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▣ ORIGINAL ARTICLE

Prevalence of Soil-Transmitted Helminths and Molecular Clarification of Hookworm Species in Ethnic Ede Primary Schoolchildren in Dak Lak Province, Southern Vietnam

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Abstract: To know the infection status of helminths in primary schoolchildren of southern parts of Vietnam, we performed an epidemiological study in Krong Pac district, Dak Lak Province, Vietnam. A total of 1,206 stool specimens were collected from ethnic Ede schoolchildren in 4 primary schools in 2015 and examined by the Kato-Katz technique. In addition, stool cultures were done by the Harada-Mori method to obtain hookworm larvae and then to clarify the species of hookworms infected. The results showed that the helminth infection rate was 25.0%, including 2.0% *Ascaris lumbricoides*, 0.33% *Trichuris trichiura*, and 22.8% hookworm infections. The average intensity of infection was 102.0 eggs per gram of feces (EPG) for *Ascaris*, 36.0 EPG for *Trichuris*, and 218.0 EPG for hookworms. ITS1 gene sequences of the hookworm larvae were identical with those of *Necator americanus* (100% homology) reported in GenBank. It has been confirmed in this study that the hookworm, *N. americanus*, is a dominant helminth species infected in primary schoolchildren of a southern part of Vietnam. Public health attention is needed for control of hookworm infections among schoolchildren in surveyed areas of Vietnam.

Key words: *Ascaris lumbricoides*, *Trichuris trichiura*, hookworm, soil-transmitted helminth, prevalence, intensity, schoolchildren, Vietnam

INTRODUCTION

It is estimated that 576-740 million individuals are infected with hookworms worldwide [1,2]. Of the infected individuals, about 80 million are severely affected [2]. The major etiology of hookworm infections is *Necator americanus* which is found in the Americas, sub-Saharan Africa, and Asia [3]. *Ancylostoma duodenale* is found in more scattered focal environments, namely Europe and the Mediterranean [3]. Most infected individuals are concentrated in sub-Saharan Africa, East Asia, and the Pacific Islands with each region having estimates of 198 million and 149 million infected individuals, respectively. Other affected regions include South Asia (59 million), Latin America and the Carribeans (50 million), and Middle East/North Africa (10 million) [1]. In the United States, 95% of human hookworm cases are caused by *N. americanus*, primarily in young schoolchildren in economically deprived rural areas. Juvenile hookworms cannot survive freezing temperatures, so the highest prevalence occurs in areas with warmer temperatures and greater rainfall [4,5]. The greatest incidence of infections occurs in Asia and sub-Saharan Africa, especially in poverty-stricken areas with poor sanitation [3]. *A. duodenale* infections occur at a lesser rate and are seen primarily in Europe and the Mediterranean.

In Vietnam, several studies have been reported regarding the prevalence of hookworm infections [6-10]. In 2006, 65 million individuals were reported to be infected with helminths, including *Ascaris lumbricoides*, *Trichuris trichiura*, and hookworms. Hookworms are distributed in the whole country, and the prevalence was 68% in the south and 85% in the north [6]. In the northern part, 95-98% of human hookworm cases were caused by *N. americanus* [7], whereas in the south there were no reports on this information. In Nghe An Province (in the north), the helminthic infection was 98%, which included *Ascaris* infection 83.6%, *Trichuris* infection 85.1%, and hookworm infection 30.3% [8]. Identification of species for *A. duo-*

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denale or *N. americanus* by morphology of adult worms is easy, but using eggs is difficult; however, one can use the third-stage filariform larvae for identification [4,5].

The present study was undertaken to determine the prevalence of soil-transmitted helminths, including *Ascaris*, *Trichuris*, and hookworms, among schoolchildren in 4 primary schools in an ethnic community Ede in Krong Pac district, Dak Lak Province, South Vietnam, using the Kato-Katz technique and stool cultures by the Harada-Mori method. In addition, the species of hookworms prevalent in this area was determined using a molecular analysis of the filariform larvae.

MATERIALS AND METHODS

Stool examination and stool culture

Stool examinations were performed on 1,206 schoolchildren using the Kato-Katz technique. In this technique, the smears were examined in a systematic manner, and the eggs of each species were reported to give the number of the eggs per gram of feces (EPG). Stool cultures were performed by the Harada-Mori method for 100 positive samples with hookworms, with the highest infection (incubating fecal material on a filter paper strip in a test tube containing water for the purpose of culturing and recovering hookworm larvae) for identification of the hookworm species (after 10 days from culture).

