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UV-press method versus artificial digestion method to detect Anisakidae L3 in fish fillets: Comparative study and suitability for the industry

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ABSTRACT

To screen the presence of Anisakidae third stage larvae (L3) in fish, fast methods such as the visual inspection and candling have been widely used by the industry over the last 50 years, and they are regulated by the European Parliament and the Council. These methods are ineffective to detect L3 embedded in fish muscles, consequently alternative methods, such as the artificial digestion (AD) and the UV-Press (UVP) are increasingly applied, but their performance needs to be evaluated. The aims of the present work were: 1) to compare the performance of AD and UVP. methods by a Ring Trial (RT) involving highly experienced laboratories; and 2) to evaluate the potential transferability of the best performing method to the industry by a collaborative study involving industrial partners (β -testing). For RT, each participating laboratory ($n=5$) received 6 samples of 100 g of fish fillets spiked with 1 L3 (1 sample), 3 L3 (2 samples), 7 L3 (2 samples), and a negative control sample (without L3). In each positive sample, there were live *Anisakis pegreffii* L3 collected from a naturally infected fish. The result evaluation was based on the agreement between the number of reported and the number of spiked L3. No false positive sample was detected. The L3 number detected by the UVP method showed higher (90%) level of agreement with the number of spiked L3 than the number of L3 detected by the AD method (83.3%); however, no significant difference in terms of accuracy ($p=0.32$) was detected when the two methods were compared. Moreover, considering only the presence/absence of L3 in the samples, the UVP reached 100% of accuracy and 100% of sensitivity; whereas, AD showed 98% of accuracy and 96% of sensitivity. The variability of the UVP method was lower than that of the AD method, indicating a better reproducibility. On the basis of the RT results, the UVP method was selected for the β -testing. Each industrial partner ($n=3$) received 15 samples of 100 g of fish fillet spiked with 1 L3 (2 samples), 2 L3 (2 samples), 3 L3 (2 samples), 4 L3 (2 samples), 5 L3 (2 samples) and 6 L3 (2 samples) and three negative control samples (without L3). The number of L3 counted in 34 out of 45 samples (75.6%) by the UVP method was in agreement with the number of spiked L3. One company reached 93.3% of agreement; whereas the other two companies reached an agreement of 66.7%. Two false negative results were found; whereas, no false positive results were obtained. Moreover, at the industrial level, considering only the presence/absence of larvae in the samples, the UVP reached 97% of accuracy, 94.4% of sensitivity, and 100% of specificity. However, the UVP method, in spite of its accuracy, needs further investigations to provide new time –temperature combinations that could allow a reduction of the testing time and its integration in the fishing deck.

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1. Introduction

The fish-borne parasitic zoonosis caused by nematodes of the genera *Anisakis* and *Pseudoterranova* known as anisakidosis, has been recognized as an important public health problem (Audicana and Kennedy, 2008; EFSA, 2010; Pozio, 2013). In addition to infections caused by ingestion of live larvae, allergic reactions to *Anisakis* proteins can also occur after ingestion of cooked or canned fish, although a prior exposure to live larvae seems to be necessary (Nieuwenhuizen and Lopata, 2014).

Currently, the geographical limits and human populations at risk of anisakidosis are expanding because of popularization of ethnic food (e.g. Oriental cuisine), human migration, growing international markets and improved transportation systems (Chai et al., 2005). Therefore, there is a change of the control responsibility in the fishery chain, which must be taken into account (D'amico et al., 2014). In the European Union, General Provisions of the Hygiene Package establish co-responsibility of the Food Business Operator (FBO) to produce safe fishery products (EC/178, 2002). Nevertheless, FBOs are not always properly trained in the use of techniques for the control of anisakid infections.

Over the last 50 years, non-invasive and fast methods, like visual inspection and candling, regulated by the European Parliament and the Council (EC/853, 2004; EC/854, 2004), have been widely used by industry for parasite screening in seafood. These methods are relatively ineffective and have the disadvantage that they cannot be applied for the analysis of processed products (Levsen et al., 2005). Even if the EU legislation (EC 2074/2005; EC 853/2004) recommends the visual inspection of fish gut for Anisakidae larvae, Llaena-Reino et al. (2012) evidenced the low efficiency of this method for predicting nematode larvae in fish fillets.

