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Comparison of mini-flotac and Kato-Katz methods for detecting soil-transmitted helminth eggs in 10% formalin preserved stools stored ≥12 months

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Abstract. In Soil-Transmitted Helminth (STH) control programs, microscopic examination is applied as a standard method for detecting the presence and the number of STH eggs. The time limitations of fresh specimen processing, especially for an accurate quantitative diagnosis, cause the specimen processing to be delayed or should be performed at a referral laboratory. This deferment requires preservatives to keep the stool integrity without reducing the accuracy. The aims of this study were: 1) to compare the proportion of positive samples and the intensity of *A. lumbricoides*, *T. trichiura,* and hookworm infection based on the examination of fresh samples and stool preserved by 10% formalin for ≥12 months and 2) to determine the most reliably accurate between Kato-Katz and mini-FLOTAC methods in detecting *A. lumbricoides*, *T. trichiura*, and hookworm eggs in preserved stools both qualitatively and quantitatively. Seventy-eight (78) stool samples were examined by mini-FLOTAC, and Kato-Katz methods. Proportion of positive samples of *A. lumbricoides*, *T. trichiura*, and hookworms in fresh and in ≥12 months 10% formalin preserved stools had no significant difference. Helminths density (eggs per gram of stool/EPG) in fresh samples was fewer compared to EPG in preserved samples ($p < 0.05$) which leads to a lower proportion of moderate and high level groups in fresh stools samples compared to those in preserved samples ($p < 0.05$). In preserved samples, as qualitative method, mini-FLOTAC detected more *A. lumbricoides* and *T. trichiura eggs* than Kato-Katz, while hookworm eggs were detected more by Kato-Katz than the mini-FLOTAC. As a quantitative detection, Kato-Katz showed higher calculation of STH EPG than mini-FLOTAC. Using 10% formalin preservation for stool samples, the STH eggs' morphology could still be well identified. Homogenization process and low number of samples tested, were acknowledged as the limitation of this study.

BACKGROUND

Helminth infections are commonly known as the most common infections worldwide caused by intestinal helminth parasites. Those parasites commonly defined as Soil-Transmitted Helminth (STH) are identified as a group of parasitic worms causing human infection through soil contaminated by eggs or larvae. The STH that most commonly cause human infection are *Ascaris lumbricoides*, *Trichuris trichiura*, and the two hookworm species, *Necator americanus* and *Ancylostoma duodenale* (Bethony *et al.*, 2006).

Globally, more than one billion people worldwide suffer from STH infection, especially in tropical and subtropical areas with poverty, poor hygiene and sanitation, and inadequate water supply (Debalke *et al.*, 2013; Strunz *et al.*, 2014). The prevalence of STH infection in Indonesia in 2015 was varied from 20 to 86% (Kemenkes RI, 2015). STH infection causes nutritional deficiencies

in the form of calories, protein and blood loss. This results in morbidity which effects on growth disorders, physical development and cognitive developmental delays in children (Hotez *et al.*, 2007).

In helminth control programs, microscopic examination is applied as a standard method for detecting the presence and the number of eggs of helminthes (PATH, 2015). Kato-Katz is the standard microscopic method for surveying the prevalence of helminth infection including STH infection (WHO, 2015). Kato-Katz method is practical and has been widely used for the diagnosis of helminth infections. However, several studies reported varied sensitivity (Santos *et al.*, 2005; Lamberton *et al.*, 2015). Other widely used quantitative microscopic methods are FLOTAC and mini-FLOTAC (WHO, 1994). The mini-FLOTAC is simpler than the FLOTAC method, since the FLOTAC requires a centrifuge in the preparation process and a longer preparation time than the mini-FLOTAC method, making it more difficult to apply in the field (Knopp *et al.*, 2014). The reading process of mini-FLOTAC preparations is easier than Kato-Katz due to the floatation solution. In addition, the enclosed mini-FLOTAC apparatus system is also an advantage of this method. It reduces the direct contact of the examiner to the stool sample preparation during inspection under a microscope (Maurelli *et al.*, 2014). A shorter preparation time is another advantage of this method compared to Kato-Katz (Ng'etich *et al.*, 2016). The time required for mini-FLOTAC examination is about 10 to 15 minutes, while Kato-Katz takes about 20 to 30 minutes after preparation. Similar to Kato-Katz, this method has a variation of sensitivity (Barda *et al.*, 2013; Knopp *et al.*, 2014; Nikolay *et al.*, 2014). However, Kato-Katz is the most widely used method for diagnosing STH infection because the preparation is simple and inexpensive, and does not require special equipment compared to the mini-FLOTAC method. Therefore, Kato-Katz is the recommended method by WHO as the standard method of STH infection diagnosis (WHO, 2015).

