Short Report: Molecular Identification of a Case of *Paragonimus pseudoheterotremus* Infection in Thailand

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Abstract. Paragonimiasis is an important food-borne parasitic zoonosis caused by infection with lung flukes of the genus *Paragonimus*. In Southeast Asia, *Paragonimus heterotremus* is the only proven causative pathogen. Recently, a new *Paragonimus* species, *P. pseudoheterotremus*, was found in Thailand. This species is genetically similar to *P. heterotremus* and is considered as a sister species. However, infectivity or pathogenicity of *P. pseudoheterotremus* to humans remains unclear. We report the first confirmed human pulmonary paragonimiasis case caused by *P. pseudoheterotremus* infection. After polymerase chain reaction/sequencing of the DNA extracted from *Paragonimus* eggs in the sputum of the patient, partial internal transcribed spacer 2 and cytochrome c oxidase subunit 1 sequences were approximately identical (98–100%) with those of *P. pseudoheterotremus*. For *P. heterotremus*, the partial internal transcribed spacer 2 sequence was approximately identical (99–100%), but the partial mitochondrial cytochrome c oxidase subunit 1 sequence showed a similarity of 90–95%.

Paragonimiasis is a food-borne zoonosis caused by infection with lung flukes of the genus *Paragonimus*. Only 7 of approximately 40 species can infect humans: 2 species (*Paragonimus kelikotti* and *P. mexicanus*) in the Western Hemisphere, 2 (*P. africanus* and *P. uterobilateralis*) in Africa, and 3 (*P. westermani*, *P. skrjabini*, and *P. heterotremus*) in Asia.^{1–3} Among them, *P. westermani* is the major pathogenic fluke for human paragonimiasis in eastern Asia where 20.7 million people (20 million in China) were infected.⁴ In addition to *P. westermani* infection, sporadic cases of *P. skrjabini* infection have been reported from China and Japan (as *P. miyazakii* infection in Japan). From southwestern China to northeastern India including the Indochina Peninsula, *P. heterotremus* is the only proven species responsible for human infection.^{1,2} In Thailand, although 6 *Paragonimus* species were registered in wild life and/or experimental animal infections, *P. heterotremus* is the only confirmed pathogen for human paragonimiasis by the recovery of worms from patients.⁵

In 2007, a new *Paragonimus* species, *P. pseudoheterotremus* was described as the seventh species in Thailand mainly on the basis of the morphologic difference of metacercariae (*P. pseudoheterotremus* metacercariae were oval shape and smaller than those of *P. heterotremus*).⁶ Although ribosomal internal transcribed spacer 2 (ITS2) sequences of *P. pseudoheterotremus* and *P. heterotremus* were almost completely identical, (only one base difference), the partial sequence of mitochondrial cytochrome c oxidase subunit 1 (*cox1*) genes of these parasites were significantly different from each other, suggesting their sister species relationship.⁷ Although adult worms of *P. pseudoheterotremus* were obtained by experimental infection in a cat,⁶ the pathogenicity of this new species to humans remains unsolved. We report a case of human pulmonary paragonimiasis caused by *P. pseudoheterotremus* in Thailand. Diagnosis was confirmed by molecular evidence using *Paragonimus* eggs in the sputum of the patient.

The study was approved by the Human Ethics Committee of Khon Kaen University (Reference no. HE551071). Oral informed consent was obtained from the patient. A 57 year-old man (monk) from Thailand was admitted to our hospital on July 1994 because of chronic productive cough with bloody sputum and dyspnea for the past eight months. He stated that one year before development of his respiratory symptoms, he occasionally ate mountainous crabs while staying in a cave as a part of his meditation training in Loei Province in northeastern Thailand.

A chest radiograph showed a faint pulmonary nodule at the left upper lobe with minimal pleural effusion with pleural thickening of both lower lung fields and generalized thickening of lung markings. Bronchoscopic examination showed nodular obstruction at the posterior basal segment of the left lower lung field. Many *Paragonimus* sp. eggs were found in bronchoalveolar lavage fluid. *Mycobacterium tuberculosis* was not detected in bronchoalveolar lavage fluid. After bronchoscopy, *Paragonimus* eggs were detected in his sputum (Figure 1). The eggs ($n = 5$) had a mean $\pm$ SD length of $79.6 \pm 5.4 \mu\text{m}$ and a mean $\pm$ SD width of $45.0 \pm 3.0 \mu\text{m}$. Eggs were slightly asymmetric in shape and had an operculum at one end but lacked a thickening at the abopercular pole. *Paragonimus* eggs in the sputum sample were kept at -70°C in the Department of Parasitology, Faculty of Medicine, Khon Kaen University, Thailand, until a polymerase chain reaction (PCR) and DNA sequencing were performed.

