Food Control 20 (2009) 623–626

Contents lists available at ScienceDirect

Food Control

journal homepage: www.elsevier.com/locate/foodcont

Molecular genotyping of *Anisakis* species from Korean sea fish by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP)

Min Hwa Lee [a], Doo-Sung Cheon [b,*], Changsun Choi [a,*]

[a] Department of Food and Nutrition, College of Human Ecology, Chung-Ang University, 72-1 Nae-ri, Daeduk-myun, Ansung-si, Kyounggi-do 456-756, Republic of Korea
[b] Division of Enteric and Hepatitis Viruses, Korea Centers for Disease Control and Prevention, Nokbun 5, Tongil-ro, Eunpyung-gu, Seoul, Republic of Korea

ARTICLE INFO

Article history:
Received 28 December 2007
Received in revised form 1 September 2008
Accepted 11 September 2008

Keywords:
Anisakis simplex
Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP)
Internal transcribed spacer (ITS)
Scomber japonicus
Trichiulus lepturus
Todarodes pacificus

ABSTRACT

The anisakid nematode is a prevalent foodborne pathogen that causes gastroenteritis in humans. The aim of this study was to identify the molecular genotype of *Anisakis* worms found in Korean sea fish and squid. Sixty DNA samples were prepared from third stage larvae of *Anisakis* spp. collected from *Scomber japonicus*, *Trichiurus lepturus*, and *Todarodes pacificus*. An approximately 1000 base pair fragment in the internal transcribed spacer (ITS) regions, including ITS-1, ITS-2, and the 5.8S subunit, was amplified and digested with HinfI or HhaI. We identified restriction fragment length polymorphism patterns of *Anisakis pegreffii*, *Anisakis simplex* sensu stricto, *Anisakis typica*, and a hybrid genotype. Among the 60 isolates, 47 were identified as *A. pegreffii*, 10 as *A. typica*, one as *A. simplex* sensu stricto, and two as hybrid genotypes. *A. pegreffii* was determined to be the most prevalent molecular genotype of *A. simplex* complex found in Korean sea fish.

1. Introduction

Anisakis is a genus of parasitic nematodes belonging to the family Anisakidae. Anisakids are foodborne pathogens which cause anisakiasis in human hosts. Marine mammals (whales, dolphins, etc.) are the definitive host for *Anisakis*. Its lifecycle is complex. Larvae are consumed by small crustaceans. Marine fishes become infected after ingesting infected crustaceans and the larvae are typically found in the gut and muscle tissue. When infected fish are consumed by marine mammals, the larvae develop into adult worms. Humans become accidental hosts by eating infected raw seafood (sushi, sashimi, etc.) or undercooked fish (Sakanari & McKerrow, 1989). Human anisakiasis occurs worldwide, the majority of cases are reported in Asian countries where consumption of raw seafood is commonplace (Abe, Tominaga, & Kimata, 2006; D'Amelio et al., 1999; Im, Shin, Kim, & Moon, 1995; Umehara, Kawakami, Araki, & Uchida, 2007). In the study by Im et al. (1995), *Anisakis* species isolated from Korean marine fish or human anisakiasis cases were classified by morphological characteristics and no molecular epidemiology was reported.

Several molecular techniques have been developed to differentiate between species of the parasitic *Anisakis* nematode. Among them, polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) was used successfully to confirm the genotypes of anisakid larvae (Abe, Ohya, & Yanagiguchi, 2005; D'Amelio et al., 1999; Marques, Cabral, Busi, & D'Amelio, 2006; Szostakowska, Myjak, & Kur, 2002; Umehara et al., 2007; Zhu, D'Amelio, Paggi, & Gasser, 2000). Based on genetic analysis of the morphospecies *Anisakis simplex* by multilocus enzyme electrophoresis, *A. simplex* complex is defined as three sibling species, *Anisakis pegreffii*, *A. simplex* sensu stricto, and *A. simplex* C, which have differences in genetic structure, host preference, and geographical distribution (Abollo, Paggi, Pascual, & D'Amelio, 2003; Mattiucci et al., 1997).

