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Research Note

Prevalence, protein analysis and possible preventive measures against zoonotic anisakid larvae isolated from marine *Atherina* fish

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Summary

Anisakidosis is a zoonotic infection caused by ingestion of raw or undercooked marine fish including *Atherina*. In some parts of Egypt, the prevalence of anisakid larvae in marine *Atherina* still, largely, undetermined. Moreover, the currently used procedures are insufficient to avoid human infection. The aim of this study was to identify the prevalence of anisakid larvae in marine *Atherina* and to test the effect of several treatment procedures on the viability of anisakid larvae. The infection rate of marine *Atherina* with anisakid larvae was 23.12 %. Anisakid larvae protein revealed molecular weights ranged from 11.5 – 118.5 Kilo Dalton (KDa.). Moreover, freezing at -20 °C for 21 hours (hrs.) as well as vinegar (5 %) treatment for 3 days were the most effective ways to inactivate the larvae. This study states the zoonotic risk of consuming raw or undercooked marine *Atherina* and highlights the importance of freezing and vinegar treatment in preventing human infection.
Keywords: Marine *Atherina*; Anisakid larvae; Prevalence; Viability; Zoonoses; SDS-PAGE

Introduction

The growing preference to consume raw or inadequately cooked fish may favor the infection with fish borne parasites (Chai *et al*., 2005). Fish borne parasitic zoonoses affect the health of more than 40 million people around the globe and impact fish Industry by causing economic losses in aquaculture production and/or mortalities (Barber, 2007). Members of anisakidae family can infect a wide variety of marine fishes such as horse mackerel (Zurak, 2010), Chub mackerel (*Scomberjaponicus*) (Bak *et al*., 2014), anchovies and Sardines (Serracca *et al*., 2014), rainbow trout (Skov *et al*., 2014), spotted mackerel (Chen *et al*., 2014), Baltic sea cod (Mehrdana *et al*., 2014) and shad (Bao *et al*., 2015). In many coastal countries including Egypt, *Atherina* (Mediterranean sand smelt) is a locally harvested small fish and increasingly consumed as an inexpensive source of animal protein (Henderson, 1987). Previous investigations have reported that marine *Atherina* can harbor third stage larvae of anisakidae family (L3) (Dezfuli *et al*., 1990; Colak, 2013; Magda, 2010). Additionally, *Atherina boyeri* infected with anisakid L3 was found to be infective in an experimental mice model (Diab *et al*., 2010). A closer look into the available data indicates the high variation of the infection rate of marine *Atherina* with anisakid L3 in the Egyptian water (Amany, 2007; Amin, 2009). Accordingly, the infection rate of marine *Atherina* with anisakid larvae still largely undetermined and requires further investigation. The ingestion of raw or undercooked L3-infected marine *Atherina* might result in gastrointestinal signs and in some cases, anaphylactic shock (Eskesen *et al*., 2001). Moreover, the anisakid allergens embded within fish tissue might triggers allergic reactions in fish consumers (Audicana *et al*., 2002; Audicana & Kennedy, 2008). Protein analysis is widely used to characterize anisakid allergens. Most of the studies have thus far focused on the protein analysis of anisakid nematode isolated from large fish (Amany, 2007; Moneo *et al*., 2005, Moneo; 2000, Rodero *et al*., 2007; Rodriguez-Perez *et al*., 2008) and very less data are available regarding the characteristics of anisakid larvae protein in marine *Atherina*. Indeed, various measures can be implemented during or after fish processing in order to reduce the risk of allergy and gastrointestinal symptom in fish consumers (Butt *et al*., 2004). Previous studies showed that anisakid larvae could withstand the majority of current inactivation treatments (Vidacek *et al*., 2011; Vidacek *et al*., 2010). Data yielded by Caballero and Moneo provided evidences that *Anisakis* *simplex* (*A. simplex*) allergens were resistant to heat and pepsin (Caballero & Moneo, 2004). Additionally, *A. simplex* allergens autoclaved for 80 min still retain their capacity to activate basophils (Carballeda-Sangiao *et al*., 2014). In the light of these studies, it is important to test the effect of other treatment procedures on the viability of anisakid L3. The objectives of this study was to determine the infection rate of marine *Atherina* with anisakid L3. Additionally, SDS-PAGE was performed to identify the protein-banding patterns of anisakid L3. Moreover, the viability of anisakid L3 was evaluated after applying various treatment processes with an overall aim of defining the most effective procedure to reduce human infection. The results of this study state the possibility of the zoonotic risk upon consuming marine *Atherina* parasitized with anisakid L3 and suggest new methods to control human anisakidosis

