平成16年秋に集団発生した肺吸虫による食中毒事例について

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緒　言

平成16年秋に，佐賀県の某ホテル内にある中華料理店において，ウェステルマン肺吸虫による集団感染が発生し，モクズガニの老酒漬（酔蟹）が原因の食中毒として，届出がなされた。本論文では，本事例の発生から食中毒としての届出に至る経緯について，報告する。

Outbreak of Paragonimiasis Caused by Ingestion of Drunken Crabs at a Hotel Restaurant in Saga Prefecture in 2004：Notification as Food Poisoning

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表1　モクズガニの老酒漬を喫食して肺吸虫に感染した患者の症状など

患者*	住所	年齢（歳）	摂食日（月/日）	発症日（月/日）	潜伏期（日）**	主な症状
A	神奈川県	49	9/25	10/2	8	発熱・頭痛・悪寒・倦怠感・咳（痰）・側腹痛・動悸
B	福岡県	66	9/16	10/12	27	発熱・悪寒・倦怠感・咳（痰）
C	福岡県	62	9/16	11/15	61	なし
D	佐賀県	35	9/30	12/1	63	なし

*：患者は何れも女性でウェステルマン肺吸虫抗原を用いた血清診断で陽性と判定された.

**：患者Aはモクズガニ喫食後8日から体調不良を訴えていたが，当初は肺結核や肺癌が疑われ，肺吸虫症の診断が遅れた．無症者である患者CおよびDは，検査日を発症日とした.

原因施設におけるモクズガニの提供状況

本事例の原因施設となった中華料理店では，平成16年9月10日～11月3日までの期間に「かにフェア」という催し物を開催し，モクズガニの老酒漬を非加熱で提供した。この料理は地元産のモクズガニを食材とし，老酒（アルコール分：20%）と醤油とを1：3の割合で混ぜた漬け汁（砂糖等で調味）に4・5日間漬け込むという，中華料理の老酒漬を模して調理したものであった。同時に，シナモクズガニ（いわゆる上海ガニ）も「かにフェア」の食材として使用したが，これは輸入品であったため，食中毒予防への配慮から，すべて加熱調理して提供していた。

探知された症例

集団感染発見の契機は，神奈川県の病院に呼吸器症状で入院していた患者（表1，A）が，ウェステルマン肺吸虫感染と血清診断されたことにある。患者のモクズガニ摂食を知った担当医が，11月10日に「ウェステルマン肺吸虫による食中毒疑いの患者が入院している」と神奈川県に連絡し，神奈川県が佐賀県に事例発見を知らせた。

佐賀県の調査により，提供された老酒漬モクズガニの皿数は138食で，喫食の可能性がある者は191名であることが明かになった。このうち114名から摂食の確認が取れ，肺吸虫感染の有無について医療機関で受診するよう要請した。その結果，感染者は患者Aを含めて4名であることが分った（表1）。内訳は，呼吸器症状を呈する有症者が2名，血清反応のみ陽性の無症者が2名であった。有症者については，糞便，喀痰，あるいは胸水を対象に虫卵の検出が試みられたが，喫食から検査までの期間は短く，虫卵はいずれも陰性であった。これら4名は全員プラジカンテルの投与を受け，抗体価や血中好酸球数の低下などを指標として，さらに有症者では臨床症状や画像所見の改善も参照して，治癒が判定された。

原因食品の特定と食中毒としての届出

原因食品を特定するために，患者4名に対して詳しい聞き取り調査を行った。その結果，肺吸虫感染に関連する食品は，当該ホテルの中華料理店で提供されたモクズガニだけで，それ以外では，サワガニやイノシシの肉も含めて，全く口にしていないことが確認された。

このように，事例の原因食品がモクズガニ（老酒漬）であり，また患者にウェステルマン肺吸虫抗原に対する抗体応答が認められたことから，原因（物質）はウェステルマン肺吸虫と判定された。これを受けて平成16年11月30日に，地元保健所長から県知事に対して，ウェステルマン肺吸虫による食中毒として届出（食中毒事件票の提出）がなされた。

届出の後，本事例の原因について更に寄生虫学的に精査し，事故発生の背景についても改めて情報収集した。これらの成績については，別途報告した[4]。

考　察

肺吸虫の感染源としてモクズガニの老酒漬が重要であることは，従来から中国・台湾で良く知られ[1)8)]，またわが国においても症例が報告されてきた[5)~9)]。これらの症例の中には，集団感染例も含まれているが[5)9)]，食中毒として届出されたものはない。

本論文の肺吸虫症例は，食品衛生法に基づき食中毒として届出された。食品衛生法とは，「飲食に起因する衛生上の危害の発生を防止する」ことを目的とした法律である。本法の条文にある上述の「衛生上の危害」を「健康被害」と読み替え，その上で「飲食に起因する健康被害」を「食中毒」として広くとらえる，これが昨今の行政上の方針となっている[3)]。

「飲食に起因する健康被害」すなわち「食中毒」を起こす病原微生物に関しては，病因となり得る細菌やウイルスが食中毒事件票に順に記されている。一方寄生虫に関しては，食中毒事件票では「その他」という項目にまとめられ，クリプトスポリジウム，サイクロスポーラ，アニサキスが例として表示されている[3)]。これは寄生虫も，細菌やウイルスと同様に，食中毒の病因物質として取り上げるべきことを意味する。本事例はこれに該当するものとして，ウェステルマン肺吸虫を病因とする食中毒として取り扱われ，アニサキス以外の寄生蠕虫による食中毒として初めて届出された。飲食に起因する健康被害の病因が寄生虫であっても，患者（食中毒患者）を診断した医師が食品衛生法に則して届け出ることで，本事例のような食品媒介寄生虫症の発生実態がより正確に把握されるようになり，また再発の予防にも役立つと期待される[2)]。

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A NEW RECORD OF *PARAGONIMUS* OTHER THAN *P. WESTERMANI* IN SOUTHERN THAILAND

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Abstract. Field surveys of *Paragonimus* in Surat Thani Province, southern Thailand, revealed a new record of a lung fluke species other than *P. westermani*. The metacercariae were obtained from the crab, *Ranguna smalleyi*. The cysts of the metacercariae were spherical in shape and the larval body in the cysts contained pinkish granules. Fully mature adult worms were obtained from experimental infections with a rat and a ferret. The adult worms from the two host animals resembled each other, except for size, and had the anatomical characteristics of *P. bangkokensis*, *ie* the cuticular spines were arranged mainly in groups, the ovaries were highly branched, while the testes were more simply divided. Chromosomal preparations of the testes showed a haploid number of 11. As no sequence data of *P. bangkokensis* has been deposited in the GenBank/EMBL/DDBJ nucleotide database, the ITS2 region was sequenced using the metacercariae as starting material. A similarity search of *P. bangkokensis* ITS2 sequence using the BLAST program revealed that there was only one base difference between this population and *P. harinasutai* occurring in central Thailand. The result may suggest a close relationship between *P. bangkokensis* and *P. harinasutai*. This is the first description of *Paragonimus* species other than *P. westermani* occurring in southern Thailand.

INTRODUCTION

At present, there are at least 6 species of *Paragonimus* in Thailand: *Paragonimus westermani*, *P. siamensis*, *P. heterotremus*, *P. bangkokensis*, *P. macrorchis*, and *P. harinasutai* (Blair *et al*, 1999). *Paragonimus bangkokensis*, the fourth species discovered in Thailand, was reported in the central part (Nakhon Nayok: Miyazaki and Vajrasthira, 1967, and Saraburi: Srisont *et al*, 1997) as well as in the eastern part (Chantaburi: Srisont *et al*, 1997). In China, He *et al* (1982) published their first discovery of this species in Hainan Island. To date, studies concerning P. bangkokensis in Thailand have included morphology (Sugiyama *et al*, 1989; Komalamisra *et al*, 2004) and molecular genetics (Intapan et al, 2004). In all studies mentioned, only specimens obtained from central Thailand were employed.

