Strongyloide Species Determinants in Vietnamese Human Stool Samples by Molecular Methods

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Article

Strongyloide Species Determinants in Vietnamese Human Stool Samples by Realtime PCR Method

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Abstracts: *Background*: Strongyloidiasis, a neglected disease caused by intestinal nematodes of the genus *Strongyloides*, is endemic to tropical and subtropical areas such as Vietnam. The morphological diagnosis of larvae by Hara Mori culture technique and microscopicare are considered the standard diagnostic procedures in the endemic areas of *Strongyloides* spp. However, they could only identify the genus, not the species of *Strongyloides.* DNA-molecular techniques which are highly sensitive and more cost-effective have been increasingly utilized in detection of *Strongyloides* species. This study aims to determine prevalence and the species of *Strongyloides* among resident population in Duc Hoa district, Long An province, Southern Vietnam. *Methods*: A cross-sectional study was conducted using 1,190 stool samples collected in Duc Hoa district, Long An province, Vietnam, from July, 2017 to November 2018. The stool specimens were transported to the Laboratory of Medical Parasitology, Pham Ngoc Thach University of Medicine within two hours of collection at an appropriate temperature of 25 °C. All samples were stored at 2 - 8°C and processed within 48 hours for microscopic examination. Molecular detection was carried out at Laboratory of Molecular Biology, Pham Ngoc Thach University of Medicine, Hochiminh city, VietNam. Results: Of the 1,190 samples tested, *Strongyloides* spp. larvae were detected in 79 specimens (6.6%) by two classical parasitological methods, namely direct microscopy and the modified Harada-Mori filter paper culture. DNA was extracted from 70 of the 79 samples of *Strongyloides* spp. larvae, which was subsequently characterized by real-time PCR amplification of the 18S and 28S regions of the rDNA gene. The results showed that 97.1% of the DNA samples were *S. stercoralis*, 2.9% were co-infections with *S. ratti* and *S. stercoralis*, and 2.9% belonged to *S. ratti*. For all 14 isolates, nucleotide sequencing was compared with other human pathogenic species of *Strongyloides* whose sequences are available in GenBank. The identity of 12/14 sequences were confirmed as *S. stercoralis* with a high level of similarity (91.3% - 100%) and over 98% for *S. ratti.* Between the two co-infection samples, the higher similarity belonged to *S. stercoralis*. Conclusion: A molecular amplification of small subunit ribosome RNA followed by sequence analysis has been proved to be a suitable method for discrimination of *Strongyloides* spp. retrieved from stool samples.

Keywords: Strongyloidiasis; S*trongyloidesstercoralis*; *Strongyloidesratti;* DNA-molecular techniques

1. Introduction

Strongyloides spp. (Nematode, Strongyloididae) or threadwormis, a chronic causative infectious agent, is one of the neglected tropical infectious pathogens. This causes a serious public health issue due to the worm's capacity to complete its entire lifecycle in the human host through autoinfection[1]. It can eventually lead to a hyper-infection syndrome in immunosuppressed patients, which can be fatal[1-4].

People acquire strongyloidiasis through penetration of the infective filariform larvae (L3) during contact contaminated soil, such as agricultural cultivation and recreation activities[1,4,5]. The clinical symptoms of strongyloidiasis range from absence of symptoms to gastrointestinal symptoms,e.g., abdominal pain, diarrhea, and colitis and urticaria from the penetration of the larvae through the skin causingchronic inflammatory response. Disseminated infections with mortality rates as high as 87% can occur specially among immunocompromised patients [8,9].

Diagnosis of strongyloidiasis is traditionally based on wet mount microscopy to detect rhabditiform/filariform of *Strongyloides* spp. larvae in faeces[7,10]. However, a low parasite load in patients with chronic infection affects the sensitivity of microscopic diagnosis. Serological methods such as Enzyme-linked immunosorbent assay (ELISA) have been used to detect human immunoglobulin G (IgG) against secreted antigen of filariform larvae during migration in the blood vessels. This is one of the most widely used techniques for the diagnosis of strongyloidiasis in a chronically infected population, with the sensitivity ranging from 73% to 100%[7,11,12]. However, this technique lacks specificity due to the cross-reactivity with antigen of other helminths, such as those that cause filariasis, schistosomiasis, ascariasis, hookworm, toxocariasis, and fascioliasisamong others [7,13,14]. Recently, molecular techniques have been developed for the detection and differentiation of *Strongyloides* spp. in fecal samples which are highly sensitive and more cost-effective for developing countries where *Strongyloides* spp are endemic [15-18].