Fig. 1. Marine *Atherina* fish

Material and Methods

Marine Atherina fish

A total of 679 marine *Atherina* fish was collected from local fish markets at Zagazig City, El-Sharkia province, Egypt. (Fig. 1)

Larvae recovery and identification

Larvae recovery was carried out either by macroscopic or microscopic examination. Macroscopic examination was done by visual inspection or by the aid of magnifying hand lens. The microscopic examination was conducted by muscle compression technique and artificial tissue digestion method.

Muscle compression technique

In this method, the viscera and muscles of the collected marine *Atherina* were compressed between two glass slides and examined under a dissecting microscope (Park *et al*., 2004).

Artificial tissue digestion method

The muscles, viscera and tissue of the collected marine *Atherina* were weighted, grounded using blender, then added to the artificial digestive fluid (pepsin, 5 gm /L; HCL, 7ml/L then completed to 1000 ml distilled water) at a rate of l part fish tissue/20 parts digestive fluid then incubated at 37 °C for 12 – 24 hrs. (Garcia, 2001). Afterwards, a tea sieve was used to purify the whole mixture. The supernatant was then pipetted off and the sediment was washed 3 times with saline solution and centrifuged at 1000 revolutions per minute (R/min) for 5 minutes until the supernatant becomes clear. The sediment was poured into small Petri dishes and the encysted larvae were recovered under the microscope. The collected larvae were washed in distilled water, cleared in lacto-phenol and permanently mounted in polyvinyl alcohol. The larvae were left to dry in hot air oven at 40 – 50 °C for 24 hrs and then examined microscopically (Pritchard & Kruse, 1982; Moravec, 1994) and micro photographed. The mounted larvae were identified according to (Amany, 2007; Asmaa, 2009; Chai *et al*., 2005).

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis

Ninety anisakid L3 were collected from viscera and muscles of infected marine *Atherina* using the previously mentioned methods. Afterwards, the collected larvae were ground together in a mortar containing 5 ml of phosphate buffer saline (PBS), centrifuged at 10000 R/min for 5 minutes. While the resultant pellet was discarded, the supernatent was considered as a crude extract of anisakid larvae (Rodriguez-Perez *et al*., 2008). The supernatant was used as a starting material for the SDS-PAGE. Electrophoresis under non-denaturating condition was performed in 10 gm/100 L (w/v) acrylamide slab gel using a Tris-glycine buffer (pH 8.3) (Laemmli, 1970). Protein samples of 10 – 50 μl were denatured at 100 °C for 5 minutes in the sample buffer (l volume of sample : 2 volume of sample buffer). A marker protein, obtained from BioRad laboratories; Catalogue No. 452, was also denatured with the same sample buffer. The marker protein is composed of B. galactosidase, 118 KDa.; Bovine serum albumin, 92 KDa.; Ovalbumin, 52.2 KDa.; Carbonic anhydrase, 35.7 KDa.; Soyabean trypsin inhibitors, 28.9 KDa.; Lysozymes, 20.8 KDa.; Aprotinin, 6.8 KDa. The samples and marker proteins were inoculated followed by an electrophoretic run and gel photography. The polymorphic peptide bands among the samples were scored as positive (+) for present and negative (-) for absent using Lab image program.

