA CCA GTA TTG GAC CAA GGA GAT TTG GGG ACA GTTC GAG GGT CAG TGG TTC GGT AAG ACT GGG GGT CAG TGG TTC CGT AAG ACT GGG GGT CTA CTA GGT AAG CTT	0.482	Fwd: 5'- GCG CGC GTC GAC CAA TTT TCC GAC CTG ACC AGT GAG -3' Rev: 5'- GCG CGC AAG CTT ACC TAG TAG ATC CCC AGT CTT ACG -3'	261
The selected nucleotide sequence included t	he forward and reverse primers priming sites (ur	derlined). The restriction sites were incorporate	d in the specific	orimers (bold and underlined)	

Table 1 (continued)

\sim
2
Ĕ.
1
ğ
5
q
an
σ
<u></u>
ਤ
S
Ĕ
÷.
0
Ĕ
5
ğ
a,
국
.⊆
σ
Ę
Jra
ă
õ
ы
<u>е</u> .
ē
Ś
S
Sit
ç
<u>e</u> .
t
Ľ.
ő
ē
F
eq
.⊆
-
Ŀ
nder
under
s (under
ites (under
g sites (under
ing sites (under
ming sites (under
oriming sites (under
s priming sites (under
ers priming sites (under
imers priming sites (under
primers priming sites (under
e primers priming sites (under
erse primers priming sites (under
everse primers priming sites (under
l reverse primers priming sites (under
nd reverse primers priming sites (under
and reverse primers priming sites (under
Ird and reverse primers priming sites (under
ward and reverse primers priming sites (under
orward and reverse primers priming sites (under
e forward and reverse primers priming sites (under
he forward and reverse primers priming sites (under
d the forward and reverse primers priming sites (under
led the forward and reverse primers priming sites (under
uded the forward and reverse primers priming sites (under
ncluded the forward and reverse primers priming sites (under
included the forward and reverse primers priming sites (under
ice included the forward and reverse primers priming sites (under
ence included the forward and reverse primers priming sites (under
quence included the forward and reverse primers priming sites (under
equence included the forward and reverse primers priming sites (under
e sequence included the forward and reverse primers priming sites (under
ide sequence included the forward and reverse primers priming sites (under
otide sequence included the forward and reverse primers priming sites (under
leotide sequence included the forward and reverse primers priming sites (under
ucleotide sequence included the forward and reverse primers priming sites (under
1 nucleotide sequence included the forward and reverse primers priming sites (under
ed nucleotide sequence included the forward and reverse primers priming sites (under
scted nucleotide sequence included the forward and reverse primers priming sites (under
elected nucleotide sequence included the forward and reverse primers priming sites (under

The PCR products were verified using 1% agarose gel electrophoresis, and the lengths of DNA fragments were estimated by comparison with VC 100 bp Plus DNA Ladder (Vivantis Technologies, Malaysia). The DNA fragments were purified from the agarose gel using the GeneJET Gel Extraction Kit (Thermofisher Scientific). The purified PCR products were digested with the respective restriction enzymes (Thermofisher Scientific) at 37 °C for 3–5 h. Then, the digested PCR products were dissolved on 1% agarose gel electrophoresis, and DNA was purified from agarose gel using GeneJET Gel Extraction Kit (Thermofisher Scientific).

The pET32a+vector was digested with the respective restriction enzymes before ligation with the selected DNA fragments of OvCB, OvAEP, and OvCF. Briefly, the pET32a+vector in *Escherichia coli* strain TOP10 was propagated overnight in Luria–Bertani (LB) broth at 37 °C in a shaking incubator at 200 rpm. Then, the bacterial pellet was collected and extracted for pET32a+vector using PureLinkTM Quick Plasmid Miniprep Kit (InvitrogenTM, Thermofisher Scientific). The pET32a+vector was digested with the corresponding restriction enzymes with the DNA fragments of OvCB, OvAEP, and OvCF, respectively. The digested vector was verified by 1% agarose gel electrophoresis and the linearized vector was purified from agarose gel using GeneJET Gel Extraction Kit (Thermofisher Scientific).

The OvCB, OvAEP, and OvCF DNA fragments were ligated into the linearized pET32a+vector (Fig. 1) using T4 ligase (Thermofisher Scientific) following the manufacturer's protocol. The ligation products confirmed the integration of DNA fragments with a pair of T7 sequencing primers before being transformed into *E. coli* competent cells (strain TOP10) using a chemical transformation method. Positively transformed colonies were selected on LB agar containing 100 μ g/mL of ampicillin. Colony PCR was performed with a set of T7 sequencing primers and specific primers to verify integration of the DNA fragments. The plasmid DNA clones were subsequently sequenced, and the results were analyzed using BioEdit version 7.2.

The recombinant DNA OvCB_OvAEP_OvCF_ pET32a+was constructed. DNA fragments of OvAEP and OvCF were cleaved from recombinant OvAEP_ pET32a+ and OvCF_pET32a+ DNA, respectively. The DNA fragments OvAEP and OvCF were ligated into the recombinant DNA, OvCB_pET32a+, to construct OvCB OvAEP OvCF pET32a+(Fig. 1). Then, the ligated OvCB_OvAEP_OvCF_pET32a+ was transformed into a bacterial expression host, *E. coli* strain BL21(DE3), using the chemical transformation method. Positive colonies were then selected and sequenced to verify the correct annotation and nucleotide sequences.

Induction of multiantigenic recombinant protein

The clone of OvCB_OvAEP_OvCF in pET32a+ in E. coli strain BL21(DE3) was cultured overnight (16-18 h) at 37 °C in 50 mL LB broth containing 100 µg/mL of ampicillin with agitation at 200 rpm. The bacterial pellet was collected as a starter for further induction of protein expression in 200 mL fresh LB broth containing 100 μ g/mL of ampicillin and 1 mM isopropyl β -D-1-thiogalactopyranoside (IPTG). After induction of the protein expression for 8-10 h, the bacterial pellet was harvested and carried out to break in denaturing binding buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, 8 M urea, pH 7.9) by freeze-thaw and sonication on ice (4 °C) at 25% amplitude for 5 min with a pulse on and off every 5 s using an ultrasonic sonicator (VCX 750 W, Sonics). The bacterial debris was then removed, and the supernatant was collected for further purification.

The supernatant containing the desired multi-antigenic recombinant protein was purified in denaturing conditions using nickel–nitrilotriacetic acid (Ni–NTA) column (HisPur[™] Ni–NTA resin, Thermo Scientific[™]). Then, the purified protein was analyzed using 15% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and the protein concentration was measured using Nan-oDrop Microvolume Spectrophotometer (Thermofisher Scientific). Finally, the purified recombinant protein was aliquot at 1 mg/mL and stored at −20 °C for further analysis.

Evaluation of the multi-epitope recombinant protein for diagnosing opisthorchiasis using immunoblotting

The multi-epitope recombinant protein was used as the target antigen to establish an immunoblotting assay for the diagnosis of opisthorchiasis. Briefly, 50 μ g of recombinant protein was separated on 15% SDS-PAGE at 100 V, 200 mA using Bio-Rad electrophoresis system. Separated proteins were transferred onto a nitrocellulose membrane (0.45 μ m, Thermofisher Scientific) using a wet tank transfer system (Cleaver Scientific, United Kingdom) at 50 V and 200 mA for 3 h. The transferred membrane was cut into 5 mm wide strips estimated to contain approximately 5 μ g of protein per strip for immunoblotting.

The strips were washed several times with phosphatebuffered saline with Tween 20 (PBST) before blocking non-specific binding sites with PBST-3% BSA in a mosied chamber for overnight at 4 °C. Subsequently, the strips were proceeded to washing steps and carried out to incubated with the sera from patients with parasitic infections at a dilution of 1:250 in PBST-1.5% BSA for 2 h at room temperature (25 °C) with agitation. The strips were then washed and proceeded to incubate with goat antihuman IgG antibody (Rockland) at dilution of 1:2000 in PBST-1.5% BSA for 2 h at room temperature (25 °C) with

