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

April, 2009

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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 and to count *Trichinella* larvae in pork samples according to one of the approved methods reported in the Annex 1 of the Commission Regulation EC No 2075/2005.

3 Time frame

The proficiency test (PT) was announced to NRLs by email on February 2nd, 2009 and the dead line to send the participation form was February 13th, 2009. On April 6th, 2009, the samples were dispatched to participants by an international courier. Reporting deadline was 24th April, 2009.

4 Test material

The test material forwarded to each laboratory, consisted of 10 meat balls made with 100 ± 5 grams of minced pork. Nine 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 added into the meat balls was of three different sizes: 1, 6 and 10 larvae.
Larvae were obtained by a partial artificial digestion of *T. spiralis*-infected mice (isolate code ISS03), according to the EURLP Guideline. 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.
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 envelop containing the meat ball was then stored at +4°C until the forwarding.
The 10 meat balls under vacuum were forwarded in a polystyrene box containing ice boxes. In the package, the 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. An envelop with the hard copies of the 5 forms (see Annex 1) and an accompanying letter was also included in the box.
To check the material stability during the time, and to estimate the suitability of the packing and forwarding conditions under which the meat balls were forwarded, two groups of 10 meat balls each, were stored in the package (and the package was stored at room temperature) as those that were forwarded, and tested at the EURLP three and five days after the forwarding.

The hard copies of the forms which should be filled in and send back to EURLP were:
1) general information on the proficiency test and its purpose (form 1, Annex 1);
2) laboratory description (form 2, Annex 1);

3) instruments, reagents and materials required for the detection of *Trichinella* larvae in pork according to the Commission Regulation No 2075/2005 (form 3, Annex 1);
4) check of the package content and its condition of preservation (form 4, Annex 1);
5) results of sample digestion (form 5, Annex 1);
6) code assigned to the laboratory.

5 Instructions to participants

Practical instructions were given to all the participants in the form 3 that accompanied the samples. A list of instruments as well as a list of chemicals and disposable material required to perform the digestion procedures, were also included. To make comparable the results obtained by the different laboratories involved in the proficiency tests, all the participants should follow the protocol step by step or, on the contrary, describe the variation.

6 Statistical Analysis

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

7 Participating laboratories

Of the 27 MS, Luxembourg appointed the Belgium NRL for parasites, the NRL of Malta was unable to perform the test even if they requested and received the samples and both the two NRLs for parasites of Romania participated at the proficiency tests, consequently 26 laboratories agreed to participate and sent the results.

Participating NRL for parasites	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
Lab. études et recherches en pathol animale et zoonoses, AFSSA, Maison Alfort	France
Federal Institute for Risk Assessment, BfR, Berlin	Germany
Centre of Athens Veterinary Institutions, Athens	Greece
Laboratories for Parasitology, Fish and Bee Diseases, Budapest	Hungary
Central Meat Control Laboratory, Celbridge, County Kildare,	Ireland
Istituto Superiore di Sanità, Rome	Italy
Laboratory of Food and Environmental Investigations (LFEI), Riga	Latvia
National Veterinary Laboratory, Vilnius	Lithuania
National Institute of Public Health and the Environment, RIVM, Bilthoven	Netherlands
National Veterinary Research Institute, Pulawy	Poland
Laboratório Nacional de Investigação Veterinária, Lisboa	Portugal
Hygiene and Public Veterinary Health Institute, Bucharest	Romania
Institute for diagnosis and animal health, Bucharest	Romania
State Veterinary and Food Institute, Bratislava	Slovak Rep.
National Veterinary Institute, Ljubljana	Slovenia
Centro Nacional de Alimentación, Agencia Española de Seguridad Alimentaria y Nutrición, Majadahonda	Spain
Statens Veterinärmedicinska Anstalt, Uppsala	Sweden
Veterinary Laboratories Agency, Weybridge	UK

8 Results

8.1 NRL description

Of the 26 NRLs which agreed to participate at the proficiency test, 22 (85%) are accredited according to ISO/IEC 17025:2005 and have validated and/or accredited the digestion test according to the Commission Regulation 2075/2005. The experience of the laboratories in the digestion test (considered as number of samples analyzed during the previous year) was very variable (from zero to several thousands).