Verweij JJ (2009), and Stefanie Krammeet *al*. (2011) have developed a real-time PCR method targeting the small subunit of the rRNA (18S and 28S) gene for specific detection of *Strongyloides* spp. DNA in fecal samples[19,20]. It was introduced as a promising alternative diagnostic approach that could determine species components, monitor the true prevalence and intensity of *Strongyloides* spp. infection in community[18,21].

Strongyloidiasis was firstly discovered in 1876 in the stools of French soldiers on duty in Southern Vietnam who had severe diarrhea, which was later proved to be endemic in Vietnam[7,22]. A recent meta-analysis reviewed the prevalence of *Strongyloides* spp. infection, from 0.2 to 2.5% in the northern and 1.19% in the southern, using stool examination and 7.6% using sera-immunological tests[22-24]. However, studies on species distinction of *Strongyloides* spp. infecting human are still limited.

Duc Hoa is a rural district of Long An Province in the Mekong Delta region. The district shares its borders with the administrative units such as Trang Bang District of Tay Ninh Province and Cu Chi District, Hoc Mon District of Ho Chi Minh city[25]. There has not been any research on strongyloidiasis or the prevalence of dominant *Strongyloides* species in this region. This study aims to determine the species of *Strongyloides* that infect the resident population in Duc Hoa district, Long Anprovince, Southern Vietnam.

2. Materials and methods

2.1. Study design, area and population

A cross-sectional study was carried out from July, 2017 to November, 2018 among the general population in four communes and one town in Duc Hoa district, Long An province, Vietnam. The study sites were randomly selected from the list of 17 communes and 3 towns of Duc Hoa (total number of residences: 245,617) (Figure 1)

Selection criteria: The study was conducted on the whole population, regardless of gender, race, over six-month residence at the study sites, and fluency in Vietnamese language. The subjects joined voluntarily and learned about this study before signing their consent to participate. Moreover, the study participants had not taken any deworming medication for 6 weeks. A total number of 1,190 participants distributed in 4 communes and 1 town in Duc Hoa satisfied the research criteria and were recruited in this study.

Sample containers were distributed one day before collection. The participants' fecal samples were colected in the wide-mouth screw-capped plastic containers, pre-labelled with the general information about the participants such as name, ID code of site, sex, and age. Stool samples were

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stored at the temperature of 25°C and transported to the Laboratory of Medical Parasitology, Pham Ngoc Thach University of Medicine within two hours of sampling. All samples were stored at 2 - 8°C and processed within 48 hours for microscopic examination. Molecular detection was carried out at laboratory of molecular biology, Pham Ngoc Thach University of Medicine, Hochiminh city, Vietnam.

