# EVALUATION OF A COMMERCIAL STOOL CONCENTRATOR KIT COMPARED TO DIRECT SMEAR AND FORMALIN-ETHYL ACETATE CONCENTRATION METHODS FOR DIAGNOSIS OF PARASITIC INFECTION WITH SPECIAL REFERENCE TO *OPISTHORCHIS VIVERRINI* SENSU LATO IN THAILAND

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**Abstract.** Opisthorchiasis and cholangiocarcinoma (CCA) are major public health problems in Thailand and countries in the lower Mekong Subregion. Elimination of opisthorchiasis will be an important step toward the prevention, control and reduction of CCA. In order to achieve this goal, a sensitive and robust diagnostic method is required to identify people with current *Opisthorchis viverrini* sensu lato infection as the parasite is a group 1 carcinogen believed to be an etiology of CCA. To date, sensitive parasitological methods, such as formalin-ethyl acetate concentration technique (FECT) is preferred, but it is not practical in a remote primary care setting. In this study, we evaluated the diagnostic accuracy of a commercial stool concentrator kit with that of a direct simple smear method and a modified FECT. In diagnosing parasite infection and opisthorchiasis, the commercial kit had greater sensitivity (43.8-58.5%) than direct smear method (12.5-31.7%), but was less sensitive than FECT (73.2-75%). In a separate sample population, similar results were obtained when comparing the diagnostic accuracy of the commercial kit and FECT. However, the commercial kit was more effective in a field setting than FECT, and had better accuracy than direct smear method, which suggests that the kit could have potential utility in epidemiological studies and control programs of opisthorchiasis, as well as other parasitic infections. The design of

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the self-contained one-tube kit plus its long storage time after sample preparation provides a considerable advantage over other methods, such as direct or Kato thick smear method, under similar field conditions.

**Keywords:** *Opisthorchis viverrini* sensu lato, formalin-ethyl acetate concentration method, simple direct smear method, stool concentrator kit

## INTRODUCTION

Infections with food-borne trematodes (FBT) are important neglected tropical diseases (NTDs) of public health importance in many Southeast Asian countries. There are an estimated 750 million people at risk of FBT infection worldwide (Keiser and Utzinger, 2009).

Liver fluke, *Opisthorchis viverrini* sensu lato, is a FBT endemic to the Mekong Subregion of Southeast Asia where approximately 10 million people are infected (WHO, 1995; Sithithaworn *et al*, 2012), and more than 60 million people are at risk of infection (Keiser and Utzinger, 2005). Liver fluke infection (opisthorchiasis) causes hepatobiliary disease and is the major risk factor for bile duct cancer cholangiocarcinoma (CCA) (IARC, 2011). In Thailand, prevalence and intensity of *O. viverrini* infection and incidence of CCA are highest in the northeastern and northern regions, and to a substantially lesser extent in the central and southern regions (Jongsuksuntigul and Imsomboon, 2003; Sriamporn *et al*, 2004; Sripa *et al*, 2011; Khuntikeo *et al*, 2015).

As a consequence of long-standing control programs, opisthorchiasis has been transformed from a high and moderate to moderate and low prevalent infection (Kongs *et al*, 2001; Sithithaworn *et al*, 2003; Sithithaworn *et al*, 2012; Khieu *et al*, 2013; Sayasone *et al*, 2015b). Diagnosis using formalin-ethyl acetate concentration technique (FECT) is commonly used in epidemiological studies and for assessment of chemotherapy efficacy (Elkins *et al*, 1990; Sayasone *et al*, 2015b), but the method is not practical in a field setting as it has a long turn-around time and requires specific materials and equipment. There have been attempts to circumvent these drawbacks of FECT by using an all-in-one stool concentrator Parasep SF kit (Saez *et al*, 2011) but tests on the diagnoses of helminthiasis have yielded variable results (Lier *et al*, 2009; Zeeshan *et al*, 2011; Funk *et al*, 2013; Kaewpitoon *et al*, 2016). The kit has not been evaluated for *O. viverrini* and other zoonotic fish-borne trematodes (FZT) in an endemic community where intestinal and liver flukes are common (Phongluxa *et al*, 2013; Sayasone *et al*, 2015a).

