

Rapid Method for Detection of Anisakidae Larvae in Marine Fishes, Based on UV Transillumination

Gaetano Vitale Celano, Antonello Paparella, Armida Fransvea, Claudia Balzaretti, and Giuseppe Celano

Abstract—A rapid and sensitive detection method is proposed to evaluate the presence and vitality degree of Anisakidae larvae in marine fishes by UV diaphanoscopy. 923 fish samples belonging to seven fish species (*Merluccius merluccius*, *Molva elongata*, *Sardina pilchardus*, *Scomber scomber*, *Triglia lucerna*, *Engraulis encrasicolus*, and *Trachurus trachurus*) were evaluated for the presence of Anisakidae larvae by using direct observation, peptic digestion, and UV transillumination. The results obtained indicate that UV transillumination can be considered a rapid and accurate tool for routine analysis of marine fishes even under field condition.

Index Terms—Anisakidae, larvae, detection, UV, transillumination.

I. INTRODUCTION

Anisakiasis, first reported in the Netherlands in the early 1960s, is a fish-borne parasitic disease caused by consumption of raw or undercooked fish or cephalopods contaminated by third larval stage of the Anisakidae family parasites, in particular *Anisakis simplex*, *A. pegreffii* and *Pseudoterranova decipiens* [1].

The prevalence and intensity of *Anisakis* infection varies with fish species, fishing area, and season [2]. Marine mammals or piscivorous birds are typically used as definitive hosts, while planktonic or benthic crustaceans act as intermediate hosts, and fish as principal transport hosts. Infection is caused by the larval stages carried by numerous fish species. Adult *A. simplex* usually live in the gastrointestinal tract of cetaceans, while the adults of *Pseudoterranova* spp. and *Phocascaris* spp. are mainly found in pinnipeds, although the definitive host range of many anisakid species is not well understood [3]. Alternative transmission routes have been suggested, such as direct infection of fish by larval stages ingestion, or the transfer of larvae from crustaceans [4]. Female worms lay eggs, which are shed in the faeces of infested marine mammals and embryonate in seawater to second-stage larvae that hatch out [5]. Then, the first intermediate hosts

are eaten by fish and cephalopods, where the third-stage larvae encyst. Finally, the life cycle is completed when marine mammals eat infected fish or cephalopods.

Human infection may be considered accidental, as humans are not suitable hosts for anisakids. However, these parasites have been involved in foodborne infections caused by consumption of raw or undercooked seafood. Reports about detection of anisakids in different fish batches, in particular in the autumn-winter season, have drawn public attention to their potential impact on public health.

Several types of parasite detection methods have been proposed for anisakids, but they are all time-consuming and difficult. In fact, different DNA-based techniques are available [6]-[8], although their applicability in production environment is debatable. In the European Union, the official method for larvae detection (Council Directive 91/493/EEC) is direct observation (Reg. 2074/2005/EC), sometimes coupled with chlorine-peptic digestion. The aim of this study was to compare the official protocol with a new observation method, based on UV transillumination, in order to evaluate larvae location and degree of infestation in seven fish species.

II. MATERIALS AND METHODS

923 fish samples belonging to seven fish species (*Merluccius merluccius*, *Molva elongata*, *Sardina pilchardus*, *Scomber scomber*, *Triglia lucerna*, *Engraulis encrasicolus*, and *Trachurus trachurus*) were analysed. Anisakidae larvae location and degree of infestation were evaluated by using direct observation, chlorine-peptic digestion and UV transillumination.

In all samples, the count was determined separately in the coelomic cavity, in the hypoaxial and epiaxial muscles, by careful dissection and direct observation of the various anatomical districts, according to the procedure described in Reg. 2074/2005/EC.

Peptic digestion was carried out by means of Trichomatic 35 (Foss Electric, Denmark). The samples were subjected to digestion with hydrochloric acid 9% (30 ml) and pepsin 1/10000 (5 g) for each cycle of 8 min. Digested samples were laid on membranes, and observed by optical microscopy at 10 x.

