

Application of Recombinant *Gnathostoma spinigerum* Matrix Metalloproteinase-Like Protein for Serodiagnosis of Human Gnathostomiasis by Immunoblotting

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Abstract. Matrix metalloproteinase (MMPs) is the extracellular zinc-dependent endopeptidase and is secreted for degrading extracellular matrix molecules of host tissues. A cDNA encoding MMP-like protein of *Gnathostoma spinigerum* larvae was amplified by reverse transcription-polymerase chain reaction, and was cloned into a prokaryotic expression vector, and expressed in *Escherichia coli*. Total immunoglobulin G class (total IgG) antibody responses to the recombinant MMP-like protein were analyzed by immunoblot diagnosis of human gnathostomiasis. Serum samples from proven and clinically suspected cases of gnathostomiasis, other parasitic diseases patients, and from healthy volunteers were tested. The immunoblotting gave high sensitivity (100%) and specificity (94.7%). Positive and negative predictive values were 85.4% and 100%, respectively. Recombinant MMP-like protein can be used as a diagnostic antigen and potentially replace native parasite antigens to develop a gnathostomiasis diagnostic kit.

INTRODUCTION

Human gnathostomiasis is an important food-borne parasitic zoonosis caused by the spirurid nematode *Gnathostoma* spp. and the disease is endemic in Asia and the Americas,^{1–4} and in returned travelers who had visited the endemic areas of this harmful parasite.^{5,6} Humans acquire infection by consuming raw or undercooked meat, i.e., freshwater fish, frogs, chicken, etc., which harbor *Gnathostoma* advanced third-stage larvae (AL3). *Gnathostoma spinigerum* is a causative agent mainly in Asian countries, i.e., Thailand, Japan, Vietnam, etc.^{2,7–9} The *Gnathostoma* AL3 migrates into the subcutaneous tissue and causes intermittent migratory swelling. Sometimes the worm migrates to vital organs, i.e., brain, eye, etc., producing severe pathologic signs and symptoms that can lead to harmful problems and death.^{6,10–12} Definitive diagnosis for human gnathostomiasis can be made by detecting the migrating larvae from the human body. Because direct detection of the parasite is difficult and often unsuccessful, diagnosis of gnathostomiasis is practically made by relying upon clinical features, history of eating parasite-contaminated foods, blood eosinophilia, and serological outcomes, i.e., enzyme-linked immunosorbent assays^{13–16} or immunoblotting using *Gnathostoma* AL3 extract, including an antigenic peptide with an approximate molecular mass of 24 kDa^{17–19} and below 27–29 kDa.²⁰ Two-dimensional gel electrophoresis (2-DE) and immunoblotting revealed that *G. spinigerum* AL3 antigenic spots with an approximate molecular mass of 23–25 kDa and pI of 8.3–8.5 revealed a high potential for the serodiagnosis of human gnathostomiasis spinigerum.²¹ The amino acid sequence of these antigenic spots was determined by liquid chromatography tandem mass spectrometry (LC/MS-MS) and the LC/MS-MS spectra²² and one of the peptide sequences showed high similarity with a matrix metalloproteinase (MMP)-like protein of *G. spinigerum* database (GenBank accession no. AAF82802).²³

Cloning and expression of *G. spinigerum* genes such as MMP-like protein,²³ cathepsin L-like cysteine protease,²⁴

and cyclophilin protein²⁵ have been reported. However, the diagnostic values of those recombinant proteins for human gnathostomiasis have not been validated. In this study, we produced a recombinant MMP-like protein of *G. spinigerum* and evaluated its sensitivity and specificity in immunodiagnosis for human gnathostomiasis. We selected MMP-like protein because its molecular mass and pI corresponded well with the 2-DE immunoreactive spots detected by the confirmed human gnathostomiasis sera.²² The goal of this study is to setup a stable mass-production system for the standardized immunodiagnostic kit with recombinant *G. spinigerum* MMP-like protein antigen.

