differed for frozen and ethanol-preserved worms and were the same as those described by Umehara *et al* (2006, 2007). Amplification products were separated on agarose gels and excised bands were sequenced using BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, USA) in an automated sequencer (ABI3100, Applied Biosystems). Sequence alignment and comparison were analyzed using GENETYX-WIN program (ver.7.0, Software Development, Japan).

RFLP analysis of PCR products

Amplicons of the entire ITS region were digested with *Hin*fI (D Amelio *et al*, 2000) and of mitochondrial *cox1* with *Sfc*I. The digested samples were then separated by electrophoresis in 3.0% (w/v) agarose gels.

RESULTS

Sequence and RFLP analysis of rDNA ITS region

Amplification of the rDNA ITS region produced a single band of about 950 bp for all samples. Digestion of PCR products by *Hin*fI produced three different RFLP patterns, corresponding to that of *A. simplex* s. str. (ca. 610 and 230 bp, Fig 1 lane 1), *A. pegreffii* (ca. 330, 280 and 230 bp, Fig 1 lane 2) or the hybrid genotype (ca. 610, 330, 280 and 230 bp, Fig 1 lane 3). The RFLP pattern produced by the hybrid genotype was identical to the combination of the RFLP patterns of *A. simplex* s. str. and *A. pegreffii*.

Sequences of the ITS amplicons were almost identical among samples; only two

Table 1
Identification of *A. simplex* at the sibling species-level.

Sources of parasites	Collection site[a]	Life cycle stage	No. of parasite identified[b] as		
			As	Ap	H
Fish					
Arabesque greenling	North	L3	20	0	0
Alaska pollack	North	L3	19	0	0
Chub mackerel	North	L3	16	0	0
Surf smelt	North	L3	10	0	0
Chub mackerel	South	L3	0	37	1 (Ap-type)
Marine mammal					
Minke whale	North	Adult	45	0	3 (2: As-type) (1: Ap-type)
Human					
5 patients	NJpn	L3	5	0	0
80 patients	SJpn	L3	94	1	0

[a] North = North Pacific Ocean; South = Southern Sea of Japan; NJpn = Northern Japan; SJpn = Southern Japan

[b] Identification for sibling species was based on rDNA RFLP patterns. For the hybrid genotype, mtDNA RFLP patterns were used.

As = *A. simplex* s. str.; Ap = *A. pegreffii*; H = Hybrid genotype

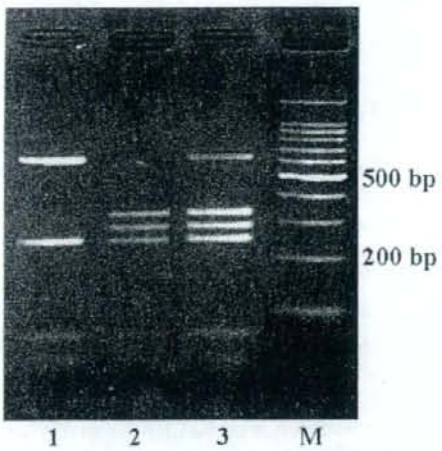

Fig 1- RFLP analysis of rDNA ITS amplicons from *A. simplex* s. str., *A. pegreffii* and hybrid genotype. Amplicons of *A. simplex* s. str. digested with *Hinf*I produced two bands (ca. 610 and 230 bp, lane 1), while those of *A. pegreffii* produced three bands (ca. 330, 280 and 230 bp, lane 2). Amplicons of hybrid genotype produced four bands (ca. 610, 330, 280 and 230 bp, lane 3). A 100-bp DNA ladder marker was used to estimate the size of the bands (lane M).

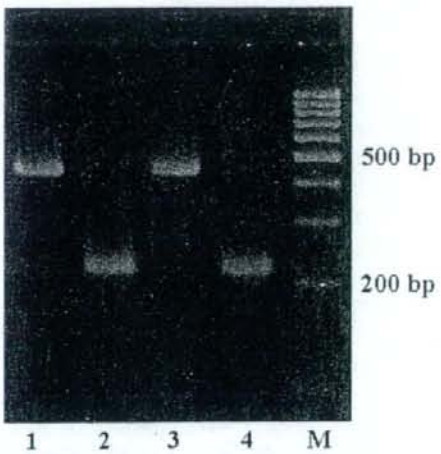

Fig 2- RFLP analysis of mitochondrial *cox1* amplicons from *A. simplex* s. str., *A. pegreffii* and hybrid genotype. Amplicons of *A. simplex* s. str. were not digested with *Sfc*I (ca. 440 bp, lane 1), whereas those of *A. pegreffii* produced a single digested band (ca. 220 bp, lane 2). Hybrid genotype amplicons were not digested (lane 3) or digested to produce two 200 bp bands (lane 4). A 100-bp DNA ladder marker was used to estimate the size of the bands (laneM).

thymine to cytosine transitions in the ITS1 region were observed between *A. simplex* s. str. and *A. pegreffii* (data not shown). Similarity searches of the GenBank/EMBL/DDBJ nucleotide database revealed that the sequences with two thymine bases and those with two cytosine bases were identical to sequence of *A. simplex* s. str. (GenBank/EMBL/DDBJ accession number: AB277822) and *A. pegreffii* (AB277823), respectively. Electropherograms of the hybrid genotype showed two double peaks at these transition sites (data not shown).

Sequence and RFLP analysis of mitochondrial *cox1*

PCR amplification of mitochondrial *cox1* produced a single band of about 440 bp for all samples. Sequence analysis revealed a high level of conservation among samples with base differences occurring at only seven sites between *A. simplex* s. str. and *A. pegreffii* (data not shown). At the amino acid level, no differences were observed between *A. simplex* s. str. and *A. pegreffii*.

Restriction enzyme *Sfc*I was selected for RFLP analysis based on the sequence differences between *A. simplex* s. str. and *A. pegreffii*. A single undigested 440 bp band was obtained for *A. simplex* s. str. (Fig. 2, lane 1), while digested 220 bp for *A. pegreffii* (Fig 2, lane 2). Amplicons from hybrid genotype were either not digested (Fig 2, lane 3, *A. simplex* s. str.-type) or digested to produce two 220 bp bands (Fig 2, lane 4, *A. pegreffii*-type).

