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研究課題名（和文）土壌環境DNA解析に基づく土壌伝播蠕虫感染症ダイナミクスの解明

研究課題名（英文）Elucidating transmission dynamics for soil-transmitted helminths based on environmental DNA analysis

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研究成果の概要（和文）：本研究には検出対象となるSTHは、Alumbricoides、Ttrichiura、および鉤虫（Aduodenale、N americanus）。全種並べられたcox1遺伝子の配列から、特定の可変/保存領域を決定し、マルチプレックスqPCRで使用するプライマー/プローブのセットを設計します。次に、風土病地域の土壌サンプルを使用しました。土壌eDNAを抽出してテストし、STH陽性症例と環境汚染との相関関係というこの研究プロジェクトの目標を完了しました。さらに、より多くの土壌サンプルを分析してテスト数を増やし、それを使用して風土病地域のSTH感染サイトの正確なリスクマッピングを設計しています。

研究成果の学術的意義や社会的意義
The results showed one sample from a house surrounding where the residents presented positive result for Ascaris lumbricoides (presenting a relationship on environment and human parasitic status). This is the first study showing the usefulness of eNA in soils samples for determination of STH.

研究成果の概要（英文）：In this study, the STH to be detected were A lumbricoides, T trichiura and Hookworms (A duodenale, and N americanus). From the aligned sequences of cox1 gene by species of parasite we determine specific variable/conserved areas and design sets of primers/probes to be used in multiplex qPCR. The assay was screened in silico for specificity. Worms' DNA were used to attest the usefulness of the system. Then, soil samples from endemic area were used. The soil eDNA was extracted and tested, completing the goals of this research project with the correlation of positive STH cases with environmental contamination. Furthermore, we are still analyzing more soil samples to increase the number of tests, and use it to design an accurate risk mapping of STH infection sites from endemic areas.

研究分野：one-health

キーワード：ecohealth　one-health　environmental DNA　helminths　pPCR　STH

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様　式　Ｃ－１９、Ｆ－１９－１（共通）

１．研究開始当初の背景

Soil-transmitted helminthiasis is one of the "Neglected Tropical Diseases (NTDs)" defined by the WHO as "tropical diseases that humanity must eradicate". It is prevalent mainly in economically developing countries and among the poor, and it is estimated that there are more than 1.5 billion patients worldwide. In addition, soil-transmitted helminthiasis not only reduces the quality of life of patients, but also has a negative impact on the economic development and stability of entire societies in endemic areas. Humans are infected by soil-transmitted helminths such as human roundworms and hookworms, and humans are infected by these worm eggs and larvae that exist in the soil. Soil-transmitted helminths mainly intestinal parasites, causing various symptoms such as abdominal pain, diarrhea, anemia, protein deficiency, rectal prolapse, stunted growth, and delayed cognitive development.

In many areas where soil-transmitted helminthiasis is endemic, the countermeasure taken is to "administer anthelmintics to treat all local population without identifying soil-transmitted helminth infections individually" and "prevention of infection" is not emphasized. Considering the emergence of drug-resistant parasites due to frequent administration of anthelmintics, unnecessary medication for uninfected people, and animal measures to control soil-transmitted helminths of animal origin, the applicants believe that it may be time to change the countermeasure policy and realize that "prevention of soil-transmitted helminthiasis is not impossible."

Therefore, establishing a method to prevent soil-transmitted helminthiasis is extremely important for the "clinical practice and public health" of endemic areas, and ultimately for "economic development and stability". The **academic "question" at the core of this research project is, "Can we establish the basis for an effective method to prevent infectious diseases, including soil-transmitted helminthiasis?"** In other words, "Focusing on the environment, which is one of the three elements that determine the establishment of an infectious disease, evaluate the environmental infection risk with the aim of blocking the infection route, and devise an effective prevention method."