MATERIALS AND METHODS

Human sera. All serum samples were supplied by the serum bank of the Faculty of Medicine, Khon Kaen University. Serum samples consisted of three groups: 1) Negative control group, which included samples from healthy adult volunteers who were free from any intestinal parasitic infection and checked by stool examination by the formalin ethyl acetate concentration technique²⁶ at the time of blood collection. A pooled sera from all those healthy individuals was also used as negative control for each assay. 2) Gnathostomiasis group, which included samples from parasitologically confirmed gnathostomiasis patients and from patients showing clinical symptoms of suspected cutaneous and visceral gnathostomiasis,^{2,27,28} with a history of eating food possibly contaminated with *Gnathostoma* larvae and were positive 24 kDa *G. spinigerum* antigen by immunoblotting.²⁹ 3) The third group ($N = 83$) consisted of serum samples from patients with other parasitic infections than gnathostomiasis. Their infections were confirmed by parasitological methods except that cysticercosis cases were diagnosed by a computerized tomography scan and found positive by the immunological method Table 1. Informed consent was obtained from all human adult participants and from parents or legal guardians of minors. The study protocol was approved by the Khon Kaen University Ethics Committee for Human Research (HE541293).

Parasites, total RNA isolation, and synthesis of cDNA encoding MMP-like protein. The *G. spinigerum* AL3 were

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TABLE 1

Type of human sera and immunoblotting results against the purified fusion-tagged recombinant MMP-like protein*

Type of serum	Group	No. positive/no. total
Healthy control	1	0/30
Confirmed gnathostomiasis	2	13/13
Suspected gnathostomiasis	2	22/22
Cysticercosis	3	0/8
Taeniasis	3	0/5
Opisthorchiasis viverrini	3	2/8
Fascioliasis	3	3/10
Paragonimiasis	3	0/10
Angiostrongyliasis	3	0/9
Strongyloidiasis	3	0/5
Hookworm infection	3	0/5
Capillariasis	3	1/9
Ascariasis	3	0/5
Trichinosis	3	0/9

*MMP = matrix metalloproteinase.

collected from mice inoculated orally with early third-stage larvae recovered from copepod.³⁰ The worms ($N = 40$) were then placed into RNAlater (Promega, Madison, WI). The total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA) and was finally dissolved in diethylpyrocarbonate-treated deionized water and stored at -70°C unit use. Based on the DNA sequence of a *G. spinigerum* MMP-like protein from the published data²³ (GenBank accession no. AF277294), we designed a primer pair to obtain the complete open reading frame of the MMP-like sequence. The primers used were as follows: GS-F1 5'-CAGTAAAGATGAAACTACAGAGTGTG-3' and GS-R1 5'-GACGTTTACGGCATTGGAG-3' (The start and stop codons are indicated in bold). A reverse transcription-polymerase chain reaction (RT-PCR) was performed using the RobusT II RT-PCR kit (Finnzymes, Espoo, Finland) according to the manufacturer's protocol. The PCR parameters were as follows: cDNA synthesis at 40°C for 60 minutes and at 94°C for 2 minutes; and then 35 cycles of 30 seconds at 94°C , 30 seconds at 55°C , and 1 minute at 72°C ; and a final step at 72°C for 10 minutes. The PCR product obtained was checked by electrophoresis using 1% agarose gel, purified, and subcloned into pCR4-TOPO Vector using a TOPO TA Cloning kit (Invitrogen), and transformed into TOP10 competent cells (Invitrogen) for sequence confirmation.

DNA sequencing and analysis. The DNA sequencing was performed using the MegaBACE 1000 DNA analysis system (GE Healthcare, Piscataway, NJ), and the sequence obtained was analyzed using software programs including BLAST (www.ncbi.nlm.nih.gov), Multalin (<http://bioinfo.genotoul.fr/multalin/multalin.html>), BioEdit version 7.0.9 (www.mbio.ncsu.edu/BioEdit/BioEdit.html), and Compute pI/Mw tool (http://web.expasy.org/compute_pi/).