Other methods as the artificial digestion (Tejada et al., 2007) and UV-Press (UVP) of fish fillets for Anisakidae parasites (Karl and Leinemann 1993; Levsen and Lunestad 2010) are increasingly applied, especially in large-scale scientific surveys. The CODEX Alimentarius presents a digestion method aimed at assessing the viability of nematodes detected in fish, applicable only to salted Atlantic herring (*Clupea harengus*) and salted sprat (*Sprattus sprattus*) (CODEX STAN 244-2004). Llaena-Reino et al. (2013) performed an optimization of the pepsin digestion method for anisakid inspection in the fishing industry, which reduces the assay time and resulted to be more handy and efficient than the method reported by the CODEX. However, the performance in terms of accuracy, sensitivity and specificity of all these procedures are unknown. Therefore, within the 7th FP European project PARASITE Risk Assessment with Integrated Tools in EU fish production value chains (PARASITE, <http://parasite-project.eu/>), an inter-laboratory collaborative (Ring Trial, RT) study was organized. This study was carried out among experienced laboratories and was aimed to determine and comparing the performance of both the artificial digestion (AD) and UVP methods in detecting Anisakidae larvae (L3) in fish fillets. Moreover, to test the potential transfer of the best performing method to the industry, a β -testing was organized. Before the participation in the β -testing exercise, industrial partners were trained through the participation in specific training workshops.

2. Materials and methods

2.1. Ring trial (RT)

2.1.1. RT test material

The material forwarded to each laboratory consisted of a panel of 6 samples of ~100 g of fish fillet sandwiches from Anisakidae-free rainbow trout (*Oncorhynchus mykiss irideus*) farmed in freshwater. Each sandwich was spiked in the core with a different number of

Table 1

Ring trial samples and results. L3, *Anisakis pegreffii* larva.

Lab Code	Sample code	No. of L3 in the sample	No. of L3 detected by UVP*	No. of L3 detected by AD**
P1	P01	7	7	7
	P02	0	0	0
	P03	3	3	3
	P04	3	3	3
	P05	7	6 [#]	7
	P06	1	1	1
P2	P07	7	7	7
	P08	1	1	0
	P09	7	7	7
	P10	3	3	3
	P11	0	0	0
	P12	3	3	2+1 [‡]
P3	P13	3	3	3
	P14	7	7	7
	P15	7	5+2 [‡]	5+2 [‡]
	P16	1	1	1
	P17	0	0	0
	P18	3	2	2
P4	P19	3	3	3
	P20	0	0	0
	P21	7	7	7
	P22	1	1	1
	P23	7	7	7
	P24	3	3	2
P5	P25	0	0	0
	P26	7	7	7
	P27	1	1	1
	P28	3	3	3
	P29	3	3	3
	P30	7	7	7

*UVP: UV-Press method.

**AD: artificial digestion method.

[#]Boxed: L3 number found in disagreement with the L3 spiked number.

[‡]Entire L3 plus one or two L3 fragments.

live L3 *Anisakis pegreffii* (Table 1) collected from the coelomic cavity of highly infected fish (silver scabbard fish). A known number of L3 live larvae free of their capsule, were carefully transferred by tweezers into an opened pocket in a fish fillet and a sandwich was prepared with another fish fillet. Single fish fillet sandwiches were individually preserved in vacuum-sealed plastic bags and stored at +4 °C, allowing the preservation of fish fillet freshness and assuring the L3 survival. Each sample was labelled with a unique code without any indication of the level of contamination or any information on the identity of the testing laboratory. Before shipping, each fillet sandwich was weighted and the value registered. Since samples for the detection of L3 were made by individually spiked samples, homogeneity was ensured by an accurate control of the number of L3 spiked into each sample by two operators.