1	ATG	CAC	CAT	CAT	CAT	CAT	CAT	TCT	TCT	GGT	CTG	GTG	CCA	CGC	GGT	45
1	Met	His	His	His	His	His	His	Ser	Ser	Glv	Leu	Val	Pro	Arq	Glv	15
46	TCT	GGT	ATG	AAA	GAA	ACC	GCT	GCT	GCT	AAA	TTC	GAA	CGC	CAG	CAC	90
16	Cor	Clu	Mot	Tur	Clu	The	712	712	712	Tura	Dho	Clu	7 rg	Cln	Uic	20
10	AMC	GLY	nec	гда	GIU	CILC	ALA	ALA	ALA	цуз	CDC	GIU	ALG	GIN	niis Mmc	105
91	ATG	GAC	AGC	CCA	GAT	CTG	GGT	ACC	GAC	GAC	GAC	GAC	AAG	GUU	ATG	135
31	Met	Asp	Ser	Pro	Asp	Leu	Gly	Thr	Asp	Asp	Asp	Asp	Lys	Ala	Met	45
136	GCT	GAT	ATC	GGA	TCC	GAA	TTC	ACT	GGA	GCA	CGA	TGG	ATA	TCT	GGA	180
46	Ala	Asp	Ile	Gly	Ser	Glu	Phe	Thr	Gly	Ala	Arg	Trp	Ile	Ser	Gly	60
181	AGA	CAT	TCG	AAA	ATA	TTC	GAA	TCT	GAC	GGC	CTA	ATT	CAC	ACT	TTC	225
61	Ara	His	Ser	Lvs	Ile	Phe	Glu	Ser	Asp	Glv	Leu	Ile	His	Thr	Phe	75
226	GGA	GCC	AAG	AGG	GAA	ACT	GCA	GAA	CAA	AAA	GCG	CAG	AGG	CCA	ACG	270
76	Cly	Al >	Twe	Arg	Clu	Thr	Al >	Glu	Cln	Tue	Al >	Gln	Arg	Pro	Thr	90
271	GTY	ALA	СУШ	CTC	GIU		CAM	CAM	UCC	LYS	ATA	CCA	ALG	DAC		215
271	GIC	AAG	CAI	GIG	GGI	TTT	GAI	GAI	ICG	CGC	AIC	CCA	AAG	AAC	TTT	313
91	val	LYS	HIS	val	GIY	Pne	Asp	Asp	Ser	Arg	lle	Pro	Lys	Asn	Pne	105
316	GAT	GCA	CGA	ACT	AAA	TGG	CCG	CAT	TGC	TCG	TCC	ATC	AGT	GAG	ATC	360
106	Asp	Ala	Arg	Thr	Lys	Trp	Pro	His	Cys	Ser	Ser	Ile	Ser	Glu	Ile	120
361	AGA	GAT	CAA	TCC	AGT	TGT	GGA	TCG	TGT	TGG	GCG	TTC	GGG	GCA	GTG	405
121	Arg	Asp	Gln	Ser	Ser	Cys	Gly	Ser	Cys	Trp	Ala	Phe	Gly	Ala	Val	135
406	GAA	GCC	ATG	AGT	GAT	CGA	CTG	TGC	ATT	CAT	TCA	AAT	GGT	TCT	TTC	450
136	Glu	Ala	Met	Ser	Asp	Ara	Leu	CVS	Tle	His	Ser	Asn	Glv	Ser	Phe	150
451	AAC	AAA	AGC	CTC	AGT	GCG	GTA	GAC	TTC	CTC	TCC	TGT	TGT	AAG	GAC	195
151	Aan	TIC	Con	Tan	Cox	710	Vol	7 an	Tau	Tan	Con	Cura	Cura	Turo	D an	1 65
100	ASII	Lys	Jer	Leu	Jer	ALA	CCD	ASP	плп	Leu	COM	Cys	Cys	цуз	ASP	105 E 4 0
496	TGT	GGA	TTC	GGT	TGT	CGT	GGA	GGA	TAT	CCT	GCT	GIG	GCG	TGG	GAC	540
166	Cys	GLY	Phe	Gly	Cys	Arg	Gly	Gly	Tyr	Pro	Ala	Val	Ala	Trp	Asp	180
541	TAT	TGG	AGG	ACT	CAC	GGC	ATT	GTC	ACA	GGT	GGT	TCA	AAA	GAA	TAT	585
181	Tyr	Trp	Arg	Thr	His	Gly	Ile	Val	Thr	Gly	Gly	Ser	Lys	Glu	Tyr	195
586	CCA	AGT	GGA	TGC	AGG	TCT	TAT	CCA	TTT	CCG	AAA	TGT	GAC	CAT	CAT	630
196	Pro	Ser	Glv	Cvs	Ara	Ser	Tvr	Pro	Phe	Pro	Lvs	Cvs	Asp	His	His	210
631	GTT	CAA	GGA	CAC	TAT	CCG	CCA	TGT	CCA	CAT	CAT	TAC	TAC	CCC	ACA	675
211	Val	Gln	Glv	Hig	Tur	Pro	Pro	CVG	Pro	Hig	Hig	Tur	Tyr	Pro	Thr	225
676	CCC	CDD	TCC	CTC	CAC	CAT	TCT	CAC	ACC	CCA	CAA		CCT	TAC	TTC	720
226	Dwo	Clu	Cura	Val	CAG	Uia	Cura	DAD	The w	Dwo	Clu	Tan	Clar	TAC	Tau	240
220	Pro	GIU	Cys	val	GIN	HIS	Cys	Asp	Thr	Pro	GIU	Leu	GIY	TAL	Leu	240
121	GAG	GAT	AAG	ACG	AGA	GC.I.	AAC	ATC	TCC	GAG	CTC	GAG	CAT	CAC	GAT	165
241	Glu	Asp	Lys	Thr	Arg	Ala	Asn	Ile	Ser	Glu	Leu	Glu	His	His	Asp	255
766	CTG	TCG	CAT	CGT	ACA	CTG	GAT	GAC	CAG	TTC	CAA	TGG	GTG	AAA	CAG	810
256	Leu	Ser	His	Arg	Thr	Leu	Asp	Asp	Gln	Phe	Gln	Trp	Val	Lys	Gln	270
811	AAT	ACC	AAG	CAA	AGT	CAC	GTA	TCG	AGA	TTC	GGG	GAA	CTG	aam	CAG	855
271	Asn	Thr	Twe	Cln	0	TT: m								CCT	0110	
856			LYS	GTH	Ser	HIS	Val	Ser	Arg	Phe	Gly	Glu	Leu	Pro	Gln	285
286	GTA	CTT	CAT	AGC	CAT	TCA	Val TCA	Ser CGC	Arg TGG	Phe GCA	Gly CAT	Glu TTG	Leu ATC	Pro	Gln ATG	285 900
200	GTA Val	CTT	CAT	AGC	CAT	TCA Ser	Val TCA Ser	Ser CGC Arg	Arg TGG Trp	Phe GCA Ala	Gly CAT His	Glu TTG Leu	Leu ATC	Pro ACC	Gln ATG Met	285 900 300
901	GTA Val	CTT Leu	CAT His	AGC Ser	CAT His	TCA Ser	Val TCA Ser	Ser CGC Arg	Arg TGG Trp	Phe GCA Ala	Gly CAT His	Glu TTG Leu	Leu ATC Ile	Pro ACC Thr	Gln ATG Met	285 900 300
901	GTA Val GTC	CTT Leu CGA	CAT His CGA	AGC Ser ATG	CAT His ATG	TCA Ser AAA	Val TCA Ser GCC	Ser CGC Arg GAA	Arg TGG Trp ACC	Phe GCA Ala GAG	Gly CAT His GAA	Glu TTG Leu GAA	Leu ATC Ile CAT	Pro ACC Thr GAA	Gln ATG Met TTG	285 900 300 945
901 301	GTA Val GTC Val	CTT Leu CGA Arg	CAT His CGA Arg	AGC Ser ATG Met	CAT His ATG Met	TCA Ser AAA Lys	Val TCA Ser GCC Ala	Ser CGC Arg GAA Glu	Arg TGG Trp ACC Thr	Phe GCA Ala GAG Glu	Gly CAT His GAA Glu	Glu TTG Leu GAA Glu	Leu ATC Ile CAT His	Pro ACC Thr GAA Glu	Gln ATG Met TTG Leu	285 900 300 945 315
901 301 946	GTA Val GTC Val GCA	CTT Leu CGA Arg TCC	CAT His CGA Arg CGA	AGC Ser ATG Met AAA	CAT His ATG Met CTA	TCA Ser AAA Lys TAT	Val TCA Ser GCC Ala CGT	Ser CGC Arg GAA Glu GCA	Arg TGG Trp ACC Thr CTT	Phe GCA Ala GAG Glu CAG	Gly CAT His GAA Glu CTT	Glu TTG Leu GAA Glu GCC	Leu ATC Ile CAT His CAG	Pro ACC Thr GAA Glu ATC	Gln ATG Met TTG Leu GTC	285 900 300 945 315 990
901 301 946 316	GTA Val GTC Val GCA Ala	CTT Leu CGA Arg TCC Ser	CAT His CGA Arg CGA Arg Arg	AGC Ser ATG Met AAA Lys	CAT His ATG Met CTA Leu	TCA Ser AAA Lys TAT Tyr	Val TCA Ser GCC Ala CGT Arg	Ser CGC Arg GAA Glu GCA Ala	Arg TGG Trp ACC Thr CTT Leu	Phe GCA Ala GAG Glu CAG Gln	Gly CAT His GAA Glu CTT Leu	Glu TTG Leu GAA Glu GCC Ala	Leu ATC Ile CAT His CAG Gln	Pro ACC Thr GAA Glu ATC Ile	Gln ATG Met TTG Leu GTC Val	285 900 300 945 315 990 330
901 301 946 316 991	GTA Val GTC Val GCA Ala AAA	CTT Leu CGA Arg TCC Ser GAA	CAT His CGA Arg CGA Arg ACA	AGC Ser ATG Met AAA Lys TTC	CAT His ATG Met CTA Leu GAA	TCA Ser AAA Lys TAT Tyr GAA	Val TCA Ser GCC Ala CGT Arg ATC	Ser CGC Arg GAA Glu GCA Ala GTC	Arg TGG Trp ACC Thr CTT Leu ACG	Phe GCA Ala GAG Glu CAG Gln GAT	Gly CAT His GAA Glu CTT Leu GTA	Glu TTG Leu GAA Glu GCC Ala ACA	Leu ATC Ile CAT His CAG Gln ACC	Pro ACC Thr GAA Glu ATC Ile TTC	Gln ATG Met TTG Leu GTC Val TAT	285 900 300 945 315 990 330 1035
901 301 946 316 991 331	GTA Val GTC Val GCA Ala AAA Lys	CTT Leu CGA Arg TCC Ser GAA Glu	CAT His CGA Arg CGA Arg ACA Thr	AGC Ser ATG Met AAA Lys TTC Phe	CAT His ATG Met CTA Leu GAA Glu	TCA Ser AAA Lys TAT Tyr GAA Glu	Val TCA Ser GCC Ala CGT Arg ATC Ile	Ser CGC Arg GAA Glu GCA Ala GTC Val	Arg TGG Trp ACC Thr CTT Leu ACG Thr	Phe GCA Ala GAG Glu CAG Gln GAT Asp	Gly CAT His GAA Glu CTT Leu GTA Val	Glu TTG Leu GAA Glu GCC Ala ACA Thr	Leu ATC Ile CAT His CAG Gln ACC Thr	Pro ACC Thr GAA Glu ATC Ile TTC Phe	Gln ATG Met TTG Leu GTC Val TAT Tyr	285 900 300 945 315 990 330 1035 345
