Short Report: Molecular Evidence of *Trichostrongylus colubriformis* and *Trichostrongylus axei* Infections in Humans from Thailand and Lao PDR

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Abstract. Human trichostrongylosis has been reported in Thailand. Recent reports in Lao People's Democratic Republic concerning species identification urged us to investigate species distribution in Thailand. We report eight human cases in Thailand and Lao People's Democratic Republic that were found to be infected by *Trichostrongylus colubriformis* and *T. axei* identified and confirmed by molecular techniques. This evidence is the first molecular evidence of human *T. colubriformis* and *T. axei* infection in Thailand. Infection by these two species was apparently epidemic in these areas. It is necessary to proceed with more comprehensive veterinary and epidemiologic studies to enable the practical prevention and control of this parasitic zoonosis.

Trichostrongylus (Strongylida and Trichostrongylidae) is an intestinal parasite of ruminants, birds, and primitive rodents with a worldwide distribution.¹ Of more than 30 species infecting mammals, only 10 species have been reported infecting human. Human infections seem to coincide with close contact with farm animals.² Infected humans present with stomachaches, abdominal bloating, diarrhea, and eosinophilia.^{3,4} *T. orientalis* has been reported from humans in China, Japan, Korea, and the Americas.⁵ In Iran, *T. orientalis* and *T. colubriformis* have been most frequently reported to infect humans, whereas other species, *T. vitrinus*, *T. axei*, *T. capricola*, *T. probolulus*, and *T. skrijabini*, are involved as well.⁶ Recently, *T. colubriformis*-infected cases were reported in Lao People's Democratic Republic (PDR) using molecular methods for species identification.^{7,8} In Thailand, a neighboring country, sporadic cases were reported at a genus level in the past^{9,10} without concerning molecular identification at species level. Here, we sought out cases of human trichostrongylosis and identified worms to a species level. *T. colubriformis* and *T. axei* were consequently identified as causative agents of human trichostrongylosis in eight cases—five cases in Thailand and three cases in Lao PDR. Phylogenetic trees were constructed to investigate of the relationships between the two species between the two countries.

This study was approved by the Khon Kaen University Ethics Committee for Human Research (HE551247). Eight trichostrongylosis cases from outpatient departments and hospitalized patients at Srinagarind Hospital, Faculty of Medicine, Khon Kaen University were diagnosed during year 2011–2012 after a routine stool examination by formalin ethyl acetate concentration technique.¹¹ Informed consent was obtained from all patients. There were five males and three females, with age ranging from 42 to 77 years. Five of them were residents of northeastern Thailand (Khon Kaen, Roi Et, Loei, and Sakon Nakhon provinces), and the other three

patients came from Lao PDR (Bolikhamsai, Khammouan, and Savannakhet provinces) (Figure 1A). Their fecal samples showed *Trichostrongylus*-like eggs (Figure 1B). The eggs looked like hookworm eggs, but they had longer and narrower shapes with tapered ends (egg size: $79.36 \pm 3.88 \mu\text{m}$ in length and $42.30 \pm 2.96 \mu\text{m}$ in width; $N = 24$). These features urged us to identify by molecular markers.

Fecal samples were subjected to an agar plate culture for parasitological confirmation.¹² Mature third-stage larvae were observed after 3 days of culture at room temperature. The larvae showed long esophagus, and their post-anal region is attenuated and terminated in a minute knob (larvae size: $825 \pm 24.86 \mu\text{m}$ in length and $27.10 \pm 2.30 \mu\text{m}$ in width; $N = 24$) (Figure 1C). Patients were treated with albendazole at 400 mg two times daily for 10 days.

One larva was isolated from each case. Its DNA was extracted using a Genomic DNA Mini Kit (Macherey-Nagel GmbH & Co., Düren, Germany) according to the manufacturer's instructions. A polymerase chain reaction (PCR) specific for the ribosomal DNA internal transcribed spacer 2 (ITS2) region was conducted with primer set JhTsp: 5' TTA TGT GCC ACA AAT GAA GA 3' and NC2: 5' TTA GTT TCT TTT CCT CCG CT 3',⁸ resulting in a *Trichostrongylus* spp. amplicon size of 482 bp. The ribosomal DNA ITS1 of *Trichostrongylus* spp. was amplified using a subset of the primer set NC5-F1: 5' GTA GGT GAA CCT GCG GAA GGA TCA T 3' and NC1r: 5' AAC AAC CCT GAA GCA GAC GTG C 3'¹³ with some modification, resulting in an amplicon size for *Trichostrongylus* spp. of 567 bp.

