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Comparative assessment of immunochromatographic test kits using somatic antigens from adult *Opisthorchis viverrini* and IgG and IgG4 conjugates for serodiagnosis of human opisthorchiasis

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Abstract

Chronic infections of humans with *Opisthorchis viverrini* and *Clonorchis sinensis* spanning decades may lead to life-threatening pathology prior to cholangiocarcinoma (CCA), which usually has a poor prognosis. Serological tools can support the parasitological examination in clinical diagnosis and support screening for risk of CCA. We developed novel immunochromatographic test kits using a soluble, somatic tissue extract of adult *O. viverrini* worms as an antigen and colloidal gold-labeled conjugates of IgG and IgG4 antibodies, and evaluated the diagnostic values of both the OvSO-IgG and OvSO-IgG4 kits. For diagnosis of human opisthorchiasis individually, the diagnostic sensitivity, specificity, and positive and negative predictive values with 95% confidence intervals in the OvSO-IgG kit were 86.6% (78.9–92.3), 89.5% (84.2–93.5), 82.9% (74.8–89.2), and 91.9% (87.0–95.4), respectively, while the 75% (65.9–82.7), 98.4% (95.5–99.7), 96.6% (90.3–99.3), and 87% (81.7–91.2), respectively, for the OvSO-IgG4 kit at the prevalence of infection of 37.1%. Twenty-three (76.7%) and 14 (46.7%) of 30 clonorchiasis sera showed positive reactivity with the OvSO-IgG and OvSO-IgG4 kits, respectively. There was 84.1% (κ-value = 0.649) concordance between the two kits, which was statistically significant ($p < 0.001$). Both ICT kits can be employed as quick and easy point-of-care diagnostic tools, and hence, the OvSO-IgG and OvSO-IgG4 kits can support expanded capacity for clinical diagnosis of human opisthorchiasis and clonorchiasis. These kits may find utility in large-scale surveys in endemic areas where there are limited sophisticated medical facilities or capacity.

Keywords Opisthorchiasis · Serodiagnosis · Somatic antigen · IgG · IgG4 · Immunochromatographic test kits

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Introduction

Chronic infections in humans with the fish-borne liver flukes, *Opisthorchis viverrini* and *Clonorchis sinensis*, may cause fatal bile duct cancer (cholangiocarcinoma; CCA) and liver cancer (hepatocarcinoma) (Honjo et al. 2005; Sripa et al. 2010; IARC 2012; Zheng et al. 2017). Approximately 600 million people globally are at risk of infection with *O. viverrini* or *C. sinensis* (Sripa et al. 2010). More than 10 million people are estimated to be infected with *O. viverrini* in the lower Mekong River sub-region, i.e., Cambodia, Lao People's Democratic Republic, southern Vietnam, Myanmar, and Thailand (Sripa et al. 2010; Aung et al. 2017). In clonorchiasis, over 200 million people are at risk of clonorchiasis, 15–20 million are infected and 1.5–2 million people (approximately 10% of infected people) present symptoms and complications in Northeast and East Asia (northern Vietnam, China, and Korea) (Sripa et al. 2010). Diagnosis

of human infections is accomplished by the demonstration of eggs in feces, bile, or duodenal contents, and it is the gold standard. However, this tool is laborious and requires proficient, practiced technicians and microscopists (Sripa et al. 2010). Serologic and molecular tools have been used to support diagnosis and screening (McCarthy et al. 2012; Saijuntha et al. 2018) of opisthorchiasis (Wongratanacheewin et al. 1988a, 2003; Akai et al. 1995; Laha et al. 2008; Sripa et al. 2012; Teimoori et al. 2015) and clonorchiasis (Sirisinha et al. 1990; Hong et al. 1999; Zhao et al. 2004; Li et al. 2011, 2012; Kim et al. 2019).

