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An immunochromatographic device to detect antibodies for rapid diagnosis of human angiostrongyliasis in whole-blood and cerebrospinal fluid samples

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ABSTRACT

Background: Human angiostrongyliasis is caused by the rat lung worm, *Angiostrongylus cantonensis* and is often a cause of eosinophilic meningitis (EOM) or meningoencephalitis. Humans are infected by consuming infective larvae from intermediate hosts (snails and slugs), paratenic hosts or contaminated vegetables. Here, a simple and rapid antibody-detection lateral-flow immunochromatographic test (ICT) was developed as a point-of-care tool for supporting diagnosis of human angiostrongyliasis. We also tested the ICT with cerebrospinal fluid (CSF) samples.

Methods: We evaluated a new ICT format using a recombinant *A. cantonensis* galectin-2 (rAcGal2) protein as a target for anti-*A. cantonensis* IgG antibodies in simulated whole-blood samples (WBSs) and compared results with those obtained using corresponding serum samples.

Results: The sensitivity and specificity values for IgG antibody detection in simulated WBSs were 82.0 (95 % CI 73.1–89.0) and 97.1 (95 % CI 93.3–99.0), while in serum samples these were 92.0 (95 % CI 84.8–96.5), and 97.1 (95 % CI 93.3–99.0), respectively. Results between simulated WBSs and serum samples did not differ significantly with a concordance of 96.3 % (Cohen's kappa 0.9177). Anti-*Angiostrongylus* antibodies were also detected in CSF samples: 12 out of 16 EOM cases were positive while all 10 CSF samples from control cases were negative.

Conclusion: The ICT is easy to implement and can provide supportive diagnosis at the bedside or in local and remote hospitals with limited facilities. An additional benefit is that it can be used with CSF samples obtained by lumbar puncture for management of high intracranial pressure of EOM patients in intensive care units.

1. Introduction

Human angiostrongyliasis is mainly caused by *Angiostrongylus cantonensis*, a metastrongyloid nematode. Rats are the definitive hosts, in which worms mature in the pulmonary arteries, while snails and slugs act as intermediate hosts of the parasite.[1] Human infection occurs through eating the third-stage larvae in undercooked snails, slugs or paratenic hosts (e.g. shrimps, lizards etc.) infected with the parasite, or from eating contaminated vegetables.[1,2] The parasite commonly causes a neurological disease, eosinophilic meningitis (EOM) or meningoencephalitis.[3,4] Cases have been recorded globally, but most often from the Asia-Pacific region.[1,5,6] Imported human angiostrongyliasis cases have been reported in Europe.[7]

Diagnosis is generally based on clinical history, laboratory findings, and cerebrospinal fluid (CSF) eosinophilia. Definitive diagnosis is confirmed by worm discovery in CSF after lumbar puncture or in the eyes during surgery, but direct detection of the parasite in patients is rare.[6,8,9] Differential diagnosis is difficult because the symptoms are often confused with those caused by other parasitic diseases i.e. paragonimiasis, gnathostomiasis, and cysticercosis.[10] A DNA-detection method has been described and is a highly sensitive and specific diagnostic tool, as assessed by quantitative real-time PCR assay.[8,11] This

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method is used in research laboratories but no commercially available kit exists. Antigenic proteins of *A. cantonensis* at approximate molecular masses of 29–31 kDa have been used as diagnostic markers for human angiostrongyliasis in enzyme-linked immunosorbent assays and immunoblot tests.[12–15] However, these serological tests are laborious to perform. Recently, a simple and rapid antibody-detection lateral-flow immunochromatographic test (ICT) using human sera was developed for supportive diagnosis[16,17] but has not been evaluated for use in whole-blood samples (WBSs). In the present study, we tested a new ICT format using a recombinant *A. cantonensis* galectin-2 (rAcGal2) protein[18] for the detection of anti-*A. cantonensis* IgG antibodies in simulated WBSs and compared its efficacy with antibody detection in corresponding serum samples. This format was also used with human CSF samples for evaluation of its diagnostic utility.