Infection rates of sibling species of *A. simplex* in Japan

The identification of the *A. simplex* worms at the sibling species-level is summarized in Table 1. L3 larvae from fish collected in North Pacific Ocean were all identified as *A. simplex*

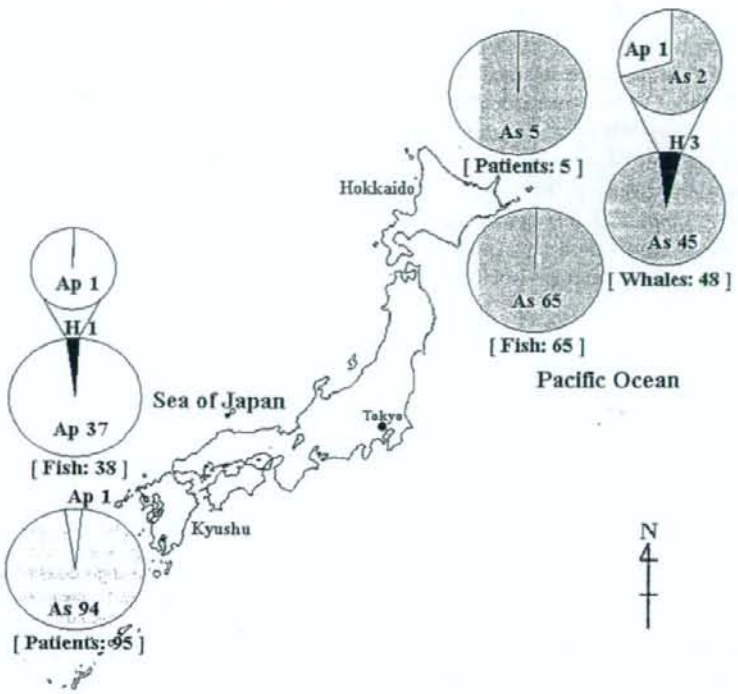

Fig 3- Distribution in Japan of *A. simplex* s. str. (As), *A. pegreffii* (Ap) and hybrid genotype (H) isolated from fish, marine mammals and patients with anisakiasis. Large pie charts show the results of identification of *A. simplex* based on analysis of the rDNA ITS region. Small pie charts show the results of typing of the hybrid genotype based on analysis of mitochondrial *cox1*. Numbers in square brackets and after the abbreviations of the species (As, Ap and H) represent the numbers of worms examined and identified, respectively.

s. str. However, almost all L3 larvae from fish captured in the southern Sea of Japan were identified as *A. pegreffii*, while only one L3 larva was identified as the hybrid genotype. However, the latter's genotype was determined to be *A. pegreffii*-type by RFLP analysis of mitochondrial *cox1*.

Adult worms from marine mammals in North Pacific Ocean were identified as *A. simplex* s. str. or the hybrid genotype. Of the three hybrid genotype worms, two were determined to be *A. simplex* s. str.-type and one *A. pegreffii*-type by RFLP analysis of mitochondrial *cox1*.

Five larvae from 5 patients in northern Japan were all identified as *A. simplex* s. str. In contrast, 94 of 95 larvae from 80 patients in southern Japan were identified as *A. simplex* s. str., while the remaining single larva was identified as *A. pegreffii*.

Mixed infections with the hybrid genotype and either *A. simplex* s. str. or *A. pegreffii* were found in marine mammals collected from North Pacific Ocean and in fish from southern Sea of Japan. However, no example of mixed infection with both *A. simplex* s. str. and *A. pegreffii* in a single host was detected.

Distribution of *A. simplex* sibling species in Japan

The distribution in Japan of *A. simplex* identified at the sibling species-level is shown in Fig 3. Almost all worms from fish were

classified into one of the two sibling species corresponding to the geographical location from which the samples were obtained; worms from north Pacific Ocean were all identified as *A. simplex* s. str., while almost all worms from the southern Sea of Japan were *A. pegreffii*. In contrast, worms from patients were mainly identified as *A. simplex* s. str. even though they were obtained from southern Japan where *A. pegreffii* predominates in samples isolated from fish.

DISCUSSION

A. simplex worms occurring worldwide show no obvious variation in morphology. However, Nascettii *et al* (1983, 1986) divided *A. simplex* worms isolated from Mediterranean Sea and North Atlantic Ocean into two sibling species, *A. pegreffii* and *A. simplex* s. str., respectively, based on difference observed in isozyme electrophoretic patterns. In addition to these two sibling species, Mattiucci *et al* (1997) differentiated some *A. simplex* worms showing another electrophoretic pattern as *A. simplex* C. Consequently, *A. simplex* worms have come to be regarded as a complex composed of three sibling species, namely, *A. pegreffii*, *A. simplex* s. str. and *A. simplex* C.

This classification has been generally accepted because the three sibling species were also unequivocally discriminated based on nuclear rDNA sequence differences (D Amelio *et al*, 2000). In this study, we applied molecular methods for sibling species-level identification of Japanese *A. simplex* worms and confirmed the usefulness of RFLP analysis of rDNA for this purpose. Thus Japanese *A. simplex* worms were classified into *A. simplex* s. str., *A. pegreffii* or the hybrid genotype.

It is well known that mitochondrial DNA (mtDNA) evolves at a faster rate than nuclear DNA and is useful for differentiating cryptic species (Blouin, 2002). Therefore, we used mitochondrial *cox1* in developing an RFLP method that is capable of classifying *A. simplex* into sibling species. Initially, the enzyme *Hin*fI was used for this discrimination (Umehara *et al*, 2006), but based on the detection of intraspecific variations at the recognition sites of *Hin*fI in mitochondrial *cox1* sequence in one of 12 *A. pegreffii* worms examined, we selected another enzyme, *Sfc*I, that discriminates between *A. simplex* s. str. and *A. pegreffii*. By sequence analysis, we confirmed that there were no intraspecific variations at the *Sfc*I recognition site in the sequences of 39 worms examined so far (20 *A. simplex* s. str. and 19 *A. pegreffii* worms).

The hybrid genotype worms were clearly classified into either *A. simplex* s. str.-type or *A. pegreffii*-type by the RFLP method employing *Sfc*I. Since the vast majority of mitochondrial genomes are inherited uniparentally from the female parent, the results obtained with the hybrid genotype worms were as predicted. The mitochondrial genome is effectively haploid (Moore, 1995) and, thus, may be advantageous for study of the evolutionary history of *A. simplex*.

The results of this study showed that *A. simplex* s. str. is primarily distributed in fish and marine mammals in North Pacific Ocean and *A. pegreffii* is predominantly distributed in fish in the southern Sea of Japan. However, worms from patients were identified as *A. simplex* s. str. even though they were obtained from southern Japan where *A. pegreffii* is the predominant species in fish. The reason for this discrepancy between the predominant sibling species in fish and in patients remains unresolved. Studies are now in progress to identify the fish species that is responsible for human infection. This information is crucial for initiating prevention measures against human anisakiasis, especially in southern Japan.

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Multiplex PCR for the identification of *Anisakis simplex* sensu stricto, *Anisakis pegreffii* and the other anisakid nematodes

Azusa Umehara [a,b,*], Yasushi Kawakami [b], Jun Araki [c], Akihiko Uchida [b]

[a] *Department of Parasitology, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-ku, Tokyo, 162-8640, Japan*
[b] *Laboratory of Medical Zoology, College of Environmental Health, Azabu University, 1-17-71 Fuchinobe, Sagamihara-shi, Kanagawa, 229-8501, Japan*
[c] *Meguro Parasitological Museum, 4-1-1, Shimomeguro, Meguro-ku, Tokyo, 153-0064, Japan*

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Abstract

A multiplex PCR method was established for the rapid identification of *Anisakis simplex* sensu stricto, *A. pegreffii*, *A. physeteris*, *Pseudoterranova decipiens*, *Contracaecum osculatum* and *Hysterothylacium aduncum*. The sequence alignment of the internal transcribed spacer 1 region (ITS-1) between *A. simplex* s. str. and *A. pegreffii* showed a high degree of similarity, but only two C–T transitions were observed. To differentiate *A. simplex* s. str. from *A. pegreffii*, an intentional mismatch primer with an artificial mismatched base at the second base from the primer 3′ end was constructed. This intentional mismatch primer, which produced a PCR band only from *A. pegreffii* DNA, was able to differentiate the two morphologically indistinguishable sibling species of *A. simplex*. Specific forward primers for other anisakid species were also designed based on the nucleotide sequences of the ITS region. The multiplex PCR using these primers yielded distinct PCR products for each of the anisakid nematodes. The multiplex PCR established in this study would be a useful tool for identifying anisakid nematodes rapidly and accurately.