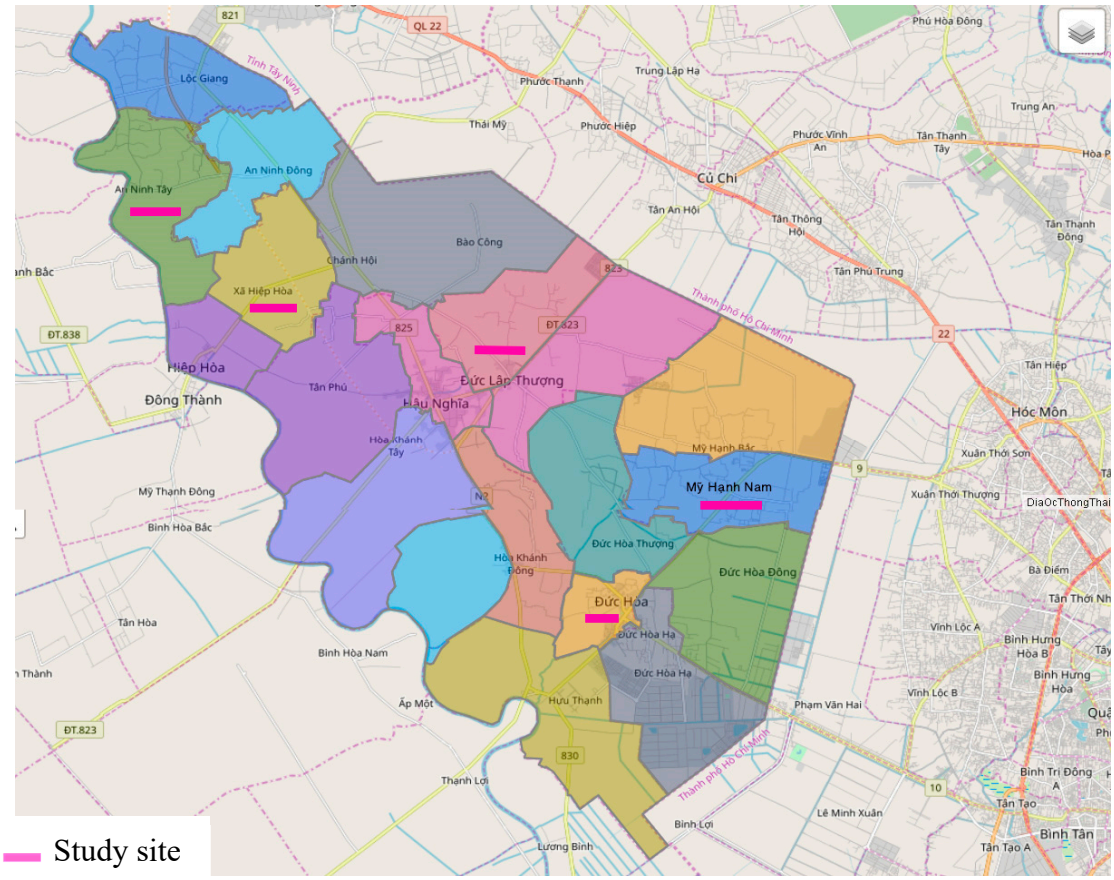

Figure 1. The geographical distribution of study site in Duc Hoa, Long An, Vietnam.

2.2. Laboratory detection of Strongyloides in fecal samples

Stool samples were checked in terms of the quality, volume, sampling time, research location in the laboratory and each was divided into two halves. The first half was tested by direct wet mount smear for microscopicmethod to detect motile protozoan trophozoites, helminth's eggs and larvae under a light microscope[26,27]. The second half was adapted from the Harada-Mori filter paper culture (HMFPC) to identify *Strongyloides* spp. and hookworm larvae[28,29]. In short, each sample was tested in duplicate; and if the result was positive in either or both of the methods above, it was considered a positive result. This procedure complianced the guideline on detection of Strongyloides in fecal samples developed by the Laboratory of Medical Parasitology and Laboratory of Molecular Biology, Pham Ngoc Thach University of Medicine, Hochiminh city, Vietnam

Morphological characteristics of all larval forms of *Strongyloides* spp. development stages were photographed in details by using Dp26 camera connected to Olympus BX53 microscope system, at magnifications of 100, 200 and 400 times. The pictures were analyzed using CellSens 5.0 software. Worms/larvae were measured in terms of body length, body width, esophageal length, abdominal cavity length, tail shape and transverse size at the tip of the tail. The developmental stages are classified based on the open shape of the mouth, esophagus, genital spines, ovaries, and intestines[30,26,31]. The *Strongyloides* spp. larvae were washed 3 times with distilled water and preserved in 75% ethanol solution. Each sample (2 larvae/100 µl) had its own code and was kept at ambient temperature for DNA extraction.

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2.3. A RealTime PCR molecular detection of Strongyloides spp.

Approximately 500 µl of the 2nd stage *Strongyloides*spp larvae (2 larvae/100 µl), preserved in 75% ethanol solution, were used for DNA extraction by using the High Pure PCR Template Preparation Kit (Product No: 11796828001, Roche Life Science, USA) in accordance with the manufacturer's instructions. The concentration and purity of the extracted DNA were determined by using an Amersham Pharmacia's GeneQuant *pro* spectrophotometer (Amersham Pharmacia Biotech). Each DNA sample was measured 3 times, and an average was calculated for evaluation in accordance with the company's regulations. The criteria for evaluating the purity of DNA samples after extraction were based on the OD_{260}/OD_{280} ratio. The DNA sample was considered suitable for realtime PCR assay when the ratio was in the range of 1.6-2.0. Samples were stored at -4^{0}C for further experiments.

A realtime PCR targeting the 18Sribosomal RNA gene was developed for identification of the genus *Strongyloides* including species such as *Strongyloidesstercoralis* and *Strongyloidesrati*. The assay in this study used three sets of species-specific primers that obtained from previous studies[19,20,32] and performed in two separate reactions in the thermal cycler Applied Biosystems 7500 Real-Time PCR (Applied Biosystems, USA). The primers sequence details were described in Table 1.

Table 1. The species-specific primers set for identification of *Strongyloides* genus and species.

