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RESEARCH ARTICLE

Detection of the carcinogenic liver fluke, *Opisthorchis viverrini*: comparison of two coprological methods versus the automatic feces analyzer

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ABSTRACT

Liver fluke infection, particularly *Opisthorchis viverrini*, poses a significant public health risk in Thailand, where it is closely associated with cholangiocarcinoma and contributes to substantial mortality in the northeastern region. Diagnosis of this condition employs various parasitological approaches. This research aims to compare the diagnostic accuracy of three parasitological techniques: the Kato Katz technique (KKT), the formalin-ethyl acetate concentration technique (FECT), and the Fully Automatic Feces Analyzer (FAFA) for *O. viverrini* identification. A total of 455 fecal specimens were collected from rural areas across five provinces in northeastern Thailand. The specimens were processed according to each method and examined through microscopy for KKT and FECT, and by utilizing an artificial intelligence-based machine for FAFA. Data analysis was conducted to assess parasitic infection rates and observe diagnostic accuracy. The results revealed a parasitic infection rate of 19.34%, with the majority of infections attributed to *O. viverrini* (18.02%), followed by *Strongyloides stercoralis* (0.88%). FECT exhibited the highest positive detection of *O. viverrini* eggs (16.48%), followed by FAFA (10.55%), and KKT (8.57%), respectively. Statistical analysis indicated sensitivity and specificity values for *O. viverrini* detection by KKT (100% and 89.21%), FECT (98.67% and 97.63%), and FAFA (97.92% and 91.15%). The positive predictive value, negative predictive value, and kappa were reported for FECT (89.16%, 99.73%, 0.92), FAFA (56.63%, 99.73%, 0.67), and KKT (45.78%, 100%, 0.58). Additionally, the preparation time for KKT, FECT, and FAFA was 30, 15, and 10 min, respectively. In conclusion, this study highlights FECT, KKT, and FAFA as comparably sensitive in diagnosing *O. viverrini*. The FAFA machine emerges as a potentially valuable tool for detecting *O. viverrini* and other parasitic infections, showcasing promise for clinical use. The findings provide valuable insights into the diagnostic landscape and underscore the potential of FAFA in enhancing efficiency and accuracy in parasitological assessments.

Keywords: *Opisthorchis viverrini*; fully automatic feces analyzer; Kato-Katz technique; formalin-ethyl acetate concentration technique.

INTRODUCTION

Opisthorchis viverrini (*O. viverrini*) infection poses a significant public health challenge in Thailand and Southeast Asian countries (Hotez *et al*., 2015; Buathong *et al*., 2017; Sripa *et al*., 2021). Designated as a group 1 carcinogen by the World Health Organization (WHO, 1994), this infection is closely linked to hepatobiliary diseases, notably cholangitis, periductal fibrosis, cholecystitis, cholelithiasis, and cholangiocarcinoma (CCA) (Pungpak *et al*., 1985; Elkins *et al*., 1990; Kaewpitoon *et al*., 2008; Sripa *et al*., 2018). The highest prevalence of *O. viverrini* infection, affecting approximately eight million individuals, has been identified in northern and northeastern Thailand (Hotez *et al*., 2015; Kaewpitoon *et al*., 2015; Wattanawong *et al*., 2021). Consumption of raw or undercooked cyprinoid fish, harboring infective-stage metacercariae, remains a recurring risk factor for infection (Sithithaworn *et al*., 2012; Painsing *et al*., 2016). Consequently, *O. viverrini* infection persists as a sporadic issue in certain rural areas of Thailand, necessitating active screening as a crucial intervention. Presently, microscopic-based stool examination remains a valuable tool for detecting various parasitic infections, including helminths and protozoa, both in community and laboratory settings. The gold standard among parasitological techniques is the microscopic examination, specifically for diagnosing *O. viverrini* eggs in fecal specimens (Bogoch *et al*., 2016). Common techniques employed for detecting eggs or larvae of intestinal helminths, including *O. viverrini*, in stool samples encompass the direct simple smear technique, Kato-Katz technique (KKT), and the formalin-ethyl acetate concentration technique (FECT) (Bergquist

et al., 2009; Qian *et al*., 2013). However, FECT and Kato-Katz are labor-intensive, with potential risks of infection and fire outbreaks due to ether use in an open system. Additionally, these methods face challenges in differentiating eggs from those of other human liver flukes (*Clonorchis sinensis* and *Opisthorchis felineus*) and small intestinal flukes (Sripa *et al*., 2011).