Expression, purification, and cleavage of the recombinant MMP-like protein. The primers carried restriction sites of *Eco*RI (GS-F2 5'-TGGCGTGGAA**TT**CTATGAAAC TACAGAGTGTG -3') and *Hind* III (GS-R2 5'CGGAGGA **AA**GCTTTTACGGC ATTTGGAG -3') (Restriction sites are indicated in bold) were designed. The PCR parameters were as follows: initial heating at 94°C for 3 minutes; and then 35 cycles of 30 seconds at 94°C , 30 seconds at 69°C , and 1 minute at 72°C ; and a final step at 72°C for 10 minutes. The PCR product was subcloned into a pET-43.1(+) expression vector

(Novagen, Darmstadt, Germany). The recombinant plasmids were then transformed into *Escherichia coli* JM 109 and the accuracy of the nucleotide sequence harbored in the bacterial clones was verified by sequencing. The plasmid DNA presenting the correct codons was used to transform in *E. coli* Rosetta-gami 2(DE3) expression host (Novagen). Protein expression was induced by 1 mM isopropyl- β -D-thiogalactopyranoside. Suspension of bacterial cell in a lysis buffer (50 mM Tris-HCl, pH 8.0, 5% glycerol, 50 mM NaCl, 0.5 mg/mL lysozyme) was sonicated on ice, and the recombinant protein fused with N utilization substance A (NusA)-tagged and 6-Histidine (6-His)-tagged residues, and was purified using Ni-NTA His Bind Resin (Novagen). The recombinant MMP-like protein was separated from fusion-tagged proteins by cleaving with recombinant enterokinase (rEK) (Novagen) according to the manufacturer's instructions.

Antigenicity of the purified recombinant MMP-like protein by immunoblotting. The purified and rEK-cleaved MMP-like fusion-tagged proteins were electrophoresed on a 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli,³¹ and then transferred to a nitrocellulose membrane³² that was cut into strips for immunoblotting. The purified fusion-tagged protein was detected by a reaction with an anti-NusA mouse monoclonal antibody (Novagen), according to the manufacturer's protocol. Briefly, after blocking the membrane strips with (3%) bovine serum albumin (BSA) in phosphate buffered saline (PBS, pH 7.5) incubating with an anti-NusA antibody, and then probing with horseradish peroxidase (HRP)-labeled goat anti-mouse IgG (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), the reaction was visualized with 3,3'-diaminobenzidine tetrahydrochloride (DAB) substrate. For demonstration of antigenicity of the cleaved recombinant MMP-like protein, the reaction was probed with a 1:100 diluted pooled sera of gnathostomiasis patients or pooled negative control sera (in 1% skimmed milk in PBS, pH 7.5) absorbed with *E. coli* lysate for 2 hours at room temperature. The membranes were washed with 1% skimmed milk in PBS, pH 7.5, containing 0.1% Tween-20 (PBST) (5 times), and incubated with goat anti-human IgG (H+L) HRP conjugate (Invitrogen) at a dilution of 1:4,000 (in 1% skimmed milk in PBST) for 2 hours at room temperature. After 5 washes with 1% skimmed milk in PBST, the strips were then developed with DAB substrate, and the reaction stopped with distilled water.

Evaluation of the purified fusion-tagged recombinant MMP-like protein as a diagnostic antigen for human gnathostomiasis. The purified fusion-tagged recombinant MMP-like protein was electrophoresed on 10% SDS-PAGE and then electrotransferred to a nitrocellulose membrane. After blocking nonspecific binding sites with 1% skimmed milk in PBST, pH 7.5 for 30 minutes, the membrane was cut into ~3 mm wide strips (9.8 μg protein/strip). Each strip was incubated with individual human serum samples absorbed with *E. coli* lysate and processed as described previously. The diagnostic parameters of sensitivity, specificity, and positive and negative predictive values were calculated as previously described.³³

RESULTS

Synthesis of the complete cDNA encoding a MMP-like protein. We successfully amplified cDNA encoding a MMP-like protein of *G. spinigerum* (Figure 1A). The gene consisted

