Paragonimiasis is a food-borne 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. 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%.

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 ± SD length of 79.6 ± 5.4 μm and a mean ± SD width of 45.0 ± 3.0 μm. 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 polypyrrole pestle (Bellco Glass Inc., Vineland, NJ), and the DNA was extracted by using a NucleoSpin® Tissue Kit (Macherey-Nagel GmbH and Co., Düren, Germany). DNA was eluted in 100 μL of distilled water and stored at −20°C until used.
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 ClustalW®.
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) until the recent discovery of P. pseudoheterotremus as the seventh species. 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 blot or a binding inhibition enzyme-linked immunosorbent assay using homologous and heterologous antigens 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, Vietnam, Laos, and India. 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. 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, 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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