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

March-April 2015

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A Report of the NRL Proficiency Testing 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 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 and amendments, 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 testing, 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 and amendments.

3 Proficiency testing organization

The proficiency testing (PT) registration, result submission, and reporting, was organized online on a dedicated section (*restricted area*) of the EURLP web site (http://www.iss.it/crlp/index.php?lang=2&id=53&tipo=10).

4 Time frame

The PT was announced to NRLs by email on 11 February, 2015 and the deadline to make the online registration was February 20, 2015. On March 16, 2015, the samples were dispatched to participants by an international courier. Reporting deadline was April 10, 2015.

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. All meat balls were spiked with 3 viable *Trichinella spiralis* larvae each.

Larvae were obtained by a partial artificial digestion of *T. spiralis*-infected mice (isolate code ISS003), according to the EURLP POP-02. Experimental infection of mice by *Trichinella* spp. muscle stage larvae. 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 and under vacuum sealed, a code was added on the envelop. 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 packs. In the box, ice packs were separated from the meat samples by a cardboard separator to

avoid a direct contact. 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 forwarded, stored at room temperature, and tested at the EURLP three and five days after packaging.

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

6 Instructions to participants

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

7 Qualitative evaluation

To successfully pass the PT, each participant had to correctly identify all the samples.

8 Participating laboratories

Of the 28 MS, Luxembourg appointed the Belgium NRL for parasites, all the other NRLs (#27) participated in the PT. In addition, NRLs from Iceland, The former Yugoslav Republic of Macedonia, Norway, Serbia and Switzerland, agreed to participate. Consequently, a total of 32 labs were involved in the PT.

9 Results

11.1 Delivery of the package to NRLs

Out of 31 packages (Italian package not included), 22 were delivered within 24 h, 8 within 48 h, 1 within 72 h, and one was the NRL of Italy located in the same Institute of the EURLP. At delivery, the internal temperature of packages delivered within 48 h was less than 10 °C, whereas for packages delivered within 72 h the internal temperature was between 10 °C and 15 °C. The time elapsed from the arrival of the package at the NRL and the control of its content was within 3 h for all NRLs.

11.2 Digestion methods

The magnetic stirred method for the pooled sample digestion was the most used (28 NRLs, 87.5%); the mechanically assisted pooled sample digestion method/sedimentation technique was used in 1 NRLs (3.2%), and the automatic digestion method for pooled samples up to 35 g was used in 3 NRLs (9.3%).

11.3 Type of meat samples

Out of 32 participating laboratories, 27 labs (84.3%) requested pork samples of 100 ± 5 g, 2 labs (6.2%) pork samples of 35 ± 2 g, 2 labs (6.2%) horse meat samples of 100 ± 5 g, and 1 lab (3%) horse meat samples of 35 ± 2 g.

11.4 Results

Out of 32 participating laboratories, 28 (87.5%) passed the PT, while 4 labs (12.5%) failed because one false negative was detected (Annex 3).

11.5 Overtime comparison

In the 2007-2010 period, the percentage of labs that passed the PT raised from 83.3% to 96.5%, witnessing an improvement in the ability of labs to perform the artificial digestion test. In the 2011-2015 period, the percentage of labs that passed the PT fluctuated from 86.2% to 100% (Annex 4).

10 Conclusions

Four participant laboratories failed the PT due to one false negative result. The four labs were invited to investigate the causes of the failure and to implement the appropriate corrective actions. The shipment date of a new sample panel was arranged. The four participating labs were informed that the new panel was part of a corrective action. This corrective action was considered as an External Quality Assessment (EQA) scheme, therefore only an Individual Report was provided to each participant. All the four labs successfully passed the EQA.

11 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 Testing 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 boar; 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 boar 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 18 MS (excluding Belgium, Cyprus, Greece, Ireland, Latvia, Luxembourg, Malta, Portugal, Romania, and Slovenia). The identification of the *Trichinella* species is extremely important for 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 (Identification of Trichinella Muscle Stage Larvae at the species level by Multiplex PCR).

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 be stored in 90% ethyl alcohol and identified at the species level at the EURLP or NRLs. During the NRL Workshop of 2014, 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 and those which can be introduced from other continents.

3 Time frame

The proficiency testing (PT) was announced to NRLs by email on 11 February, 2015 and the dead line to send the participation form was February 20, 2015. On March 16, 2015, the samples were dispatched to participants by an international courier. Reporting deadline was April 10, 2015.