Keywords: *Anisakis*; *Pseudoterranova*; *Contracaecum*; *Hysterothylacium*; Multiplex; Polymerase chain reaction

1. Introduction

Anisakiasis is caused by the ingestion of larval nematodes belonging to the genera *Anisakis*, *Pseudoterranova*, *Contracaecum* and *Hysterothylacium* in raw or undercooked seafood. Regional eating habits are recognized as a factor in the high prevalence of anisakiasis [1]. With the increased popularity of eating undercooked or raw fish dishes, the number of anisakiasis cases may be expected to increase. The species of Anisakidae most often associated with anisakiasis is *Anisakis simplex*. Recently, it has been recognized that the morphospecies *A. simplex* does not consist of only a single species but a complex of three sibling species, namely, *A. simplex* sensu stricto, *A. pegreffii* and *A. simplex* C. Especially, *A. simplex* s. str. and *A. pegreffii* widely extend across geographic ranges and the numbers of hosts [2,3].

To date, anisakid larvae recovered from patients have been identified based on their morphology. However, it is very difficult to identify worms that have been partly destroyed or lack key morphological features. Thus, DNA differential diagnosis is considered very useful for the definitive identification of clinically obtained worms. Several methods for identification of anisakid species such as PCR–RFLP [4–10] and sequencing of rRNA gene [4,7–9,11] or mitochondrial DNA [12] have been developed. Furthermore, RFLP of the ITS and the 5.8 subunit rRNA gene have been successfully employed for identification of sibling species of *A. simplex* [5,13–20]. We report the establishment of a multiplex PCR for the differential diagnosis of anisakid nematodes as an alternative to conventional methods.

Abbreviations: rRNA, ribosomal RNA; ITS, internal transcribed spacer; SNP, single nucleotide polymorphism; RFLP, restriction fragment length polymorphism.

* Corresponding author. Department of Parasitology, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-ku, Tokyo, 162-8640, Japan. Tel.: +81 3 5285 1111; fax: +81 3 5285 1173.
E-mail address: umeazu@nih.go.jp (A. Umehara).

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2. Materials and methods

2.1. Parasite materials

A total of thirty-four anisakid nematodes were collected from fish caught in waters off the Japanese coast. The parasites were identified to the genus level based on the morphological characteristics described by Koyama et al. [21] and Kagei [22]. Species identification was carried out by sequencing 18S–28S rRNA genes (see Section 2.4). The anisakids used in this study and the locality of their hosts are summarized in Table 1.

2.2. DNA extraction

DNA from individual worms was extracted using QIAamp DNA Mini Kit (Qiagen, Tokyo, Japan) according to the manufacturer's instruction. DNA was eluted in elution buffer and kept at − 20 °C until use.

2.3. PCR–RFLP analysis

Larvae were distinguished based on the genetic markers described by Zhu et al. [4], Amelio et al. [5] and Abe and Yagi [9]. The ITS region including 5.8S rRNA gene was amplified using two universal primers NC5 (forward; 5′-TAGGTGAACCTGCGGAAGGATCATT-3′) and NC2 (reverse; 5′-TTAGTTTCTTTTCCTCCGCT-3′). All PCR reactions were carried out in a final volume of 30 μl containing 1 μl of genomic DNA, 0.75 units TaKaRa Ex *Taq* (Takara Bio Inc., Japan), 1× PCR buffer (10 mM Tris–HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$), 0.2 mM dNTP mixture and 0.3 μM forward and reverse primers. An initial denaturation step at 94 °C for 5 min was followed by 26 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s and extension at 72 °C for 30 s with a final extension step at 72 °C for 7 min. The amplification was performed in a RoboCycler Gradient 96 with Hot Top (Stratagene Japan K.K., Japan). The PCR products were digested with restriction enzymes, *Hinf* I, *Hha*I, *Rsa*I and *Hae*III (Toyobo, Japan). The digested products were analyzed by electrophoresis on a 3.0% Seakem GTG agarose gel (Cambrex Bio Science, USA) and visualized by illumination with shortwave ultraviolet light after ethidium bromide staining.

Table 1
Anisakid nematodes used in this study and locality of their hosts

Anisakid species[a]	Host species	No. of worms	Locality
Anisakis simplex sensu stricto	*Pleurogrammus azonus*	10	Hokkaido
A. pegreffii	*Scomber japonicus*	10	Kyushu
A. physeteris	*Scomber japonicus*	1	Kyushu
Contracaecum osculatum	*Pleurogrammus azonus*	3	Hokkaido
Hysterothylacium aduncum	*Hypomesus pretiosus japonicus*	5	Hokkaido
Pseudoterranova decipiens	*Pleurogrammus azonus*	5	Hokkaido

[a]Parasites were first identified to the genus level, then species were identified by sequencing of 18S–28S rRNA genes.

2.4. DNA sequencing

We analyzed 18S–28S rRNA gene sequences of each parasite. The following pair of primers for PCR reactions was used: nemspec 18SF (forward; 5′-TCTAGCCTACTAAATAGTCATC-3′), complementary to the region coding for the 18S rRNA, and NC2 (reverse; 5′-TTAGTTTCTTTTCCTCCGCT-3′), complementary to the region coding for the 28S rRNA. All PCR reactions were carried out in a final volume of 30 μl containing 3 μl of genomic DNA, 0.75 units TaKaRa Ex *Taq* (Takara), 1× PCR buffer, 0.2 mM dNTP mixture, and 0.3 μM forward and reverse primers. An initial denaturation step at 93 °C for 1 min was followed by 30 cycles of denaturation at 93 °C for 30 s, annealing at 52 °C for 1 min and extension at 72 °C for 75 s with a final extension step at 72 °C for 7 min. The PCR products were purified, ligated into the pGEM-T easy vector (Promega, USA), and transformed into competent *Escherichia coli* DH5α cells. Plasmids were extracted using a Wizard Plus SV Minipreps DNA Purification System (Promega). The DNA sequences were defined using Big Dye Terminator v3.1 Cycle Sequencing Kit and ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, USA). Nucleotide sequences obtained from each species were aligned using the multiple sequence alignment program Clustal W (http://clustalw.ddbj.nig.ac.jp/top-j.html).

2.5. Primer design

Based on the aligned 18S–28S rRNA gene sequences, the following forward primers were designed to amplify different size products; APY (5′-GGCTGGTTGATGAACTGTTG-3′) specific for *Anisakis physeteris*; PD (5′-CGAGTACTTTTTATGGTCGTGAAGT-3′) specific for *Pseudoterranova decipiens*; AC (5′-GACATTGTTATTTCATTGTATGTGTTGAAAATG-3′) specific for *A. simplex* but, common to *A. simplex* sensu stricto and *A. pegreffii*; COS (5′-TGATATGCTTGAAAGGCAGG-3′) specific for *Contracaecum osculatum* and HAD (5′-GCCTTCCATATGCGCGTATA-3′) specific for *Hysterothylacium aduncum*. Moreover, we also designed two intentional mismatch primers to obtain PCR product only from *A. pegreffii* DNA. The sequences of these primers were APE1: 5′-GAGCAGCAGCTTAAGGCAGAGGC-3′ and APE2: 5′-GAGCAGCAGCTTAAGGCAGATGC-3′. The underlined nucleic acid bases indicate artificial mismatched sites (Fig. 1). Artificial mismatched bases were introduced into the terminal region using the methods previously described [23,24]. Universal primer B (5′-GCCGGATCCGAATCCTGGTTAGTTTCTTTTCCT-3′) [5] was used as reverse primer.