A previous study suggested the superiority of FECT over KKT for *O. viverrini* screening, but its logistical complexity, involving centrifugation, poses practical disadvantages compared to KKT (Charoensuk *et al*., 2019). Furthermore, the FA280 fully automatic feces analyzer (FAFA), based on artificial intelligence (AI) technology, has been developed to accurately distinguish liver flukes, hookworm, roundworm, whipworm, pinworm, and tapeworm (Sichuan Orienter Bioengineering Co., Ltd, Orienter, Chengdu, China, 2019). The FAFA machine exhibits superior performance, including a test speed of ≥80 tests/hr, a detection rate of ≥95%, and an accuracy deviation in the counting of ≤20% (50~100 units/μL simulation samples). To ensure safety, complete sealing of sample collection tubes post-sampling, maintenance of a fully sealed pipeline system during analysis, and secure sealing of waste samples, test kits, and liquids are imperative measures. Despite these advancements, the widespread integration of the FAFA for routine examination of *O. viverrini* in Thai laboratories remain constrained by cost, and it has not attained widespread popularity for detecting parasites in stool samples. Therefore, this study aims to conduct a cross-sectional investigation of liver fluke infection in northeastern Thailand. It seeks to evaluate the diagnostic performances of fecal examination methods, including KKT, FECT, and FAFA, for diagnosing *O. viverrini*. The study will provide insights into the current status of the infection in the region and offer additional findings on the analytical performance and feasibility of commercial methods for application in larger field studies aimed at surveilling and controlling opisthorchiasis across endemic countries in Southeast Asia.

MATERIALS AND METHODS

Ethical statement

The procedure was approved by the Human Ethics Committee of the health office in Nakhon Ratchasima Province (Reference number NRPH013), and the ethical aspects of fecal examination were approved by the Bioethics Committee of Suranaree University of Technology, Thailand (Reference number SUT-IBC-019/2022).

Sample size calculation and study areas

An established framework was used to calculate the sample size needed for a comparison between diagnostic tests (Buderer, 1996). The sample size was calculated using the equation: $n = [Z_{\frac{\alpha}{2}}\sqrt{2\,x\,\overline{P}(1-\overline{P})} + Z_{\beta}\sqrt{P_1(1-P_1)+P_2(1-P_2)}\,]^2\ /(P_1-P_2)^2$, where n = sample size, $Z_{\frac{\alpha}{2}}$ = statistic for a level of confidence (1.96), Z_{β} = statistic for a level of confidence (0.84), $\overline{P}$ = the average of P_1 and P_2, and P_1,P_2 = the specificity of two diagnostic tests (Hajian-Tilaki *et al.,* 2014). The required sample size for this study comprised 455 individuals. A cross-sectional design was employed to gather fecal specimens, the study population mostly comprised a high-risk group or area where parasitic infections have been reported, and a simple random sampling technique was utilized to select the 455 participants from the population in Nakhon Ratchasima (n=146), Chaiyaphum (n=204), Kalasin (n=54), Roi-Et (n=25), and Nong Khai (n=26) in northeastern Thailand during 2021 to 2023 (Figure 1). Male and female aged 15 years or older were recruited. After the participants informed consent, they were registered for demographic information and fecal specimens' collection, respectively.

Kato-Katz technique

One gram of feces was pressed through a mesh screen to remove large particles, and then a portion of the sieved sample was transferred to the hole of a template on a slide. After filling the hole, the template was removed, and the remaining sample was covered