Recently, a rapid diagnostic immunochromatographic test (ICT) kit using an excretory-secretory (ES) antigen from adult *O. viverrini* was developed for specific IgG detection in human opisthorchiasis and clonorchiasis (Sadaow et al. 2019). The preparation of ES antigen is comparatively tedious and expensive due to the necessity of in vitro maintenance of the adult parasites, which in turn are sourced from experimental infection of laboratory hamsters. The yield is also limited. By contrast, the preparation of somatic antigen is less expensive than ES antigen and yields greater quantities of usable antigen, and worm culture is not needed. In this study, the ICT kit (OvSO-kit) using somatic extract from adult *O. viverrini* worms was developed for the detection of specific IgG antibody, with a key we have termed the OvSO-IgG kit, in sera from human cases confirmed with opisthorchiasis. IgG antibody responses predominate in opisthorchiasis sera (Wongratanacheewin et al. 1988b) while IgG4 antibody provides high diagnostic specificity for human opisthorchiasis (Tesana et al. 2007). Accordingly, an OvSO-kit termed the OvSO-IgG4 kit also was developed for the detection of specific IgG4 antibody. The diagnostic performance of these two kits was investigated and assessed in this study.

Materials and methods

Parasite and antigen

Opisthorchis viverrini metacercariae were collected from naturally infected cyprinid fish from a water reservoir in Amphur Muang, Khon Kaen Province, Thailand. The fish were purchased from a local fish market in Khon Kaen, Thailand. Following collection, *O. viverrini* metacercariae were examined under a dissecting microscope and identified as described (Sripa et al. 2010) and used for infection of laboratory hamsters. The experimental infection of hamsters were performed as described (Intapan and Maleewong 2006; Sadaow et al. 2019). Adult *O. viverrini* worms were recovered from the livers and bile ducts of the hamsters 3 months later. Adult somatic antigen was obtained as described (Intapan and Maleewong 2006). Briefly, the adult worms were homogenized with tissue grinder in a small volume of 0.1 M phosphate-buffered saline, pH 7.4, containing proteinase inhibitors (cOmplete^{TM} ULTRA Tablets, Mini EASYpack Protease Inhibitor Cocktail Tablets, Roche, Basel, Switzerland). The suspension was sonicated with an ultrasonic disintegrator and centrifuged at 10,000 × *g* for 30 min at 4 °C. The protein concentration of the antigen was determined (Bradford 1976). The supernatant as the antigen was aliquoted and stored at − 80 °C until used.

Human sera

Human opisthorchiasis sera used in this study were obtained from the serum biobank of the Department of Parasitology, Faculty of Medicine, Khon Kaen University. Serum samples from cases of clonorchiasis had been stored at the Department of Parasitology, National Institute of Infectious Diseases, Tokyo, Japan. There were four categories of sera included in our study: (1) 30 samples of negative control from healthy volunteers from northeastern Thailand who were proven to be free of any intestinal parasitic infection at the time of blood collection; (2) 112 samples of opisthorchiasis sera from persons infected *O. viverrini* in northeastern provinces of Thailand, a region endemic area for *O. viverrini*; (3) 30 samples of clonorchiasis from persons infected with *C. sinensis* in Guangi Zhuang Autonomous Region, China, a locality endemic from infection with *C. sinensis* (Yu et al. 2003); and (4) 160 samples from cases diagnosed with other parasites besides *O. viverrini* and *C. sinensis*. The healthy volunteers, opisthorchiasis, paragonimiasis, taeniasis saginata, ascariasis, hookworm infections, trichuriasis, capillariasis phillipinensis, strongyloidiasis, giardiasis, blastocystosis, and minute intestinal fluke infections were confirmed by parasitological detection in stool samples using the modified formalin ethyl acetate concentration method (Elkins et al. 1986). Clonorchiasis was confirmed by the modified Kato-Katz technique (Katz et al. 1972) and ELISA (Yu et al., 2003). Fascioliasis was confirmed by recovery of worms and serological methods (Wongkham et al. 2005), cysticercosis was confirmed by serology and computed tomography (Intapan et al. 2008), gnathostomiasis was confirmed by serological methods with clinical manifestation and histories of dietary preferences (Intapan et al. 2010), angiostrongyliaisis cantonensis was diagnosed by serological methods with clinical manifestation (Somboonpatarakun et al. 2020), trichinellosis cases were confirmed by detection of intramuscular larvae and serological methods (Morakote et al. 1991), and sparganosis was confirmed by detection of plerocercoids (Table 1). Sera pooled from the healthy individuals and opisthorchiasis cases served as the negative and positive controls, respectively, to ensure between day precision. Informed consent was obtained from adult participants and from parents or legal guardians of minors. The reporting