For UV transillumination, the method described by [9] was modified as follows: 1. saline solution (1:5 w/v) at 30 °C was added to fish samples, which were then homogenized in stomacher for 1-2 min; 2. the homogenate was pressed to a thin layer of 5 mm and examined under UV light (Transilluminator Fotodine 3-3102, Celbio, Italy) at 366 nm in a dark room. In such operating conditions, the larvae

Manuscript received February 10, 2013; revised April 11, 2013.

G. V. Celano is with the Veterinary Medicine Department, University of Bari, Italy (email: gaetanovitale.celano@uniba.it).

A. Paparella is with the Faculty of Bioscience and Technology for Food, Agriculture and Environment, University of Teramo, Italy (email: apaparella@unite.it).

A. Fransvea and G. Celano are with Med & Food C.Q.S. Srl, Valenzano (BA), Italy (email: armidafransvea@gmail.com, g.celano1@gmail.com).

C. Balzaretti is with the Department of Veterinary Science for Health, Animal Production, and Food Safety, University of Milan, Italy (email: claudia.balzaretti@unimi.it).

remain intact, and *Anisakis* spp. shows bluish-white fluorescence [10].

chlorine peptic digestion, and UV transillumination, are shown in Table I. Our data indicate that peptic digestion was not suitable for anisakids detection, because only fragments of larvae were recovered.

III. RESULTS AND DISCUSSION

The results obtained by comparing direct observation,

TABLE I: DISTRIBUTION OF ANISAKIDAE LARVAE IN DIFFERENT ANATOMICAL REGIONS OF SEVEN FISH SPECIES

Species	Samples (total number)	Method of analysis											
		Direct observation				Chlorine peptic digestion				UV light observation			
		Total number of larvae	Coelomic cavity	Hypoaaxial muscle	Epiaxial muscle	Total number of larvae	Coelomic cavity	Hypoaaxial muscle	Epiaxial muscle	Total number of larvae	Coelomic cavity	Hypoaaxial muscle	Epiaxial muscle
<i>Engraulis encrasicolus</i>	95	-	-	-	-	N.D.	N.D.	N.D.	N.D.	11	6	2	3
<i>Sardinapilchardus</i>	106	2	2	-	-	N.D.	N.D.	N.D.	N.D.	14	9	3	2
<i>Trigialucerna</i>	54	6	6	-	-	N.D.	N.D.	N.D.	N.D.	27	22	5	-
<i>Molvaelongata</i>	96	7	7	-	-	N.D.	N.D.	N.D.	N.D.	49	39	11	-
<i>Trachurustrachus</i>	103	132	130	2	-	N.D.	N.D.	N.D.	N.D.	243	275	18	-
<i>Scomberscomber</i>	207	312	273	33	6	N.D.	N.D.	N.D.	N.D.	462	358	85	19
<i>Merlucciusmerluccius</i>	262	632	581	48	3	N.D.	N.D.	N.D.	N.D.	937	465	378	74