901 301 946 316 991 331 1036	GTA Val GTC Val GCA Ala AAA Lys CAG	CTT Leu CGA Arg TCC Ser GAA Glu CCA	CAT His CGA Arg CGA Arg ACA Thr ACC	AGC Ser ATG Met AAA Lys TTC Phe ATG	CAT His ATG Met CTA Leu GAA Glu CGC	TCA Ser AAA Lys TAT Tyr GAA Glu ATG	Val TCA Ser GCC Ala CGT Arg ATC Ile TTG	Ser CGC Arg GAA Glu GCA Ala GTC Val TCA	Arg TGG Trp ACC Thr CTT Leu ACG Thr AAG	Phe GCA Ala GAG Glu CAG Gln GAT Asp TCG	Gly CAT His GAA Glu CTT Leu GTA Val GAG	Glu TTG GAA Glu GCC Ala ACA Thr GAA	Leu ATC Ile CAT His CAG Gln ACC Thr CTC	Pro ACC Thr GAA Glu ATC Ile TTC Phe CAG	Gln ATG Met TTG Leu GTC Val TAT Tyr GTC	285 900 300 945 315 990 330 1035 345 1080
901 301 946 316 991 331 1036 346	GTA Val GTC Val GCA Ala AAA Lys CAG Gln	CTT Leu CGA Arg TCC Ser GAA Glu CCA Pro	CAT His CGA Arg CGA Arg ACA Thr ACC Thr	AGC Ser ATG Met AAA Lys TTC Phe ATG Met	CAT His ATG Met CTA Leu GAA Glu CGC Ara	TCA Ser AAA Lys TAT Tyr GAA Glu ATG Met	Val TCA Ser GCC Ala CGT Arg ATC Ile TTG Leu	Ser CGC Arg GAA Glu GCA Ala GTC Val TCA Ser	Arg TGG Trp ACC Thr CTT Leu ACG Thr AAG Lvs	Phe GCA Ala GAG Glu CAG Gln GAT Asp TCG Ser	Gly CAT His GAA Glu CTT Leu GTA Val GAG Glu	Glu TTG Leu GAA Glu GCC Ala ACA Thr GAA Glu	Leu ATC Ile CAT His CAG Gln ACC Thr CTC Leu	Pro ACC Thr GAA Glu ATC Ile TTC Phe CAG Gln	Gln ATG Met TTG Leu GTC Val TAT Tyr GTC Val	285 900 300 945 315 990 330 1035 345 1080 360
901 301 946 316 991 331 1036 346 1081	GTA Val GTC Val GCA Ala AAA Lys CAG Gln	CTT Leu CGA Arg TCC Ser GAA Glu CCA Pro	CAT His CGA Arg CGA Arg ACA Thr ACC Thr	AGC Ser ATG Met AAA Lys TTC Phe ATG Met	CAT His ATG Met CTA Leu GAA Glu CGC Arg	TCA Ser AAA Lys TAT Tyr GAA Glu ATG Met	Val TCA Ser GCC Ala CGT Arg ATC Ile TTG Leu	Ser CGC Arg GAA Glu GCA Ala GTC Val TCA Ser AGT	Arg TGG Trp ACC Thr CTT Leu ACG Thr AAG Lys	Phe GCA Ala GAG Glu CAG Gln GAT Asp TCG Ser GAG	Gly CAT His GAA Glu CTT Leu GTA Val GAG Glu	Glu TTG Leu GAA Glu GCC Ala ACA Thr GAA Glu AAG	Leu ATC Ile CAT His CAG Gln ACC Thr CTC Leu ACG	Pro ACC Thr GAA Glu ATC Ile TTC Phe CAG Gln CGG	Gln ATG Met TTG Leu GTC Val TAT Tyr GTC Val TAT	285 900 300 945 315 990 330 1035 345 1080 360
901 301 946 316 991 331 1036 346 1081 361	GTA Val GTC Val GCA Ala AAA Lys CAG Gln GAC	CTT Leu CGA Arg TCC Ser GAA Glu CCA Pro CAA	CAT His CGA Arg CGA Arg ACA Thr ACC Thr TTT	AGC Ser ATG Met AAA Lys TTC Phe ATG Met TCC	CAT His ATG Met CTA Leu GAA Glu CGC Arg GAC	TCA Ser AAA Lys TAT Tyr GAA Glu ATG Met CTG	Val TCA Ser GCC Ala CGT Arg ATC Ile TTG Leu ACC	Ser CGC Arg GAA Glu GCA Ala GTC Val TCA Ser AGT	Arg TGG Trp ACC Thr CTT Leu ACG Thr AAG Lys GAG	Phe GCA Ala GAG Glu CAG Gln GAT Asp TCG Ser GAG	Gly CAT His GAA Glu CTT Leu GTA Val GAG Glu TTC Pbe	Glu TTG Leu GAA Glu GCC Ala ACA Thr GAA Glu AAG	Leu ATC Ile CAT His CAG Gln ACC Thr CTC Leu ACG	Pro ACC Thr GAA Glu ATC Ile TTC Phe CAG Gln CGG	Gln ATG Met TTG Leu GTC Val TAT Tyr GTC Val TAT	285 900 300 945 315 990 330 1035 345 1080 360 1125 375
901 301 946 316 991 331 1036 346 1081 361	GTA Val GTC Val GCA Ala AAA Lys CAG Gln GAC Asp	CTT Leu CGA Arg TCC Ser GAA Glu CCA Pro CAA Gln	CAT His CGA Arg CGA Arg ACA Thr ACC Thr TTT Phe	AGC Ser ATG Met AAA Lys TTC Phe ATG Met TCC Ser	CAT His ATG Met CTA Leu GAA Glu CGC Arg GAC	TCA Ser AAA Lys TAT Tyr GAA Glu ATG Met CTG Leu	Val TCA Ser GCC Ala CGT Arg ATC Ile TTG Leu ACC Thr	Ser CGC Arg GAA Glu GCA Ala GTC Val TCA Ser AGT Ser	Arg TGG Trp ACC Thr CTT Leu ACG Thr AAG Lys GAG Glu	Phe GCA Ala GAG Glu CAG Glu CAG GAT Asp TCG Ser GAG Glu	Gly CAT His GAA Glu CTT Leu GTA Val GAG Glu TTC Phe	Glu TTG Leu GAA Glu GCC Ala ACA Thr GAA Glu AAG Lys CAC	Leu ATC Ile CAT His CAG Gln ACC Thr CTC Leu ACG Thr	Pro ACC Thr GAA Glu ATC Ile TTC Phe CAG Gln CGG Arg	Gln ATG Met TTG Leu GTC Val TAT Tyr Cal TAT Tyr 200	285 900 300 945 315 990 330 1035 345 1080 360 1125 375
901 301 946 316 991 331 1036 346 1081 361 1126	GTA Val GTC Val GCA Ala AAA Lys CAG Gln GAC Asp TTG	CTT Leu CGA Arg TCC Ser GAA Glu CCA Pro CAA Gln AGG	CAT His CGA Arg CGA Arg ACA Thr ACC Thr ACC Thr Phe ATG	AGC Ser ATG Met AAA Lys TTC Phe ATG Met TCC Ser CGA	CAT His ATG Met CTA Leu GAA Glu CGC Arg GAC Asp	TCA Ser AAA Lys TAT Tyr GAA Glu ATG Met CTG Leu GAT	Val TCA Ser GCC Ala CGT Arg ATC Ile TTG Leu ACC Thr GAG	Ser CGC Arg GAA Glu GCA Ala GTC Val TCA Ser AGT Ser CCG	Arg TGG Trp ACC Thr CTT Leu ACG Thr AAG Lys GAG Glu ATT	Phe GCA Ala GAG Glu CAG Gln GAT Asp TCG Ser GAG Glu GTC	Gly CAT His GAA Glu CTT Leu GTA Val GAG Glu TTC Phe AAT	Glu TTG GAA Glu GCC Ala ACA Thr GAA Glu AAG Lys GAG	Leu ATC Ile CAT His CAG Gln ACC Thr CTC Leu ACG Thr GAT	Pro ACC Thr GAA Glu ATC Ile TTC Phe CAG Gln CGG Arg CCC	Gln ATG Met TTG Leu GTC Val TAT Tyr GTC Val TAT Tyr ACC	285 900 300 945 315 990 330 1035 345 1080 360 1125 375 1170
901 301 946 316 991 331 1036 346 1081 361 1126 376	GTA Val GTC Val GCA Ala AAA Lys CAG Gln GAC Asp TTG Leu	CTT Leu CGA Arg TCC Ser GAA Glu CCA Pro CAA Gln AGG Arg	CAT His CGA Arg CGA Arg ACA Thr ACC Thr TTT Phe ATG Met	AGC Ser ATG Met AAA Lys TTC Phe ATG Met TCC Ser CGA Arg	CAT His ATG Met CTA Leu GAA Glu CGC Arg GAC Asp TTT Phe	TCA Ser AAA Lys TAT Tyr GAA Glu ATG GAT Leu GAT Asp	Val TCA Ser GCC Ala CGT Arg ATC Ile TTG Leu ACC Thr GAG Glu	Ser CGC Arg GAA Glu GCA Ala GTC Val TCA Ser AGT Ser CCG Pro	Arg TGG Trp ACC Thr CTT Leu ACG Thr AAG Lys Glu ATT Ile	Phe GCA Ala GAG Glu CAG Gln GAT Asp TCG Ser GAG Glu GTC Val	Gly CAT His GAA Glu CTT Leu GTA Val GAG Glu TTC Phe AAT Asn	Glu TTG GAA Glu GCC Ala ACA Thr GAA Glu AAG Lys GAG Glu	Leu ATC Ile CAT His CAG Gln ACC Thr CTC Leu ACG Thr GAT Asp	Pro ACC Thr GAA Glu ATC Ile TTC Phe CAG Gln CGG Arg CCC Pro	Gln ATG Met TTG Leu GTC Val TAT Tyr GTC Val TAT Tyr ACC Thr	285 900 300 945 315 990 330 1035 345 1080 360 1125 375 1170 390
901 301 946 316 991 331 1036 346 1081 361 1126 376 1171	GTA Val GTC Val GCA Ala AAA Lys CAG Gln GAC Asp TTG Leu CCA	CTT Leu CGA Arg TCC Ser GAA Glu CCA Pro CAA Gln AGG Arg CAA	CAT His CGA Arg CGA Arg ACA Thr ACC Thr TTT Phe ATG Met GAA	AGC Ser ATG Met AAA Lys TTC Phe ATG Met TCC Ser CGA Arg GAT	CAT His ATG Met CTA Leu GAA Glu CGC Arg GAC Asp TTT Phe GTG	TCA Ser AAA Lys TAT Tyr GAA Glu ATG GAT Leu GAT Asp ACG	Val TCA Ser GCC Ala CGT Arg ATC Ile TTG Leu ACC Thr GAG Glu ATG	Ser CGC Arg GAA Glu GCA Ala GTC Val TCA Ser CCG Pro GAT	Arg TGG Trp ACC Thr CTT Leu ACG Thr AAG Glu ATT Ile AAC	Phe GCA Ala GAG Glu CAG Glu GAT ASP TCG GAG GLu GTC Val AGC	Gly CAT His GAA Glu CTT Leu GTA Val GAG Glu TTC Phe AAT Asn AAT	Glu TTG GAA Glu GCC Ala ACA Thr GAA Glu AAG Lys GAG Glu TTT	Leu ATC Ile CAT His CAG Gln ACC Thr CTC Leu ACG Thr GAT Asp GAT	Pro ACC Thr GAA Glu ATC Ile TTC Phe CAG Gln CGG Arg CCC Pro TGG	Gln ATG Met TTG Leu GTC Val TAT Tyr ACC Thr CGA	285 900 300 945 315 990 330 1035 345 1080 360 1125 375 1170 390 1215