Testing the viability of anisakid larvae under various treatments

In this experiment, we used parts of the previously collected marine *Atherina* to isolate live anisakid L3 using the previously mentioned methods. Ninty nine viable anisakid L3 were placed in PBS and then observed for viability by microscopic examination (Huang, 2005, Karl *et al*., 1994, Solas, 2009, Vidacek *et al*., 2009, Rodriguez-Mahillo *et al*., 2008). The isolated larvae were exposed to the following treatments:

- Vinegar (5 %): Forty five viable anisakid larvae were divided into 3 groups, each contains 15 viable larvae. Each group was immersed, separately, in Vinegar solution 5 % (Acetic acid) at room temperature for 21 hrs., 48 hrs. and 72 hrs.
- Salt solution (NaCl 10 %): Nine viable anisakid larvae were suspended in NaCl solution (10 %) at room temperature for 21 hrs.
- Refrigeration (4 °C): Fifteen viable anisakid larvae were placed in PBS and exposed to refrigeration temperature (4 °C) for 24 hrs. Since we found that all the larvae viable after 24 hrs., the same larvae were further placed in refrigerator for 72 hrs. and one week.

- Freezing (-20 °C): Fifteen viable anisakid larvae were placed in PBS and incubated at a freezing temperature (-20 °C) for 21 hrs.
- Pepsin/HCL mixture: Fifteen viable anisakid larvae were immersed in pepsin/HCL mixture (Artificial tissue digestion fluid) at 37 °C for 72 hrs.

Each of the previously mentioned treatments were conducted in separate dishes. Motility of the recovered larvae under the microscope was considered as a viability indicator. The larvae that show spontaneous movement are considered viable and those which were found non-motile are counted as dead. Both the numbers and the percentages of the viable and dead anisakid larvae were calculated.

Results and Discussion

Identification and prevalence of anisakid L3 in marine Atherina:
Human anisakidosis has been reported with increased frequency after consumption of raw or undercooked infected marine fish such as mackerel, squid, cod, anchovy, salmon and tuna (Noh *et al*., 2003). Recent data indicates that Surmullet (*Mullus surmuletus*) and common pandora (*Pagellus erythrinus*) as well as sardine (*Sardina pilchardus*) that are captured from Mediterranean coasts can harbor anisakid larvae with varying prevalence (Pulleiro-Potel *et al*., 2015; Molina-Fernández *et al*., 2015). Marine *Atherina* is considered to be a reservoir for anisakid larvae. It can harbor anisakid L3 and human is known to be a susceptible host. Upon infection, the clinical disease manifests itself as abdominal pain, nausea, vomiting and diarrhea (Kobayashi *et al*., 2007). In this study, we aimed at investigating the prevalence of anisakid larvae in marine *Atherina* that were collected from local markets in Zagazig city in El-Sharkia province, Egypt, where it represents a major source of low-cost protein. The muscle and viscera of 679 marine *Atherina* were examined macroscopically and microscopically using muscle compression and artificial tissue digestion methods. Microscopically, the identified larvae were cylindrical in shape, 20 – 36 mm in length and 0.4 – 0.45 mm in width. Upon magnification, boring tooth was prominent at the anterior end and the mucron was markedly located at the caudal end of the identified larvae (data not shown). As documented by (Nada & Abd El-Ghany, 2011; Koinari *et al*., 2013), the morphology of the identified larvae holds similarities with that of anisakid family and suggests a close species relationship with *A. simplex* that has been descriped previously (Arafa *et al*., 2009).

The infection rate of marine *Atherina* with anisakid L3 was 23.12 %. Quite similar results have been reported by (Amin, 2009) where the infection rate was (23.4 %). Lower infection rates were reported in other studies (Valero, 2006; Amany, 2007; Amer, 2007; Asmaa, 2009; Chaligiannis *et al*., 2012; Farjallah *et al*., 2008; Koinari *et al*., 2013; Marty, 2008). Higher infection rates were documented by others (Nada & Abd El-Ghany, 2011; Jabbar *et al*., 2013; Ma *et al*., 1997; Setyobudi *et al*., 2011). These remarkable variations in the aforementioned studies may be attributed to diverse biological factors, for instance the abundance of the intermediate hosts, fish feeding habits (pellets versus raw feed), length and weight of fish. Indeed, the larger and heavier the fish, the more the accumulation of anisakid larvae in its body, particularly when the large fish pray the small parasitized one (Bernardi, 2009; Ma *et al*., 1997; Valero, 2006).

