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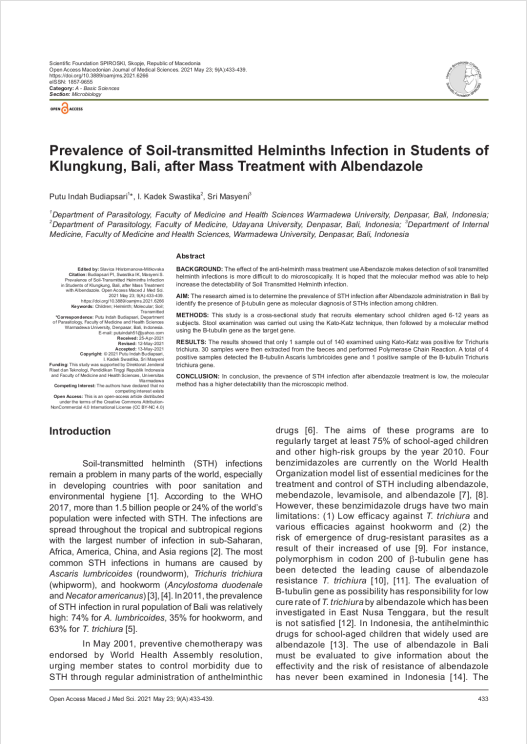

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Prevalence of Soil-transmitted Helminths Infection in Students of Klungkung, Bali, after Mass Treatment with Albendazole

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Abstract

BACKGROUND: The effect of the anti-helminth mass treatment use Albendazole makes detection of soil transmitted helminth infections is more difficult to do microscopically. It is hoped that the molecular method was able to help increase the detectability of Soil Transmitted Helminth infection.

AIM: The research aimed is to determine the prevalence of STH infection after Albendazole administration in Bali by identify the presence of β-tubulin gene as molecular diagnosis of STHs infection among children.

METHODS: This study is a cross-sectional study that recruits elementary school children aged 6-12 years as subjects. Stool examination was carried out using the Kato-Katz technique, then followed by a molecular method using the B-tubulin gene as the target gene.

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CONCLUSION: In conclusion, the prevalence of STH infection after albendazole treatment is low, the molecular method has a higher detectability than the microscopic method.

Introduction

Soil-transmitted helminth (STH) infections remain a problem in many parts of the world, especially in developing countries with poor sanitation and environmental hygiene [1]. According to the WHO 2017, more than 1.5 billion people or 24% of the world's population were infected with STH. The infections are spread throughout the tropical and subtropical regions with the largest number of infection in sub-Saharan, Africa, America, China, and Asia regions [2]. The most common STH infections in humans are caused by *Ascaris lumbricoides* (roundworm), *Trichuris trichiura* (whipworm), and hookworm (*Ancylostoma duodenale* and *Necator americanus*) [3], [4]. In 2011, the prevalence of STH infection in rural population of Bali was relatively high: 74% for *A. lumbricoides*, 35% for hookworm, and 63% for *T. trichiura* [5].

In May 2001, preventive chemotherapy was endorsed by World Health Assembly resolution, urging member states to control morbidity due to STH through regular administration of anthelminthic drugs [6]. The aims of these programs are to regularly target at least 75% of school-aged children and other high-risk groups by the year 2010. Four benzimidazoles are currently on the World Health Organization model list of essential medicines for the treatment and control of STH including albendazole, mebendazole, levamisole, and albendazole [7], [8]. However, these benzimidazole drugs have two main limitations: (1) Low efficacy against *T. trichiura* and various efficacies against hookworm and (2) the risk of emergence of drug-resistant parasites as a result of their increased of use [9]. For instance, polymorphism in codon 200 of β-tubulin gene has been detected the leading cause of albendazole resistance *T. trichiura* [10], [11]. The evaluation of B-tubulin gene as possibility has responsibility for low cure rate of *T. trichiura* by albendazole which has been investigated in East Nusa Tenggara, but the result is not satisfied [12]. In Indonesia, the antihelminthic drugs for school-aged children that widely used are albendazole [13]. The use of albendazole in Bali must be evaluated to give information about the effectivity and the risk of resistance of albendazole has never been examined in Indonesia [14]. The

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research aimed is to determine the prevalence of STH infection after albendazole administration in Bali, by identify the presence of β-tubulin gene as molecular diagnosis of STHs infection among children who treated by albendazole.