Qualitative STH examination based on microscopic methods can be applied directly in the field or study area. However, to do an accurate quantitative examination of STH, there are technical constraints such as support facilities, large number of samples or limited human resources that cause the examination cannot be performed in the field or promptly. Thus, to maintain the integrity of the sample, preservative is required to keep the STH contained in it, so that both the species type and its eggs number are similar as when the sample is fresh. In this study, we evaluated the effect of 10% formalin that has been used as preservative for stool samples collected $\geq$ 12 months previously on STH eggs and compared the sensitivity of mini-FLOTAC and Kato-Katz methods. The aim of this study was to determine the incidence and the intensity of *A. lumbricoides*, *T. trichiura*, and hookworm infection of preserved stool compare with its incidence and intensity while the samples were still in fresh condition and we also observed the morphology of *A. lumbricoides*, *T. trichiura*, and hookworm eggs detected in preserved stools. This study also aimed to determine the most highly accurate method of detecting *A. lumbricoides*, *T. trichiura*, and hookworm eggs in preserved stools both qualitatively and quantitatively.

MATERIAL AND METHODS

Study samples

This study was an observational study of diagnostic STH testing using a cross-sectional design. The research was conducted at the Parasitology Laboratory of Faculty of Medicine, Public Health and Nursing (FK-KMK) Universitas Gadjah Mada (UGM). This study has been approved by Ethics Commission of the FK-KMK, UGM with number: KE/FK/1181/EC/2017. The samples of this study were stool samples from previous study in Alor, East Nusa Tenggara and Central Maluku, preserved and stored in the Parasitology Laboratory of FK-KMK UGM (Ndona, 2015; Lalangpuling, 2017). The samples chosen in this study were preserved stool samples that met inclusion and exclusion criteria. The inclusion criteria in

this study were positive stool samples containing STH eggs based on the results of the previous study, while the exclusion criteria were dried and or insufficient amount of samples to determine using mini-FLOTAC and Kato-Katz methods.

Microscopic Examination

All preservative samples were analyzed qualitatively and quantitatively by mini-FLOTAC and Kato-Katz methods. The mini-FLOTAC was performed using saturated sodium chloride (NaCl) as the floatation solution (FS2). For the mini-FLOTAC technique, 2 grams of stool were weighed and diluted by 2 ml of 5% formalin (Polman *et al.*, 2015). After being homogenized and filtered, the suspension was directly added to 40 ml of the FS2. The suspension (stool + 5% formalin + FS2) was poured slowly into the chamber of reading disc of mini-FLOTAC. Ten to 15 minutes were needed for the eggs of parasite to float before microscopic examination. Egg per gram (EPG) was obtained by multiplying the total number of eggs from chamber 1 and chamber 2 of the mini-FLOTAC disc by a factor 10 (Ng'etich *et al.*, 2016). The Kato-Katz was performed using the 41.7 mg template, according to the WHO recommendations and EPG for each helminth was calculated as described previously (WHO, 1994; Polman *et al.*, 2015). Examination of fixed stool samples resulted in proportion and infection intensity for each species of *A. lumbricoides*, *T. trichiura*, and hookworm. Intensity of STH infection was categorized according to WHO guidelines (WHO, 2011). Kato-Katz results found in this study (preserved stools) were compared with Kato-Katz results examined in previous studies (fresh stools) (Ndona, 2015; Lalangpuling, 2017).