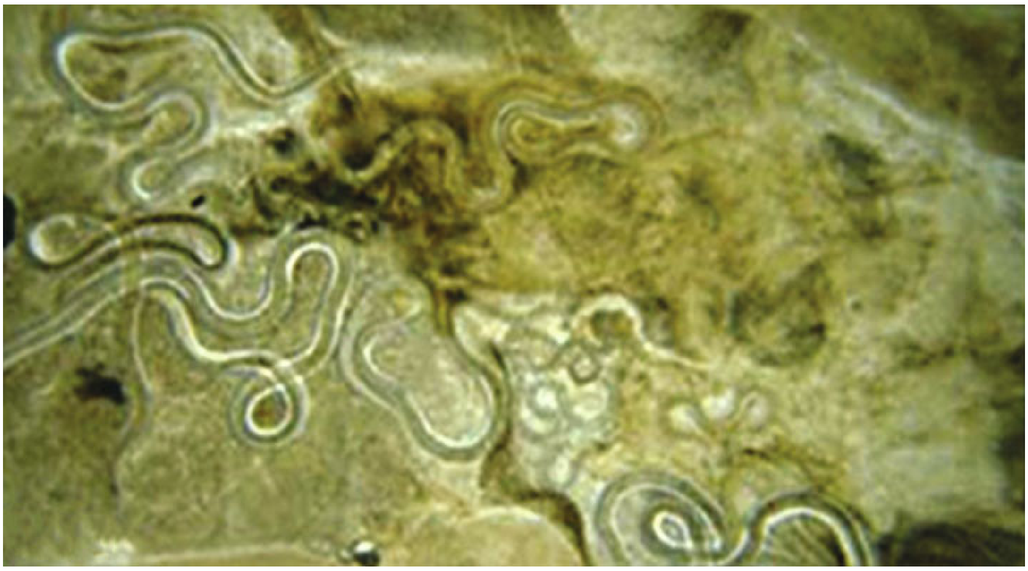

Fig. 2. High intensity of anisakid larvae in the viscera of examined marine *Atherina* (×100)

Interestingly, we found higher density of anisakid larvae in the viscera of the examined marine *Atherina* (Fig. 2) compared to fish muscle. There are ample evidences that the larvae of anisakidae family tend to have a different tissue preference. This might be due to either differences in the fish host (Quiazon *et al*., 2011) or due to the location-associated abundance of certain nutrients. (Smith, 1984) found that anisakid larvae can parasitized the viscera more than other fish parts of marine telosts. In chum Salmon, most of anisakid larvae prefere to infect the muscle more than other parts of the fish body (Setyobudi *et al*., 2011). The presence of this nematode with such a high intensity in the viscera of infected marine *Atherina* species points out to the great risk for human if this fish is consumed without prior treatments. The results of this experiment laid out an evidence that marine *Atherina* can harbor infective anisakid larvae. Accordingly, this type of fish is a suitable reservoir

Table 1. Protein banding patterns of anisakid larvae isolated from marine *Atherina* using SDS- PAGE

Molecular weight of anisakid L3 protein (KDa*)	Marker protein	Sample protein
118.5	***–	**+
118	+	–
92	+	–
52	+	–
47	–	+
35.7	+	–
30.7	–	+
28.9	+	–
22.8	–	+
20.8	+	–
11.5	–	+
6.8	+	–

*KDa: Kilo Dalton; **+: Present; ***–: Absent

host for anisakid worm and the ingestion of undercooked infected marine *Atherina* probably lead to human anisakidosis. Further studies are recommended to identify the species of zoonotic anisakid larvae and the distribution of the larvae in different parts of the fish body.

