Reports on the Proficiency Tests for *Trichinella* spp. organised by the EURLP for the NRLs

March, 2013

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A Report of the NRL Proficiency Test to detect *Trichinella spiralis* larvae in pork and/or horse meat samples according to the EU directive 2075/2005

1 Introduction

Nematode worms of the genus *Trichinella* are zoonotic parasites circulating in most of the European countries in both wild and domestic animals (Pozio et al., 2009). Humans acquire the infection by the consumption of raw or undercooked meat from pigs, horses, wild boars and other game animals (Pozio et al., 2003). According to the Commission Regulation (EC) No 2075/2005, all animals which are potential carrier of *Trichinella* spp. infective larvae should be tested at the slaughterhouse according to one of the approved test.

2 Scope

One of the core duties of the EURL for Parasites is to organise proficiency tests, 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 comparison is to test the competence of the appointed NRLs to detect *Trichinella* larvae in pork and/or horse meat samples according to one of the approved methods reported in the Annex 1 of the Commission Regulation EC No 2075/2005.

3 Proficiency test organization

For the first time, the proficiency test (PT) registration, result submission, and reporting, have been organized online on a dedicated section of the EURLP web site (http://www.iss.it/crlp/test/index.php?lang=2&tipo=28).

4 Time frame

The PT was announced to NRLs by email on 15 February, 2013 and the dead line to make the online registration was 4 March, 2013. On March 18, 2013, the samples were dispatched to participants by an international courier. Reporting deadline was 28 March, 2013.

5 Test material

The test material forwarded to each laboratory consisted of 3 meat balls made with 100 ± 5 grams (or 35 ± 2 grams) of minced pork or 3 meat balls made with 100 ± 5 grams (or 35 ± 2 grams) of minced horse meat. Two meat balls contained in the core a known number of viable *Trichinella spiralis* larvae; whereas, one meat ball, which did not contain any larva, was the negative control. To evaluate the NRL competence and skill and the sensitivity of the digestion method in each participating laboratory, the number of larvae spiked into the meat balls was of two different sizes: 3 or 5.

Larvae were obtained by a partial artificial digestion of *T. spiralis*-infected mice (isolate code ISS003), according to the EURLP Guidelines. Larvae were counted under a stereo-microscope using a watch glass of 2 cm of diameter and transferred to the meatball rinsing the watch glass with PBS. To ensure that no larva remained on the glass, it was examined twice under the stereo-microscope and rinsed with PBS allowing the PBS to reach the core of the ball (see Annex 2).

Each meat ball was close in a plastic bag under vacuum, a code was added on the envelop and the same code with the number of larvae was reported in an Excel file. Each envelope containing the meat ball was then stored at +4°C until the forwarding.

The 3 meat balls under vacuum were forwarded in a polystyrene box containing ice boxes. In the package, ice boxes were separated from the meat samples by a cardboard separator to avoid a direct contact between meat samples and ice boxes. The packages were forwarded according to the international forwarding regulations by an international courier.

To check the sample stability over time, and to estimate the suitability of the packing and forwarding conditions under which the meat balls were sent, two groups of 3 meat balls each, were packaged as those that were forwarded, stored at room temperature, and tested at the EURLP three and five days after packaging.

Each participant lab was invited to add the information about package content and its condition of preservation in the online "shipment form".

6 Instructions to participants

The instructions were available to all the participants at the "instruction" page of the web site.

7 Qualitative evaluation

To successfully pass the PT each participant had to correctly identify the positives and the negatives samples.

8 Quantitative Evaluation

Because of the low number of samples it was impossible to correctly apply a Z-score or any other statistical parameter. The results were evaluated according to the following criteria:

- the detection of at least 2 larvae was considered acceptable for sample spiked with 4-5 larvae;
- the detection of at least 1 larva was considered acceptable for sample spiked with 1-3 larvae.

Overestimation:
- a maximum overestimation of 2 larvae was considered acceptable for sample spiked with 4-5 larvae;
- a maximum overestimation of 1 larva was considered acceptable for sample spiked with 1-3 larvae.

The difference between expected and observed results was reported to allow the lab to monitor their results overtime and to compare them with the performance of the other labs.

9 Statistical analysis

The analysis of data was performed using the STATA software; the statistical significance of the results was confirmed by the Kruskal-Wallis test.