For the shipping, each sample panel was further sealed under vacuum in a larger bag, which was inserted in a polystyrene box. Ice packs were placed into the box to maintain the temperature between 4 °C and 10 °C during transportation. The stability of the samples in this range of temperature and packing conditions had been previously evaluated by ad hoc experiments carried out at the European Union Laboratory for Parasites (Rome, Italy), in which L3 in the vacuum-sealed samples stored at 4 °C–10 °C were viable for at least 2 weeks after the preparation.

2.1.2. Participating laboratories

Five highly qualified experienced laboratories participated to the RT, namely: L'Agence Nationale de Sécurité Sanitaire de l'Alimentation, Boulogne sur Mer (France); Department of Public

Health and Infectious Diseases, Sapienza University, Rome (Italy); Instituto de Investigaciones Marinas –CSIC, Vigo (Spain); Max Rubner Institute, Hamburg (Germany); and the National Institute of Nutrition and Sea Food Research, Bergen (Norway).

2.1.3. Instructions to participants and detection methods

Each laboratory received four forms as follows: 1. “Package check” to verify the content and the condition of the RT samples at the arrival; 2. “Instruments and Materials” needed to perform the methods; 3. “Procedure” step by step description of AD and UVP (Fig. 1); and 4. “Results” in which the number of L3 recorded in each sample should be reported. The code assigned to each laboratory was sent to participants by an individual email the day of the shipment. To allow comparison between the results obtained by the laboratories, all the participants should follow the protocol step by step or describe the variation.

2.1.4. Criteria for result evaluation

The result evaluation was based on the agreement between the number of reported larvae by each method and the number of spiked L3, evaluated using Kappa statistic (Landis and Koch, 1977). Results were also evaluated based on the presence/absence of larvae in the sample, independently of the number. Thus, the accuracy, based on the Receiver Operating Characteristic, sensitivity and specificity of both UVP and AD methods were estimated (OIE, 2012) together with the 95% confidence intervals (95% CI).

2.2. β -testing

2.2.1. Training workshops on UVP method

Two training workshops were organized to guarantee attendance of all industrial partners in the project. The methods and the critical control points (CCP) were presented in detail and discussed. Moreover, a didactic video and a 3D-animation on UVP were shown. Attendants were given the opportunity to gain hands on experience with similar samples to those that would have to be analysed later on in their own premises. The first training workshop was attended by representatives from ARVI and some of their associates, as well as by the staff from the XENOTECHS company hired to provide support to ARVI for this task. The second training workshop was attended by representatives from the other industrial partners in the project, namely NEDERLOF'S and Hermes.

2.2.2. β -testing test material

The material forwarded to each industrial partner consisted in a panel of 15 samples of ~100 g of fish fillets from Anisakidae-free sea bass (*Dicentrarchus labrax*). Each fish fillet was spiked in its core with a different number of live L3 *Anisakis pegreffii* (Table 5) collected from the coelomic cavity of highly infected fish (blue whiting, *Micromesistius poutassou*). Samples were prepared to be sent as reported for the RT.

2.2.3. Industrial partners

Three industrial partners participated in the β -testing exercise, namely: Cooperativa de Armadores de Pesca del Puerto de Vigo-ARVI (Spain), HERMES (Norway) and NEDERLOF's (The Netherlands).

2.2.4. Instructions to participants and detection methods

Each participant received five forms, four of which were the same as the reported for the RT and the number five was a blank sheet to collect end user's comments about the applicability of the method that was tested. The “Procedure” description is shown in Fig. 2. The results were evaluated as described for RT.

Table 2

Performance of the UV-Press and Artificial Digestion methods in the Ring Trial.

	UV-Press	Artificial Digestion
Accuracy	100% (95% CI: 88.4%–100%)	98% (95%CI: 82.8%–99.1%)
Sensitivity	100% (95% CI: 86.3%–100%)	96% (95%CI: 79.6%–99.9%)
Specificity	100% (95% CI: 47.8%–100%)	100% (95%CI: 47.8%–100%)

Table 3

Ring Trial: Kappa coefficients between the gold standard (i.e., the number of spiked *Anisakis pegreffii* larva) and the UV-Press method (UVP) and between the number of *A. pegreffii* larva and the artificial digestion method (AD).