Human anisakiasis cases still occur with low incidence in Korea. Human anisakiasis was diagnosed in 107 Korean patients with acute gastritis symptoms between 1989 and 1992. The etiological agents collected by endoscopy were classified as *A. simplex*, *Contracaecum* type A, and *Pseudoterranova decipiens* based solely on morphological examination. A number of marine fish including yellow corvina, sea eel, ling and yellowtail were suggested as the source of infection (Im et al., 1995). However, the molecular characteristics of *Anisakis* species in Korea have not been elucidated.

The object of this study was to determine the molecular genotypes of anisakid larvae isolated from sea fish including *Scomber japonicas* (chub mackerel), *Trichiurus lepturus* (ribbon fish), and *Todarodes pacificus* (pacific squid) which are commonly consumed in Korea and to investigate the prevalence of *Anisakis* larvae found in these species.

* Corresponding authors. Tel.: +82 31 670 4589; fax: +82 31 676 8741 (C. Choi), tel.: +82 2 380 1493; fax: +82 2 380 1495 (D.-S. Cheon).
E-mail addresses: cheonds@hanmail.net (D.-S. Cheon), cchoi@cau.ac.kr (C. Choi).

0956-7135/$ - see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodcont.2008.09.007

2. Materials and methods

2.1. Samples

Ninety sea fish and 15 squid from wholesale and retail markets were collected between March and July of 2006. Forty eight *Scomber japonicus*, 42 *T. lepturus*, and 15 *T. pacificus* were submitted for macroscopic examination. Sixty anisakid larvae were collected from the three different hosts. Among the 60 larvae, 34 were isolated from *S. japonicus*, 21 from *T. lepturus*, and five from *T. pacificus*. Larvae were examined microscopically and the morphology of all isolates was compatible with *Anisakis* species as previously described (Sakanari & McKerrow, 1989).

2.2. DNA extraction

Individual Anisakid larva isolated from sea fish samples were transferred to sterile Eppendorf tubes. Each larva was washed with phosphate-buffered saline (PBS, pH 7.0) three times and larval DNA was extracted using a modified phenol–chloroform based method. Briefly, 300 μl of 0.1 N sodium hydroxide solution was added to each sample and the samples were boiled for 10 min until the larva dissolved. Five hundred microliters of phenol (Catalog Number, C-9015, Bioneer, Daejeon, Korea) was added to each sample and the tubes were vortexed vigorously and centrifuged at 8500 rpm for 10 min. The aqueous phase of each tube was transferred to a clean tube and mixed with 500 μl of phenol:chloroform (1:1) (Bioneer). Tubes were vortexed vigorously and centrifuged at 8500 rpm for 10 min. The aqueous phase was transferred to a new tube and mixed with 500 μl of cold ethanol (Cat. No. 200-578-6, Hayman, Essex, England) and 50 μl of 3 M sodium acetate (Cat. No. S7899, Sigma, MA, USA). The tubes were kept at −20 °C overnight and centrifuged at 14,000 rpm for 30 min. DNA pellets were washed with 70% cold ethanol and centrifuged at 14,000 rpm for 30 min. The supernatant was discarded and each DNA pellet was carefully air-dried and resuspended in 30 μl of distilled water.

2.3. Polymerase chain reaction (PCR)

The forward primer A (5′-GTC GAA TTC GTA GGT GAA CCT GCG GAA GGA TCA TT-3′) and reverse primer B (5′-GCC GGA TCC GAA TCC TGG TTA GTT TCT TTT CCT CCG CT-3′) were used to amplify the entire internal transcribed spacer (ITS) region which includes ITS-1, the 5.8S ribosomal DNA and ITS-2 (D'Amelio et al., 1999; Pontes, D'Amelio, Costa, & Paggi, 2005; Umehara, Kawakami, Matsui, Araki, & Uchida, 2006; Umehara et al., 2007).

PCR was performed in a 20 μl reaction volume containing 2 μl of extracted DNA, 1× PCR buffer, 2 mM $MgCl_2$, 250 μM of each dNTP (ATP, GTP, TTP, and CTP), 0.5 μM of each primer, and 1.25 U of *Taq* DNA polymerase (Cat. No. E-2011, Bioneer). PCR was performed in an MJ mini cycler (Bio-Rad, CA, USA) using the following conditions: 94 °C for 5 min (initial denaturation), 35 cycles of denaturing at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 30 s (main cycles), and 72 °C for 10 min (final extension).