10 Participating laboratories

Of the 27 MS, Luxembourg appointed the Belgium NRL for parasites, all the other NRLs (#26) participated at the PT. In addition, NRLs from Iceland, Macedonia, Norway, and Serbia, agreed to participate. Consequently, a total of 30 labs were involved.

11 Results

11.1 Delivery of the package to NRLs

Out of the 30 packages, 21 were delivered within 24 h, 6 within 48 h, one within 72 h, one within 96 h and one was the NRL of Italy which is located in the same Institute of the EURLP. At the delivery, the internal temperature of 29 packages was less than 10°C, whereas the internal temperature was higher in one package. The time elapsed from the arrival of the package at the NRL and the control of its content was within 1 h for 29 NRLs, and within 18 h for 1 NRLs; in this last case, the parcel was stored in a refrigerator at +4°C.

11.2 Digestion methods

The magnetic stirred method for the pooled sample digestion was the most used (26 NRLs, 86.2%); the mechanically assisted pooled sample digestion method/sedimentation technique was used in 2 NRLs (6.8%) and the automatic digestion method for pooled samples up to 35 g was used in 2 NRLs (6.8%).

11.3 Type of meat samples

Out of the 30 participating laboratories, 25 labs (83.3%) requested pork samples of 100 ± 5 g, 3 labs (10%) pork samples of 35 ± 2 g, and 2 labs (6.6%) horse meat samples of 100 ± 5 g.

11.4 Qualitative results

Out of the 30 participating laboratories, 26 labs (86.6%) passed the PT and 4 labs (13.3%) failed it because found false negatives (Table 2). One of the labs which failed the PT, detected also one false positive sample. The three samples which were stated as negative, had been spiked with 3 and 5 larvae (Table 2).

Table 2 - Number of larvae in the sample considered to be negative by NRLs

Laboratory code	15	16	18	42
No. of larvae in the samples considered negative	3	3 5	5	3

11.5 Quantitative results

The 26 labs that passed the PT obtained a positive evaluation for all the analysed samples; the 4 labs that failed the PT, obtained one or more negative evaluations. No participant overestimated the number of larvae spiked in the samples.

11.6 Descriptive statistic of the results

For each laboratory, the difference between the expected and observed number of larvae for each sample, and the mean value, calculated over the three samples, were evaluated (Annex 3). The graphical representation of the mean values obtained by each lab and the overall mean value calculated over all the laboratories is shown in the Annex 4. The mean values obtained by laboratories in the 2007-2013 period are shown in the Annex 5. All labs show an improvement in the digestion test from 2007 to 2008, while labs showed fluctuating results from 2008 to 2013. The comparison of the overall mean difference (i.e. the mean of relative difference values considering all samples and all laboratories) confirms that a strong improvement was made from the first (2007) to the second (2008) PT, while the improvement is less evident in the

further years (Table 1). In 2012, the overall mean difference value increased a little bit comparing to the previous years, but, in 2013, the overall mean difference value decreased.

Table 1 - Overall mean difference comparison for the 2007-2013 period

Year	2007	2008	2009	2010	2011	2012	2013
Relative difference values	0.41	0.25	0.23	0.22	0.20	0.26	0.21

12 Conclusions

The comparison of the 2007-2013 results shows that there has been an improvement in the laboratory performances from the first (2007) to the second (2008) PT, while during the following years the improvement was less evident. The absence of laboratories reporting a number of larvae greater than the number of larvae spiked into the sample witness an increased accuracy in the count but it can be also explained by the reduced number of samples and the reduced number of larvae in each sample. The fact that there are still laboratories reporting false negative results, means that there is still a need for improvement. The new approach used for the quantitative evaluation seems to be more suitable than the Z-score to evaluate the lab performance in PTs with small amount of samples (lower than 5).

13 References

Pozio E., Rinaldi L., Marucci G., Musella V., Galati F., Cringoli G., Boireau P., La Rosa G. (2009) Hosts and habitats of *Trichinella spiralis* and *Trichinella britovi* in Europe. Int. J. Parasitol. 39:71-79.

Pozio E., Gomez Morales M.A., Dupouy Camet J. (2003). Clinical aspects, diagnosis and treatment of trichinellosis. Expert Review of Anti-infective Therapy 1:471-482.

Commission Regulation (EC) No 2075/2005 of 5 December 2005 laying down specific rules on official controls for Trichinella in meat. OJ L338/60-82.

Commission Regulation (EC) No. 882/2004 of the European Parliament and of the Council of 29 April 2004 on official controls performed to ensure the verification of compliance with feed and food law, animal health and animal welfare rules. OJ L191/1-59.

