Report of the second NRL proficiency test on the "Detection of Anisakidae L3 larvae in fish fillets"

March, 2013

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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 for the human consumption that are obviously contaminated with parasites, on the market.

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 was to test the competence of the appointed NRLs to detect Anisakidae larvae in fish fillets. The PT has been organized following the request of some NRLs during the 2012 EURLP annual workshop and after 4 years from the first PT on the detection of Anisakidae larvae in fish fillets.

3 Time frame

The PT was announced to NRLs by email on February 15th, 2013, and the dead line to send the participation form (MO_MAQPVI_01_PT request form PT anisakidae) was March 1st, 2013. On March 18th, 2013, the samples were dispatched to participants by an international courier. Reporting deadline was 26th March, 2013.

4 Test material

According to the ISO 17043 (par. 4.4.2.3), the "PT items should match in terms of matrix, measurands and concentrations, as closely as practicable, the type of items or materials encountered in routine testing or calibration". The test material forwarded to each laboratory, consisted of 3 fish fillet containing in the core a different number of Anisakidae L3 larvae. In order to mimic as much as possible a routine analysis, the fillets of horse mackerel (*Trachurus trachurus*), commonly marketed in the EU and at risk for infection with Anisakidae, were used. Although a pool (~10% of fillets' stock) was analyzed by the digestion method and no larvae were detected, the potential presence of Anisakidae larvae in the muscles could not be excluded. A panel of 3 samples of ~ 100g fish fillet sandwich were prepared and, to guarantee positive fillets, two samples out of the three were spiked with one or three live larvae of *A. pegreffii. Anisakis pegreffii* L3 larvae, free of their capsule, were recovered from the body cavity of a heavily parasitized silver scabbardfish (*Lepidopus caudatus*) from the Mediterranean sea. All the collected larvae were identified at the genus level by a microscope and 50 randomly collected larvae were identified at the species level by PCR-RFLP of the ITS region. L3 larvae were carefully transferred by tweezers in the middle of each fish fillet sandwich. The fish sandwiches were then put individually in a plastic bag sealed under vacuum, identified by a numeric code stuck on the plastic bag, without any indication of the level of contamination or any information on the identity of the testing laboratory, and stored at +4°C. The same code with the number of larvae was reported in an Excel file (MO/POPVI-00/06, Rev. 0). The same day of preparation, the 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. The stability of the samples in the package was previously evaluated by ad hoc experiments made at the EURLP. Larvae in the samples sealed under vacuum and stored between +4 and +10°C, were viable up to 7 days from the date of preparation.

5 Instructions to participants

From February 19th, 2013, general information on the PT organization were made available to the NRLs on the EURLP web site (http://www.iss.it/crlp/test/index.php?lang=2&tipo=28).
Electronic copies of the forms which should be filled in and send back to EURLP were send by e-mail on March 12th 2013; they were (MO_POPVI-00 from 01 to 04) (see Annex 1):

(01) "The package check". This form was to verify the content and the condition of the PT samples at the arrival.
(02) "Instruments and Material" needed to perform the suggested methods.
(03) "Procedure" step by step description of the four suggested methods.
(04) "Result" in which the number of recorded larvae in each sample should be reported.

The code assigned to the laboratory was send independently to each participant by e-mail on March 12th 2013.
To make comparable the results obtained by the different laboratories, 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. artificial digestion.

6 Criteria for the result evaluation and statistical analysis

The evaluation was only qualitative (presence or absence of larvae). After the discussion followed the presentation of the PT results during the 8th NRLs annual workshop (Rome, 23- 24 May, 2013) the terminology used in the PT Report was modified. The result was "correct" if the laboratory detected Anisakidae larvae in the two spiked samples and no larva in the non-spiked sample. The result was "incorrect" if the laboratory did not detect any larva in the spiked samples. Since fillets from fished fish at risk of natural Anisakidae infection were used for the sample preparation, the presence of one or more larvae in the non-spiked sample cannot be excluded; therefore, this result was not taken into account for the PT evaluation and expressed as "not applicable". The PT was considered "positive" if no "incorrect" results were obtained; the PT was considered "negative" if at least one "incorrect" result was obtained. Due to the use of wild fish, low number of samples and spiked larvae, no statistical analysis of the results was performed.
The EURLP provided a PT Report (see Annex 2) including the results of the qualitative evaluation. In the report, the following information were displayed: i) the number of spiked larvae per sample; ii) the number of larvae detected by the laboratory in the sample; and iii) the final evaluation.

7 Participating laboratories

Twenty-three NRLs from 22 MS (two NRLs from Romania) and the NRL of Norway, agreed to participate to the PT. The NRL of Bulgaria, Cyprus, Malta, Norway, and UK, participated to this PT for the first time. Only 23 out of the 24 participants, sent the results.

