Report of the NRL proficiency test to detect *Anisakis* sp. larvae in fish fillets

April, 2009

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1 Introduction

Nematode worms of the Anisakidae family are zoonotic parasites circulating in most of marine fish and cephalopods at the larval stage and in marine mammals at the adult stage (Audicana and Kennedy, 2008). Humans acquire the infection by the consumption of raw, undercooked or marinated fish. According to the Commission Regulation (EC) No 853/2004, food business operators must ensure that fishery products have been subjected to a visual examination for the purpose of detecting visible parasites before being placed on the market. They must not place fishery products that are obviously contaminated with parasites on the market for human consumption.

2 Scope

One of the core duties of the EURL for Parasites is to organise proficiency tests (PT), as it is stated in the Regulation (EC) No 882/2004 of the European Parliament and of the Council (Commission Regulation No. 882/2004). The scope of this PT is to test the competence of the appointed NRLs to detect Anisakidae larvae in fish fillets.

3 Time frame

The PT was announced to NRLs by email on February 2nd, 2009 and the dead line to send the participation form was February 13th, 2009. On April 6th, 2009, the samples were dispatched to participants by an international courier. Reporting deadline was 24th April, 2009.

4 Test material

The test material forwarded to each laboratory, consisted of 1 fish fillet containing in the core three anisakidae larvae. Briefly, L3 *Anisakis* sp. larvae were added to fish sandwich samples of 50 g each of fish muscles *Anisakis*–free. Larvae free of their capsule, were obtained by microscopical visualization of a fish sample highly infected with Anisakidae larvae. Larvae were identified to the genus level microscopically. Three Anisakis L3 were carefully transferred and included in the fish sandwich. The fish sandwich was immediately packed in a plastic envelop under vacuum and stored at +4°C. A code was added on the envelop and the same code with the number of larvae was reported in an Excel file. Samples were forwarded to each participant laboratory by an international courier in a polystyrene box with cooler packages to maintain a temperature lower than 10°C during the shipping. The packages were forwarded according to the international forwarding regulations. An envelop with the hard copies of the 5 forms (see ANNEX 1) was also included in the box. To check the material stability during the time, and to estimate the suitability of the packing and forwarding conditions under which the fish fillets were forwarded, two samples were stored in the package (and the package was stored at room temperature) as those that were forwarded, and tested at the EURLP three and five days after the forwarding.

The hard copies of the forms which should be filled in and send back to EURLP were:

1) general information on the PT and its purpose (form 1, Annex 1);
2) laboratory description (form 2, Annex 1);
3) instruments, reagents and materials required for the detection of Anisakidae larvae in fish fillets (form 3, Annex 1);
4) check of the package content and its condition of preservation (form 4, Annex 1);
5) results of sample examination (form 5, Annex 1);
6) code assigned to the laboratory.

5 Instructions to participants

Practical instructions were given to all the participants in the form 3 that accompanied the samples. A list of instruments as well as a list of chemicals and disposable material required to perform the digestion procedures, were also included. To make comparable the results obtained by the different laboratories involved in the PT, all the participants should follow the protocol step by step or, on the contrary, describe the variation. Four tests were suggested and described: 1. candling; 2. compressorium; 3. UV examination after freezing; and 4. digestion.

6 Statistical Analysis

Since only one sample was forwarded to the participants laboratory, no statistical analysis of the results was performed.

7 Participating laboratories

Of the 27 MS, only 20 laboratories (two NRL from Romania) from 19 MS, agreed to participate and sent the results.