Studies of the second intermediate hosts of *P. bangkokensis* revealed 3 species of waterfall crabs: *Potamiscus smithianus, Larnaudia beusekomae*, and *Apotamonautes hainanensis*. There was one report of *P. bangkokensis* natural infection in Indian mongooses. Experimental infections were carried out and adult worms were obtained from cats and a bandicoot (Miyazaki and Vajrasthira, 1967; He *et al*, 1982).

In the present study, we focused on *P. bangkokensis* occurring in southern Thailand. We extensively surveyed the distribution of the metacercariae in waterfall crabs in Surat Thani Province. Experimental infections with metacercariae were carried out in mammalian hosts, other than cats and bandicoots. Morphological, chromosomal, and molecular data were obtained either from the metacercarial or adult stages, or both.

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MATERIALS AND METHODS

Collection of crab intermediate hosts

Waterfall crabs were collected along mountain streams in Pa Nom district, Surat Thani Province during the period January 2003-May 2004. The crabs were identified as *Ranguna smalleyi* according to Naiyanetr (1988). These crabs were further examined for *Paragonimus* metacercariae.

Examination of *Paragonimus* metacercariae

The crabs were ground and digested with pepsin (1%, w/v) and concentrated hydrochloric acid (0.7%, v/v). The samples were filtered through wire sieves (pore sizes 300 and 500 μm), and 0.9% physiological saline was added. They were allowed to stand for approximately one hour and the sediment was

examined under a stereomicroscope for *Paragonimus* metacercariae. Morphological observations and measurements of the metacercariae obtained were carried out.

Adult worms from test rat and ferret

The metacercariae were infected into a Wistar rat and a ferret to raise them to adult worms. The feces of these test animals were examined for *Paragonimus* eggs after approximately 60 days using the formalin-ether concentration method. The results of the experimental infections are shown in Table 1. To prepare permanent specimens for morphological observation, recovered worms were pressed between two glass slides, fixed in 70% ethanol, stained with borax carmine, and mounted with Canada balsam.

Chromosomal preparations from adult worms

The Giemsa air-dried preparation of chromosomes from testes followed Sugiyama *et al* (1985). Photomicrographs of a total of 30 cells with well-spread meiotic metaphase chromosomes were taken and a haploid number of chromosomes obtained.

DNA amplification and sequencing of the ITS2 region

DNA samples were prepared from the metacercariae, as previously described by Sugiyama *et al* (2002). DNA was amplified by PCR using primers 3S (Bowles *et al*, 1995) and A28 (Blair *et al*, 1997) for the ITS2 region of the nuclear ribosomal DNA. The conditions for the PCR reactions followed Sugiyama *et al* (2002). The resultant PCR products were sequenced using the corresponding primers and the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, USA) on an automated DNA sequencer (ABI310, Applied Biosystems). The consensus sequence obtained was compared to sequences in a database (http://www.ncbi.nlm.nih.gov/BLAST/).

RESULTS

New crab intermediate host of *P. bangkokensis*

In January 2003 and May 2004, field surveys of *P. bangkokensis* were carried out in Surat Thani Province. A total of 1,001 freshwater crabs were examined. These crabs, belonging to the family Potamidae, called *Ranguna smalleyi* (Fig 1), are recorded as a new crustacean host of *P. bangkokensis*.

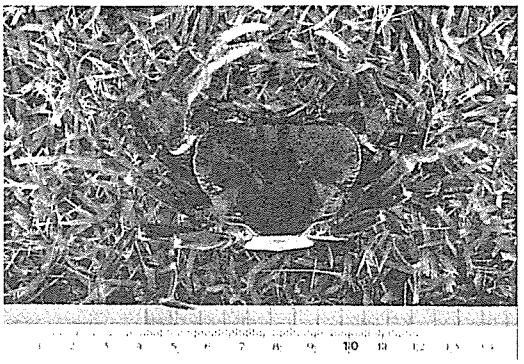

Fig 1- *Ranguna smalleyi*, a new crab intermediate host of *P. bangkokensis* collected in southern Thailand.

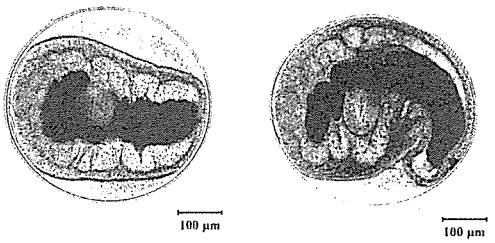

Fig 2- *Paragonimus bangkokensis* metacercariae obtained in southern Thailand. Scale bar = 100 µm.

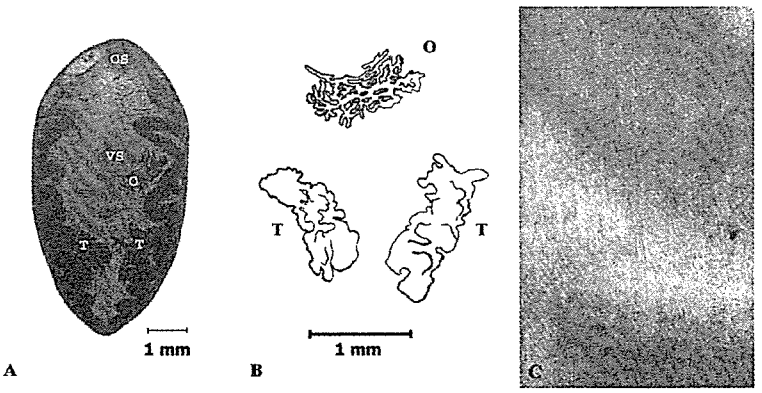

Fig 3- Adult worm obtained from a test rat. A: Photomicrograph of the adult worm (OS: oral sucker, VS: ventral sucker, O: ovary, T: testes). B: Drawing of the ovary and testes. C: Photomicrograph of cuticular spines. Scale bar = 1 mm.

Table 1
Experimental infections of rat and ferret with metacercariae of *P. bangkokensis*.

Test animals	Dose of mc[a]	Duration of infection (days)	Rate of mc[a] recovered as adult flukes (%)	No. of adult flukes recovered		
				Lungs	Liver	Total
Rat	11	111	18	1	1	2
Ferret	7	239	43	3	-	3

[a] mc = metacercariae

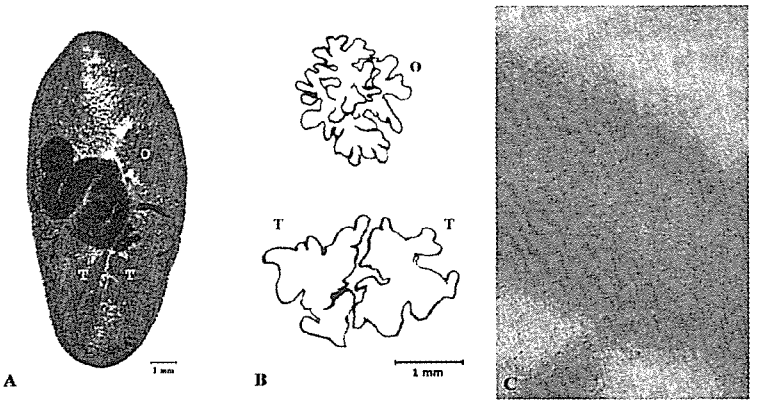

Fig 4- Adult worm obtained from a test ferret. A: Photomicrograph of the adult worm (O: ovary, T: testes). B: Drawing of the ovary and testes. C: Photomicrograph of cuticular spines. Scale bar = 1 mm.

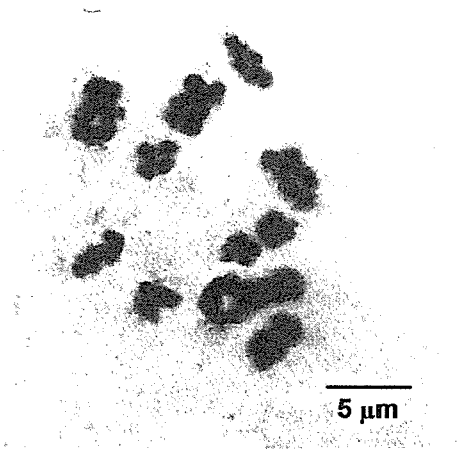

Fig 5- A photomicrograph of the meiotic metaphase chromosomes with a haploid number of 11 (n = 11). Scale bar = 5 µm.

