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The Use of Ziehl Neelsen Staining to Differentiate the Morphology of Taenia Spp. Eggs

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***Abstract*— Currently, distinguishing Taenia species depends on the morphology of the scolex or proglottid, which is often unavailable. This study aimed to determine whether Ziehl Neelsen staining can distinguish the eggs of Taenia solium and Taenia saginata. This cross-sectional study was conducted using stool samples with 5% formalin-PBS as a preservative. Forty pots of stool samples were examined to detect Taenia species eggs using the Kato-Katz technique. Ten slides were made for each pot, then stained with Ziehl Neelsen's stain. All slides were examined for differences in color and eggs morphology using a light microscope. From 400 slides, 154 slides showed that Taenia saginata eggs were magenta colored (38.63%) and 90 slides showed Taenia solium eggs were blue/purple (22.5%). Taenia saginata eggs are larger and oval shaped, while Taenia solium egg is smaller and most of them are round in shape. Our finding showed that Ziehl Neelsen stain was more sensitive and specific to differentiate Taenia solium and Taenia saginata mature egg.**

Keywords*— *Taenia solium; Taenia saginata; Ziehl Neelsen

I. INTRODUCTION

Taeniasis is the intestinal infection of humans caused by adult stages of Taenia solium, Taenia saginata, and Taenia asiatica. [1] These parasites have a worldwide distribution, and the highest burden is in developing countries, which cause a health problem in several regions in Indonesia. [2,3] Humans were the only definitive host for these three species, so they can turn into their mature form in humans. [4,5] Larval stage of these parasites is called cysticercosis that refers to the tissue infection of the intermediate host, cattle for Taenia saginata and swine for Taenia solium and Taenia asiatica. [5,6] Humans can get infected with larval stage of these parasites by ingesting eggs accidentally that are contaminated in soil, released by themselves or by another carrier. [5,7] Furthermore, gravid proglottid acts as a new infection source for intermediate host particularly in developing countries with poor sanitation conditions. [8,9]

Human cysticercosis is endemic in most developing countries and frequently attacks the human central nervous system (CNS), causing a variety of neurological symptoms. [10] In contrast to cysticercosis, taeniasis is relatively harmless because the adult worm stage of this cestode infects the human small intestine and causes specific symptoms, such as abdominal pain and nausea. [1,5]

It is difficult to differentiate Taenia solium and Taenia saginata by parasitology examintaion because their eggs are very similar. [11] The right identification is very important because the consequences of infection in humans by these two parasites are very different. Taenia saginata is relatively harmless, because only the intestinal tapeworm phase occurs in humans, whereas Taenia solium infection has a wider and more severe health effect, mainly resulting in extraintestinal infections by larval phase or cysts in the CNS. [1,6] Taenia solium has the capacity to infect the human brain with a larval form causing neurocysticercosis, the leading cause of acquired epilepsy worldwide. [5,12] Therefore, adequate early detection and treatment of taeniasis is essential for the prevention of cysticercosis infection. Furthermore, a reliable epidemiological information is necessary for the use in effective control of taeniasis or cysticercosis, including accurate tools for parasite identification, is urgently needed. [1-5]

Currently, the identification of the two human Taenia species is based on the number of uterine branches in intact gravid proglottids, and the absence or presence of hooks in the scolex of tapeworms. [13] Obtaining gravid proglottids or scolex that are still solid and intact after treatment is often difficult because most gravid proglottids are damaged or only immature proglottids are present in the stool. [14] Several studies reported that treatment with nyclosamide and ricin oil laxative only resulted in immature proglottids in nearly half of the patients. Other methods, such as the biochemical analysis of total protein or the zymogram method, have been explored but are difficult to interpret and the results are inconsistent. [15,16]

Taenia asiatica is endemic in North Sumatra Province with prevalence ranging from 1.9-20.7%. [2] Considering that this study was conducted in Jakarta, it is only limited to diagnosing taeniasis caused by Taenia solium and Taenia saginata due to regional endemicity. Molecular techniques have been used to differentiate between Taenia solium and Taenia saginata. However, despite providing precise diagnostic results, the procedure is somewhat complicated and these techniques require expensive specialized equipment. In Indonesia as a developing country, a simple, easier, fast and inexpensive diagnostic method is needed for the identification of these two Taenia species. Therefore, this study was aimed to differentiate Taenia solium and Taenia saginata based on egg morphology using Ziehl Neelsen staining and its comparison to Kato-Katz method.