In this study, we evaluated the diagnostic accuracy of a commercial kit in comparison with conventional examination methods, namely, direct smear detection and quantitative FECT, for diagnosis of parasitic infection including opisthorchiasis.

## MATERIALS AND METHODS

### Sample population and specimen collection

A sample population from an area endemic for opisthorchiasis in Khon Kaen Province, Thailand, was recruited to provide fecal samples. Approximately 10 g of fecal matter were collected from each individual in a 50-ml plastic container. These were kept in an icebox during transportation from the study site to the laboratory. Fecal samples collected in

March 2013 (Set 1) ($n$ = 130; age range 25-67 years) were examined by simple smear method, FECT and the commercial kit; and samples collected in June 2013 (Set 2) ($n$ = 190; age range 28-70 years,) were examined by FECT and a commercial kit.

The study protocol was approved by Khon Kaen University Ethics Committee for Human Research based on the Declaration of Helsinki and ICH Good Clinical Practice Guidelines (Reference No. HE551342). All participants identified as infected by parasites were treated with appropriate anthelminthic drugs.

**Fecal examination methods**

**Direct smear method.** Standard direct smear method was prepared by spreading in duplicate on a microscopic slide fresh feces (about 2 mg) together with one drop of normal saline and one drop of iodine solution (Beaver, 1950). The slides were examined under a light microscope (100x - 400x magnification).

**Quantitative FECT.** Fecal examination using a modified FECT was conducted as previously described (Elkins *et al*, 1990). In brief, 2 g of fresh fecal sample were mixed with 7 ml of 10% formalin solution, filtered through two layers of gauze, vigorously mixed with 3 ml of ethyl acetate for 30-60 seconds, and then centrifuged at 500*g* for 5 minutes. The pellet was re-suspended in 1 ml of 10% formalin solution and examined in duplicate under a light microscope (100x and 400x magnifications). The number of eggs per gram of feces (epg) was calculated as follows: (number of eggs counts/drop x total drops of fecal solution)/(g of feces).

**Commercial kit method.** Fecal samples were examined using Mini Parasep SF fecal parasite concentrator kit (Diasys Europe, Berkshire, UK) according to the manufacturer's instructions. In short, fecal sample (~0.5 g) was introduced into a tube containing 3.3 ml of 10% formalin solution and one drop of Triton x-100 then was added. The sample was vortexed to emulsify the mixture and centrifuged at 500*g* for 2 minutes. The pellet was re-suspended in 1 ml of 10% formalin solution and two drops, mixed with Lugol's iodine solution, were examined under a light microscope (100x - 400x magnification).

**Data analysis**

Statistical analyses of data were performed using Fisher's exact test or a chi-square test. For all statistical analyses, a $p$-value < 0.05 is considered significant. Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and 95% confidence intervals (95% CI) for total parasites and for *O. viverrini* separately were determined using MedCalc v. 16.2.1. Agreement between methods using the Kappa statistical test ($k$) was performed to determine consistency. Kappa values ranged from 0 to 1, which indicate the level of agreement between methods, with value < 0 indicating poor agreement, 0-0.2 slight, 0.21-0.4 fair, 0.41-0.6 moderate, 0.61-0.8 substantial, and 0.81-1 almost perfect (Landis and Koch, 1977).

## RESULTS

**Comparison between direct smear method, FECT and mini Parasep SF kit**

A total of 130 fecal samples were individually examined by three methods: direct smear, FECT and mini Parasep SF kit. Seven different species of parasites were identified, of which the most common parasite was *Strongyloides stercoralis*, followed by *O. viverrini* (Table 1). Direct smear method yielded four parasite species, namely, *O. viverrini*, *S. stercoralis*, hookworm and *Capillaria philippinensis*.

