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Research paper

Scavenging hagfish as a transport host of Anisakid nematodes

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ABSTRACT

Hagfish are the most primitive craniates and scavengers, feeding on dead organisms as well as fish and invertebrates. Hagfish play an important ecological role in recycling nutrients, helping to recycle biomass from the upper water column. We investigated 265 specimens of four hagfish species, including *Eptatretus burgeri*, *Eptatretus* yangi, *Eptatretus sheni* and *Eptatretus taiwanae* from northeastern Taiwanese waters of the northwestern Pacific from November 2013 to June 2014. Eight species of Anisakid nematodes were identified: *Anisakis pegreffii*, *Anisakis simplex* s.s., a recombinant genotype of *A. pegreffii* and *A. simplex* s.s., *Anisakis typica*, *Anisakis* sp., *Anisakis brevispiculata*, *Anisakis physeteris* and *Hysterothylacium amoyense*. *Anisakis* sp. and *H. amoyense* represented new locality records. The prevalence, mean intensity and mean abundance of anisakid nematodes for all specimens were 21.51%, 5.39 larvae per fish and 1.16 larvae per fish, respectively. *A. pegreffii* was the most frequent species in *E. burgeri*, *E. yangi* and *E. taiwanae*, whereas in *E. sheni*, the dominant species was *Anisakis* sp. The number of nematodes was significantly related to the host length for *E. burgeri* and *E. sheni*, but was not related to the sex of the four hagfish species. This report of scavenging hagfish infected with Anisakid larvae suggests hagfish as a transport/paratenic host between cetaceans and piscivores. Anisakiasis may be caused by the consumption of infectious third-stage larvae in raw or undercooked hagfish.

1. Introduction

There are 78 extant hagfish species, Superclass Agnatha, Class Myxini, in six genera around the world (Froese and Pauly, 2014). Hagfish are found in the family Myxinidae, which contains 13 described species in three different genera, including nine species of the genus *Eptatretus*, two of *Paramyxine* and two of *Myxine* in Taiwan (Kuo et al., 1994, 2010; Kuo and Mok, 1994; Mok, 2002; Mok and Chen, 2001; Mok and Kuo, 2001). Hagfish have a cartilaginous skeleton and eel-like body; they are jawless and scaleless, without paired or dorsal fins or eyes (Fernholm, 1998). Hagfish species occur almost worldwide in subtropical, temperate and cold temperate ocean waters in both hemispheres (Jorgensen et al., 1998; Gorbman et al., 1990). The optimal conditions are a salinity around 34 ppt and a temperature below 20 °C. The fish are collected from depths ranging from 20 to 5000 m (Jorgensen et al., 1998).

Hagfish are prey for marine mammals, sharks and larger cephalopods (Martini and Flescher, 2002). They are also important scavengers that feed on dead organic matter, including teleosts, cetaceans, benthic invertebrates and discards from fisheries (Zintzen et al., 2011). They feed on marine worms and other invertebrates, such as prawns and polychaete worms (Zintzen et al., 2011). Hagfish play an important role in ecosystems because of their widespread abundance in the deep sea, scavenger behavior and role in turning over the substrate in benthic environments and recycling of organic matter (Martini and Flescher, 2002).

Anisakid nematodes belong to the family Anisakidae (Railliet and Henry, 1912), that have a life cycle, which is associated with aquatic organisms (invertebrates, fish and marine mammals) and piscivorous birds. Human Anisakiasis are caused by the genera *Anisakis* and *Pseudoterranova*, and to a lesser degree, the genera *Hysterothylacium* and *Contracaecum* (Sakanari and Mckerrow, 1989; Yagi et al., 1996; Tejada et al., 2013). The genus *Anisakis* (Dujardin, 1845) contains marine parasitic nematodes with global distribution in tropical, temperate (cold and warm) and polar waters (Nagasawa, 1990). The life cycle of *Anisakis* spp. consists of four larval stages (L1–L4) and the fifth stage, the adult worm. Larvae undergo two molts in the eggs (L1–L3) before hatching. Then the infective third-stage larvae are eaten by small crustaceans, such as copepods and euphausiaceans (Smith, 1983). Infected crustaceans are consumed by cephalopods and smaller fish, and piscivorous fish