8.2 Delivery of the package to NRLs

All the packages were delivered to NRLs within 24-72 hours. At the delivery, the internal temperature of all the packages was less than 10°C. The time elapsed from the arrival of the package at the NRL and its control was of 1 hour for 21 NRLs, of 3 hours for 2 NRLs, and of 4 hours for 3 NRLs.

8.3 Digestion methods

The magnetic stirred method for the pooled sample digestion was the most used (25 NRLs, 97%); the mechanically assisted pooled sample digestion method/sedimentation technique was used in one NRL only (3%).

8.4 Descriptive statistic of the results

The descriptive statistic of the difference between expected and reported counts by laboratory, shows a statistically significant variation among laboratories on the difference between expected and reported count according to the Kruskal-Wallis test (p=0.001) (Annex 2). The descriptive statistic of the difference between the expected and reported larval count by laboratory and the comparison between 2007, 2008 and 2009 results are shown in the Annex 3. The box-plots of the absolute difference between expected and reported count by laboratory is shown in the Annex 4. Fourteen (54%) laboratories show a constant trend toward improvement in the digestion test during the 2007-2009 period. Five (19%) laboratories show an improvement considering only 2008 vs 2009 results. The remaining 6 (23%) laboratories do not seem to have a particular trend, their results show a fluctuation in scores during the examined period. One laboratory participated for the first time at the proficiency test. The comparison of the overall mean difference (i.e. the mean of relative difference values considering all samples and all laboratories) shows a general improvement in the proficiency test results during the 2007-2009 period; the relative difference values were 0.40 for 2007; 0.22 for 2008; and 0.14 for 2009.

8.5 Not consistent results

NRLs did not detect any false positive sample; however, 8 laboratories of 26 (31%) found false negatives (Table 2). All samples stated negatives, contained only one larva.

Table 2. No. of larvae in the sample considered to be negative by NRLs

Laboratory code	11	14	15	18	22	24	25	34
No. of larvae in the samples considered negative	1	1	1 1	1	1	1 1	1 1 1	1

8.6 NRL experience

The NRL "experience" based on the number of sample analyzed in the previous year (2008), does not shows any influence on the score value of the NRL. No difference was observed between accredited and non-accredited laboratories.

9 Conclusions

More homogeneous results among NRLs were observed in the 2009 proficiency test, compared to the 2007 and 2008 proficiency tests. Fourteen laboratories (54%) of 26 performed better than in 2008. The presence of laboratories reporting a number of larvae greater then the real number of larvae spiked into the sample could suggest: 1) a problem to count the larvae present in the sediment; and 2) cross contamination from one digestion process to the next. No false positive has been found, whereas 8 (31%) laboratories found false negatives (last year the value was 37.5%). There is an urgent need for criteria to evaluate single laboratory results for the artificial digestion in the course of proficiency tests. However, according to the EC Regulation 2075/2005, technicians must be able to identify positive samples irrespective to the amount of larvae contained in the sample.

10 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/.

Annex 1

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Form 1 Laboratory code ____

Ring Trial on the Digestion Method to detect *Trichinella* Larvae in Pork Samples

1. **Purpose**: To test a panel of pork samples (positive and negative for *Trichinella spiralis* larvae) by one of the approved digestion method according to the EU directive 2075/2005, to assess and compare the sensitivity and reproducibility of these tests in different laboratories.
2. **Procedure**: The detailed protocols are described in separate sheets. The panel of pork samples includes both positive and negative samples. All packages have been shipped to all the participating laboratories on the same day. All participating laboratories should digest the samples within 3 days after delivery of the package and should provide the results (both as hard copy and electronic copy) <u>within the end of April, 2007.</u>

To test the samples we sent you, have you carefully followed one of the protocol reported in the Form 4, according to the Commission Regulation 2075/2005?