Methods

Study area

The research was conducted in Gelgel village, Klungkung Regency in the period of May–July 2020 (Figure 1).

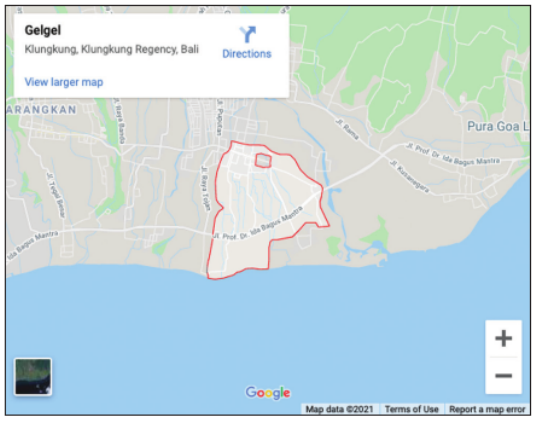

Figure 1: Location of Gelgel village, Klungkung, Bali (Google Map, 2021)

Procedure

This study was community based, subjects were recruited from some elementary school in Klungkung, Bali, demographic data were collected by questionnaire, stool samples were collected for further STHs species. First- to sixth-degree pupils who meet the criteria were offered an inform consent to participate in this study. In this study, 250 school-aged children from elementary school in Gelgel village, Klungkung Regency, Bali, were recruited to get a representative of STH infection.

This type of research is a cross-sectional analytic study. Elementary school students who have been given a single dose of albendazole 400 mg by the Puskesmas (local health center) were examined for stool 21 days after administration of the drug using the Kato-Katz method [15]. The cure rate was assessed by the percentage of children who were positively infected divided by the total number of children given therapy. Infections were classified by worm species. The research was conducted in Gelgel village, Klungkung Regency in the period of May–July 2020. Worm eggs were examined using the Kato-Katz quantitative method. The total number of worm eggs is the number of worm eggs obtained multiplied by 24 equal to the number of worm eggs in 1 g of stool [16]. All demographic data were translated into frequency tables and presentations.

Inclusion and exclusion criteria

The sample of this study was elementary school students which Grades I–VI, who had no previous disease history. Elementary students who refused to take the worm medicine that were gave, who did not take a stool examination on day 21, and those who refused to participate in this study were excluded from the study. The inclusion criteria are subject who positive for STH infection based on fecal examination with albendazole as the treatment. Subjects who taken other drugs besides albendazole were excluded from this research.

Data collection

Parents of subjects that met the above criteria were gave an informed consent form subsequently. On consent, a demographic, medical, nutrition status, and relevant clinical information was obtained from the participant. Single dose of albendazole was given to all students. Stool container was given and the sample collected 21 days later, stool samples from the subject were collected to assess the effectivity of this drug, to assess the STH species, and to detect the B-tubulin gene [17].