2.6. PCR and multiplex PCR

To select the optimal position for the artificially mismatched bases in the intentional mismatch primers, PCR reactions were carried out with forward primers having artificial mismatched bases in different positions in the terminal region, APE1 or APE2, and universal reverse primer.

In multiplex PCR, six specific forward primers and one universal reverse primer were used. Six separate tubes containing

A. simplex s. str. GAGCAGTAGCTTAAGGCAGAGTT
A. pegreffii GAGCAGCAGCTTAAGGCAGAGTC
APE1 GAGCAG**C**AGCTTAAGGCAGAG**GC**
APE2 GAGCAG**C**AGCTTAAGGCAGA**TGC**

Fig. 1. The sequences of the intentional mismatch primers (APE1 and APE2) compared with those of *A. simplex* s. str. and *A. pegreffii* in the corresponding region. The primers were constructed based on the sequence of *A. pegreffii* and mismatches were introduced at the second base for APE1 (T to G) and at the second and third bases for APE2 (GT to TG). There are two more base differences between *A. simplex* s. str. and the primers (boxed C).

template DNA of each anisakid species were prepared. Following optimization of the annealing temperatures of primers by gradient analysis and primer ratios by titration, a multiplex PCR protocol was established. All reactions were carried out in a final volume of 20 μl containing 0.5 units TaKaRa Ex *Taq* (Takara), 0.2 mM each dNTP, 0.07 μM HAD primer, 0.3 μM other five forward primers, 0.6 μM reverse primer, and various amounts of template DNA (1–40 ng) in 1× PCR buffer. PCR reactions were performed as follows: initial denaturation at 95 °C for 3 min, followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 56 °C for 30 s, extension at 72 °C for 45 s, and a final extension at 72 °C for 7 min. All products were electrophoresed in 2.0% agarose gel, followed by staining with ethidium bromide and then visualized under ultraviolet light.

3. Results

3.1. PCR–RFLP analysis

PCR products were digested with restriction enzymes to identify the characteristic of RFLPs. The larvae were distinguished based on combinations of different RFLP patterns. *A. simplex* s. str., *A. pegreffii* and *A. physeteris* were identified based on *Hin*fI and *Hha*I restriction profiles (Table 1). On the other hand, *C. osculatum*, *H. aduncum* and *P. decipiens* were distinguished based on *Rsa*I and *Hae*III restriction profiles (Fig. 2).

3.2. Sequence analysis

The nucleotide sequences determined in this study have been deposited in DDBJ/EMBL/GenBank databases under accession numbers AB277821 to AB277826.

A total of 1300 bp sequence of *A. physeteris* (AB277821), 1353 bp sequences of both *A. simplex* s. str. (AB277822) and *A. pegreffii* (AB277823), 1305 bp sequence of *P. decipiens* (AB277824), 1364 bp sequence of *C. osculatum* (AB277825) and 1433 bp sequence of *H. aduncum* (AB277826) were obtained. The aligned sequences of six anisakids including sibling species showed high sequence differences in the ITS region. Sequence differences were 58.4% and 91.3% in the ITS-1 and ITS-2 regions, respectively. However, *A. simplex* s. str.

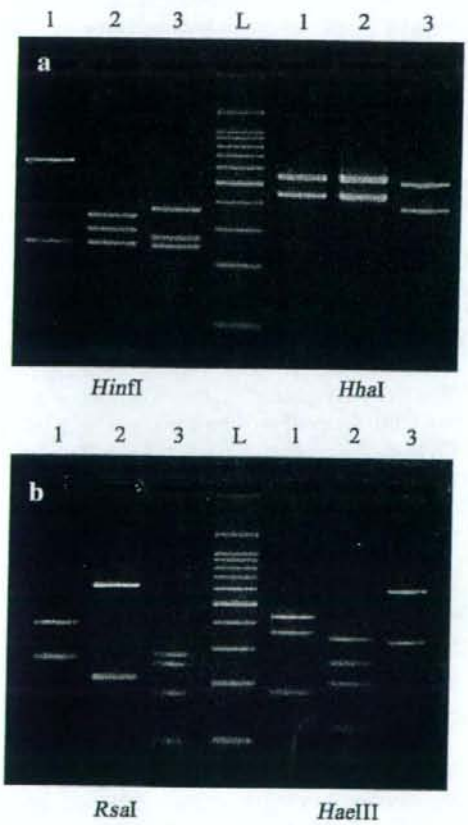

Fig. 2. (a) RFLP analysis using *Hin*fI and *Hha*I enzymes. Lane 1, *A. simplex* s. str.; Lane 2, *A. pegreffii*; Lane 3, *A. physeteris*; L, 100 bp ladder. (b) RFLP analysis using *Rsa*I and *Hae*III enzymes. Lane 1, *C. osculatum*; Lane 2, *H. aduncum*; Lane 3, *P. decipiens*; L, 100 bp ladder.

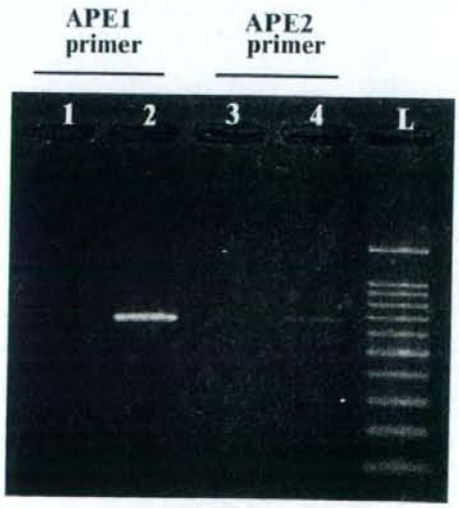

Fig. 3. Specificity test of intentional mismatch primers for *A. simplex* s. str. and *A. pegreffii*. PCR was performed using the intentional mismatch primers APE1 and APE2 (see Fig. 1) as the former, and universal primer B as the reverse primer. Lanes 1, 3, *A. simplex* s. str.; Lanes 2, 4, *A. pegreffii*; L: 100 bp ladder.

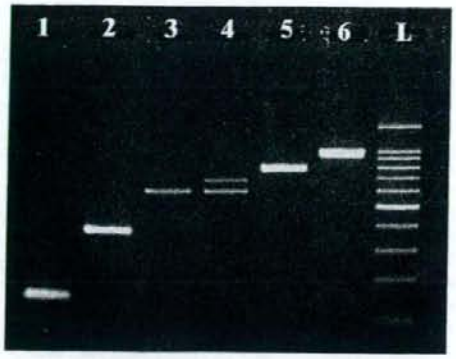

Fig. 4. Molecular identification of anisakid nematodes by multiplex PCR. Lane 1, *A. physeteris*; Lane 2, *P. decipiens*; Lane 3, *A. simplex* s. str.; Lane 4, *A. pegreffii*; Lane 5, *C. osculatum*; Lane 6, *H. aduncum*; L: 100 bp ladder.

exhibited a high level of similarity to *A. pegreffii*, these two sibling species differing only by two base pairs. We detected two C–T transitions in the ITS-1 region between two sibling species.