2. Materials and method

2.1. Clinical specimens and processing

All procedures performed involving human participants were in accordance with the Khon Kaen University Ethics Committee for Human Research, permit HE664044 approved November 30, 2023. In total, 270 leftover serum samples were obtained from the frozen biobank at the Department of Parasitology, Faculty of Medicine, and Mekong Health Science Research Institute Biobank Project, Khon Kaen University. These included 100 samples of angiostrongyliasis sera (group 1), including 7 from patients with proven angiostrongyliasis confirmed by recovery of the worms from the patients' eyes, and 93 from patients with EOM-associated angiostrongyliasis diagnosed with angiostrongyliasis based on immunoblotting.[13] All cases had a history of consuming raw or undercooked snails possibly contaminated with *A. cantonensis* third-stage larvae. EOM-associated angiostrongyliasis was diagnosed based on the presence of more than 10 % eosinophils in the CSF, along with negative results for gram staining, acid-fast *Bacillus* staining, India-ink staining, cryptococcal antigen testing, and CSF culture[19] The incubation period for EOM-associated angiostrongyliasis ranged from 1 to 90 days, with a median of 15 days. In addition, 25 serum samples from healthy volunteers (group 2) who were free from any intestinal parasites (stool samples were negative based on the formalin ethyl-acetate concentration method[20]; at the time of blood collection, and 145 serum samples from patients with other parasitic diseases (group 3) which included 10 samples each of gnathostomiasis, capillariasis, strongyloidiasis, trichinosis, trichuriasis, ascariasis, hookworm infections, paragonimiasis, fascioliasis, opisthorchiasis, cysticercosis, taeniasis, amoebiasis, blastocystosis, and five samples of sparganosis. Cases of capillariasis, strongyloidiasis, trichuriasis, ascariasis, hookworm infections, opisthorchiasis, taeniasis, amoebiasis, and blastocystosis had been confirmed by parasitological examination of stool samples.[20] Fascioliasis cases were confirmed by serological methods and clinical manifestations.[21] Paragonimiasis was confirmed by the presence of eggs in sputa or feces and by Western blot.[22] Cysticercosis cases were confirmed by serology and computed tomography.[23] Sparganosis cases were confirmed using histopathological investigation and PCR.[24] Trichinosis cases were confirmed using the enzyme-linked immunosorbent assay (ELISA) technique.[25] Gnathostomiasis cases were confirmed by serological methods with clinical manifestations and history of dietary preferences, as previously described.[26]

Simulated whole blood was prepared with type O red blood cells (RBCs) that were used to spike serum samples, as previously reported.[27] In short, a 500 μL sample of type O whole blood was centrifuged at 13,200×*g* for 10 min at 4 °C, followed by removal of the plasma. The packed RBCs were rinsed using phosphate-buffered saline (PBS, pH 7.4) and centrifuged three times at 13,200×*g* for 10 min at 4 °C each time. The packed RBCs were then re-suspended with PBS (pH 7.4) to a final volume of 500 μL. Aliquots of 10 μL of the suspension were taken in fresh tubes and centrifuged at 13,200×*g* for 10 min at 4 °C and the supernatant (6.5 μL) discarded, leaving 3.5 μL of packed RBCs. The simulated WBSs were prepared by adding 6.5 μL of each serum sample to 3.5 μL of packed RBCs to simulate the normal levels of human blood components.