Table 1
Positive detection rates of parasites determined by direct smear method, FECT and mini Parasep SF kit (Kit) and statistical comparisons among the three methods ($n$ = 130).

| Parasite | No. positive (%) | | | Comparison ($p$-value)[a] | | |
|---|---|---|---|---|---|---|
| | Direct smear | FECT | Kit | Direct smear *vs* FECT | Direct smear *vs* Kit | FECT *vs* Kit |
| *Opisthorchis viverrini* | 2 (1.5) | 12 (9.2) | 7 (5.4) | 0.011[b] | >0.05[b] | >0.05 |
| *Strongyloides stercoralis* | 9 (6.9) | 14 (10.8) | 11 (8.5) | >0.05 | >0.05 | >0.05 |
| Other parasites | | | | >0.05[b,c] | >0.05[b,c] | >0.05[b,c] |
| Hookworm egg | 1 (0.8) | 1 (0.8) | 1 (0.8) | | | |
| Minute intestinal fluke | 0 (0) | 1 (0.8) | 1 (0.8) | | | |
| *Taenia* sp | 0 (0) | 0 (0) | 3 (2.3) | | | |
| *Capillaria philippinensis* | 2 (1.5) | 4 (3.1) | 3 (2.3) | | | |
| *Trichuris trichiura* | 0 (0) | 1 (0.8) | 0 (0) | | | |
| Total | 13 (10.0) | 30 (23.1) | 24 (18.5) | 0.005 | 0.051 | >0.05 |

[a]Chi square test. [b]Fisher's exact test. [c]Comparison of other parasites between methods.

Table 2
Diagnostic accuracy of direct smear method, FECT and mini Parasep SF kit (Kit) in diagnosis of *Opisthorchis viverrini* and other parasite species in fecal samples using combined methods as a reference diagnosis ($n$ = 130).

| Method | Sensitivity[a] | Specificity[a] | PPV[a] | NPV[a] | Kappa value[b] |
|---|---|---|---|---|---|
| Total parasites | | | | | |
| Direct smear | 31.7 (18.6-48.2) | 100 (94.8-100) | 100 (71.7-100) | 76.1 (67.1-83.3) | 0.39 (0.23-0.55) |
| FECT | 73.2 (56.8-85.2) | 100 (94.8-100) | 100 (85.9-100) | 89.0 (80.8-94.1) | 0.79 (0.67-0.91) |
| Kit | 58.5 (42.2-73.3) | 100 (94.8-100) | 100 (82.2-100) | 82.8 (75.3-90.1) | 0.66 (0.52-0.80) |
| *Opisthorchis viverrini* | | | | | |
| Direct smear | 12.5 (2.2-39.6) | 100 (95.9-100) | 100 (19.8-100) | 89.1 (82.0-93.7) | 0.20 (-0.04-0.44) |
| FECT | 75.0 (47.4-91.7) | 100 (95.9-100) | 100 (69.9-100) | 96.6 (91.0-98.9) | 0.84 (0.69-0.99) |
| Kit | 43.8 (20.8-69.4) | 100 (95.9-100) | 100 (56.1-100) | 92.7 (86.2-96.4) | 0.58 (0.34-0.82) |

[a]% (95% CI). [b]Mean (range) of pair-wised agreement between diagnostic methods.

Six different parasite species were detected by both FECT and mini Parasep SF kit. *Taenia* eggs were found only using the mini Parasep SF kit and *Trichuris trichiura* was found only by FECT. For detection of all parasite species, FECT and mini Parasep SF kit yielded comparable positive rate. Positive detection rate by FECT is significantly greater than for direct smear method ($p$ = 0.005). Positive detection rate determined by the kit was slightly higher than the direct smear method ($p$ = 0.051). For the detection of *O. viverrini*, positive rate was highest with FECT (9.2%), followed by mini Parasep SF kit (5.4%) and direct smear method (1.5%). Difference

Table 3
Comparison of detection rates of parasitic infections by FECT and mini Parasep SF kit (Kit) ($n$ = 190).