Table 1 Comparison of reactivities using OvSO-IgG and OvSO-IgG4 kits

Type of samples	Number of positive cases/total cases examined (intensity level of bands[a])	
	OvSO-IgG kit	OvSO-IgG4 kit
Thai healthy control	1/30 (0.5)	0/30
Opisthorchiasis	97/112 (0.5–4)	84/112 (0.5–8)
Clonorchiasis	23/30 (0.5–3)	14/30 (0.5–3)
Paragonimiasis	6/10 (0.5)	0/10
Fascioliasis	4/10 (0.5–2)	0/10
Cysticercosis	0/10	0/10
Sparganosis	0/10	1/10 (1)
Taeniasis saginata	0/10	0/10
Ascariasis	0/10	0/10
Hookworms	0/10	0/10
Angiostrongyliasis	2/10 (0.5)	1/10 (0.5)
Trichinellosis	0/10	1/10 (2)
Trichuriasis	0/10	0/10
Capillariasis philippinensis	0/10	0/10
Gnathostomiasis	2/10 (0.5–1)	0/10
Strongyloidiasis	1/10 (0.5)	0/10
Giardiasis	1/10 (1)	0/10
Blastocystosis	1/10 (2)	0/10
Infection with minute intestinal flukes	2/10 (0.5–1)	0/10

[a]The intensity level of band at the T-line was evaluated according to the interpretation card reference

of experimental data has been performed using the criteria of the STARD 2015 list for reporting diagnostic accuracy studies (Cohen et al. 2016).

Immunochromatographic tests

The OvSO-IgG kit was optimized according to the method previously reported (Sadaow et al. 2019) with some modification by using the antigen (1 mg/mL) absorbed in the T-line at a flow rate of 0.1 µL/mm. The anti-mouse IgG (Lampire Biological Laboratories, Pipersville, PA) was dispensed onto nitrocellulose membrane (Sartorius Stedim Biotech SA, Goettingen, Germany) to serve as the C-lines. The colloidal gold-conjugated mouse monoclonal anti-human IgG (Kestrel BioSciences Co., Pathumthani, Thailand) was sprayed onto a glass microfiber filter (GF33; Whatman Schleicher & Schuell, Dassel, Germany) to form the conjugate pad. Samples of sera were diluted with sample buffer at a ratio of 1:30, and 5 µL of diluted serum was loaded into the port (S) and 90 µL of chromatography buffer was added into the buffer well (B), as marked in Supplementary Fig. 1. The OvSO-IgG4 kit was also optimized using a method like that described above, except the concentration of antigen (2 mg/mL), colloidal gold-conjugated monoclonal anti-human IgG4 (ZyMAX, Invitrogen, Camarillo, CA), and the sera were undiluted when tested in the kit. The intensity of positive bands with both kits was estimated visually (unaided) by comparison with the reference board with level ≥ 0.5 as the cutoff level (Supplementary Fig. 1).

Statistical analysis

The diagnostic values were calculated as described (Galen, 1980). These values were calculated and expressed as follows: sensitivity was equal to the number of true positives/(number of true positives + number of false negatives) × 100; specificity was equal to the number of true negatives/(number of false positives + number of true negatives) × 100; positive predictive value was equal to the number of true positives/(number of false positives + number of true positives) × 100; negative predictive value was equal to the number of true negatives/(number of false negatives + number of true negatives) × 100; accuracy was equal to the number of true positives + number of true negatives/(number of true positives + number of false negatives + number of false positives + number of true negatives) × 100; true negative was the number of control samples (other parasitoses and healthy controls) that were negative by the assay; true positive was the number of proven opisthorchiasis or clonorchiasis or boths 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 proven opisthorchiasis or clonorchiasis or both samples that were negative by the assay. Positive likelihood ratio was calculated as sensitivity/(100 − specificity) and negative likelihood ratio as (100 − sensitivity)/specificity. Sensitivity, specificity, and cross-reactivity of OvSO-IgG and OvSO- IgG4 kits were compared using McNemar's test (The SPSS Statistics Data Editor, SPSS Inc., Chicago, IL). Total concordance was calculated using Cohen's kappa coefficient. Stata version 13.1 was used to perform the analysis.