Community Reference Laboratory for Parasites (2007). Guideline for the detection of *Trichinella* larvae at the slaughterhouse or connected laboratory in a Quality Assurance System, pp. 1-14, www.iss.it/crlp/docu/.

B Report of the NRL Proficiency Test on *Trichinella* spp. larva identification at the species level by a molecular method

1 Introduction

In the MS of the European Union, four species of the genus *Trichinella* are circulating among domestic and/or wild animals: *Trichinella spiralis*, *Trichinella nativa*, *Trichinella britovi* and *Trichinella pseudospiralis*. *Trichinella britovi* is the most widespread species infecting prevalently carnivore mammals but also wild boars; whereas, it is seldom detected in domestic pigs. This species is circulating in most of MS excluding Cyprus, Denmark, Ireland, Luxembourg, Malta and UK (Pozio et al., 2009). *Trichinella spiralis* infecting prevalently domestic pigs and wild boars is more scattered in single foci present in Austria, Bulgaria, Finland, Germany, Hungary, Lithuania, Poland, Romania, Ireland and North Ireland (UK), Slovak Rep., Spain and Sweden (Pozio et al., 2009). *Trichinella nativa* is circulating among carnivore mammals living in cold regions (Estonia, Finland, Latvia, Lithuania and Sweden) but recently it has been detected in one red fox of Poland and in three red foxes of Germany. *Trichinella pseudospiralis* is the only species infecting both mammals and birds (Pozio and Zarlenga, 2013). The main reservoir host is the wild boar; seldom this species has been detected in carnivore mammals (foxes and raccoon dogs). This species has been detected in 12 MS of the EU (Bulgaria, Czech Rep., Denmark, Estonia, Finland, France, Germany, Hungary, Italy, Netherlands, Slovak Rep. and Sweden). The identification of the *Trichinella* species is extremely important for the epidemiological investigation and to trace back the origin of infections in animals and humans. These parasites can be distinguished among them only by molecular methods (Pozio and La Rosa, 2010). Several protocols are today available in the literature, but only few of them allow to identify single organisms (www.iss.it/binary/crlp/cont/PCR_method_WEB_SITE.pdf).

2 Scope

According to of the Commission Regulation (EC) No 2075/2005 (Annex 1, Chapter I), when *Trichinella* spp. larvae are detected by digestion of animal muscles, the larvae should beare to be stored in 90% ethyl alcohol and identified at the species level at the EURLP or NRLs. During the NRL Workshop of 2010, participants requested the EURLP to organise a PT on the identification of *Trichinella* spp. larvae at the species level. The scope of this PT is to test the competence of NRLs to correctly identify the species circulating in Europe.

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 March 4, 2013. On March 18, 2013, the samples were dispatched to participants by an international courier. Reporting deadline was April 2, 2013.

4 Participating laboratories

Of the 27 MS, Luxembourg appointed the Belgium NRL for parasites, only 15 MS agreed to participate at the PT (Austria, Belgium, Bulgaria, Estonia, Finland, France, Germany, Greece, Italy, Latvia, Lithuania, Netherland, Poland, Slovak Rep., and Spain). In addition, NRL from Serbia agreed to participate (see Annex 1). A total of 16 NRLs participated at the PT.

5 Test material

The test material forwarded to each laboratory consisted of *Trichinella* muscle larvae in ethanol. According to the lab requests, two different type of samples were forwarded: pool of larvae or single larvae. The panel of pooled larvae consisted of four vials containing 10 larvae of *T. spiralis, T. nativa, T. britovi,* and *T. pseudospiralis.* The single larva panel consisted of 12 vials containing one single larva each (i.e., three vials for each of the four species). Larvae were obtained by artificial digestion of mouse carcasses infected with *T. spiralis* (isolate code ISS003), *T. nativa* (isolate code ISS70), *T. britovi* (isolate code ISS002), and *T. pseudospiralis* (isolate code ISS013), according to the EURLP Guidelines. Participating labs were invited to use one of the published methods available in the international literature. Each laboratory received, together with the samples, the appropriate form to register the results (Annex 6).

6 Results

6.1 Type of samples

Out of 16 participating laboratories, 10 labs (62.5%) requested a pool of larvae and 6 labs (37.5%) requested single larvae.