| Parasite | No. positive (%) | | Chi-square test | |
|---|---|---|---|---|
| | FECT | Kit | $\chi^2$ | $p$-value |
| *Opisthorchis viverrini* | 43 (22.6) | 24 (12.6) | 6.541 | 0.011 |
| *Strongyloides stercoralis* | 21 (11.1) | 15 (7.9) | 1.105 | >0.05 |
| Other parasites | | | 0.269[a] | >0.05[a] |
| Minute intestinal fluke | 8 (4.2) | 4 (2.1) | | |
| Hookworm egg | 4 (2.1) | 4 (2.1) | | |
| *Taenia* sp | 1 (0.5) | 5 (2.6) | | |
| *Ascaris lumbricoides* | 1 (0.5) | 0 (0) | | |
| *Echinostome* eggs | 1 (0.5) | 1 (0.5) | | |
| *Capillaria philippinensis* | 4 (2.1) | 3 (1.6) | | |
| *Trichuris trichiura* | 1 (0.5) | 0 (0) | | |
| Total | 68 (35.8) | 50 (26.3) | 3.982 | 0.045 |

[a]Comparison of other parasites between methods.

between FECT and direct smear method is statistically significant (Fisher's exact test, $p = 0.011$), but not between FECT and mini Parasep SF kit. As for the detection of *S. stercoralis* and other parasite species, there is no significant difference among all three methods.

Diagnostic performance of the three methods in comparison with the combined results as the gold standard showed, for all parasite species, that the sensitivity of FECT was highest, followed by mini Parasep SF kit and then by direct smear method (Table 2). Specificity of the three methods was 100%. The agreement between each method compared with the combined methods varied from high for FECT ($k$-value = 0.79), substantial for mini Parasep SF kit ($k$-value = 0.66) and fair for the direct smear method ($k$-value = 0.39).

For detection of *O. viverrini*, sensitivity of FECT, mini Parasep SF kit and direct smear method was 75.0%, 43.8% and 12.5%, respectively. Specificity of the three methods was 100%. The observed agreement was almost perfect for FECT ($k$-value = 0.84), moderate for mini Parasep SF kit ($k$-value = 0.58) and slight for direct smear method ($k$-value = 0.2).

**Comparison of FECT and mini Parasep SF kit**

An additional 190 fecal specimens were examined using FECT and mini Parasep SF kit. Nine different parasites were identified, of which the most common were *O. viverrini* and *S. stercoralis* (Table 3). All nine were identified by FECT and seven by mini Parasep SF kit. For all parasite species, the positive detection rate by FECT (35.8%) is significantly higher than for mini Parasep SF kit (26.3%) ($\chi^2 = 3.98$, $p = 0.045$). Positive detection rate for *O. viverrini* by FECT (22.6%) is significantly higher than for mini Parasep SF kit (12.6%) ($\chi^2 = 6.54$, $p = 0.011$). In the case of *S. stercoralis* and the other parasite species, positive detection rates were not different among the two methods.

Table 4
Performance of FECT and mini Parasep SF kit (Kit) for diagnosis of total parasitic infection and *Opisthorchis viverrini* in fecal samples with reference to a combined diagnosis ($n$ = 190).

| Method | Sensitivity[a] | Specificity[a] | PPV[a] | NPV[a] | Kappa[b] |
|---|---|---|---|---|---|
| Total parasite | | | | | |
| FECT | 82.9 (72.7-90.0) | 100 (95.7-100) | 100 (93.3-100) | 88.5 (81.2-93.4) | 0.85 (0.77-0.92) |
| Kit | 61.0 (49.5-71.4) | 100 (95.7-100) | 100 (91.1-100) | 77.1 (69.1-83.6) | 0.64 (0.53-0.75) |
| *Opisthorchis viverrini* | | | | | |
| FECT | 84.3 (70.9-92.5) | 100 (96.6-100) | 100 (89.8-100) | 94.6 (89.2-97.4) | 0.89 (0.81-0.96) |
| Kit | 47.1 (33.2-61.4) | 100 (96.6-100) | 100 (82.8-100) | 83.7 (77.0-88.8) | 0.57 (0.43-0.70) |

[a]% (95% CI). [b]Mean (range) of pair-wised agreement between diagnostic methods.