Participating NRL for parasites	Country
Institut für Veterinärmedizin, Innsbruck	Austria
Institute of Tropical Medicine, Antwerp	Belgium
State Veterinary Institute, Olomouc	Czech Rep
Estonian Veterinary and Food Laboratory, Tartu	Estonia
Finnish Food Safety, Evira, Oulu	Finland
Lab. études et recherches en pathol animale et zoonoses, AFSSA, Maison Alfort	France
Bundesanstalt fur Ernahrung und Lebennsmittel, Karlsruhe	Germany
Centre of Athens Veterinary Institutions, Athens	Greece
Veterinary Laboratory Department of Agriculture & Food Laboratories, Celbridge	Ireland
National Reference Laboratory for Anisakiasis, Ist. Zooprof. Sper. Sicilia, Palermo	Italy
Laboratory of Food and Environmental Investigations (LFEI), Riga	Latvia
National Veterinary Laboratory, Vilnius	Lithuania
National Institute of Public Health and the Environment, RIVM, Bilthoven	Netherlands
National Veterinary Research Institute, Pulawy	Poland
Hygiene and Public Veterinary Health Institute, Bucharest	Romania
Institute for diagnosis and animal health, Bucharest	Romania
State Veterinary and Food Institute, Bratislava	Slovak Rep.
National Veterinary Institute, Ljubljana	Slovenia
Centro Nacional de Alimentación. Agencia Española de Seguridad Alimentaria y Nutrición, Majadahonda	Spain
Statens Veterinärmedicinska Anstalt, Uppsala	Sweden

8 Results

8.1 NRL description

Of the 20 NRLs which agreed to participate at the ring trial, 15 (75%) are accredited according to ISO/IEC 17025:2005 since an average of seven years (range 3-12 years), but only six of them have accredited one or more tests to detect anisakidae larvae in fish. Out of 20 laboratories which participated at the PT, one laboratory has validated three tests (candling, compressorium and digestion), one laboratory two tests (UV examination after freezing and digestion) and all the other 18 laboratories only one test. Concerning the personnel working on fish parasites, there were only one scientist in 9 NRLs, two scientists in 10 NRLs and three scientists in only one NRL; concerning

technicians, there were 5 in one NRL, 3 in three NRLs, 2 in four NRLs, 1 in ten NRLs and no technician was present in two NRLs.

8.2 Delivery of the package to NRLs

All the packages were delivered to NRLs within 24-96 hours (one day for nine NRLs; two days for seven NRLs; three days for three NRLs; and four days for one NRL). At the delivery, the internal temperature of all the packages was less than 10°C in 18 parcels (90%). The time elapsed from the arrival of the package at the NRL and its control was of less than one hour for 16 NRLs, of 1 hour for 1 NRL, of 5 and 18 hours for 2 NRLs, and of 4 days for one NRL (but the package was stored at +4°C).

8.3 Test methods

Out of 20 NRLs, 13 (56.5%) laboratories used the digestion method, 6 (26.0%) candling, 3 (13.0%) the compressorium, and 1 (4.3%) the UV examination after freezing.

8.4 NRL experience

The NRL "experience" based on the number of sample analyzed in the previous year (2008), was very variable (from zero for eight laboratories to one-thousand for one laboratory) and it does not shows any influence on number of larvae detected in the sample. No difference was observed between accredited and non-accredited laboratories.

