Report on the fifth NRL Proficiency Test to detect adult worms of *Echinococcus* sp. in the intestinal mucosa of the definitive host

March, 2013

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1 Introduction

Cestode worms of the genus *Echinococcus* are zoonotic parasites circulating in most of the European countries in both wild and domestic animals (Eckert et al., 2001). Humans acquire the infection by the ingestion of eggs shed by dogs with their faeces which can contaminate raw or undercooked vegetables and fruits, fomities, and the dog coat. Herbivore and omnivore animals (e.g. sheep, goats, cattle, pigs) are the intermediate hosts of parasites belonging to the *Echinococcus granulosus* complex; whereas, sylvatic rodents are the intermediate hosts of parasites belonging to *Echinococcus multilocularis*. Humans can accidentally acquire the infection as an intermediate host, even if they represent a dead end of the parasite cycle. Domestic dogs and sylvatic canids (e.g. red foxes and raccoon dogs) act as final hosts of *E. multilocularis*. Domestic and stray dogs and rarely wolves, act as final hosts of *E. granulosus*. The incidence of infection greatly varies from one to another MS. In endemic EU countries the incidence can reach 6.3 cases for 100,000 inhabitants (Pozio, 2008).

2 Scope

One of the core duties of the EURLP is to organise proficiency tests (PTs), 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 identify adult worms or their parts, e.g. proglottids and rostrellum, of *Echinococcus* sp. in the intestinal mucosa collected from the gut of the final host, and to differentiate this worm from other material of parasitic and non-parasitic origin present in the matrix.

3 Time frame

The proficiency test (PT) was announced to NRLs by email on 15 February, 2013 and the dead line to send the participation form was 28 February, 2013. From 19/02/2013, the general information on the PTs organization was available on the EURLP web site at the following address: http://www.iss.it/crlp/test/index.php?lang=2&tipo=28. On 18 March, 2013, the PT packages were dispatched to participants by an international courier. The due date to submit the PT results was 2 April, 2013. PT reports with the PT evaluations were delivered to NRLs on 10 April, 2013.

4 Test material

From November 2012 to March 2013, carcasses of red foxes shot by hunters in Italy, and sent to the “Istituto Zooprofilattico Sperimentale delle Venezie” (Padova, Italy) and to the “Istituto Zooprofilattico Sperimentale del Lazio e Toscana” (Rome, Italy) were used for the collection of the intestinal mucosa. Carcasses were forwarded in individual plastic bags at +4 °C. The intestinal tract was removed and stored at −20 °C. Then for safety reasons (i.e. to kill the *Echinococcus* embryos eventually present in the gut), the intestinal tract was frozen at −80 °C for 7 days before examination. After freezing, the gut was thawed at room

temperature and the middle and posterior parts of the intestine were collected and tested by sedimentation and counting technique (SCT) according to a previous published protocol (Mathis et al., 1996). If the sample resulted negative, the anterior third part of the intestine was opened and the mucosa was scraped and autoclaved for the reduction of bacterial activity. The mucosa of the small intestine of 30 foxes found to be negative was spiked with 12 or 32 worms in order to prepare weakly or highly positive samples, respectively (**Annex 1**). No spiked mucosa was used as negative control sample. Adult worms were kindly provided by Dr. T. Sreter of the NRL of Hungary.

The test material forwarded to each laboratory consisted of three vials containing:

1. *Echinococcus* negative mucosa, this sample being considered the negative control;
2. *Echinococcus* mucosa spiked with 12 worms, this sample being considered a weakly infected sample;
3. *Echinococcus* mucosa spiked with 32 worms, this sample being considered a highly infected sample (**Annex 1**).

All samples were delivered within 24-36 hours. The following forms were included in the package:

1) information on PT and its purpose (**Annex 2**);
2) package content and its condition of preservation (**form 1, Annex 2**)
3) instructions for the detection of *Echinococcus* sp. adult worms (**form 3, Annex 2**);
4) results (**form 4, Annex 2**);
5) laboratory code.

5 Instructions to participants

Practical instructions were given to all the participants in the form 3 and in the accompanying letter. To make the results comparable among the laboratories, all participants had to follow the protocol step by step or describe the modification made, if any. It was requested to qualitatively and quantitatively evaluate the samples by SCT (Eckert et al., 2001).

6 Participating laboratories

Twenty-three NRL laboratories agreed to participate (**Annex 3**).

7 Evaluation criteria

7.1 qualitative evaluation

The PT result evaluation was expressed as "**correct**" (detection of one or more *Echinococcus* sp. adult worms in spiked samples or no worm in not spiked samples) or "**incorrect**" (false positive or false negative results), irrespective of the number of worms in the sample/s. The **final evaluation** was only based on qualitative evaluation and was

expressed as "**positive**" if the results of all samples were correct or "**negative**" if at least one result was incorrect.