Yes ❑ NO ❑

If the answer is **NO**, please, write in detail on a separate sheet what has been changed. The changes, step by step, should be also written in the Form 3.

You have received both a hard copy (in the package) and an electronic copy of the same forms by email. You should fill in both and send us back by courier or priority mail as well as by email. The hard copy should be signed by you.

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Form 2 Laboratory code ____

Ring Trial on the Digestion Method to detect *Trichinella* Larvae in Pork Samples

Laboratory description

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

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

How many meat samples have you tested by digestion last year? ____________

- of pig origin ____________
- of horse origin ____________
- of wild boar origin ____________
- of other origin (specify) ____________

What kind of approved test (CR No 2075/2005) have you used for digestion?

Magnetic stirrer method for pooled sample digestion ❑
Mechanically assisted pooled sample digestion method/sedimentation technique ❑
Mechanically assisted pooled sample digestion method/on filter isolation technique ❑
Automatic digestion method for pooled samples of up to 35g ❑

Have you modify the test in your lab? Yes ❑ No ❑

If yes, please, specify in an attached sheet what has been modified.

Was the digestion test you are routinely used validated and accredited in your lab? Yes ❑ No ❑

____

How many scientists and technicians are working in the lab? Scientists ____ Technicians ____

How many scientists and technicians are working on the digestion? Scientists____ Technicians____

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

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Form 3 Laboratory code ____

Ring Trial on the Digestion Method to detect *Trichinella* Larvae in Pork Samples

Instruments, reagents and materials required for the detection of *Trichinella* larvae in pork according to the Commission Regulation No 2075/2005

1 Instruments

The following instruments are needed to perform one of the approved digestion method. To make comparable the results obtained by laboratories involved in the ring trial, we have to verify which instruments have been used. Thus you are requested to mark the column YES in case of use of the relevant instrument or, on the contrary, describe the variation in the following column.

1.1 <u>Magnetic stirrer method for pooled sample digestion</u>

DESCRIPTION	YES	VARIATION
A blender with a sharp chopping blade		
Magnetic stirrers with thermostatically controlled heating plate and teflon-coated stirring rods approximately 5 cm long		
Conical glass separation funnels, capacity of at least 2 litres		
Stands, rings and clamps		
Sieves, mesh size 180 microns, external diameter 11 cm, with stainless steel mesh		
Funnels, internal diameter not less than 12 cm, to support the sieves		
Glass beakers, capacity 3 litres		
Glass measuring cylinders, capacity 50 to 100 ml, or centrifuge tubes (specify)		
A trichinoscope with a horizontal table or a stereo-microscope, with a substage transmitted light source of adjustable intensity (specify)		
A balance accurate to at least 0.1 g		
Pipettes of different sizes (1, 10 and 25 ml) and pipette holders		
A thermometer accurate to 0,5 °C within the range 1 to 100 °C		
Siphon for tap water.		

1.2 <u>Mechanically assisted pooled sample digestion method/sedimentation technique</u>

DESCRIPTION	YES	VARIATION
A stomacher lab-blender 3 500 thermo model		
Conical separation funnels, capacity 2 litres		
Stands, rings and clamps		
Sieves, mesh size 180 microns, external diameter 11 cm, with stainless steel mesh		
Funnels, internal diameter not less than 12 cm, to support the sieves		
100 ml glass measuring cylinders		
A trichinoscope with a horizontal table or a stereo-microscope, with a substage transmitted light source of adjustable intensity (specify)		
A balance accurate to at least 0.1 g		
A vibrator, e.g. an electric shaver with the head removed		
A relay which will switch on and off at one-minute intervals		
A larval counting basin and a number of 9 cm diameter petri dishes		
Pipettes of different sizes (1, 10 and 25 ml) and pipette holders		
A thermometer accurate to 0,5 °C within the range 1 to 100 °C		

