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

March-April, 2012

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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, 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* group; 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 matrice.

3 Time frame

The proficiency test (PT) was announced to NRLs by email on 19 January, 2012 and the dead line to send the participation form was 3 February, 2012. On March 19, 2012, the samples were dispatched to participants by an international courier. Reporting deadline was 28 March, 2012.

4 Test material

From November 2011 to February 2012, carcasses of red foxes shot by hunters in Italy, were sent to the “Istituto Zooprofilattico Sperimentale delle Venezie” (Padova, Italy) and to the “Istituto Zooprofilattico Sperimentale del Lazio e Toscana” (Rome, Italy).

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 25 foxes found to be negative was spiked with 11 or 31 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* mucosa spiked with 31 worms, this sample being considered a highly infected sample (**Annex 1**);
2. *Echinococcus* negative mucosa, this sample being considered the negative control;
3. *Echinococcus* mucosa spiked with 11 worms, this sample being considered a weakly infected sample.

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) laboratory description (**form 2, Annex 2**);
4) instructions for the detection of *Echinococcus* sp. adult worms (**form 3, Annex 2**);
5) results (**form 4, Annex 2**);
6) 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 obtained by laboratories comparable, 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-five NRL laboratories agreed to participate (**Annex 3**).

7 Evaluation criteria

7.1 qualitative evaluation

The SCT results were reported as "positive" (i.e., detection of one or more *Echinococcus* adult worms) or "negative" (i.e., no detection of *Echinococcus* worms), irrespective of the number of worms in the sample/s. The final evaluation is considered as "positive" if the highly spiked sample (sample # 1) and the non-spiked samples (negative control, sample # 2) were correctly identified.

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 is ≤ |3|, the laboratory result is positive; however, if the |2| < z-score ≤ |3|, the technician performing the test should be alerted; if the z-score > |3|, the laboratory result is unsatisfactory.

8 Results

Out of the 25 NRLs which agreed to participate to the PT, 20 (80%) were accredited according to ISO/IEC 17025:2005 and 4 (16%) had accredited a diagnostic test to detect *Echinococcus* larvae/adults in the intermediate/definitive hosts. Ninety-four persons are currently working with *Echinococcus* in the 25 NRLs: 48 scientist and 46 technicians.

The average recovery rate of adult worms from the weakly spiked (n=11) sample was 5 (% of recovery 45.5; range 1-10), whereas in the highly spiked sample (n=31), the average recovery rate was 16 (% of recovery 52; range 2-30).

Qualitative evaluation obtained by the NRLs (Annex 4):

- sample 1 (positive): 25 laboratories (100%) obtained a positive evaluation;
- sample 2 (negative): 24 laboratories (96%) obtained a positive evaluation;
- sample 3 (positive): 20 laboratories (80%) obtained a positive evaluation.

Quantitative evaluation obtained by the NRLs (Annex 5):

- sample 1 (spiked with 31 worms): 21 laboratories (84%) obtained a positive evaluation;
- sample 2 (negative sample): 24 laboratories (96%) obtained a positive evaluation;
- sample 3 (spiked with 11 worms): 20 laboratories (80%) obtained a positive evaluation.

9 Conclusions

The results of the fourth PT for *Echinococcus* show that the NRL personnel are skill to detect this parasite both in a qualitative and quantitative tests.

This year a quantitative evaluation was introduced using the z-score as statistical approach for the evaluation of the performance. 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

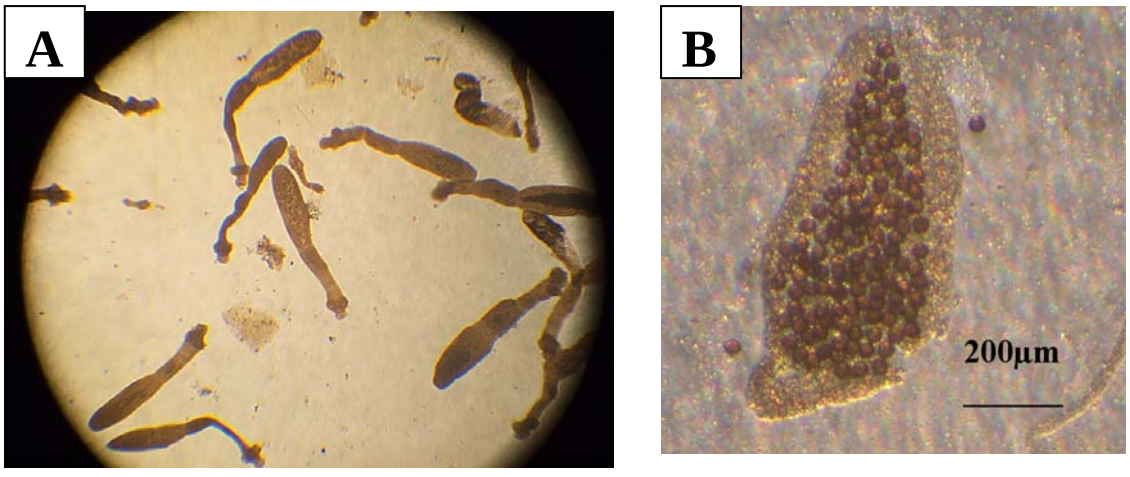
Eckert, J., Gemmell, M.A., Meslin, F.X., Pawlowski, Z.S. (2001). WHO/OIE manual on echinococcosis in humans and animals: a public health problem of global concern. World Organisation for Animal Health, Paris, France, pp. 1- 265.