6.2 Methods of analysis

Out of the 16 labs which received the PT samples, only 15 (93.7%) sent the results. Of them, 9 labs followed the EURLP protocol, 3 labs followed the protocol published by Pozio and La Rosa (2010), 2 lab followed the protocol published by Zarlenga et al. (1999), and 1 lab did not communicate this information.

6.3 Molecular identification

6.3.1 PCR products

Out of 10 labs which analyzed pool of larvae, 9 were able to obtain PCR products and one failed because of technical problems encountered during the DNA extraction step. Out of the six labs which analyzed single larvae, two laboratories (code 7 and 22) failed to obtain PCR products from 3 and 7 samples, respectively.

6.3.2 Species identification

Independently of the type of sample (single larva or pool of larvae), three labs failed to correctly identify some larvae at the species level (Annex 8).

7 Conclusions

Out of 16 participants, 12 (75%) were able to correctly identify all the larvae at the species level; of them, 9 labs analyzed pools of larvae and 3 labs single larvae. The non-EU lab correctly identified all the samples. The failure to obtain PCR products and the mistakes in *Trichinella* spp. larva identification were mainly due to the use of not suitable diagnostic tools (reagents and apparatuses) and to the lack of an appropriate training.

8 References

De Bruyne A, Yera H, Le Guerhier F, Boireau P, Dupouy-Camet J, 2005. Simple species identification of *Trichinella* isolates by amplification and sequencing of the 5S ribosomal DNA intergenic spacer region. Vet Parasitol. 132, 57-61.

Pozio E, Hoberg E, La Rosa G, Zarlenga DS, 2009. Molecular taxonomy, phylogeny and biogeography of nematodes belonging to the *Trichinella* genus. Infect Genet Evol. 9, 606-616.

Pozio E, La Rosa G, 2010. *Trichinella*. In: Dongyou, L. (ed.), Molecular detection of foodborne pathogens. Taylor and Francis, CRC Press, Boca Raton, London, New York, pp. 851-863.

Pozio E, Zarlenga DS, 2013. New pieces of the *Trichinella* puzzle. Int J Parasitol (in press).

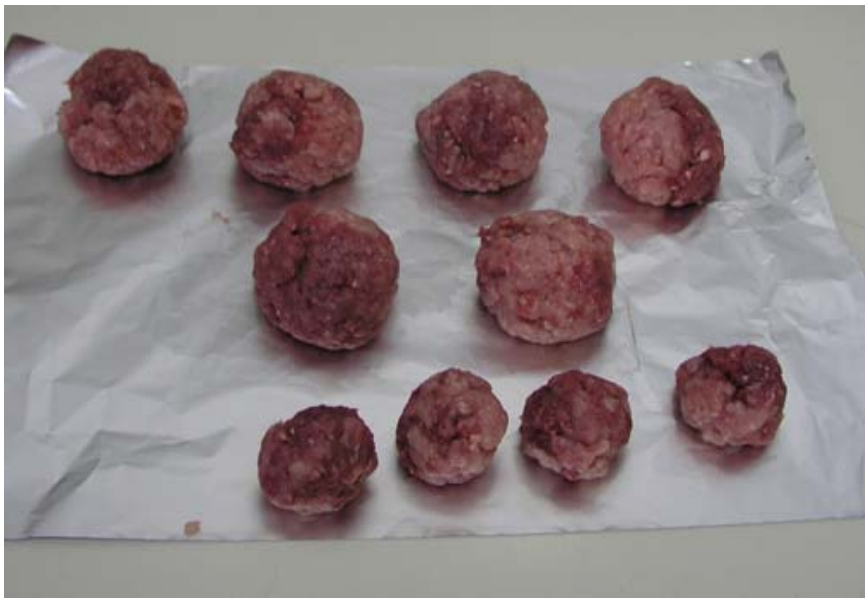
Zarlenga DS, Chute MB, Martin A, Kapel CM, 1999. A multiplex PCR for unequivocal differentiation of all encapsulated and non-encapsulated genotypes of *Trichinella*. Int J Parasitol. 29, 1859-1867.