7.2 quantitative evaluation

No consistent data exist in the scientific literature for the quantitative evaluation of the sedimentation and counting technique; therefore, a z-score was established using the standard deviation of the sample from the average PT values. This statistical approach shows the laboratory performance in comparison to the average performance of this PT.

The Z score was calculated by the formula: $$z = \frac{X_{lab} - X_{\mathrm{Ref}}}{\hat{\sigma}}$$

where:
X_{lab} is the number of worms found in the sample by the laboratory;
X_{ref} is the number of worms spiked in the sample;
σ is the standard deviation (i.e., 16 for the sample # 1; and 5 for the sample # 3) calculated from the quantitative results obtained in this PT.

Evaluation criteria:
If the z-score was "≤ |3|", the laboratory result was "**positive"**; however, if the z-score was "|2| < z-score ≤ |3|", the result was still positive but the laboratory should be alerted to start preventive actions to avoid a future negative performance; if the z-score was "> |3|", the laboratory result was "**negative"**.

8 Results

Twenty-nine laboratories were invited to participate to the PT on the detection of adult worms of *Echinococcus* sp. in the intestinal mucosa of the definitive host. Twenty-three (79%) laboratories agreed to participate to *Echinococcus* PT.

The average recovery rate of adult worms from the weakly spiked (n=12) sample was 7 (% of recovery 58.3; range 0-12), whereas in the highly spiked sample (n=32), the average recovery rate was 18 (% of recovery 56; range 2-32).

The qualitative evaluation obtained by the NRLs was(Annex 4):

- sample 1 (negative sample): 23 laboratories (100%) obtained a positive evaluation;
- sample 2 (spiked with 12 worms): 22 laboratories (96%) obtained a positive evaluation;
- sample 3 (spiked with 32 worms): 23 laboratories (100%) obtained a positive evaluation.

The quantitative evaluation obtained by the NRLs was (Annex 5):

- sample 1 (negative sample): 23 laboratories (100%) obtained a positive evaluation;
- sample 2 (spiked with 12 worms): 21 laboratories (91%) obtained a positive evaluation;
- sample 3 (spiked with 32 worms): 22 laboratories (96%) obtained a positive evaluation.

9 Conclusions

The experience derived from the fifith PT carried out in 2013, showed that the personnel of NRLs were skill to detect this parasite both by a qualitative and quantitative test. This year for the evaluation of the laboratory performance, a quantitative evaluation was introduced using the z-score as statistical approach. Since no consistent data exist in the scientific literature on the detection limits of these parasites in the intestinal content by SCT, data originating from this and further PTs will be very important to establish a detection limit of this technique.

10 References

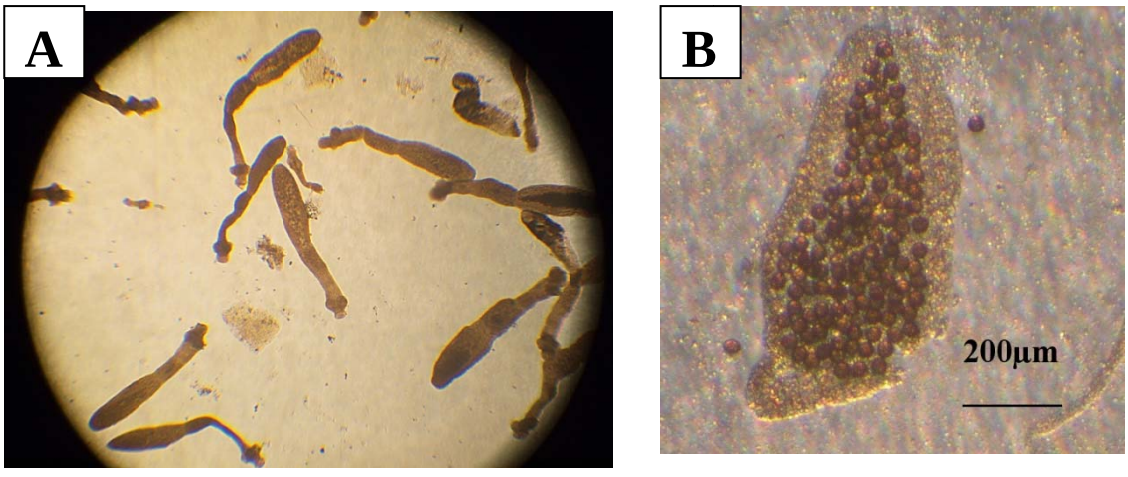
Eckert, J., Gemmell, M.A., Meslin, F.X., Pawlowski, Z.S. (2001). WHO/OIE manual on echinococcosis in humans and animals: a public health problem of global concern. World Organisation for Animal Health, Paris, France, pp. 1- 265.