Name of primers	Targeting gene (Accession No.)	Nucleotide sequence
	***Strongyloides* genus**	
StroS-F	28S rRNA	5'-TTA GAG TCG TGT TGC TTG GAA-3'
StroS-R		5'-GTG CAA CTG GCT CTG TAT GC-3'
StroS-Probe	U39490	HEX-5'-CTG TGA AGG AAA ATT GCA AAG TAC TCC GGA-3'-TAMRA
	Strongyloidesstercoralis	
1530 F	18S rRNA	5'-GAATTCCAAGTAAACGTAAGTCATTAGC-3'
1630 R		5'-TGC CTC TGG ATA TTG CTC AGT TC-3'
1586T Probe	AF279916	FAM-5'-ACA CAC CGG CCG TCG CTG C-3'-BHQ
	Strongyloidesratti	
SratF	28S rRNA	5'-GCA GCC TTG AAA ATG GAT GG-3'
SratR		5'-CTG TTG CGG ATA TGG GTA CG-3'
SratP probe	DQ14570	Cy5-5'-CGAAGTGGAAAAGGGTTTCACG-3'-TAMRA

The optimized realtime PCR mixture that was used in a 25 µL reaction was characterized as follows: 12.5 µL qPCR Master mix (Absolute®QPCR, Aligen-Abgen, UK), 0.6 µl (300nmol) each of primers (IDT, Singapore), 0.6 µL (200nmoL) each of labelled probes (IDT, Singapore), 5 µL of extracted DNA template from larvae and deionized water. The condition for realtime PCR involved denaturation at 95 °C for 15 minutes and was followed by 40 cycles at 95 °C for 15s and the final one at 60 °C for 1 minute.

The positive control DNA for *Strongyloides stercoralis* was obtained from the larvae culture medium and negative control was deionized water without DNA. Amplification, detection and data analysis were performed in StratageneMx3005P system (Agilent Technologies Division, Germany). Fluorescence was measured at the end of each extension step and the threshold cycle or Cut-off value of each positive samples were calculated.

2.4. Nested PCR and Sequencing

18S rDNA gene of 70 positive samples was amplified by a nested-polymerase chain reaction (nested-PCR). Primers were designed for 18S ribosomal DNA small subunit based on the *Strongyloides* spp. sequence deposited in GenBank (accession numbers: AJ417023)[33]. All PCR products were purified and sent to the First BASE Laboratories-Axil Scientific, Malaysia for sequencing.

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2.5. Data analysis

Data were analyzed using the statistical package for social sciences (SPSS) software for windows version 20.0 (SPSS, IL, USA). Frequencies were expressed as percentages.

DNA sequences were edited by using the Bioedit v.2.6 program[34]. Multiple sequence alignments were performed with Clustal W method and databases (http://www.ncbi.nlm.nih.gov/). Phylogenetic tree of identified *Strongyloides* spp. was was constructed by using MEGA v6.0 with the neighbourjoining (NJ) cluster algorithm and Tamura-3-Parameter. Bootstrap with 1000 replications was utilized for determining the topology reliability of the tree.

3. Results

3.1. Microscopic diagnosis

A total of 79 samples among 1,190 stool samples (6.6%) were found to be positive for the larvae *Strongyloides* spp. using both techniques. The direct smear microscopy detected 58.2% of total cases, much lower than the mHMFPC technique (93.7%).

3.2. Morphological characteristics of Strongyloidesspp larvae stages

All of the 1st stage larvae had pointed tail with an average body length of 279.9 µm and the esophageal length was 27.1% of the body's. When cultured at day 3, the 2nd larvae stage had slender shape, with the tip of the tail blunt or notched. Free-living male of *Strongyloides* spp. showed a pointed tail at an average length of 778.8 µm. In contrast, free-living female of *Strongyloides* spp. had an average length of 916.7µL with the vulva located near the middle of the body (Table 2).

Table 2. Morphological index of *Strongyloides* spp. isolated from faecal samples.