4 Participating laboratories

Of the 28 MS, Luxembourg appointed the Belgium NRL for parasites, 15 MS agreed to participate at the PT (Austria, Belgium, Estonia, Finland, France, Germany, Greece, Italy, Latvia, Lithuania, Netherland, Poland, Romania, Slovak Rep., and Spain). In addition, 2 labs from Serbia and 1 NRL from Switzerland, agreed to participate (see Annex 1). A total of 18 NRLs participated in 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 POP-02. Participating labs were invited to use one of the published methods available in the international literature. Each laboratory received, together with samples, the appropriate form to register the results (Annex 5).

6 Results

6.1 Type of samples

Out of 18 participating labs, 7 labs (39%) requested a pool of larvae and 11 labs (61%) requested single larvae.

6.2 Methods of analysis

Out of 18 labs which received PT samples, 11 labs followed the EURLP protocol, 3 labs followed the protocol published by Pozio and La Rosa (2010) (one of them made minor modification to the original protocol), 3 lab followed the protocol published by Zarlenga et al. (1999) (one of them made minor modification to the original protocol), and 1 lab followed both the protocol published by Liu et al. (1996) and the protocol of Rombout et al. (2001).

6.3 Molecular identification

6.3.1 PCR products

Two labs failed to obtain PCR products. One lab missed to amplify one sample because of a technical problem encountered during the protocol, the other lab, that participated for the first time, reported problems to purify DNA for all the 4 samples included in the PT panel.

6.3.2 Species identification

Independently of the type of sample (single larva or pool of larvae), all labs which obtained PCR product (16) correctly identified all the species included in the PT panel (Annex 6).

6.3.3 Overtime comparison

The comparison of results obtained in the 2011-2015 period show an increase of the percentage of laboratories, which passed the PT (Annex 7).

7 Conclusions

Out of 18 labs, 16 (89%) correctly identified all the larvae at the species level; of them, 8 labs analyzed pools of larvae and 8 labs single larvae. The reasons for the PT failure can be ascribed in one case to an accidental error, and in the second case to the lack of experience in manipulating the samples during the DNA purification step.

8 References

Liu, L.X., Blaxter, M.L., Shi, A., 1996. The 5S ribosomal RNA intergenic region of parasitic nematodes: variation in size and presence of L1 RNA. Mol. Biochem. Parasitol. 83, 235–239.

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 43, 983-997.

Rombout, Y.B., Bosch, S., Van Der Giessen, J.W.B., 2001. Detection and identification of eight Trichinella genotypes by reverse line blot hybridization. J. Clin. Microbiol. 39, 642–646.

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.

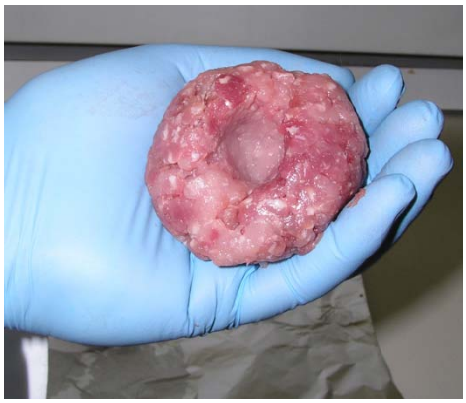
Annex 1

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

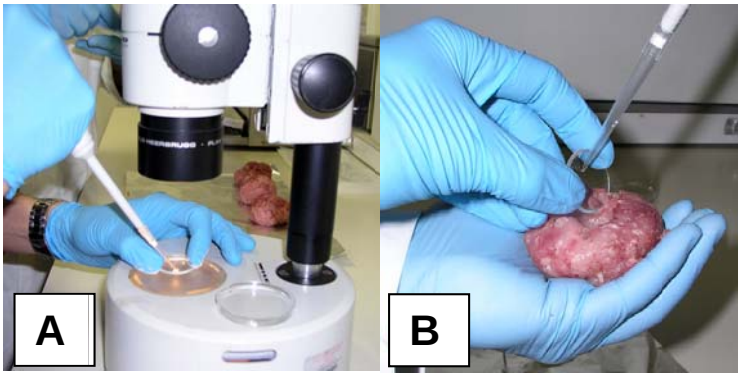
NRL for parasites	Country	PT digestion	PT PCR
Institut für Veterinärmedizin, Innsbruck	Austria	yes	yes
Institute of Tropical Medicine, Antwerp	Belgium	yes	yes
National Diagnostic and Research Veterinary Institute, Sofia	Bulgaria	yes	no
Croatian Veterinary Institute Vinkovci	Croatia	yes	no
State Veterinary 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, 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	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	yes
State Veterinary and Food Institute, Bratislava	Slovakia	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	no
Institute for Laboratory Diagnostic INEP, Belgrade	Serbia	yes	yes
Institute for Medical Research, University of Belgrade, Belgrade	Serbia	no	yes
Department of Parasitology, University of Skopje, Skopje	FYROM	yes	no
Institute of Parasitology, University of Bern, Bern	Switzerland	yes	yes