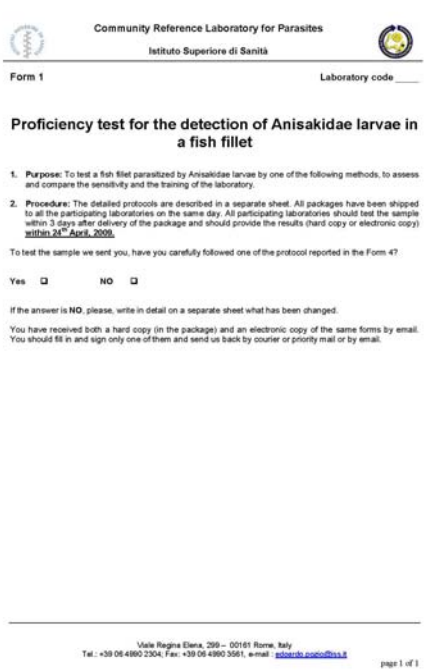
9 Conclusions

As far as we know, this is the first organized PT in Europe, but probably at the world level, to detect anisakidae larvae in fish fillets. Out of the 27 MS of the EU, 19 countries participated at the PT and two NRLs in Romania. Most of NRLs which did not participate at the PT, claimed that anisakiasis does not represent a problem in their country, because the amount of consumed seawater fish is very limited or people do not consume raw fish. Most (70%) of the participating laboratories detected all the three larvae present in the PT sample, five (25%) NRLs detected two larvae and only one (5%) NRL detected only one larva. These results suggest that most of persons working in the NRLs are skill to detect Anisakidae worms in marine fish products; however, since in the routine at the fish markets, veterinarians test only few marine fish from each stock to detect Anisakidae larvae, this approach cannot prevent that infected fish escape from the veterinary controls and that Anisakidae worms could reach the human beings.

10 References

Audicana MT, Kennedy MW. 2008. *Anisakis simplex*: from obscure infectious worm to inducer of immune hypersensitivity. Clin Microbiol Rev 21, 360-379.

Annex 1



Community Reference Laboratory for Parasites

Istituto Superiore di Sanità

Form 1 Laboratory code ____

Proficiency test for the detection of Anisakidae larvae in a fish fillet

1. **Purpose:** To test a fish fillet parasitized by Anisakidae larvae by one of the following methods, to assess and compare the sensitivity and the training of the laboratory.
2. **Procedure:** The detailed protocols are described in a separate sheet. All packages have been shipped to all the participating laboratories on the same day. All participating laboratories should test the sample within 3 days after delivery of the package and should provide the results (hard copy or electronic copy) **within 24th April, 2009.**

To test the sample we sent you, have you carefully followed one of the protocol reported in the Form 4?

Yes ☐ NO ☐

If the answer is **NO**, please, write in detail on a separate sheet what has been changed.

You have received both a hard copy (in the package) and an electronic copy of the same forms by email. You should fill in and sign only one of them and send us back by courier or priority mail or by email.

Viale Regina Elena, 299 – 00161 Rome, Italy
Tel.: +39 06 4990 2304; Fax: +39 06 4990 3561, e-mail : edoardo.pozio@iss.it

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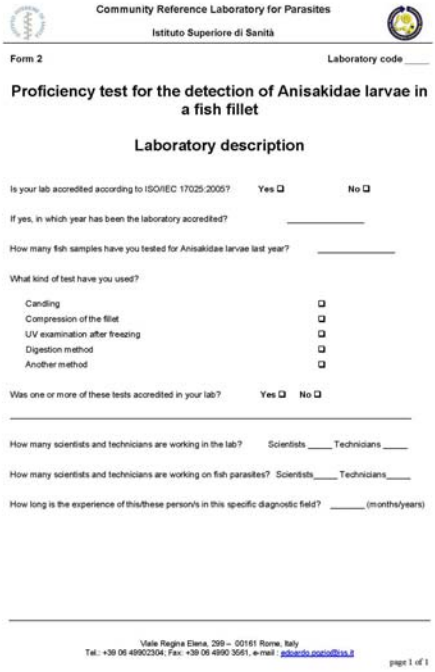

Community Reference Laboratory for Parasites

Istituto Superiore di Sanità

Form 2 Laboratory code ____

Proficiency test for the detection of Anisakidae larvae in a fish fillet

Laboratory description

Is your lab accredited according to ISO/IEC 17025:2005? Yes ☐ No ☐

If yes, in which year has been the laboratory accredited? _____

How many fish samples have you tested for Anisakidae larvae last year? _____

What kind of test have you used?

- Candling ☐
- Compression of the fillet ☐
- UV examination after freezing ☐
- Digestion method ☐
- Another method ☐

Was one or more of these tests accredited in your lab? Yes ☐ No ☐

How many scientists and technicians are working in the lab? Scientists ____ Technicians ____

How many scientists and technicians are working on fish parasites? Scientists____ Technicians____

How long is the experience of this/these person/s in this specific diagnostic field? _____(months/years)

Viale Regina Elena, 299 – 00161 Rome, Italy
Tel.: +39 06 49902304; Fax: +39 06 4990 3561, e-mail : edoardo.pozio@iss.it

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Form 3 Laboratory code _____

Proficiency test for the detection of Anisakidae larvae in a fish fillet

Instruments, reagents and materials required for the detection of Anisakidae larvae in fish fillets

1 **Instruments**
The following instruments are needed to perform one of the methods. To make comparable the results obtained by laboratories involved in the ring trial, we have to verify which instruments have been used. Thus, you are requested to mark the column YES in case of use of the relevant instrument or, on the contrary, describe the variation in the following column.