Structure	Mean ± SD	Min – Max
The 1st stage larvae		
Body length (µm)	279.9 ± 17.5	240.6 – 320.3
Horizontal size (µm)	18.47 ± 0.61	16.5 – 20.0
Length of esophagus (µm)	75.7 ± 5.1	64 – 90.1
Buccal cavity length (µm)	4.4 ± 0.3	3.9 – 5.3
Ratio of esophagus /body length (%)	27.1 ± 2.1	21.0 – 34.0
Pointed tail shape	79/79 (100%)	
The 2st stage larvae		
Body length (µm)	576.4 ± 24.9	510.0 – 632.0
Horizontal size (µm)	16.9 ± 1.1	15.3 – 19.6
Length of esophagus (µm)	244.7 ± 17.9	210.3 – 132.0
Buccal cavity length (µm)	4.5 ± 0.5	4.0 – 6.0
Ratio esophagus length/ body length (%)	42.5 ± 3.8	36.0 – 53.0
Horizontal size at endpoint of tail (µm)	2.6 ± 0.2	2.2 – 3.4
Endpoint of tail (blunt-end/notched)	11/68 (13.9 %/ 86.1 %)	
Free-living male (n = 5)		
Body length (µm)	778.8 ± 27.7	740.8 – 812.6
Horizontal size (µm)	45.1 ± 1.7	43.4 – 47.6
Length of esophagus (µm)	131.3 ± 6.9	120.0 – 136.2
Buccal cavity length (µm)	7.1 ± 0.6	6.6 – 8.1
Length of genital spines (µm)	33.4 ± 0.9	32.1 – 34.4
Ratio esophagus length/body length (%)	17.0 ± 1.1	16.9 – 18.0
Pointed tail shape	(100%)	
Free-living female (n = 3)		
Body length (µm)	916.7 ± 21.6	892.6 – 934.2

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Horizontal size (µm)	46.2 ± 1.7	44.2 – 47.5
Length of esophagus (µm)	130.6 ± 4.6	127.4 – 135.9
Buccal cavity length (µm)	6.8 ± 0.4	6.5 – 7.2
Ratio esophagus length/body length (%)	14.3 ± 1.2	14.0 – 15.0
Distance between vulva with head (% of body length)	49 ± 1.0	48.0 – 50.0