Twenty-six leftover CSF samples from the frozen biobank at the Faculty of Medicine, Khon Kaen University were also evaluated. These CSF samples had been collected from EOM-associated angiostrongyliasis ($n = 16$) patients and control ($n = 10$) people. All EOM patients had presented with severe headaches after eating raw shrimps, snails or monitor lizards (*Varanus bengalensis*). The diagnostic criterion of EOM was based on finding ≥10 % eosinophils in the CSF.[19] All stains and bacterial cultures of the CSF samples turned out negative, nor was any parasite found. The serum syphilis-venereal disease research laboratory test was non-reactive in every case, but all EOM patients were positive for specific IgG antibody to *A. cantonensis* antigen in the CSF by ELISA.[28] Also, all EOM sera reacted to a specific *A. cantonensis* antigenic band by immunoblotting.[13] The control group ($n = 10$) consisted of four patients without meningitis whose CSF was sampled during lumbar puncture for anesthesia before surgery and six symptomatic meningitis-like patients (tension headache) with normal CSF profiles. All control patients were negative for specific IgG antibody to *A. cantonensis* antigen in the CSF samples by ELISA.[28] The CSF controls were also non-reactive for the specific *A. cantonensis* antigenic band by immunoblotting.[13] More details about EOM patients and control persons were reported previously.[29]

2.2. Recombinant antigen-based immunochromatographic test kit

The rAcGal2 protein was produced as previously described.[18] Briefly, the full-length cDNA of *A. cantonensis* galectin-2 (GenBank accession AEK98124.1) was subcloned into a pET43.1b(+) expression vector (Novagen, Darmstadt, Germany) and transformed into *Escherichia coli* Rosetta-gami 2(DE3) expression host (Novagen). The soluble rAcGal2 fusion protein, tagged with NusA-tag, 6 × His-tags (N and C term), S-tag, and HSV-tag was purified using Ni-NTA His Bind Resin (Novagen) according to the manufacturer's instructions. The resulting protein, with a molecular mass of 91 kDa,[18] was dialyzed against distilled water and stored at −80 °C until used in the ICT.

The diagnostic ICT device for human angiostrongyliasis diagnosis was developed using the rAcGal2 antigen. The construction was as follows: rAcGal2 antigen at a concentration of 2 mg/mL was immobilized onto a nitrocellulose membrane (Sartorius Stedim Biotech, Goettingen, Germany) at the test line (T), and 1 mg/mL of goat anti-mouse IgG antibodies (Lampire Biological Laboratories, Pipersville, PA) were also immobilized on the control line (C) of the nitrocellulose membrane using a XYZ3210 Dispenser (Bio-Dot, Irvine, CA) at a flow rate of 0.1 μL/mm. Gold (40 nm diameter) -conjugated mouse anti-human IgG antibody was then sprayed onto a conjugate pad (a glass microfiber filter; Whatman Schleicher & Schuell, Dassel, Germany) at a flow rate of 1 μL/mm) as a detector reagent. The strip was housed in a plastic housing (Adtec Inc., Oita, Tokyo, Japan) and sealed with a desiccant in an aluminum foil pouch for storage or transport. The test kit consisted of an immunochromatographic device, sample buffer for diluting the test sample, and running buffer for chromatography.

Each simulated WBS or serum sample was diluted (1:50) with buffer (Fig. 1A). Five microliters of each diluted simulated WBS or serum sample was added into the sample well, and 60 μL of chromatography buffer was added into the buffer well (Fig. 1B). The result was recorded at 15 min (Fig. 1C). The within-day and between-day precision of the ICT kit was determined via analysis of pooled positive and negative reference samples. The positive reference sample was prepared by mixing equal volumes of sera from 10 patients with EOM-associated angiostrongyliasis (diagnosed based on positive immunoblotting) and the negative sample using sera from 10 healthy volunteers. Packed RBCs, as described above, were added to these reference samples.