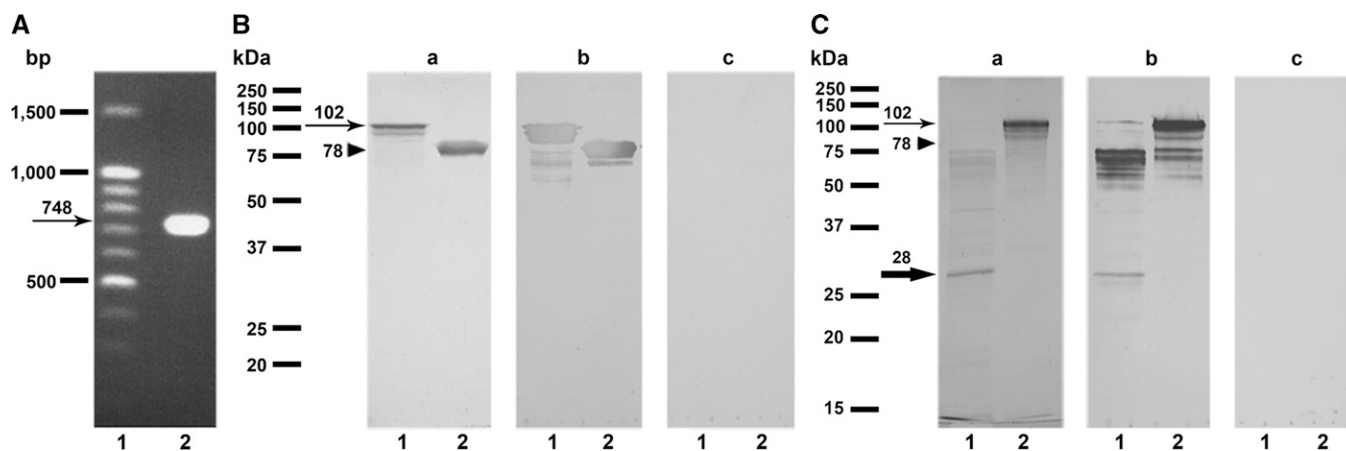


FIGURE 1. (A) Ethidium bromide stain patterns of the *Gnathostoma spinigerum* complete cDNA encoding a matrix metalloproteinase (MMP)-like protein on a 1.0% agarose gel. An arrow indicates band at approximate of 748 base pairs. Lane 1, DNA size markers (ZipEnzyme, Novosibirsk, Russia) and lane 2, the complete cDNA encoding a MMP-like protein amplified by reverse-transcriptase polymerase chain reaction (PCR) with gene-specific primers. (B) Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis of the fusion-tagged protein with and without recombinant MMP-like protein expressed in *Escherichia coli*. Coomassie Brilliant Blue stained (a) and immunoblotting probed with (b) and without (c) an anti-NusA antibody. Lane 1, the fusion-tagged protein containing the recombinant MMP-like protein (102 kDa, arrow) and lane 2, the fusion-tagged protein alone (78 kDa, arrow head). (C) SDS-PAGE analysis of the recombinant MMP-like protein cleaved with recombinant enterokinase (rEK) and the recombinant fused-tagged MMP-like proteins. Coomassie Brilliant Blue stained (a) and immunoblotting probed with sera from pooled gnathostomiasis patients (b) and pooled healthy negative controls (c). Lane 1, the rEK-cleaved MMP-like protein and lane 2, the fusion-tagged MMP-like recombinant protein. A thin arrow indicates expression of the fusion-tagged MMP-like protein (102 kDa). An arrow head indicates 78 kDa band of the fusion-tagged protein alone. A thick arrow indicates band of the rEK-cleaved MMP-like protein (28 kDa).

of a single open reading frame of 735 basepairs encoding 244 amino acids with a predicted molecular mass of 28 kDa and a theoretical *pI* of 7.8. The sequence was identical to the nucleotide sequence of a *G. spinigerum* MMP-like gene (GenBank accession no. AF277294).

Expression, purification, cleavage, and immunocharacterization of the recombinant MMP-like protein. A complete cDNA encoding the MMP-like protein was cloned into an expression vector and the recombinant MMP-like fusion-tagged protein was expressed in an *E. coli* expression system. The purified recombinant protein gave a single band in SDS-PAGE (Figure 1B, panel a). By immunoblot analysis, the fusion-tagged MMP-like protein and fusion-tagged protein alone were visualized by anti-NusA antibody at approximate molecular masses of 102 and 78 kDa, respectively (Figure 1B, panel b). The rEK-cleaved MMP-like protein has a molecular mass of ~28 kDa (Figure 1C, panel a). The cleaved MMP-like protein showed strong positive reactivity with pooled gnathostomiasis patient serum (Figure 1C, panel b) but did not react with the pooled negative control serum (Figure 1C, panel c) by immunoblotting.