He was treated with praziquantel, 25 mg/kg of body weight, 3 times/day for 2 consecutive days. Two days after treatment, *Paragonimus* eggs in sputum disappeared. His clinical symptoms and laboratory data markedly improved without any complication by two months after treatment.

For accurate identification of the causative species of *Paragonimus* in this patient, a PCR/sequencing method was used for diagnosis. Genomic DNA was extracted from the eggs in the sputum. In brief, egg-containing sputum was homogenized in a disposable polypropylene pestle (Bellco Glass Inc., Vineland, NJ), and the DNA was extracted by using a NucleoSpin® Tissue Kit (Macherey-Nagel GmbH and Co., Dürren, Germany). DNA was eluted in 100 μL of distilled water and stored at -20°C until used.

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FIGURE 1. *Paragonimus pseudoheterotremus* egg from the sputum of a patient, Thailand.

The ITS2 sequence was amplified by using species-specific primers PhITS2-F (5'-CTG TGT GAA TTA ATG TGA ACT GC-3') and PhITS2-R (5'-AGT GAT ATG CTT AAG TTC AGC G-3'), which were designed from the known ITS2 sequence of *P. heterotremus* (GenBank accession no. AB308377). Because the ITS2 sequence of eggs from the patient was

completely or almost completely identical with those of *P. heterotremus* and *P. pseudoheterotremus*, a partial fragment of *cox1* gene was amplified by using the species-specific primers PphCOI-F (5'-CCG GGT TTG GTG TTG TG-3') and PphCOI-R (5'-ACA ACG AAC CAA GTG TCA TG-3'), which were designed from a specific region of *P. pseudoheterotremus cox1* gene (GenBank accession no. EF446315).

The PCR was conducted by using a GeneAmp® PCR System 9700 (Applied Biosystems, Singapore). The reaction was carried out in a 25-μL volume containing PCR buffer (60 mmol/L Tris sulfate, pH 8.4, 18 mmol/L ammonium sulfate, 1.5 mmol/L MgCl₂), 200 μmol/L of each deoxyribonucleotide triphosphate, 0.2 μmol/L of each primer, and 0.625 units of *Taq* DNA polymerase. The DNA template was initially denatured at 94°C for 5 minutes. The amplification procedure comprised 35 cycles at 95°C for 30 seconds (denaturation), 55°C for 30 seconds (annealing), and 72°C for 30 seconds (extension), with a final extension at 72°C for 10 minutes. The amplified product was subjected to electrophoresis on a 1.5% agarose gel; the *cox1*- and ITS2-fragments were then removed from the gel and purified for DNA sequencing, which was performed by using the MegaBACE™ 1000 DNA Analysis System (GE Healthcare, Piscataway, NJ).

The ITS2 and *cox1* gene sequences of the *Paragonimus* eggs from the patient were analyzed by BLAST-N search (National Center for Biotechnology Information, Bethesda, MD), and DNA alignment was performed by using ClustalW⁸

		10	20	30	40	50	60	
<i>P. pseudoheterotremus</i> THA patient	1	AGACATATTT	GCATGACTTT	AACTAACAAA	GATTCTCTGT	TTGGGTATTA	TGGTTTGGTT	60
<i>P. pseudoheterotremus</i> EF014339 THA	1					C.....		60
<i>P. heterotremus</i> AB325519 IND	1		.T.....	G.....		T.....	C.....	60
		70	80	90	100	110	120	
<i>P. pseudoheterotremus</i> THA patient	61	TTTGCTATGG	GGGCTATTGT	GTGTTTGGGG	AGGGTTGTTT	GAGCGCATCA	TATGTTTATG	120
<i>P. pseudoheterotremus</i> EF014339 THA	61		C..					120
<i>P. heterotremus</i> AB325519 IND	61		C..					120
		130	140	150	160	170	180	
<i>P. pseudoheterotremus</i> THA patient	121	GTTGGTTTAG	ATGTTAAGAC	TGCTGTCTTT	TTTAGCTCTG	TTACTGGTGT	GATTGGCATT	180
<i>P. pseudoheterotremus</i> EF014339 THA	121							180
<i>P. heterotremus</i> AB325519 IND	121					C..A..		180
		190	200	210	220	230	240	
<i>P. pseudoheterotremus</i> THA patient	181	CCTACGGGGA	TTAAGGTTT	TTCTTGATTG	TTTATGTTGG	GAGGAACG	TTTACGATT	240
<i>P. pseudoheterotremus</i> EF014339 THA	181							240
<i>P. heterotremus</i> AB325519 IND	181	T.....		G...		C.....	G...	240
		250	260	270	280	290	300	
<i>P. pseudoheterotremus</i> THA patient	241	TGAGATCCAG	TGGTTTGATG	GATTTTGGGC	TTTATCTTTT	TGTTTACTAT	CGGTGGTGTT	300
<i>P. pseudoheterotremus</i> EF014339 THA	241							300
<i>P. heterotremus</i> AB325519 IND	241	G.	G..	T		T.....	G	300
		310	320	330	340			
<i>P. pseudoheterotremus</i> THA patient	301	ACTGGAATTA	TTTTGTCTTC	CTCTATTTTG	GATAGTTTGC	TG		342
<i>P. pseudoheterotremus</i> EF014339 THA	301							342
<i>P. heterotremus</i> AB325519 IND	301	G.....	C...	T				342