Annex 2

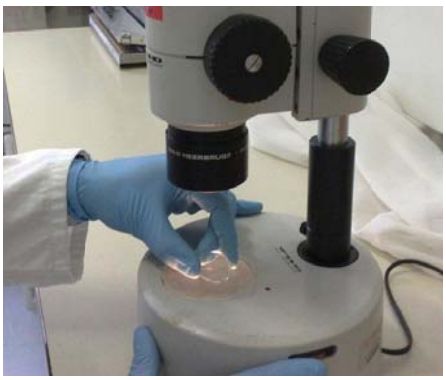
Meatball samples (35g and 100g)



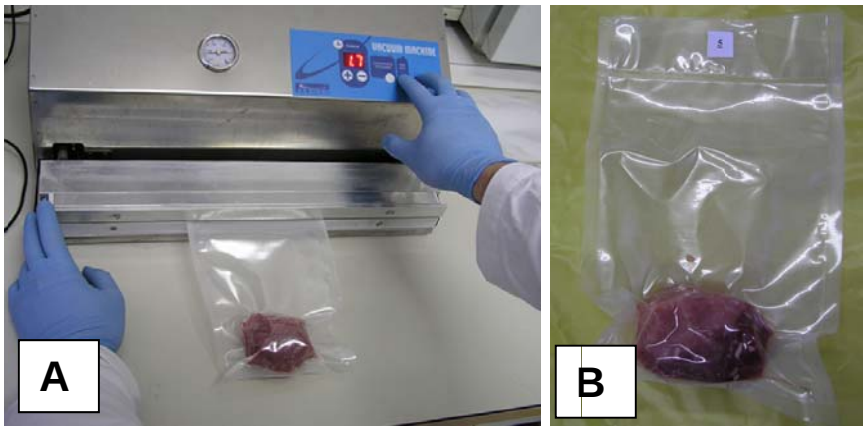
An hollow is made in the center of each meatball to house the larvae



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

PT to detect Trichinella spiralis larvae in pork and/or horse meat samples according to the EU Directive 2075/2005

2015 results

Lab code	False negatives	False positives	PT final evaluation
NRL1	0	-	positive
NRL2	0	-	positive
NRL3	0	-	positive
NRL4	0	-	positive
NRL5	0	-	positive
NRL6	0	-	positive
NRL7	0	-	positive
NRL8	0	-	positive
NRL9	0	-	positive
NRL10	0	-	positive
NRL11	0	-	positive
NRL12	0	-	positive
NRL13	0	-	positive
NRL14	0	-	positive
NRL15	0	-	positive
NRL16	0	-	positive
NRL17	0	-	Positive
NRL18	0	-	positive
NRL19	1	-	negative
NRL20	0	-	positive
NRL21	0	-	positive
NRL22	0	-	positive
NRL23	1	-	negative
NRL24	0	-	positive
NRL25	1	-	negative
NRL26	0	-	positive