Evaluation of the diagnostic values of the purified fusion-tagged recombinant MMP-like protein. Immunoblot analysis employing the diagnostic values of the purified fusion-tagged recombinant MMP-like protein for human gnathostomiasis was evaluated using individual serum from healthy control, gnathostomiasis patients, and the patients with other parasitic diseases (Table 1; Figure 2A). All serum samples from the confirmed ($N = 13$) and suspected ($N = 22$) gnathostomiasis patients strongly reacted with the purified recombinant MMP-like fusion protein. In contrast, none of the 30 healthy control sera showed positive seroreactivity to this recombinant antigen. Some cross-reactivity was observed in serum samples of capillariasis (1 of 9), opisthorchiasis viverrini (2 of 8), and fascioliasis (3 of 10). The calculated diagnostic sensitivity, specificity, and positive and negative predictive values were 100%,

94.7%, 85.4%, and 100%, respectively. Non-reactive band was shown when all sera reacted with the fusion-tagged protein alone (78 kDa) (Figure 2B).

DISCUSSION

For the immunodiagnosis of gnathostomiasis, many attempts have been made³⁴ to establish a specific diagnostic test system for the disease caused by *Gnathostoma* spp., such as *Gnathostoma binucleatum*,^{16,35} *Gnathostoma doloresi*,³⁶ and *G. spinigerum*.¹⁷ However, the limitation is the small amount production of the specific antigen from native worm extracts. Maintenance of the lifecycle of *Gnathostoma* spp. in experimental animals in the laboratory is expensive and time consuming. The cDNA encoding MMP-like protein of *G. spinigerum* AL3 was cloned and the deduced amino acid sequence was correlated with that of the immunodominant spots²² and this protein was considered as the potential antigen for detecting specific antibodies in infected patients. Thus, in this study, we intended to produce the recombinant *G. spinigerum* MMP-like protein for massive antigen supply for the serodiagnosis of human gnathostomiasis in Asian countries. The MMP-like gene was expressed in *E. coli* using pET 43.1(+), which contained the Nus A and 6-His tagged residues as fusion proteins. This expression system resulted in a high yield (~140 mg/one L of *E. coli* culture) and the fusion protein was obtained in the soluble fraction, as evaluated by Coomassie Brilliant Blue staining (shown in Figure 1B, panel a, lane 1). The fusion protein was seen as a molecular mass of ~102 kDa, which was somewhat greater than the theoretical molecular mass of 89 kDa (fusion tag, 61 kDa; MMP-like protein, 28 kDa). This could possibly be caused by the alteration of the constant charge: mass ratio in binding between the SDS and the protein carrying a large size of the fusion tag.