II. RESEARCH METHODOLOGY

A. *Study design*

The study was cross-sectional and conducted at the Parasitology Laboratory of the Medical Faculty of Universitas Trisakti Jakarta and carried out from January to June 2018. Research Ethics Commission of Universitas Trisakti reviewed and approved the research protocol.

The samples of forty pots of stool from confirmed patients with taeniasis was purchased from the Parasitology laboratory of the National Central hospital in Jakarta. The sample of stool from taeniasis patients had been preserved in a 5% formalin-PBS solution. Stool samples were taken to the parasitology laboratory of Universitas Trisakti for examination of Taenia species eggs in all stool pots using the Kato-Katz technique until it reached a total of 40 pots positive for Taenia species eggs.

All samples were positive for Taenia species on examination with the Kato-Katz technique, then a new preparation was made from each pot for Ziehl Neelsen staining. Ten new slides were made for each pot, then stained with Ziehl Neelsen's stain. All slides were examined for differences in color and morphology of eggs under light microscopy using 100x and 400x magnifications.

B. Direct microscopic examination using the Kato-Katz method [17]

The tools and materials used are distilled water, glycerine, malachite green, object glass, cellophane tape 2.5 cm wide, 2 mm thick perforated, filter wire, 20 - 15 cc plastic pot, toothpick-size stick cardboard, oil paper, filter paper, waterproof marker, rubber bottle cap, metal scissors, small plastic washbasin, soap and detergent, washcloth, rubber gloves, 5 - 10% formaldehyde, and microscope.

Procedure: Wear gloves to reduce contamination. Then remove the stool from the refrigerator (temperature 4°C) and weigh it to determine the amount of stool contained in each bottle (40 bottles) of about 100 mg. Write the Code Number on the object glass with a marker as written on the stool bottle. Then use stick to collect stool approximately the size of green beans, and place it on the object glass. Then cover it with cellophane which has been soaked in Kato-Katz solution. After the stool has formed a smooth layer under the cellophane with a rubber bottle cap or glass slide. Let it sit for 20-30 minutes. Then perform an examination with a weak magnification of 100x (objective 10x and ocular 10x) and if necessary it can be increased by 400x (objective 40x and ocular 10x). Finally, determine the results of a positive or negative stool containing Taenia spp. eggs.

C. Ziehl Neelsen Staining [18]

The tools and materials used were stool containing Taenia eggs in pots; object glass; cover glass; wood clamp; Bunsen burner; disposal container; coloring shelf; immersion oil; xylol; microscope; and reagents consisted of 3% Carbol Fuchsin Solution; 70% Ethanol 1% HCL; 3% Methylene Blue Solution.

Procedure: Clean the object glass until there is no grease attached then write down the sample identification on the object glass using a permanent marker. Stool containing Taenia spp. eggs are collected using a stick. Then the stool sample is smeared on the object glass and the sample is flattened until it is even, making sure that the stool is not too thick or too thin and then dry it in air. Fixate the stool by passing the preparation over a flame three times.

Staining procedure: Put the preparation on the stain rack (so that the surface is even) then drip 3% carbol fuchsin dye to cover the stool smear, and let it sit for 15 minutes. Then the slide is heated until steam is formed (± 5 minutes) but do not let it boil or the slide will dry out. Then let it cool at room temperature. Then the dye is drained and then washed with running water until its clean. The remaining water is drained and then the slide is dripped with 70% Ethanol 1% HCL until no more dye has dissolved. Next, wash the slide with running water until clean and drain the water followed by dropping the stool with 3% methylene blue dye to cover the entire smear and let it stand for ± 10-20 seconds. Wash the slide with running water until clean, then drain the slide and let it dry at room temperature. Do not place the slide in direct sunlight. Subsequently, the slide was dripped with immersion oil and then observe the preparation under a microscope with objective of 10x ocular magnification. When finished, clean the objective lense with xylol.