Results

The diagnostic values and intensities of positive bands in the OvSO-IgG kit and the OvSO-IgG4 kit calculated for diagnosis of opisthorchiasis, clonorchiasis, and both diseases were compared (Tables 1 and 2). Representative results obtained in OvSO-IgG kit and the OvSO-IgG4 kit were shown in Supplementary Fig. 2. Low-level cross-reactive intensities were also detected and graded using the reference board (Supplementary Fig. 1). For diagnosis of opisthorchiasis individually, the sensitivity, specificity, and positive and negative predictive values with 95% confidence intervals in the OvSO-IgG kit were 86.6% (78.9–92.3), 89.5% (84.2–93.5), 82.9% (74.8–89.2), and

Table 2 The diagnostic test statistical parameters of the OvSO-IgG and OvSO-IgG4 test kits

Number	OvSO-IgG kit			OvSO-IgG4 kit		
	OV[a]	CS[b]	OV and CS[c]	OV[a]	CS[b]	OV and CS[c]
True positive	97	23	120	84	14	98
False positive	20	20	20	3	3	3
True negative	170	170	170	187	187	187
False negative	15	7	22	28	16	44
Diagnostic values (95% confidence interval)						
Sensitivity (%)	86.6 (78.9–92.3)	76.7 (57.7–90.1)	84.5 (77.5–90.0)	75.0 (65.9–82.7)	46.7 (28.3–65.7)	69.0 (60.7–76.5)
Specificity (%)	89.5 (84.2–93.5)	89.5 (84.2–93.5)	89.5 (84.2–93.5)	98.4 (95.5–99.7)	98.4 (95.5–99.7)	98.4 (95.5–99.7)
Positive predictive value (%)	82.9 (74.8–89.2)	53.5 (37.7–68.8)	85.7 (78.8–91.1)	96.6 (90.3–99.3)	82.4 (56.6–96.2)	97.0 (91.6–99.4)
Negative predictive value (%)	91.9 (87.0–95.4)	96.0 (92.0–98.4)	88.5 (83.2–92.7)	87.0 (81.7–91.2)	92.1 (87.5–95.4)	81.0 (75.3–85.8)
Positive likelihood ratio	8.2 (5.4–12.5)	7.3 (4.6–11.5)	8.0 (5.3–12.2)	47.5 (15.4–146.7)	29.6 (9.0–96.8)	43.7 (14.1–135.0)
Negative likelihood ratio	0.15 (0.09–0.24)	0.26 (0.14–0.50)	0.17 (0.12–0.26)	0.25 (0.18–0.35)	0.54 (0.39–0.76)	0.31 (0.25–0.40)
Accuracy (%)	88.4 (84.3–91.8)	87.7 (82.6–91.7)	87.3 (83.3–90.7)	89.7 (85.7–92.9)	91.4 (86.8–94.7)	85.8 (81.6–89.4)

[a]OV as an opisthorchiasis individually; [b]CS as clonorchiasis individually; [c]OV and CS as opisthorchiasis/clonorchiasis as a combined disease entity

91.9% (87.0–95.4), respectively, while for the OvSO-IgG4 kit were 75% (65.9–82.7), 98.4% (95.5–99.7), 96.6% (90.3–99.3), and 87% (81.7–91.2), respectively, at the prevalence of infection of 37.1%. Twenty-three (76.7%) and 14 (46.7%) of 30 clonorchiasis sera were positively reactive as assessed with the OvSO-IgG and OvSO-IgG4 kits, respectively.The differences in performance between both tests were statistically significant ($p < 0.001$) with Cohen's kappa agreement 84.1% (κ-value = 0.649) for opisthorchiasis individually (Table 3), 84.6% (κ-value = 0.363) for clonorchiasis individually (Table 4), and 81.6% (κ-value = 0.608) for opisthorchiasis/clonorchiasis as a combined disease entity (Table 5).

Discussion

POC tests based on chromatographical lateral flow approaches have found utility in a broad range of applications including the diagnosis of infections including parasitic diseases (Drancourt et al. 2016). Recently, an ICT kit for serodiagnosis of human opisthorchiasis and clonorchiasis was developed based on IgG antibody detection in sera using soluble ES antigen from adults *O. viverrini* worms (Sadaow et al. 2019). The diagnostic sensitivity, specificity, and positive and negative predictive values of the ICT based on ES antigen were 94.6%, 91.2%, 89.7%, and 95.4%, respectively (Sadaow et al. 2019). In the present study, we developed new ICT kits, termed OvSO-kits, using soluble lysates of adult *O. viverrini* somatic tissues as the antigen for detection of IgG and IgG4 antibodies. Both the OvSO-IgG and OvSO-IgG4 kits can be used for serodiagnosis of opisthorchiasis. These kits can also detect some clonorchiasis cases. This is expected given the close phylogenetic relationship of these two fish-borne trematodes and, consequently, their shared antigenic epitopes (Sirisinha et al. 1990). The sensitivity values of both OvSO-kits were lower than for ES antigen while the specificity of OvSO-IgG4 kit gave higher values. However, the limitations of preparing *O. viverrini* ES antigen include