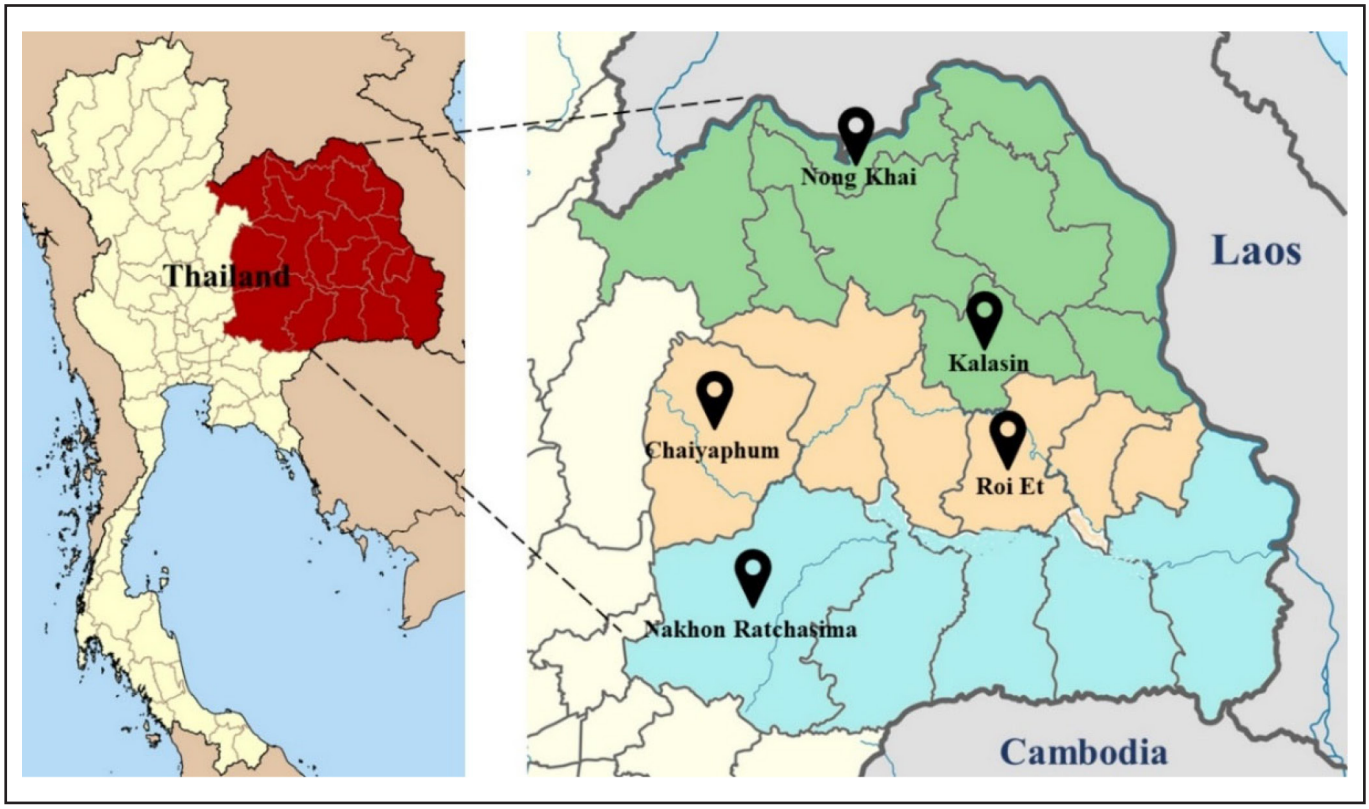

Figure 1. Map of fecal specimens collection areas. The representation of five provinces (Nong Khai, Kalasin, Chaiyaphum, Roi Et, and Nakhon Ratchasima) was marked with a black dropped pin (Modified from Atlas of Thailand, 2010).

with a piece of cellophane pre-soaked in a glycerol-malachite green solution. The number of eggs was counted under a light microscope and multiplied by 24 to calculate the eggs per gram (EPG) (Kato & Miura, 1954; Katz *et al*., 1972).

Formalin-ethyl acetate concentration technique

Three grams of stool were dissolved in 10 ml of 0.85% saline, and the debris was strained on gauze to bring the volume in the centrifuge tube to 15 ml and then centrifuged at 1,500 rpm for 5 min. Thereafter, 7 ml of 10% formalin and 3 ml of ethyl acetate were added to the sediment and mixed thoroughly. The sample was centrifuged at 1,500 rpm for 5 min and then the top layers of the supernatant were decanted. A cotton-tipped applicator was used to remove the debris from the sides of the centrifuge tube. The resulting sediment was fixed with 1 ml of 10% formalin. The final fecal suspension was examined with two drops of 40 µl per sample by the same microscopist using a compound microscope at 100′ and 400′ magnifications with the results combined and multiplied by the number of drops in the suspension and divided by the mass of stool in grams to calculate the number of EPG (Truant *et al*., 1981; Charoensuk *et al*., 2019; Kopolrat *et al*., 2022).