Parasite identification

Stool samples were collected by researcher and laboratory assistant, then filled by ethanol 85%. Specimens were transported in a sample box to the laboratory. The fecal sample was examined using Kato-Katz technique to diagnose and to count egg per gram of feces. The intensity of infection was determined by multiplicate egg count 24 times, based on the WHO standard procedure. Furthermore, the subject who suffers persistence infection after treatment of albendazole was confirmed by polymerase chain reaction (PCR) under PCR condition as prescribed on the previous study to detect the β-tubulin gene [18]. To classify infection intensity, we used the World Health Organization cutoffs (light intensity infections were defined as <5000 egg per gram (epg) for *A. lumbricoides*, <1000 epg for *T. trichiura*, and <2000 epg for hookworm; moderate-intensity infections were defined as 5000 < epg < 50,000 for *A. lumbricoides*, 1000 < epg < 10,000 for *T. trichiura*, and 2000 < epg < 4000 for hookworm; and heavy intensity infections were defined as > 50,000 epg for *A. lumbricoides*, > 10,000 epg for *T. trichiura*, and > 4000 epg for hookworm [19].

Isolation of DNA stool

Fecal sample which preserves by ethanol 85% then placed in freezer −-80°C. Extracted genomic DNA from fecal samples was used to amplify a 472 bp fragment of *T. trichiura* β-tubulin isotype 3 gene, including codon positions 167, 198, and 200. Genomic DNA extracted from *A. lumbricoides* adult worms was used to amplify a 564 bp fragment of *Ascaris* β-tubulin isotype 1 gene, including codon positions 167, 198, and 200. Before the isolation, 180–220 mg of stool was weighed and placed in a tube on ice. The procedure of DNA isolation was performed according to the Qiagen QIAamp Fast DNA Stool Mini Kit. A total of 1 ml InhibitEX buffer were added to the samples then vortexed until thoroughly homogenized. The samples were then incubated at 95°C for 5 min and followed by vortexed 15 s. Next the samples were centrifuge for 1 min. A total of 15 ul proteinase K were added into a new 1.5 ml microcentrifuge and 200 ul of supernatant was added followed by 200 ul buffer AL and vortexed for 15 s. Next, the samples were incubated at 70°C for 10 min. Then, 200 ul ethanol (96–100%) was added to the lysate and mixed by vortex. A total of 600 ul lysate were placed in a QIAamp spin column and were centrifuged with a speed of 14.000 rpm for 1 min. The filtrate was then discarded, and 500 µl of AW1 buffer was added. Then the QIAamp spin columns were centrifuged with a speed of 14.000 rpm for 1 min. The filtrate was then discarded, and 500 µl of AW2 buffer was added. Next, the QIAamp spin columns were centrifuge with a speed of 14.000 rpm for 3 min. The QIAamp spin columns were placed into a new microcentrifuge tube and 200 ul buffer ATE was added. The microcentrifuge tubes were incubated at room temperature for 1 min and were centrifuged with a speed of 14.000 rpm for 1 min. The DNA samples were stored at −20°C before use [20], [21].

PCR and gel electrophoresis

In this study, the primer A. lumbricoides 5'-AGAGCCACAGTTGGTTTAGATACG-3' (Forward); 5'-AGGGTCCTGAAGCAGATGTC-3'(reverse) and for T. trichiura, the primer 5'-GAGTAACGACATGCCTACGC-3' (Forward); 5'-TTGCGTCGAACATTTGCTGA-3' (reverse). PCR protocol for each species a total of 12,5 µl GoTaq Green Master Mix, 2x (Promega, Madison, WI, USA) was placed in a PCR tube and was added with 1 µl forward primer, 1 µl reverse primer, 9,5 µl nuclease-free water, and 1 µl samples. The tube was placed in PCR machine. PCR was conducted at an initial denaturation temperature of 94°C for 3 min, followed by denaturation temperature of 94°C for 45 s, annealing temperature of 54°C for 45 s, extension temperature of 68°C for 1 min (denaturation temperature to extension temperature was then repeated 35 times), and a final extension temperature of 68°C for 10 min. The PCR results were visualized using electrophoresis [23].

The PCR results were visualized using electrophoresis. A total 2.5 of 100 bp DNA Ladder (Jena Bioscience) and each PCR product were added 5 ul to the wells with 1.5% agarose gel. The electrophoresis machine was run with 200 mA, 150 V for 30 min. The results were read in the Gel Documentation Machine [24].