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.

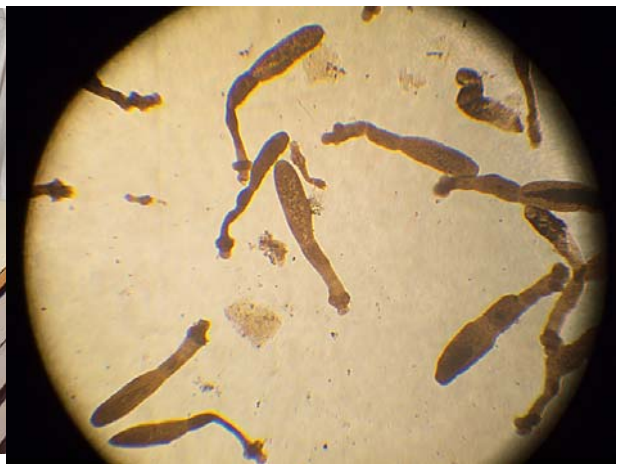
Pozio, E. (2008). Epidemiology and control prospects of foodborne parasitic zoonoses in the European Union. Parassitologia 50:17-24.

Annex 1

Necroscopy of a fox carcass: collection of the gut.



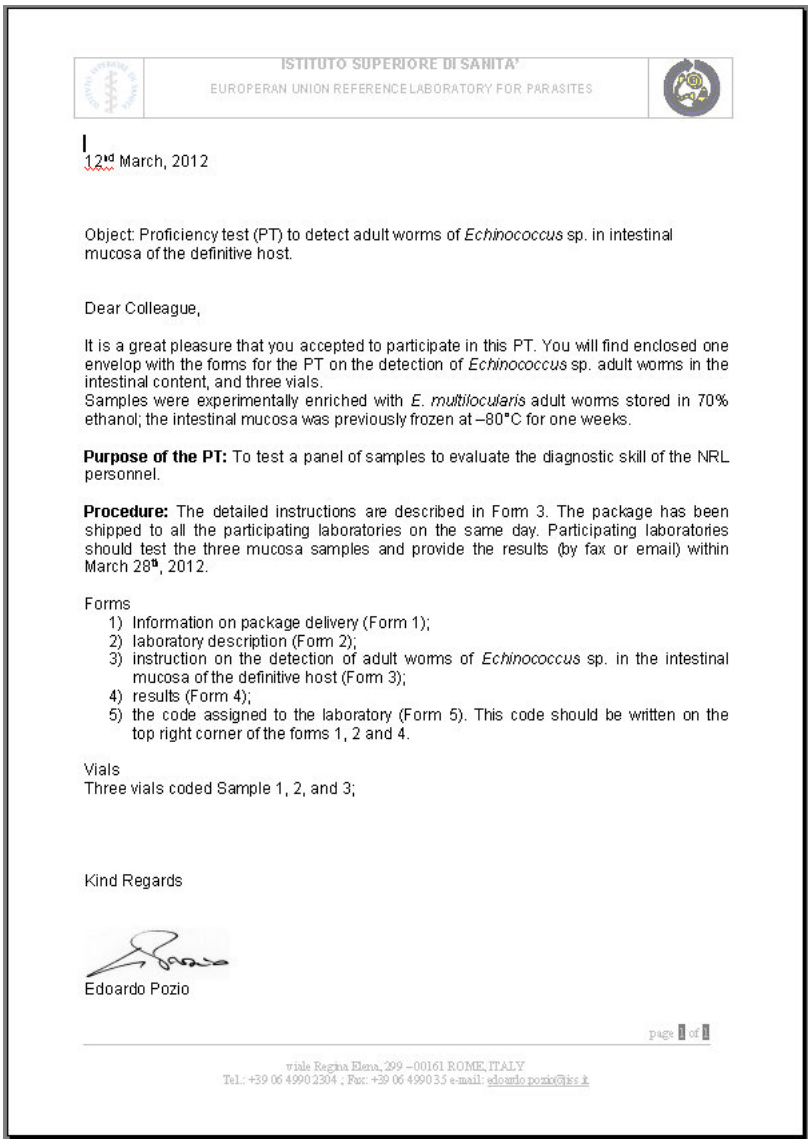
Adult worms of *Echinococcus* (a) and a proglottid (b) isolated from the intestinal mucosa of a fox