1.1 Digestion

DESCRIPTION	YES	VARIATION
A blender with a sharp chopping blade		
Magnetic stirrers with thermostatically controlled heating plate and teflon-coated stirring rods approximately 5 cm long		
Sieves, mesh size 180-250 microns, with stainless steel mesh		
Glass beakers, capacity 3 litres		
A stereo-microscope, with a substage transmitted light source of adjustable intensity		
A balance accurate to at least 0.1 g		
Pipettes of different sizes (1, 10 and 25 ml) and pipette holders		
A thermometer accurate to 0,5 °C within the range 1 to 100 °C		

1.2 UV on frozen and squeezing of the fillet

DESCRIPTION	YES	VARIATION
An UV-light transilluminator		
A freezer – 20°C		
A (stereo-) microscope with transmitted light (magnification 15 to 60 times)		

1.3 Compression of the fillet

DESCRIPTION	YES	VARIATION
A compression system		
A (stereo-) microscope with transmitted light (magnification 15 to 60 times)		

Viale Regina Elena, 299 – 00161 Rome, Italy
Tel.: +39 06 4990 2304; Fax: +39 06 4990 3561, e-mail : edoardo.pozio@iss.it

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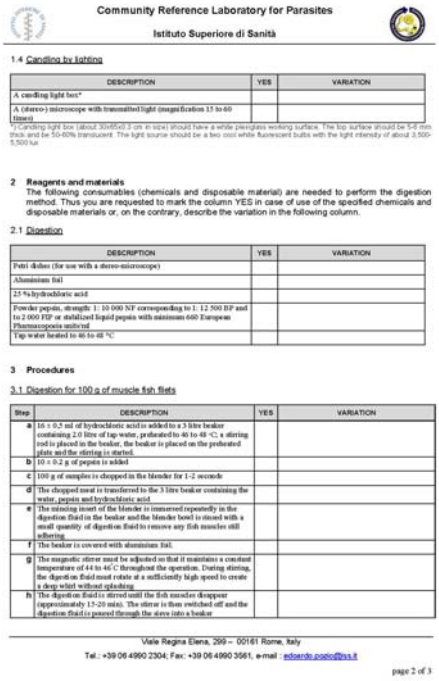

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1.4 Candling by lighting

DESCRIPTION	YES	VARIATION
A candling light box*		
A (stereo-) microscope with transmitted light (magnification 15 to 60 times)		

*Candling light box (about 30x65x0.3 cm in size) should have a white plexiglass working surface. The top surface should be 5-8 mm thick and be 50-60% translucent. The light source should be a two cool white fluorescent bulbs with the light intensity of about 3,500-5,500 lux

2 **Reagents and materials**
The following consumables (chemicals and disposable material) are needed to perform the digestion method. Thus you are requested to mark the column YES in case of use of the specified chemicals and disposable materials or, on the contrary, describe the variation in the following column.

2.1 Digestion

DESCRIPTION	YES	VARIATION
Petri dishes (for use with a stereo-microscope)		
Aluminium foil		
25 % hydrochloric acid		
Powder pepsin, strength: 1: 10 000 NF corresponding to 1: 12 500 BP and to 2 000 FIP or stabilized liquid pepsin with minimum 660 European Pharmacopoeia units/ml		
Tap water heated to 46 to 48 °C		