The PCR products were amplified by using the GeneAmp PCR System 9700 (Applied Biosystems, Singapore). For the ITS2 gene, the DNA template was initially denatured at 94°C for 5 minutes. The amplification procedure consisted of 35 cycles at 95°C for 30 seconds (denaturation), 54°C for 30 seconds (annealing), and 72°C for 30 seconds (extension), with a final extension at 72°C for 10 minutes. For ITS1, the DNA template was initially denatured at 94°C for 5 minutes. The amplification procedure consisted of 35 cycles at 95°C for 30 seconds (denaturation), 65°C for 30 seconds (annealing), and 72°C for 30 seconds (extension), with a final extension

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FIGURE 1. (A) Distribution of trichostrongylosis cases in Thailand: (1) Khon Kaen, (2) Roi Et, (3) Loei, and (4 and 5) Sakon Nakhon. Distribution of trichostrongylosis cases in Lao PDR: (6) Bolikhamxai, (7) Khammouan, and (8) Savannakhet. (B) Egg of *Trichostrongylus* from patient's feces under the microscope. (C) Third-stage larva of *Trichostrongylus* from agar plate culture. Scale bar: 50 µm.

FIGURE 2. Phylogenetic trees of *Trichostrongylus* spp. were constructed by the neighbor-joining method with MEGA version 3.1. The left tree was from ITS1 gene sequences of *Trichostrongylus* spp., and the right tree was from ITS2 gene sequences. The sequences were deposited in GenBank, and bootstrap scores expressed as percentages of 1,000 replications are given at each node. Others from DNA database are indicated with species and accession numbers. The numbers of cases from the present study are indicated with human numbers 1–8 as follows: Thailand: Khon Kaen (human 1), Roi Et (human 2), Loei (human 3), and Sakon Nakhon (humans 4 and 5); Lao PDR: Bolikhamxai (human 6), Khammouan (human 7), and Savannakhet (human 8). Thailand (THA) and Lao PDR (LAO) are represented with country codes (ISO 3166-1 α-3 codes).

at 72°C for 10 minutes. The PCR product was run on a 1% agarose gel. DNA sequencing was performed using the MegaBACE 1000 DNA Analysis System (GE Healthcare, Piscataway, NJ). The sequences were aligned and compared with the sequence data for *Trichostrongylus* spp. in the GenBank database using the multiple sequence alignment program of Clustal W.¹⁴ A phylogenetic tree was constructed by using the neighbor-joining method with MEGA version 3.1.¹⁵ The tree was evaluated by a bootstrap test based on 1,000 resamplings. All sequences were submitted to the GenBank database (Figure 2).

For ITS2 sequences, 100% similarities were obtained with known databases for adult *T. colubriformis* from sheep (GenBank accession nos. S69220, X78063, and EF427624). The nematode sequences also revealed 100% similarities with *T. colubriformis* found in humans from Lao PDR (GenBank accession nos. AB503250, AB503251, and AB503252).⁸ One sample from Sakon Nakhon, Thailand (Human4; KC 337066) (Figure 2) showed 99% similarity with *T. axei* from sheep (GenBank accession no. AY439026). For ITS1 sequences, 99% similarities were obtained with known database for adult *T. colubriformis* from sheep (GenBank accession no. JF680985). One sample from Sakon Nakhon, Thailand (Human4; KC337056) (Figure 2) showed 100% similarity with *T. axei* from Soay sheep (GenBank accession no. AY439026). Intraspecific variability level was shown within the *T. colubriformis* nematode when larvae from two geographical areas were compared with parasites from the GenBank database (National Center for Biotechnology Information, GenBank Flat File Release 192.0, October 15, 2012).

Species identification using molecular approaches has revealed *T. colubriformis* infection in humans in Lao PDR,^{7,8} whereas molecular evidence of *Trichostrongylus* species in infected humans in Thailand awaits investigation. The present study is the first molecular evidence of human *T. colubriformis* and *T. axei* infection in Thailand. The combined sequence data from both ITSs could be useful for increasing accuracy of species identification. For *T. colubriformis*, the sequences showed low intraspecific variability level within nematodes from Lao PDR and Thailand. The phylogenetic reconstruction (Figure 2) revealed close phylogenetic associations between two populations of *T. colubriformis* from Thailand and Lao PDR.