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1.3 <u>Mechanically assisted pooled sample digestion method/on filter isolation technique</u>
The same apparatuses as at 1.2 plus the following equipments

DESCRIPTION	YES	VARIATION
1 litre Gelman funnel, complete with filter holder (diameter 45 mm)		
an Erlenmeyer flask, capacity 3 litres, fitted with a side tube for suction		
a filter pump		

1.4 <u>Automatic digestion method for pooled samples of up to 35g</u>

DESCRIPTION	YES	VARIATION
A Trichomatic 35® blender with filtration insert		
A balance accurate to 0,1 g		
a thermometer accurate to 0,5 °C within the range 1 to 100 °C		
A (stereo-)microscope with transmitted light (magnification 15 to 60 times) or a trichinoscope with a horizontal table		

2 Reagents and materials

The following consumables (chemicals and disposable material) are needed to perform one of the approved digestion method. To make comparable the results obtained by laboratories involved in the ring trial, we have to verify which consumables have been used. Thus you are requested to mark the column YES in case of use of the specified chemicals and disposable materials or, on the contrary, describe the variation in the following column.

2.1 <u>Magnetic stirrer method for pooled sample digestion</u>

DESCRIPTION	YES	VARIATION
A number of 9 cm diameter petri dishes (for use with a stereo-microscope), marked on their undersides into 10 × 10 mm square examination areas using a pointed instrument or A larval counting basin (for use with a trichinoscope), made of 3 mm thick acrylic plates		
Aluminium foil		

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DESCRIPTION	YES	VARIATION
25 % hydrochloric acid		
Pepsin, strength: 1: 10 000 NF corresponding to 1: 12 500 BP and to 2 000 FIP		
Tap water heated to 46 to 48 °C		

2.2 Mechanically assisted pooled sample digestion method/sedimentation technique

DESCRIPTION	YES	VARIATION
Plastic bags suitable for the stomacher lab-blender		
A larval counting basin and a number of 9 cm diameter petri dishes		
17,5 % hydrochloric acid		
Pepsin, strength 1: 10 000 NF corresponding to 1: 12 500 BP and to 2 000 FIP		
A number of 10 litre bins to be used for decontamination of apparatus, e.g. with formol, and for digestive juice remaining where specimens test positive		

2.3 Mechanically assisted pooled sample digestion method/on filter isolation technique
The same materials as at 2.2 plus the following consumables

DESCRIPTION	YES	VARIATION
filter discs, consisting of a circular stainless steel mesh with an aperture of 35 microns (disc diameter: 45 mm)		
two rubber rings 1 mm thick (external diameter: 45 mm; internal diameter: 38 mm), the circular mesh being placed between the two rubber rings and bonded to them using a two component glue suitable for the two materials		
plastic bags, capacity at least 80 ml		
equipment for sealing the plastic bags		
rennilase, strength 1: 150 000 soxhlet units per gram		

2.4 Automatic digestion method for pooled samples of up to 35g

DESCRIPTION	YES	VARIATION
Hydrochloric acid 8,5 ± 0,5 % weight		
Transparent polycarbonate membrane filters with a diameter of 50 mm and a pore size of 14 microns		
Pepsin, strength 1: 10 000 NF corresponding to 1: 12 500 BP and to 2 000 FIP		

3 Procedures

3.1 Magnetic stirrer method for pooled sample digestion

Step	DESCRIPTION	YES	VARIATION
a	16 ± 0,5 ml of hydrochloric acid is added to a 3 litre beaker containing 2.0 litre of tap water, preheated to 46 to 48 °C; a stirring rod is placed in the beaker, the beaker is placed on the preheated plate and the stirring is started.		