The diagnostic performance of FECT and mini Parasep SF kit in comparison with the combined methods (gold standard) showed a sensitivity of FECT higher than that of the kit (Table 4). Specificity of the two methods was 100%. The observed agreement between the two methods was substantial for mini Parasep SF kit ($k$-value = 0.64) and almost perfect for FECT ($k$-value = 0.85). Regarding *O. viverrini* detection, sensitivity of FECT was higher than for mini Parasep SF kit while specificity of the two methods was 100%. The observed agreement between the two methods was moderate for mini Parasep SF kit ($k$-value = 0.57) and almost perfect agreement for FECT ($k$-value = 0.89).

**Effect of intensity of infection on mini Parasep SF kit performance**

When the fecal specimens were separated according to intensity of infection (epg) by FECT, mini Parasep SF kit detected 23 out of 27 samples with epg < 100 and 1 out of 10 samples with epg 101-200. In 6 cases with epg > 200, all were negative by the kit (data not shown).

## DISCUSSION

In this study we evaluated the performance of a stool concentrator (mini Parasep SF) kit for detection of parasite with special reference to *O. viverrini* in endemic communities of opisthorchiasis in Northeast Thailand. In comparison with direct smear method and FECT, which are commonly used in laboratory diagnosis and epidemiological studies, the diagnostic accuracy of the kit in terms of sensitivity was higher than the direct smear method but lower than FECT. The highest positive detection rate of *O. viverrini* was obtained using FECT. These observed differences may be due to the amount of fecal matter used by each technique, as well as the filtration and fat removal procedure in FECT. In this study, 2 g of fecal sample was used for FECT while 4-fold less sample (0.5 g) was used for the kit and only 0.002 g for the direct smear method.

Direct smear method as well as Kato-Katz (KK) thick smear technique require fresh fecal samples for microscopy examination, which may cause considerable constraint in a large scale study. A previous study revealed that for diagnosis of opisthorchiasis, a modified KK technique is slightly more sensitive than the direct smear method but is lacking in reproducibility in comparison with Stoll's dilution

method (Viyanant *et al*, 1983). A combination of repeated stool examination by KK method and single FECT was suggested as a gold standard diagnosis for helminth infection including *O. viverrini* (Sayasone *et al*, 2015b). For clonorchiasis, caused by the liver fluke, *Clonorchis sinensis*, which is found in central Vietnam and north through to China and Korea (Petney *et al*, 2013), it was demonstrated that among three fecal examination methods, namely, KK method, formalin-ether technique (FE) and direct smear method, the KK method proved to be most sensitive for samples with various parasite intensities. In the case of light intensity of infection, FECT was found to be more sensitive than the KK method (Hong *et al*, 2003). Recently, a report from China suggested that multiple KK thick smears should be examined for an accurate diagnosis and for assessing drug efficacy against *C. sinensis* infection (Qian *et al*, 2013). However, when considering practicality in a mass epidemiological survey and control activity, the traditional FECT using a fat extraction solvent to separate fat content and the requirement of a centrifugation instrument is not ideal in a field setting (Allen and Ridley, 1970).