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act as paratenic hosts after subsequent ingestion (Lile, 1998; Chen and Shih, 2015). These larvae enter the intestinal tract and migrate into the mesenteries, viscera, or muscles of the host teleosts. The life cycle is completed when infected fish and invertebrates are eaten by marine mammals and the larvae develop into adults. The nematode genus *Hysterothylacium*, which consists of 72 species, is one of the largest of the ascaridoid nematodes (Moravec et al., 2012; Li et al., 2012, 2013; Xu et al., 2014; Moravec and Justine, 2015). The life cycle of *Hysterothylacium* shows that the two molts occur in the egg, and the third larval stages are known to parasitize numerous fish species and over 100 invertebrate species (Hurst, 1984; Marcogliese, 1996; Navone et al., 1998). The adult worms mostly live in the digestive tract of fishes (Deardorff and Overstreet, 1980).

The aim of this study was to investigate the parasitic fauna of hagfish in an open and deep-water environment in Taiwan. We investigated infection parameters (i.e., prevalence, mean abundance and mean intensity) as well as the association of parasitism with fish body length and sex. Finally, we surveyed the occurrence of larval *Anisakis* nematodes in scavenger hagfish to provide information on a new pathway for *Anisakis* transmission.

2. Material and methods

2.1. Sampling

Hagfish were purchased from the Tashi fishing port in northeastern Taiwan (121°94′E; 24°84′N) between November 2013 and June 2014. We obtained 265 hagfish of four species – *Eptatretus burgeri* ($n = 66$), *E. yangi* ($n = 96$), *E. sheni* ($n = 81$), and *E. taiwanae* ($n = 22$) – identified by morphologic characteristics (Kuo et al., 1994). The fish were numbered, measured (total length = TL), weighed, and their sex determined.

A longitudinal section was cut in the fish from the cloaca to the gill apertures to expose the internal organs. Candling systems were used to reveal nematodes deeply embedded in the fish fillet and intestinal wall. The body skin, body cavity, digestive tract and viscera were examined for nematodes and other metazoan parasites with the naked eye or under a stereomicroscope. In addition, the gut contents were flushed and endoparasites and food items were examined under a dissecting microscope.

Nematode samples were fixed and preserved in 70% ethanol. The species and developmental stage of anisakid nematodes were pre-identified by morphological characteristics described previously (Smith, 1983; Shih, 2004; Chen and Shih, 2015).

2.2. Molecular identification of anisakid nematodes

Genomic DNA was isolated from larvae by using the Geno Plus Genomic DNA Extraction Miniprep System (Viogene, Taiwan). The ITS region (ITS1-5.8S-ITS2) was amplified with primer sequences for NC5 (5′-GTA GGT GAA CCT GCG GAA GGA TCA TT-3′) and NC2 (5′-TTA GTT TCT TTT CCT CCG CT-3′) (Zhu et al., 1998). PCR reactions were carried out in a total volume of 50 μl including 15 μl genomic DNA, 0.25 μl TaKaRa Ex Taq (5 U/μl; Takara, Kyoto, Japan), 4 μl dNTP (2.5 mM each), 1 μl each primer (10 μM), 5 μl of 10× Ex Taq Buffer and 23.75 μl ddH_2O. PCR cycling parameters included denaturation at 95 °C for 10 min, then 35 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °Cfor 75 s and final extension at 72 °C for 10 min.

The PCR products were analyzed by the RFLP technique with the restriction enzymes *Hha*I and *Hinf*I (Thermo Scientific, USA). All reactions were performed at 37 °C for 60 min and digested products were analyzed by electrophoresis in 2.0% agarose gel stained by GelRed Nucleic Acid Stain and visualized under UV light.