901 301 946 316 991 331 1036 346 1081 361 1126 376 1171 391	GTA Val GTC Val GCA Ala AAA Lys CAG Gln GAC Asp TTG Leu CCA Pro	CTT Leu CGA Arg TCC Ser GAA Glu CCA Pro CAA Gln AGG Arg CAA Gln	CAT His CGA Arg CGA Arg ACA Thr ACC Thr TTT Phe ATG Met GAA Glu	AGC Ser ATG Met AAA Lys TTC Phe ATG Met Ser CGA Arg GAT Asp	CAT His ATG Met CTA Leu GAA Glu CGC Arg GAC Asp TTT Phe GTG Val	TCA Ser AAA Lys TAT Tyr GAA Glu ATG GAT Leu GAT ASp ACG Thr	Val TCA Ser GCC Ala CGT Arg ATC Ile TTG Leu ACC Thr GAG Glu ATG Met	Ser CGC Arg GAA Glu GCA Ala GTC Val TCA Ser CCG Pro GAT Asp	Arg TGG Trp ACC Thr CTT Leu ACG Thr AAG Lys Glu ATT Ile AAC Asn	Phe GCA Ala GAG Glu CAG Glu GAT GAG GLu GTC Val AGC Ser	Gly CAT His GAA Glu CTT Leu GTA Val GAG Glu TTC Phe AAT Asn AAT	Glu TTG Leu GAA Glu GCC Ala ACA Thr GAA Glu AAG Lys GAG Glu TTT Phe	Leu ATC Ile CAT His CAG Gln ACC Thr CTC Leu ACG Thr GAT Asp GAT Asp	Pro ACC Thr GAA Glu ATC Ile TTC Phe CAG Gln CGG Arg CCC Pro TGG Trp	Gln ATG Met TTG Leu GTC Val TAT Tyr ACC Thr CGA Arg	285 900 300 945 315 990 330 1035 345 1080 360 1125 375 1170 390 1215 405
901 301 946 316 991 331 1036 346 1081 361 1126 376 1171 391 1216	GTA Val GTC Val GCA Ala AAA Lys CAG Gln GAC Asp TTG Leu CCA Pro GAT	CTT Leu CGA Arg TCC Ser GAA Glu CCA Pro CAA Gln AGG Arg CAA Gln CAT	CAT His CGA Arg CGA Arg ACA Thr ACC Thr TTT Phe ATG Met GAA Glu GGT	AGC Ser ATG Met AAA Lys TTC Phe ATG Met TCC Ser CGA Arg GAT Asp GCA	CAT His ATG Met CTA Leu GAA Glu CGC Arg GAC Asp TTT Phe GTG Val GTC	TCA Ser AAA Lys TAT Tyr GAA Glu ATG Met CTG Leu GAT ASp ACG Thr GGA	Val TCA Ser GCC Ala CGT Arg ATC Ile TTG Leu ATC GAG Glu ATG Met CCA	Ser CGC Arg GAA Glu GCA Ala GTC Val TCA Ser CCG Pro GAT Asp GTA	Arg TGG Trp ACC Thr CTT Leu ACG Thr AAG Lys Glu ATT Ile AAC Asn TTG	Phe GCA Ala GAG Glu CAG Glu CAG GAT ASP TCG Ser GAG GIU GTC Val AGC Ser GAC	Gly CAT His GAA Glu CTT Leu GTA Val GAG Glu TTC Phe AAT Asn AAT Asn CAA	Glu TTG Leu GAA Glu GCC Ala ACA Thr GAA Glu AAG Lys GAG Glu TTT Phe GGA	Leu ATC CAT His CAG Gln ACC Thr CTC Leu ACG Thr GAT Asp GAT	Pro ACC Thr GAA Glu ATC Phe CAG Gln CGG Arg CCC Pro TGG Trp TGT	Gln ATG Met TTG Leu GTC Val TAT Tyr Cal TAT Tyr ACC Thr ACC Arg GGT	285 900 300 945 315 990 330 1035 345 1080 360 1125 375 1170 390 1215 405 1260
901 301 946 316 991 331 1036 346 1081 361 1126 376 1171 391 1216 406	GTA Val GTC Val GCA Ala AAA Lys CAG Gln CAG CAG Leu CCA Pro GAT Asp	CTT Leu CGA Arg TCC Ser GAA Glu CCA Pro CAA Gln AGG Arg CAA Gln CAT His	CAT His CGA Arg CGA ACA Thr ACC Thr TTT Phe ATG GAA Glu GGT Gly	AGC Ser ATG AAA Lys TTC Phe ATG Met CGA Arg GAT Asp GCA Ala	CAT His ATG Met CTA GAA GLC Arg GAC Asp TTT Phe GTG Val GTC Val	TCA Ser AAA Lys TAT Tyr GAA GAU ATG Met CTG Leu GAT ASp ACG Thr GGA Gly	Val TCA Ser GCC Ala CGT Arg ATG TTG Leu <i>ACC</i> Thr GAG Glu ATG Met CCA Pro	Ser CGC Arg GAA Glu GCA Ala GTC Val TCA Ser CCG Pro GAT Asp GTA Val	Arg TGG Trp ACC Thr CTT Leu ACG Thr AACG Thr AAG Glu ATT Ile AAC Asn TTG Leu	Phe GCA Ala GAG Glu CAG Glu CAG GAT Asp TCG Ser GAU GTC Val AGC Ser Asp	Gly CAT His GAA Glu CTT Leu GTA Val GAG Glu TTC Phe AAT Asn AAT Asn CAA	Glu TTG Leu GAA Glu GCC Ala ACA Thr GAA Glu AAG Glu TTT Phe GGA Gly	Leu ATC Ile CAT His CAG Gln ACC Thr CTC Leu ACG Thr GAT Asp GAT Asp	Pro ACC Thr GAA Glu ATC TTC Phe CAG Gln CGG Arg CCC Pro TGG Trp TGT Cys	Gln ATG Met TTG Leu GTC Val TAT Tyr GTC Val TAT Tyr CGA Arg GGT Gly	285 900 300 945 315 990 330 1035 345 1080 360 1125 375 1170 390 1215 405 1260 420
901 301 946 316 991 331 1036 346 1081 361 1126 376 1171 391 1216 406 1261	GTA Val GTC Val GCA Ala AAA Lys CAG GIn GAC TTG CCA Pro GAT Asp TCG	CTT Leu CGA Arg TCC Ser GAA Glu CCA Glu CCA Gln AGG Arg CAA Gln CAT His TGC	CAT Hiss CGA Arg CGA Arg CGA Arg ACG Thr Thr ACC Thr TTT Phe ATG GAA GIU GGT GGIY TGG	AGC Ser ATG Met AAA Lys TTC Phe ATG Met TCC Ser CGA Asp GCA Ala GCA	CAT Hiss ATG Met CTA Leu GAA Glu CGC GAA GGU CGC CAS Phe GTG GTG Val TTT TTT TTT	TCA Serr AAA Lyss TAT Tyr GAA ATG GAU ATG GAU CTG CAS ACG Thr CAS GAU TCT	Val TCA Ser GCC Ala CGT Arg ATC Ile TTG GAC CT TTG GAG Glu ATG GAG Glu ATG GTG GTG	Ser CGC Arg GAA Glu GCA Ala GTC Val TCA Ser CCG GAT Asp GTA Val ATT	Arg TGG Trp Acc Thr CTT Leu ACG Thr AACG Thr AAG Glu ATT Ile AAC Glu AAC CAS GLU GGG GGGG GGGG	Phe GCA Ala GAG Glu CAG GAT Asp TCG GAG GLU GTC Val AGC Val Ser GAC Asp AAT	Gly CAT His GAA Glu CTT Leu GTA Val GAG Glu TTC Phe AAT ASN AAT ASN CAA GIN GTC	Glu TTG GAA Glu GCC Ala ACA Thr GAA GAU Lys GAG Glu TTT Phe GGA GAG GAG	Leu ATC Ile CAT His CAG Gln ACC Thr CTC Leu ACG Thr GAT Asp GAT Asp GGT	Pro ACC Thr GAA Glu ATC Ile TTC Phe CAG Gln CGG Arg CCC Pro TGT TGT CYS CAG	Gln ATG Met TTG Leu GTC Val TAT Tyr GTC Val TAT Tyr ACC TGG GGT GGT TGG	285 900 300 945 315 990 330 1035 345 1080 360 1125 375 1170 390 1215 405 1260 420 1305
901 301 946 316 991 331 1036 346 1081 361 1126 376 1171 391 1216 406 1261 421	GTA Val GTC Val Ala AAA AAA Lys CAG Gln CAG Gln TTG Leu CCA Pro GAT TCG Ser	CTT Leu CGA Arg TCC Ser GAA Glu CCA Pro CAA Gln AGG Arg CAA Gln CAT TGC CAS TGC CVS	CAT His CGA CGA Arg CGA Arg CGA Arg ACA Thr ACC Thr TTT Phe ATG GAA Glu GGT TGG TTC	AGC Ser ATG Met Lys TTC Phe ATG Met TCC Ser CGA Arg GAT Asp GCA Ala	CAT Hiss ATG Met CTA Leu GAA Glu CGC Arg GAC Asp TTT Phe GTG Val GTC Val TTT TTT Phe	TCA Ser AAA Lys TAT Tyr GAA Glu ATG GAT ACG GAT ASp ACG GAT Thr GGA Gly TCT Ser	Val TCA Ser GCC Ala CGT ATC TILe TTG CAT GAG Glu ATG CA Pro GTG GTG Val	Ser CGC Arg GAA Glu GCA Ala GTC Val TCA Ser CCG Fro GAT Asp GTA Val TCA	Arg TGG Trp ACC Thr CTT Leu ACG Thr AAG Glu ATT Ile AAC Glu ATT Ile GGG GG V	Phe GCA Ala GAG Glu CAG GIU CAG GAT ASP TCG GAG GAC Val AGC Ser GAC ASP AAT	Gly CAT His GAA Glu CTT Leu GTA Val GAG Glu TTC Phe AAT ASN AAT ASN Gln CAA Gln GTC Val	Glu TTG GAA Glu GCC Ala ACA Thr GAA Glu TTT GAA Glu TTT Phe GAG Gly GAG Glu	Leu ATC Ile CAT His CAG Gln ACC Thr CTC Leu ACG Thr GAT Asp GAT Asp GGT GGIv	Pro ACC Thr GAA Glu ATC Ile TTC Phe CAG Gln CGG Arg CCC Pro TGG TGT TGT TGT Cys CAG Gln	Gln ATG Met TTG Leu GTC Val TAT Tyr GTC Val TAT Tyr CGA GGT GGT GGT TGG TCD	285 900 300 945 315 990 330 1035 345 1080 360 1125 375 1170 390 1215 405 1260 420 1305 435
