Guideline for the detection of *Trichinella* larvae at the slaughterhouse or connected laboratory in a Quality Assurance System

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1. Introduction

In order to prevent human Trichinellosis due to consumption of infested meat, the European Commission adopted a regulation stating specific rules aimed at the detection of *Trichinella* in fresh meat (Commission Regulation No. 2075/2005. Specific rules on official controls for *Trichinella* in meat. Official Journal of the European Union 338:60-82.). Obligations of competent authorities and of food business operators involve sampling of carcases, training of personnel performing the test, methods of detection, inspection on *Trichinella*-free holdings, monitoring programmes and import health requirements. For the acronyms see ANNEX 1.

The pooled-sample digestion method for the detection of *Trichinella* larvae in meat is recommended for routine use as reference method, and is considered satisfactory if the limit of detection of 3 larvae per 100 grams of meat is achieved in the 75% of tests. Equivalent methods, such as mechanically assisted pooled sample digestion method/sedimentation technique and automatic digestion are also set out, together with trichinoscopic method which should be used only in exceptional circumstances. Validation of the digestion method on pork and horse meat was performed by an accredited laboratory of Canada (Forbes and Gajadhar, 1999), while similar or different methods employed for the same purpose were not, thus preventing comparison among them. It is desirable that each NRL carries out validation studies to asses the performance equivalency for different or modified methods. In this way, it will be possible to provide a scientifically valid rationale for reliable tests at the country level for the same purpose, such as meat or herd certification.

2. Scope and field of application

The aim of this document is to provide specific guidance to implement an appropriate *Trichinella* digestion method by a laboratory accredited according to the ISO/IEC 17025:2005 standard (hereafter referred as ISO 17025). A laboratory performing the digestion assay, or equivalent, in a QAS falls within the requirements for quality and competence provided by the international standard *EA-04/10:2002. Accreditation for microbiological laboratories.* This standard provides additional guidance for those laboratories performing microbiological testing (as stated in point 1.2) thus a "*Trichinella* laboratory" should follow this standard for those points fitting its area of competence, even though ISO 17025 remains the authoritative document.

To help to implement the indications provided in this document in a QAS system, the paragraphs of the afore mentioned standards related to the subjects concerned, are indicated. To prevent the exclusion of a number of laboratories which are working on these tests, if their requirements do not fit with those reported in the ISO 17025, the accreditation process should be introduced step by step in a reasonable period of time favouring at the same time the routine work and the improvement of the test sensibility, reproducibility, repeatability, reproducibility and robustness. The NRL should help the laboratories in these processes and a network among laboratories performing these tests should be established under the NRL control and supervision at the country level.

3. Personnel (ISO 17025, paragraph 5.2 – EA-4/10, paragraph 2)

3.1 Competence requirements.

3.1.1 technician: The pooled-sample digestion method for the detection of *Trichinella* larvae in meat should be performed by technicians with extensive relevant experience in this specific field. He/she should have basic knowledge of *Trichinella* parasites, and has to be familiar with the use of microscopy and the evaluation of *Trichinella* larvae morphology, to distinguish them from other nematode larvae or from undigested debris. The personnel should follows ad hoc courses at least every two years and should participate at proficiency tests at least one time per year.

3.1.2 Training. The adequate training for technicians should include all aspects of the method, including procedure, pre- and post-testing requirements, reporting, *Trichinella* biology and safety. Each training course should have theoretic and

practical lessons. The training should be provided by qualified persons (e.g. from the personnel of the NRL or from other skilful person under the supervision of the NRL), and the acquired competence should be demonstrated by successful testing of control samples (see ANNEX 2)

4. Environment (ISO 17025, paragraph 5.3 – EA-4/10, paragraph)

Additional provisions reported in EA-4/10, paragraph 3, are suitable for a "*Trichinella* laboratory", in terms of requirements of premises and hygiene.