3 **Procedures**

3.1 Digestion for 100 g of muscle fish filets

Step	DESCRIPTION	YES	VARIATION
a	16 ± 0,5 ml of hydrochloric acid is added to a 3 litre beaker containing 2.0 litre of tap water, preheated to 46 to 48 °C; a stirring rod is placed in the beaker, the beaker is placed on the preheated plate and the stirring is started.		
b	10 ± 0.2 g of pepsin is added		
c	100 g of samples is chopped in the blender for 1-2 seconds		
d	The chopped meat is transferred to the 3 litre beaker containing the water, pepsin and hydrochloric acid		
e	The mincing insert of the blender is immersed repeatedly in the digestion fluid in the beaker and the blender bowl is rinsed with a small quantity of digestion fluid to remove any fish muscles still adhering		
f	The beaker is covered with aluminium foil.		
g	The magnetic stirrer must be adjusted so that it maintains a constant temperature of 44 to 46 C throughout the operation. During stirring, the digestion fluid must rotate at a sufficiently high speed to create a deep whirl without splashing.		
h	The digestion fluid is stirred until the fish muscles disappear (approximately 15-20 min). The stirrer is then switched off and the digestion fluid is poured through the sieve into a beaker		

Viale Regina Elena, 299 – 00161 Rome, Italy
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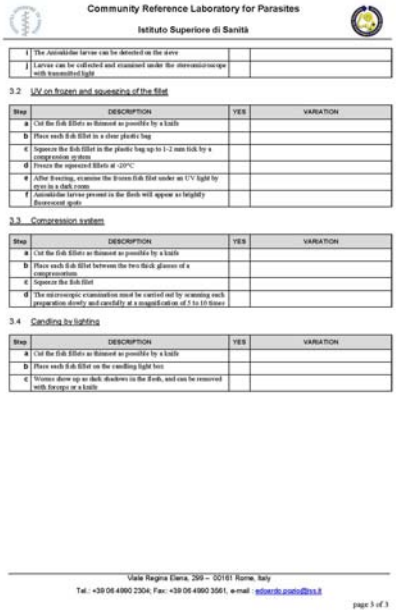

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i	The Anisakidae larvae can be detected on the sieve		
j	Larvae can be collected and examined under the stereomicroscope with transmitted light		

3.2 UV on frozen and squeezing of the fillet

Step	DESCRIPTION	YES	VARIATION
a	Cut the fish fillets as thinnest as possible by a knife		
b	Place each fish fillet in a clear plastic bag		
c	Squeeze the fish fillet in the plastic bag up to 1-2 mm tick by a compression system		
d	Freeze the squeezed fillets at -20°C		
e	After freezing, examine the frozen fish fillet under an UV light by eyes in a dark room		
f	Anisakidae larvae present in the flesh will appear as brightly fluorescent spots		

3.3 Compression system

Step	DESCRIPTION	YES	VARIATION
a	Cut the fish fillets as thinnest as possible by a knife		
b	Place each fish fillet between the two thick glasses of a compression		
c	Squeeze the fish fillet		
d	The microscopic examination must be carried out by scanning each preparation slowly and carefully at a magnification of 5 to 10 times		

3.4 Candling by lighting

Step	DESCRIPTION	YES	VARIATION
a	Cut the fish fillets as thinnest as possible by a knife		
b	Place each fish fillet on the candling light box		
c	Worms show up as dark shadows in the flesh, and can be removed with forceps or a knife		

Viale Regina Elena, 299 – 00161 Rome, Italy

Tel.: +39 06 4990 2304; Fax: +39 06 4990 3561, e-mail : edoardo.pozio@iss.it

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Form 4 Laboratory code ____

Proficiency test for the detection of Anisakidae larvae in a fish fillet

Check of the package content and its condition of preservation

1. One fish sample under vacuum ☐
2. Hard copies of forms which should be filled in and send back to CRLP ☐

The content of the package has been forwarded refrigerated

- When did you receive the package? Date _____hour____
- When did you open it? hour____
- Was the sample still refrigerated in the package (temperature <10°C)? Yes ☐ No ☐

The fish sample should be stored refrigerated at 4°C or better on ice before testing.