In the case of the commercial kit, this is a self-contained fixative and filtration apparatus for single use. As opposed to direct smear or the KK-based methods, the kit allows for extended storage time allowing samples to be processed and examined with more accuracy at a later period. Recently, other types of kits have been applied for parasite diagnosis in human and animal studies (Levecke *et al*, 2009; Razmi, 2009; Kurup and Hunjan, 2010). Despite the kit used in this being less effective in detecting higher parasite intensities, more comprehensive studies are required using larger sample sizes to ascertain whether the kit can be used for quantitative examination, especially for monitoring drug efficacy. To date, monitoring drug efficacy is becoming indispensable to detect emergence of resistance or to identify confounding factors affecting drug efficacy (Geerts and Gryseels, 2000; Albonico *et al*, 2004, 2007). Thus, the efficacy of anthelmintic drugs is usually monitored qualitatively based on the cure rate. However, fecal egg count reduction test has been suggested for monitoring drug efficacy quantitatively (Keiser and Utzinger, 2008), thus requiring the need for a sensitive detection technique that will allow an accurate estimation of infection intensity based on fecal egg counts. In addition, diagnosis problems persist due to the widespread use of chemotherapy for parasite control, and this may increase the proportion of light infections. To date, there has been little attention paid to the feasibility of using different methods for mass diagnosis under field conditions in poorly equipped laboratories, inadequately trained personnel and the ability of these personnel to estimate the efficacy of the administered drugs in different settings of pre-drug administration infection intensities.

In spite of the practical advantages listed above, the kit has potential drawbacks compared with FECT. In the latter, fecal debris and fat are efficiently removed and the final sedimented material contains less fecal matter, most of which is of small size. On the other hand, the final sediment from the kit often is constituted of thick and larger size debris, which may interfere with the detection of parasites. Similar observations were made in a previous study (Levecke *et al*, 2009). A further limitation of the kit is the volume of fixative solution (3 ml) in a 5-ml tube, which limits the maximum amount of fecal sample to

0.5 g. Inadequate fixation may occur if a larger amount of sample is used because to facilitate fixation the sample must be mixed thoroughly by mechanical shaking or using a vortex mixer.

It is well known that for diagnosis of helminthiasis, including opisthorchiasis, a gold standard is lacking and the chance for a false negative diagnosis is high when the intensity of infection is light (Johansen *et al*, 2015). Evidence from an autopsy study demonstrated that as many as 70% of false negatives occur in individuals with <10 worms in the biliary system (Sithithaworn *et al*, 1991). There have been proposals to circumvent this problem by using latent class analysis in which the results from different methods are combined and considered to be a gold standard (Dendukuri *et al*, 2009). In this study, a similar approach was used by combining the results from each method (direct smear, commercial kit and FECT), and used as a gold standard to calculate the diagnostic performance of each technique. Another approach to circumvent the problem of a lack of gold standard diagnosis is to use more sensitive diagnostic methods, such as molecular and immunological diagnoses (Duenngai *et al*, 2008; Parvathi *et al*, 2008; Duenngai *et al*, 2013). For instance, copro and urine antigen detection methods have resulted in a considerable number of antigen positives (up to 47%) in cases of egg-negative using conventional methods (Watwiengkam *et al*, 2013; Worasith *et al*, 2015). These studies correspond well with the previous autopsy report and may approach the true prevalence of infection (Sithithaworn *et al*, 1991).

In this study, the observed sensitivity for *O. viverrini* detection was highest using FECT (75-84%) followed by the commercial kit (44-47%) and then the direct smear method (13%). The choice of method to be used for diagnosis, therefore, has important implications for determining the prevalence of infection and subsequent chemotherapeutic control. Moreover, in this study a single stool sample was collected for examination by FECT and the commercial kit. Although duplicate examinations were performed, repeated sample collection for examination, *ie*, over 3 days has shown a higher prevalence of infection than from single stool examinations (Sayasone *et al*, 2015b). How many repeated sample collections are required to determine the true prevalence of infection is not clear, but a combination of methods, such as molecular and/or immunological analyses, in addition to classical parasitological methods may contribute toward a gold standard diagnosis.

In conclusion, the large number of samples collected for surveillance and control in remote communities and the long distance to the laboratory make methods that require fresh fecal samples, such as direct smear and KK techniques, not feasible. A kit with a closed concentrator system provides a substantial increased ease of use and presents a reduced health hazard compared to conventional concentration methods. Fixing stool samples enables a longer time before sample processing and examination. Although the kit offers a safer and faster way to conduct the concentration procedure, there are considerable disadvantages in terms of cost and diagnostic accuracy compare with KK thick smear method that is commonly used in field studies, and adoption of the kit needs to be evaluated.

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