For antibody detection in human CSF, each sample was diluted (1:25) with buffer solution and 5 μL added into the sample well. The rest

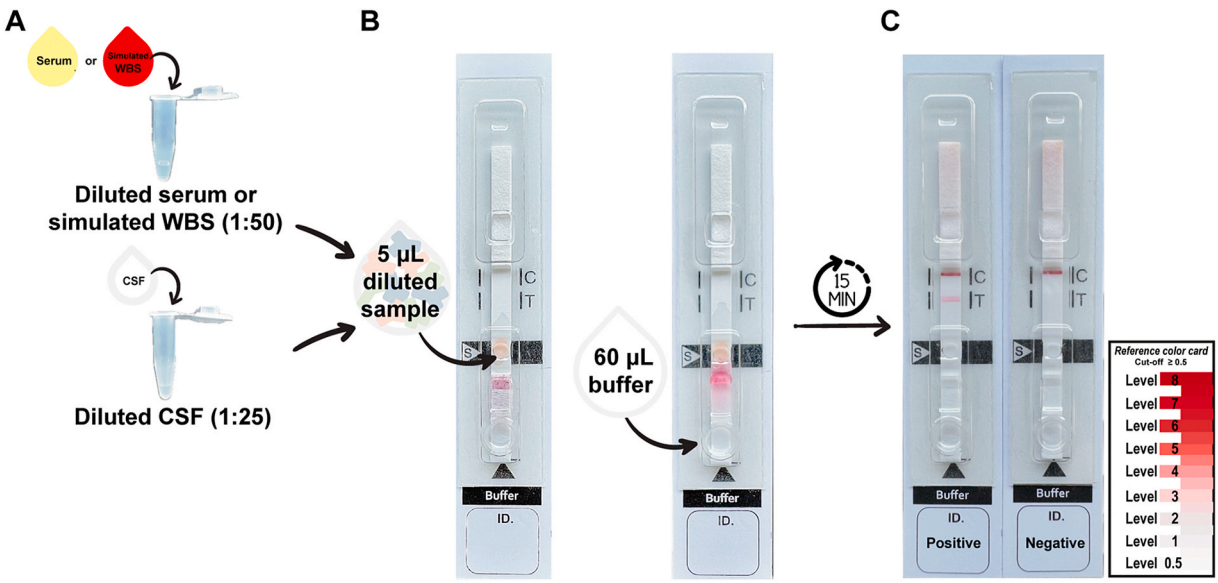

Fig. 1. Processing of samples in the recombinant antigen-based immunochromatographic test kit. (A) Optimum dilution of serum, simulated WBS, and CSF samples. (B) Apply diluted sample and buffer into their respective wells. (C) Kits showing positive and negative results. Results were read, at 15 min, by the naked eye with reference to the color card. For a positive sample, the test band and control band turn red whereas only the control band turns red in a negative sample. Test bands with a color intensity ≥0.5 indicate a positive result. WBS = simulated whole-blood sample. CSF = cerebrospinal fluid.

of the procedure was the same as for simulated WBSs and serum samples (Fig. 1).

Results were read by the naked eye with reference to a color card, evaluated independently by two laboratory personnel whose interpretations agreed in every case. The results were compared between simulated WBSs and the corresponding serum samples. A test line of any visible color at or above level 0.5 was considered positive (Fig. 1C). The test was regarded as negative if only the control line was visible (Fig. 1C).

2.3. Diagnostic performance

The diagnostic values were calculated as previously described.[30] Sensitivity was the number of true positives/(number of true positives + number of false negatives) x 100; specificity was the number of true negatives/(number of false positives + number of true negatives) x 100; accuracy was the number of true positives + number of true negatives/(number of total samples) x 100; true negative was the number of control samples (healthy control and other parasitosis) that were negative by the assay; true positive was the number of angiostrongyliasis serum samples that were positive by the assay; false positive was the number of control samples that were positive by the assay; and false negative was the number of angiostrongyliasis serum samples that were negative by the assay. Stata Statistical Software: Release 10 (StataCorp LLC, Lakeway Drive College Station, TX) was used to calculate positive (sensitivity/(100 – specificity) and negative (100 – sensitivity)/specificity) likelihood ratios, receiver operating characteristic areas, and Cohen's kappa value. The diagnostic parameters of the ICT kit were compared when used with simulated WBSs and with the corresponding serum samples: the percentage of total concordance (Cohen's kappa value) was used to assess agreement.