901 301 946 316 991 331 1036 346 1081 361 1126 376 1171 391 1216 406 1261 421 1306	GTA Val GTC Val GCA Ala AAA Lys CAG Gln Lsy CAG Gln TTG CAG Fro GAT Asp TCG Ser TTC	CTT Leu CGA Arg TCC Ser GAA Glu CCA Glu CCA Gln AGG Arg CAA Gln CAT His TGC Cys C C	CAT Hiss CGA Arg CGA Arg CGA Arg ACA Thr ACC Thr TTT Phe ATG Glu GGT Gly TGG Gly Trp AAG	AGC Ser ATG Met Lys TTC Phe ATG Met TCC Ser CGA Arg GAT Asp GCA Ala ACT	CAT Hiss ATG Met CTA Leu GAA Glu CGC Arg GAC Asp TTT Phe GTG GTC Val TTT Phe GCG GGC	TCA Ser AAA Lys TAT Tyr GAA Glu ATG GAT CTG CAG CAG CAT	Val TCA Ser GCC Ala CGT Arg ATC TI E E U U E U T TG GAG Glu ATG GAG GIU ATG GTA CCA CTA	Ser CGC Arg GAA Glu GCA Ala GTC Val TCA Ser CCG Fro GAT Asp GTA Val ATT Ile CT2	Arg TGG Trp ACC Thr CTT Leu ACG Thr AAG Glu ATT Ile AAC Glu ATT Ile GGG GGT	Phe GCA Ala GAG Glu CAG Glu CAG GAT TCG GAG GAC Val AGC Ser GAC Asp AAT	Gly CAT His GAA Glu CTT Leu GTA GAG GAG GAG GAG AAT ASN AAT ASN CAA GIN GTC CTT	Glu TTG GAA Glu GCC Ala ACA Thr GAA GAU AAG GLU SAG GAU TTT Phe GAG Glu GCG GCG GCG	Leu ATC Ile CAT His CAG Gln ACC Thr CTC Leu ACG Thr GAT Asp GAT Asp GAT GAT GIY GCC	Pro ACC Thr GAA Glu ATC Ile TTC Phe CAG Gln CGG Arg CCC Pro TGG TCT Cys CAG Gln CGS CAG	Gln ATG Met TTG Leu GTC Val TAT Tyr Cal TAT Tyr ACC Thr ACG GGT GGT GGT GGT CTC	285 900 300 945 315 990 330 1035 345 1035 345 1125 375 1170 390 1215 405 1260 420 1305 435 1350
901 301 946 316 991 331 1036 346 1081 361 1126 376 1171 391 1216 406 1261 1306 436	GTA Val GTC Val GCA Ala AAA Lys CAG Gln TTG GAT Asp TCG GAT Asp TCG Ser TTC	CTT Leu CGA Arg TCC Ser GAA Glu CCA Glu CCA AGG Arg CAA Gln CAT His TGC CysT CYST	CAT Hiss CGA Arg CGA Arg ACA Thr ACC Thr TTT Phe ATG Glu GGT Gly TGG Trp AAG	AGC Ser ATG Met AAA Lys TTC Phe ATG Met TCC Ser CGA Arg GAT Asp GCA Ala GCA Ala	CAT Hiss ATG Met CTA Leu CGC Arg GAC Asp TTT Phe GTG Val TTT Phe GTC Val TTT Phe GCC CCL	TCA Ser AAA Lys TAT Tyr GGA Glu ATG GAT CTG GAT CTC Ser CAT	Val TCA Ser GCC Ala CGT Arg ATC Ile TTG Leu ATC Thr GAG Glu ATG Met CCA Pro GTG Val	Ser CGC Arg GAA Glu GCA Ala GTC Val TCA Ser CCG GAT Asp GTA Val ATT Ile C	Arg TGG Trp ACC Thr CTT Leu ACG Thr AAG Glu ATT Ile AAC Glu ATT Ile GGG GLY CCL	Phe GCA Ala GAG Glu CAG GIU GAT ASp GAG GAC Val AGC Ser GAC Asp AAT Asn AAA	Gly CAT His GAA Glu CTT Leu GTA Val GAG Glu TTC Phe AAT Asn AAT Asn CAA GIn GTC Val TCC	Glu TTG GAA Glu GCC Ala ACA Thr GAA GAU GAG Glu TTT Phe GGA Gly GAG Glu GLY GAG	Leu ATC Ile CAT His CAG Gln ACC Thr CTC Leu ACG Thr ACC Thr GAT ASp GAT ASp GGT Gly GCC	Pro ACC Thr GAA Glu ATC Ile TTC Phe CAG Gln CGG Arg CCC Pro TGG Trp TGT Cys CAG Gln Gln ACC Arg	Gln Gln ATG Met TTG GTC Val TAT Tyr GTC Val TAT Tyr CVal TAT Tyr CGA GGT Gly TGG Gly TGG CLau	285 900 300 945 315 990 330 1035 345 1080 360 1125 375 1170 390 1215 405 1260 420 1305 435 1350
901 301 946 316 991 331 1036 346 1081 361 1126 376 1171 391 1216 406 1261 421 1306 436	GTA Val GTC Val GCA Ala AAA Lys CCAG Gln CCAG Gln TTG CCA Pro GAT Asp TCG Ser TTC Phe	CTT Leu CGA Arg TCC Ser GAA Glu CCA Glu CCA Arg Gln CAA Gln CAA Gln CAA GIN CAA Arg CAA Arg CAA	CAT His CGA Arg CGA Arg ACA Thr ACC Thr TTP Phe ATG Glu GGT TGG Trp AAG Lys	AGC Ser ATG Met AAA Lys TTC Phe ATG Phe ATG CF ATG GAT Asp GCA Ala GCA Ala ACT Thr	CAT Hiss ATG Met CTA Leu GGA GIU CGC Arg GAC Asp TTT Phe GTG Val GTC Val TTT Phe GGG GCC CCC	TCA Ser AAA Lys TAT Tyr GAA Glu ATG GAT CTG GAT CTG GAT CT Ser GAT	Val TCA Ser GCC Ala Arg Arc Ile TTG GAC Chr GAG Glu ATG Met CCA Pro GTG GTG Val Leu	Ser CGC Arg GAA Glu GCA Ala GTC Val TCA Ser CCG GAT ASP GTA Val ATT Ile CTA	Arg TGG Trp ACC Thr CTT Leu ACG Thr AAG Glu ATG Leu GGG Glu GGG Gly GIY GIY CTT	Phe GCA Ala GAG Glu CAG Glu CAG GIN GAT CG Ser GAG GAG GAC Val AGC Ser GAC Asp AAT ASn AA	Gly CAT His GAA Glu CTT Leu GTA GAA Glu TTC Phe AAT ASn AAT ASn GIC CTA CTA CTT Leu	Glu TTG GAA Glu GCC Ala ACA Thr GAA Glu CJys GAG Glu TTT Phe GGA Gly GAG Glu GCG Ala	Leu ATC Ile CAT His CAG Gln ACC Thr CTC Leu ACG Thr ASP GAT ASP GAT ASP GGT GIY GCC Ala	Pro ACC Thr GAA Glu ATC Ile TTC Phe CAG Gln CGG CCC Pro TGG TGG TGG TGG CYS CAG Gln CCA Ala	Gln Gln ATG Met TTG GTC Val TAT Tyr CGA GGT GGT CGA GGT CTC Leu	285 900 300 945 315 990 330 1035 345 1080 360 1125 375 1170 390 1215 405 1260 420 1305 435 1350 450
901 301 946 316 991 331 1036 346 1081 361 1126 376 1171 391 1216 406 1261 421 1306 436 1351	GTA Val GTC Val GCA Ala AAA Lys CAG GIn CAG GIN TTG CAG CAG CAS TTG Ser TTC Fhe GAC	CTT Leu CGA Arg TCC Ser GAA Glu CCA Glu CCA Arg CAA Gln Arg CAA Gln CAT His TGC Cys CGT	CAT His CGA Arg CGA Arg ACA Thr ACC Thr TTT Phe AAG Glu GGT GGQ Trp AAG Lys CAC	AGC Ser ATG Met AAA Lys TTC Phe ATG Met TCC CGA Arg GAT Asp GCA Ala GCA Ala ACT Thr CCAC	CAT His ATG Met CTA Leu GAA Glu CGC Arg GAC Asp TTT Phe GTG GTG Val TTT Phe GGG Gly CAC	TCA Ser AAA Lys TAT Tyr GAA Glu ATG GAT ASD GAT ASD GGA GLY Ser GAT	Val TCA Ser GCC Ala CGT Arg ATC Ile TTG Leu TTG GAG Glu ATG GAU ATG CCA Pro GTG Val Leu CCA	Ser CGC Arg GAA Glu GCA Alac GTC Val TCA Ser CCG GAT ASP GTA Val ATT Ile CTA Leu	Arg TGG Trp ACC Thr CTT Leu ACG Thr AAG Glu ATT Ile AAC GGU ATT Ile GGG Gly GGT GJY 4.7	Phe GCA Ala GAG Glu CAG Glu CAG GAT Asp TCG GAG GAC GAC GAC GAC Ser GAC Ser AAT Asn AAC Lys	Gly CAT His GAA Glu CTT Leu GAG GIU CTA Phe AAT AAT AAT AAT CAA Gln GTC Val CTT Leu	Glu TTG GAA Glu GCC Ala ACA Thr GAA Glu TTT CAA GAG GAG GAG GIU GAG GIU GCG Ala	Leu ATC Ile CAT His CAG Gln ACC Thr CTC Leu ACG Thr GAT Asp GAT Asp GAT GAT GAT Asp GAT Asp GAT Asp GAT	Pro ACC Thr GAA Glu ATC Ile TTC Phe CAG Gln CGG Arg CCC Pro TGG TGG TGG TGG TGG CCS CAG Gln CAA Ala	Gln ATG Met TTG GTC Val TAT Tyr Cal TAT Tyr CGA GGT GGT CGG Trp CTC Leu	285 900 300 945 315 990 330 1035 345 1080 360 1125 375 1170 390 1215 405 1260 420 1305 435 1350 450