3.3. The specificity of intentional mismatch primers and multiplex PCR

Intentional mismatch primers APE1 and APE2 (Fig. 1) in PCR were tested experimentally using *A. simplex* s. str. and *A. pegreffii* genomic DNA as a template. Each primer was tested separately. The expected size of the PCR product (672 bp) was obtained only when the template DNA of *A. pegreffii* was present. However, comparison of the sensitivity of each intentional mismatch primer resulted in PCR reactions with the APE2 primer producing only a faint band (Fig. 3). Therefore, six specific forward primers, including APY, PD, APE1, AC, COS and HAD and one reverse primer (primer B) were used in the multiplex PCR reaction. The multiplex PCR products with molecular sizes of 143, 370, 799, and 991 bp were amplified in *A. physeteris* (Lane 1), *P. decipiens* (Lane 2), *C. osculatum* (Lane 5) and *H. aduncum* (Lane 6), respectively (Fig. 4). PCR products with a molecular size of 588 bp were amplified in both *A. simplex* s. str. (Lane 3) and *A. pegreffii* (Lane 4). PCR products of 672 bp were amplified only for *A. pegreffii* (Lane 4) (Fig. 4). The PCR product amplified by APE1 primer can distinguish between *A. simplex* s. str. and *A. pegreffii*. The most reliable SNP genotyping could be obtained using 1 ng samples of genomic DNA.

4. Discussion

Genetic markers provide specific identification of anisakid larvae that lack morphologically distinct characteristics. Molecular techniques using PCR amplification [7,9] and restriction enzyme digestion [4–10] to differentiate anisakid species have been described. In this study, we established a multiplex PCR assay for easy identification of six anisakid species, including two sibling species of *A. simplex*.

We confirmed the presence of specific nucleotide sequences of *A. simplex* s. str., *A. pegreffii*, *A. physeteris*, *C. osculatum*, *P. decipiens* and *H. aduncum* in the ITS region. Therefore, we designed forward primers according to the specific sequences in the ITS region. However, between *A. simplex* s. str. and *A. pegreffii*, 18S–28S rRNA gene sequences are highly conserved; sequence comparison by alignment showed only two C–T transitions in the ITS-1 region. This finding corresponds to those in previous reports [15,17]. For SNP genotyping, we designed intentional mismatch primers containing one or two artificial mismatched bases within the third position from the primer 3′ end. The specificity of these primers to discriminate between two sibling species was increased due to the introduction of artificial mismatched bases. Based on this study, it was shown that a mismatch incorporated at the second base from the 3′ end in the primer could optimize PCR specificity and reactivity. Greater duplex thermal stability was observed for a duplex containing one mismatch than that containing two mismatches. In addition, we found that the amount of template DNA strongly influenced the result of the multiplex PCR reaction. When the amount of template DNA was greater than 1 ng, nonspecific amplicons were observed.

The PCR product of predicted size (672 bp) was observed only in *A. pegreffii* as a result of introducing an artificial mismatched base. It is possible to differentiate between *A. simplex* s. str. and *A. pegreffii* in multiplex PCR. However, it is not determined yet whether some recombinant genotypes of *A. simplex* s. str. and *A. pegreffii* could be detectable by the present method. Nevertheless, the six multiplex PCR amplicons of different sizes could be visualized by agarose gel electrophoresis. The identity of individual multiplex PCR products was confirmed by specific PCR with each of the single primer pair. Our results indicated that this novel assay is rapid, accurate and cost-effective for the identification of anisakid species. Therefore, multiplex PCR is useful for diagnostic purposes and molecular epidemiological studies of anisakid species.

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Paragonimiasis in India : a newly emerging food borne parasitic disease

Department of Microbiology, Sikkim Manipal Institute of Medical Sciences, 5th mile, Tadong, Gangtok, Sikkim, India
T. Shantikumar Singh

Department of Parasitology, National Institute of Infectious Diseases
Hiromu Sugiyama

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Introduction

Paragonimiasis is a food borne parasitic zoonosis caused by trematode species of the genus *Paragonimus*. The disease, primarily affects the lungs, but can involve other organs and tissues of the body. Humans acquire the infection by ingestion of raw or undercooked fresh water crabs and / or crayfish containing metacercariae, the infective form of the parasite. Sometimes, infection can occur by ingestion of raw or undercooked meat of pig or wild boar which serve as paratenic hosts[1]. The disease is widely distributed, mainly in Asia, but also in Africa and Americas. Although India is the first known region in Asia of the oldest lung flukes such as *P. compactus* (Cobbold, 1859) and *P. westermani* (Kerbert, 1878), very little attention has been paid to this important trematode because paragonimiasis was never considered a public health problem in India until 1982 when the first case of pulmonary paragonimiasis had been reported from Manipur[2]. Subsequent studies revealed paragonimiasis was indeed an important emerging food borne parasitic zoonosis endemic in the northeast states of India.

Distribution

The disease is endemic in Manipur[3], and Arunachal Pradesh[4]. A case of pulmonary paragonimiasis was also reported from Maharastra State in 1983[5]. The prevalence rates in Manipur and Changlang District of Arunachal Pradesh were 6.7% (2% to 35.6%) and 52% (in children of < 15 yrs), respectively[3,6]. In March 27−29, 2008, an investigation conducted on paragonimiasis resulted in the discovery of 7 new cases and 38 old cases of paragonimiasis in Pfutsero town of Phek district and Kohima, Nagaland, respectively (unpublished data).

The parasite and the hosts

The *Paragonimus* species described in India numbered five : *P. compactus* and *P. westermani* from wild mammals during 19th century and *P. heterotremus*, *P. skrjabini* and *P. hueit'ungensis* during 20th century[7,8,9,10,11]. *P. heterotremus* is the common causative species of human paragonimiasis in Manipur[12], Arunachal Pradesh[6] and Nagaland, whereas *P. skrjabini* was also found as a causative agent of human

paragonimiasis in Manipur. The role of *P. westermani* in human paragonimiasis in India is yet to be established. The natural mammalian hosts of *Paragonimus* species in India were leopards, tigers, civet cats, toddy cats, dogs and mongoose. Whereas the second intermediate hosts were the mountainous fresh water crabs, *Potamiscus manipurensis* and *Barytelphusa lugubris*, the first intermediate molluscan hosts are yet to be determined.

Transmission of infection

Children between 5 to 15 years of age have higher incidences of infection, which is more common among boys. The major mode of infection was consumption of undercooked/smoked freshwater crabs. Although less common, ingestion of raw crab extract for cure of fever, jaundice and allergic conditions, as practiced traditionally by certain communities in the hills, could cause severe infections. Consumption of improperly cooked or smoked meat of pig and wild bore could be another mode of infection. The traditional practice of topical application of crushed crabs to cure skin lesions could possibly cause cutaneous paragonimiasis.

Clinical manifestations

In Manipur, paragonimiasis patients have presented in 3 distinguishable clinical groups.