Automatic Feces Analyzer

The techniques employed by the FA280 Fully Automatic Feces Analyzer entail the utilization of artificial intelligence (AI)-based machine. The collection tube was added 0.5 g of fecal specimen. The machine operates with the following steps of liquidation. Briefly, sample dilution was added into the sample container for liquidation. After mixing, the liquidized samples were passed to the concentrated specimen. The microscope of the machine was located and identified the terminator of parasite detection and then the results reporting showed the images and other parameters such as color and character of feces.

Statistical analysis

Statistical analysis was performed using SPSS version 26.0 for Windows (SPSS Inc., Chicago, USA). The gold standard was defined as the combined method. Positive results referred to the presence of parasite eggs or larvae in the examined fecal specimen of three methods; KKT, FECT, and FAFA. The positive result regardless of the technique was considered a true positive. Therefore, specificity would be judged 100% from this setting. *O. viverrini* eggs were identified and counted to report the prevalence of infection with 95% confidence interval (CI). Each examination technique was evaluated for sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV), and percent agreement was reported as Cohen's kappa (0.00-0.19; none, 0.20-0.39; minimal, 0.40-0.59; weak, 0.60-0.79; moderate, 0.80-0.89; strong, 0.90-1.00; almost perfect). Data was reported as a percentage with 95% CI. The statistical significance level was predetermined at *P*-value < 0.05.

RESULTS

The examination of fecal specimens from a total of 455 samples revealed an overall prevalence of parasitic infection at 19.34% (88/455). The most prevalent infection was *O. viverrini* at 18.02% (82/455), followed by *Strongyloides stercoralis* at 0.88% (4/455), *Taenia* spp. and *Enterobius vermicularis* at 0.22% each (1/455). Parasite eggs and larvae were identified using KKT, FECT, and the FAFA machine, as illustrated in Figure 2. Upon provincial analysis, Kalasin province exhibited the highest infection rate at 55.55% (30/54), followed by Chaiyaphum at 22.55% (46/204), and Roi Et at 16.0% (4/25) (Table 1).

