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Research Note

Histology as a Valid Tool To Differentiate Fresh from Frozen-Thawed Marinated Fish

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ABSTRACT

European Commission Regulation (EU) 1276/2011 requires that fishery products intended for raw consumption be frozen at –20°C for not less than 24 h or at –35°C for at least 15 h in order to kill viable parasites other than trematodes. But because marinating processes are not always effective in destroying nematode larvae, raw marinated fish preparations should be frozen before consumption. This study evaluated the performance of a standardized histological method to distinguish between fresh and frozen-thawed raw marinated fish. Sixty anchovy (*Engraulis encrasicolus*) fillets were sampled: 30 were marinated at +4°C for 24 h, and 30 were frozen at –20°C for 24 h before being marinated for 24 h. All 60 samples were fixed in formalin, processed for paraffin embedding, cut, and stained with hematoxylin and eosin. The slide preparations were examined microscopically by three independent histopathologists and classified as frozen-thawed or negative according to standard operating procedure criteria in use at our laboratory. Performance evaluation of the method showed 100% sensitivity (95% confidence interval [CI], 88.4 to 100%) and 100% specificity (95% CI, 88.4 to 100%), and the interrater agreement (Cohen's kappa) was 1 (95% CI, 0.85 to 1). Histology proved a valid and reliable tool to distinguish fresh from frozen-thawed marinated fish. It can be applied to deliver safe raw fishery products to consumers in order to minimize the risk of anisakidosis.

Key words: Fresh; Frozen-thawed; Histology; Marinated fish

Anisakidosis is a zoonotic disease caused by nematodes belonging to the genera *Anisakis* and *Pseudoterranova*. Human infestation occurs by ingestion of third-stage *Anisakis* larvae present in cephalopods, raw fish, and almost-raw fish, i.e., undercooked, marinated, pickled, smoked, or salted seafood. The prevalence of *Anisakis simplex* infection in fish varies widely between geographical areas or fishing grounds and fish host species *(10)*. A study carried out in the middle Adriatic *(4)* reported that of 5,696 anchovies sampled, 5.72% were infected with Anisakidae larvae: 4.79% with *Anisakis* larvae, 1.23% with *Hysterothylacium* larvae, and 0.3% with the larvae of both genera. A more recent study *(6)* investigating the prevalence of Anisakidae larvae in anchovies fished off the Tyrrhenian coast of central Italy estimated a prevalence of 2.3%. No recent data are available for Sicily.

Data from 2010 show that the average fish consumption in the European Union is 23.1 kg per capita per year *(9)*. About 5% of the world's anchovy catch (563,000 metric tons) is from the Mediterranean and the Black Sea. Italy is one of the largest supplier countries and a major producer of prepared and preserved anchovies (6,600 metric tons in 2008) *(7)*. The marked worldwide rise in the prevalence of anisakidosis in humans in the last 35 years has been linked to increased application of diagnostic techniques, greater global demand for seafood, and growing consumer preference for raw or lightly cooked food, which together have increased the risk of parasite exposure *(10)*. In Italy alone, 54 cases of anisakidosis in humans were reported between 1996 and 2011, most often in coastal areas *(14)*, in addition to sporadic anisakidosis cases after the consumption of marinated anchovies *(11, 15, 16)*. Because the marinating process is not always effective in destroying nematode larvae, these preparations should be frozen before consumption.

European Union Regulation 1276/2011 *(8)* require that fishery products intended for raw consumption and, also, "almost-raw fish" be frozen at –20°C for not less than 24 h or –35°C for at least 15 h in order to kill viable parasites other than trematodes. Declaration of fish as fresh or frozen-thawed is compulsory; however, differentiating between fresh and frozen-thawed fish is not easy. In response to this problem, a histological method to identify fish as fresh or frozen-thawed was validated and accredited at the State Veterinary Research Institute of Piedmont, Liguria, and the Valle D'Aosta (IZSPLV), Italy, in 2009. This simple, rapid, and cost-effective method has a sensitivity of 90.70% (95% confidence interval [CI], 82.49 to 95.90%) and a specificity of 92.59% (95% CI, 75.71 to 99.09%) *(3)*; it was validated

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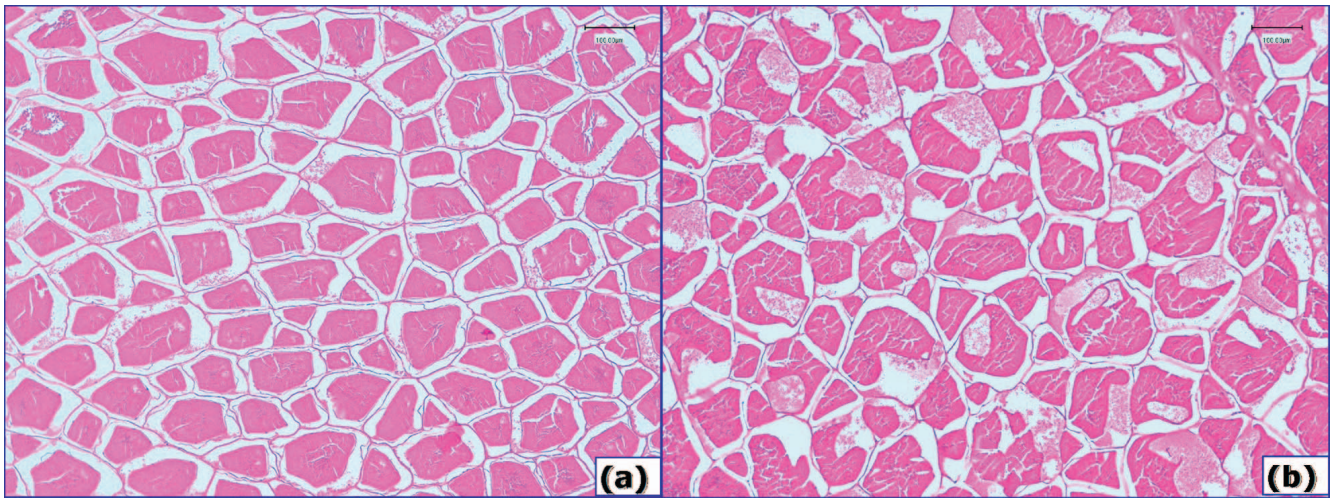