Annex 1

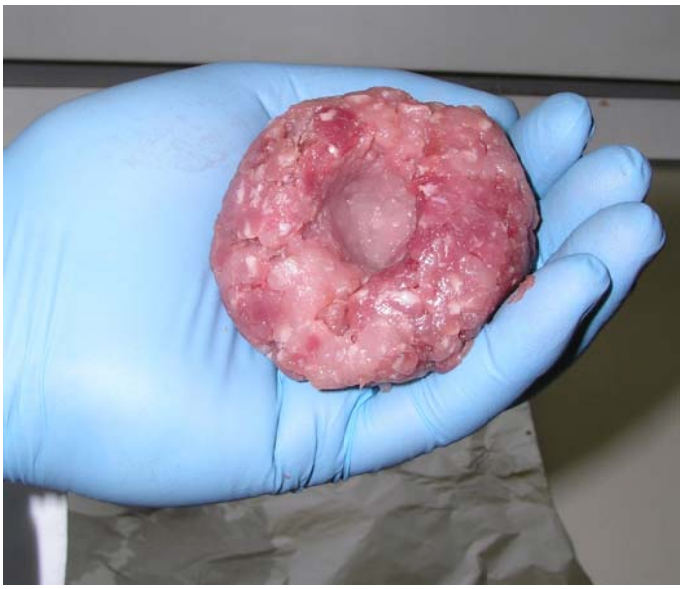
National Reference Laboratories (NRL) participating at the proficiency tests for *Trichinella*

NRL for parasites	Country	PT digestion	PT PCR
Institut für Veterinärmedizin, Innsbruck	Austria	yes	yes
Institute of Tropical Medicine, Antwerp	Belgium	yes	yes
National Diagnostic and Research Veterinary Institute, Sofia	Bulgaria	yes	yes
State Veterinary Laboratory, Nicosia	Cyprus	yes	no
State Veterinary Institute, Olomouc	Czech Rep	yes	no
National Veterinary Institute, Copenhagen	Denmark	yes	no
Estonian Veterinary and Food Laboratory, Tartu	Estonia	yes	yes
Research Unit Finnish Food Safety Authority, Evira, Oulu	Finland	yes	yes
Food borne parasite NRL & UMR BIPAR ANSES, ENVA, UPEC, ANSES-Laboratoire de Santé Animale, Maison Alfort	France	yes	yes
Federal Institute for Risk Assessment, BfR, Berlin	Germany	yes	yes
Centre of Athens Veterinary Institutions, Athens	Greece	yes	yes
Central Veterinary Institute, Budapest	Hungary	yes	no
Central Meat Control Laboratory, Celbridge, County Kildare,	Ireland	yes	no
Istituto Superiore di Sanità, Rome	Italy	yes	yes
Laboratory of Food and Environmental Investigations, Riga	Latvia	yes	yes
National Food and Veterinary Risk Assessment Institute, Vilnius	Lithuania	yes	yes
National Veterinary Laboratory, Albertown, Marsa	Malta	yes	no
National Institute of Public Health and the Environment, Bilthoven	Netherlands	yes	yes
National Veterinary Research Institute, Pulawy	Poland	yes	yes
Laboratório Nacional de Investigação Veterinária, Lisboa	Portugal	yes	no
Hygiene and Public Veterinary Health Institute, Bucharest	Romania	yes	no
State Veterinary and Food Institute, Bratislava	Slovak Rep.	yes	yes
National Veterinary Institute, Ljubljana	Slovenia	yes	no
Centro Nacional de Alimentación, Agencia Española de Seguridad Alimentaria y Nutrición, Majadahonda	Spain	yes	yes
Statens Veterinärmedicinska Anstalt, Uppsala	Sweden	yes	no
Veterinary Laboratories Agency, Weybridge	UK	yes	no
Keldur - Institute for Experimental Pathology, Reykjavík	Iceland	yes	no
Norwegian Veterinary institute, Oslo	Norway	yes	yes
Institute for Laboratory Diagnostic INEP, Belgrade	Serbia	yes	yes
Department of Parasitology, University of Skopje	Macedonia	yes	no