Morphological identification of hookworm larvae

Identification of hookworm species was done by the morphology of filariform larvae according to the key of Sasa et al. [11] reported in 1958 (Table 1).

Identification of hookworm larvae by molecular method

Species identification of hookworm larvae was also done by PCR using the gene ITS1 (internal transcribed spacer 1) of rDNA and comparison with these genes reported in GenBank. For DNA isolation, Qiagen kits (DNeasy Blood & Tissue Kit, Qiagen Sciences, Germantown, Maryland, USA) were used for extraction of the total DNA from the hookworm *N. americanus* in accordance with the manufacturer's protocol. In brief, worm homogenates were resuspended in 100 µl of manufacturer's lysis buffer containing >8 mM EDTA, >0.5% SDS, and 20 µl proteinase K, which was then incubated at 56℃ for 30 min. Thereafter, 4 µl RNase and 200 µl lysis buffer were added and treated in accordance with the manufacturer's protocol (for a microfuge scale preparation).

Table 1. Identification key[a] of hookworm species by the morphology of filariform larvae

Organ	*Ancylostoma duodenale*	*Necator americanus*
Mouth	Dim and thin	Dark and exclamation mark
Tail	Straight tail	Bent tail

[a]Sasa et al. [11].

PCR amplification of ITS1

PCR for amplification of the 250 bp fragment of the ITS1 of *Necator* was performed in a 50 µl volume. PCR reactions were performed in 10 mM Tris-HCl (pH 8.4), 50 mM KCl, 3.0 mM $MgCl_2$, 250 µM each of dATP, dCTP, dGTP, and dTTP, and 50 pmol of each primer with 1 U Taq polymerase (Promega, Madison, Wisconsin, USA). The following primers were used in separate reaction mixes: NC1 (5′-ACGTCTGGTTCAGGGTTGTT-3′) and NC2 (5′-TTAGTTTCTTTTCCTCCGCT-3′); OB (5′-TATATTGCAACAGGTATTTTGGTAC- 3′) and NC2; NA (5′-ATGTGCACGTTATTCACT-3′) and NC2 [13]. Amplification was first conducted for 25 cycles using the primer set NC1-NC2. Then, 2 µl of each NC1-NC2 amplicon was transferred to a fresh tube containing the same PCR reaction buffer with the primer set OB-NC2 and another with the primer set NA-NC2, and amplified for another 35 cycles. Cycling was performed in a Genius Thermal Cycler (Techne, Cambridge, UK) using the following parameters: initial denaturation at 94℃ for 5 min, followed by 25 cycles (35 cycles in the second PCR) of 94℃ for 30 sec (denaturation), 55℃ for 30 sec (annealing), and 72℃ for 30 sec (extension), followed by a final extension at 72℃ for 5 min.

Sanger sequencing

Dideoxy sequencing was performed using BigDye™ Terminator Chemistry v. 3.1 (Applied Biosystems, Foster City, California, USA) according to the manufacturer's instructions, as described previously. Forward and reverse primers for *N. americanus* were used as sequencing primers (section 2.3) using the ABI 3130 Bioanalyzer (Applied Biosystems).

Phylogenetic analysis

Multiple sequence alignments were performed by using the ATGC software version 7.0.2 and the Clustal W program to determine the nucleotide and amino acid sequence similarities. Phylogenetic trees were constructed in MEGA 6.06 using the neighbor-joining (NJ) cluster algorithm with evolutionary distances estimated using the Kimura 2-parameter model; boot-

Table 2. Helminthic infections in schoolchildren of 4 primary schools

School code	No. exam.	Helminthic infection		*Ascaris*		*Trichuris*		Hookworms	
		(+)	%	(+)	%	(+)	%	(+)	%
1	316	113	35.8	10	3.2	1	0.32	103	32.6
2	298	97	32.6	7	2.4	1	0.34	89	29.9
3	301	54	17.9	2	0.66	1	0.33	52	17.3
4	291	37	12.7	5	1.7	1	0.34	31	10.7
Total	1,206	301	25.0	24	2.0	4	0.33	275	22.8