	Agreement	Expected Agreement	Kappa	95% CI
UVP	90.0%	24.4%	0.87	0.82–1.00
AD	83.3%	23.3%	0.78	0.68–0.82

3. Results

3.1. RT results

Packages were delivered to laboratories within 48 h. At the delivery, the internal package temperature was in the range of +6 °C ÷ +8.3 °C. The time elapsed from the arrival of the package at the laboratories and the package control was less than 20 h. During this time, packages were stored at +4 °C.

Laboratories performed the two methods, first UVP and then AD, on each sample, with small changes of the AD protocol: the flesh was not chopped or it was hand-chopped in two and three laboratories, respectively.

The RT results are shown in Table 1. By UVP, the L3 recovery was correct for 27 out of 30 samples (90%, 95% CI: 73.5–97.9%). The diagnostic accuracy to discriminate positive and negative samples measured by the ROC area was 1.00 (95% CI: 0.88–1.00) with a 100% agreement with the presence of spiked L3. By AD, the L3 recovery was correct for 25 out of 30 samples (83.3%, 95% CI: 65.3–94.4%). The diagnostic accuracy to discriminate positive and negative samples measured by the ROC area was 0.98 (95% CI: 0.83–1.00) with a 96.7% agreement with the presence of spiked L3 (1 false negative). When comparing the two methods, no significant difference in terms of accuracy ($p = 0.32$) was observed. Accuracy, sensitivity and sensibility of the UVP and AD methods to discriminate positive and negative samples are shown in Table 2. The Kappa statistic between UVP and the number of spiked L3 and between AD and the number of spiked L3 on identifying the exact number of larvae are shown in Table 3.

Only one laboratory (code P5) reached a 100% agreement with the number of spiked L3 by the two methods; two laboratories (codes P1 and P4) reached a 92% agreement with Kappa statistic equal to 0.89 (95% CI: 0.76–1.00); one laboratory (code P2) reached a 84% agreement (Kappa: 0.78; 95% CI: 0.56–1.00); and one laboratory (P3) reached a 67% agreement (Kappa: 0.60; 95% CI: 0.33–1.00).

The highest agreement was found by UVP when samples with 1 spiked L3 were tested (100%, 95% CI: 69.2–100%). The lowest (70%, 95% CI 34.8–93.3%) agreement was found by AD when samples with 3 spiked L3 were tested. A 90% (95% CI: 55.5–99.7%) agreement was found by UVP testing samples spiked with 3 L3 as well as by AD testing samples spiked with 7 (95% CI: 55.5–99.7%) larvae. An 80% (95% CI: 44.4–97.5%) agreement was found by UVP testing samples spiked with 7 L3 as well as by AD testing samples spiked 1 L3. Table 4 shows the CV calculated for each set of samples with the same number of spiked L3 and analysed by the same method.

3.2. β -testing

Table 5 shows the results obtained by industrial partners. Among the 36 positive samples, 34 (sensitivity: 94.4%; 95% CI: 81.3–

**PARASITE
WP 6.5**

Department of Infectious, Parasitic and Immunomediated Diseases
Unit of Gastroenteric and Tissue Parasitic Diseases
Istituto Superiore di Sanità

**Form 3****Laboratory code** _____**First Ring Trial on "DETECTION OF ANISAKIDAE L3 LARVAE IN FISH FILLETS"****Procedure**

To make comparable the results obtained by laboratories involved in the RT, the operative protocol must be carefully followed. Thus, you are requested to mark the column YES if you strictly follow the protocol or, alternatively, describe any variation at each step.