Fig. 1 The deduced amino acids sequence of recombinant DNA of OvCB_OvAEP_OvCF in pET32a+. The start codon, Met, and the stop codon (*) of the vector were highlighted. The 6 × His sequence tags were shown in both N- and C-terminals of the peptide (bold and underlined were restriction sites, GAA TCC: EcoR I; GAG CTC: Sacl; GTC GAC: Sall and AAG CTT: HindIII, bold and italics were primer priming sites)

agitation. After removing the secondary antibodies, the strips were washed with PBST and PBS. The reactivity of the protein bands was detected using TMB-Blotting Substrate Solution (Thermofisher Scientific). The reactivity was stopped by washing with tap water. Positive reactivity was indicated by the blue color of the protein bands. Positive (pool-positive *O. viverrini* infection human sera) and negative controls (pooled non-parasitic infection human sera) were used for quality control immunoblotting and validation of the results.

Statistical analysis

Immunoblot performance in the diagnosis of opisthorchiasis was evaluated. The performance characteristics of the test, including sensitivity (true-positive rate), specificity (true-negative rate), false-positive rate (FPR), false-negative rate (FNR), positive predictive value (PPV), negative predictive value (NPV), and diagnostic accuracy, were analyzed on the basis of the categorization of the results obtained in a 2×2 table [18].