Statistical Analysis

The comparison between proportion of *A. lumbricoides*, *T. trichiura*, and hookworm infection based on the results of fresh and preserved stools examined by Kato-Katz was analyzed by *Mc Nemar* test. The comparison between EPG and infection intensity of *A. lumbricoides*, *T. trichiura*, and hookworms in fresh and preserved stool examined by Kato-Katz was calculated by *Wilcoxon Sum Rank* test, as well as the comparison of EPG and intensity of STH infection in preserved stool based on Kato-Katz and mini-FLOTAC methods. The level of significance was set at p value <0.05. Sensitivity of the mini-FLOTAC was calculated by comparing the results of preserved stool examination using the Kato-Katz method. Morphology of *A. lumbricoides*, *T. trichiura*, and hookworm eggs were described based on microscopic observations by mini-FLOTAC and Kato-Katz methods.

RESULTS

Seventy-eight (78) preserved stool samples, consisting of 28 samples from Alor, East Nusa Tenggara Province and 50 samples from Central Maluku, Maluku Province, Indonesia were used in this study.

Proportion of positive samples and intensity of Soil-Transmitted Helminth infection

The proportion of positive samples infected by *A. lumbricoides*, *T. trichiura* and hookworm showed no significant difference between fresh and preserved stool examined using the Kato-Katz method (p >0.05) (Table 1). No significant difference of positive sample proportion was found for

Table 1. The proportion of STH infection in fresh and preserved stools with Kato-Katz

Species STH	Fresh stools	Preserved stools	p value
	n	n	
A. lumbricoides	67	60	1.996
T. trichiura	52	53	1.750
Hookworm	30	37	0.511

A. lumbricoides and *T. trichuris* when the two methods, i.e Kato-Katz and mini-FLOTAC were applied on preserved samples, meanwhile the difference was significant for hookworm samples, i.e. higher proportion was found when examined using the Kato-Katz method compared to the mini-FLOTAC method (p <0.05) (Table 2).

When comparing the helminth density (EPG) using the Kato-Katz method, those in fresh stools showed fewer helminths EPG than those in preserved ones (p <0.05) (Table 3). The difference might be due to changes in intensity level of infection as the proportion of moderate and high level group increased in preserved samples compared to those in the fresh stools (i.e. 48, 42 and 4 compared to 40, 25, 2 for *A. lumbricoides*, *T. trichiura* and hookworm, respectively) (Figure 1). When comparing helminth EPG using Kato-Katz and Mini-FLOTAC of the preserved stools, examination using the Kato-Katz method resulted in higher EPG compared to the the mini-FLOTAC method

Table 2. The proportion of STH infection in preserved stools with Kato-Katz and mini-FLOTAC

Species STH	Kato-Katz	Mini-FLOTAC	p value
	n	n	
A. lumbricoides	60	62	0.508
T. trichiura	53	56	0.250
Hookworm	35	21	0.001

Table 3. The median EPG of STH in fresh and preserved stools with Kato-Katz

Spesies STH	Fresh stools	Preserved stools	p value
	Median (max-min)	Median (max-min)	
A. lumbricoides	5,328 (178584-0)	13,176 (321,096-0)	0.000
T. trichiura	462 (14484-0)	1,620 (150,608-0)	0.000
Hookworm	0 (4680-0)	0 (3,300-0)	0.003