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

The three PT samples forwarded to the 25 participating labs in 2012

Annex 2



ISTITUTO SUPERIORE DI SANITA'
EUROPERAN UNION REFERENCE LABORATORY FOR PARASITES

12nd March, 2012

Object: Proficiency test (PT) to detect adult worms of *Echinococcus* sp. in intestinal mucosa of the definitive host.

Dear Colleague,

It is a great pleasure that you accepted to participate in this PT. You will find enclosed one envelop with the forms for the PT on the detection of *Echinococcus* sp. adult worms in the intestinal content, and three vials.
Samples were experimentally enriched with *E. multilocularis* adult worms stored in 70% ethanol; the intestinal mucosa was previously frozen at –80°C for one weeks.

Purpose of the PT: To test a panel of samples to evaluate the diagnostic skill of the NRL personnel.

Procedure: The detailed instructions are described in Form 3. The package has been shipped to all the participating laboratories on the same day. Participating laboratories should test the three mucosa samples and provide the results (by fax or email) within March 28th, 2012.

Forms
1) Information on package delivery (Form 1);
2) laboratory description (Form 2);
3) instruction on the detection of adult worms of *Echinococcus* sp. in the intestinal mucosa of the definitive host (Form 3);
4) results (Form 4);
5) the code assigned to the laboratory (Form 5). This code should be written on the top right corner of the forms 1, 2 and 4.

Vials
Three vials coded Sample 1, 2, and 3;

Kind Regards

Edoardo Pozio

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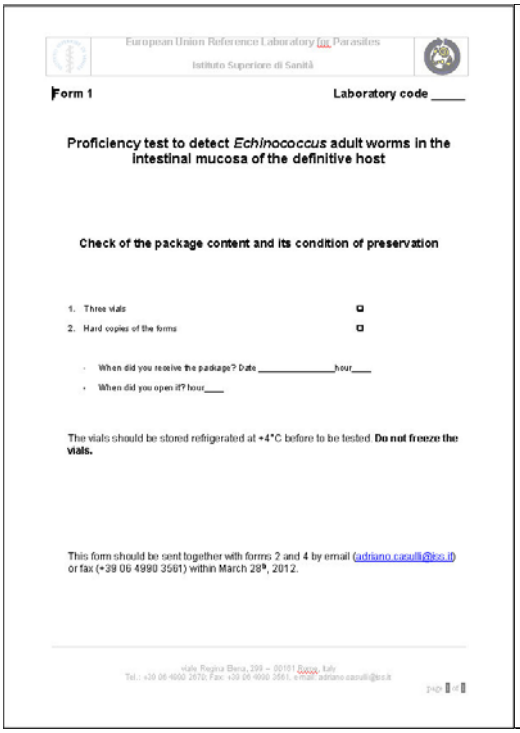

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Form 1 **Laboratory code** _____

Proficiency test to detect *Echinococcus* adult worms in the intestinal mucosa of the definitive host

Check of the package content and its condition of preservation

1. Three vials ☐
2. Hard copies of the forms ☐

- When did you receive the package? Date _____hour____
- When did you open it? hour____

The vials should be stored refrigerated at +4°C before to be tested. **Do not freeze the vials.**

This form should be sent together with forms 2 and 4 by email (adriano.casulli@iss.it) or fax (+39 06 4990 3561) within March 28th, 2012.

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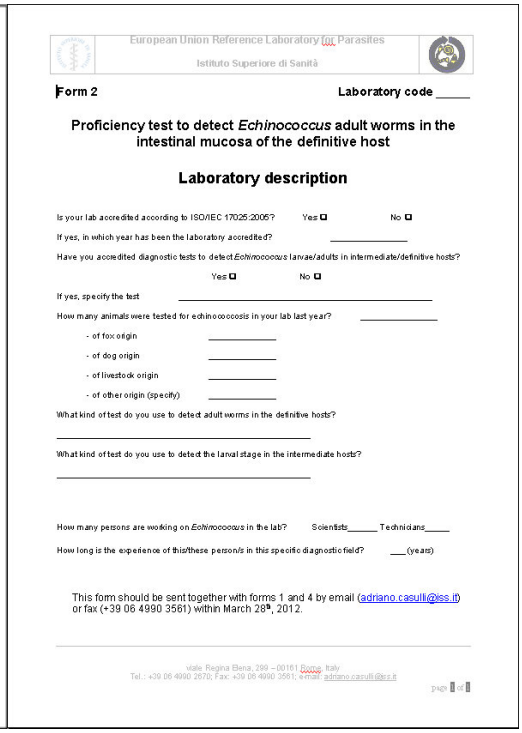