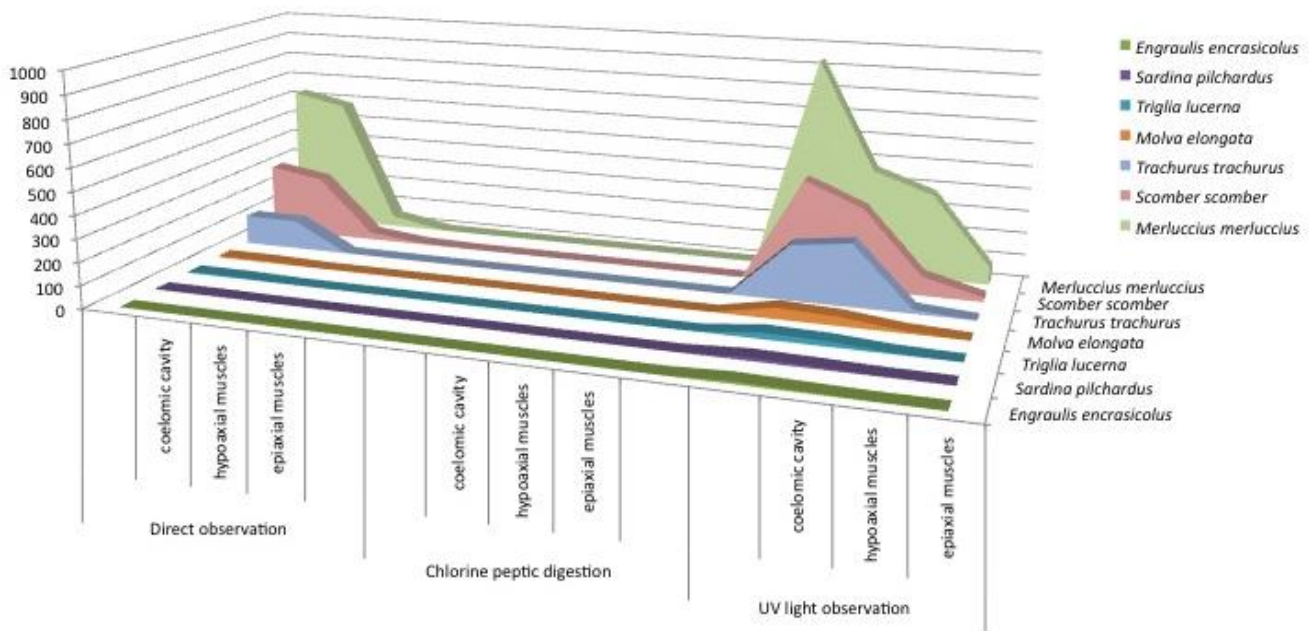


Fig. 1. Effect of fish species on the number of recovered larvae.

By comparing data obtained with visual inspection and UV transillumination, it is clearly evident that visual inspection was less effective in larvae detection. In UV transillumination, the larvae with a size from 1 to 3 cm were white fluorescent and could be easily differentiated from muscle fibres. In a large number of samples (data not shown), the nematodes were moving actively and were clearly visible under UV transillumination. All positive samples were identified by light microscopy as *Anisakis simplex*, following the morphological criteria proposed by [11].

Fig. 1 shows the distribution of positive samples in the different anatomical regions of the seven fish species, analysed by the three methods used in this study. Our data suggest that the degree of infestation of the coelomic cavity is not in correlation with the degree of infestation and localization in the muscle, but may be related to fish species.

In particular, the nematodes detected in the coelomic cavity were 93.9% of total larvae in *Trachurustrachus*,

81.5% in *Trigialucerna*, 78% in *Molvamacrophtalma*, 77.5% in *Scomberscombrus*, 64.3% in *Sardinapilchardus*, 54.5% in *Engraulis encrasicolus*, and 50.7% in *Merlucciusmerluccius*. Conversely, in *Oncorhynchus salmon* from Alaska, more than 90% of *Anisakis* larvae were found in the flesh, mainly in the hypaxial muscle [12].

In our study, the recovery from muscle tissues was remarkably higher in the samples analysed by UV transillumination. The same method can also be used to distinguish viable from dead larvae by colouring the larvae with several dyes or adding tetrazolium chloride [13].

According to our data, visual inspection of the coelomic cavity is not a suitable method to predict the presence of nematodes in the edible tissues. Our results are in agreement with [14], who have recently found a poor statistical significance between the number of *Anisakis* larvae in the abdominal cavity and the number of parasites in the edible part of *Micromesistiuspoutassou* and *Scomberscombrus*.