２．研究の目的

Based on the concept of ecohealth, this study aims to: 1) develop a method for detecting soil-transmitted helminths from the environmental element "soil" using environmental DNA techniques, 2) establish a method for evaluating the infection risk in areas where soil-transmitted helminthiasis is prevalent, and 3) present data that will contribute to the prevention of soil-transmitted helminth infection in humans and animals.

３．研究の方法

Local of the Study

Based on the previous field surveys in Thailand, and the availability of the Thai local collaborators, we defined the area of the study in the STH endemic region of Tha Song Yang district of Tak province (Fig. 1). Tha Song Yang district is located at 500km distance from Bangkok bordering to Myanmar.

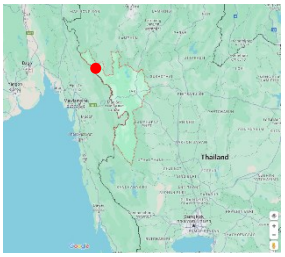

Fig. 1. Map of Thailand and the study site (red circle) located in Tak province, bordering to Myanmar.

According to a parasitological study conducted by Mahidol University in the area, the STH occurring in the area are *Ascaris lumbricoides*, *Trichuris trichiura* and the hookworms *Ancylostoma duodenale* and *Necator americanus*.

Development of a soil-transmitted helminth-specific DNA detection method

Primers and probes design

Since the sample used is "DNA from soil", which is relatively difficult to handle possessing great quantity of inhibitors and free-living organisms DNA, the length of the desired amplicon was set to be short (approximately 100-200bp). The target genes were mitochondrial COX1 gene, often used to distinguish parasite species. The probe used will be a hydrolysis probe (dual-labeled probe). DNA sequences of from *Ascaris lumbricoides*, *Ascaris suum*, *Toxocara canis*, *Toxocara cati*, *Ancylostoma duodenale*, *Ancylostoma caninum*, *Ancylostoma braziliense*, *Ancylostoma ceylanicum* and *Trichuris trichiura* were obtained from the NCBI GenBank, and aligned using MEGA software (version 11.0.13) and BioEdit. Several sets of primers and probes were designed for the species-specific detection of eDNA using Primer-BLAST (Primer3 and BLAST) by Ye et al., 2012 and PrimerExplorer V5 (https://primerexplorer.jp/lampv5e/index.html). The primer optimal melting temperatures (Tm) were set at 60°C, and the differences in Tm between the forward and reverse primers (ΔTm) were less than 2°C. Additionally, sequences with a GC content within 40–60% were selected. To check the specificity of the selected oligos *in silico*, a new Primer-BLAST with default settings was performed to confirm whether any species other than the target were potentially amplified. For in vitro tests, DNA samples from adult worms were used as templates for PCR. The PCR mixture consisted of 1 x Ex Taq™ (Probe qPCR) Master Mix (Takara, Japan), 900 nM of each primer, 125 nM of each probe, 2 µL of template DNA, and ultrapure water to a total volume of 20 µL. The experiments were conducted in triplicate, with three negative controls, on a Thermal Cycler Dice® Real Time System III (Takara, Japan). The initial step was 50°C for 2 min and 95°C 10 min, followed by 55 cycles of 95°C 15s and 59°C 1 min. The sensitivity of the soil-transmitted helminth-specific DNA detection method will be evaluated using samples whose DNA concentration (1 to 10^7 copies) was confirmed using the DNA Copy Number and Dilution Calculator (Thermofisher). The detection limit will be set at approximately 20 copies. Regarding specificity, a different parasite species from the one to be detected will be used to check whether each primer and probe shows no cross-reaction.