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Form 2 **Laboratory code** _____

Proficiency test to detect *Echinococcus* adult worms in the intestinal mucosa of the definitive host

Laboratory description

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

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

Have you accredited diagnostic tests to detect *Echinococcus* larvae/adults in intermediate/definitive hosts?

Yes ☐ No ☐

If yes, specify the test _____

How many animals were tested for echinococcosis in your lab last year? _____

- of fox origin _____
- of dog origin _____
- of livestock origin _____
- of other origin (specify) _____

What kind of test do you use to detect adult worms in the definitive hosts?

_____

What kind of test do you use to detect the larval stage in the intermediate hosts?

_____

How many persons are working on *Echinococcus* in the lab? Scientists_____ Technicians_____

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

This form should be sent together with forms 1 and 4 by email (adriano.casulli@iss.it) or fax (+39 06 4990 3561) within March 28th, 2012.

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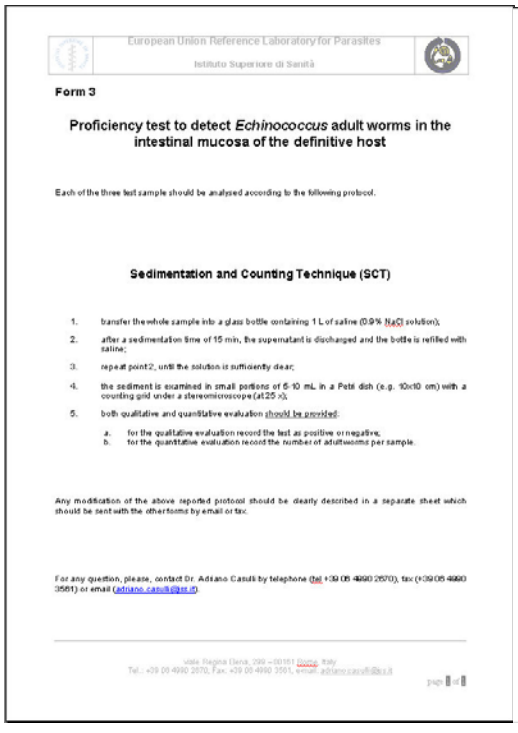

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Form 3

Proficiency test to detect *Echinococcus* adult worms in the intestinal mucosa of the definitive host

Each of the three test sample should be analysed according to the following protocol.

Sedimentation and Counting Technique (SCT)

1. transfer the whole sample into a glass bottle containing 1 L of saline (0.9% NaCl solution);
2. after a sedimentation time of 15 min, the supernatant is discharged and the bottle is refilled with saline;
3. repeat point 2, until the solution is sufficiently clear;
4. the sediment is examined in small portions of 5-10 mL in a Petri dish (e.g. 10x10 cm) with a counting grid under a stereomicroscope (at 25 x);
5. both qualitative and quantitative evaluation should be provided:
   a. for the qualitative evaluation record the test as positive or negative;
   b. for the quantitative evaluation record the number of adult worms per sample.

Any modification of the above reported protocol should be clearly described in a separate sheet which should be sent with the other forms by email or fax.

For any question, please, contact Dr. Adriano Casulli by telephone (tel +39 06 4990 2670), fax (+39 06 4990 3561) or email (adriano.casulli@iss.it).

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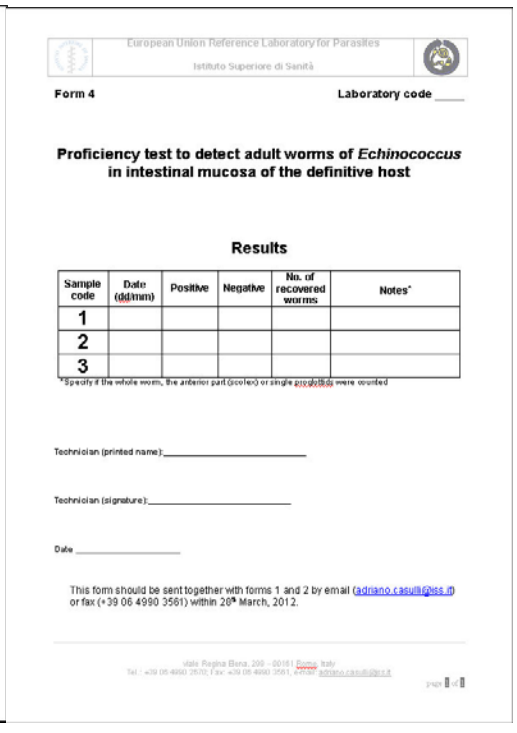