Ethical considerations

The study, the collection of clinical and epidemiological data was submitted for ethical approval. All steps on this research ruled according to the permission of the Ethics Committee of Udayana University/Sanglah Central Hospital Denpasar, Bali, ethical clearance number 789/UN14.2.2.VII.14/LT/2020. Enrolment of the study participants is conditional on appropriate consent. Written consent for children/minors was sought from the parents or legal guardian. Deidentified biological material collected in this study was maintained under strict quality-controlled conditions in locked freezers. All biological materials were processed and stored within the territory of Indonesia.

Data analysis

Statistical analyses performed using SPSS 18. All demographic data were translated into frequency tables following by descriptive explanation. Molecular identification was described by figure and descriptive narrations.

Results and Discussion

Characteristics of research subjects

A total of 250 research subjects consisted of elementary school students and toddlers were recruited as samples, as many as 140 subjects collected stool samples and completed questionnaires with the consent of their parents. Stool samples were then examined in the laboratory by the Kato-Katz method and found only one positive sample that was infected with *T. trichiura*, the remaining 139 were negative for STH infections. Most of the research subjects were male 75 (53.6%), judging by the family income, most of them earned less than 250 thousand with the main occupation of parents mostly in the private sector and farming, considering that farmers are a profession with uncertain income that is dependent to yield. The majority of parents were high school graduates, but there were some who only went to elementary school, and some did not even receive any formal schooling. With the 9-year compulsory school program that is free of charge by the government, most of the population can at least finish up to junior high. Data on the characteristics of research subjects are described in Table 1.

The observed prevalence was higher for qPCR than Kato-Katz for *A. lumbricoides* and *T. trichiura* but

Table 1: Characteristics of research subjects

Characteristics	N (%)
Infection status	
None	139 (99.3)
Yes	1 (0.7)
Gender	
Male	75 (53.6)
Female	65 (46.4)
Family income	
<250 k IDR	54 (38.6)
250–500 k IDR	33 (23.6)
>1 k IDR	53 (37.9)
Parent's level of education	
Bachelor	13 (9.3)
Elementary school	10 (7.1)
Senior high school	97 (69.3)
Junior high school	17 (12.1)
No school	3 (2.1)
Parent's profession	
Labor	2 (1.4)
Farmer	47 (33.6)
Government employees	5 (3.6)
Private business	86 (61.4)

no difference for hookworm (Table 2). The prevalence of *A. lumbricoides* was 0% using Kato-Katz and 13.3% using PCR. The prevalence of hookworm was 0% using Kato-Katz and PCR. For *T. trichiura*, the observed prevalence was 0.007% by Kato-Katz and 3.33% by PCR. Using Kato-Katz, one of children was infected with *T. trichiura*, with 24 epg that was categorized in light intensity infection.

Table 2: Comparison of STH prevalence, Kato-Katz, and PCR

Species	Kato-Katz			PCR	
	Number of positive samples	Prevalence (%)	EPG	Number of positive samples	Prevalence (%)
Ascaris lumbricoides	0	0	0	4	13.33
Hookworm	0	0	0	0	0
Trichuris trichiura	1	0.007	24	1	3.33

EPG: Egg per gram, STH: Soil-transmitted helminth, PCR: Polymerase chain reaction.

Molecular diagnosis

We analyzed 30 fecal samples from 30 patients collected in two elementary schools in Gelgel village. *A. lumbricoides* infection was found in four samples, than *T. trichiura* was found in one sample (Figures 2-5).