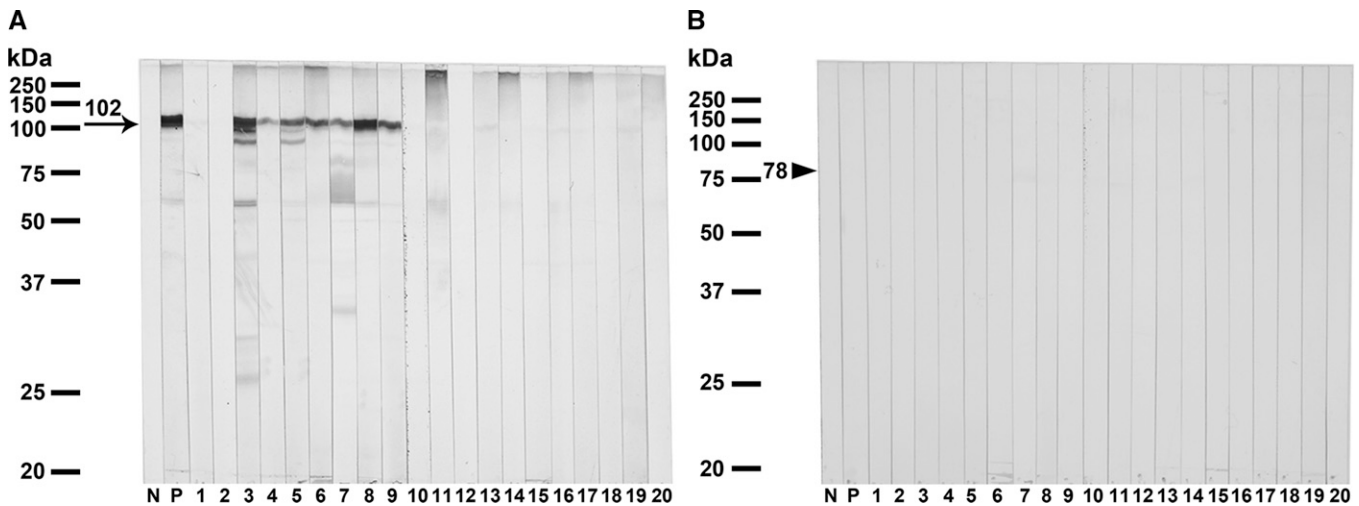


FIGURE 2. Representative immunoblotting patterns reacted with the purified fusion-tagged MMP-like protein (A) compared with the fusion-tagged protein alone (B) expressed in *Escherichia coli*. Antigens were probed with 1:100 diluted sera from pooled negative controls (N), pooled human gnathostomiasis patients (P), healthy control (1, 2), parasitologically confirmed gnathostomiasis (3–7), clinically suspected visceral gnathostomiasis (8) and cutaneous gnathostomiasis (9), cysticercosis (10), angiostrongyliasis (11), paragonimiasis (12), fascioliasis (13), trichinosis (14), ascariasis (15), opisthorchiasis viverrini (16), strongyloidiasis (17), taeniasis (18), capillariasis (19), and hookworm infection (20). The arrow indicates the specific immunoreactive band at ~102 kDa and arrow head indicates the fusion-tagged protein alone at ~78 kDa.

The immunodominant antigenicity of the recombinant fusion protein against human sera was shown by immunoblotting. The recombinant fusion protein specifically reacted with the gnathostomiasis patient sera, but not with sera from healthy control or from patients infected with other parasites. Only faint cross-reactivity was observed with the sera of capillariasis (1 of 9), opisthorchiasis viverrini (2 of 8), and fascioliasis (3 of 10) patients. These cross-reactions are possibly explained because these patients might have a previous history of subclinical infection with *G. spinigerum* and mixed infections with these parasites. Even when cross-reactions with fascioliasis, capillariasis, and opisthorchiasis sera were observed, it does not cause a real problem in the clinical setting because these parasitic infections usually present with clinical features different from those of gnathostomiasis. However, subclinical infection with *G. spinigerum* and mixed infections sera with other parasites need to be evaluated with more samples. In addition, we have also tested the immunoblotting patterns using various human sera as revealed in Table 1 and reacted with the recombinant MMP-like protein cleaved with rEK (28 kDa) (see Supplemental Figure 1), the diagnostic sensitivity and specificity were quite similar and revealed the results as presented when testing with the purified fusion-tagged MMP-like protein (102 kDa). These results ensure both types of antigen can be used for supportive diagnostic purpose.

Previous immunoblotting reports have shown that the native 24 kDa *G. spinigerum* larval antigen reacting to total IgG antibody could contribute to the reliable diagnosis of human gnathostomiasis. The demonstrated sensitivity ranged from 83.3% to 100%, whereas the specificity ranged from 87.8% to 100%.^{17,19,21} The native 21 kDa antigenic band of *G. spinigerum* AL3 reacting faintly to IgG4 antibody gave the 100% sensitivity and specificity,¹⁸ whereas the 24 kDa *G. spinigerum* antigen revealed the sensitivity and specificity that ranged from 75% to 92.9% and 93.4% to 93.9%, respectively.^{18,19} In another study, the antigenic bands below 27–29 kDa of *G. spinigerum* AL3 showed the 100% sensitivity and specificity.²⁰ This study showed high sensitivity and specificity of an

immunoblot technique against the recombinant *G. spinigerum* MMP-like protein for detection of IgG antibody are quite similar with previous reports as described above.

In conclusion, we report the successful cloning of the *G. spinigerum* MMP-like gene and the fusion-tagged protein expressed as a soluble form in the *E. coli* cytoplasmic compartment. The recombinant *G. spinigerum* MMP-like protein has the potential for development of a serodiagnostic kit for human gnathostomiasis in endemic areas as a stable mass production system.

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