III. RESULT AND DISCUSSION

Indonesia and most countries in Southeast Asia are endemic areas for the Taenia tapeworm that infects humans; Taenia solium, Taenia saginata, and Taenia asiatica. [19] Currently, an accurate and important diagnostic tool is needed for the identification of taeniasis to the species level. In addition, it can also be used to assess the infection intensity, anthelmintic drug efficacy, as well as monitoring and elimination of deworming drugs. [20,21]

The most common diagnostic approach for helminth infection in epidemiological studies is the copro-microscopic detection of worm eggs using the Kato-Katz technique. This method is recommended by the World Health Organization (WHO) due to its simplicity and relatively low cost, since most of the equipment is reusable. However, this technique has limitations in terms of sensitivity and nonspecific in identifying Taenia spp. eggs. [20-22]

Diagnosis of taeniasis is routinely carried out by direct examination of the eggs of Taenia spp. in stool samples, which have a low detection sensitivity. [23] This is because the eggs of Taenia solium and Taenia saginata have identical morphology and cannot be differentiated by microscopy. [4,24] The cattle tapeworm, Taenia saginata, does not cause human cysticercosis; thus, it can be distinguished with T. solium both epidemiologically and clinicaly. [25]

A. Identification of Taenia species by parasitological examination using the Kato-Katz

The Kato-Katz technique is a common method and widely used for diagnosing soil-transmitted helminth infections. [20-22] Multiple Kato-Katz examinations are usually performed to improve diagnostics. [20,26] In this study, the Kato-Katz Technique was examined twice by two different researchers to reduce the subjectivity factor. Forty pots of stool samples were examined using direct examination (microscope) for identifying Taenia spp. eggs.

All of the samples were made into slides, 10 slides respectively. From direct examination of 400 slides, 81 positive slides were found for Taenia spp. eggs and 319 slides representing other worms. The eggs of Taenia species in the Kato-Katz method appear to be round-oval with a size of about 35 μ, brownish yellow color, and the visible contents are hexacanth embryo covered with two layers of walls with a structure resembling a cart-wheel (Fig.1).

The examination results of this study found several worm eggs from Nematode and Cestode class that often infect humans. The intestinal nematode class eggs, which is a soil-transmitted helminth, were frequently found and the largest number of species was Trichuris trichiura, 286 eggs (28,6%), followed by 258 (25.8%) Ascaris lumbricoides and 104

(10.4%) Hookworms. Meanwhile, at least 46 eggs (4,6%) were found to be Hymenolepis diminuta from the Cestoda group. The results of the Kato-Katz method examination found that 244 eggs were positive for Taenia spp. (Table 1).

These results were similar to Qian et al., [26] that reported among 397 samples, 77 samples were positive for Clonorchis sinensis, 20 samples were positive for Trichuris trichiura and 3 samples were positive for Ascaris lumbricoides. Keller et al., [20] identified 1,020 samples that were positive for Trichuris trichiura and 167 samples positive for Ascaris lumbricoides out of a total of 1,636 samples.

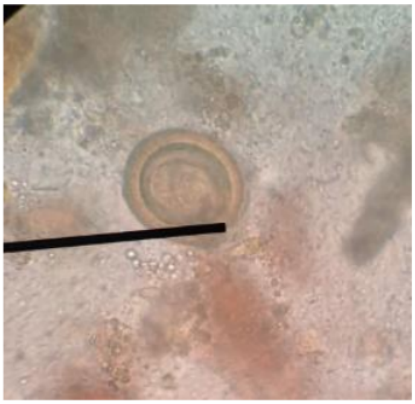

Fig. 1. Taenia species eggs in the Kato-Katz method

In line with Dunn et al., [27] the most prevalent soil-transmitted helminth was Trichuris trichiura (22,84%), followed by Necator americanus (22,69%) and Ascaris lumbricoides (8,80%). However, in contrast to Oishi et al., [28] Ascaris lumbricoides (2,4%, 13/549) was the most prevalent soil-transmitted helminth detected.