Results

Selection of DNA encoded B-cell epitope-rich peptides

OvCB, OvAEP, and OvCF carry several peptide sequences that represent B-cell epitopes (Table 1). Peptide sequences with the highest epitope probability scores were selected. Five epitopes of OvCB included amino acids from 39 to 227, six epitopes of OvAEP included amino acids from 249 to 357, and two epitopes of OvCF included amino acids from 78 to 163 were selected for cloning (Table 1).

Construction of recombinant DNA

The recombinant OvCB_OvAEP_OvCF_pET32a+DNA was constructed. The total length of the recombinant OvCB OvAEP OvCF DNA in the pET32a+vector was 1224 nucleotides, and the expected molecular weight of the recombinant protein was 54.7 kDa. The deduced amino acid sequence of recombinant OvCB_ OvAEP OvCF pET32a+was constructed, and the order of nucleotides and integration of DNA fragments in pET32a+were verified via sequencing using T7 sequencing primers (Fig. 1), which revealed several point mutations in the nucleotide sequence of recombinant OvCB_OvAEP_OvCF_pET32a+. However, the mutation was silent and the subsequent amino acid sequence of the protein remained unaffected (Additional file 1: Fig. S1).

Recombinant protein production and purification

The recombinant protein OvCB_OvAEP_OvCF_ pET32a+ was produced in *E. coli* BL21(DE3). The recombinant protein with His-tag at the amino- and carboxyl-terminal ends was expressed in an insoluble form. The expressed proteins were dissolved in 8 M ureabinding buffer and partially purified on a Ni–NTA column. However, the high content of acidic amino acids in the protein causes purified recombinant proteins to have a molecular weight larger than their predicted size [19]. Thus, using SDS-PAGE and immunoblotting with an anti-His tag antibody, the molecular weight of the recombinant protein was determined to be approximately 70 kDa (Fig. 2).



Fig. 2 The recombinant OvCB_OvAEP_OvCF protein fused with 6×His in both N- and C-terminals in pET32a+system. The recombinant protein expressed in *Escherichia coli*, BL21(DE3), was larger than what was expected. The correspondence of molecular weight of the recombinant protein on 15% SDS-PAGE (**A**) and immunoblot with anti-His tag antibody (**B**) was observed at approximately 70 kDa

Evaluation of recombinant protein in the diagnosis of opisthorchiasis using immunoblotting

The potential of the recombinant protein OvCB_OvAEP_ OvCF_pET32a+as a diagnostic antigen for opisthorchiasis was evaluated using immunoblotting. The strong immunoreactivity of all opisthorchiasis human sera (35 cases; 100% sensitivity) at the recombinant protein band of approximately 70 kDa was observed. The strong reactivity of opisthorchiasis sera to recombinant proteins was unrelated to the intensity of infection.

The specificity of the recombinant protein was evaluated using 20 serum samples from subjects with other parasitic infections and 17 negative serum samples from subjects with non-parasitic infections in an endemic area. Among samples from subjects with other parasitic infection, the serum IgG antibodies from taeniasis (T) (two cases), strongyloidiasis (SS) (one case), giardiasis (GL) (one case), E. coli (EC) infection (two cases), enterobiasis (EV) (one case), and mixed infection of *Echinostome* spp. and Taenia spp. (E&T) (one case) were reacted with the recombinant protein. No reactivity was observed in the sera of the subjects with non-parasitic infections (Additional file 3: Fig. S3). The cross-reactivity with other parasitic infections and no reactivity with non-parasitic infections showed 78.4% specificity for detection by immunoblotting. In addition, FNR, FPR, PPV, and NPV were 0%, 21.6%, 81.4%, and 100%, respectively. Moreover, the diagnostic accuracy was 88.9% (Table 2).

Discussion

The shift to light infections necessitates sensitive methods for diagnosing opisthorchiasis in many endemics [1-4]. Antigen–antibody reactivity can be effectively exploited to detect light-intensity *O. viverrini* infection. In addition, using multi-epitope proteins as target antigens can increase the detection sensitivity.

The multi-B-cell epitope protein BSjPGM-BSjRAD23-1-BSj23 is an example of a constructed antigen with potential as a diagnostic target. BSjPGM-BSjRAD23-1-BSj23 showed high sensitivity (97.8%, 89/91) and

 Table 2 2×2
 table
 represents
 the
 performance
 of

 immunoblotting in detecting specific IgG antibodies against the
 recombinant protein OvCB_OvAEP_OvCF_pET32a+
 0vCF_pET32a+

Immunoblotting	FECT	Total	
	Positive opisthorchiasis	Negative	
Positive	35 (TP)	8 (FP)	43
Negative	0 (FN)	29 (TN)	29
Total	35	37	72

specificity in diagnosing goat schistosomiasis with reduction of cross-reactivity with goat hemonchosis and orientobilharziasis [17].

In this study, the single immunogenic molecules, OvCB, OvAEP, and OvCF, contained several B-cell epitopes. The combination of OvCB, OvAEP, and OvCF epitopes for B cells contributes to a higher number of antigenic protein targets, which increases the sensitivity of diagnosing opisthorchiasis.

The high levels of antibodies specific to *O. viverrini* infection, particularly total IgG antibody in the serum [20], as well as the immunogenicity of the recombinant OvCB_OvAEP_OvCF protein, provided a sensitivity of 100% and specificity of 78.4% for diagnosing opisthorchiasis. The strong reactivity with total IgG antibodies in the serum from light-infection opisthorchiasis observed in this study allows for efficient screening of light *O. viverrini* infections in both endemic and nonendemic areas. In addition, the high immunogenicity of the OvCB_OvAEP_OvCF protein could promote its detection in chronic and heavy infection cases where immunosuppression has been indicated [21].

Nevertheless, the recombinant OvCB OvAEP OvCF protein also showed strong reactivity to total IgG antibodies against other endemic helminth and protozoan infections. The highly conserved OvCB, OvAEP, and OvCF across parasitic helminths and protozoa allow for cross-reactivity with sera from patients with other parasite-infected [14, 16, 22]. Furthermore, IgG antibodies persisted in O. viverrini-infected hosts after antihelmintic treatment [23], and patients with a light infection excreting fewer than 50 eggs/g feces, which were undetectable by FECT, showed positive reactivity with the recombinant protein [24]. The detection of IgG4 antibodies [25] or the engineering of recombinant protein targets using rational design and directed evolution can improve the affinity and specificity of protein target binding to specific antibodies, thereby increasing the sensitivity and specificity of diagnosis [26, 27]. Moreover, other immunoglobulin isotypes or specific antibodies should be detected in alternative samples, such as saliva and urine.

Furthermore, the high immunogenicity of the recombinant OvCB_OvAEP_OvCF protein, which promotes high sensitivity in the detection of opisthorchiasis, could facilitate practical screening. The high sensitivity of multi-epitope protein target detection may enable patients with mild infections to be enrolled in confirmatory tests. Thus, OvCB_OvAEP_OvCF may be another target for controlling and preventing opisthorchiasis and CCA.

Conclusions

Proteins that present epitopes on B cells can stimulate host immune responses and serve as targets for detecting specific host humoral immune responses that diagnose infections. Combining multiple proteins that present B-cell epitopes, including OvCB, OvAEP, and OvCF, could result in a highly immunogenic protein with high sensitivity for the diagnosis of low-intensity O. viverrini infections. Although the prevalence of opisthorchiasis has decreased, mild infections continue to be reported and are difficult to diagnose using traditional microscopic methods. Thus, detecting the specific antibody to a single multi-B cell epitope protein, OvCB_OvAEP_OvCF, which demonstrated a high sensitivity of 100% and specificity of 78.4% for diagnosing light-intensity O. viverrini infection, was found to be an efficient screening method before proceeding with the confirmation test. The high immunogenicity of the OvCB OvAEP OvCF protein suggests that it can be used as a screening target in both endemic and nonendemic areas. Nonetheless, further studies are needed to increase the specificity of OvCB OvAEP OvCF detection. Additionally, the development of a screening test capable of detecting different immunoglobulins or infections in different sample types should be considered.

Abbreviations

PCR	Polymerase chain reaction
dNTP	Deoxyribonucleotide triphosphate
IPTG	Isopropyl β-D-1-thiogalactopyranoside
rpm	Revolutions per minute
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PBST	Phosphate-buffered saline with Tween 20
BSA	Bovine serum albumin
Ni–NTA	Nickel–nitrilotriacetic acid

Supplementary Information

The online version contains supplementary material available at https://doi. org/10.1186/s13071-024-06285-7.

Additional file 1: Fig. S1. Nucleotide sequence alignment. The graphic represents the nucleotide sequence alignment of the original OvCB, OvAEP, and OvCF sequences and the recombinant DNA obtained from sequencing. Edited nucleotides in the consensus sequence are indicated by lowercase letters.

Additional file 2: Fig. S2. Alignment of OvCB, OvAEP, and OvCF protein sequences with the recombinant protein sequence OvCB_OvAEP_OvCF_pET32a + .