IV. CONCLUSIONS

Our findings indicate that the official method used in the European Union for anisakids detection in fish samples, based on visual inspection of the whole fish abdominal cavity and gut, does not provide a sufficient guarantee of larvae recovery, particularly in epaxial and hypaxial muscles.

To control the risk of viable parasites, accurate inspection of fish muscles appears the only scientific criterion to estimate infestation in edible tissues. In this respect, UV transillumination can be considered a rapid alternative tomolecular methodswhen large sample amounts need to be examinedquantitatively. In future studies, we will evaluate the effectiveness of UV transillumination in processed fish samples. Moreover, investigating how this method applies to industrial environment would be of great interest.

In conclusion, we believe that the method proposed in this study can be applied to upgrade detection of *Anisakis* spp. in the edible part of the fish. Furthermore, the procedure described in this paper is rapid and accurate, does not modify parasite morphology, and does not seem to affect larvae viability. This method can be considered a valuable tool for routine analysis of marine fishes even under field condition.

REFERENCES

- [1] V. Pravettoni, L. Primavesi, and M. Piantanida, "Anisakis simplex: current knowledge," *Eur. Ann. Allergy Clin. Immunol.*, vol. 44, no. 4, pp. 150-156, 2012.
- [2] I. Mladineo, V. Simat, J. Miletić, R. Beck, and V. Poljak, "Molecular identification and population dynamic of *Anisakis pegreffii* (Nematoda: Anisakidae Dujardin, 1845) isolated from the European anchovy (*Engraulis encrasicolus* L.) in the Adriatic Sea," *Int. J. Food Microbiol.*, vol. 157, no. 2, pp. 224-229, 2012.
- [3] R. C. Anderson, *Nematode Parasites of Vertebrates: Their Development and Transmission*, Wallingford, UK: CAB International, 1992.
- [4] S. Klimpel and H. W. Palm, "Anisakid nematode (Ascaridoidea) life cycles and distribution: increasing zoonotic potential in the time of climate change?" in *Progress in Parasitology, Parasitology Research Monographs*, H. Mehlhorn, Ed., B. Heidelberg, D: Springer Verlag, 2011, ch. 11, pp. 201-222.
- [5] M. Koie, B. Berland, and M. D. B. Burt, "Development to third-stage larvae occurs in the eggs of *Anisakis simplex* and *Pseudoterranova decipiens* (Nematoda, Ascaridoidea, Anisakidae)," *Can. J. Fish. Aqu. Sci.*, vol. 52, pp. 134-139, 1995.
- [6] A. T. Santos, P. Sasal, O. Verneau, and P. Lenfant, "A method to detect the parasitic nematodes from the family Anisakidae, in *Sardinia pilchardus*, using specific primers of 18 S DNA gene," *Eur. Food Res. and Tech.*, vol. 222, no. 1-2, pp. 71-77, 2006.
- [7] C. Mossali, S. Palermo, E. Capra, G. Piccolo, S. Botti, C. Bandi, S. D'Amelio, and E. Giuffra, "Sensitive detection and quantification of anisakid parasite residues in food products," *Foodborne Pathog. Dis.*, vol. 7, no. 4, pp. 391-397, April 2010.
- [8] W. Fang, F. Liu, S. Zhang, J. Lin, S. Xu, and D. Luo, "Anisakis pegreffii: a quantitative fluorescence PCR assay for detection in situ," *Exp. Parasitol.*, vol. 127, no. 2, pp. 587-592, Feb. 2011.
- [9] H. Karl and M. Leinemann, "A fast and quantitative detection method for nematodes in fish fillets and fishery products," *Arch. Lebensmittelhyg.*, vol. 44, pp. 124-125, 1993.
- [10] A. Levsen and B. T. Lunestad, "Anisakis simplex third stage larvae in Norwegian spring spawning herring (*Clupea harengus* L.), with emphasis on larval distribution in the flesh," *Vet. Parasitol.*, vol. 171, pp. 247-253, 2010.
- [11] B. Berland, "Anisakis spp.," in *Parasites of the colder climates*, H. Akuffo, E. Linder, I. Ljungström, and M. Wahlgreen (eds), London: Taylor & Francis, pp. 161-168, 2003.
- [12] H. Karl, F. Baumann, U. Ostermeyer, T. Kuhn, and S. Klimpel, "Anisakis simplex (s.s.) larvae in wild Alaska salmon: no indication of

post-mortem migration from viscera into flesh," *Dis. Aquat. Org.*, vol. 94, pp. 201-209, 2011.