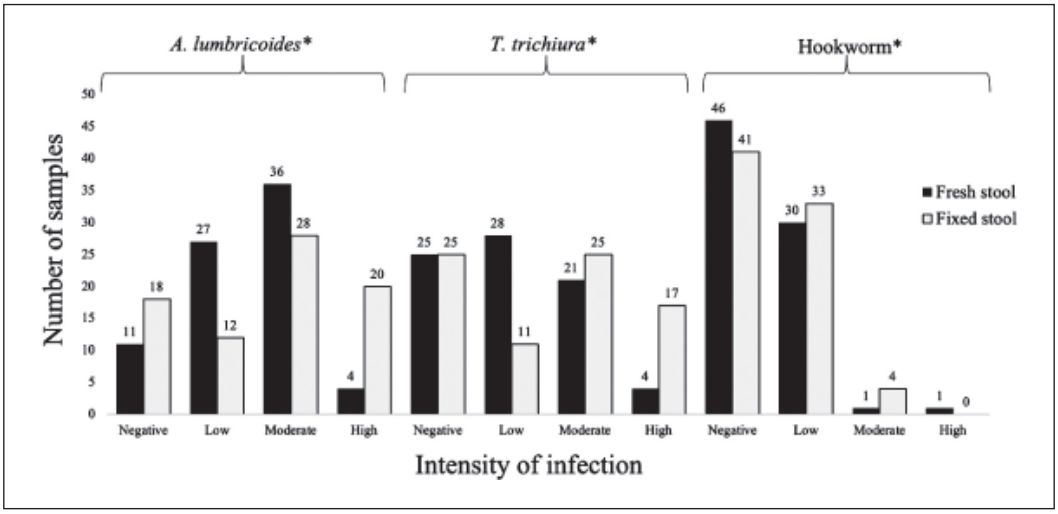

Figure 1. Level of intensity of STH infection in fresh and preserved stools with Kato-Katz, (*p <0.05).

(p <0.05) (Table 4). As a consequence, the proportions of STH that fell into moderate and high level groups were found to be higher when examined using the Kato-Katz method compared to the Mini-FLOTAC method (i.e. 48, 42, and 4 compared to 17, 36, and 0 for *A. lumbricoides*, *T. trichiura* and hookworm, respectively) (Figure 2).

Morphology of STH eggs

According to either Kato-Katz and mini-FLOTAC methods, identified and readable *A. lumbricoides* eggs were 30 samples (45.5%) in preserved stools (Figure 3 Panels A & B), although there was a defect in the lining of some of the *A. lumbricoides*'s egg shells (32 samples/48.5%) (Figure 3 Panels E & F). A total of 4 samples (6.1%) of infertile *A. lumbricoides* eggs were not spherical or oval (Figure 3 Panel D); however, the identification process could still be performed well. The observations by mini-FLOTAC and Kato-Katz showed that the shape and the layer structure of *T. trichiura* eggs did not change in preserved stools (Figure 3 Panels C & I). The clearly defined egg shell of hookworm was identified briefly by mini-FLOTAC (Figure 3 Panel C), and hence, the faded egg shell of hookworm was detected by the Kato-Katz method (Figure 3 Panels G, H & I).

The Sensitivity of the Method

The mini-FLOTAC method detected more *A. lumbricoides* and *T. trichiura* eggs than the Kato-Katz method i.e. 63 vs 60 samples for *A. lumbricoides* and 56 vs. 53 samples for *T. trichiura* in preserved stool samples. However, hookworm eggs were detected more by Kato-Katz than the mini-FLOTAC method i.e. 32 vs 21 respectively (Figure 4). The sensitivity of mini-FLOTAC (Kato-Katz as a 'gold' standard method) in preserved stools was 95% for *A. lumbricoides* and 100% for *T. trichiura*, while the sensitivity was low (54.05%) for detecting hookworm.

Table 4. The median EPG of STH in preserved stools with Kato-Katz and mini-FLOTAC

Spesies STH	Kato-Katz	Mini-FLOTAC	p value
	Median (max-min)	Median (max-min)	
A. lumbricoides	13,176 (321,096-0)	643 (116,590-0)	0.000
T. trichiura	1,620 (150,608-0)	865 (50,685-0)	0.000
Hookworm	0 (3,300-0)	0 (165-0)	0.000