3.1 UV-PRESS METHOD

Step	DESCRIPTION	YES	VARIATION
1	Weight each sample and report the weight in Form 4.		
2	Open each sample and carefully check the presence of larvae outside the fillet sandwich or in the plastic bag		
3	Place each fillet sandwich in a transparent plastic bag		
4	Squeeze the fillet sandwich in the plastic bag up to 2 mm tick by a press-vacuum system		
5	Deep freeze the squeezed fillets at -20°C for 24h and keep them frozen until examination		
6	Before examining, samples have to be thawed		
7	Examine the thawed fish fillet under a 366 nm UV light, preferably with both up and down light, by eyes in a dark room		
8	Anisakidae larvae present in the flesh will appear as brightly fluorescent spots, they have to be counted		

3.2 ARTIFICIAL DIGESTION METHOD (FOR 100g OF FISH FILLET)

Step	DESCRIPTION	YES	VARIATION
1	10.5 ml of 37% hydrochloric acid (0.063 M final concentration) in 1.0 L of tap water, preheated to 37°C; in a 3 L beaker. A stirring rod is placed in the beaker, the beaker is placed on the preheated plate (37°C) and the stirring is started.		
2	Pepsin is added to a 0.5% final concentration (2.0 L final volume) and volume adjusted to 2.0 L with 37°C preheated tap water.		
3	The pressed fish fillet must be completely transferred and gently chopped by: the blender, for 1-2 seconds, or by scissors or by hands. Please specify the chopping method used.		
4	The chopped fillet is transferred to the 3 L beaker containing the digest solution.		
5	The mincing insert of the blender, or the scissors, is immersed repeatedly in the digest fluid in the beaker and the blender bowl is rinsed with a small quantity of digest fluid to remove any fish muscles still adhering		
6	The beaker is covered with aluminium foil		
7	The magnetic stirrer must be adjusted so that it maintains a constant temperature of 37°C throughout the operation. During stirring, the digest fluid must rotate at a sufficiently high speed to create a deep whirl without splashing		
8	The digest fluid is stirred until the fish muscles disappear (approximately 30 min). The stirrer is then switched off and the digest fluid is poured through the sieve into a beaker		
9	Anisakidae larvae can be detected on the sieve		
10	Larvae can be gently collected by tweezers, counted and examined under the stereomicroscope with transmitted light		

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Fig. 1. Step by step description of the artificial digestion and UV-Press methods sent to the Ring Trial participants.

99.3) tested positive by UVP. All the nine negative samples were correctly classified as negative (specificity 100%; 95% CI: 66.4–100). The accuracy measured by the ROC area was overall 0.97 (95% CI:

0.88–0.99). The agreement between the number of L3 spiked in the samples and the L3 number counted by UVP was 75.6% with a Kappa statistic of 0.71 (95% CI: 0.68 – 0.76). Two companies



β -testing on Detection of Anisakidae L3 Larvae in Fish Fillets

Instructions

At the arrival of the samples, please check the package content and its condition of preservation

The following remarks are to be taken into account before performing the test:

1. in order to follow the same procedure, all the participants should freeze the samples, as soon as they arrive to their premises, at -20°C for at least 24h
2. afterwards, the samples have to be analyzed following the procedure described below
3. samples have to be handled by the personnel following the routine safety procedures requested for infectious biological material, i.e. wearing individual protection devices (coat, mask and gloves). Specific safety measures must be followed according to the test procedure applied (i.e. use of UV-protection glasses; etc.)
4. the due date to submit (by e-mail) the results of sample analysis will be indicated to participants.

UV-Press Method

Procedure:

- a. Freeze each sample at -20°C as soon as they arrive to your premises for at least 24h
- b. Prior to the analysis take them out the freeze and let them thaw in the fridge or at room temperature
- c. Open each sample and carefully check the presence of larvae outside the fillet
- d. Place each fish fillet in a new transparent plastic bag
- e. Squeeze the fish fillet in the plastic bag up to 2 mm thick by a press-vacuum system
- f. Examine the fish fillet under an UV light by eyes in a dark room
- g. Anisakidae larvae present in the flesh will appear as brightly fluorescent spots
- h. The number of larvae counted in each fish fillet has to be registered in the form provided by CETMAR.
- i. Moreover, all inconveniences and problems faced during the process have to be stated in the form also

For further information about the beta-testing exercise, please contact:

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Fig. 2. Step by step description of the UV-Press method sent to the β -testing participants.

Table 4
Ring Trial: Coefficient of variation for each method. L3, *Anisakis pegreffii* larva.