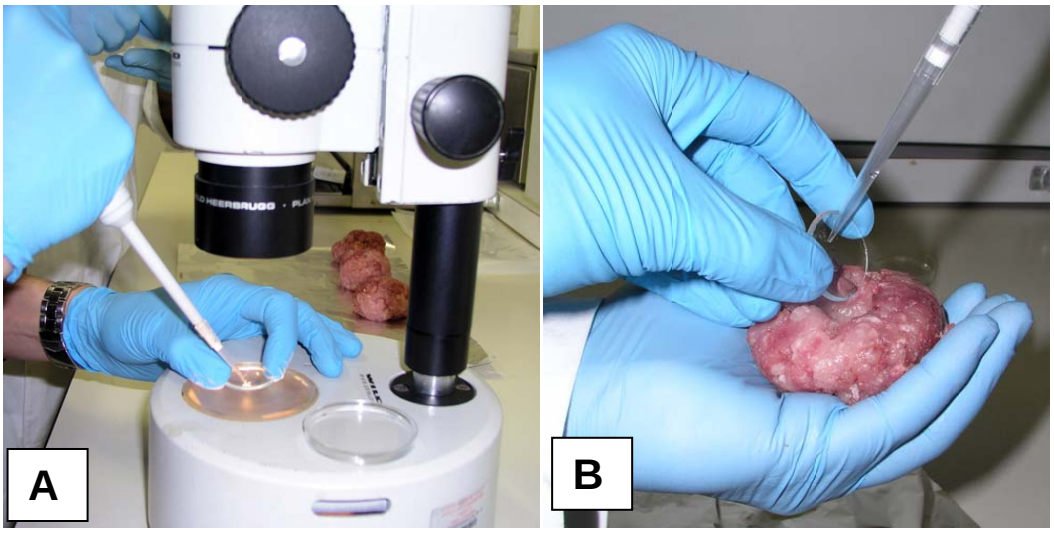
Annex 2



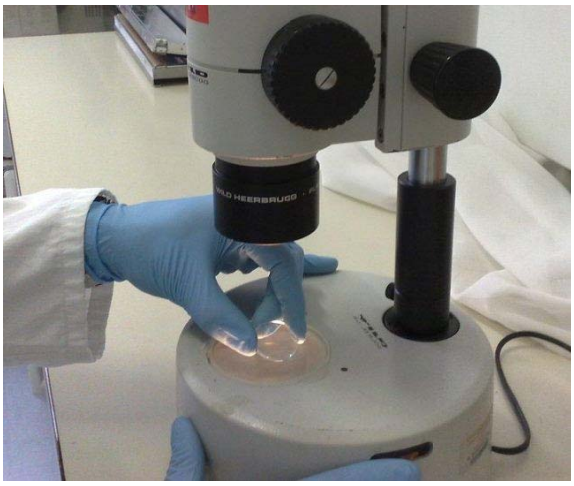
Meatball samples (35g and 100g)



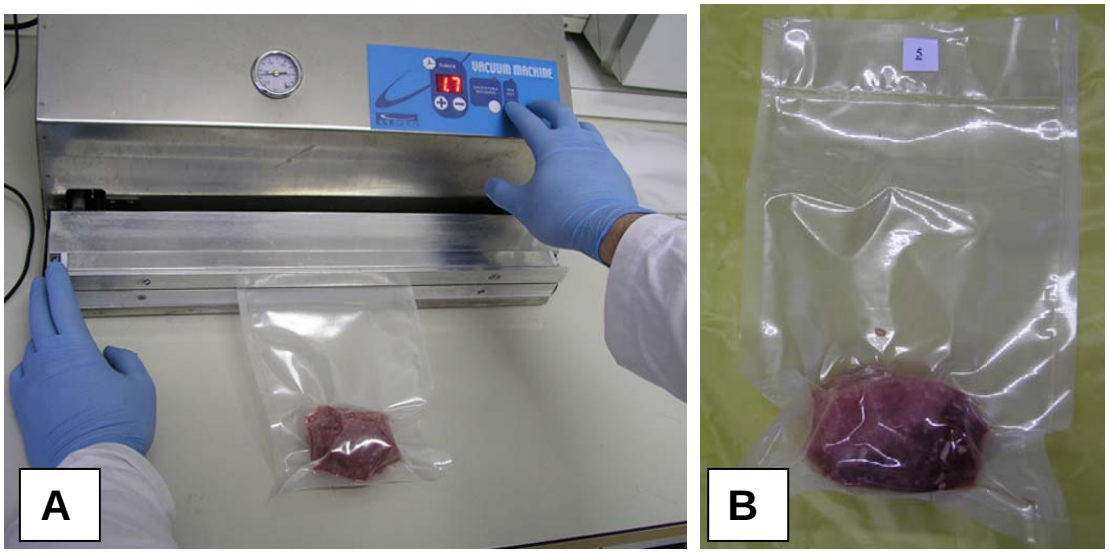
An hollow is made in the center of each meatball to house the larvae



Larvae are counted under a stereo-microscope using a watch glass (A) and transferred to the meatball rinsing the watch glass with 200 µl of PBS (B)



The watch glass is examined under the stereo-microscope to ensure that no larva remains on it



Each sample is under vacuum sealed (A) and labeled with a numeric code (B)