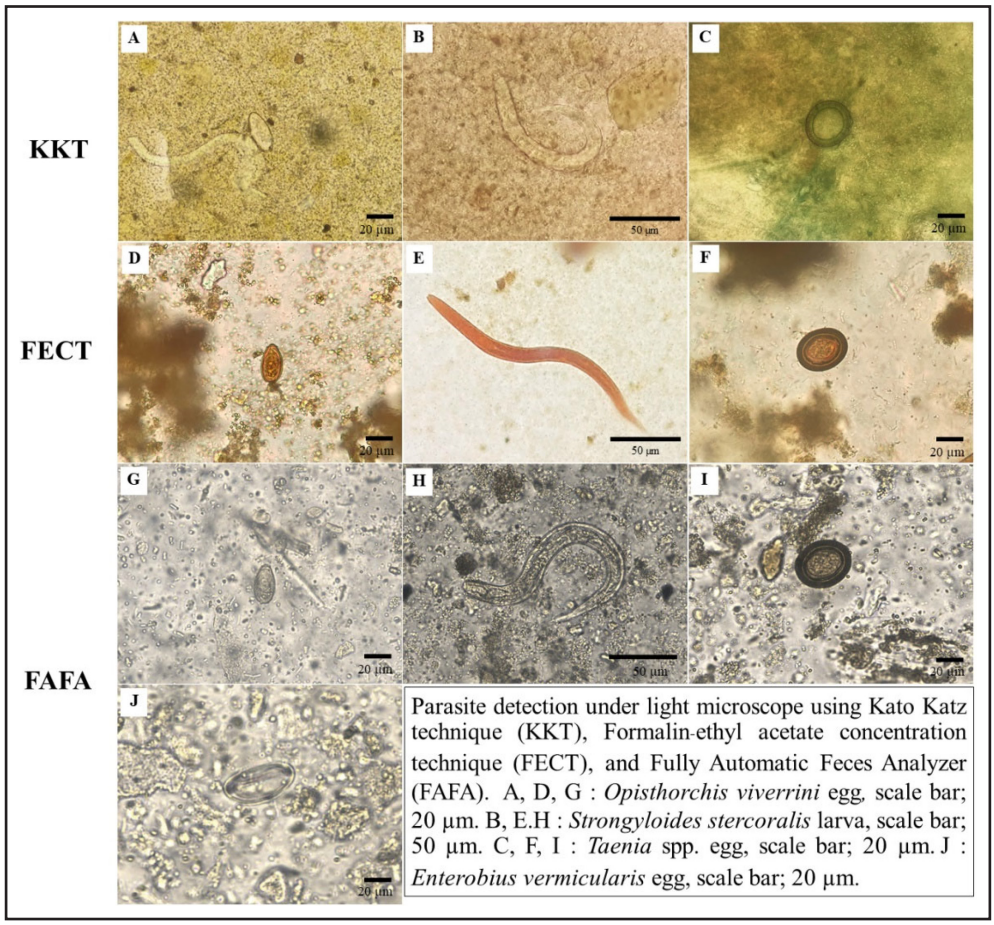

Figure 2. Intestinal helminth eggs and larva in fecal specimens under light microscopy.

Table 1. Parasitic infections among the population in five provinces through the examination of fecal specimens using KKT, FECT, and FAFA (*n*=455)

Parasite	Province					Total (%)
	NR (*n*=146) (%)	CP (*n*=204) (%)	KL (*n*=54) (%)	RE (*n*=25) (%)	NK (*n*=26) (%)	
O. viverrini	2 (1.37)	46 (22.55)	30 (55.55)	4 (16.00)	0	82 (18.02)
S. stercoralis	3 (2.05)	0	1 (1.85)	0	0	4 (0.88)
Taenia spp.	0	0	0	0	1 (3.85)	1 (0.22)
E. vermicularis	1 (0.68)	0	0	0	0	1 (0.22)
Total	**5 (4.79)**	**46 (22.55)**	**31 (57.41)**	**4 (16.00)**	**1 (3.85)**	**88 (19.34)**

NR= Nakhon Ratchasima, CP= Chaiyaphum, KL=Kalasin, RE= Roi-Et and NK= Nong Khai.

Table 2. Identification of parasitic infections through the examination of fecal specimens using KKT, FECT, and FAFA (*n*=455)

Parasites	KKT		FECT		FAFA		*P*-value
	n(%)	95%CI	*n*(%)	95%CI	*n*(%)	95%CI	
O. viverrini	39 (8.57)	0.54–0.72	75 (16.48)	0.88–0.97	48 (10.55)	0.62–0.79	0.000
S. stercoralis	2 (0.44)	0.45–1.00	4 (0.88)	1.00–1.00	3 (0.66)	0.58–1.00	0.000
Taenia spp.	1 (0.22)	1.00–1.00	1 (0.22)	1.00–1.00	1 (0.22)	1.00–1.00	0.002
E. vermicularis	–	–	–	–	1 (0.22)	1.00–1.00	0.002
Double infection	–	–	1 (0.22)	1.00-1.00	1 (0.22)	1.00–1.00	0.002

Table 3. Diagnostic accuracy of KKT, FECT, and FAFA for *O. viverrini* examination in fecal specimens

Method	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Kappa (95%CI)
KKT	100.00	89.21	45.78	100.00	0.58 (0.47–0.68)
FECT	98.67	97.63	89.16	99.73	0.92 (0.87–0.97)
FAFA	97.92	91.15	56.63	99.73	0.67 (0.56–0.77)

The identification of *O. viverrini* eggs through the FECT predominantly yielded a detection rate of 16.48% (75/455). Subsequently, the FAFA machine demonstrated a rate of 10.55% (48/455), while the KKT exhibited a rate of 8.57% (39/455). Moreover, *S. stercoralis* larvae were primarily identified at 0.88% (4/455) using FECT, 0.66% (3/455) using FAFA, and 0.44% (2/455) using KKT. *Taenia* spp. eggs were detected at 0.22% (1/455) using FECT, FAFA, and KKT. *E. vermicularis* was identified at 0.22% (1/455) using FAFA. The same specimens, total of 23 cases were positively detected the helminths by FAFA, KKT, and FECT (22 cases of *O. viverrini* eggs and 1 case of *S. stercolaris* larvae).Additionally, double infection (*O. viverrini* eggs and *S. stercoralis* larvae) was observed at 0.22% (1/455) using both FECT and FAFA. (Table 2).