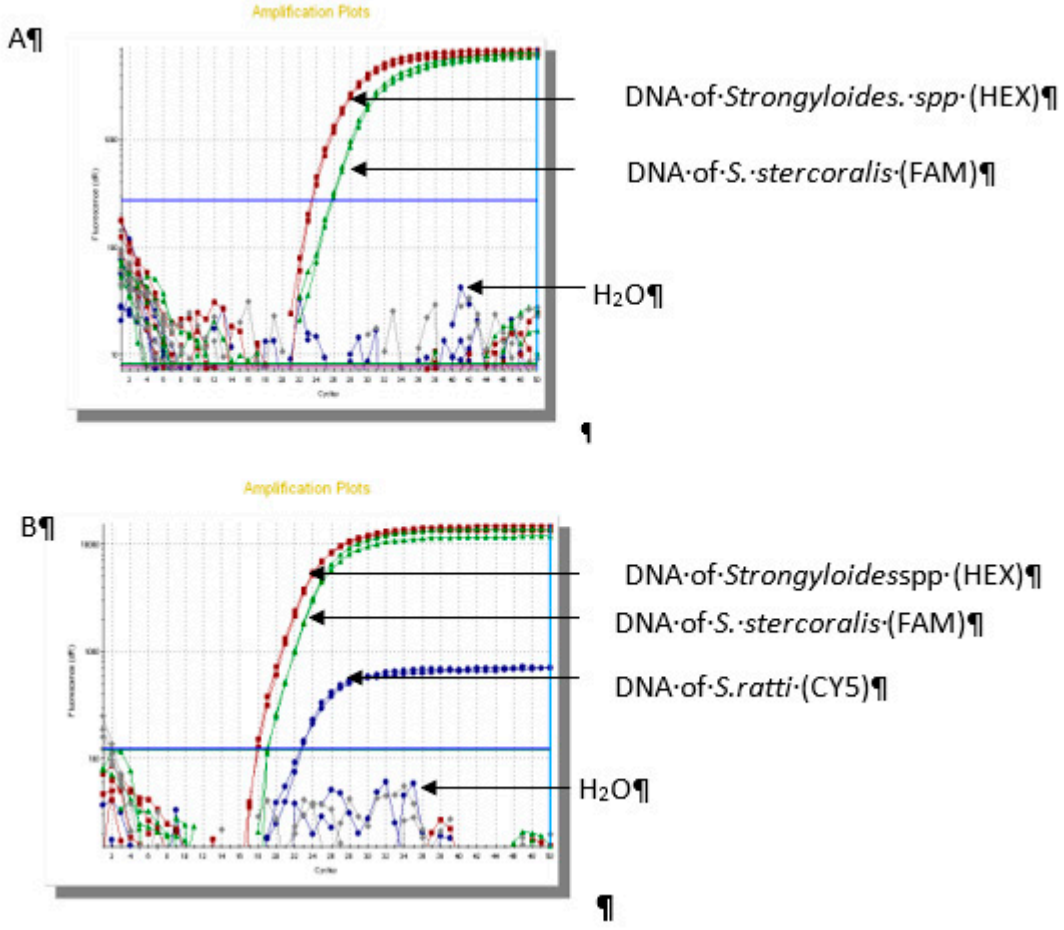
3.3. Realtime PCR technique

70 of the 79 extracted DNA samples (88.6%) had the $OD_{260/280}$ ratio, greater than 1.7 and passed the quality needed for realtime PCR (*Supplementary data 1*). Nine others were detected in DNA negative sample.

The results of real-time PCR assay showed that 66/70 (94.2%) samples were positive for *Strongyloidesstercolaris*, 2/70 (2.9%) samples were *Strongyloides ratti* and 2/70 (2.9%) samples were co-infection (Figure 2 and Supplementary data 2).

3.4. Nested - PCR and sequencing

A total number of 14 samples were tested by nested PCR, including 10 out of 66 of *Strongyloidesstercolaris* and 4 samples of *Strogyloides ratti* DNA. The result product was electrophoresed on agarose gel shown in Fig. 3 with the expected size (975 bp). No DNA product was detected in negative sample.



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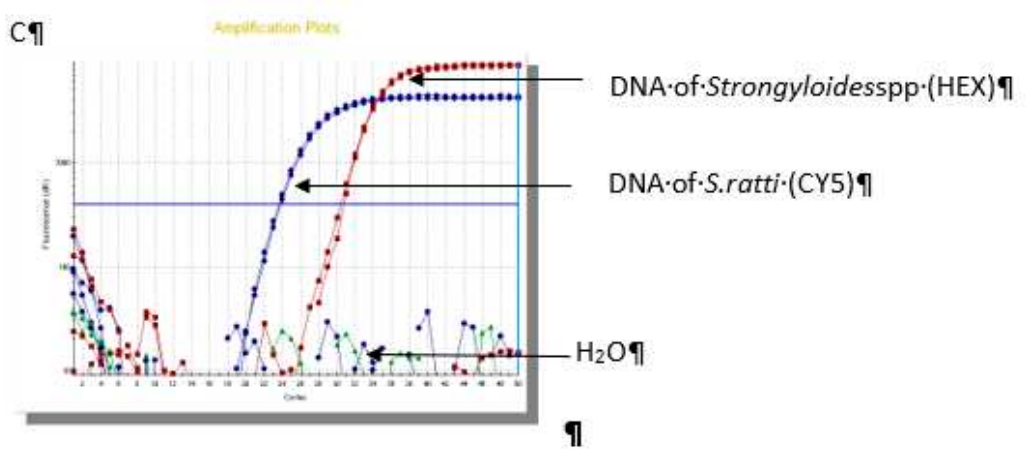

Figure 2. Result of real-time PCR in identifiication of *Strongyloides*spp.(A): Sample No.1 *S. stercoralis*; (B): Sample No.25 coinfection of *S. stercoralis* - *S. ratti*; (C): Sample No.54 *S. ratti.*

All 14 nested-PCR products were purified, sequenced and compared with the 18S rRNA of the *Strongyloides* spp. deposited in Genbank by using BLAST program. The result showed that the genetic homologysequence at AJ417023 location of *Strongyloide*s spp. dominated in Duc Hoa. While the sequence homology elsewhere (AB453329.1, AB923889.1, LN609412.1, AB923888.1, LL999065.1, LM528082.1, LL999063.1, MK369923.1 and LL999126.1) was 93-100% for *S. stercoralis* and more than 98% for *S.ratti* (table 3 and supplementary data 3).

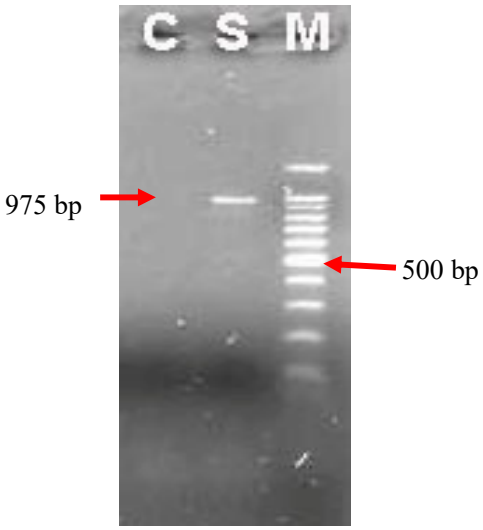

Figure 3. Electrophoresis products of nested PCR on agarose gel 1.5%.M: scale of DNA 100 bp; C: Negative control (H_2O); S: DNA sample of Strongyloidesspp.