Characteristics of metacercariae

A total of 55 metacercariae were recovered from all crabs examined. Fig 2 shows representative metacercariae, which were spherical in shape; the inner membrane was approximately 13 µm thick. In 25 fresh specimens, excluding the outer membrane, the longitudinal and transverse diameters of the inner cyst ranged from 521-379 x 469-365 µm (average 437 x 422 µm). The larval bodies contained pinkish granules.

Adult worms from test animals; morphology and number of chromosomes

After *Paragonimus* eggs were detected in feces, the rat and ferret were sacrificed. Table 1 illustrates the number of adult flukes obtained at necropsy.

Five adult worms were obtained from the rat and ferret; thus, these mammals became the new definitive hosts of *P. bangkokensis*. In 3 mounted specimens of adult worms from the rat (Fig 3A) and ferret (Fig 4A), the sizes of the worms were different. Two specimens from the rat had an average size of 7.60 x 3.75 mm, with length 7.5-7.7 mm and width 3.7-3.8 mm. The average transverse diameters of the oral and ventral suckers measured 590 µm and 597 µm, respectively. The ovary was highly branched, while the testes were much more simply divided (Fig 3A and 3B). The cuticular spines were arranged in groups (Fig 3C). One specimen from the ferret showed a larger body size, with length and width 13.40 x 6.30 mm. The transverse diameters of the oral and ventral suckers were 1.06 and

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Pb: TGTCGATGAAGAGCGCAGCCAACTGTGTGAATTAATGTGAACTGCATACTGCTTTGAACA
Ph: TGTCGATGAAGAGCGCAGCCAACTGTGTGAATTAATGTGAACTGCATACTGCTTTGAACA

Pb: TCGACATCTTGAACGCATATTGCGGCCACGGGTTAGCCTGTGGCCACGCCTGTCCGAGGG
Ph: TCGACATCTTGAACGCATATTGCGGCCACGGGTTAGCCTGTGGCCACGCCTGTCCGAGGG

Pb: TCGGCTTATAAACTATCGCGACGCCCAAAAAGTCGCGGCTTGGGTTTTGCCAGCTGGCGT
Ph: TCGGCTTATAAACTATCGCGACGCCCAAAAAGTCGCGGCTTGGGTTTTGCCAGCTGGCGT

Pb: GATTTCCCCGATCTGACCTTGTGTCGGTGGGGTGCCAGATCTATGGCGTTTCCCTAACCT
Ph: GATTTCCCCGGTCTGACCTTGTGTCGGTGGGGTGCCAGATCTATGGCGTTTCCCTAACCT
                 *

Pb: GTCCGGGCGTACCCATGTTGTGGCTGAAGGCCTTGGTGGGGATGTGGCAACGGAATCGTG
Ph: GTCCGGGCGTACCCATGTTGTGGCTGAAGGCCTTGGTGGGGATGTGGCAACGGAATCGTG

Pb: GCTCAGTGAATTATTTATGTGCGCGTTCCGTCGTCCTATCATCATCTATGGTTGATGCTG
Ph: GCTCAGTGAATTATTTATGTGCGCGTTCCGTCGTCCTATCATCATCTATGGTTGATGCTG

Pb: CGTGTGGTGTGCGTCTGATGCTGACCTGAGTATGTGCCATGTGGCTCATTCTCCTGACCT
Ph: CGTGTGGTGTGCGTCTGATGCTGACCTGAGTATGTGCCATGTGGCTCATTCTCCTGACCT

Pb: CGGATCAGACGTGAGTACCCGCTGAACTTAAGCATATCACTAA
Ph: CGGATCAGACGTGAGTACCCGCTGAACTTAAGCATATCACTAA
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Fig 6- Similarity search result of the ITS2 sequences from *P. bangkokensis* (Pb) and *P. harinasutai* (Ph). An asterisk (*) indicates a variable region.

1.18 mm, respectively. Other structures, including the ovary, testes (Fig 4A and 4B), and cuticular spines (Fig 4C) resembled those of the rat.

In the present study, chromosomal preparations of the testes from one adult worm showed many well-spread meiotic metaphase chromosomes. Fig 5 is a photomicrograph of the chromosomes with a haploid number of 11 (n = 11).

Genetic variation of *P. bangkokensis* based on the ITS2 region

The result of the ITS2 sequence similarity search of *P. bangkokensis* showed only one base difference between this population and *P. harinasutai* occurring in central Thailand (GenBank accession number AF159609) (Fig 6).

DISCUSSION

In southern Thailand, *P. bangkokensis* metacercariae were discovered in the new crab intermediate host, *R. smalleyi*. Similar to the previous report by Miyazaki and Vajrasthira (1967), the metacercariae were spherical in shape and the average longitudinal and transverse diameters of the inner cyst were 437 x 422 μm. The larval bodies contained pinkish granules. In addition, the morphological characteristics of the adult worms obtained from the new mammalian hosts (the rat and ferret) were also identical to those described by Miyazaki and Vajrasthira (1967). These worms possessed a highly branched ovary, more simply divided testes, clustered cuticular spines, and a haploid chromosome number of 11. In terms of the genetic variation of *P. bangkokensis* based on the ITS2 region, there was only a single base difference between this population and *P. harinasutai* occurring in central Thailand. The result may suggest a close relationship between *P. bangkokensis* and *P. harinasutai*. This agreed with the morphological characteristics of both species, except for the arrangement of the cuticular spines.

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【实验报道】

哈氏并殖吸虫 ITS2 基因和 CO1 基因序列分析

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【摘要】 **目的** 进一步确认浙江宁海哈氏并殖吸虫(*Paragonimus harinasutai*)分类地位及其遗传变异。 **方法** 从溪蟹中分离获得哈氏并殖吸虫囊蚴，单个囊蚴经 PCR 扩增，进行 ITS2 基因和 CO1 基因序列分析；ITS2-PCR 扩增产物 *Bsa*HI、*Stu*I 酶切。 **结果** ITS2-PCR 扩增产物经 *Bsa*HI 和 *Stu*I 酶切 (PCR-RFLP)，琼脂糖凝胶电泳分离图谱基本一致。浙江宁海哈氏并殖吸虫 ITS2 基因有 366 碱基对，与泰国种群 (AF159609) 核酸同源性为 95.6%，CO1 基因有 390 碱基对，与泰国种群 (AF159600)核酸同源性为 89.5%。 **结论** 本研究从分子生物学角度进一步确认浙江宁海哈氏并殖吸虫分类地位,但与泰国种群(Nakorn-nayok 省)相比较，存在较大变异。

【关键词】 哈氏并殖吸虫；分类；序列分析

中图分类号：R383.3 文献标识码:A

Sequence Analysis of ITS2 and CO1 Genes of *Paragonimus harinasutai*

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【**Abstract**】 **Objective** To identify *Paragonimus harinasutai* from Ninghai, Zhejiang Province, China. **Methods** Metacercariae were collected from the crabs *Sinopotamon chekiangenes* in Xixi villages of Ninghai County for ITS2 sequence analysis, CO1 sequence analysis and endonuclease *Bsa*HI and *Stu*I analysis by PCR-RFLP. **Results** The fingerprintings of PCR-RFLP were virtually same to the isolate from Thailand (Nakorn-nayok). The ITS2 sequence with 366 bp and CO1 sequ-ence with 390 bp of the metacercariae collected from Ninghai revealed a nucleotide identity 95.6% and 89.5% respectively to the Thai isolate. **Conclusion** The study confirmed that *Paragonimus harinasutai* is present in Ninghai, China, with certain variation on molecular biology in comparison to the Thai isolate.