Step	DESCRIPTION	YES	VARIATION
b	10 ± 0.2 g of pepsin is added		
c	100 g of samples is chopped in the blender for ....seconds		
d	The chopped meat is transferred to the 3 litre beaker containing the water, pepsin and hydrochloric acid		
e	The mincing insert of the blender is immersed repeatedly in the digestion fluid in the beaker and the blender bowl is rinsed with a small quantity of digestion fluid to remove any meat still adhering		
f	The beaker is covered with aluminium foil.		
g	The magnetic stirrer must be adjusted so that it maintains a constant temperature of 44 to 46°C throughout the operation. During stirring, the digestion fluid must rotate at a sufficiently high speed to create a deep whirl without splashing		
h	The digestion fluid is stirred until the meat particles disappear (approximately 30 min). The stirrer is then switched off and the digestion fluid is poured through the sieve into the sedimentation funnel		
i	The digestion process is considered satisfactory if not more than 5 % of the starting sample weight remains on the sieve		
j	The digestion fluid is allowed to stand in the funnel for 30 min		
k	After 30 minutes, a 40 ml sample of digestion fluid is quickly run off into the measuring cylinder or centrifuge tube		
l	The digestion fluids and other liquid waste are kept in a tray until reading of the results is completed		
m	The 40 ml sample is allowed to stand for 10 minutes. 30 ml of supernatant is then carefully withdrawn by suction to remove the upper layers and leave a volume of not more than 10 ml		
n	The remaining 10 ml sample of sediment is poured into a larval counting basin or petri dish		
o	The cylinder or centrifuge tube is rinsed with not more than 10 ml of tap water, which has to be added to the sample in the larval counting basin or petri dish. Subsequently, the sample is examined by trichinoscope or stereo-microscope at a 15 to 20 times magnification. Visualisation using other techniques is allowed. In all cases of suspect areas or parasite like shapes, higher magnifications of 60 to 100 times must be used		
p	Digests are to be examined within 30 min		
q	If the sediment is found to be unclear on examination, the sample is poured into a measuring cylinder and made up to 40 ml with tap water and then the above procedure is followed. The procedure can be repeated 2 to 4 times until the fluid is clear enough for a reliable reading		

3.2 Mechanically assisted pooled sample digestion method/sedimentation technique

Step	DESCRIPTION	YES	VARIATION
a	The stomacher lab-blender 3 500 is fitted with a double plastic bag and the temperature control set at 40 to 41°C		
b	One and a half litres of water preheated to 40 to 41 °C is poured into the inner plastic bag		
c	25 ml of 17,5 % hydrochloric acid is added to the water in the stomacher		
d	100 samples weighing approximately 1 g each (at 25 to 30 °C) taken from each individual sample in accordance with 2 are added		
e	Lastly, 6 g pepsin is added. This order must be followed strictly to avoid decomposition of the pepsin		
f	The stomacher is then allowed to pound the content of the bag for 25 minutes		
g	The plastic bag is removed from the stomacher and the digestion fluid is filtered through the sieve into a 3 litre beaker		
h	The plastic bag is washed with approximately 100 ml of water, which is then used to rinse the sieve and lastly added to the filtrate in the beaker		

Step	DESCRIPTION	YES	VARIATION
i	Ice (300 to 400 g of ice flakes, scaly ice or crushed ice) is added to the digestion fluid to bring its volume up to about 2 litres. The digestion fluid is then stirred until the ice has melted		
j	The chilled digestion fluid is transferred to a 2 litre separation funnel, equipped with a vibrator in an extra clamp		
k	Sedimentation is allowed to proceed for 30 min, during which time the sedimentation funnel is vibrated intermittently, i.e. one minute vibration followed by a one-minute pause		
l	After 30 minutes, a 60 ml sample of the sediment is quickly run off into a 100 ml measuring cylinder (the funnel is rinsed with detergent solution after use)		
m	The 60 ml sample is allowed to stand for at least 10 minutes, after which time the supernatant is withdrawn by suction to leave a volume of 15 ml, to be examined for presence of larvae		
n	For suction, a disposable syringe, equipped with a plastic tube, can be used. The length of the tube must be such that 15 ml remains in the measuring cylinder when the flanges of the syringe rest on the cylinder's rim		
o	The remaining 15 ml is poured into a larval counting basin or two petri dishes and examined using a trichinoscope or stereo-microscope		
p	The measuring cylinder is washed with 5 to 10 ml of tap water and the washings are added to the sample		
q	Digests are to be examined within 30 min		
r	If the sediment is found to be unclear on examination, the final sample of 60 ml is poured into a measuring cylinder and allowed to stand for 10 minutes; 45 ml of supernatant fluid is then removed by suction and the remaining 15 ml is made up to 45 ml with tap water, after a further settling period of 10 minutes, 30 ml of supernatant fluid is removed by suction and the remaining 15 ml is poured into a petri dish or larval counting basin for examination, the measuring cylinder is washed with 10 ml of tap water and these washings are added to the sample in the petri dish or the larval counting basin for examination		