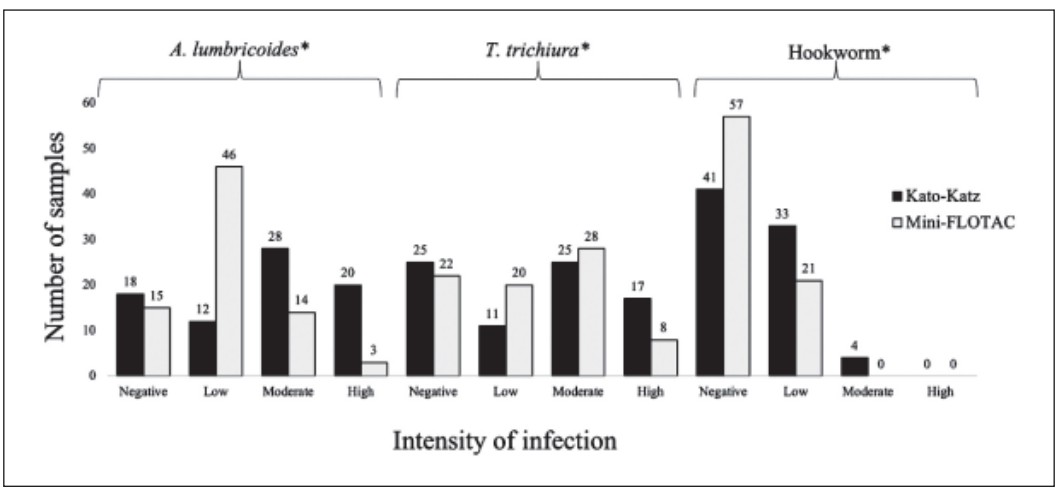

Figure 2. Level of intensity of STH infection in preserved stools with Kato-Katz and mini-FLOTAC (*p <0.05).