3. Results

The diagnostic values of the rAcGal2 recombinant antigen-based ICT for human angiostrongyliasis were assessed using individual simulated WBSs and corresponding serum samples from angiostrongyliasis patients, normal healthy subjects, and patients with other parasitoses (Table 1, Fig. 2). Most of the simulated WBSs (77/93) and the corresponding serum samples (87/93) from patients with EOM-associated angiostrongyliasis were positive. Five out of seven paired samples from confirmed ocular angiostrongyliasis sera were also positive. All samples from 25 healthy controls were negative. Cross-reactivity was detected in simulated WBSs and serum samples from cases of hookworm infections (1/10), taeniasis (1/10), sparganosis (2/5), and blastocystosis (1/10) (Table 1; Supplementary file 1). The calculated diagnostic accuracy, sensitivity, and specificity for IgG antibody detection in simulated WBSs were 91.5 %, 82.0 %, and 97.1 %, respectively and in corresponding serum samples were 95.2 %, 92.0 %, and 97.1 %, respectively (Table 2). The positive and negative likelihood

Table 1
The results of the rAcGal2 recombinant antigen-based ICT kit for diagnosis of human angiostrongyliasis were compared between simulated WBSs and the corresponding serum samples.

Type of Samples (n = 270)	Simulated WBSs (No. Positive/Total)	Serum samples (No. Positive/Total)
Group 1 (n = 100)		
Ocular angiostrongyliasis	5/7	5/7
Eosinophilic meningitis	77/93	87/93
Group 2 (n = 25)		
Healthy controls	0/25	0/25
Group 3 (n = 145)		
Gnathostomiasis	0/10	0/10
Capillariasis	0/10	0/10
Strongyloidiasis	0/10	0/10
Trichinosis	0/10	0/10
Trichuriasis	0/10	0/10
Ascariasis	0/10	0/10
Hookworm infections	1/10	1/10
Paragonimiasis	0/10	0/10
Fascioliasis	0/10	0/10
Opisthorchiasis	0/10	0/10
Cysticercosis	0/10	0/10
Taeniasis	1/10	1/10
Sparganosis	2/5	2/5
Amoebiasis	0/10	0/10
Blastocystosis	1/10	1/10

ICT, immunochromatography test; WBSs, Whole-blood samples.

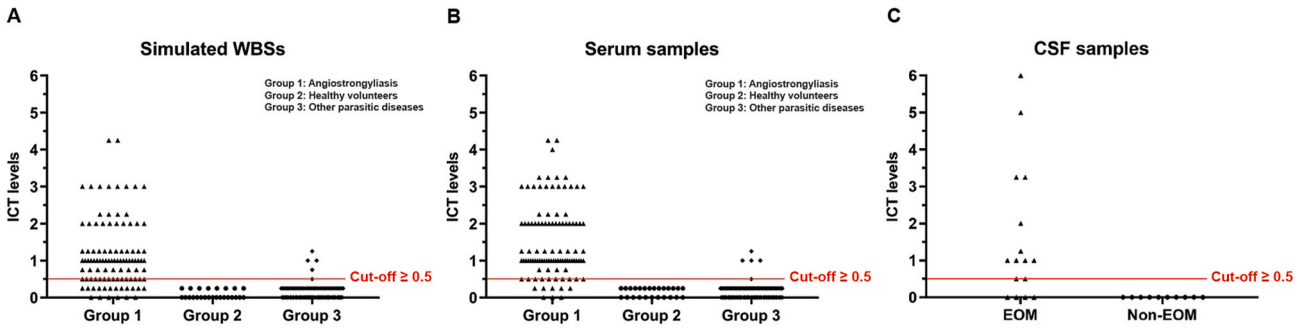

Fig. 2. Graphical representation of test line color intensity level for each sample in the immunochromatographic test (ICT). (A) Simulated whole-blood samples (WBSs). (B) Serum samples. (C) cerebrospinal fluid (CSF) samples. The cut-off level for a positive result was 0.5 (horizontal line). EOM refers to eosinophilic meningitis associated with angiostrongyliasis. Non-EOM refers to samples from patients without angiostrongyliasis. (See more information in materials and methods).