3.3 Mechanically assisted pooled sample digestion method/on filter isolation technique

Step	DESCRIPTION	YES	VARIATION
a	The digestion procedure is the same above reported (see 3.2, from 'a' to 'h')		
b	Ice (300 to 400 g of ice flakes, scaly ice or crushed ice) is added to the digestion fluid to bring its volume up to about 2 litres		
c	The digestion fluid is stirred until the ice has melted. The chilled digestion fluid is then left for at least three minutes to let the larvae coil		
d	The Gelman funnel, fitted with a filter holder and filter disc, is mounted on an Erlenmeyer flask connected to a filter pump		
e	The digestion fluid is poured into the Gelman funnel and filtered. Towards the end of filtration, the digestion fluid can be helped to pass through the filter by applying suction with the filter pump. Suction must cease before the filter becomes dry, i.e. when 2 to 5 ml of fluid is left in the funnel		
f	Once all the digestion fluid has been filtered, the filter disc is removed and placed in an 80 ml capacity plastic bag, together with 15 to 20 ml of rennilase solution. The rennilase solution is made by adding 2 g of rennilase to 100 ml of tap water		
g	The plastic bag is sealed twice and placed between the inner and outer bags in the stomache		
h	The stomacher is allowed to pound for three minutes, e.g. while it is working on a complete or incomplete pool		
i	After three minutes, the plastic bag, complete with filter disc and rennilase solution, is removed from the stomacher and opened with scissors. The liquid contents are poured into a larval counting basin or petri dish. The bag is washed out with 5 to 10 ml of water, which is then added to the		

Step	DESCRIPTION	YES	VARIATION
	larval counting basin for examination by trichinoscope or to the petri dish for examination by stereo-microscope		
j	Digests must be examined as soon as they are ready. Under no circumstances is examination to be postponed until the following day		

3.4 Automatic digestion method for pooled samples of up to 35g

Step	DESCRIPTION	YES	VARIATION
a	Place the blender with the filtration insert, connect the waste tube and place the tube so it drains into the waste bin		
b	When the blender is switched on, heating will start		
c	Before this is done, the bottom valve located below the reaction chamber must be opened and closed		
d	Up to 35 samples weighing approximately 1 g each (at 25 to 30°C) taken from each individual sample in accordance with point 2 are then added		
e	Pour water up to the edge of a liquid chamber connected to the blender (approximately 400 ml)		
f	Pour about 30 ml hydrochloric acid (8,5 %) to the edge of the smaller, connected liquid chamber		
g	Place a membrane filter under the coarse filter in the filter holder in the filter insert		
h	Lastly, add 7 g of pepsin. This order must be followed strictly to avoid decomposition of the pepsin		
i	Close the lids of the reaction and liquid chambers		
j	Select the period of digestion		
k	When the start button on the blender is turned on, the process of dispensing and digestion starts automatically, followed by filtration After 10 to 13 minutes the process is completed and stops automatically		
l	Open the lid of the reaction chamber after checking that the chamber is empty. If there is foam or any digestion liquid remaining in the chamber, repeat the procedure		
m	Remove the filter holder and transfer the membrane filter to a slide or Petri dish.		
n	Examine the membrane filter using a (stereo-) microscope or a trichinoscope		

Notes

For any other aspect of the digestion methods, which was not detailed above, refers to the Annex I of the Commission Regulation No. 2075/2005.