【**Key words**】 *Paragonimus harinasutai*; Classification; Sequence analysis

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哈氏并殖吸虫 (*Paragonimus harinasutai*) 于 1968 年 Miyazaki 等[1]在泰国 Nakorn-nayok 省 Sarika 村首次报道并订为新种。当时从 *Potamon smithianus* 溪蟹中获得大型(平均 601 μm×579 μm)微红色囊蚴，人工感染猫和狗获得成虫。此后 Blair 等[2]对该种进行 CO1 和 ITS2 序列分析。至今未见人体感染报道。1985 年 11 月间，作者在浙江省宁海县进行肺吸虫病流行病学调查时，从浙江华溪蟹(*Sinopotamon chekiangenes* Taiet Sung, 1975)分离出一种微红色的大型囊蚴。分离 1 000 只溪蟹，获这种囊蚴 79 只。感染 3 只猫后，从肺脏获成虫 44 条。标本经初步鉴定暂定名为并殖科哈氏并殖吸虫[3]。中国大陆并殖吸虫种类繁多，鉴定需十分慎重。于 2004 年将模式标本送往泰国 Mahidol 大学热带医学学院重新鉴定。从形态学上确认为哈氏并殖吸虫。模式标本(一正二付)保存于浙江省医学科学院生物工程研究所。本研究对浙江宁海县哈氏并殖吸虫分子生物学初步研究，从分子生物学角度进一步确定浙江宁海哈氏并殖吸虫的分类地位及其遗传变异。

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材料和方法

1 标本采集

浙江华溪蟹于 2004 年采自浙江宁海西溪冷风洞。从 100 只溪蟹中获得 5 只哈氏并殖吸虫囊蚴。分离囊

蚴 70%酒精固定备用。

2 DNA 提取

DNA 抽提和纯化按 Sugiyama等[4]方法进行：单个囊蚴经双蒸水洗涤后置 100 μl 含蛋白酶 K 的 DNA 抽提液[10 mmol/L Tris-HCl，PH 8.0，10 mmol/L 乙二胺四乙酸钠(EDTA)，0.1%十二烷基硫酸钠(SDS)，0.2 mg/ml 蛋白酶 K]60 ℃ 2 h，95 ℃灭活 10 min。酒精纯化 DNA，三羟基氨基甲烷乙二胺四乙酸(TE 缓冲液)保存。

3 ITS2、CO1 基因扩增和序列分析

ITS2 基因 PCR 扩增引物选用：3S′ 5′-GGTAC C-GGTG GATCA CTCGG CTCGT G-3′，A28 5′-GGGAT CCTGG TTAGT TTCTT TTCCT CCGC-3′[4]。CO1 基因 PCR 扩增引物选用：JB3：5′-TTTTT TGGGC ATCCT GAGGT TTAT-3′；JB4：5′-TAAAG AAAGA ACATA ATGAA AATG-3′[5]。PCR 扩增条件为 98 ℃，5s；55 ℃，10 s；72 ℃，10 s；35 个循环。PCR 产物经 QIAEXII (德国 Qiagen 公司) 纯化，在自动序列分析仪进行序列分析 (AB1310，Applied Biosysteme，日本，国家感染病研究所寄生动物部)。

4 PCR 扩增产物酶切(PCR-RFLP)

ITS2-PCR 扩增产物经 *Bsa*HI 和 *Stu*I 酶切(37 ℃，12 h)，2%琼脂糖凝胶电泳分离，100 bp DNA 序列(美国 Invitrogen 公司)为分子标记。

5 资料分析

PCR-RFLP 琼脂糖凝胶电泳分离图谱用 Kodak 1D 软件分析 DNA 片断长度；序列分析结果与 Gen-Bank 中哈氏并殖吸虫 ITS2 和 CO1 基因序列进行对比，并计算核酸同源性。

结 果

1 ITS2-PCR 扩增产物经 *Bsa*HI 和 *Stu*I 酶切

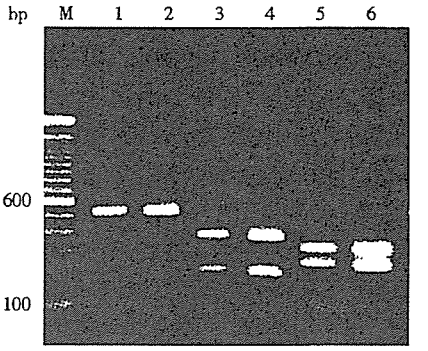
浙江哈氏并殖吸虫种群 PCR-RFLP 琼脂糖凝胶电泳分离图谱，与泰国哈氏并殖吸虫种群基本一致 (图1)。ITS2-PCR 扩增产物约 560 bp，*Bsa*HI-PCR 扩增产物约 340bp 和 220bp，*Stu*I-PCR 扩增产物约 300bp 和 260bp。

2 ITS2 基因序列分析

浙江宁海哈氏并殖吸虫 ITS2 基因有 366 碱基对，与泰国种群(AF159609)对比：有 16 个碱基发生了变化，浙江宁海种群 268-270 位点(GTC)碱基为泰国种群所没有，同源性为 95.6%(图 2)。

3 CO1 基因序列分析

浙江宁海哈氏并殖吸虫 CO1 基因有 390 碱基对，与泰国种群 (AF159600)对比：有 41 个碱基发生了变化，同源性为 89.5% (图 3)。



M：DNA 标志物，1：浙江宁海种群 ITS2 基因 PCR 产物，2：泰国种群 ITS2 基因 PCR 产物，3：浙江宁海种群 *Bsa*HI 酶切图谱，4：泰国种群 *Bsa*HI 酶切图谱，5：浙江宁海种群 *Stu*I 酶切图谱，6：泰国种群 StuI 酶切图谱。

M: DNA marker, 1: PCR product of ITS2 from China, 2: PCR product of ITS2 from Thailand, 3: The fingerprinting for *Bsa*HI from China, 4: The fingerprinting for *Bsa*HI from Thailand, 5: The fingerprinting for *Stu*I from China, 6: The fingerprinting for *Stu*I from China.

图 1 哈氏并殖吸虫 PCR 扩增产物经 *Bsa*HI 和 *Stu*I 酶切琼脂糖凝胶电泳分离图谱

Fig.1 The fingerprinting of PCR-RFLP by endonuclease *Bsa*HI and *Stu*I

```
PhariZJ     ATATTGCGGC CACGGGTTAG CCTGTGGCCA CGCCTGTCCG AGGGTCGGCT
AF159609    ..........  ..........  ..........  ..........  ..........

51 TATAAACTAT CGCGACGCCC AAAAGGTCGC GGCTTGGGTT TTGCCAGCTG
   ..........  ..........  .....A....  ..........  ..........

101 GCGTGATTTC CCCAATCTGA CCTTGTGTCA GTGGGGTGCC AGATCTATGG
    ..........  .....GG....  ..........  .....G....  ..........

151 CGTTTCCCTA ACCTATCCGG GCGTACCCAT GTTGTGGCTG AAGGCCTTGG
    ..........  .....G....  ..........  ..........  ..........

201 TGGGGATGTG GCAACGGAAT CGTGGCTCAG TGAAGTATTT ATGTGCGCGT
    ..........  ..........  ..........  .....T....  ..........

251 TCCGTCGTTT TATCATCGTC ATCTATGGTT GATGCTGCGT ATGGTGTGCG
    ..........CC..........  ..........  ..........  .....G....

301 TCCGATGCTA ACCTACGTAT GTGCCATGTG GCTCATTCTC CTGACCTCGG
    ...T.....G  .....GA....  ..........  ..........  ..........

351 ATCAGACGTG AGTACC
```

图 2 浙江宁海和泰国二地哈氏并殖吸虫 ITS2 基因序列比较

Fig.2 ITS2 nucleotide sequence of *P. harinastai* from China and Thailand

```
AF159600    1 TTTGATTTTA CCGGGATTTG GAGTTGTAAG TCATATTTGC ATGACTTTGA CTAATAAAGA
PhariZJ-CO1 .....T....G.....G.....G.....T.....

61 TTCGCTGTTT GGTTATATG GGTTGGTGTT CGCTATGGGG GCGATTGTTT GTTTGGGTAG
   .....T.....T.....A.....T.....G.....A....

121 TGTTGTGTGA GCCCATCATA TGTTTATGGT TGGTTTAGAT GTTAAGACCG CGGTGTTTTT
    .....T.....C.....C..T.....T.....

181 TAGATCTGTT ACTGGGGTGA TAGGTATCCC GACAGGGATT AAGGTTTTTT CCTGGTTGTT
    .....T.....G.....T.....T.....A.....

241 TATGTTGGGT GGCACTCGTT TGCGGTTTTG AGATCCTGTA ATATGGTGAA TTTTGGGGTT
    .....C.....G.....A.....G.....A....

301 TATTTTTCTG TTTACTATTG GTGGTGTGAC TGGAATTATT TTGTCTTCCT CTATTTTGGA
    .....T.....G.....A.....C C..A.....C..

361 TAGTTTGTTG CATGATACCT GATTTGTGTT
    .....AC.....C.....G.....
```