FIGURE 1. *Microscopic characteristics of fresh (a) and frozen-thawed (b) marinated fish. Transverse muscle sections with hematoxylin and eosin staining.*

irrespective of the fish species analyzed. It has been applied in official monitoring plans since 2012. The aim of this study was to evaluate the histological method's reliability in distinguishing between fresh and frozen-thawed raw marinated fish.

MATERIALS AND METHODS

Anchovy (*Engraulis encrasicolus*) was chosen as the model species. Sixty anchovies caught in Sicily (FAO fishing area 37) were manually filleted and marinated in a vinegar and water (1:1) marinade containing 1% citric acid and 3% NaCl, according to the marinating protocols in use in Italy *(5)*. Thirty anchovies were marinated at refrigeration temperature (+4°C) for 24 h, and 30 samples were frozen for 24 h at −20°C (i.e., the time-and-temperature combination stated in European Union Regulations) before being marinated for 24 h. These 60 samples (30 fresh and 30 frozen samples) were the reference materials.

After marinating, before the usual conservation in seed oil, all 60 samples were fixed in 10% neutral buffered formalin for 24 h, placed in numbered plastic boxes, and routinely processed. Paraffin-embedded blocks were cut on a microtome into 3-μm (±2-μm) sections and stained with hematoxylin and eosin. Three expert histopathologists independently examined the slide preparations by optical microscopy at increasing magnification (×100, ×200, and ×400) and classified them as frozen-thawed or fresh according to the standard operating procedure criteria in use at the IZSPLV laboratory. Frozen-thawed samples were identified when vacuoles of various dimensions, optically empty or filled with eosinophilic material caused by ice crystals, were observed in the cytoplasm of muscle cells; fresh samples were identified when these microscopic changes were absent. A "nonconclusive'' classification, owing to unspecific microscopic findings according to the standard operating procedure criteria in use, was also considered as a result. The sensitivity and specificity of the histological method were calculated, with corresponding 95% CIs; the combined Cohen's kappa for the three experts was estimated.

RESULTS

All 60 fresh and frozen samples (Fig. 1) were correctly identified as such. Optically empty spaces or spaces filled with eosinophilic material were easily observed in the frozen samples. No samples were classified as "nonconclusive."

Statistical analysis showed 100% sensitivity and 100% specificity. The combined Cohen's kappa of 1 (95% CI, 0.85 to 1) indicated perfect agreement between the readers.

DISCUSSION

We selected anchovy (*E. encrasicolus*) as the species model because it is similar in size to other commonly consumed raw marinated fish products, such as pilchards (*Sardina pilchardus*) and round sardinellas (*Sardinella aurita*). All samples were correctly classified: the microscopic changes were unequivocally identified in the samples frozen at −20°C for 24 h. The method had optimal sensitivity and specificity and a high interrater agreement (Cohen's kappa of 1). A further validation study is in progress to evaluate microscopic lesions after freezing at −35°C for 15 h in fish and raw marinated fish.

Marinating is one of the oldest and most popular methods for preserving fish; however, because traditional marinating methods are not effective in killing *A. simplex* larvae, marinated fish should be considered as "almost raw" and frozen before consumption to protect against potential anisakidosis infection. Therefore, a valid analytical tool to differentiate fresh from frozen-thawed marinated fish is needed.

Studies investigating the effect of marinating on inactivation of *Anisakis* larvae showed that the larvae are inactivated in anchovies marinated in 6% vinegar and 12% NaCl and conserved at +4°C for 13 days *(17)* and in pilchards treated with 6% acetic acid and 10% NaCl for 24 h and then conserved in oil at +4°C for 13 days *(2)*. Both methods, as well as a variant marinade with 2.4% acetic acid and 6% NaCl for 35 days *(1)*, are reported by the European Food Safety Authority *(10)* as being able to inactivate *Anisakis* larvae. Recently, Giarratana et al. *(13)* evaluated the effect of allyl isothiocyanate on *Anisakis* larvae during the anchovy marinating process and concluded that allyl isothiocyanate is a promising biocidal agent against *Anisakis* larvae in industrial marination processes. The same group conducted a preliminary study on the activity of *R*-limonene

against *Anisakis* larvae *(12)*. Our results show that histology is a reliable and highly accurate method to differentiate fresh from frozen-thawed marinated fish. It may be usefully applied as a screening tool to ensure food safety and minimize the risk of anisakidosis, as well as to assess the effect of freezing treatment on fishery products.

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