Annex 3

Descriptive statistics of the absolute difference between expected and reported count by laboratory for all samples (horse meat and pork) in 2013

Laboratory code	No. of samples	Mean	SD
NRL1	3	0.33	0.58
NRL2	3	0	0
NRL3	3	0.33	0.58
NRL4	3	0	0
NRL5	3	0.33	0.58
NRL6	3	0	0
NRL7	3	0	0
NRL8	3	0.66	1.15
NRL9	3	0.66	1.15
NRL10	3	0.33	0.58
NRL11	3	1	1
NRL12	3	0.33	0.58
NRL13	3	0.33	0.58
NRL14	3	1	1
NRL15	3	2	1.73
NRL16	3	2.6	2.52
NRL17	3	0	0
NRL18	3	2	2.64
NRL19	3	0.66	0.58
NRL20	3	0	0
NRL21	3	0.33	0.58
NRL22	3	0.33	0.58
NRL23	3	0.66	1.15
NRL24	3	0.33	0.58
NRL25	3	0.33	0.58
NRL26	3	0.66	1.15
NRL34	3	0	0
NRL40	3	0.33	0.58
NRL41	3	0.33	0.58
NRL42	3	1	1.73
All	90	0.56	1.04

Kruskal-Wallis test, p =0.9809

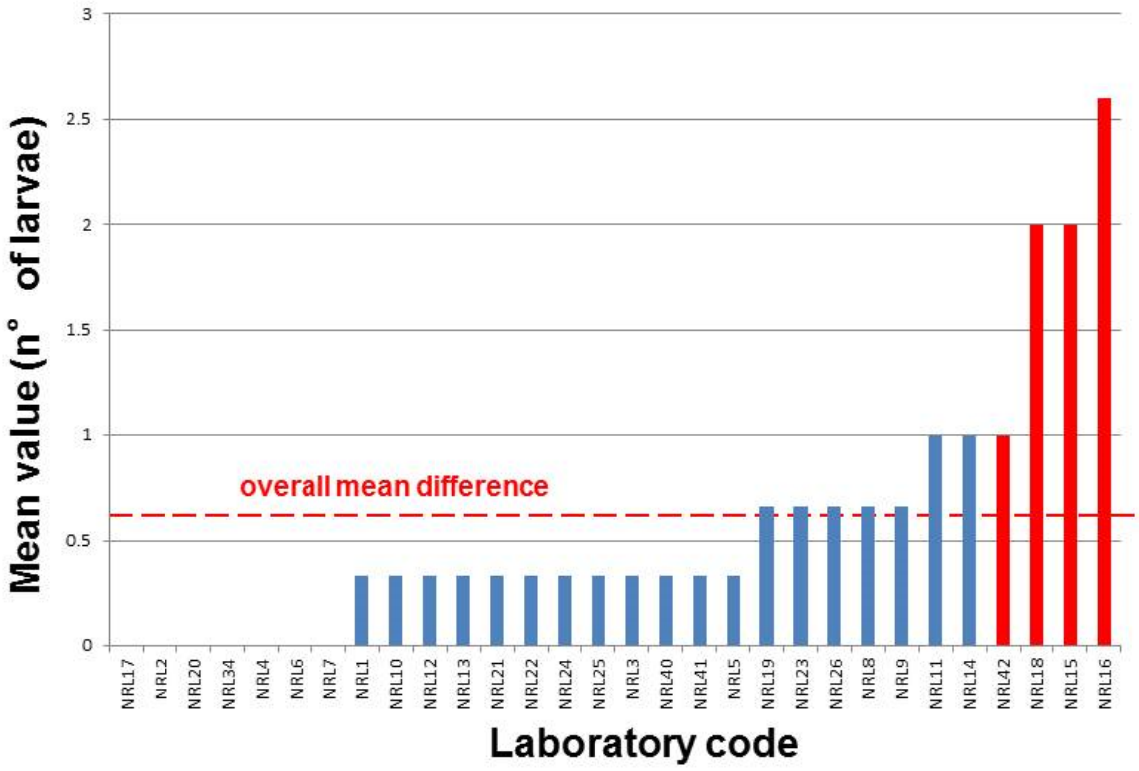
Annex 4

Descriptive statistic of the difference between the expected and reported larval count by laboratory and comparison among the years 2007-2013.