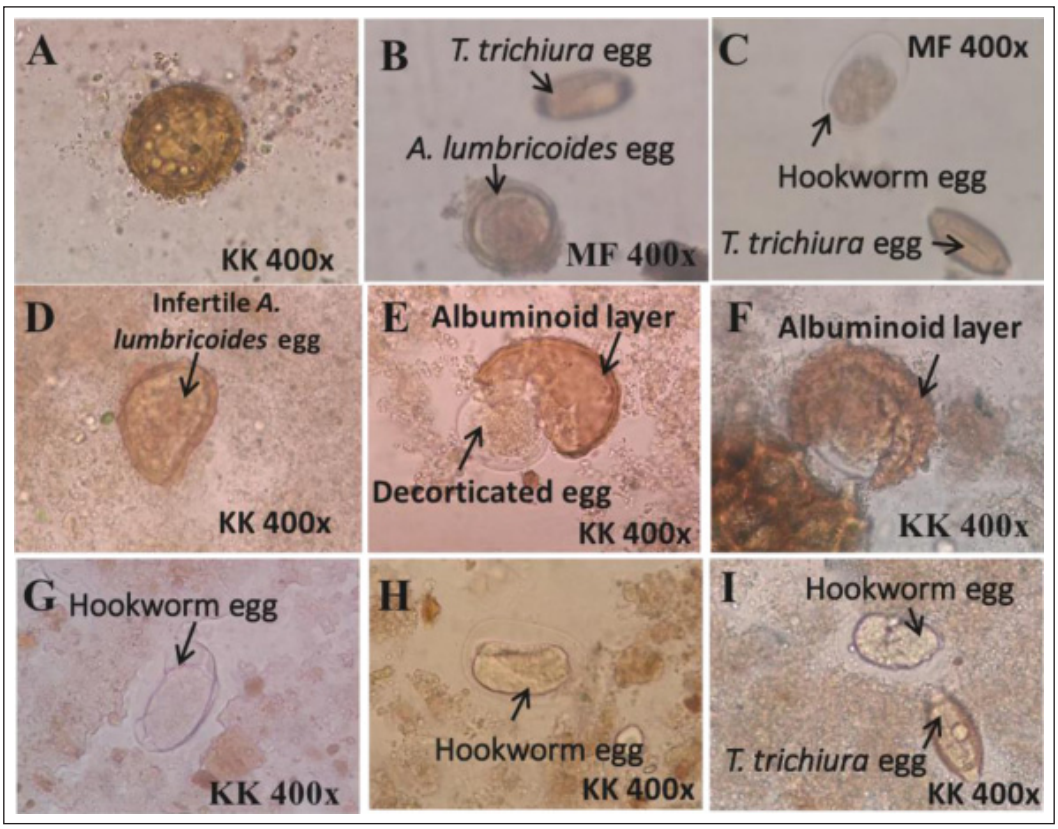

Figure 3. Morphology of Soil-Transmitted Helminths (STH) eggs in 10% formalin preserved stools. (A) A clearly visible of *A. lumbricoides* egg, (B) A clearly visible of *A. lumbricoides* and *T. trichiura* eggs, (C) The clearly visible of hookworm and *T. trichiura* egg, (D) A non-rounded infertile *A. lumbricoides* egg, (E & F) A damaged albuminoid layer of *A. lumbricoides* egg, (G & H), A faded hookworm eggshell, (I) A faded hookworm eggshell and a clearly visible of *T. trichiura* egg (KK: Kato-Katz, MF: mini-FLOTAC).

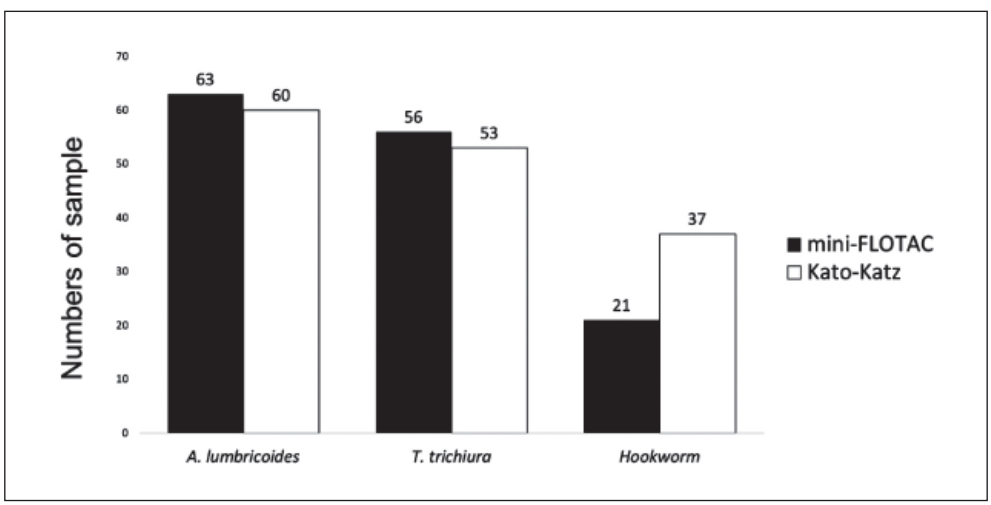

Figure 4. Comparison of the proportion of Soil-Transmitted Helminth (STH) infection in 10% formalin preserved stools (12 months) between mini-FLOTAC (MF) and Kato-Katz (KK) methods.

DISCUSSION

Stool preservative plays an important role to maintain parasites' integrity in the stool samples thus STH eggs identification results obtained after preservation are close to the fresh ones. This study then aimed to compare the incidence and intensity of *A. lumbricoides*, *T. trichiura*, and hookworm infection based on the comparison of the results of fresh and preserved stools for ≥ 12 months in 10% formalin and describe the morphology of STH eggs detected in preserved stools. The results of this study are expected to be a guide for choosing the most accurate microscopic method for detecting STH eggs both qualitatively and quantitatively in epidemiological studies or surveys when the specimen examination is delayed.

Our study showed no significant changes in the proportion of *A. lumbricoides* and *T. trichiura* infection in preserved stools. This result was in line with a previous study (Barda *et al.*, 2015). It seems that 10% formalin as a stool preservative was able to maintain egg morphology of *A. lumbricoides*, *T. trichiura*, and hookworm for more than 12 months. Commercial formalin solution contains formaldehyde which is capable to bind proteins in the structure of a tissue thus preventing autolysis process and causing the tissue to become more resistant to changes in the surrounding environment (Kiernan, 2000).