2.3. DNA sequencing

Single DNA bands containing PCR products were isolated and purified by using the Gel/PCR DNA Fragments Extraction Kit (Geneaid, Taiwan). Sequencing involved the Applied Biosystems 3730xl DNA Analyzer.

2.4. Statistical analysis

The prevalence (the% of infected fish), mean intensity (the mean number of larvae per infected host) and mean abundance (the mean number of larvae per host) of anisakid nematodes found in hagfish were calculated as described previously (Bush et al., 1997). Differences in prevalence of nematodes among the four hagfish species were tested by Pearson's chi-square test, and mean intensity and abundance were analyzed by Mann–Whitney *U* test. The relationship between intensity of nematodes and total length of hagfish was analyzed by Pearson's correlation and simple linear regression. Chi-square test was used to compare the prevalence of anisakid nematodes by sex, and mean intensity and abundance were analyzed by the Mann–Whitney *U* test. All statistical analyses involved use of R (Venables and Smith, 2009).

3. Results

3.1. Detection of nematodes in hagfish

A total of 307 anisakid larvae were collected from the four different fish species, *E. burgeri*, *E. yangi*, *E. sheni* and *E. taiwanae*. Nematodes were isolated from the inner intestinal wall and were rarely found in the muscle tissue (Fig. 1). The gut contents were grouped as shrimp, fish, cephalopod remains and unidentifiable food items. The prevalence, mean intensity and mean abundance of nematodes for the total fish sample were 21.5%, 5.4 ± 18.4 (range 1–134) and 1.2 ± 8.8, respectively (Table 1). The prevalence of the four species of hagfish was 48.5% for *E. burgeri*, 9.4% for *E. yangi*, 14.8% for *E. sheni* and 18.2% for *E. taiwanae*. The prevalence and mean abundance were higher for *E. burgeri* than the other three species ($p < 0.05$). Mean intensity did not differ among the four species.

3.2. RFLP analysis

Molecular genotypes of 307 anisakid larvae were determined by PCR-RFLP with *Hinf*I and *Hha*I restriction endonucleases. We obtained seven species of the genus *Anisakis* – *A. simplex* sensu stricto (s.s.), *A. pegreffii*, *A. typica*, *A. brevispiculata*, *A. physeteris*, *Anisakis* sp., a recombinant genotype of *A. simplex* s.s. and *A. pegreffii* – and one of the genus *Hysterothylacium*, *H. amoyense*. Restriction with *Hinf*I produced two fragments each in *A. simplex* s.s. (~620 and 250 bp), *A. typica* (~620 and 350 bp), and *H. amoyense* (~660 and 350 bp), three in *A. pegreffii* (~370, 300 and 250 bp) and *A. physeteris* (~380, 270,250 bp), four in a recombinant genotype (~620, 370, 300 and 250 bp) and no cutting site in *Anisakis* sp. and *A. brevispiculata* (~900 bp). With *Hha*I, *A. simplex* s.s., *A. pegreffii* and the recombinant genotype exhibited two fragments (~550 and 430 bp) and *A. physeteris* (~520 and 390 bp); *A. brevispiculata* exhibited three fragments (~360, 310 and 190 bp); and *A. typica* (~320, 240, 180, 160, 100 bp), *Anisakis* sp. (~360, 210, 160, 110 bp) and *H. amoyense* (~360,330, 130, 80 bp) exhibited four fragments. Subsequently, the PCR product obtained for Anisakis sp. was sequenced (KT964236), and it was found to have 100% similarity to *Anisakis* sp. KMAQ-2013 (KC342892-KC342894) in *Kogia sima* in southern Philippines (Quiazon et al., 2013).

Among 250 anisakid larvae from *E. burgeri*, 172 individuals were *A. pegreffii* (68.9%), 63 *Anisakis* sp. (25.2%), three *A. typica* (1.2%),