5. Validation of test methods (ISO 17025, paragraph 5.4 – EA-4/10, paragraph 4)

Detection method/s used at the slaughterhouse have to be validated by confirmation, through the provision of objective evidence, that the requirements of the detection of larvae of *Trichinella* spp. in muscles have been fulfilled according to at least one of the methods recognised by the EU legislation 2075/2005.

To validate the detection method, the sensitivity, specificity, repeatability, intermediate repeatability, reproducibility and robustness of the test/s should be evaluated and documented.

- The sensitivity should be equal or higher than 3-5 larvae per 100 grams of pooled meat samples (1g x pig x 100 animals); for this purpose, spike samples containing a known number of larvae should be added to the meat samples before digestion and the number of recognised larvae should be evaluated after digestion (see Annex 4).

- The specificity should be evaluated by a trained technician capable to distinguish *Trichinella* larvae from other nematode larvae or from debris, which could be present in the sediment after digestion. For any question, the technicians may send the larvae or suspected larvae to the NRL for the identification.

- The repeatability should be evaluated at least 2 times with the same system (technician, apparatuses, working day, etc.).

- The intermediate repeatability should be evaluated comparing the results obtained by different technicians, in different days, with different muscle samples collected from pigs and/or horses and/or wild boars if these animal species are routinely tested in the slaughterhouse.

- The reproducibility should be evaluated by ring testing among a group of qualified laboratories, which have to test a panel consisting of a minimum of 10 samples including 1 negative sample, 3 samples containing 3–5 larvae, 3 samples containing 10–20 larvae and 3 samples containing 20–50 larvae.

- The robustness should be evaluated by varying in a controlled way the operative conditions in order to assess how much the system can afford procedure variations without affecting the result.

6. Equipment (ISO 17025, paragraph 5.5 – EA-4/10, paragraph 6)

6.1 The number of each type of apparatuses [those reported in the ANNEX 1 of the Commission Regulation No. 2075/2005] should be related to the size of the laboratory and the number of samples, which should be tested per day. At least one apparatus for each type should be available for an emergency situation. It is recommended that also this supply should be maintained in working order with periodical calibration.

6.2 Materials subject to wear and tear (e.g. knife, scissors, tweezers, blender, meat mincer, magnetic stirrer, sieves, glass containers) should be periodically changed and a stock should always be available.

6.3 The bottle containing hydrochloric acid (HCl) should be stored in an appropriate cabinet with filters or with an appropriate system to remove the fumes and it should be added to the digestion fluid under a chemical hood or with a close system which avoids the fume dispersion. The personnel working with HCl should be provided with protective clothing for face and hands.

6.4 Consumable materials (both disposables and chemicals) should be stored in appropriate cabinets and a good amount of stock should be available.

7. Reagents (ISO 17025, paragraph 4.6, 5.5 – EA-4/10, paragraph 7)

Since the pepsin is a critical material to perform the test, special attention should be paid to store it: the jar with the powder pepsin should be stored in a closed container in the dark, at a temperature between +4 and +15°C, in a dry environment for no more than two years, according to the European Pharmacopoeia. In this condition, the enzymatic activity can be considered to be stable for at least two years. Furthermore, since pepsin powder can be carcinogenic and allergenic for humans, it should be handled under a chemical hood or on a ventilated table and the personnel should wear a protective mask and gloves. A commercial liquid solution of pepsin can be used as an alternative, stored according to the manufacturers instructions. A pepsin solution cannot be prepared in the laboratory from the powder and then stored for later (days/weeks) use. The technicians should carefully follow the storage conditions according to the manufacturer. Each new batch of pepsin needs to be tested for its efficiency to digest the meat and to free the larvae from their collagen capsule.

8. Reference standard and reference material (ISO 17025, paragraph 5.6.3 – EA-4/10, paragraph 8.1)

Each laboratory should have access to the following reference materials through the NRL:

8.1 *Trichinella* infested fresh meat. Samples can originate from small laboratory animals (e.g. mice and rats), large laboratory animals (e.g. pigs) or from naturally infected animals (both domestic and sylvatic). These meat samples can be preserved at +4°C for 7-10 days. It is important to stress that the digestibility of muscle tissues varies from one animal to another. Meat from mice and rats has a higher digestibility than that of domestic pigs which, in their turn, is easier digestible than that from wildlife.