图 3 浙江宁海和泰国二地哈氏并殖吸虫 CO1 基因序列比较

Fig.3 Co1 nucleotide sequence of *P. harinastai* from China and Thailand

讨 论

浙江宁海哈氏并殖吸虫成虫标本狭长的体型，腹吸盘大于口吸盘，腹吸盘位于虫体前三分之一，卵巢、睾丸形态，囊蚴带微红色及其大小等特征，与 Miyazaki 等[1]在泰国首次报道哈氏并殖吸虫特征相符。但我国虫种有一些不甚相同的特征[6]：如混生型皮棘，泰国标本通过实验感染 381 d 获得，其皮棘仍为单生型。而浙江标本，从胸腔获一不成熟虫体，从腹吸盘后已有丛生棘分化，可见我国标本混生棘的特征较为稳定。又如囊蚴壁也不同。囊蚴壁的有无，层数和厚薄在种的鉴别上有着重要的意义。但另一方面，囊壁的厚薄也可能受到中间宿主的影响，不同的中间宿主对后尾蚴会有不同影响，可能因此有所区别。两地虫种的卵巢、睾丸也可能有不同变异，但因新种描述十分简单，难以确切比较。

一个种和另一个种的区别不能单纯根据孤立的特征决定，应该考虑虫体的变化是外因和内因经过漫长的时间所造成的[7,8]。泰国与我国浙江省相隔遥远，长期的地理隔离和不同中间宿主，可能造成同一虫种的变异，要肯定一种新种最好通过生活史各期发育的比较以及生物化学和分子生物学等方面的研究。利用分子生物学技术比传统的形态学分类具有客观性。研究并殖吸虫种间和种内遗传差异主要通过对核糖体基因第二间隔区(ITS2)和部分 CO1 基因序列分析。ITS2 属于核基因组，相对保守稳定，已广泛应用于并殖吸虫种间变异研究，而 CO1 基因属于线粒体基因组，应用于并殖吸虫种内变异研究[9]。为进一步从分子生物学方面确定浙江宁海虫株的分类地位，本研究于 2004 年再次从西溪冷风洞采集哈氏并殖吸虫囊蚴进行分子生物学研究。结果表明 ITS2-PCR 扩增产物经 *Bsa*HI 和 *Stu*I 酶切，PCR-RFLP 琼脂糖凝胶电泳分离图谱与泰国哈氏并殖吸虫基本一致；浙江宁海哈氏并殖吸虫 ITS2 基因有 366 碱基对，而泰国种群(AF159609)仅 363 对碱基，有 16 个碱基位点不同，同源性为 95.6%；浙江宁海哈氏并殖吸虫 CO1 基因有 390 对碱基，与泰国种群(AF159600)同源性为 89.5%，有 41 个碱基位点不同。研究结果从分子生物学角度进一步确认浙江宁海虫株为哈氏并殖吸虫，与泰国虫株相比较，两者存在较大遗传变异。

以往的实验表明，人工感染家猫成功证明猫为适宜终末宿主，3 只家猫感染后成虫回收率分别为 60.0%、83.3%和 85.7%[3]。虫体在宿主体内发育良好，并最后寄生于肺脏。浙江宁海县是以卫氏并殖吸虫为主的肺吸虫病流行区，哈氏并殖与卫氏并殖二种吸虫囊蚴同时存在于浙江华溪蟹内，因此对哈氏并殖吸虫在浙江自然终宿主及对人的致病性，以及该虫在我国的分布情况有待于进一步研究。

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浙江宁海卫氏并殖吸虫ITS2序列分析*

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摘要 为了进一步了解浙江宁海卫氏并殖吸虫（*Paragonimus westermani*）的遗传变异情况，对卫氏并殖吸虫不同种群进行ITS2序列分析研究。分别自浙江宁海小汀、宁海西溪（冷风洞）和宁海余山采集浙江华溪蟹，分离卫氏并殖吸虫囊蚴并进行ITS2序列PCR扩增分析。结果表明：浙江宁海三地卫氏并殖吸虫ITS2序列有363个碱基对，肺型肺吸虫和非肺型肺吸虫的ITS2序列完全相同；与泰国种群（AF159604，Nakorn Nayok省）核酸同源性为97.5%，两地卫氏并殖吸虫遗传变异较大。浙江宁海卫氏并殖吸虫与日本、韩国等地的卫氏并殖吸虫的核酸同源性均较高，属亚洲东北组群。

关键词 卫氏并殖吸虫；ITS2；中国；泰国

浙江省宁海市是肺吸虫病流行区，其中小汀村是典型肺型肺吸虫病流行区。为了进一步了解浙江宁海卫氏并殖吸虫遗传背景，对宁海肺型肺吸虫和非肺型肺吸虫种群进行ITS2序列分析研究。

1 材料与方法

1.1 标本采集

浙江华溪蟹（*Sinopotamon chekiangenes* Taiet Sung，1975）分别采自浙江宁海小汀、宁海西溪（冷风洞）和宁海余山。分离卫氏并殖吸囊蚴大小为360～400 μm，囊蚴70%酒精固定备用。

1.2 DNA提取

DNA抽提按Sugiyama方法进行（Sugiyama *et al*., 2002）：单个囊蚴水化后置100uL含蛋白酶K的lysis缓冲液（10 mol/L Tris-HCl，pH 8.0，10 mol/L EDTA，0.1% SDS，0.2mg/mL Proteinase K），60℃ 2h裂解抽提DNA，95℃灭活蛋白酶K10min。酒精纯化DNA，TE缓冲液保存。

1.3 PCR扩增和ITS2分析

ITS2序列PCR扩增引物选用3S′和A28：3S′ 5′-GGTAC CGGTG GATCA CTCGG CTCGT G-3′，A28 5′-GGGAT CCTGG TTAGT TTCTT TTCCT CCGC-3′。PCR扩增条件为：98℃，5s；55℃，10s；72℃，10s。35个循环。PCR产物经QIAEXII（Qiagen，Germany）纯化，在自动序列分析仪进行序列分析（AB1310，Applied Biosysteme，日本，国家感染病研究所寄生动物部）。

1.4 资料分析

序列分析结果与GenBank中卫氏并殖吸虫ITS2序列进行对比，并计算核酸同源性。

2 结果

浙江宁海小汀、宁海西溪（冷风洞）和宁海余山三地卫氏并殖吸虫ITS2序列有363个碱基对，序列完全相同：

ATATTGCGGC CACGGGTTAG CCTGTGGCCA
CGCCTGTCCG AGGGTCGGCT TATAAACTAT
CGCGACGCCC AAAAAGTCGC GGCTTGGGTT
TTGCCAGCTG GCGTGATCTC CCCAATCTGG
TCTTGTGCCT GTGGGGTGCC AGATCTGTGG
CGTTTCCCTA ACATACTCGG GCGCACCCAC
GTTGCGGCTG AAAGCCTTGA CGGGGATGTG
GCAACGGAAT CGTGGCTCAG TGAATGATTT
ATGTGCGCGT TCCGCTGTCC TGTCTTCATC
TGTGGTTTAT GTTGCGCGTG GTCTGCTTTC

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GATGCTGACC TACGTATGTG CCATGTGGTT CATTCTCCTG ACCTCGGATC AGACGTGAGT ACC

浙江宁海卫氏并殖吸虫 ITS2 序列与泰国种群（AF159604，Nakorn Nayok 省）核酸同源性为 97.5%，与亚洲其它国家（地区）卫氏并殖吸虫种群核酸位点差异的比较见表 1。

表 1　浙江宁海卫氏并殖吸虫 ITS2 序列与亚洲其它国家种群的比较

Tab.1　ITS2 sequence comparison of *Paragonimus westermani* populations from Ninghai Zhejiang and some Asian countries