Table 2
The diagnostic values of the rAcGal2 recombinant antigen-based ICT kit were compared between simulated WBSs and the corresponding serum samples.

Diagnostic values	Simulated WBSs	Serum samples
Sensitivity (%)	82.0 [73.1–89.0]	92.0 [84.8–96.5]
Specificity (%)	97.1 [93.3–99.0]	97.1 [93.3–99.0]
Accuracy (%)	91.5 [87.5–94.5]	95.2 [91.9–97.4]
Positive likelihood ratio	27.9 [11.7–66.4]	31.3 [13.2–74.3]
Negative likelihood ratio	0.19 [0.12–0.28]	0.08 [0.04–0.16]
Receiver Operating Characteristic area	0.895 [0.855–0.935]	0.945 [0.916–0.975]

ICT, immunochromatography test; WBSs, Whole-Blood Samples.

ratios for antibody detection in simulated WBSs were 27.9 and 0.19, and in corresponding serum samples were 31.3 and 0.08, respectively. The agreement evaluation between these sets of results yielded a Cohen's kappa value of 0.92, indicating an almost perfect agreement, and the concordance was 96.3 % (260/270).

For the CSF samples, 12 out of 16 EOM cases (75 %) were positive. In contrast, none of the 10 control cases showed positive results (Supplementary file 2).

4. Discussion

In any clinical setting, but especially in resource-limited facilities, point-of-care (POC) testing tools to support diagnosis in clinical care are required. Recently, an antibody detection-based lateral-flow immunochromatographic assay exploiting a 31-kDa A. *cantonensis* antigen purified from native worm extract[16] was reported, as was a similar kit using rAcGal2 recombinant antigenic protein.[17] The assay using native 31-kDa A. *cantonensis* antigen gave 100 % sensitivity and 98.7 % specificity in human sera, with cross-reactivity in one out of four hookworm infection cases.[16] The recombinant rAcGal2 antigenic protein-based ICT showed 87.0 % sensitivity and 96.5 % specificity[17] with cross-reactivity reported among opisthorchiasis (1/10), trichinellosis (1/10), ascariasis (1/9), hookworm infections (1/9) and sparganosis (1/5) serum samples. In addition, the recombinant rAcGal2 protein-based ICT also gave a positive result in 11 out of 12 (91.7 %) positive control sera from *Angiostrongylus costaricensis*-infected patients diagnosed with abdominal angiostrongyliasis.[31]

The ICT kit described here can be used with both simulated WBSs and serum samples. Comparison between results obtained using simulated WBSs and the corresponding serum samples revealed almost perfect agreement (Cohen's kappa value = 0.92). The sensitivity of IgG detection (92.0 %) was higher among serum samples than among simulated WBSs (82.0 %), but both types of samples had the same value for specificity (97.1 %). Thus, the use of whole blood is virtually as effective as using sera, and is simpler, saving both time and equipment. Slight differences between the diagnostic values reported by Somboonpatarakun et al.[17] and the ICT discussed here, both of which used the same recombinant antigen, could have several possible explanations. As pointed out by Boonroumkaew et al.,[32] these include differences in the panels of samples used, membranes and cover housing, buffer types and antigen concentrations. We suspect that the use of different plastic housings in the two studies could play a role. The distance between the sample drop area and the T line was 0.5 cm in the previous housing[17] but was 1 cm in the present study, which used a newly developed housing (Adtec Inc.).