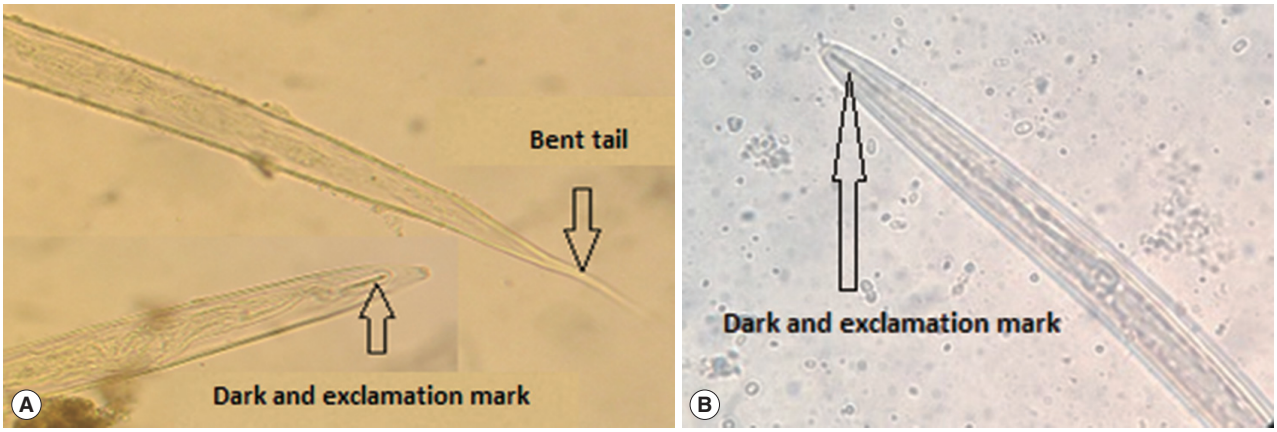

Fig. 1. Eggs (A: before culture; B: egg after hatching) and larvae of *Necator americanus*.

strapping was performed using 1,000 pseudo-replicates.

RESULTS

Prevalence of helminths

Out of 1,206 stool samples collected from schoolchildren and examined by the Kato-Kaz technique, the overall prevalence of helminth eggs was 25.0% (12.7-35.8% by school), including 22.8% hookworm infections (10.7-32.6%), 2.0% *Ascaris* infections (0.66-3.2%), and 0.33% *Trichuris* infections (0.32-0.34%) (Table 1). By gender, the prevalence of helminthic infections was not different between boys (27.1%) and girls (22.8%) with $P > 0.05$. The average intensity of infection was relatively light; 218.0 EPG for hookworms (122.3-261.9 EPG by school), 102.0 EPG for *Ascaris* (24.0-172.8 EPG), and 36.0 EPG for *Trichuris* (24.0-48.0 EPG) (Table 2).

Morphological characteristics of hookworm larvae

Of 100 stool samples, which were cultured by the Harada-Mori method, at 10 days after culture, a total of 1,250 filariform hookworm larvae were collected. All (100%) of the larvae were identified as those of *N. americanus* (Fig. 1). Their morphological characters included their body length of 520-580 μm (n = 10) not including the sheath, the presence of a dark exclamation mark around the mouth part, short and bent tail (50-70 μm), and conspicuous transverse striations on the sheath of the tail region.

Table 3. Intensity of helminth infections in schoolchildren of 4 primary schools

School code	*Ascaris*		*Trichuris*		Hookworms	
	No. (+)	EPG	No. (+)	EPG	No. (+)	EPG
1	10	117.6	1	48.0	103	261.9
2	7	51.4	1	24.0	89	251.9
3	2	24.0	1	24.0	52	130.2
4	5	172.8	1	48.0	31	122.3
Total	24	102.0	4	36.0	275	218.0

Molecular identification of hookworm larvae

A portion of ITS1 of Vietnamese *Necator* was sequenced and compared with different *N. americanus* species from GenBank (Table 3). The results showed a comparison of 185 nucleotides of ITS1 genome between Vietnamese *Necator* (Veca-VN) and other *N. americanus* species, including Lao *N. americanus* (Neca1), Japanese *N. americanus* (Neca2), unknown *N. ameri-*

Table 4. Sequencing of the portion of internal transcribed spacer 1 (ITS1) of different *Necator americanus* isolates from GenBank compared with *N. americanus* from Vietnam[a]