Table 3 Comparison of diagnostic parameters in both ICT kits: opisthorchiasis individually

Test type and results[a]	OvSO-IgG4 kit		
OvSO-IgG kit	Number of positives	Number of negatives	Total
Number of positives	78	39	117
Number of negatives	9	176	185
Total	87	215	302

[a]These results showed statistically significant differences between both kits (Exact McNemar's test; $p < 0.001$), with Cohen's kappa agreement 84.1% (κ-value = 0.649, ($p < 0.001$)

Table 4 Comparison of diagnostic parameters in both ICT kits: clonorchiasis individually

Test type and results[a]	OvSO-IgG4 kit		
OvSO-IgG kit	Number of positives	Number of negatives	Total
Number of positives	13	30	43
Number of negatives	4	173	177
Total	17	203	220

[a]These results showed statistically significant differences between both kits (Exact McNemar's test; $p < 0.001$), with Cohen's kappa agreement 84.6% (κ-value = 0.363, $p < 0.001$)

Table 5 Comparison of diagnostic parameters in both ICT kits: opisthorchiasis/clonorchiasis as a combined disease entity

Test type and results[a]	OvSO-IgG4 kit		
OvSO-IgG kit	Number of positives	Number of negatives	Total
Number of positives	90	50	140
Number of negatives	11	181	192
Total	101	231	332

[a]These results showed statistically significant differences between both kits (Exact McNemar's test; $p < 0.001$), with Cohen's kappa agreement 81.6% (κ-value = 0.608, $p < 0.001$)

its expense, and its labor-intensive and time-consuming nature, and low yield of ES product (~ 0.3 μg/adult worm). By contrast, desirable attributes of the new OvSO-kits that use *O. viverrini* somatic antigen include its less laborious aspect, higher yield of soluble antigen, and that it obviates the requirement for in vitro culture of the flukes. Notably, the yield for somatic antigen (~ 30 μg/adult worm) is ~ 100 times greater the yield of the ES products from similar numbers of adult *O. viverrini*.

Elevated IgG, IgA, and IgE antibody titers in serum and bile have been reported during opisthorchiasis especially in serum IgG antibody which predominates in opisthorchiasis (Wongratanacheewin et al. 1988b) and clonorchiasis (Li et al. 2018). IgG4 antibody levels are also correlated with chronic helminth infections and intensity of infection (Grogan et al. 1996; Shin et al. 2016). Moreover, IgG and IgG4 antibodies have been used to diagnose of other helminth infections including gnathostomiasis (Anantaphruti et al. 2005), strongyloidiasis (Boonroumkaew et al. 2020), and fascioliasis (Wongkham et al. 2005). IgG4 antibody has a high diagnostic specificity for human opisthorchiasis (Tesana et al. 2007) and clonorchiasis (Hong et al. 1999). With respect to heavy opisthorchiasis or clonorchiasis, the disease manifestation may be subclinical but often induces jaundice, cholangitis, cholecystitis, and enlarged liver and gallbladder, and in chronic stages, frequently leads to biliary adenocarcinoma (Honjo et al. 2005; Sripa et al. 2010; IARC 2012; Zheng et al. 2017). Accordingly, access to rapid detection of *O. viverrini* or *C. sinensis* using OvSO-IgG and OvSO-IgG4 kits can provide valuable tools to diagnose and monitor for treatment and reduce the risk of development of CCA.

As noted, the OvSO-IgG kit exhibited a higher sensitivity than the OvSO-IgG4 kit, which may be due to the IgG antibody produced in both stages of early/acute and chronic infection stages (Kanamura et al. 1979). Whereas IgG4 represents only one subclass of IgG in human sera (Hamano et al. 2001), it predominates in the chronic infection stage (Aalberse et al. 1983). However, the diagnostic usefulness/availability from both tests was markedly different ($p < 0.001$) (Table 2): the OvSO-IgG kit exhibits high sensitivity that should be suitable for use in the initial screening of early *O. viverrini* and possibly also of *C. sinensis* infections, whereas the OvSO-IgG4 kit, with its high specificity, can support and ensure serodiagnosis. Therefore, both kits can be together used for serodiagnosis of opisthorchiasis and/or clonorchiasis at the bedside and for sero-epidemiological investigations. Moreover, negative results were seen with opisthorchiasis sera (Table 1), perhaps being the consequence of light infection.