SDS-PAGE analysis

Accidental ingestion of raw or undercooked seafood contain live anisakid larvae could triggers a typical hypersensitivity reaction (Rodriguez-Perez *et al*., 2008). This is due to the fact that the excretory/secretory products (ESPs) of anisakid larvae are recognized by the host as foreign antigens (Raybourne *et al*., 1986). These proteins might invoke gastro-allergic symptoms and sensitivity in infected human (Moneo *et al*., 2005; Park *et al*., 2011; Park *et al*., 2012; Cho & Lee, 2006). A number of anisakid proteins have been recognized and listed in the Allergome database (Mari *et al*., 2009; Cho *et al*., 2014). Additionally, species-specific antigens are elicited from anisakid L3 and constitute the basis for currently used diagnostic tests (Del Pozo *et al*., 1997; Daschner *et al*., 2000). In order to elucidate the properties of anisakid larvae protein, protein analysis using polyacrylamide gel electrophoresis (SDS-PAGE) was performed. It permits fractionation of the larval protein into different bands with various molecular weights. As shown in Table 1 and Fig. 3, the SDS-PAGE of anisakid L3 revealed five protein bands with different molecular weights of (11.5, 22.8, 30.7, 47 and 118.5 KDa.). Although further *in vivo* experiments could substantiate the idea, we could claim that the identified proteins might contribute to allergy cases in human since the minced larvae were previously determined to be of zoonotic nature. Lower molecular weight protein (9 KDa.) was identified in other studies (Moneo *et al*., 2005). Rodero *et al* detected proteins of similar molecular weight ranged from 14 – 184 KDa. (Rodero *et al*., 2007). Moreover, it was verified that the *A. simplex* crude extract antigens showed protein bands with molecular weights of 205, 120, 66 – 45, 40, 31 – 21 and 14 KDa. (Rodero *et al*., 2002). Also, Kobayashi *et al*. obtained a new heat-stable allergen (Anis 8) from *A. simplex* larvae with a molecular weight of 15 KDa.(Kobayashi *et al*., 2007). In another study, it was found that analysis of *A. simplex* crude extract revealed a protein with a molecular weight of 24 KDa. This study put forward the claim that this protein is considered a potent allergen released from the excretory gland of anisakid larvae (Moneo, 2000). The gene coding for this protein was isolated from *A. simplex* L3 cDNA library by expressed sequence tag analysis (Park *et al*., 2004). Other study reported several protein bands of *A. simplex* larvae with molecular weights of 43.90, 35.14, 32.18, 27.85, 25.60, 20.10, 18.78, 18.10, 17, 15.20 and 14.30 KDa (Amany, 2007). As pointed out by Rodriguez-Perez *et al*., the crude extract protein of *A. simplex* larvae had a molecular weight of 14 KDa. (Rodriguez-Perez *et al*., 2008). The observed diversity in the molecular weights of anisakid L3 proteins might be due to differences in the type of the prepared antigen, the maturity stage of the isolated larvae, gels and the condition of SDS-PAGE (Amany, 2007). Although we do not have sufficient evidence to determine

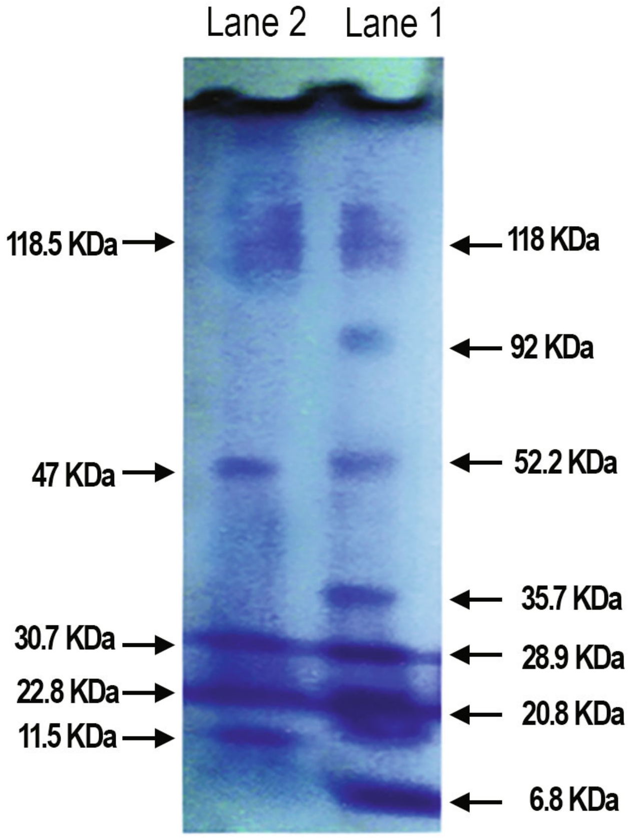

Fig. 3. SDS-PAGE indicates the protein bands of anisakid larvae isolated from marine *Atherina*
Lane 1 indicates marker protein and Lane 2 indicates sample protein