Viale Regina Elena, 299 – 00161 Rome, Italy

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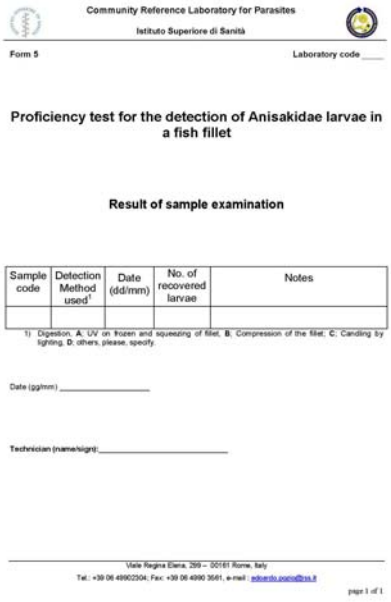

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Form 5 Laboratory code ____

Proficiency test for the detection of Anisakidae larvae in a fish fillet

Result of sample examination

Sample code	Detection Method used[1]	Date (dd/mm)	No. of recovered larvae	Notes

1) Digestion, **A**; UV on frozen and squeezing of fillet, **B**; Compression of the fillet; **C**; Candling by lighting, **D**; others, please, specify.

Date (gg/mm) _____

Technician (name/sign): _____

Viale Regina Elena, 299 – 00161 Rome, Italy

Tel.: +39 06 49902304; Fax: +39 06 4990 3561, e-mail : edoardo.pozio@iss.it

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Annex 2

Main features of National Reference Laboratories for Parasites participating at the PT

Lab code	Accredi-tation	Year of accredi-tation	N. of fish tested per year	Test[1]	Test accredi-tation	N. of scientists working on fish parasites	N. of technicians working on fish parasites
1	y	n.r.	0	D	no	2	3
2	y	2004	1	D	no	2	2
3	y	1998	75	CO	y	2	1
4	y	2004	1	D	no	2	2
6	y	2005	0	D	y	1	1
7	y	2005	0	D	no	3	1
9	y	1998	0	D	no	1	3
10	no	-	2	D	no	1	3
12	y	2000	105	D	no	1	1
16	y	2002	0	CA	no	1	1
17	y	2006	40	CA	y	2	1
18	y	2004	2	CO	no	2	5
21	y	1999	4	CA	y	1	0
22	no	-	0	D	no	2	0
23	y	1997	10	CA	y	2	2
24	no	-	0	D	no	1	2
25	y	2002	5	CA/CO/D	y	2	1
28	no	-	1000	UV/D	no	1	1
29	y	1999	400	CA	no	2	1
30	no	-	0	D	no	1	1

[1]D = digestion; CO = compressorium; CA = candling; UV = UV examination after freezing

Annex 3

Forwarding time and condition of preservation of the package content

Laboratory code	Forwarding days	Time (hours) between package arrival and opening	Was the sample refrigerated at the arrival?
1	3	0.00	y
2	1	0.30	y
3	1	0.30	y
4	1	0.30	y
6	2	0.00	y
7	2	5.30	y
9	2	0.15	y
10	1	1.00	y
12	1	0.20	y
16	3	0.05	no
17	1	0.15	y
18	1	0.15	Y
21	2	4 days	y
22	2	0.15	y
23	1	0.05	y
24	2	0.00	y
25	1	0.30	y
28	2	0.00	y
29	4	0.00	no
30	3	18.00	y

Annex 4

Proficiency test results

Laboratory code	No. of recovered larvae	No. of larvae in the sample	Test method used[1]
1	2	3	D
2	3	3	D
3	3	3	CO
4	3	3	D
6	3	3	D
7	3	3	D
9	3	3	D
10	2	3	D
12	3	3	D
16	2	3	CA
17	3	3	CA
18	2	3	CO
21	3	3	CA
22	3	3	D
23	3	3	CA
24	2	3	D
25	3	3	CA/CO/D
28	1	3	UV/D
29	3	3	CA
30	3	3	D

[1]D = digestion; CO = compressorium; CA = candling; UV = UV examination after freezing.