8.2 *Trichinella* larvae after digestion preserved in ethyl alcohol. At least larvae showing two shapes should be stored in ethyl alcohol or mounted on special microscopic slides:

- those with a coil shape
- those with a comma shape

8.3 Figures and pictures showing different shapes of *Trichinella* larvae

- in muscles
- after digestion

9. Meat sampling (ISO 17025, paragraph 5.7 – EA-4/10, paragraph 10)

9.1. Animal identification

Each slaughtered animal should be unequivocally identifiable as well as each piece of its carcass and the identification code should allow to trace back the origin of the animal up to its farm/s to allow epidemiological investigations, when a *Trichinella*-positive animal is

detected. In large slaughterhouses, a specific program such as an electronic database should allow tracing back of all slaughtered animals and their origin.

9.2. Trace back procedures to carcass of origin

When an animal is slaughtered, the carcass or its parts should be unequivocally identifiable with an unique code to allow the trace back procedures to carcass of origin if the test is positive. This is one of the most important critical points. The lack of a correct procedure to trace back the carcass of origin has been the cause of a large outbreak of trichinellosis in humans in the past.

9.3 Sample acceptance/rejection criteria

A sample can be accepted for analysis if:

- the amount of muscle sample of each animal is in agreement with that reported in the Annex 1 of the current Commission Regulation (EC) No 2075/2005;
- the meat sample is free of all fascia and fat since these tissues are indigestible and do not contain *Trichinella* larvae;
- each sample can clearly be distinguished from the other samples present on the tray and identifiable by a code which should allow to trace back the carcass of origin or its parts.

A batch of samples can be accepted for analysis if:

- the amount of the muscle sample of each animal is in agreement with that reported in the Annex 1 of the current Commission Regulation (EC) No 2075/2005;
- the meat sample is free of all fascia and fat since these tissues are indigestible and do not contain *Trichinella* larvae;
- if *Trichinella*-larvae are found new samples are to be collected from the corresponding batch of animals to be analysed for *Trichinella* in agreement with that reported in the Annex 1 of the current Commission Regulation (EC) No 2075/2005;

A sample has to be rejected if:

- the amount of muscle sample is lower than that requested by the current Commission Regulation (EC) No 2075/2005 ;
- the meat sample contains fascia and fat;
- the sample cannot be clearly distinguished from the other samples present on the tray and cannot be identified by a code.

9.4 Sample collection

Muscle samples should be collected from sites of predilection for the species being tested according to the Commission Regulation (EC) No 2075/2005. If *Trichinella* predilection sites are not known for the species to be tested, either tongue or diaphragm are recommended. Sample sizes should be selected to meet the sensitivity needs of the test; individual samples of 100 g may be taken from one animal, or multiple samples may be collected from a number of animals to make a pool of up to 100 g of tissue.

The sensitivity of testing has been reported as follows:

- a 1 g sample will detect infections 3 LPG of tissue;
- a 3 g sample will detect infections 1.5 LPG of tissue;
- a 5 g sample will detect infections 1 LPG of tissue.

Note

For public health purposes, testing a 1 g sample/pig (diaphragm or tongue) has been shown to be effective in reducing the incidence of human trichinellosis in several countries. However in a small slaughterhouse, where it is possible to know that meat is not intended for thorough cooking or other post-slaughter processing, testing of sample sizes sufficient to detect infection levels of 1 LPG of tissue (e.g. a minimum 5 g sample/pig) is recommended to increase the sensitivity.