	58	147	170	217	230	232	252	278	297	298	316	330	350
浙江宁海小汀 Xiaoting, Ninghai	T	G	G	G	G	G	C	T	T	T	A	T	C
浙江宁海西溪 Xixi, Ninghai	T	G	G	G	G	G	C	T	T	T	A	T	C
浙江宁海余山 Yushan, Ninghai	T	G	G	G	G	G	C	T	T	T	A	T	C
中国台湾 U96908 Taiwan, China	T	G	G	G	A	G	C	T	T	T	A	T	C
日本 U96907 Japan	T	G	G	G	G	G	C	T	T	T	A	T	C
韩国（3n）AF333277 Korea (trip)	T	G	G	G	G	G	C	T	T	T	A	T	C
韩国（2n）AF333278 Korea (dip)	T	G	G	G	G	G	C	T	T	T	A	T	C
韩国（3n）AF333276 Korea (trip)	T	G	G	G	A	G	C	T	T	T	A	T	C
泰国 AF159604 Tailand	C	A	C	G	G	A	T	C	G	T	A	C	T
菲律宾 U96910 Philippines	T	A	G	A	G	A	T	T	G	A	T	T	C
马来西亚 U96909 Malaysia	T	A	G	A	G	A	T	T	G	A	A	T	C

3　讨论

核糖体基因第二间隔区（ITS2）属于核基因组，相对保守稳定，已广泛应用于并殖吸虫种间和种内变异研究。近年来对卫氏并殖吸虫 ITS2 和 COI 基因研究，Blair 等（1997）认为亚洲卫氏并殖吸虫可以分为两大群：遗传距离相对亲近的东北组群（中国大陆和台湾、日本、韩国）和遗传距离彼此较远的南部组群（马来西亚、泰国和菲律宾）；本项研究表明，浙江宁海小汀、宁海西溪（冷风洞）和宁海余山肺吸虫 ITS2 基因序列完全相同；浙江宁海卫氏并殖吸虫属亚洲东北组群。

卫氏并殖吸虫的致病性的品系长期以来有广泛的研究和较多争议。由于其成虫、囊蚴、虫卵等形态较一致，很难从形态上区别肺型和非肺型不同的品系，给该病的诊断和治疗带来了困难。国内部分学者提出大品系和小品系囊蚴的区别：认为大品系能引起典型肺部症状，而小品系则不能。钱宝珍对浙江省 10 县（市）卫氏并殖吸虫成虫形态观察，结果证实该观点并不完全正确（钱宝珍，2004）。Miyazaki（1978）曾用染色体和等位基因酶的差别解释致病性上的差异，提出三倍体型（triploid type）并殖吸虫能引起典型的肺部症状。但这种观点也不能在中国大陆卫氏并殖

吸虫致病种群中得到证实，国内研究人员通过浙江典型肺型吸虫病流行区绍兴兰亭、宁海小汀和浙江永嘉卫氏并殖吸虫种群染色体研究结果认为，二倍体型（diploid type）或嵌合型（mosaic type）也可能引起典型肺部症状（王芃芃等，1986；段云芬等，1994；洪家林等，2003）。随着分子生物学技术，尤其是 PCR 技术和核酸自动测序技术的迅速发展，分子生物学技术应用于并殖吸虫的研究已深入展开。Park 等（2003）对韩国的二倍体和三倍体卫氏并殖吸虫比较研究发现 ITS2 基因序列完全相同（AF333277，AF333278）或差异很小（AF333276）。Agatsuma 等（1994）应用限制性为内切酶 DNA 多态技术（RFLP，restriction fragment length polymorphism）对卫氏并殖吸虫不同种群线粒体 DNA 进行初步研究。结果表明，应用内切酶 *Pst* Ⅰ，*Hea* Ⅲ和 *Rsa* Ⅰ可区别日本二倍体和三倍体型染色体肺吸虫。但由于染色体差异并不能正确解释其致病性，该研究仍不能区分肺型和非肺型不同品系问题。

浙江宁海小汀村曾是肺型肺吸虫病流行区，44 例病人有咳血、咳痰症状，在痰液中均能检获虫卵（寿干城等，1987）。而宁海西溪村，溪蟹感染率和感染强度远高于小汀村，当地居民也有同样食用生蟹生活习惯，但长期以来未见典型肺吸虫病病人，研究结果表明浙江宁海卫氏并殖吸虫肺型肺吸虫和非肺型肺吸虫 ITS2 序列完全相同。钱宝珍和沈琦（2006）曾应用随机扩增多态 DNA（PCR-RAPD，PCR-based random amplified polymorphic DNA）技术，对浙江宁海小汀、宁海西溪、遂昌、临安等地卫氏并殖吸虫囊蚴进行遗传变异研究。结果提示 B 17～1 600 bp，A 9～680bp 可作为卫氏并殖吸虫不同致病品系的分子标记。与其他 DNA 技术相比，PCR-RAPD 具有快速、简便、敏感、可比性强的特点，特别适用于生物种群间遗传变异和种群分子标记的研究。因此稳定 PCR-RAPD 反应条件，解决 PCR-RAPD 稳定性和重演性问题；可望解决卫氏并殖吸虫致病种类分子标记问题。

本研究属国家科技部中国泰国合作项目，该项目以中国浙江省和泰国 Nakhorn Nayok 省肺吸虫病流行区为现场，研究中国和泰国双方肺吸虫病的致病种类，以期建立致病虫株分子标记和遗传变异的检测方法。用此研究方法进行中国浙江省与泰国 Nakhorn Nayok 省的卫氏并殖吸虫 ITS2 序列对比。结果表明两地卫氏并殖吸虫遗传变异较大，同源性为 97.5%。这是否可作为泰国卫氏并殖吸虫未见人群感染报道的遗传背景有待进一步研究。

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ITS2 SEQUENCE OF *PARAGONIMUS WESTERMANI* IN NINGHAI, ZHEJIANG

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Abstract In order to understand the genetic variation of *Paragonimiasis westermani* in Ninghai county, Zhejiang province, the metacecariae were sampled from Xiaoting, Xixi and Yushan village respectively for their ITS sequences analysis. Results indicated that all ITS of the three populations contained 363 base pairs nucleotide acid with the same sequence. The similarity of *P. westermani* ITS sequence was 97.5% between the *P. westermani* populations in Zhejiang and the population from Nakorn Nayok province (AF159604) in Thailand, which appeared lower compared to those between the three *P. westermani* populations in Zhejiang and populations in Japan Korea and Taiwan province of China, so the *P. westermani* populations obtained in the three villages in Ninghai should belongs to Northeast Asia group.

Key words *Paragonimus westermani*; ITS2; China; Thailand

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＜国内情報＞

2004年秋に集団発生したウェステルマン肺吸虫による食中毒事例について

モクズガニの老酒漬（酔蟹）を原因とするウェステルマン肺吸虫の集団感染事例に関して報告する。佐賀県の某ホテル内にある中華料理店が，2004年９～11月に，モクズガニの老酒漬を非加熱で提供し，本事例が発生した。老酒漬は地元産のモクズガニを食材とし，老酒（アルコール分：20%）と醤油とを１：３の割合で混ぜた漬け汁（砂糖等で調味）に４，５日間漬け込むという，中華料理を模して調理したものである。同時にシナモクズガニ（いわゆる上海ガニ）も食材として使用されたが，こちらは輸入品であるため，食中毒予防への配慮から，すべて加熱調理して提供されていた。

集団感染発見の契機は，神奈川県の病院に呼吸器症状で入院していた同県内の在住者が，2004年11月にウェステルマン肺吸虫感染と血清診断されたことにある。患者のモクズガニ喫食を知った担当医が，「ウェステルマン肺吸虫による食中毒疑いの患者が入院している。」と，まず神奈川県に連絡し，神奈川県が佐賀県に事例の発見を知らせた。