The proportions of moderate, and high intensity level of infection of *A. lumbricoides*, *T. trichiura*, and hookworm in preserved stool examined by Kato-Katz were higher than those in fresh stools ($p<0.05$). It might be caused to the homogenization process (Dacombe *et al.*, 2007; Cringoli *et al.*, 2010) in previous study that was not done evenly compared to the homogenization process in this study while preservation had been added and it is acknowledged as an uncontrolled factor of this study. Due to the low number of samples tested, the difference also might not be a significance in practical use.

The higher median results EPG of *A. lumbricoides*, *T. trichiura*, and hookworm in preserved stools detected by Kato-Katz compared to mini-FLOTAC in this study were not in line with the results of a previous study (Barda *et al.*, 2015). In their study, morphology of *A. lumbricoides* and *T. trichiura* eggs did not alter, but hookworm eggs were difficult to identify due to their distinctive shape and morphology after 31 days of preservation with 5% formalin. The egg shell of hookworm during the study period faded and was no longer clearly readable. Meanwhile in our study, some of the *A. lumbricoides* and hookworm eggs in some stool samples were altered structurally, but still could be identified based on their morphology. Some of the *A. lumbricoides* eggs were found to have all intact layers, with three layers of eggshell (lipid, chitin, and vitelin layers) and albuminoid layer that were still clearly visible, although some had a damaged egg shell, shape and the layers that did not appear in an intact condition were detected by the Kato-Katz method. Probably, higher concentration of formalin preservative could maintain STH eggs better, particularly for *A. lumbricoides* and hookworm eggs.

This study showed that the median number of *A. lumbricoides* eggs in preserved stool examined by the Kato-Katz method was higher than the results of preserved stool examined by the mini-FLOTAC method. Perhaps, the mini-FLOTAC method only allowed intact eggs to be detected, and the damaged ones could not float along with the undamaged ones, and as a consequence, thus affecting the results in EPG found by the mini-FLOTAC method. The morphology of *T. trichiura* eggs found during observation showed no visible defect of the eggshells; therefore, its identification process in preserved stools was easy to do. *Trichuris trichiura* eggs have a small, tight and intact structure, and thus easily float to the surface of the mini-FLOTAC preparation and were detected more by the mini-FLOTAC method. Therefore, the difference in the intensity of *T. trichiura* infection was not as large as the intensity of *A. lumbricoides* infection. Related to hookworm eggs, since its eggs have thin layers and fragile egg shells, examination using Mini-FLOTAC could not

detect all hookworm eggs particularly those that were damaged during preservation and storage (Waller, 1971); and consequently this affected the incidence rate and EPG in preserved stools.

In this study, the proportion of *A. lumbricoides*, *T. trichiura*, and hookworm infection based on mini-FLOTAC and Kato-Katz methods showed some variation. *Ascaris lumbricoides* and *T. trichiura* eggs in preserved stools examined by mini-FLOTAC were detected in higher numbers than those examined by Kato-Katz. Three samples with low infection (5,10, 25 EPG), that were able to be detected by the mini-FLOTAC methods (*A. lumbricoides* eggs), were undetectable when examined by the Kato-Katz method. This discrepancy was possibly caused by less weight of stools used in the Kato-Katz compared to the mini-FLOTAC method (41.7 mg vs 2 g), supporting a previous finding that the Kato-Katz method has a lower sensitivity than the mini-FLOTAC method in low infection setting (Nikolay *et al.*, 2014; Lamberton *et al.*, 2015).

CONCLUSION

The study determined that *A. lumbricoides*, *T. trichiura*, and hookworm eggs in stool preserved by 10% formalin for ≥ 12 months could still be detected qualitatively by both the mini-FLOTAC and Kato-Katz methods. For quantitative diagnosis, Mini-FLOTAC can be used as an alternative method for the Kato-Katz, especially for detecting the good morphology and intact structure of STH eggs. However, Kato-Katz showed better performance compared to the mini-FLOTAC methods for quantitative diagnosis of STH when samples were preserved in 10% formalin.

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