Table 3. Analyzing the similarity between 18S rDNA sequence of Strongyloidesspp dominated in Duc Hoa, Long An and the corresponding sequences of the other geographical areas in Genbank.

No.	Code of sample	Similarity (%)	Genbank ID	Species
1	1	99.5	AB923888.1	S. *stercoralis*
2	7	98.6	AB923888.1	*S. stercoralis*
3	11	99.4	AB923888.1	*S. stercoralis*
4	15	99.7	AB923888.1	*S. stercoralis*
5	20	95.6	MK369923.1	*S. stercoralis*
6	25	98.5/98	AB923888.1/ AB453329.1	*S. stercoralis/ S. ratti*
7	26	91.3	LL999104.1	*S. stercoralis*

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8	35	100	LL999088.1	*S. stercoralis*
9	42	100	LL999110.1	*S. stercoralis*
10	47	99.2	AB923888.1	*S. stercoralis*
11	50	100	MK369923.1	*S. stercoralis*
12	54	98.0	AB923889.1	*S. ratti*
13	65	99.3/98.0	AB923888.1/ AB453329.1	*S. stercoralis/ S. ratti*
14	66	98.0	LN609412.1	*S. ratti*

4. Discussion

Investigating the prevalence and identifying the species components of *Strongyloides*spp among the residents of Duc Hoa are of high priority due to the special ecological context of this location and suitable condition for distribution and transmission of this intestinal nematode[23-25,35].

In this study, 6.6% (79/1,190) participants were confirmed with a gastrointestinal strongyloidiasis, however, simple wet mount microscopic technique was only able to detect 58.2% (46/79). It suggested that the detection rate of direct smear preparation technique for strongyloidiasis was quite low. Therefore, it is not recommended as the main diagnostic test for routine screening of *Strongyloides*spp infection in community because of its low sensitivity.

On the other hand, modified Harada-Mori filter paper culture (mHMFPC) was able to detect more *Strongyloides*spp larvae than wet mount (93.7% vs 58.2%). This result was higher than 78.4% in a study at Cu Chi district (2005)[36] and 47.8% by Rayzan H. Z et al (2012)[15] in Egypt. There were still 5 out of 79 (6.3%) cases with negative results in the mHMFPC techniquebut positive for the direct smear technique. Therefore, it is suggested that both should be used in field studies as the combination would increase detection rates[37].

The 1st stage larvae morphology in this study tended to be longer than in the studies by Grove DI (1989)[30] and Prayong R.(2013)[31], while the horizontal size of larvae was similar with these studies. The average length of the esophagus was 75.7 µm, with the bulb forms; and the average ratio compared to the body length was 27.1%, completely consistent with the structure of stage 1stlarvae.The buccal cavity of 1st stage of *strongyloides*spp larvae had an average length of 4.4 µm, the min - max was 3.9 µm - 5.3 µm. This was an important structure to distinguish with 1st stage hookworm larvae that had long buccal cavity.This is consistent with the findings by authors Grove D. I (1989)[30], Hong TT (2017)[38] and Prayong R. (2013)[31] with the mean of buccal cavity of 4-8 µm. From the above results, it would be confirmed that all surveyed larvae in this study were 1st stage *Strongyloides*spp larvae.

The 2nd stage larve of *Strongyloides* spp. had a body length of 576.4 µm in average, the average horizontal size was 16.9 µm. The results were consistent with those by Grove D.I (1989)[30], Prayong R. (2013)[31], which reported between 450 - 600 µm, horizontally slender than 1st stage larvae. The larvae had a cylindrical esophagus with an average length of 244.7 µm, and had a body length ratio of 42.5%. In addition, none of the larvae had pointed tail, in fact, 86.1% of them had notched tail. This is entirely consistent with the structure of the 2nd stage larvae with cylindrical esophagus which covers one-third of the body length.