Laboratory code	Mean						
	2007	2008	2009	2010	2011	2012	2013
1	0.36	0.24	0.11	0.25	0.23	0.35	0.16
2	0.14	0.22	0.07	0.08	0.09	0.08	0
3	0.52	0.37	0.23	0.10	0.29	0.34	0.16
4	0.20	0.12	0.03	0.12	0.13	0.13	0
5	0.14	0.06	0.08	0.12	0.09	0.26	0.1
6	0.30	0.08	0.17	0.10	0.04	0.17	0
7	0.23	0.05	0.03	0.07	0.04	0	0
8	0.13	0.08	0.03	0.12	0.02	0.05	0.2
9	0.13	0.19	0.06	0.11	0.18	0.23	0.2
10	0.17	0.45	0.28	0.41	0.15	0.14	0.1
11	0.33	0.22	0.24	0.23	0.16	0.36	0.36
12	0.72	0.10	0.10	0.26	0.18	0.5	0.1
13	1	0.36	0.40	0.25	0.14	0.1	0.1
14	0.75	0.12	0.20	0.12	0.18	0.16	0.36
15	0.64	0.64	0.42	0.25	0.42	0.63	0.8
16	0.41	0.33	0.13	0.23	0.33	0.62	1
17	0.86	0.26	0.68	0.05	0.14	0.09	0
18	0.26	0.27	0.45	0.37	0.53	0.34	0.5
19	0.08	0.12	0.03	0.16	0.08	0.08	0.26
20	-	0.19	0.08	0.01	0.07	0.03	0
21	0.40	0.25	0.02	0.51	0.16	0.03	0.1
22	0.36	0.41	0.30	0.23	0.15	0.18	0.16
23	0.48	0.38	0.17	0.08	0.26	0.43	0.33
24	0.45	0.27	0.37	0.36	0.26	0.24	0.1
25	0.70	0.43	0.75	0.26	0.50	0.46	0.16
26	-	-	-	0.15	0.11	0.33	0.2
34	-	-	0.23	0.54	0.23	0.34	0
35	-	-	-	0.44	0.36	0.59	-
36	-	-	-	0.48	-	-	-
40	-	-	-	-	-	0.15	0.16
41	-	-	-	-	-	-	0.16
42	-	-	-	-	-	-	0.5

Annex 5

Graphical representation by histograms of the absolute difference between expected and reported count of all the samples (horse meat and pork), by laboratory



The four labs with the red histogram did not pass the PT

Annex 6

European Union Reference Laboratory for Parasites

Istituto Superiore di Sanità

Form 6A Laboratory _____

Proficiency test to identify *Trichinella* larvae at the species level by a molecular method

Identification of pool of larvae

Results

Sample code	Molecular Method[1]	Date (dd/mm)	Identified species	Notes

1) Explain briefly or add a reference for the molecular method used.

Date _____

Technician (name/sign): _____

viale Regina Elena, 299 – 00161 Rome, Italy

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

European Union Reference Laboratory for Parasites

Istituto Superiore di Sanità

Form 6B Laboratory _____

Proficiency test to identify *Trichinella* larvae at the species level by a molecular method

Identification of single larvae

Results

Sample code	Molecular Method[1]	Date (dd/mm)	Identified species	Notes

1) Explain briefly or add a reference for the molecular method used.

Date _____

Technician (name/sign): _____

viale Regina Elena, 299 – 00161 Rome, Italy

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Annex 7

PT on *Trichinella* spp. larva identification at the species level by a molecular method

Lab code	Sample type	Correct identification	Incorrect identification	Not identified	Molecular method
1	single larvae	10	-	-	EURLP protocol
3	pool of larvae	4	-	-	EURLP protocol
4	single larvae	10	-	-	EURLP protocol
6	pool of larvae	Technical problem during DNA purification			Not reported
7	single larvae	1	7	3	EURLP protocol
8	pool of larvae	4	-	-	Pozio and La Rosa 2003*
10	single larvae	9	3	-	Not reported
11	pool of larvae	4	-	-	EURLP protocol
12	single larvae	10	-	-	Pozio and La Rosa 2003
16	pool of larvae	4	-	-	Zarlenga at al. 1999*
21	pool of larvae	4	-	-	Pozio and La Rosa 2003
22	single larvae	4	1	7	EURLP protocol
23	pool of larvae	4	-	-	EURLP protocol
24	pool of larvae	4	-	-	EURLP protocol
25	pool of larvae	4	-	-	Zarlenga at al. 1999*
34	pool of larvae	4	-	-	EURLP protocol*

*some minor modifications were made to the original protocol