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Form 4 **Laboratory code** _____

Proficiency test to detect adult worms of *Echinococcus* in intestinal mucosa of the definitive host

Results

Sample code	Date (dd/mm)	Positive	Negative	No. of recovered worms	Notes*
1					
2					
3					

*Specify if the whole worm, the anterior part (scolex) or single proglottids were counted

Technician (printed name):_____

Technician (signature):_____

Date _____

This form should be sent together with forms 1 and 2 by email (adriano.casulli@iss.it) or fax (+39 06 4990 3561) within 28th March, 2012.

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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
University of Veterinary and Pharm Sciences, Brno	Czech Rep
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, Vilnius	Lithuania
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 (Positive)	Sample 2 (Negative)	Sample 3 (Positive)
1E	Positive	Negative	Negative
3E	Positive	Negative	Positive
5E	Positive	Negative	Positive
6E	Positive	Negative	Positive
7E	Positive	Negative	Positive
9E	Positive	Negative	Positive
11E	Positive	Negative	Positive
12E	Positive	Negative	Positive
13E	Positive	Positive	Negative
14E	Positive	Negative	Positive
16E	Positive	Negative	Negative
18E	Positive	Negative	Positive
19E	Positive	Negative	Negative
20E	Positive	Negative	Positive
22E	Positive	Negative	Positive
23E	Positive	Negative	Positive
24E	Positive	Negative	Positive
25E	Positive	Negative	Positive
26E	Positive	Negative	Negative
29E	Positive	Negative	Positive
30E	Positive	Negative	Positive
31E	Positive	Negative	Positive
32E	Positive	Negative	Positive
33E	Positive	Negative	Positive
39E	Positive	Negative	Positive

Annex 5

Proficiency Test Results (Quantitative evaluation)

Lab code	Sample 1 (31 worms)		Sample 2 (negative)		Sample 3 (11 worms)	
	Evaluation	z-score	Evaluation	z-score	Evaluation	z-score
1E	Positive	-2,96	Positive	n.a.	Negative	-3,39
3E	Negative	-3,37	Positive	n.a.	Positive	-0,93
5E	Positive	-1,08	Positive	n.a.	Positive	-1,54
6E	Positive	-2,83	Positive	n.a.	Positive	-1,85
7E	Positive	-2,83	Positive	n.a.	Positive	-2,16
9E	Positive	-1,21	Positive	n.a.	Positive	-1,23
11E	Positive	-2,15	Positive	n.a.	Positive	-1,23
12E	Positive	-1,75	Positive	n.a.	Positive	-1,54
13E	Positive	-2,56	Negative	n.a.	Negative	-3,39
14E	Negative	-3,10	Positive	n.a.	Positive	-2,47
16E	Positive	-2,29	Positive	n.a.	Negative	-3,39
18E	Positive	-0,13	Positive	n.a.	Positive	-0,31
19E	Negative	-3,90	Positive	n.a.	Negative	-3,39
20E	Positive	-1,21	Positive	n.a.	Positive	-1,54
22E	Positive	-1,75	Positive	n.a.	Positive	-0,31
23E	Positive	-2,02	Positive	n.a.	Positive	-1,23
24E	Positive	-2,15	Positive	n.a.	Positive	-2,78
25E	Positive	-1,21	Positive	n.a.	Positive	-1,85
26E	Negative	-3,64	Positive	n.a.	Negative	-3,39
29E	Positive	-0,13	Positive	n.a.	Positive	-2,47
30E	Positive	-2,69	Positive	n.a.	Positive	-2,16
31E	Positive	-2,02	Positive	n.a.	Positive	-2,16
32E	Positive	-0,94	Positive	n.a.	Positive	-0,93
33E	Positive	-1,35	Positive	n.a.	Positive	-1,85
39E	Positive	-2,42	Positive	n.a.	Positive	-0,31

n.a. not applicable

Z-score evaluation: if the z-score is ≤ |3|, the laboratory result is positive; however, if the |2| < z-score ≤ |3|, the technician performing the test should be alerted; if the z-score > |3|, the laboratory result is unsatisfactory.