8 Results

8.1 Delivery of the package to NRLs

All the packages were delivered to NRLs within 24-72 hours (one day for 17 NRLs; two days for 6 NRLs; three days for 1 NRL) (see Annex 3). At the delivery, the internal temperature of all the packages was less than 10°C in 22 parcels (90%). The time elapsed from the arrival of the package at the NRL and its control was of less than one hour for 20 NRLs, approximately 2 hours for 1 NRL, of 20 hours for 1 NRLs, and of 46-48 hours for 2 NRL (but the packages were stored at +4°C).

8.2 Test methods

Thirteen laboratories used the digestion method only, five laboratories used the digestion method in combination with other methods (UV, candling or compressorium) (see Annex 4). Two labs used the candling only, one lab used the compressorium only, one lab used the candling and the compressorium and, one lab used the UV only. Eight laboratories introduced small changes in the procedures, mainly in the digestion method, including the use of liquid pepsin instead of powder pepsin and hand-chopping or no chopping of the fish fillets. Four laboratories also performed the molecular analysis of the recovered larvae. Larvae were typed as *A. pegreffii* and hybrid genotype *A. simplex/pegreffii*.

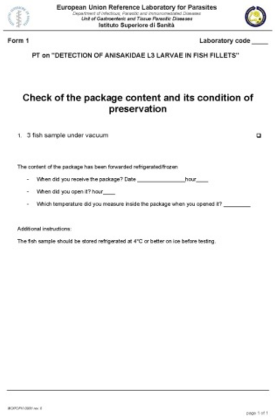
9 Conclusions

Out of the 24 participant laboratory, 23 sent the results and one failed to pass the PT since didn't find any larva in the sample spiked with one larva. All the laboratories found, at least in one sample, more larvae than the spiked ones and seven labs detected larvae in the not spiked sample, in agreement with the use of fillets from fish at high risk of natural infection with Anisakidae. Six out of the 23 labs reported the presence of motile larvae irrespective of the detection method used (3 digestion, 2 candling, and 1 compressorium). Laboratories, especially those that found larvae in the negative sample, promptly provided to the EURLP photographic records and three laboratories send larvae to the EURLP for their molecular identification. Since same of the NRLs reported to have never had the chance to test fish for the presence of Anisakidae larvae, the use of fish fillets naturally infected with Anisakidae larvae with the addition of spiked larvae has offered the advantage to analyze samples that reproduce the routine analysis. After 4 years from the previous PT, the results suggest that the majority of the participating laboratories have an high level of competence in the identification of Anisakidae larvae, irrespective to the detection method used. It is relevant to stress that the results obtained when the artificial digestion was applied after a first screening with one of the other methods (candling, compressorium, or UV examination after freezing), suggest that the digestion method has a higher sensitivity compared to the other three methods. At the fish market, veterinarians test only few marine fish from each stock to detect Anisakidae larvae. This routine 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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Form 1 Laboratory code ____

PT on "DETECTION OF ANISAKIDAE L3 LARVAE IN FISH FILLETS"

Check of the package content and its condition of preservation

1. 3 fish sample under vacuum

The content of the package has been forwarded refrigerated/frozen

- When did you receive the package? Date ____________ hour____
- When did you open it? hour____
- Which temperature did you measure inside the package when you opened it? ________

Additional instructions:

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


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Form 2 Laboratory code ____

PT on "DETECTION OF ANISAKIDAE L3 LARVAE IN FISH FILLETS"

List of instruments, reagents and materials required to perform the test

1 Instruments

1.1 DIGESTION OF FISH FILLETS

1.2 UV ON FROZEN AND SQUEEZING OF THE FILLET

1.3 COMPRESSION OF THE FILLET

1.4 CANDLING BY LIGHTING


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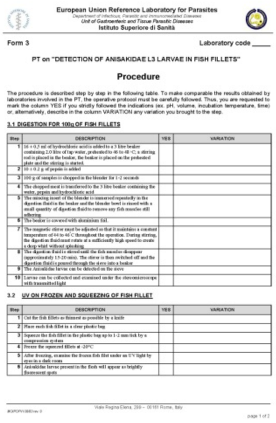
2 Reagents and materials

1.5 DIGESTION OF FISH FILLETS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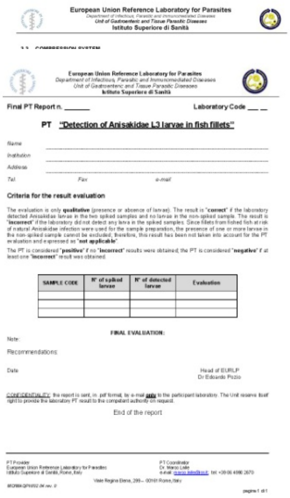
Form 3 Laboratory code ____