Only 8 adults of *Strongyloides* spp., including 5 males and 3 females, were collected and morphologically characterized in this study. The average length of the male's body was 778.8 µm, and the average horizontal size was 45.1 µm. Although this result was higher than that reported by Prayong R. (2013) of appropriately700 µm[31], it is completely consistent with those by Grove D.I. (1989)[30] from 700 to 900 µm. They had an average esophageal length of 131.3 µm, and reached a ratio of 17% of the larval body length.The average spicule of adult male worm was 33.4 µm. In addition, it was also shown that the female worm was about 916.7 µm x 46.2 µm. This result was within the threshold but at a low level compared to the reported by Grove D.I. (1989)[30]. Free-living females had a longer esophagus than males (130.6µm vs. 131.3µm). This resulted in a smaller esophagus-to-body length ratio in females, reaching 14.3% compared to 17% in males. The two branches of the uterus, filled with eggs, were located symmetrically across the vulva. Distinguishing *Strongyloides*spieces

could only be based on morphology. This was quite challenging because the internal organs of this species were similar in shape and too small in size[30].For the above reasons, in terms of morphology corresponding to the design in this study, only 1st, 2nd stage larvae, male and female adults of *Strongyloides*spp were identified.

In this study, 70 extracted DNA samples of *Strongyloides*spp isolates, originally retrieved from human, were characterized by real-time PCR amplification of the 18S, 28S region of the rDNA gene. The result showed that *S. stercoralis* accounted for 97.1%, of which 2.9% was co-infected with *S. ratti*.

The dominant *S. stercoralis* in this study was consistent with those in the studies by N.V.DE (2017)[39] and D.T. Hong (2019)[40](97.1% vs. 100%, respectively), although the authors did not use the same realtime PCR technique. Moreover, in this study the causative agent of *S.ratti*, a common gastro-intestinal parasite of the rat, has been detected by molecular biology. It means that this species could be a human pathogen and it could not be differentiated due to the fact that morphological structure of *Strongloides*spieces larvae was almost unidentical.

Among 70 DNA samples characterized, 10 positive samples with *S. stercoralis* and 4 samples with S. ratti were randomly choosen to be amplfied by the nested-PCR. All PCR results yielded an identical pattern, with a visible fragment size of 975-bp. For all 14 isolates, nucleotide sequencing was achieved; and comparison with other human pathogen *Strongyloides*spp available sequences in GenBank was performed, using BLAST program. The results confirmed the identity of 12/14 sequences as *S. stercoralis* with high level of similarity (91.3% - 100%) and over 98% for *S. ratti*. Between the two co-infection samples *S. stercoralis* and *S. ratti* (samples 25 and 65), the higher similarity belonged to *S. stercoralis*species. This result could be explained by the highert number of *S. stercoralis* pathogens in the sample, or the *S. ratti* gene segment not being amplified through reaction.

The similarity of *S. stercoralis* sequence was recorded in comparision with the reference sequence in Genbankin the study by De NV (2017)[39] and Hong DT (2019)[40]. However, both authors experimented in small groups, consisting of only 2 and 7 samples respectively, thus it is inevitable that the rate of variation would be lower.

On the other hand, the phylogenetic tree showed the *S. ratti* species in the study completely closed to the species originating from the *Rattus novegicus* and it was consistent with the findings by Polanco Campo L F. (2018)[41] in Brazil (*Supplementary data 3*). Thus, with the identity of new species - *S. ratti*, this study provided solid evidence to confirm the presence of *S. ratti* as a causative infectious disease on human at the molecular level.

This study has three main limitations. Firstly, the study sites were not representative for the ecological environment of Long An province as well as the Southern provinces in Vietnam. The second limitation is the small number of participants and positive samples, thus it is necessary for further studies to be conducted in different areas in Vietnam, in order to compare with the results of this study. Last but not least, the 18S and 28S rDNA gene would help to differentiate *Strongyloides* species, however, it is required other specific genetic markers such as cytochrome C oxidase subunit 1 *(Cox1)* gene to be used to describe the phylogenetic tree of *Strongyloides* spp. more specifically.

In conclusion, the study shows that it is not possible to make distinction among different *Strongyloides* species' larvae by performing morphologically identification because some larvae may be zoonostic pathogen. A molecular amplification of small subunit ribosome DNA and following sequence analysis is a suitable method for discrimination of *Strongyloides* spp. isolated from faecal samples. It is also the first report of zoonosis *Strongyloides ratti* infection in human in Vietnam.

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Data Availability Statement:The data that support the findings of this study are available from the corresponding author, upon reasonable request. If you have concerns about sharing the data, please contact nth14@huph.edu.vn and ducvinh@pnt.edu.vn.

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