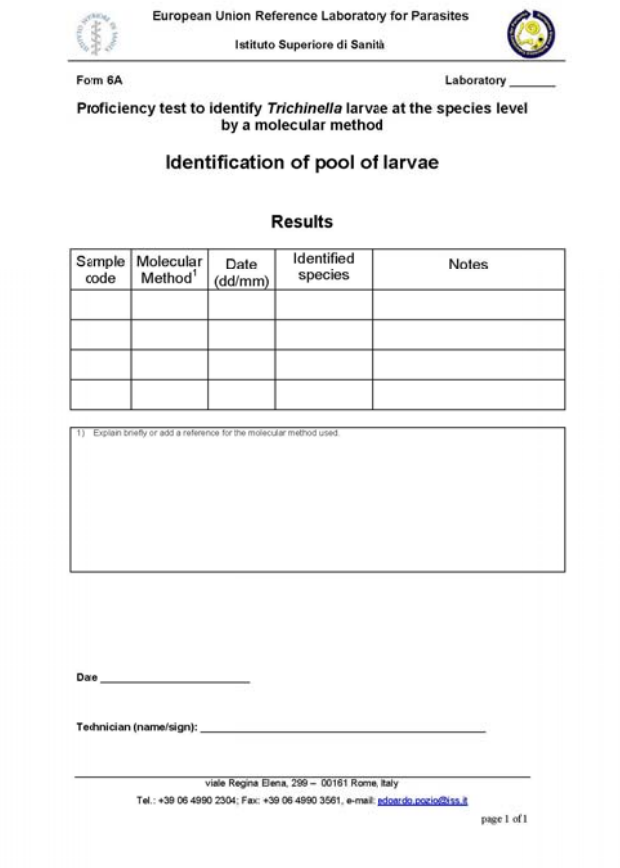
NRL34	0	-	positive
NRL35	1	-	negative
NRL40	0	-	positive
NRL41	0	-	positive
NRL42	0	-	positive
TLE6	0	-	positive

Annex 4

PT to detect Trichinella spiralis larvae in pork and/or horse meat samples according to the EU Directive 2075/2005

Overtime comparison of PT results

Annex 5



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

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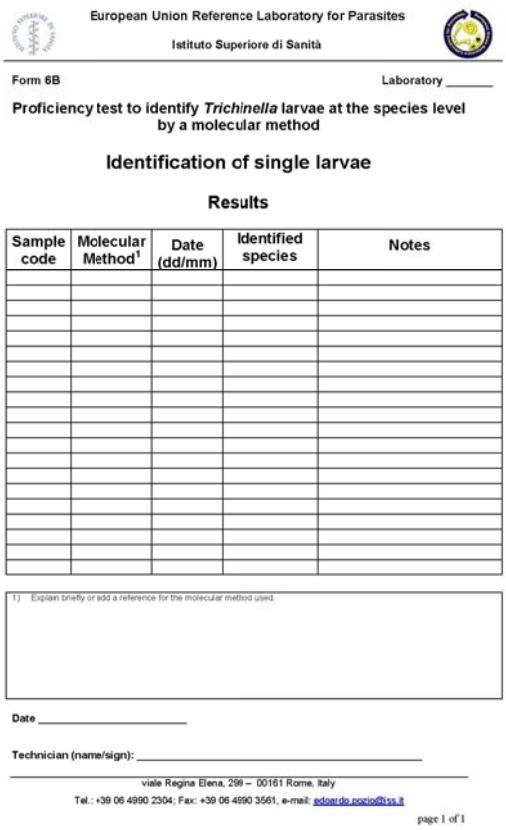

European Union Reference Laboratory for Parasites

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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): _____

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Annex 6

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

2015 results

Lab code	Sample type	Method used	N. correct identification	N. incorrect identification	N. missed identification	Final evaluation
NRL1	single	EURLP protocol	12	0	0	positive
NRL3	single	EURLP protocol	12	0	0	positive
NRL4	single	EURLP protocol	12	0	0	positive
NRL6	pool	Liu et al. 1996 and Raubout et al. 2001	3	0	1	negative
NRL7	single	EURLP protocol	12	0	0	positive
NRL8	pool	Pozio and La Rosa 2003	4	0	0	positive
NRL10	single	EURLP protocol	12	0	0	positive
NRL12	single	Pozio and La Rosa 2003, EURLP protocol	11	0	1	positive
NRL16	pool	Zarlenga et al. 1999	4	0	0	positive
NRL17	pool	EURLP protocol	4	0	0	positive
NRL21	pool	Pozio and La Rosa 2003	4	0	0	positive
NRL22	single	EURLP protocol	12	0	0	positive
NRL23	pool	EURLP protocol	4	0	0	positive
NRL24	single	EURLP protocol	12	0	0	positive
NRL25	pool	Zarlenga et al. 1999, EURLP protocol	4	0	0	positive
NRL34	pool	EURLP protocol	4	0	0	positive
NRL35	pool	Zarlenga et al. 1999	4	0	0	positive
NRL45	pool	EURLP protocol	4	0	4	negative

Annex 7

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

Overtime comparison of PT results