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Form 4 Laboratory code ____

Ring Trial on the Digestion Method to detect *Trichinella* larvae in pork samples

Check of the package content and its condition of preservation

1. Ten meat ball samples under vacuum ❑
2. Hard copies of forms which should be filled in and send back to CRLP ❑

The content of the package has been forwarded refrigerated

- When did you receive the package? Date ________________hour____
- When did you open it? hour____
- Were the meat balls still refrigerated in the package (temperature <10°C)? **Yes** ❑ **No** ❑

The meat balls should be stored refrigerated at 4°C before use.

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Form 5 Laboratory code ____

Ring Trial on the Digestion Method to detect *Trichinella* Larvae in Pork Samples

Results of Sample Digestion

Sample code	Digestion Method[1]	Date (dd/mm/yy)	No. of recovered larvae	Notes

[1]) Magnetic stirrer method for pooled sample digestion. **A**; Mechanically assisted pooled sample digestion method/sedimentation technique. **B**; Mechanically assisted pooled sample digestion method/on filter isolation technique. **C**; Automatic digestion method for pooled samples of up to 35g. **D**, others, please, specify.

Technician (name/sign):___________________________

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Annex 2

Descriptive statistics of the absolute difference between expected and reported count, by laboratory in 2009

Laboratory	N	mean	sd	min	max
1	9	0.5	1.01	-1	2
2	9	0.4	0.72	0	2
3	9	2.2	2.44	0	6
4	9	0.2	0.44	0	1
5	9	0.5	1.33	0	4
6	9	0.5	1.23	-1	3
7	9	0.3	1.00	0	3
8	9	0.2	0.44	0	1
9	9	0.4	0.53	0	1
10	9	2.4	2.79	0	8
11	9	1.1	2.02	-1	5
12	9	0.6	1.73	-1	5
13	9	2.2	2.54	-1	6
14	9	0.7	0.83	0	2
15	9	2.2	2.90	0	7
16	9	0.8	0.93	0	2
17	9	-1.0	1.50	-3	2
18	9	2.1	2.67	-1	7
19	9	0.2	0.44	0	1
20	9	0.6	0.87	0	2
21	9	0.2	0.44	0	1
22	9	1.7	1.92	0	4
23	9	1.3	1.41	0	3
24	9	1.3	1.11	0	3
25	9	3.6	2.40	1	7
34	9	1.0	0.86	0	2
All	234	1.04	1.80	-3	8

There was a statistically significant variation among laboratories on the difference between expected and reported count (p=0.001, Kruskal-Wallis test).

ANNEX 3

Descriptive statistic of the difference between the expected and reported larval count by laboratory and comparison among 2007, 2008 and 2009.

Laboratory	Mean 2007	Mean 2008	Mean 2009
1	0.36	0.21	0.08
2	0.14	0.22	0.07
3	0.47	0.30	0.23
4	0.18	0.12	0.03
5	0.14	0.00	0.08
6	0.16	0.83	0.04
7	0.20	0.51	0.03
8	0.13	0.77	0.03
9	0.13	0.15	0.06
10	0.17	0.45	0.28
11	0.33	0.21	0.20
12	0.72	0.10	0.08
13	1	0.29	0.17
14	0.75	0.09	0.20
15	0.64	0.64	0.42
16	0.40	0.11	0.13
17	0.86	0.24	0.61
18	0.26	0.27	0.23
19	0.83	0.12	0.03
20	-	0.19	0.08
21	0.40	0.25	0.02
22	0.36	0.37	0.30
23	0.48	0.38	0.17
24	0.45	0.27	0.37
25	0.69	0.43	0.75
34	-	-	0.23

Annex 4

Box-plots of the absolute difference between expected and reported count by laboratory