佐賀県の調査により，当該店においてモクズガニを喫食したことが確認された者は114名に上った。医療機関受診の結果，肺吸虫の感染者は計４名であることが分かった。上述の患者を含む２名（もう１名は福岡県在住者）は呼吸器症状を呈し，他の２名（福岡県および佐賀県在住者）は血清反応のみ陽性の無症者であった。これら４名は駆虫剤プラジカンテルの投与を受け，抗体価の低下などを指標とし，治癒が判定された。

原因食品のモクズガニは，関係者からの聞き取り調査等の結果，県北西部を流れる玉島川で捕獲されたことが明らかとなった。そこで，69匹を入手して肺吸虫の有無を調べたところ，13匹からメタセルカリアが検出された（寄生率19%）。陽性個体の中には，全身から167個のメタセルカリアが検出され，このうち124個が可食部である筋肉に寄生するカニもいた。このメタセルカリアと，試験ネコに感染させて得た成虫の形態を精査し，本事例の原因はウェステルマン肺吸虫（３倍体型）であることを確定した。

肺吸虫の感染源としてモクズガニの老酒漬が重要であることは，従来から中国・台湾で良く知られ，またわが国においてもこれを原因とする集団発生例が報告されてきた[2,3]。モクズガニは肺吸虫の感染源（中間宿主）であり，加熱調理してから喫食することが周知されていると思われたが，原因施設である料理店の調理担当者はこれを知らず，今回の事故を発生させた。このような形での肺吸虫症の発生を予防するには，料飲店関係者に対して，モクズガニ（やサワガニ，さらにシナモクズガニ）は十分に加熱して提供するように，改めて徹底した啓発を行う必要がある。

本事例は，ウェステルマン肺吸虫を原因とする食中毒として食品衛生法に基づいて取り扱われ，寄生蠕虫（多細胞の寄生虫）としてはアニサキス以外で初めて

食中毒として届け出られた。「飲食に起因する健康被害」を「食中毒」として広くとらえ，健康被害（食中毒）の病因が寄生虫である場合でも，患者（食中毒患者）を診断した医師が食品衛生法に則して届け出る[1]ことで，本事例のような食品媒介寄生虫症の発生実態がより正確に把握されるようになり，再発の予防にも役立つものと期待される。

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First Record of *Leptospira borgpetersenii* Isolation in the Amami Islands, Japan

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***Abstract*: In 2003, a *Leptospira* survey was performed on Yoroshima Island of the Amami Islands located in the southwestern part of Japan. Seven *Leptospira* strains were isolated from the field rat *Rattus rattus*, which were identified as *L. borgpetersenii* by *flaB* sequencing, 16S rDNA sequencing and *gyrB* sequencing, and serovar Javanica was determined by a microscopic agglutination test. *Not*I-long restriction fragment analysis indicated that these isolates were genetically indistinguishable from an isolate from the Okinawa Islands. The present results suggest that *L. borgpetersenii* is migrating into the Amami Islands in Japan.**

***Key words*: *Leptospira*, Amami Island, LRFP, Field rat**

Leptospirosis is a worldwide zoonosis caused by infection with spirochetes belonging to the genus *Leptospira*. Leptospirosis is acquired through direct contact with animal reservoirs and/or an environment contaminated by their urine, and causes a wide range of clinical manifestations (4). In subtropical/tropical countries, outbreaks of leptospirosis have caused a great amount of damage (3, 11, 14). Eight pathogenic *Leptospira* species are known to cause human leptospirosis worldwide (4).

Recently, huge outbreaks of leptospirosis have not been reported in Japan, but sporadic cases and a small outbreak have been recorded in the Okinawa Islands in the southwestern part of Japan (13). In the Okinawa Islands, *Leptospira interrogans* and *Leptospira borgpetersenii* have been isolated from field rats (*Rattus rattus*, *R. norvegicus*, *Suncus murinus*) and humans (10, unpublished data). However, *L. borgpetersenii* has not been isolated in the main islands of Japan (Hokkaido Island, Honshu Island, Shikoku Island and Kyushu Island).

Yoroshima Island (28°0′ N, 129°9′ E), one of the Amami Islands, is located between the main islands of Japan and the Okinawa Islands and lies within a subtropical zone. To date, there has been no recorded isolation of *Leptospira* from wild animals or domestic animals of the Amami Islands; however, no field survey has been attempted in this region. Therefore, the rate of contamination among field rats infected with dominant *Leptospira* species and serovars has remained unknown. The purpose of this study was to isolate *Leptospira* in this area and to characterize the isolates in order to determine whether or not the properties of these isolates are similar to those from the Okinawa Islands or the main islands of Japan.

Materials and Methods

Leptospira *isolation from field rats,* Leptospira *strains, and* Leptospira *cultivation.* In 2003, a field sur-

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Abbreviations: L. borgpetersenii, Leptospira borgpetersenii; L. interrogans, Leptospira interrogans; L. santarosai, Leptospira santarosai; L. weilii, Leptospira weilii; R. norvegicus, Rattus norvegicus; R. rattus, Rattus rattus.

vey was conducted on Yoroshima Island, one of the Amami Islands, as a part of the Project for the Surveillance of Zoonosis. A total of 19 *R. rattus* captured on Yoroshima Island were sacrificed, and the kidneys were used for the isolation of *Leptospira*. For the cultivation of *Leptospira*, Korthof's medium was used (4). Cultivation was continued for 2 months at 30 C and the cultures were assessed every week by dark field microscopy.

Strain OS39, *L. borgpetersenii* serovar Javanica, was isolated from *S. murinus* on Okinawa Island. *Leptospira* serogroup Javanica strains Veldrat Batavia 46 (*L. borgpetersenii* serovar Javanica), Aa3 (*L. santarosai* serovar Fluminense), 24 (*L. santarosai* serovar Vargonicas), A85 (*Leptospira* sp. serovar Mengla), Cox (*L. weilii* serovar Coxi), A102 (*L. weilii* serovar Mengrun), S590 (*L. weilii* serovar Mengma), Piyasena (*L. borgpetersenii* serovar Ceylonica), De10 (*L. borgpetersenii* serovar Dehorg), Kerala (*Leptospira* sp. serovar Menoni), L82 (*L. borgpetersenii* serovar Zhenkang), Sorex Jalna (*L. borgpetersenii* serovar Sorexjalna), and Poi (*L. borgpetersenii* serovar Poi) were kindly provided by Rudy Hartskeerl, the Royal Tropical Institute in the Netherlands. These strains were cultured in Korthof's medium at 30 C for use in this study.

DNA preparation, sequencing, and phylogenetic analysis. Genomic DNA was extracted by using a High Pure PCR template purification kit (Roche Diagnostics), which was based on the silica particles/guanidinium thiocyanate method originally reported by Boom et al. (2). Oligonucleotide primers, listed in Table 1, were used for the PCR and sequencing studies.

PCR amplification for the 16S rRNA gene (*rrs*), DNA gyrase subunit B gene (*gyrB*), and flagellin gene (*flaB*) was performed under the following conditions: 30 cycles of 95 C denaturing for 30 sec, 50 C annealing for 30 sec, and 72 C extension for 60 sec; the amplification was carried out using high-fidelity EX-*Taq* polymerase (TaKaRa Bio). The PCR products were purified with a High Pure PCR purification kit (Roche Diagnostics) according to the manufacturer's instructions. Direct cycle sequencing was performed by using a BigDye termination kit (PE Biosystems) or a Thermo Sequenase pre-mixed cycle sequencing kit (Amersham). For the sequencing of *gyrB*, Texas-red labeled sequence primer sets, LgyrF3, LgyrR3, LgyrF, and LgyrR (Proligo Japan) were used. Sequences were aligned using the CLUSTAL-W software package (19), and the neighbor joining (NJ) phylogenetic tree construction (15) and bootstrap analysis were carried out according to the Kimura 2-parameter distances method (9), supplied on the web (www.ddbj.nig.ac.jp). Pairwise alignments were performed with an open-gap penalty of 10, a gap extension penalty of 0.5, and a gap distance of 8. Multiple alignments were also performed using the same values, and the phylogenetic branches were supported by bootstrap analysis with 1,000 replications. The phylogenetic tree was constructed by using TreeView software (ver. 1.5). The *flaB* sequences of the *Leptospira* strains previously published (accession numbers AB027160, AB027165, AB027169, AB027170, AB027174, AB027175, AB027179–AB027182, AB027184, AB027185, AB030270–AB030273, AB167766) were used for the phylogenetic analysis. The *flaB* sequence of *Leptospira* isolates in this study were deposited in DDBJ/EMBL/GenBank as accession numbers AB174774–AB174780. The *gyrB* sequences of *Leptospira* strains used in this study have been assigned DDBJ/EMBL/GenBank accession numbers AB169982, AB169989–AB169995, AB169997–AB170002, and AB252857.