Notation	Origin	Host	Length	Species	GeneBank	Author
Neca-VN	VietNam	Human	185 bp	*Necator americanus**	-	This study
Neca 1	Lao	Human	185 bp	*Necator americanus*	LC036565.1	Hasegawa et al,. (2015)
Neca 2	Japan	Human	185 bp	*Necator americanus*	LC036563.1	Hasegawa et al,. (2015)
Neca 3	-	Gorilla	185 bp	*Necator americanus*	JX159772.1	Hamad et al,. (2014)
Neca 4	Malaysia	Human	185 bp	*Necator americanus*	JF960373.1	Ngui et al,. (2012)
Neca 5	Malaysia	Human	185 bp	*Necator americanus*	JF960401.1	Ngui et al,. (2012)
Neca 6	Malaysia	Human	185 bp	*Necator americanus*	JF960388.1	Ngui et al,. (2012)
Neca 7	Malaysia	Human	185 bp	*Necator americanus*	JF960397.1	Ngui et al,. (2012)

[a]Results of this study.

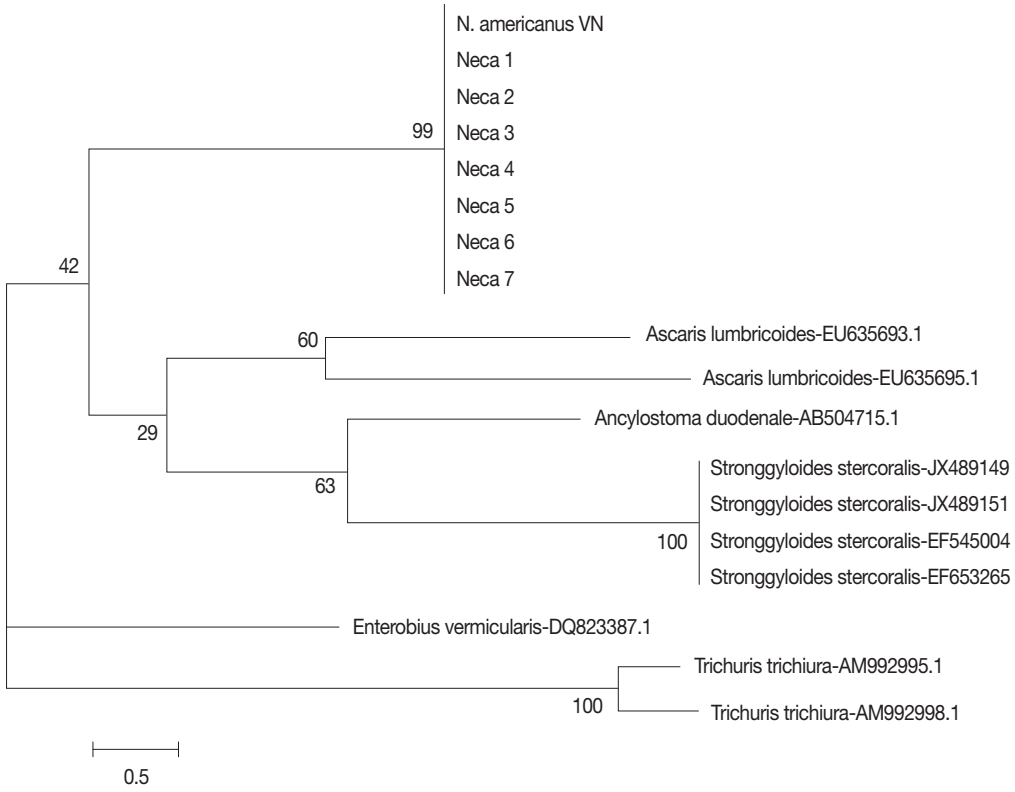

Fig. 2. Phylogenetic tree of *Necator americanus* Vietnam and other strains from part of ITS1 nucleotide sequence estimated by Neighbor-Joining (NJ) using MEGA 6.06 [12]. Note: *N. americanus*-VN=Vietnamese *Necator*; other *N. americanus* was from GenBank (no. LC036565.1, LC036563.1, JF960397.1, JF960401.1, JF960373.1, JF960388.1, and JX159772.1, respectively); *Ascaris lumbricoides* (EU635693.1 and EU635695.1); *Ancylostoma duodenale* (AB504715.1); *Strongyloides stercoralis* (JX489149, JX489151, EF545004, and EF653265); *Enterobius vermicularis* (DQ823387.1); *Trichuris trichiura* (AM992995.1 and AM992998.1).

canus (Neca3), and Malaysian *N. americanus* (Neca4, Neca5, Neca6, and Neca7) (Table 4). There were no differences between Vietnamese *Necator* (Veca-VN) and all other species; the homology (similarity) in nucleotides was 100% (Table 5).