L3 number in the sample	UV-Press CV%	Artificial Digestion CV%
7	10	9
3	11	18
1	0	49

reached an agreement of 66.7% (Kappa = 0.61, 95% CI: 0.50–0.68 for P1; Kappa = 0.61, 95% CI: 0.43–0.75 for P2), while the third company reached an agreement of 93.3% (Kappa = 0.92, 95% CI: 0.84–1.00 for P3).

In the sheet for comments, the industrial companies stressed the difficulty to integrate UVP in their routine work at the fishing deck, since the results cannot be provided on time.

4. Discussion

Considering the presence/absence of L3 in the samples as a binomial classification system, i.e. independently of the number of reported L3, the results show that the UVP performance is higher than that of AD in terms of accuracy and sensitivity (Table 2). Regarding specificity, even if it is estimated in 100%, without any false positive, it is strongly influenced by the small size of the sample (only 5 negative samples), therefore, the 95% CI are wide (Table 2). Moreover, a higher level of agreement was reached between UVP and the spiked L3 in samples than between the number of spiked L3 and AD (90%, CI 82% to 100% versus 83.3%, CI 68% to 82%); however, a statically significant difference in terms of accuracy was not found. Further, the small sample size did not permit us to evaluate the simultaneous effect of the spike level, the number of recovered larvae, the different laboratories and different detection methods. Nevertheless for both methods, the expected agreement, that could be due just to chance is low (Table 3). Finally, the UVP

variability was lower than that of AD, as indicated by the CV values calculated for each set of samples, indicating a good reproducibility (Table 4). The main conclusion of the RT was the higher performance of UVP when compared with AD, even if AD was subsequent to UVP and the labs could gain knowledge on the spiked level from the first method.

Data showing the better performance of UVP in comparison with AD are extremely important, since the current European regulations (EC/853, 2004; EC/854, 2004) for testing the presence of anisakid L3 are based on methods, like visual inspection, with a very low efficiency in predicting nematode larvae in fish fillets (Llarena-Reino et al., 2012).

Given the high UVP performance, it was selected for the β -testing. In this study (Table 5), no false positive results were obtained, indicating that industrial operators were able to correctly distinguish L3 in fish fillets. Since operators had no expertise on the detection of nematode larvae, a high efficacy of the training carried out before the β -testing exercise could be argued. Two false negative results were found. This may be due to the L3 migration out of the fillets, which therefore could not be identified by operators. We need also to stress that only in two cases more larvae than those spiked were counted, probably due to the fragmentation of some larvae after the pressing process.

UVP has been revealed as a sensible, accurate and easy to implement test. According to the comments provided by the ship-owner partners involved in the β -testing, it would be difficult to integrate UVP in their working routine at the fishing deck, since the results cannot be provided on time. It seems that this method is more appropriate for self-control of zoonotic parasite hazards in fish plants on land. Further investigations could provide time-temperature combinations that allow reducing the freezing time needed. Some kind of automation could also contribute to reduce the time needed to perform the test.

Table 5
 β -testing samples and results. L3, *Anisakis pegreffii* larva.

Company code	Sample Code	No. of spiked L3	No. of recovered L3	Sample Code	No. of spiked L3	No. of recovered L3
P1	P01	1	1	P09	0	0
	P02	3	2*	P10	5	4
	P03	5	5	P11	6	4
	P04	1	1	P12	4	5
	P05	0	0	P13	0	0
	P06	2	0**	P14	2	2
	P07	6	6	P15	3	3
	P08	4	4			
P2	P16	1	0	P24	3	3
	P17	0	0	P25	4	3
	P18	6	7	P26	2	2
	P19	3	3	P27	0	0
	P20	4	4	P28	5	4
	P21	6	6	P29	2	2
	P22	0	0	P30	1	1
	P23	5	4			
P3	P31	2	2	P39	5	5
	P32	5	5	P40	2	2
	P33	0	0	P41	4	2
	P34	1	1	P42	6	6
	P35	0	0	P43	1	1
	P36	6	6	P44	4	4
	P37	3	3	P45	0	0
	P38	3	3			

*Box: L3 number found in disagreement with the L3 spiked number.

**Grey box: false negative results.

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