Cross agglutination test. Cross agglutination analysis was performed using a microscopic agglutination test (MAT) with a panel of anti-*Leptospira* rabbit sera listed in Table 2. *Leptospirae* were incubated with serial

Table 1. PCR and sequence primers of *rrs*, *gyrB* and *flaB*

Primer	Target gene	Sequence (5'-3')[c]	Position in accession no.	Reference
Lept-16S-F1[a,b]	*rrs*	GTT TGA TCC TGG CTC AGA ACT A	11–32 (X17547)	Fukunaga et al. (5)
Lept-16S-R1[a,b]	*rrs*	TTC TTA ACT GCT GCC TCC CGT	349–329 (X17547)	Fukunaga et al. (5)
UP1TL[a,b]	*gyrB*	CAY GCN GGN GGN AAR TTY GA	301–320 (AF434658)	Yamamoto and Harayama (24)
UP2rTL[a,b]	*gyrB*	TCN ACR TCN GCR TCN GTC AT	1520–1501 (AF434658)	Yamamoto and Harayama (24)
LgyrF[b]	*gyrB*	GGT CTT TCC GGA GAA GAT G	940–958 (AF434658)	This study
LgyrR[b]	*gyrB*	GAA TTG AAT TGA GGT TGA GG	1016–997 (AF434658)	This study
LgyrF3[b]	*gyrB*	GTW TCD GGN GGW YTV CAY GG	337–356 (AF434658)	This study
LgyrR3[b]	*gyrB*	TTM CCN GGA AGV CCD CCH CC	1232–1213 (AF434658)	This study
L-flaB-F1[a,b]	*flaB*	CTC ACC GTT CTC TAA AGT TCA AC	35–57 (AF064056)	Kawabata et al. (8)
L-flaB-R1[a,b]	*flaB*	TGA ATT CGG TTT CAT ATT TGC C	825–804 (AF064056)	Kawabata et al. (8)

[a] The primer was used for amplification of DNA fragment on target gene.
[b] The primer was only used for sequencing of amplified DNA fragment.
[c] D, A or G or T; M, A or C; N, A or G or T or C; R, A or G; V, A or C or G; W, A or T; Y, C or T.

twofold dilutions of the sera starting at 1:200 in microtiter plates kept at room temperature for 2 hr. Agglutination was examined by dark-field microscopy. The titer was defined as the highest dilution giving 50% agglutination in comparison with that of the negative control.

Long restriction fragment pattern (LRFP) analysis. Aliquots (10 ml) of spirochete cultures were collected and prepared as agarose plugs, as described by Smith et al. (17), but with a minor modification. *Leptospira* cells centrifuged at 15,000×*g* for 15 min at 4 C were washed with EDTA–Saline (0.15 M NaCl–0.1 M EDTA, pH 8.0) and were resuspended in PettIV solution (1 M NaCl–0.1M EDTA, pH 8.0) to 2×10^{10} cells/ml. An equal volume of 2% InCert agarose (Bio-Rad) solution was added to prepare the gel plugs. In order to digest protein, the gel plugs were incubated with proteinase K (0.1 mg/ml) at 50 C for 18 hr, then the reaction was terminated by treatment with 0.1 M phenylmethylsulfonyl fluoride. The gel plugs were digested with 15 units of *Not*I (Nippon Gene, Osaka, Japan) at 37 C for 18 hr. The DNA was electrophoresed on 1.2% pulsed-field certified agarose (Bio-Rad) gels in 0.5% TBE (0.045 M Tris-borate and 0.001 M EDTA) using Gene Navigator (Pharmacia). Lambda phage DNA concatemers (New England Biolabs) were used as molecular size markers. The DNA samples were run at 200 V with a consecutive pulse time of 10 sec for 5 hr, 30 sec for 12 hr, and 60 sec for 7 hr. After electrophoresis, DNA bands were stained with SYBR green I (BioWhittaker).

Results and Discussion

As a part of the Project for the Surveillance of Zoonosis, a total of 19 *R. rattus* captured on Yoroshima were sacrificed, and seven of nineteen rat kidneys were found to be culture-positive (36.8%) in this study.

Previously, we discovered the *Leptospira* infection of field rats on Okinawa Island and in other regions in Japan. The rates of infection were 4.5% (10/220, 2000y–2001y) and 2.3% (40/1,766, 2000y–2002y), respectively (unpublished data). On the other hand, Akiyama and colleagues have reported that the rate of detection of *Leptospira* from field rats ranged from 19.0 to 80.6% in Miyagi Prefecture (1). Therefore, it was suspected that field rats on Yoroshima Island might be highly infected with *Leptospira*, as was the case in Miyagi Prefecture.

The seven isolates (Yoro-1 to Yoro-7) from this study were used for further analyses. In order to determine the species of the isolates, a portion of the *flaB* and the *rrs* sequence was determined and compared with previous published sequences. The *flaB* sequences determined here were identical among the seven isolates (accession numbers AB174774–AB174780). The sequence of *flaB* was also identical to those of *L. borgpetersenii* strains Poi (accession number AB027176), Castellon 3 (accession number AB027163), Veldrat Batavia 46 (accession number AB027171), and M84 (accession number AB027173). An NJ-phylogenetic tree created according to the *flaB* sequence of representative strains was constructed as shown in Fig. 1A.

Strains Yoro-1 to Yoro-7 were identified as *L. borgpetersenii*, and were distinguishable from the *L. interrogans* serovars Icterohaemorrhagiae, Copenhageni, Hebdomadis, Autumnalis, and Australis, which have been isolated in the main islands of Japan. We also found that the Amami isolates could be identified as genus *Leptospira* by *rrs*-sequencing, and that they were clearly distinguishable from *Leptospira* isolates from the main islands of Japan (data not shown).

DNA gyrase is a housekeeping enzyme for bacteria. Phylogenetic analysis of *gyrB* has been applied to the taxonomic classification of *Pseudomonas*, *Acinetobacter*, and *Mycobacterium* (6, 24, 25). Molecular evolution of *gyrB* is thought to be faster than that of *rrs*; the average base substitution rate of *rrs* is 1% per 50 million years, while that of the *gyrB* at synonymous sites is 0.7 to 0.8% per 1 million years (6, 25). The *gyrB* analysis can recognize subtle differences among closely related bacterial strains (7, 20, 22, 23), although tested strains were identical according to *rrs* sequencing. As a result, the *gyrB* sequences of Yoro-1 to Yoro-7 (accession numbers AB169982–AB169988) were identical to those of *L. borgpetersenii* strains Piyasena, Veldrat Batavia 46, De10, Kerala, and L82 (Fig. 1B). These results indicate that the field rats in the Amami Islands were infected with *L. borgpetersenii* strains.

Serovar determination was very important, because serodiagnosis in leptospirosis is dependent on the identification of the serovar of infected *Leptospira* (4). The serovar identification of isolates from Yoroshima Island was achieved by a cross-agglutination test based on MAT (Table 2). Seven isolates, Yoro-1 to Yoro-7 were agglutinated by antisera against strains *L. borgpetersenii* Veldrat Batavia 46 (serovar Javanica) and OS39 (serovar Javanica) isolated on Okinawa Island, as well as by antiserum against Yoro-1; however, the other sera tested did not agglutinate isolates from Yoroshima Island. The reactivity of the Yoroshima isolates to a panel of antisera showed similar reactivity among the isolates, thus indicating that all Yoroshima isolates belonged to the same serovar and were closely related to the serovar Javanica and Okinawa isolate OS39. Therefore, *Leptospira* isolates from the Amami Islands were determined to be of the serovar Javanica. In the past,