Bands were resolved by 1% agarose gel electrophoresis (Cat. No. 161-3102D, Bio-Rad, CA, USA) and stained with ethidium bromide. DNA was visualized on a UV transilluminator (SL-20 High Performance DNA Image Visualizer, UVP, Upland, CA, USA).

2.4. Restriction fragment length polymorphism (RFLP)

PCR products of approximately 1000 base pairs (bp) amplified with primer A and primer B were used for restriction fragment length polymorphism (RFLP) analysis. Amplicons (8 μl) were mixed with 10× reaction buffer and digested with HinfI or HhaI (10U; Bioneer) at 37 °C for 2 h. PCR-RFLP products were resolved by 1% agarose gel electrophoresis containing ethidium bromide at 100 V for 1 h. RFLP patterns were analyzed using Doc-IT®LS Image Analysis Software (UVP, Upland, CA, USA).

3. Results and discussion

3.1. Molecular genotypes based on PCR-RFLP

Molecular genotypes of 60 anisakid larvae isolated from three different hosts (*S. japonicus*, *T. lepturus*, *T. pacificus*) were determined by PCR-RFLP using HinfI and HhaI restriction endonucleases. Table 1 summarizes the sizes and number of the restriction fragments produced by PCR-RFLP of anisakid larvae obtained in this study. Upon digestion with HinfI, *A. pegreffii* clearly showed three bands of 370, 300, and 250 base pairs (bp) in length, *A. simplex* sensu stricto showed three bands of 620, 250, and 80 bp (the 80 bp band was typically very faint), *Anisakis typica* showed two bands of 620 and 350 bp, and the hybrid genotype showed bands of 620, 370, 300, and 250 bp in length. Several studies have shown the presence of a hybrid genotype which was identified as a recombinant anisakid larva between *A. simplex* sensu stricto and *A. pegreffii* by both sequencing analysis and PCR-RFLP (Abollo et al., 2003; Umehara et al., 2006). Two clear bands of 550 and 430 bp for *A. pegreffii* and *A. simplex* sensu stricto and four bands of 320, 240, 180, and 160 bp in length for *A. typica* were observed in PCR-RFLP patterns digested with HhaI (Table 1).

Among the 60 anisakid isolates collected 47 were identified as *A. pegreffii*, 10 were identified as *A. typica*, two were a hybrid genotype, and one was identified as *A. simplex* sensu stricto. Of the 34 isolates from *S. japonicus*, 32 were identified as *A. pegreffii*, and two were identified as a hybrid genotype. Of the twenty-one isolates from *T. lepturus*, 13 were identified as *A. pegreffii*, and eight were identified as *A. typica*, and among the five isolates from *T. pacificus*, two were identified as *A. pegreffii*, two were identified as *A. typica*, and one was identified as *A. simplex* sensu stricto (Table 2).

3.2. Prevalence of A. pegreffii

Forty-seven out of 60 anisakid larvae (78.3%) used in this study were matched with a typical PCR-RFLP pattern for *A. pegreffii*. Thirty-two of 47 *A. pegreffii* isolates were from *S. japonicus*, thirteen

Table 1
PCR-RFLP patterns of *Anisakis* species using HinfI or HhaI restriction endonucleases

Identified species	Sizes of the bands obtained with	
	HinfI	HhaI
A. pegreffii	370, 300, 250	550, 430
A. simplex sensu stricto	620, 250, 80	550, 430
A. typica	620, 350	320, 240, 180, 160
Hybrid genotype	620, 370, 300, 250	550, 430

Table 2
Molecular genotypes of *Anisakis* species isolated from three hosts by PCR-RFLP

Host	*A. pegreffii*	*A. typica*	*A. simplex* sensu stricto	Hybrid genotype
Scomber japonicus	32/34 (94.1%)	0/34 (0%)	0/34 (0%)	2/34 (5.9%)
Trichiurus lepturus	13/21 (61.9%)	8/21 (38.1%)	0/21 (0%)	0/21 (0%)
Todarodes pacificus	2/5 (40%)	2/5 (40%)	1/5 (20%)	0/5 (0%)