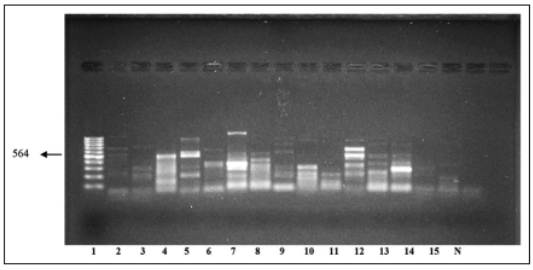

Figure 2: Amplification of A. lumbricoides b-tubulin gene of 564 bp fragment was detected in sample number 3, 4, and 11

Discussion

In this study, the prevalence of STH infections was very low that confirmed by Kato-Katz and PCR method, the result of this study was supported by research conducted in Malaysia by Sinniah *et al*., 2014, which stated that there was a dramatic reduction in STH infections due to the success of the antihelminthic mass drug administration (MDA) program [20]. Matamoros *et al*., 2019, also stated that regular treatment with antihelminthics has become one of the most popular and successful public health interventions that occur in STH endemic countries [13]. In this study, it was also found that the clean and healthy living behavior of the subjects' parents was still lacking, even though that did not necessarily affect the possibility of an increased risk of STH infection [3], [4]. This result is supported by Abraham *et al*., 2018, which stated that to reduce STH reinfection, including water and sanitation interventions. However, environmental interventions require ongoing efforts from governments or other institutions to build infrastructure and promote healthy behavior modification, and their effectiveness is often limited by deeply rooted cultural norms and behavior [1].

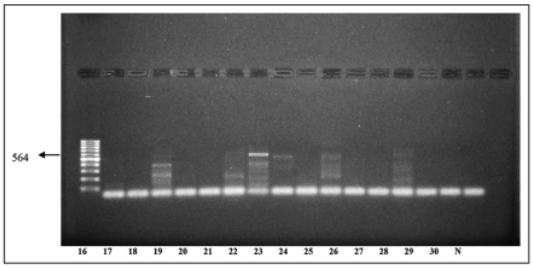

Figure 3: Amplification of A. lumbricoides b-tubulin gene of 564 bp fragment was detected in sample number 22

This study found 1 (0.007%) child who was infected with *T. trichiura*, although the cure rate is high for *A. lumbricoides* and hookworm infections, but it seems that there are still problems that require special attention for *T. trichiura* infection. This result is supported by research conducted by Moser *et al*., 2018, which stated that albendazole shows good effectiveness against hookworm infections, albendazole, mebendazole, levamisole, and pyrantel pamoate have low effectiveness against *T. trichiura*. Moreover, Moser's study *et al*, 2015, which stated that both albendazole and mebendazole only have low-to-moderate effectiveness in *T. trichiura,* especially in endemic areas [10]. Different results came from the WHO statement, 2015, which stated that albendazole chewable tablets are effective, inexpensive and could be easily provided by non-medical personnel, such as teachers. In this case, the WHO announced that medical staff can also give albendazole tablets to students and the administration is safe. Of course, the potential reinfection of *T. trichiura* after drug administration cannot be ignored in this case, similar to the study of Jia *et al*., 2012, STH reinfection occurs quickly after treatment, especially for *A. lumbricoides* and *T. trichiura*. Therefore, it is necessary to administer anthelmintic drugs as often as possible to maximize the

benefits of preventive chemotherapy. A comprehensive control approach consisting of health education and environmental sanitation is needed to stop the transmission of STH [11].

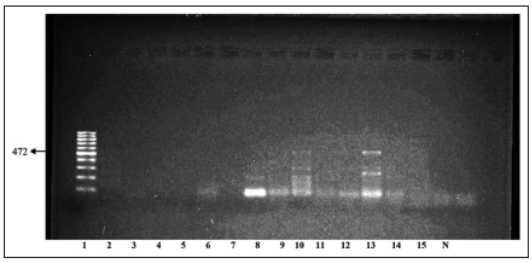

Figure 4: Amplification of Trichuris trichiura B-tubulin gene of 472 bp fragment was detected in sample number 12