FIGURE 2. Alignment of partial cytochrome c oxidase subunit I (*cox1*) gene sequence of *Paragonimus pseudoheterotremus* eggs from a patient in Thailand (*P. pseudoheterotremus* THA patient) with those of *P. pseudoheterotremus* (EF014339 from Thailand) and *Paragonimus heterotremus* (AB325519 from India) sequences from a database. ISO 3166-1 alpha-3 codes were used as the country code.

and the maximum composite likelihood tree using MEGA 4.⁹ The partial ITS2 sequence of *Paragonimus* sp. eggs from the patient was identical with that of *P. pseudoheterotremus* and various geographic isolates of *P. heterotremus* (identity = 99–100%). The partial *cox1* gene sequence also showed extremely high (98–99%) sequence similarities with those of *P. pseudoheterotremus*. The homology with *P. heterotremus* was 90–95% (Figure 2). Phylogenetic analysis of the *cox1* gene sequence (Figure 3) showed that the *Paragonimus* sp. from the patient was a member of the *P. pseudoheterotremus* clade, which is distinct from the *P. heterotremus* clade. From these results, the patient was given a diagnosis of infection with *P. pseudoheterotremus*. The partial ITS2 and *cox1* sequences were deposited in GenBank under accession numbers JQ796814 and JQ796815, respectively.

In Thailand, there were six species of *Paragonimus* (*P. westermani*, *P. siamensis*, *P. heterotremus*, *P. bangkokensis*, *P. harinasutai*, and *P. macrorchis*)¹⁰ until the recent discovery of *P. pseudoheterotremus* as the seventh species.⁶ The present results confirmed that *P. pseudoheterotremus* can be a pathogen to humans. For species identification of etiologic agents of paragonimiasis, detection of species-specific band by serum samples of patients in an enzyme-linked immunoelectrotransfer blot¹¹ or a binding inhibition enzyme-linked immunosorbent assay using homologous and heterologous

antigens¹² have been developed. Whether such immunologic or molecular methods could be applicable for the discrimination of *P. heterotremus* and *P. pseudoheterotremus* should be explored.

Paragonimus heterotremus is distributing widely from southwestern China to northeastern India, and confirmed cases of human infection have been reported in Thailand,^{13,14} Vietnam,^{15,16} Laos,^{17,18} and India.¹⁹ Conversely, metacercariae of *P. pseudoheterotremus* were first found in freshwater crabs in Kanchanaburi, Thailand, and adult worms were obtained by experimental infection in a cat.⁶ However, the natural definitive hosts and distribution or pathogenicity of this parasite to humans have not been elucidated. The present case is the first detection of human infection with *P. pseudoheterotremus*.

Because *P. pseudoheterotremus* and *P. heterotremus* are difficult to discriminate on the basis of morphology of eggs,⁶ and because *P. heterotremus* is widely distributed in Southeast Asia, molecular diagnosis using sputum eggs is necessary for accurate identification of the pathogen of paragonimiasis in Southeast Asia. The present results also suggest possible intra-species variation among geographically different isolates of *P. pseudoheterotremus*. Large scale community-based surveys along the Mekong River Basin and neighboring countries are needed to identify the human pathogenic species.

FIGURE 3. Neighbor-joining tree based on partial cytochrome c oxidase subunit I (*cox1*) gene sequences (342 bp). Bootstrap scores expressed as percentages of 1,000 replications are given at each node. Sequences of *Paragonimus heterotremus*, *P. pseudoheterotremus*, and *P. westermani* obtained from a DNA database are indicated with species accession no. country code (ISO 3166-1 alpha-3 codes).

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