- [13] M. Leinemann and H. Karl, "Untersuchungen zur Differenzierung lebender und toter Nematodenlarven (*Anisakis* sp.) in Heringen und Heringenzeugnissen," *Arch. Lebensmittelhyg.*, vol. 39, pp. 147-150, 1988.
- [14] M. Llarena-Reino, A. F. González, C. Vello, L. Outeiriño, and S. Pascual, "The accuracy of visual inspection for preventing risk of *Anisakis* spp. infection in unprocessed fish," *Food Control*, vol. 23, no. 1, pp. 54-58, 2012.



Gaetano Vitale Celano received a M. Sc. in Veterinary Medicine from the University of Bari (Italy), and a specialization in Food Microbiology at the Institut Pasteur de Lille. His major field of study is food safety, in particular foodborne pathogens and parasites.

He started his academic career at the University of Bari in 1984, where he is currently Full Professor of Food Inspection at the Veterinary Medicine Department. He is author or coauthor of over 100 scientific papers, published in national and international peer-reviewed journals.

He is member of different scientific societies and has coordinated several research projects. He is also President of the Spin-off company Med & Food C.Q.S. Srl, located in Valenzano (Italy), which is aimed to promote food quality programmes and specific certification projects in the Mediterranean region.



Antonello Paparella received a M. Sc. in Veterinary Medicine from the University of Bari (Italy). Before starting his academic career, he worked as a researcher in major food companies and gave a significant contribution to the development of new technologies for food manufacturing and control.

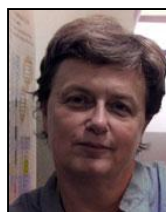
He was Visiting Professor in different universities from 1991 to 2000, when he was appointed Associate Professor. He is now Full Professor of Food Microbiology at the University of Teramo. His major field of research is food safety. In particular, his studies aim to evaluate the impact of new technologies on pathogens, as well as on the microbial ecology of foods. He is author or coauthor of over 135 scientific papers.

He is member of different scientific societies and expert of the National Departments of Agriculture and Environment. He is also very active in scientific communication and is in the staff of a popular television programme in Italy.



Armida Fransvea received her M. Sc. in Veterinary Medicine from the University of Bari (Italy). She collaborates with the research group of Food Inspection at the Veterinary Medicine Department at the same university. Her major field of study is food safety.

She is involved in teaching activities on food quality and certification, and is now Scientific Secretary of the Spin-off company Med & Food C.Q.S. Srl, located in Valenzano (Italy).



Claudia Balzaretto took a M. Sc. in Veterinary Medicine from the University of Milan (Italy). Her major field of study is food microbiology in catering, as well as exposure quantification of foodborne risks.

She was responsible for quality controls in the regional catering service in Milan. She started her academic career at the University of Milan, where she is currently Researcher at the Department of Veterinary Science for Health, Animal Production, and Food Safety. She is author or coauthor of over 135 scientific papers.

She was Quality Assurance Manager for Milano Ristorazione SpA and is an expert in food quality systems for the catering service in Lombardy region.



Giuseppe Celano took his M. Sc. in Veterinary Medicine from the University of Bari (Italy), and collaborates with the research group of Food Inspection at the Veterinary Medicine Department at the same university.

His major field of study is food safety, with a particular emphasis on animal source foods.

He is currently working in the Spin-off company Med & Food C.Q.S. Srl, located in Valenzano (Italy).