1. Pulmonary paragonimiasis
2. Extra pulmonary paragonimiasis
3. Pleuropulmonary paragonimiasis

The various clinical forms of 84 patients were analyzed, and the most clinical form was pulmonary paragonimiasis followed by pleural effusion.

1. **Pulmonary paragonimiasis** (59.2%)

The major clinical features comprised chronic cough, chest pain, difficult breathing and expectoration of rusty brown or blood stained sputum and/or recurrent haemoptysis. Normal chest X-rays were seen in 15% even in symptomatic patients with or without detectable *Paragonimus* eggs in the sputum. The most common abnormal findings seen in the chest X rays were usually ill defined patchy consolidations (63%), 5 to 60 mm in size, pleural thickening or blunting of costophrenic angle, pleural effusion during the early stage of infections and later nodular densities 1 to 4 cm in diameter, cavities, pleural effusion usually bilateral and unilateral or encysted small ring shadows in 7%[13].

2. **Extra pulmonary paragonimiasis**

a) Pleural effusion (17%) : Usually bilateral, commonly seen in children, presented with chest pain, breathlessness and cough as the main clinical manifestations.

b) Cutaneous paragonimiasis (16%) : Migratory subcutaneous nodules initially appeared on the chest wall or abdominal wall then migrated downwards through the abdominal wall to the genitalia and thigh. The nodules, usually, firm, nontender contained immature worm, rarely mature adult worms and eggs may be recovered.

c) Cardiovascular paragonimiasis (4.4%) : Clinically pericarditis associated with pulmonary paragonimiasis may be observed, especially in children. Congestive heart failure with pleuropulmonary paragonimiasis was seen in a 8-year-old boy.

d) Abdominal paragonimiasis (2.2%) : Abdominal paragonimiasis was seen in, with pain and distension in abdomen, diarrhoea and hepatosplenomegally as presenting symptoms.

e) Cerebral paragonimiasis : Pargonimiasis involving central nervous system although rare may present a serious form of the disease with high mortality rate. One patient presented with epileptic fits, headache and focal muscular paresis.

3. **Pleuropulmonary paragonimiasis** (2.2%)

Patients of pleuropulmonary paragonimiasis presented with mixed symptoms of pulmonary paragonimiasis and pleural effusion, the predominant clinical symptoms were mainly dyspnoea, pain and tightness in the chest and cough with or without blood stained sputa or haemoptysis.

Differential diagnosis

Almost all the cases of pulmonary paragonimiais were initially diagnosed as smear negative pulmonary tuberculosis and patients were put under antitubercular drugs therapy for a period from 6 months to one and half years. The patients who did not respond to antitubercular therapy were investigated first for fungal disease or carcinoma and in few cases for paragonimiais. In Manipur, pulmonary paragonimiasis were often misdiagnosed as pulmonary TB or bronchitis or bacterial pneumonia or bronchiectasis or lung cancer.

Laboratory Diagnosis

A definitive diagnosis of paragonimiasis was made by microscopy demonstration of *Paragonimus* eggs (85%) in the wet smear of clinical specimens. The presence of charcot leydon crystals (92%) and high eosinophils count (61%) in the peripheral blood were considered as strong indications of paragonimiasis in the absence of other causes of eosinophilia. The serological tests which were used for diagnosis in the absence of *Paragonimus* eggs were intradermal test, and recently dot ELISA.

Treatment

Two major antihelminthic drugs, praziquantel and bithionol, were available for the treatment of paragonimiasis. Praziquantel (Biltricide, Bayer), the drug of choice of paragonimiasis, is given in doses of 25 mg per kg body weight 3 times a day for three days. However, relapse occurred in about 2% of the cases. A 100% cure rate was obtained if the praziquantel therapy was extended up to 5 days. Bithionol (M/S Marcel Quarre), another drug, was available as an investigational drug for the treatment of paragonimiasis. This drug when given in doses of 40 mg per kg body weight in two equally divided doses on alternate days for a course of 10 to 15 doses was found as effective as praziquantel but about 50% of patients developed urticaria after 2 or 3 doses.

Discussion

Paragonimiasis has emerged as an important food borne parasitic disease in India, mainly in the northeast states. Most common mode of transmission has been the ingestion of inadequately cooked or smoked fresh water crabs and infrequently raw crab extract. The problem of paragonimiasis is likely to continue in India unless it is included in the curriculum of medical colleges and medical practitioners change their attitude in dismissing the disease as rare parasitic infection and temptation to diagnose such condition as smear negative pulmonary tuberculosis. Failure to recognize pulmonary paragonimiasis had resulted in the overdiagnosis of pulmonary tuberculosis and unwarranted antitubercular drug therapy. Since pulmonary paragonimiasis is clinically and radiologically indistinguishable from pulmonary TB, all patients presenting with pulmonary

symptoms should be investigated for paragonimaisis before concluding a case as smear negative pulmonary tuberculosis or multidrug-resistant TB. History of consumption of fresh water crabs, finding of Charcot leyden crystals even in the absence of *Paragonimus* eggs in the clinical specimens and peripheral blood eosinophilia are sufficient justifications for a provisional diagnosis of pulmonary paragonimiasis. Once diagnosed, paragonimiasis can be effectively treated with praziquantel. Recently, triclabendazole, administered at 10 mg/kg body weight as single dose therapy has been found to have efficacy, safety, and tolerability, comparable to praziquantel at recommended dose[10].

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市販サワガニを対象とした肺吸虫メタセルカリアの寄生状況調査

国立感染症研究所　寄生動物部
杉山　広・梅原梓里・森嶋康之・川中正憲・山崎　浩

Key Words：サワガニ，ウェステルマン肺吸虫，宮崎肺吸虫，食習慣

緒　言

わが国に長期間在住するアジア系外国人が，淡水産・汽水産のカニを食材とする出身地固有の料理を加熱せずに賞味し，肺吸虫に感染する事例が増加の傾向にある。飲食をともにした日本人の感染も報告されており，食習慣に起因する新たな肺吸虫症として，注意する必要が出てきた[1)2)]。これらの事例では，市販のサワガニが原因食材となった場合が多い。そこで，東京都内で販売されていたサワガニを対象に，肺吸虫の寄生状況を調べた。肺吸虫メタセルカリアが検出された場合は，形態を精査し，また塩基配列に基づく虫種同定を併せて試みた。

サワガニからの肺吸虫メタセルカリアの検出状況

東京都のスーパーマーケットやデパートの鮮魚売り場，あるいは鮮魚店で，2004年4月～2008年2月の間に8回にわたり，合計266匹のサワガニを購入し，肺吸虫の寄生状況を調べた。その結果，44匹（17%）から肺吸虫メタセルカリアが検出された（表1）。寄生率が最も高かったのは，2008年2月に購入した宮崎県産のサワガニで，その値は88%（検査した26匹中の23匹が陽性）を示し，1匹のカニから最高23個のメタセルカリアが検出された。

検出メタセルカリアの形態学的特徴

2007年4月に購入した宮崎県産のサワガニからは，直径（内嚢の外径）が平均448ミクロンのメタセルカリアを検出した（14個を計測）。このうちの11個には，外嚢の外側に膜様物の付着を認め，これらを宮崎肺吸虫と判定した。一方，2007年6月に購入した宮崎県産のサワガニからは，直径（内嚢の外径）が平均352ミクロンのメタセルカリアを検出した（4個を計測）。このうちの3個には，口吸盤の背縁に穿刺棘を確認し，ウェステルマン肺吸虫と判定した。しかしながら，膜様物や穿刺棘が明かでないメタセルカリアに関しては，大きさだけで種を決定する訳にはいかず，分子同定を試みた。