TABLE I. FREQUENCY OF WORM EGG SPECIES USING THE KATO-KATZ METHOD

Species	Quantity (n)	Percentage (%)
Trichuris trichiura	286	28.6
Ascaris lumbricoides	258	25.8
Hookworm	104	10.4
Taenia sp	244	24.4
Hymenolepis nana	62	6.2
Hymenolepis diminuta	46	4.6

B. Identification of Taenia species using Ziehl Neelsen staining

All positive slides for Taenia spp. eggs (81/400) were then examined with Ziehl Neelsen staining to distinguish between two species, Taenia solium and Taenia saginata. The samples were stained with 3% Carbol fuchsin for 15 minutes, then washed with tap water, and then the color was removed with ethanol 70% HCl 1% for 2 minutes. After washing the two slides were contrasted with 3% methylene blue for 5 minutes and washed again, then allowed to dry at room temperature. After Ziehl Neelsen staining, 51,85% (42/81) were positive for Taenia saginata, 35,80% (29/81) were positive for Taenia solium, while the remaining 12,35% (10/81) were unidentified (Table 2). This happened because the color was too dense so that the characteristics for species identification were not visible and doubtful.

TABLE II. FREQUENCY OF TAENIA SPECIES EEGS

ZIEHL NEELSEN	Taenia spp. eggs	
	Quantity (N)	*Percentage (%)*
Taenia solium	29	35.80
Taenia saginata	42	51.85
Unidentified	10	12.35
Total	81	100

Taenia saginata eggs in the Ziehl Neelsen method appear completely stained with magenta red, oval in shape, while Taenia solium eggs appear purplish blue in color and rounder in shape. The eggs size and shape of Taenia saginata were slightly larger and always round-oval, whereas Taenia solium eggs were smaller and mostly spherical in shape (Fig. 2).

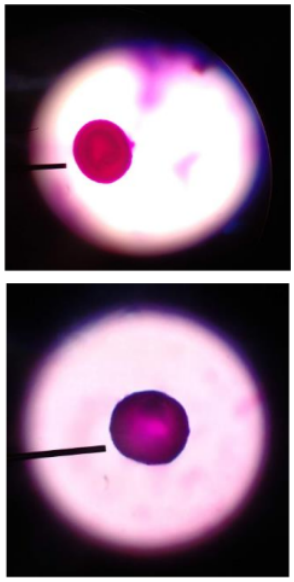

Fig. 2. Eggs of Taenia sp. in the Ziehl Neelsen method. (A) *Taenia saginata*; (B) *Taenia solium*

These results were in line with the study of Amer et al., [29] who used Ziehl Neelsen's stain for the first time to differentiate the egg morphology of Taenia saginata and Taenia solium. Jimenez et al., [30] also showed that Ziehl Neelsen's staining could fully differentiate mature Taenia solium eggs from Taenia saginata in cases where the egg wall was completely magenta in 7/13 Taenia saginata, or completely blue/purple at 4/18 Taenia solium. In this study, all eggs found by the Ziehl Neelsen technique did not show any hooks, whereas in the study of Clavel et al., [31] the hooks are colored irregularly and most of them showed only a slight pink hue. This is probably due to the heating of Carbol-fuchsin

and the length of time it had been stained so that it affected dye absorption.

C. *Sensitivity and Specificity Analysis of Taenia sp. examination between Kato-Katz Method and Ziehl Neelsen Method*

The egg detection data of Taenia species examinations using two different methods were further analyzed to determine the level of sensitivity and specificity between the Kato-Katz and Ziehl Neelsen methods. This study used a diagnostic test 2x2 table from the Cat Maker® Application (Table 3).