These are some limitations to this study. The OvSO-IgG test kit showed cross-reactions with several other parasitic infections such as paragonimiasis (6/10), fascioliasis (4/10), angiostrongyliasis (2/10), gnathostomiasis (2/10), infection with minute intestinal flukes (2/10), strongyloidiasis (1/10), giardiasis (1/10), and blastocystosis (1/10), while the OvSO-IgG4 kit showed cross-reaction with sparganosis (1/10), angiostrongyliasis (1/10), and trichinellosis (1/10). The OvSO-IgG test kit gave higher sensitivity but lower specificity than the OvSO-IgG4 kit. This outcome may have arisen because *O. viverrini* somatic antigen preparation includes more than 30 antigenic polypeptides and hence may share epitopes with other parasites. Also, subclinical infection with liver flukes may have contributed to these findings because the sera for testing the control specificity were mostly from residents from northeast Thailand, which is a region endemic for opisthorchiasis (Sripa et al. 2010). These limitations should not obstruct the application of these tools given the clinically different setting of opisthorchiasis and clonorchiasis (Blair et al. 1999; Tsai et al. 2001; Gottstein et al. 2009; Boonyasiri et al. 2014; Nawa et al. 2015; Nutman 2017). Nevertheless, it is necessary to consider the possibility of *Fasciola* infection in the regions where fascioliasis occurs, and to comprehensively diagnose the disease including other examination data, such as clinical signs, stool examination, and radio imaging (Mas-Coma et al. 2015).

Prospective users of these kits should also be aware of the following attributes and constraints. First, since some clonorchiasis cases can be detected, the kits will be useful as rapid diagnostic tests in highly *O. viverrini* endemic regions such as Cambodia and northeast Thailand but that the results should be carefully interpreted in highly *C. sinensis* endemic regions, where opisthorchiasis is not found, such as north Vietnam and southeast and northwest China. Second, since no data were available on the duration of infections of the cases in the present study, additional, control parasite-infection sera, for which more specific information on time since parasite exposure and/or treatment is available, will be needed to more fully evaluate the reliability of the assays, for example, during acute infection and following praziquantel therapy. Third, the performance of the OvSO-IgG and

OvSO-IgG4 kits may be affected between batch to batch variation during production of *O. viverrini* somatic antigen. Fourth, it will be informative to evaluate the utility of the kits for diagnosis of infection with *Opisthorchis felineus* in Western Siberia, Russian Federation, and adjacent territories (Fedorova et al. 2020). Fifth, because both positive and false negative results can affect their diagnostic values, diagnosis by parasitological approaches should be considered. And, lastly, the future identification and mass production of sensitive and specific recombinant antigens can be expected to supersede methods that use complex mixtures of native antigens as used here.

In conclusion, we have developed new ICT tools that are rapid, easy to use, and can support the stool examination in the alternative ways for clinical diagnosis of opisthorchiasis and/or clonorchiasis. The production of the OvSO-IgG and OvSO-IgG4 kits appears to be less costly, less time-consuming, and is far easier to produce than the OvES-kit. The OvSO-IgG and OvSO-IgG4 kits likely also are promising tools for antibody screening for risk of CCA at the bedside, in the hospitals, or health centers with sub-optimal medical capacity in the regions where both opisthorchiasis and clonorchiasis are endemic.

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Author contribution Conceptualization: WP, HY, VHM, WI, WM, PJB, and PMI. Methodology: WP, LS, HY, OS, RR, PJB, and PMI. Formal analysis and investigation: WP, LS, HY, RR, OS, WM, and PMI. Writing—original draft preparation: WP, RR, HY, WI, and VHM. Writing—review and editing: WP, HY, WI, PJB, WM, and PMI. Supervision: HY, WM, VHM, PJB, and PMI. All authors read and approved the final manuscript.

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Declarations

Ethics approval This study was approved by the research ethics committee of the Khon Kaen University Ethics Committee for Human Research (Ethics number: HE611507, approved 2 November 2018). Animal Ethics Committee of the Khon Kaen University, according to the Ethic of Animal Experimentation of the National Research Council of Thailand (AEMDKKU 002/2018, approved 4 January 2018).

Conflict of interest The authors declare no competing interests.

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