The sensitivity for *O. viverrini* egg detection was found to be 100.00%, 98.67%, and 97.92% for KKT, FECT, and FAFA machine, respectively. In terms of specificity, KKT, FECT, and FAFA demonstrated values of 89.21%, 97.63%, and 91.15%, respectively. The FECT exhibited the highest positive predictive value (PPV) for detecting *O. viverrini* eggs at 89.16%, followed by FAFA at 56.63%, while KKT displayed the lowest PPV at 45.78%. On the other hand, KKT demonstrated the highest negative predictive value (NPV) at 100.00%, followed by FECT, and FAFA machine, both with a NPV of 99.73%. Substantial agreement was observed between the total positive results and the three methods: FECT (kappa=0.92; almost perfect), FAFA (kappa=0.67; moderate), and KKT (kappa=0.58; weak) (Table 3).

Table 4. Considerations of preparation time, cost implications associated with individual techniques, and the extent of chemical exposure during experimentation

	Methods		
	KKT	FECT	FAFA
Preparation time (min)	30	15	10
Cost per sample (Thai Baht)	60	250	160
Chemical exposure			
Ethyl acetate	–	+	–
Formalin	–	+	–

The preparation times for KKT, FECT, and FAFA were 30, 15, and 10 min, respectively. Notably, FECT involved exposure to formalin and ethyl acetate, whereas both KKT and FAFA remained chemical-free in their processing methods. The cost of sample examinations associated with each technique was outlined in Table 4. It is important to note that the price of the FAFA machine is not included in the cost analysis.

DISCUSSION

This study conducted a comparative analysis of three parasitological methodologies-namely KKT, FECT, and FAFA-aimed at diagnosing *O. viverrini* and parasitic infections within the fecal samples of a community population in northeastern Thailand. The results of the parasitic infection examination using all three methods revealed the presence of parasitic infections in all provinces, with a higher prevalence observed in Kalasin province, followed by Chaiyaphum province. These findings align with the study conducted by Wattanawong *et al*. (2021) which reported a higher frequency of infections in the northeastern region, particularly with respresent to *O. viverrini*, *S. stercoralis*, *Taenia* spp., and *E. vermicularis*. From the findings of this study, it is evident that the prevalence of *O. viverrini* infection is higher than that reported in the national survey of 2019. This highlights the ongoing presence of infections at various community levels, emphasizing the critical necessity for continuous surveillance by relevant authorities. In assessing the qualitative and quantitative diagnosis of *O. viverrini*, the three methodologies demonstrated comparable performance, as assessed through sensitivity, specificity, PPV, and NPV. The calculated kappa coefficient also underscored substantial agreement among the three tests. In addition, when diagnosing various parasitic helminth infections, the FECT demonstrated significantly elevated diagnostic parameters in comparison to the FAFA and KKT. In the context of population screening for *O. viverrini*, FECT exhibited noteworthy performance in helminthiasis diagnosis, particularly in the case of opisthorchiasis. Although the FAFA yields lower diagnostic results than the FECT method, it is noteworthy that, when compared to the KKT method, FAFA demonstrates superior diagnostic outcomes. Nevertheless, our findings are consistent with previous research, underscoring a limitation shared by both FECT and KKT—namely, their incapacity to differentiate between minute intestinal fluke (MIF) eggs and *O. viverrini* eggs (Chai *et al*., 2005; Buathong *et al*., 2017; Lamaningao *et al*., 2017). Prior investigations in the northeastern region of Thailand have predominantly focused on *O. viverrini* infection, with a low prevalence of MIF reported (Ramsay *et al*., 1989; Elkins *et al*., 1991; Boonjaraspinyo *et al*., 2013). In contrast, the central region of the Lao People's Democratic Republic (Lao PDR) identified *Haplorchis* spp. as more abundant than *O. viverrini* (Chai *et al*., 2013). Furthermore, a study in northern Thailand employing molecular diagnostics revealed the occurrence of MIF to be 3.8 times higher than that of *O. viverrini* (Buathong *et al*., 2017). Epidemiological surveys of parasitic helminths in Southeast Asia, including Thailand, frequently indicate coinfections with multiple species (Boonjaraspinyo *et al*., 2013; Chai *et al*., 2013; Sayasone *et al*., 2015). Given this scenario, there is a preference for diagnostic methods that can reliably classify helminth eggs from different species. This underscores the importance of employing methodologies with the capacity to discriminate among various parasitic infections, facilitating a more nuanced understanding of the complex epidemiological landscape in the region. While the examination and differentiation of both *O. viverrini* and MIF using the FAFA have not been extensively studied before, this research represents the inaugural investigation assessing both types of flukes, demonstrating effective discrimination in comparison to the KKT.