Survey of *Paragonimus* Metacercariae in Freshwater Crabs Sold at Markets in Tokyo

Hiromu Sugiyama　Azusa Umehara　Yasuyuki Morishima　Masanori Kawanaka　Hiroshi Yamasaki

Department of Parasitology, National Institute of Infectious Diseases

論文請求先：杉山　広　〒162-8640　東京都新宿区戸山1-23-1　国立感染症研究所　寄生動物部

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表1　東京都で販売されていたサワガニにおける肺吸虫メタセルカリアの寄生状況

購入時期	購入先	産地	検査数	陽性数	寄生率 (%)	検出数 計(範囲[平均])[a]	同定結果[b]
2004年4月	スーパーA	静岡	48	0	0	-	-
2007年4月	デパートA	宮崎	46	0	0	-	-
4月	スーパーA	宮崎	16	7	44	29(1-12[4.1])	Pm
4月	スーパーB	長崎	21	5	24	9(1-4[1.8])	Pm
6月	スーパーC	静岡	35	0	0	-	-
6月	個人商店	宮崎	44	5	11	9(1-3[1.8])	Pw(3n)
2008年1月	デパートB	宮崎	30	4	13	6(1-3[1.5])	Pm+Pw(2n)
2月	デパートB	宮崎	26	23	88	116(1-23[5.0])	Pm
合計			266	44	17	169(1-23[3.8])	

[a] 検出されたメタセルカリアの合計数（陽性カニ1匹から検出されたメタセルカリア数の最小値と最大値および［被検カニ1匹から検出されたメタセルカリア数の平均値］）

[b] Pm：宮崎肺吸虫；Pw (2n)：ウェステルマン肺吸虫（2倍体型）；Pw (3n)：ウェステルマン肺吸虫（3倍体型）

分子同定

分子同定に当たり，PCR増幅の標的として，リボゾームDNA・ITS 2領域とミトコンドリアDNA・16Sリボゾーム DNAの部分配列を選んだ。前者は宮崎肺吸虫とウェステルマン肺吸虫との鑑別に[3]，後者はウェステルマン肺吸虫の2倍体型と3倍体型との判別に利用される配列である[4,5]。メタセルカリアからのDNAの抽出，これをテンプレートとしたPCR増幅，制限酵素による切断とパターン解析（RFLP）などの一連の手技は，既報に準じた[3]。制限酵素として，ITS 2領域には*Bss*SI（宮崎肺吸虫）と*Sna*BI（ウェステルマン肺吸虫），また16Sリボゾーム DNA配列には*Sna*BI（2倍体型）と*Bsr*DI（3倍体型）を使用した。一部のPCR産物については，塩基配列を解読して，PCR-RFLPの結果を確認した。

同定の結果は表1に示すとおりで，宮崎肺吸虫と同定されたものが多かった。しかしながら，2007年6月に購入した宮崎県産のサワガニからは，ウェステルマン肺吸虫（3倍体型）が検出された。また，2008年1月に購入した宮崎県産のサワガニには，ウェステルマン肺吸虫（2倍体型）だけが寄生するもの（1匹，3個）と，宮崎肺吸虫だけが寄生するもの（3匹，各1個ずつ）が混在していた。

考　察

本邦産のサワガニからは，宮崎肺吸虫，ウェステルマン肺吸虫（2倍体型あるいは3倍体型）および大平肺吸虫（かつて佐渡肺吸虫とされたもの[6]）が検出されており，前2種が人体寄生種として重要である[7]。この中でも宮崎肺吸虫は，東北地方から長崎・宮崎両県を含めた九州に至る広範な地域において，サワガニからメタセルカリアが検出されている[7]。市販サワガニ（名古屋市で販売）からも，すでに本虫メタセルカリアが検出されており[8]，東京で購入したサワガニからの本虫検出は，ある程度予想されたものであった。

これに加えて今回は，ウェステルマン肺吸虫のメタセルカリアが検出された。サワガニからウェステルマン肺吸虫を検出したとの報告は少なく，宮崎県からは報告がなかった[7]。さらに本虫の3倍体型がサワガニから検出された地区は，鹿児島県の北薩地方と屋久島に限定される[9,10]。

ウェステルマン肺吸虫の両型（2倍体型・3倍体型）は，生物学的な性状が相互に異なり，感染時の病態にも差異を認めることがある[11]。このため，両型の鑑別はきわめて重要で，鑑別に正確を期すためには，メタセルカリアについての検討だけではなく，これを終宿主動物へ感染させて成虫を回収する必要がある。そして得られた成虫に関して，染色体標本として染色体数と減数分裂像の有無を調べるか，染色封入標本として精子形成の有無を調べることで鑑別する[12]。われわれも，市販サワガニからウェステルマン肺吸虫のメタセルカリアを改めて分離し，さらにこのような検討を進める予定にしている。

今回の調査で寄生率が最も高かったのは，2月（初旬）に購入したサワガニであった。カニの産地である宮崎県でもこの時期は大変寒く，野外でのサワガニ採集は容易でないと聞く。そこでサワガニを購入した鮮魚売り場に尋ねると，養殖サワガニなので冬期でも安定供給できたとの回答を得た。しかしながら実際は，肺吸虫メタセルカリアが多数検出されたので，販売されていたサワガニは，養殖により作出されたのではないと考えられた。恐らく夏に野外で採集し，飼育（いわゆる蓄養）しておいて，必要に応じて出荷されたのではないかと思われた。市販のサワガニは販売の時期を問わず肺吸虫寄生の危険性がある，との考えが妥当と思われた。

本調査とは別に，福岡のスーパーマーケットでもサワガニを購入し（2008年4月・宮崎県産），肺吸虫寄生の有無を調べた。その結果，検査した30匹のうち，15匹から宮崎肺吸虫のメタセルカリアが検出された（寄生率：50%）。東京以外でも食材としてサワガニが販売され，しかも肺吸虫陽性のものが混在していると云う実態が示された。肺吸虫感染の原因食品としてサワガニへの対応が必要で，関係部局との検討を始めている。

本論文の要旨は，感染症・公衆衛生関係の専門家および地方自治体の医療保健行政担当者に対する啓発情報として，「病原微生物検出情報，29，284-285，2008（http：//idsc.nih.go.jp/iasr/29/344/kj3441.html）」に速報した。

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喀痰から虫卵が検出され塩基配列から種同定した宮崎肺吸虫症の1例

日本赤十字社和歌山医療センター　呼吸器内科部
田尻智子・堀川禎夫・西山秀樹

国立感染症研究所　寄生動物部
森嶋康之・川中正憲・山崎　浩・杉山　広

Key Words：肺吸虫症，宮崎肺吸虫，虫種同定，塩基配列

緒　言

タイ人女性の喀痰から虫卵が検出され，形態と塩基配列を精査して宮崎肺吸虫と同定した。胸部X線で結節影を認め，喀痰に検出した虫卵を用いて，塩基配列から宮崎肺吸虫が原因と同定された症例報告は少なく，本例は貴重な症例と考えられた。