Field survey and data collection in endemic areas using soil-transmitted helminth-specific DNA detection method and environmental DNA (Mahidol University)

As previously mentioned, in consultation with the Faculty of Tropical Medicine, Mahidol University, Thailand, which is our collaborating institution, the field survey site will be set in the Tha Song Yang region of Tak Province in the northwestern region of Thailand, which borders Myanmar. Mahidol University has been conducting field surveys in this area for several years and has established a coordination and cooperation system with local residents and responsible parties. Considering the local climate, the field survey is scheduled to be conducted twice a year, in the dry season and in the rainy season. Soil samples are planned to be collected from the house premises, workplaces, near livestock, and fields, and three samples of approximately 500 grams of soil will be collected at each location to a depth of less than 10 cm. DNA (environmental DNA) will then be extracted from the sample soil using a commercially available kit, and the helminth species present in the soil will be identified using a soil-transmitted helminth-specific DNA detection method. The results of fecal tests already conducted by Mahidol University will be used to

determine the status of human soil-transmitted helminth infection, but demographic data (age, family structure, livestock and pet ownership, etc.) will be collected using questionnaires.

Visualization of results using GIS and risk mapping

The results of human and animal soil-transmitted helminth infection, and the results of soil contamination by environmental DNA will be visualized on a map of the survey area, and the risk of soil-transmitted helminth infection will be evaluated for each parasite. The results will be visualized in a way that is easy to understand for the recipients of the results (local responsible organizations and residents), such as the dynamics of infection between humans and animals and soil-transmitted helminths, as well as points for infection prevention (including information on risk areas, etc.). The results are statistically processed using Microsoft Excel and EpiInfo 7 (Centers for Disease Control and Prevention).

４．研究成果

Local of the Study and sampling

The field work was jeopardized by the COVID19 pandemics, but with all the mobility restrictions we could make 2 soil collections. One in the dry season and another in the rainy season. However, the number of samples sites were limited, and the processing of the material slowed by logistic and weather conditions. The site for sampling in Tha Song Yang is located in Ban Mai, Mae Salid Village (Fig. 2)

Fig. 2. Soil sampling area (in red). Left: Wide view of the Mae Salid Village and its proximity to Myanmar border. Center: Main street of Mae Salid Village and the location of the sampling area. Right: Ban Mai where the soil samples were collected, near Mae Salid Anglican Centre (school area).

Soil sampling was performed from vegetable gardens, school's playground and local houses' surroundings (Fig.3)

Fig.3. Sampling sites at Ban Mai, Tha Song Yang, Thailand in the dry season of 2022.

Design of a multiplex assay for STH

Due to the complexity to design a multiplex PCR we opt to target the 3 main species occurring in Tha Song Yang, namely *A. lumbricoides*, *T. trichiura*, and *A. duodenale*.

Primers and probes

Analysis of the entropy within the species presented few spaces without nucleotide variation showing the potential of *cox1* gene for designing a multiplex assay (Fig. 4)

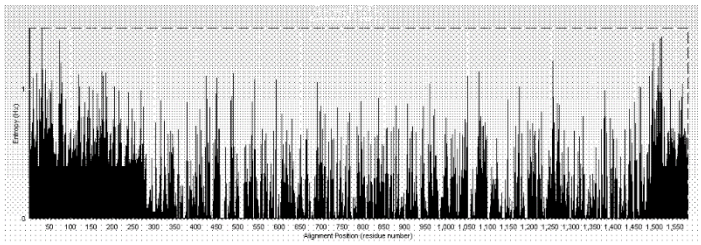

Fig. 4. Entropy (Hx) plot of the *cox1* sequences of STH used in this study retrieved from the database.

From each species, 10 best primers/probe set were selected and tested (Table 1). From the selection 1 set from each species were used in the multiplex qPCR in this study.

Table 1. The 10 best primers designed for each species of STH of this study. The red letters indicates the chosen sets for the multiplex assays.

A. lumbricoides	**Forward primer**	**Reverse primer**	**Internal oligo**	**Length**
Set 7	GTTGCCTTTGATGTTGGGGG	TTGAACTGTCTATCCTCCTTTGAGT	TGAGTTTTTGGTTGTTGCCTACTGCT	178
T. trichiura	**Forward primer**	**Reverse primer**	**Internal oligo**	**Length**
Set 2	CGGAGGACTAACCGGTGTTT	TGCCATCATAGCAAAAACCACA	ACGTAGTGGGACACCTACATTTCGT	124
A. duodenale	**Forward primer**	**Reverse primer**	**Internal oligo**	**Length**
Set 7	GGGTGCCCCAGATATGAGAT	AGCCAAATCAACCCTTCTCCC	CCTCCTTTAAGGACTTTGGGTCATCCA	175

Regarding the chosen primers probe set, specificity tests showed satisfactory results (Fig. 5). The CTs variated from 18 to 35 using DNA extracted from adult worms. No cross reactions were observed.