Thailand and Lao PDR are Asian countries where many communities are located along the Mekong River. We showed that *T. colubriformis* and *T. axei* are the species causing human trichostrongylosis, an emerging zoonosis, in the two countries. Our molecular study also revealed that the parasites from the two areas have close phylogenetic associations. Little or no intraspecific variation was detected between two geographical regions.

Infective, third-stage larvae hatch from eggs within a few days, and they contaminate vegetables and water in farms. Humans become infected with *T. colubriformis* by consumption of vegetables or drinking water contaminated with infective larvae. The prevalence of infection is high among farmers who have poor personal hygiene and live in close contact with farm animals, such as sheep and cattle.^{1,2} Human infections have been reported in many countries, and it may become an emerging disease because of the trend of increasing use of animal feces in organic farms. Our results suggest that it is essential to conduct more comprehensive veterinary and epi-

demologic studies to enable the effective prevention and control this parasitic zoonosis.

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REFERENCES

1. Anderson RC, 2000. *Nematode Parasites of Vertebrates: Their Development and Transmission*, 2nd Ed. New York, NY: CABI.
2. Tongson MS, Eduardo SL, 1982. Trichostrongyliidiosis. Steel JH, Schultz MG, eds. *CRC Handbook Series in Zoonoses. Section C: Parasitic Zoonoses, Vol II. Boca Raton, FL: CRC Press*, 331–338.
3. Lattes S, Ferte H, Delaunay P, Depaquit J, Vassallo M, Vittier M, Kokcha S, Coulibaly E, Marty P, 2011. Trichostrongylus colubriformis nematode infections in humans, France. *Emerg Infect Dis* 17: 1301–1302.
4. Wall EC, Bhatnagar N, Watson J, Doherty T, 2011. An unusual case of hypereosinophilia and abdominal pain: an outbreak of Trichostrongylus imported from New Zealand. *J Travel Med* 18: 59–60.
5. John DT, Petri WA, 2006. Markell and Voge's Medical Parasitology, 9th Ed. St. Louis, MO: Elsevier, 266–267.
6. Ghadirian E, Arfaa F, 1975. Present status of trichostrongyliasis in Iran. *Am J Trop Med Hyg* 24: 935–941.
7. Yong TS, Lee JH, Sim S, Lee J, Min DY, Chai JY, Eom KS, Sohn WM, Lee SH, Rim HJ, 2007. Differential diagnosis of Trichostrongylus and hookworm eggs via PCR using ITS-1 sequence. *Korean J Parasitol* 45: 69–74.
8. Sato M, Yoonuan T, Sanguankiat S, Nuamtanong S, Pongvongsa T, Phimmayoi I, Phanhanan V, Boupba B, Moji K, Waikagul J, 2011. Human Trichostrongylus colubriformis infection in a rural village in Laos. *Am J Trop Med Hyg* 84: 52–54.
9. Panasoponkul C, Radomyos P, Singhasivanon V, 1985. Trichostrongylus infection in a Thai boy. *Southeast Asian J Trop Med Public Health* 16: 513–514.
10. Jariya P, Lertlaituan P, Junnoo V, Bedavanij A, Tesjaroen S, 1988. One human case of trichostrongyliasis in Thailand. *J Med Assoc Thai* 71: 469–470.
11. Elkins DB, Haswell-Elkins M, Anderson RM, 1986. The epidemiology and control of intestinal helminths in the Pulicat Lake region of Southern India. I. Study design and pre-and post-treatment observations on Ascaris lumbricoides infection. *Trans R Soc Trop Med Hyg* 80: 774–792.

12. Koga K, Kasuya S, Khamboonruang C, Sukhvat K, Ieda M, Takatsuka N, Kita K, Ohtomo H, 1991. A modified agar plate method for detection of *Strongyloides stercoralis*. *Am J Trop Med Hyg* 45: 518–521.
13. Hoste H, Chilton NB, Beveridge I, Gasser RB, 1998. A comparison of the first internal transcribed spacer of ribosomal DNA in seven species of *Trichostrongylus* (Nematoda: Trichostrongylidae). *Int J Parasitol* 28: 1251–1260.
14. Thompson JD, Higgins DG, Gibson TJ, 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* 22: 4673–4680.
15. Kumar S, Tamura K, Nei M, 2004. MEGA3: Integrated software for molecular evolutionary genetics analysis and sequence alignment. *Brief Bioinform* 5: 150–163.