症例：37歳，タイ人女性。

現病歴：1991年（17年前）に来日し，千葉県に10年間，和歌山県に7年間在住している。この間に4回里帰りをしている。2007年11月下旬に胸痛を自覚し，近医を経て，精査加療目的で当院を紹介受診した。

生活歴：喫煙歴（10本/日×25～28歳）。

既往歴：高血圧。

現症：SpO_2 98%（室内気），咳嗽・喀痰なし，呼吸音に異常なし。

検査：WBC 9,800/μl (Neut 68.7%, Eo 9.4%, Baso 0.1%, Lymph 16.3%, Mono 5.6%), CRP 1.39mg/dl, ALP 138 IU/l, AST 21 IU/l, ALT 17 IU/l, LD 176 IU/l, γ-GTP 66 IU/l, BUN 15mg/dl, Cr 0.5mg/dl, Na 140mEq/l, K 4.0mEq/l, Cl 105mEq/l, 非特異的IgE 8467.0 IU/ml

画像：（胸部X線）右肺に不整な結節影を認め，左肺に浸潤影を認めた（図1a）。

（胸部CT）右肺に蛇行した構造を認め，左肺に浸潤影を認めた（図1b）。

A Case of Paragonimiasis miyazakii：Detection of Eggs in Sputum for Morphological and Molecular Identification

Tomoko Tajiri*　Sadao Horikawa*　Hideki Nishiyama*
Yasuyuki Morishima**　Masanori Kawanaka**　Hiroshi Yamasaki**　Hiromu Sugiyama**

*Department of Respiratory Diseases, Wakayama Medical Center of Red Cross Society
**Department of Parasitology, National Institute of Infectious Diseases

論文請求先：田尻智子　〒640-8558　和歌山市小松原通四丁目20番地　日本赤十字社和歌山医療センター　呼吸器内科部

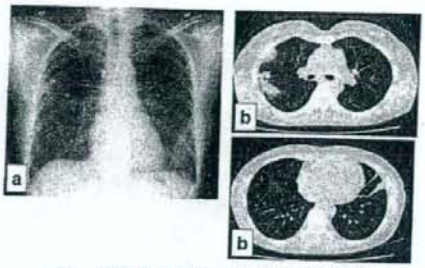

図1　胸部単純写真および胸部CT所見
a. 右上肺の結節影，左下肺の浸潤影
b1. 右肺上葉の蛇行した構造，左肺下葉の浸潤影

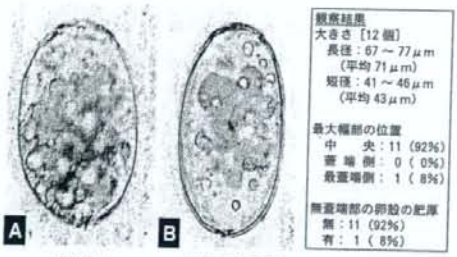

図2　喀痰中の虫卵の形態

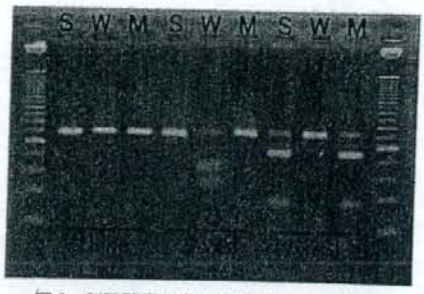

図3　制限酵素による切断パターンの解析
（PCR-RFLC）
S：検索対象の肺吸虫；W：ウェステルマン肺吸虫（2倍体型）；M：宮崎肺吸虫；各DNAを用いて増幅されたPCR産物（ITS 2領域）
検索対象の肺吸虫に由来するPCR産物は，ウェステルマン肺吸虫の配列を認識する制限酵素（*Bso*BI）では切断されず，宮崎肺吸虫の配列を認識する制限酵素（*Bss*SI）で切断され，しかもそのバンドパターンは宮崎肺吸虫のものと一致した。
塩基配列解読の結果もこの結論を支持した。検索対象の肺吸虫を宮崎肺吸虫と同定。

経過：血液検査および画像所見より肺吸虫症を疑い，喀痰および糞便を検査した。喀痰から虫卵を検出し，虫卵の形態から宮崎肺吸虫を疑ったが，免疫血清学的検査では，宮崎肺吸虫抗原およびウェステルマン肺吸虫抗原ともに抗体反応陽性を示した。プラジカンテル（75mg/kg/日×3日間）で治療を行い，自他覚所見ともに改善した。患者に食歴を尋ねると，地元のタイ料理店や日本の自宅で，サワガニ入りパパイヤの千切りサラダ（ソムタム・プー）を頻回に摂食していたことが判り，不完全加熱のサワガニが感染源になったと考えられた。

虫卵の形態学的特徴

原因虫種を推定するため，喀痰中の虫卵（12個）について計測・観察した（図2）。まず虫卵の大きさは，長径が67～77μm（平均71μm），短径は41～46μm（平均43μm）であった。次に最大幅部の位置は，中央が11個（92%），蓋端側は0個（0%），無蓋端側は1個（8%）であった。また無蓋端部における明かな卵殻の肥厚は，「認めない」が11個（92%），「認める」が1個（8%）であった。長径が長くなく，最大幅部が概ね中央部にあるという特徴から，本例の虫卵は宮崎肺吸虫ないし2倍体型ウェステルマン肺吸虫の可能性が高いと考えられた。免疫血清学的検査では種同定できず，分子生物学的手法を用い，検討を加えることにした。

塩基配列の解読と比較検索

虫卵を用い，既報[1]に準じてDNA抽出とPCR，制限酵素による切断パターンの解析，塩基配列の解読・解析を行った。PCR産物の制限酵素による切断パターンは宮崎肺吸虫のパターンを示し（図3），塩基配列解読の結果もこれに一致した。

考　察

宮崎肺吸虫は，ヒトの体内で成熟し排卵することはきわめて稀であると言われる[2]。しかし，ヒトの

肺実質に虫嚢を形成し，喀痰や胸水から排卵を認める症例も報告されるようになってきた[3]~[6]。その機序として，複数の虫体が寄生し，長期間駆虫されない場合に，生じる病態であることが示唆されている。本例でも，頻回の摂食で複数のメタセルカリアが取り込まれ，長期間駆虫されなかったため，虫体が肺実質で成熟し，排卵を認めたものと考えられた。

また，本例は，胸部写真に関しても稀な所見を呈した。一般に，宮崎肺吸虫症では，胸膜所見が目立ち，胸部写真もそれを反映して胸水貯留や気胸を呈することが多く，肺内所見は少ないと言われる[2][7]。本例は，胸膜所見が目立たず，肺野の結節・浸潤影といった肺内所見を発現し，宮崎肺吸虫症としては，非典型的な経過を辿った。

以上のような病態や胸部写真から，本例は当初，宮崎肺吸虫よりも3倍体型ウェステルマン肺吸虫を想起させるものであったが，虫卵を用いた分子生物学的検査を行うことで，宮崎肺吸虫と確定診断するに至った。本例の様に，非典型的な臨床経過を辿る肺吸虫症例もあるので，寄生虫材料を確保しての種同定は，原因を明かにするためにきわめて重要であると考えられた。

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