Mathis A, Deplazes P, Eckert J. (1996). An improved test system for PCR-based specific detection of Echinococcus multilocularis eggs. J Helminthol. 70:219-22.

Pozio, E. (2008). Epidemiology and control prospects of foodborne parasitic zoonoses in the European Union. Parassitologia 50:17-24.

Annex 1

Necroscopy of a fox carcass: collection of the gut.



Adult worms of *Echinococcus* (a) and a proglottid (b) isolated from the intestinal mucosa of a fox

Preparation of the intestinal mucosa spiked with adult worms of *Echinococcus*

The three PT samples forwarded to the 23 participating labs in 2013

Annex 2

Dear Colleagues,

according to the decisions made at the workshop of 2012, this year (2013), the EURLP will organize four proficiency testing (PTs):

- Detection of *Trichinella* larvae in meat samples by digestion
- Identification of *Trichinella* larvae at the species level by PCR
- Detection of Anisakidae larvae in fish fillets
- Detection of adult worms of *Echinococcus* sp. in the intestinal content of the definitive host

From Tuesday 19/02/2013, you can read the general information on the PTs organization on the EURLP web site at the following address: http://www.iss.it/crlp/test/index.php?lang=2&tipo=28

All PT panels will be forwarded on **Monday 18 March**. The package will be delivered to labs within 24-48 h.

If you are interested in participating in one or more of the four PTs, you shall inform the person in charge of each PT **within Friday 22 February**:

- PT *Trichinella* digestion, **Dr. Gianluca Marucci**, email: gianluca.marucci@iss.it, tel +39 06 4990 2310
- PT *Trichinella* PCR, **Dr. Gianluca Marucci**, email: gianluca.marucci@iss.it, tel +39 06 4990 2310
- PT Anisakidae, **Dr. Marco Lalle**, email: marco.lalle@iss.it, tel +39 06 4990 2670
- PT *Echinococcus*, **Dr. Adriano Casulli**, email : adriano.casulli@iss.it, tel +39 06 4990 2670

The three persons in charge for the PTs will provide you all the information to enroll the PTs of your interest.

Looking forward to hearing from you

Kind Regards,

Edoardo Pozio

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European Union Reference Laboratory for Parasites
Department of Infectious, Parasitic and Immunomediated Diseases
Unit of Gastroenteric and Tissue Parasitic Diseases
Istituto Superiore di Sanità

Form 1 **Laboratory code** ____

PT on "Detection of Echinococcus spp. adult worms in the intestinal mucosa of the definitive host

Check of the package content and its condition of preservation

Insert sample codes:

1. _____ ❑
2. _____ ❑
3. _____ ❑

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:

European Union Reference Laboratory for Parasites
Department of Infectious, Parasitic and Immunomediated Diseases
Unit of Gastroenteric and Tissue Parasitic Diseases
Istituto Superiore di Sanità

Form 3 **Laboratory code ____**

PT on "Detection of Echinococcus spp. adult worms in the intestinal mucosa of the definitive host

Method: "Sedimentation and Counting technique (SCT)"

Procedure

The procedure is described step by step in the following table. To make comparable the results obtained by laboratories involved in the PT, the operative protocol must be carefully followed. Thus, you are requested to mark the column YES if you strictly followed the indications (ex. pH, volume, incubation temperature, time) or, alternatively, cescribe in the column VARIATION any variation you brought to the step.

Step	DESCRIPTION	YES	VARIATION
1.	Transfer the whole sample into a glass bottle containing 1L of saline solution (0.9% NaCl);		
2.	After a sedimentation of 15 min, the supernatant discharged and the bottle is refilled with saline solution;		
3.	Repeat step 2, until the solution is sufficiently clear (usually 2-3 times);		
4.	Examine the sediment in 5-10 ml portions in a Petri dish with a counting grid under a stereomicroscope (25x).		

European Union Reference Laboratory for Parasites
Department of Infectious, Parasitic and Immunomediated Diseases
Unit of Gastroenteric and Tissue Parasitic Diseases
Istituto Superiore di Sanità

Form n. 4 **Laboratory Code _____**

PT on "Detection of Echinococcus spp. adult worms in the intestinal mucosa of the definitive host

Results

SAMPLE CODE	ADULT WORM NUMBERS	NOTES

The due date to submit the PT results is: 02/04/2013

Date _____

Analyst
Name _____
Surname_____
signature

Annex 3

National Reference Laboratories (NRL) participating at the proficiency test for *Echinococcus* sp.