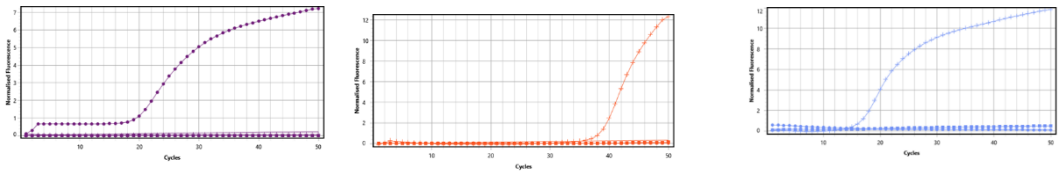

Fig. 5. Results of specificity of the primers/probe sets to (Left) *A. lumbricoides*, (Middle) *A. duodenale* and (Right) *T. trichiura* in qPCR assays.

The latter results

Samples from the field

Due to Nagoya Protocol and Japan Customs issues, soil material cannot be shipped directly to Japan. Therefore, we are working in collaboration with Trop Med Mahidol University for extracting and purifying eDNA in Thailand.

The first batch of samples from the field arrived at the Laboratory in Thailand and was processed accordingly. Until now, we could test 20 sites samples, that were subjected to a blind test. Unfortunately, only one sample from a house surrounding where the residents presented positive result for *Ascaris lumbricoides* (showing a relationship on environment and human parasitic status) and there was no detection of eDNA in other soils samples (Fig. 6).

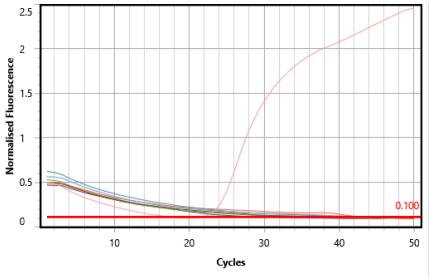

Fig. 6. Results of qPCR of soils samples eDNA from Mae Salid Village, Ban Mai, tha Song Yang, Thailand. From 20 samples tested only 1 was detected showing the presence of *Ascaris lumbricoides* in the area.

We are still processing samples, so we expect to have sound results after testing all the collected material. The risk mapping will be finished at this time.

5．主な発表論文等

〔雑誌論文〕　計1件（うち査読付論文　1件／うち国際共著　1件／うちオープンアクセス　1件）

1．著者名 Araujo MP、Sato MO、Sato M、Bandara WMKM、Coelho LFL、Souza RLM、Kawai S、Marques MJ	4．巻 10
2．論文標題 Unbalanced relationships: insights into the interaction between gut microbiota, geohelminths, and schistosomiasis	5．発行年 2022年
3．雑誌名 PeerJ	6．最初と最後の頁 e13401～e13401
掲載論文のDOI（デジタルオブジェクト識別子） 10.7717/peerj.13401	査読の有無 有
オープンアクセス オープンアクセスとしている（また、その予定である）	国際共著 該当する

〔学会発表〕　計1件（うち招待講演　1件／うち国際学会　1件）

1．発表者名 Marcello Otake Sato
2．発表標題 The use of environmental approaches in NTDS: Schistosomiasis and STH
3．学会等名 Joint International Tropical Medicine Meeting（招待講演）（国際学会）
4．発表年 2023年

〔図書〕　計0件

〔産業財産権〕

〔その他〕

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6．研究組織

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7．科研費を使用して開催した国際研究集会

〔国際研究集会〕　計0件

８．本研究に関連して実施した国際共同研究の実施状況

共同研究相手国	相手方研究機関