This ICT yielded false negative results in simulated WBSs and serum samples of two (of seven) ocular angiostrongyliasis cases. This could be a consequence of the parasite's location in the eye, an immunologically privileged site where the immune response is limited.[33] Another factor to consider when evaluating sensitivity is that some EOM-associated angiostrongyliasis samples, which were positive by immunoblotting,[13] tested negative in simulated WBSs and serum samples—16 samples (17.2 %) and 6 samples (6.4 %), respectively. This discrepancy may be due to the use of different antigen types: the current ICT kit employs the recombinant rAcGal2 protein antigen, whereas immunoblotting utilizes the native 29–31 kDa antigen from young adult *A. cantonensis*.[13] However, a significant advantage is that the use of recombinant antigens allows for the mass production of antigenic material.

Some simulated WBSs and serum samples showed cross-reactivities—with cases of hookworm infections, taeniasis, blastocystosis and sparganosis. Cross-reactivities may be attributable to concomitant or previous subclinical infections by these parasites (many angiostrongyliasis sera used in the present study came from an area where these other parasitoses were also endemic). Another reason for cross-reactivities could be shared antigenicity among the causative agents. However, these should not produce diagnostic problems in practice because clinical symptoms and signs of these parasitoses usually differ from those of angiostrongyliasis.[34] This is certainly true for clinical symptoms of hookworm infections, taeniasis, and blastocystosis, which normally also reveal a diagnostic stage in fecal samples. Clinicians can differentially diagnose sparganosis based on clinical symptoms and signs, including stool examination, and a history of eating hazardous foods, in combination with other laboratory blood and CSF findings.

One benefit of this diagnostic ICT kit is that it can be used to detect anti-*Angiostrongylus* antibodies in CSF samples such as might be obtained from lumbar puncture for management of high intracranial pressure of EOM patients in intensive care.[8,35] This POC testing tool can be used instead of the time-consuming diagnostic ELISA and immunoblotting.[13,36]

Limitations of this study: clinicians and laboratory personnel should be aware that, until now, the current ICT test kit has only been evaluated under laboratory conditions using a defined set of frozen samples in a single-site study. The performance of the test will depend on the types of

diseases that potentially cross-react in localities where the kit might be used. Sensitivity and specificity might vary when evaluating acute cases because seroconversion might be delayed. Clinicians working in endemic areas should carefully interpret the diagnostic results. For example, when suspected eosinophilic cases are serologically negative, patients should be repeatedly tested over ensuing days or weeks. The kit still needs to be assessed using more samples from other parasitosis cases in the real world and using fresh whole blood and CSF samples (our samples were based on archived frozen sera and CSF).

In conclusion, this POC ICT is optimized for the detection of anti-*Angiostrongylus* antibodies in human sera and simulated WBSs. The latter type of sample requires no centrifugation for serum separation and whole-blood hemolysis and should be applicable to fingerstick blood obtained in the field in future. The kit can also be applied to CSF samples and be used in poorly serviced remote areas.

CRediT authorship contribution statement

Patcharaporn Boonroumkaew: Writing – original draft, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Lakkhana Sadaow:** Visualization, Investigation, Conceptualization, Writing – original draft, Methodology, Formal analysis. **Nongnapas Kanchanangkul:** Visualization, Investigation, Conceptualization, Methodology, Formal analysis. **Rutchanee Rodpai:** Formal analysis, Writing – original draft, Validation, Methodology, Investigation, Conceptualization. **Oranuch Sanpool:** Writing – original draft, Validation, Methodology, Formal analysis, Conceptualization. **Pewpan M. Intapan:** Writing – review & editing, Validation, Supervision, Formal analysis, Conceptualization. **David Blair:** Writing – review & editing, Validation, Conceptualization. **Wanchai Maleewong:** Writing – review & editing, Validation, Project administration, Investigation, Formal analysis, Writing – original draft, Supervision, Methodology, Funding acquisition, Conceptualization.

Availability of data and materials

All data generated or analyzed during this study are included in this manuscript and its supplementary information files.

Declaration of generative AI in scientific writing

We state that any help from generative AI or AI assisted technology has not been obtained in writing of this manuscript.

Declaration of competing interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jmii.2025.06.006.

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