PT on "DETECTION OF ANISAKIDAE L3 LARVAE IN FISH FILLETS"

Procedure

3.1 DIGESTION FOR 100g OF FISH FILLETS

3.2 UV ON FROZEN AND SQUEEZING OF FISH FILLET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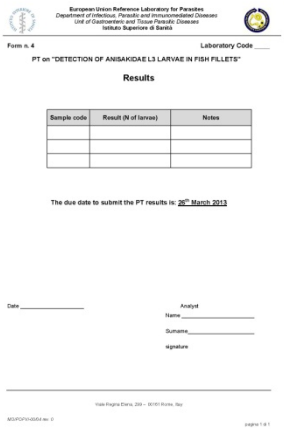
Final PT Report n. ______ Laboratory Code __ __

PT "Detection of Anisakidae L3 larvae in fish fillets"

Criteria for the result evaluation

FINAL EVALUATION:

End of the report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Form n. 4 Laboratory Code ____

PT on "DETECTION OF ANISAKIDAE L3 LARVAE IN FISH FILLETS"

Results

Sample code	Result (N of larvae)	Notes

The due date to submit the PT results is: 26th March 2013

Annex 2

Participating NRLs	Country
Institut für Veterinärmedizin, Innsbruck	Austria
Institute of Tropical Medicine, Antwerp	Belgium
National Diagnostic and Research Veterinary Institute, Sofia	Bulgaria
Cyprus Veterinary Services, Nicosia	Cyprus
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
Max Rubner-Institut, Institut für Sicherheit und Qualität bei Milch und Fisch, Hamburg	Germany
Institute of Infectious and Parasitic Diseases , Athens	Greece
Veterinary Laboratory Department of Agriculture & Food Laboratories, Celbridge	Ireland
National Reference Laboratory for Anisakiasis, Ist. Zooprof. Sper. Sicilia, Palermo	Italy
Institute of Food Safety, Animal Health and Environment "BIOR",Riga	Latvia
National Food and Veterinary Risk Assessment Institute, Vilnius	Lithuania
National Veterinary Laboratory, Albertown - Marsa	Malta
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.
Centro Nacional de Alimentación. Agencia Española de Seguridad Alimentaria y Nutrición, Majadahonda	Spain
Statens Veterinärmedicinska Anstalt, Uppsala	Sweden
Cefas Weymouth Laboratory, Weymouth	UK
Norwegian Veterinary Institute, Oslo	Norway

Annex 3

Forwarding time and condition of preservation of the package content

Laboratory code	Forwarding days	Time (hours) between package arrival and opening	Temperature inside of the package at the arrival?
A1	1	0.30	7°C
A2	1	0.10	5°C
A3	1	0.10	11°C
A4	1	0.30	4°C
A5	1	0.00	-
A6	1	0.00	9°C
A7	1	0.30	-
A8	1	0.05	8.8°C
A9	1	0.05	2°C
A10	1	0.00	5°C
A11	1	20	4°C
A12	1	0.25	4°C
A13	2	46	4°C
A14	1	2.15	cool
A15	1	0.05	4°C
A16	2	0.00	8°C
A17	1	0.00	3.5°C
A18	2	0.05	3.8°C
A20	3	0.05	5.9°C
A21	2	0.00	-
A22	2	0.15	10°C
A23	1	0.15	8.8°C
A24	1	48	cool

Annex 4

Proficiency test results

Lab Code	Spiked larvae[1]			Method(s)[2]	Final Evaluation
	0	1	3		
A1	9(1f)	8(4f)	6(2f)	D	Positive
A2	2	6	9	D	Positive
A3	0	8	11	D	Positive
A4	0	3	5	Co	Positive
A5	0	0	5	C+Co	Positive
A6	0	12	20	D	Positive
A7	1	9	14	C+D	Positive
A8	0	1(1f)	2(4f)	UV+D	Positive
A9	0	10	8	D	Positive
A10	0	2(2f)	5(8f)	C	Positive
A11	0	14	8	D	Positive
A12	0	3	10(1f)	C	Positive
A13	0	5	12	D	Positive
A14	4 (3f)	8(2f)	9(3f)	D	Positive
A15	0	7	6	D	Positive
A16	0	10	8	D	Positive
A17	1	5	7	Co+UV+D	Positive
A18	0	9 (3f)	4(4f)	UV	Positive
A19	-	-	-	-	-
A20	0	3(2f)	7(3f)	D	Positive
A21	1	3	6(6f)	D	Positive
A22	3	8	11	C+D	Positive
A23	0	3(1f)	16(1f)	C+D	Positive
A24	0	2(2f)	2(3f)	D	Positive

1) Number of recovered larvae are reported. Number of larval fragment are reported in brackets.
2) D = digestion; Co = compressorium; C = candling; UV = UV examination after freezing.