The FAFA machine employs the sedimentation concentration technique to process fecal specimens, resembling the FECT. This method demonstrates reliability in automatically concentrating stool samples to detect parasites commonly endemic in Thailand. Both preserved and fresh specimens are compatible with the FAFA machine, and our findings align with the established reliability demonstrated in other published studies on commercial kits (Sawangkla *et al*., 2022). This machine proves particularly suitable for laboratory use, offering an advantageous solution for regions lacking specialized personnel. In terms of safety, the FAFA machine ensures the processing of specimens within a sealed container, mitigating potential hazards for operating staff. The standardized operation of the machine contributes to minimal errors compared to human-induced errors. Furthermore, the turnaround time for specimens processed by the FAFA machine is notably shorter than conventional methods, enhancing efficiency in diagnostic procedures. However, a notable drawback pertains to the associated costs, encompassing the specimen container and the requisite training for machine operation. Several factors may contribute to the observed variance among distinct tests. Firstly, the FAFA machine processes a greater amount of fecal specimen (0.5 g) compared to FECT (3 g) and KKT (1 g). Secondly, procedural stages within FECT involving the filtration of fecal debris and fat play a pivotal role in enhancing the isolation of eggs, thereby amplifying the likelihood of detecting parasite eggs or larvae (Kopolrat *et al*., 2022). Moreover, the long-standing use of the KKT in screening and control programs in Thailand (Jongsuksuntigul & Imsomboon, 2003; Wattanawong *et al*., 2021) may be associated with underreporting in current epidemiological settings, given the prevalence of light infections of *O. viverrini*. While FECT requires specific laboratory equipment and proves impractical in resource-poor settings, the FAFA machine's design as an all-in-one tube with a fixative and built-in filtration apparatus removes fecal debris akin to the FECT procedure. Importantly, the FAFA allows for sample storage after preparation, providing a distinct advantage over KKT, which necessitates immediate reading by technicians within 30 min of slide preparation. Our findings indicate that the FAFA performs equally well compared to FECT and outperforms KKT. This suggests that the FAFA may emerge as the method of choice for future field applications in parasite surveys, particularly in the northeastern region of Thailand (Charoensuk *et al*., 2019). Advancements in science and technology have led to the development of commercial kits with accuracy comparable to conventional techniques (Soares, 2020). Numerous studies evaluating the clinical use of such kits, including Feconomics®, Mini Parasep®, and Sciendox 50®, have demonstrated promising results when compared with FECT (Kurt *et al*., 2012; Sanprasert *et al*., 2016; Sawangkla *et al*., 2022). Additionally, the turnaround time for specimens processed by the FAFA machine was comparatively shorter than the conventional method mentioned above.These developments underscore the evolving landscape of diagnostic tools and the potential integration of innovative technologies for improved parasite detection in various settings.

In summary, a noteworthy prevalence of *O. viverrini* infection is evident in northeastern Thailand. The outcomes of this study reveal that the FAFA, FECT, and KKT methodologies demonstrate comparable diagnostic sensitivity for *O. viverrini*. Specifically, the FAFA machine emerges as a potentially valuable tool for the detection of *O. viverrini* and other parasitic infections. Its aptness for clinical application as a concentration technique device is underscored by heightened safety for laboratory technicians and efficient processing times. To further enhance our comprehension, prospective studies should systematically evaluate the reliability of the FAFA machine in comparison to alternative stool concentration techniques, especially considering that our investigation primarily juxtaposed it with FECT and KKT. Furthermore, assigning precedence to the utilization of fresh specimens in clinical assessments can reinforce the validation of this machine's reliability.

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Conflict of Interest

The authors declare that there are no conflicts of interest.

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