whether these proteins could play a role as an allergen, this remains possible. The results of this experiment are considered level-one demonstration for ani-sakid L3 – derived proteins. It suggests that the anisakid L3 crude protein has molecular weights ranged from 11.5 – 118.5 KDa. The next goal would be to conduct additional *in vivo* experiments and immunology oriented studies to provide an insight as to the allergenic properties of these proteins. Furthermore, the species and stage associated differences in terms of the allergic characteristics of the produced proteins still an interesting phenomenon, which warrants for further investigation.

Studying the viability of anisakid L3 under different treatment conditions

Human anisakidosis is most commonly associated with consumption of raw or lightly cooked seafood. In Mediterranean countries, the traditional fish dishes are usually non-thermally treated and hence, constitute an important public health problem. This might be, in part, due to the heat resistance of *A. simplex* allergen (Moneo *et al*., 2005), but also can be a result of the physical damage caused by the migrating larvae after consumption of infected fish (Audicana & Kennedy, 2008). Various food-processing treatments can be applied on fish to reduce the risk of human infection (Butt *et al*., 2004). Although microwave treatment can kill anisakid larvae faster than normal waterbath heating, it does not penetrate all areas of fish tissue depending on the thickness and was not able to inactivate anisakid allergen (Vidacek *et al*., 2011). However, chilling, freezing and heat treatments have been tested for their effect on the anisakid L3 (Tejada *et al*., 2006). Going forward with the question of what is the most effective methods to inactivate the anisakid larvae, the effect of several food processing treatments on the viability of anisakid L3 isolated from marine *Atherina* eas evaluated. According to our results, anisakid larvae were quite resistant to vinegar solution 5 % (acetic acid) over different treatment periods. Immersion of anisakid larvae in vinegar solution 5 % for 21 and 48 hrs. resulted in death of only 20 % (3 out of 15) of the larvae and the remaining 80 % (12 out of 15) were found viable. Extending the incubation time to 72 hrs resulted in death of all the exposed larvae (100 %) (Table 2). There are some similarities between our results and that found in other studies (Sanchez-Monsalvez *et al*., 2005). They argued that further washing of marinated fish would reduce the acid concentration to a level that is acceptable for the consumers. The actual selection of the acetic acid concentration in the marinating process depends on the cost and the processing time available. We have tested a concentration of 5 % of acetic acid since this is the most commonly used one in the commercial products. When the live anisakid L3 were incubated in salt solution (NaCl 10 %) for 21 hrs., 7/9 (77.7 %) larvae were found dead and 2/9 (22.2 %) were found viable. Our results also verified that incubating the larvae at 4 °C in the home refrigerator for 24, 72 hrs. and one week had no effect of the larvae viability. Similar findings

Table 2. Effect of different treatment procedures on the viability of anisakid larvae recovered from marine *Atherina* fish

Treatments	Number (N) of exposed live larvae	N of viable larvae	% of viable larvae	N of non-viable larvae	% of non -viable larvae
Vinegar 5 % for 21 hours (hrs.)	15	12	80	3	20
Vinegar 5 % for 48 hrs.	15	12	80	3	20
Vinegar 5 % for 72 hrs.	15	-	0	15	100
Salt solution (NaCl) 10 % for 21 hrs.	9	2	22.2	7	77.7
Refrigeration temperature (4˚C) for 24 hrs.		15	100	-	0
Refrigeration temperature (4˚C) for 72 hrs.	15	15	100	-	0
Refrigeration temperature (4˚C) for one week		15	100	-	0
Freezing (-20˚C) for 21 hrs.	15	-	0	15	100
Pepsin/HCl mixture for 72 hrs.	15	15	100	-	0