Additional file 3: Fig. S3. Immunoblotting with specific IgG antibodies against the recombinant protein OvCB_OvAEP_OvCF_pET32a+. All sera from patients with opisthorchiasis (A and B) showed a band of specific reactivity corresponding to the molecular weight of the recombinant protein. Cross- and false-reactivity were observed in sera with taeniasis (T), *E. coli* infection (EC), enterobiasis (EV), giardiasis (GL), strongyloidiasis (SS), and mixed infection with *Echinostome* spp. and *Taenia* spp. (E&T) (C). The absence of reactivity was observed in sera from negative subjects (D).

Acknowledgements

The authors wish to thank Professor Banchob Sripa from the Tropical Disease Research Center, Faculty of Medicine, Khon Kaen University, for providing a cDNA library of adult *Q. viverrini* cDNA library and the pET32a+ vector. The authors would also like to thank the technicians of the laboratory of the College of Medicine and Public Health, Ubon Ratchathani University, for their help in providing resources to run this work. The authors would like to thank Editage (www.editage.com) for the English language editing.

Author contributions

JS designed the study, performed the experiments, interpreted the results, and wrote the manuscript. TC wrote the manuscript. All authors read and approved the final manuscript.

Funding

This research was supported by National Science, Research and Innovation Fund, and Ubon Ratchathani University, Research and Innovation Grant.

Availability of data and materials

The original data supporting the findings of this study are available within the article and its supplementary materials.

Declarations

Ethics approval and consent for participation

This study adhered to the guidelines of the Declaration of Helsinki. All procedures involved in participants in this study were approved by the Human Ethics Committee of Ubon Ratchathani University (UBU-REC-46–2563).

Consent for publication

All authors have read and agreed to the published version of the manuscript.

Competing interests

The authors declare that they have no competing interests.

Received: 17 January 2024 Accepted: 17 April 2024 Published online: 07 May 2024

References

- Sithithaworn P, Andrews RH, Nguyen VD, Wongsaroj T, Sinuon M, Odermatt P, et al. The current status of opisthorchiasis and clonorchiasis in the Mekong Basin. Parasitol Int. 2012;61:10–6.
- Sayasone S, Utzinger J, Akkhavong K, Odermatt P. Multiparasitism and intensity of helminth infections in relation to symptoms and nutritional status among children: a cross-sectional study in southern Lao People's Democratic Republic. Acta Trop. 2015;141:322–31.
- Miyamoto K, Kirinoki M, Matsuda H, Hayashi N, Chigusa Y, Sinuon M, et al. Field survey focused on *Opisthorchis viverrini* infection in five provinces of Cambodia. Parasitol Int. 2014;63:366–73.
- Boondit J, Suwannahitatorn P, Siripattanapipong S, Leelayoova S, Mungthin M, Tan-Ariya P, et al. An epidemiological survey of *Opisthorchis viverrini* infection in a lightly infected community, Eastern Thailand. Am J Trop Med Hyg. 2020;102:838–43.
- Jamornthanyawat N. The diagnosis of human opisthorchiasis. Southeast Asian J Trop Med Public Health. 2002;33:86–91.
- Buathong S, Leelayoova S, Mungthin M, Ruang-Areerate T, Naaglor T, Suwannahitatorn P, et al. Molecular discrimination of *Opisthorchis*-like eggs from residents in a rural community of central Thailand. PLoS Negl Trop Dis. 2017;11:e0006030.
- Worasith C, Wangboon C, Duenngai K, Kiatsopit N, Kopolrat K, Techasen A, et al. Comparing the performance of urine and copro-antigen detection in evaluating *Opisthorchis viverrini* infection in communities with different transmission levels in Northeast Thailand. PLoS Negl Trop Dis. 2019;13:e0007186.
- 8. Worasith C, Kamamia C, Yakovleva A, Duenngai K, Wangboon C, Sithithaworn J, et al. Advances in the diagnosis of human Opisthorchiasis:

development of *Opisthorchis viverrini* antigen detection in urine. PLoS Negl Trop Dis. 2015;9:e0004157.

- Sawangsoda P, Sithithaworn J, Tesana S, Pinlaor S, Boonmars T, Mairiang E, et al. Diagnostic values of parasite-specific antibody detections in saliva and urine in comparison with serum in opisthorchiasis. Parasitol Int. 2012;61:196–202.
- Watthanakulpanich D, Waikagul J, Anantaphruti MT, Dekumyoy P. Evaluation of *Bithynia funiculata* snail antigens by ELISA-serodiagnosis of human opisthorchiasis. Southeast Asian J Trop Med Public Health. 1997;28:593–8.
- Waikagul J, Dekumyoy P, Chaichana K, Thairungroje Anantapruti M, Komalamisra C, et al. Serodiagnosis of human opisthorchiasis using cocktail and electroeluted *Bithynia* snail antigens. Parasitol Int. 2002;51:237–47.
- Pakdee W, Waikagul J, Kalambaheti T, Ito A, Dekumyoy P. Iso-electricfocusing of *Bithynia* snail antigens for IgG- and IgG(1–4)-ELISA detection of human opisthorchiasis. Southeast Asian J Trop Med Public Health. 2010;41:813–20.
- Chanawong A, Waikagul J, Thammapalerd N. Detection of shared antigens of human liver flukes *Opisthorchis viverrini* and its snail host. *Bithynia* spp. Trop Med Parasitol. 1990;41:419–21.
- Sripa J, Brindley PJ, Sripa B, Loukas A, Kaewkes S, Laha T. Evaluation of liver fluke recombinant cathepsin B-1 protease as a serodiagnostic antigen for human opisthorchiasis. Parasitol Int. 2012;61:191–5.
- Teimoori S, Arimatsu Y, Laha T, Kaewkes S, Sereerak P, Tangkawattana S, et al. Immunodiagnosis of opisthorchiasis using parasite cathepsin F. Parasitol Res. 2015;114:4571–8.
- Laha T, Sripa J, Sripa B, Pearson M, Tribolet L, Kaewkes S, et al. Asparaginyl endopeptidase from the carcinogenic liver fluke, *Opisthorchis viverrini*, and its potential for serodiagnosis. Int J Infect Dis. 2008;12:e49-59.
- Lv C, Hong Y, Fu Z, Lu K, Cao X, Wang T, et al. Evaluation of recombinant multi-epitope proteins for diagnosis of goat schistosomiasis by enzymelinked immunosorbent assay. Parasit Vectors. 2016;9:135.
- Almeida M, Pizzini C, Damasceno L, Muniz M, Paes R, Peralta R, et al. Validation of western blot for *Histoplasma capsulatum* antibody detection assay. BMC Infect Dis. 2016;16:1–18.
- Guan Y, Zhu Q, Huang D, Zhao S, Jan Lo L, Peng J. An equation to estimate the difference between theoretically predicted and SDS PAGEdisplayed molecular weights for an acidic peptide. Sci Rep. 2015;5:13370.
- Wongratanacheewin S, Bunnag D, Vaeusorn N, Sirisinha S. Characterization of humoral immune response in the serum and bile of patients with opisthorchiasis and its application in immunodiagnosis. Am J Trop Med Hyg. 1988;38:356–62.
- Haswell-Elkins MR, Sithithaworn P, Mairiang E, Elkins DB, Wongratanacheewin S, Kaewkes S, et al. Immune responsiveness and parasitespecific antibody levels in human hepatobiliary disease associated with *Opisthorchis viverrini* infection. Clin Exp Immunol. 1991;84:213–8.
- Pinlaor P, Kaewpitoon N, Laha T, Sripa B, Kaewkes S, Morales ME, et al. Cathepsin F cysteine protease of the human liver fluke. *Opisthorchis viverrini*. PLoS Negl Trop Dis. 2009;3:e398.
- Akai PS, Pungpak S, Chaicumpa W, Kitikoon V, Ruangkunaporn Y, Bunnag D, et al. Serum antibody responses in opisthorchiasis. Int J Parasitol. 1995;25:971–3.
- Kopolrat KY, Singthong S, Khuntikeo N, Loilome W, Worasith C, Homwong C, et al. Performance of Mini Parasep[®] SF stool concentrator kit, Kato-Katz, and formalin-ethyl acetate concentration methods for diagnosis of opisthorchiasis in Northeast Thailand. Parasit Vectors. 2022;15:234.
- Phupiewkham W, Rodpai R, Inthavongsack S, Laymanivong S, Thanchomnang T, Sadaow L, et al. High prevalence of opisthorchiasis in rural populations from Khammouane Province, central Lao PDR: serological screening using total IgG- and IgG4-based ELISA. Trans R Soc Trop Med Hyg. 2021;115:1403–9.
- Lee JH, Seo HS, Song JA, Kwon KC, Lee EJ, Kim HJ, et al. Proteinticle engineering for accurate 3D diagnosis. ACS Nano. 2013;7:10879–86.
- Blackstock D, Park M, Sun Q, Tsai S-L, Chen W. Engineering protein modules for diagnostic applications. Curr Opin Chem Eng. 2013;2:416–24.

Publisher's Note

Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.