The phylogenetic tree of *N. americanus* Vietnam and other geographical strains derived from partial ITS1 nucleotide sequences estimated by NJ method using MEGA 6.06 [12] showed that the Vietnamese strain of *N. americanus*, together with other geographical strains compared, is one group (Fig. 2).

DISCUSSION

In the present study, the overall prevalence of helminths was 25.0%, including 2.0% *Ascaris*, 0.33% *Trichuris*, and 22.8%

Table 5. Percent identity of *ITS1* nucleotide sequences of Vietnamese *N. americanus* and other *N. americanus* isolates in GenBank

	Neca-VN	Neca1	Neca2	Neca3	Neca4	Neca5	Neca6	Neca7
Neca-VN		100	100	100	100	100	100	100
Neca 1	100		100	100	100	100	100	100
Neca 2	100	100		100	100	100	100	100
Neca 3	100	100	100		100	100	100	100
Neca 4	100	100	100	100		100	100	100
Neca 5	100	100	100	100	100		100	100
Neca 6	100	100	100	100	100	100		100
Neca 7	100	100	100	100	100	100	100	

Veca-VN is Vietnamese *Necator*; Neca1 is Lao *Necator americanus* (GenBank no. LC036565.1), Neca2 is Japanese *N. americanus* (LC036563.1), Neca3 is unknown origin *Necator* (JX159772.1), and Neca4, Neca5, Neca6, and Neca7 are Malaysian *N. americanus* (JF960373.1, JF960401.1, JF960388.1, and JF960397.1, respectively).

hookworms. In comparison with our study, the results of other authors in north Vietnam showed that the prevalence was higher than in our study. For example, in Thai Binh Province (in the north), a study in 1996 targeting a primary schoolchildren revealed that the overall helminthic infection was 74.3%, including 61.0% *Ascaris* infection, 45.9% *Trichuris* infection, and 1.6% hookworm infection [10]. Another study in Nghe An Province (in the north) in 2005 showed that the prevalence of overall helminths was 98.0%, including 83.6% *Ascaris*, 85.1% *Trichuris*, and 30.3% hookworms [6]. It is difficult to explain the reason why the prevalence of *Ascaris* and *Trichuris* was lower in this study; a speculation may include that the soil nature in the subjected area (i.e., sandy soil) may be different from that in previous studies (i.e., clay soil).

In Vietnam, hookworm infections in humans included *A. duodenale* and *N. americanus*, but changed according to the lapse of time. For example, in 1960, hookworm infections included 36.1% *A. duodenale* and 54.9% *N. americanus*, and 9.0% mixed-infection with both species [14]. Similarly, in 1968-1970, hookworm infections included 24.3-34.4% *A. duodenale* and 65.6-75.7% *N. americanus* [15]. However, in 1981, hookworm infections contained only 3% *A. duodenale* and the majority (97%) was *N. americanus* [16]. Similarly, in 1995, hookworm infections included 3.1% *A. duodenale* and 96.9% *N. americanus* [6]. Since that time up to now, no reports have shown such information in Vietnam. This present study showed that all 1,250 cultured hookworm larvae obtained from 100 infected schoolchildren were identified as 100% *N. americanus*. Comparatively, in Bangladesh, Shahid et al. [9] reported that *A. duodenale* was 11.5% and *N. americanus* was 88.5% in 2010. In the USA, 95% of human hookworm cases in 2005 were *N. americanus* infection [3]. No much information is available from other countries.

The results of the present study may indicate possible disappearance of *A. duodenale* from north Vietnam. The reason is difficult to explain; however, a suggestion could be given as follows: *N. americanus* may be more resistant than *A. duodenale* to changing environment, including temperature and rainfall, as well as in their susceptibility to anthelmintics such as albendazole, mebendazole, and pyrantel pamoate.

In conclusion, the surveyed area in Krong Pac district, Dak Lak Province, Vietnam was confirmed to be an endemic area of soil-transmitted helminths, particularly, hookworms. The hookworm species determined morphologically after culture of eggs to larvae and molecular analysis of ITS1 sequence was confirmed to be 100% *N. americanus*. This may mean disappearance of *A. duodenale* from north Vietnam. Studies on chronological changes in hookworm species in endemic areas will be helpful for understanding the epidemiological transition of hookworm infections in each area.

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CONFLICT OF INTEREST

We have no conflict of interest related to this work.

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