were reported earlier by Huang (2005) and Vidacek *et al*. (2009). The authors clarified that the anisakid L3 can survive the chilling temperature for more than 8 months. It is likely that refrigeration at 4 °C is not the method of choice to protect the fish consumers from marine *Atherina*-borne anisakid larvae. Since -20 °C is the most commonly used freezer temperatures either in manufacturers of salt-fermented seafood or in house hold settings, we tested the viability of the larvae this condition. The study revealed that exposing the larvae to -20 °C for 21 hours killed all larvae. Other studies stated that L3 larvae were killed within 48 hours under -20 °C (Oh *et al*., 2014). Similar findings were reported by previous studies (Beldsoe, 2001), where anisakid L3 were found dead by freezing after 24 hrs. Here, we reported that the same larvicidal effect could be attained in a shorter time. The underlying mechanism behind the larvicidal effect of freezing on the anisakid L3 still a debatable issue. Remodeling of the body shape of frozen larvae were observed by environmental scanning electron microscope (SEM) suggesting that freezing might alter the permeability of the larval cuticle (Tejada *et al*., 2006). However, (Archer, 2004) reported that freezing process induces physical and chemical modifications and possibly genetic changes that eventually lead to larval death. Another plausible explanation is that when the temperature around the nematode is dramatically declined, damage from thermal shock might be initiated. Indeed, frozen cells can be mechanically disrupted by the formation of intra-and extracellular ice crystals. During freezing, the concentration of cell solutes could mediate dissociation of cellular lipoproteins (Elkest & Marth, 1992). The lethal effect of freezing seems to be common for all nematode larvae as stated by Shaver and Mizelle, who found that the larvae of *Trichenella spiralis* were inactivated upon exposure to -22 °C for one minute (Shaver & Mizelle, 1955). It is worth mentioning that the fish should be frozen for a sufficient time to ensure that the larvae are killed. There are other factors that might affect the whole process such as the temperature of the freezer and the mass of the fish in the container (Wharton, 2002). Live anisakid larvae exposed to pepsin/HCl treatment remain viable after an exposure time of 72 hrs. The resistance of the larvae to pepsin treatment raises up a possibility that the larvae may survive in the acidic condition of the human stomach. Although we stated that both freezing and vinegar treatments has a larvicidal action, we expect the same lethal effect on the larvae embedded in fish body. This is because the within-fish larvae are accessible and easily affected by the applied treatment. This notion is support by the fact that the connective tissue capsule that surround the larvae, as a host defense mechanism, did not provide any barrier against ice nucleation (Wharton, 2002). Marine *Atherina* is a small size fish (ranged from 1.6 cm to 12.5 in other species) (Pombo, 2005) and has low protein and fat content. Therefore, these treatments might have the same effect if applied to the fish containing the anisakid L3. From a public health point of view, it is assumed that freezing and vinegar treatment of infected marine *Atherina* would contribute more to the reduction of anisakid associated gastrointestinal symptoms than reducing the allergy-related manifestation because *A. simplex* allergens, which invoke a hypersensitivity reaction, seems to be highly resistant to heat and freezing (Vidacek *et al*., 2009). *A. simplex* allergens are preserved in long-term frozen storage (-20 degrees C +/- 2 degrees C for 11 months) of parasitized hakes (Rodriguez-Mahillo *et al*., 2010). Moreover, Several allergens from *Anisakis simplex* are highly resistant to heat and pepsin treatments (Caballero & Moneo, 2004). Therefore, these procedures might not be adequate to avoid allergy in individuals previously sensitized to *A. simplex*. Nevertheless, it would be interesting to explore the effect of these treatments on the allergen secreted by anisakid nematode.

Based on the data presented in this study, it is recommended that marine *Atherina*, intended for human consumption should be either frozen at -20 °C for 21 hrs. or immersed in vinegar solution 5 % for at least 72 hrs. to ensure the death of anisakid L3 and subsequently reduce the risk of human anisakidosis.

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