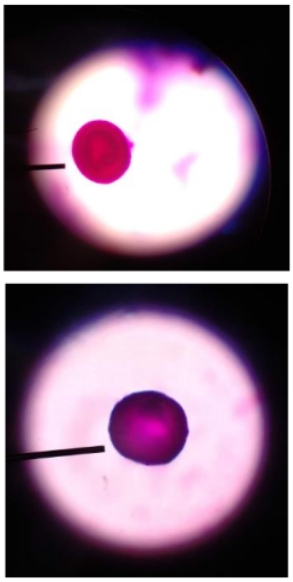

Fig. 3. Eggs of Taenia sp. in the Ziehl Neelsen method. (A) *Taenia saginata*; (B) *Taenia solium*

Sensitivity value shows the level of positivity of a method in measuring with certainty the observed variable. While the specificity value shows the level of negativity of a method in measuring with certainty the observed variable.

With the formula:

Sensitivity	= a: (a+c) = 88%
Confidence Interval 95%	= 80 – 95 %
Specificity	= d: (b+d) = 21%
Confidence Interval 95%	= 12 – 30 %
Positive Predicted Value	= a: (a+b) = 53%
Negative Predicted Value	= d: (c+d) = 63%

TABLE III. SENSITIVITY AND SPECIFICITY TEST

Ziehl Neelsen	**Kato-Katz Method**			
	Positif		***Negatif***	
	n	**%**	***n***	**%**
Positif	71	87.65	64	79.01
Negatif	10	12.35	17	20.92

The sensitivity value shows the level of positivity of a method in measuring with certainty the observed variables. In this study the Ziehl Neelsen method was used to identify Taenia spp. eggs. While, the specificity value shows the level of negativity of a method in identifying Taenia spp. eggs. Sensitivity and specificity values were calculated using the Cat Maker® statistical test.

The sensitivity test results of the Ziehl Neelsen method had a value of 88%, This showed the ability of the Ziehl Neelsen method to stay positive for all positive samples based on the results of Kato-Katz method in identifying Taenia spp. eggs. The specificity value is the ability of the Ziehl Neelsen method to read the negative results of Taenia spp. eggs by 63% against the Kato-Katz method which also was negative. The confidence interval for sensitivity is 80 – 95 %, which means that the confidence level of the detected positive identification remained within the range (95% statistical confidence interval), whereas for the confidence interval for specificity of 12 - 30%, Ziehl Neelsen's ability to stay negative in the slide was within the range (95% statistical confidence interval). The positive predictive value is 53%, meaning that if the Ziehl Neelsen method detects positivity, the Kato-Katz method will also detect 53% positivity, while the negative predictive value is 63%, meaning that if the Ziehl Neelsen method did not detect or had a negative result, the Kato-Katz method was also 63% detected as negative.

Several studies reported that the Kato-Katz technique sensitivity for soil-transmitted helminths was in the range of 40% to 65%. [22,26,32] Whereas a study by Levecke et al., [33] reported that the sensitivity of the Kato-Katz method (88,1%) was more sensitive to detect Ascaris lumbricoides infection, sensitivity for hookworm 78,3%, and Trichuris trichiura 82,6%. There are no studies that have clearly reported on the sensitivity and specificity of Ziehl Neelsen's staining. However, one study reported that 78 laboratories using Ziehl Neelsen stain to diagnose taeniasis, found 17 (22%) laboratories that could detect Taenia solium eggs and 50 (64%) laboratories that identified Taenia saginata taeniasis, the rest used Carmine staining and performed an ELISA method as well. [11]

CONCLUSION

This study concluded that Ziehl Neelsen's stain was more sensitive and specific than the Kato-Katz method, 88% and 21% respectively. The parasitological examination with Ziehl Neelsen staining can be used for the identification of Taenia species due to its simplicity and is relatively inexpensive. However, in-depth studies are needed to determine the sensitivity and specificity of Ziehl Neelsen staining compared to other techniques so that a definite diagnosis of taeniasis can be established.

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