The massive use of antihelminthic drugs in the past 50 years ago is also a special concern, this was also stated by Welch *et al*., 2010, which stated the possibility of imminent drug resistance, a threat recognized since the beginning of the era of modern chemotherapy. Therefore, there is an urgent need for joint efforts to find and develop the next generation of anthelminthic drugs. Oral single-dose albendazole, mebendazole, and pyrantel pamoate showed a high cure rate for *A. lumbricoides* [25]. For hookworm infections, albendazole was more effective than mebendazole and pyrantel pamoate. Treatment of *T. trichiura* with a single oral dose of anthelmintic is currently unsatisfactory[24]. Research conducted by Speich *et al*., 2012 on other antihelmintic drugs stated that nitazoxanide has no effect on *T. trichiura* infection. The low efficacy of albendazole against *T. trichiura* in the current worm medicine program encourages researchers to develop new anthelmintic drugs to treat trichuriasis [23].

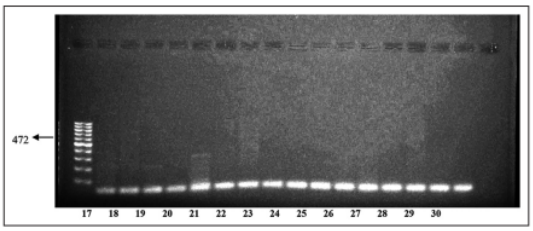

Figure 5: Amplification of Trichuris trichiura B-tubulin gene of 472 bp fragment was not detected in sample

We get one positive case of *T. trichiura* with low infection intensity from a total sample of 130 elementary schoolchildren in Gelgel village, Klungkung Regency. MDA programs have resulted in large reductions in the prevalence and intensity of STH infections to date. As the intensity and prevalence of STH infections decrease, increasingly sensitive diagnostic methods are needed to determine whether STH transmission has been interrupted and when interventions can be reduced or discontinued [27].

The use of molecular methods is increasing in cases with low or undetectable infection intensity by microscopic examination [17]. We tested the b-tubulin gene on a sample of primary schoolchildren receiving albendazole therapy every 6 months. We successfully amplified the b-tubulin gene from *A. lumbricoides* and *T. trichiura*. The b-tubulin gene *A. lumbricoides* was detected in four samples, while *T. trichiura* b-tubulin gene was detected in only one sample. This could be due to the fact that the amount of amplified *Ascaris* DNA was directly proportional to the number of *Ascaris* eggs which were more than other species, making it relatively easier to detect than *T. trichiura* eggs. In addition, it can also be caused by several factors, the outer layer of the egg is more easily denatured in *A. lumbricoides* than *T. trichiura*. A positive correlation also found between the amount of DNA and the number of worms removed from the body after treatment using albendazole [19].

We successfully detected *T. trichiura* B-tubulin gene in one sample. In contrast to *T. trichiura*, compared to other STH, this species is more difficult to extract its DNA from eggs so that a more specific homogenization technique is needed for the PCR method in this species [18]. Molecular-based diagnostic methods, especially qPCR, proved to be more sensitive to detect STH infection even in subjects who had received therapy, and microscopically, no eggs were detected in feces. Even if it is compared to the microscopic PCR method, the cost is higher [7]. In terms of the method of extracting DNA from worm eggs, it does require more effort than extracting DNA from viruses or bacteria, this includes the freeze-thaw cycle and repeated heating [14], [15]. The advantages of using the PCR method are increased sensitivity and specificity compared to the Kato-Katz method [5], Species and strain level identification of parasites is possible, molecular epidemiology to monitor transmission patterns. While the limitation of PCR method is required well-equipped laboratory infrastructure and well-trained personnel, more expensive compared to the Kato-Katz technique, contamination can lead to false positive [13].

Conclusion

Albendazole is still effective against STH infection among school-aged children. The greater detectability and precision of PCR relative to Kato-Katz make it an attractive diagnostic for monitoring the successes of STH control programs deploying mass deworming since the infection intensity tends to decline after several years of intervention.

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