National Reference Laboratories	Country
Institut für Veterinärmedizin, Innsbruck	Austria
Institute of Tropical Medicine, Antwerp	Belgium
National Diagnostic and Research Veterinary Institute, Sofia	Bulgaria
State Veterinary Laboratory, Nicosia	Cyprus
Danish Food and Veterinary Institute, Copenhagen	Denmark
Estonian Veterinary and Food Laboratory, Tartu	Estonia
Finnish Food Safety, Evira, Oulu	Finland
Technopole Agricole et Vétérinaire, Malzeville	France
Friedrich-Loeffler-Institut, Institut für Epidemiologie	Germany
Centre of Athens Veterinary Institutions, Athens	Greece
Laboratories for Parasitology, Fish and Bee Diseases, Budapest	Hungary
Veterinary Laboratory Department of Agriculture & Food Laboratories	Ireland
Istituto Zooprofilattico Sperimentale of Sardinia, Sassari	Italy
Laboratory of Food and Environmental Investigations, National Diagnostic Centre	Latvia
National Veterinary Laboratory	Malta
National Institute of Public Health and the Environment	Netherlands
National Veterinary Institute	Norway
National Veterinary Research Institute, Pulawy	Poland
National Veterinary Institute	Portugal
Institute for Diagnosis and Animal Health	Romania
National reference Laboratory for parasites State Veterinary and Food Institute	Slovak Republic
National Veterinary Institute	Sweden
Veterinary Laboratories Agency	UK

Annex 4

Proficiency Test Results (Qualitative evaluation)

LAB CODE	SAMPLE 1 (N=0)	SAMPLE 2 (N=12)	SAMPLE 3 (N=32)	FINAL EVALUATION
E1	Correct	Correct	Correct	Positive
E2	Correct	Correct	Correct	Positive
E3	Correct	Correct	Correct	Positive
E4	Correct	Correct	Correct	Positive
E5	Correct	Correct	Correct	Positive
E6	Correct	Correct	Correct	Positive
E7	Correct	Correct	Correct	Positive
E8	Correct	Correct	Correct	Positive
E9	Correct	Correct	Correct	Positive
E10	Correct	Correct	Correct	Positive
E11	Correct	Correct	Correct	Positive
E12	Correct	Not Correct	Correct	Negative
E13	Correct	Correct	Correct	Positive
E14	Correct	Correct	Correct	Positive
E15	Correct	Correct	Correct	Positive
E16	Correct	Correct	Correct	Positive
E17	Correct	Correct	Correct	Positive
E18	Correct	Correct	Correct	Positive
E19	Correct	Correct	Correct	Positive
E20	Correct	Correct	Correct	Positive
E21	Correct	Correct	Correct	Positive
E22	Correct	Correct	Correct	Positive
E23	Correct	Correct	Correct	Positive

Annex 5

Proficiency Test Results (Quantitative evaluation)

	SAMPLE 2 (N=12)			SAMPLE 3 (N=32)		
LAB CODE	OBSERVED	EVALUATION	z-score	OBSERVED	EVALUATION	z-score
E1	7	Positive	-1,46	17	Positive	-1,66
E2	8	Positive	-1,17	8	Positive	-2,65
E3	12	Positive	0,00	30	Positive	-0,22
E4	11	Positive	-0,29	29	Positive	-0,33
E5	10	Positive	-0,58	30	Positive	-0,22
E6	5	Positive	-2,04	12	Positive	-2,21
E7	5	Positive	-2,04	2	Negative	-3,31
E8	3	Positive	-2,62	23	Positive	-0,99
E9	5	Positive	-2,04	13	Positive	-2,10
E10	8	Positive	-1,17	13	Positive	-2,10
E11	5	Positive	-2,04	23	Positive	-0,99
E12	0	Negative	-3,50	7	Positive	-2,76
E13	2	Positive	-2,91	12	Positive	-2,21
E14	8	Positive	-1,17	13	Positive	-2,10
E15	12	Positive	0,00	20	Positive	-1,33
E16	5	Positive	-2,04	22	Positive	-1,10
E17	10	Positive	-0,58	23	Positive	-0,99
E18	11	Positive	-0,29	34	Positive	0,22
E19	1	Negative	-3,21	7	Positive	-2,76
E20	9	Positive	-0,87	26	Positive	-0,66
E21	8	Positive	-1,17	5	Positive	-2,98
E22	6	Positive	-1,75	22	Positive	-1,10
E23	9	Positive	-0,87	25	Positive	-0,77