9.5 Sample preparation

Samples should be trimmed free from all fat and fascia since these tissues are indigestible and do not contain *Trichinella* larvae. Samples are then blended or ground to facilitate digestion. Insufficient blending will result in poor digestion, while too much blending could disrupt larvae in the muscle. Blending should be continued just until no visible pieces of meat remain. Preparation of samples using a meat grinder is an acceptable method, provided the pore size of the grinding plate does not exceed 3mm in diameter.

10. Quality assurance of results/quality control of performance (ISO 17025, paragraph 5.9 – EA-4/10, paragraph 12)

10.1 Internal quality control

Periodic checks (every six months) on consistency of test results should be performed by the laboratory by replicate testing and the use of reference material.

10.2 External quality control

To demonstrate continued proficiency, certified laboratories should regularly participate in proficiency testing provided by a reference laboratory. Guidelines for the evaluation of proficiency sample results are based on expected performance of the method, supported by scientifically derived data.

Proficiency testing panels should consist of at least 10 samples, including 1 negative sample, 5 samples containing 3–5 larvae and 4 samples containing 10–20 larvae; however, another group of samples containing 20-50 larvae could be useful at least in the years before the laboratory will reach the accreditation, when the expected detection level could be very low. Acceptable results of testing are:

- a test sensitivity of 75% (with a 95% confidence level) for samples containing 3–5 larvae;

- recovery of a minimum of 75% of total larvae from samples containing 10–20 larvae.

ANNEX 1

List of abbreviations

QAS	Quality Assurance System
LPG	Larvae Per Gram
ISO	International Organization for Standardization
IEC	International Electrotechnical Commission
EA	European co-operation for Accreditation

ANNEX 2

Training and evaluation procedures of analysts performing the tests

Analysts from each laboratory receive a training course at the NRL or at another laboratory officially recognised by the NRL to be in a position to do the training. Subject areas include parasite biology, the assay, QAS, records, reporting and the relevance of the test, animal health and food safety purposes. Trainees must pass a written exam as well as a practical exam using proficiency samples.

Analysts must successfully test two proficiency panels, each containing three positive samples and one negative sample. The first panel is tested at the NRL or an equivalent laboratory as part of the training process, and the second panel is tested at the candidate laboratory. The positive samples contain known numbers of live *Trichinella* larvae and 75% of these larvae must be recovered from each sample. Samples are prepared using validated procedures (see ANNEX 5).

Follow-up proficiency testing to maintain accredited status is conducted yearly using samples provided by NRL or by an equivalent laboratory. These proficiency panels alternate between spiked meat samples as described in the ANNEX 5, and purified, excysted larvae in PBS, with identical assessment criteria.

Reference

Forbes LB, Scandrett WB, Gajadhar AA. 2005. A program to accredit laboratories for reliable testing of pork and horse meat for *Trichinella*. Vet. Parasitol. 132, 173-177.

ANNEX 3

Protocol to spike meat samples with *Trichinella spiralis* larvae

1. Materials

Laboratory mice or rats experimentally infected with *Trichinella spiralis* larvae at least since 2 months.

Digestion apparatuses and reagents for HCl-pepsin digestion according to the ANNEX I of the Commission Regulation No 2075/2005.

2. Procedure

2.1 Preparation of encysted larvae

The protocol is available in the paper of Vallee et al. (2007).

2.2 Preparation of calibrated samples

The Ts capsules should be examined in a Petri dish with a stereomicroscope at 40-50 magnification. To prepare the calibrated samples onto a counting agar matrix.

Briefly, the matrix is a disc (1.5 cm diameter, 3 mm thick) of 2% agar-agar in distilled water. Ts capsules should be harvested with a glass Pasteur pipette and put one by one on the agar-agar disc.

The number of Ts capsules deposited on each disc should be evaluated by at least three counting with the stereomicroscope at 40-50 magnifications. If a Ts capsule contains two or more larvae, it should be discarded.

When the established number of Ts capsules on the disc will be reached, the disc should be transferred in the meat matrix.

The meat matrix is made by two 10 g meatballs prepared by crushing fat-free pork in a mincer.

One meatball will be used as support of the agar-agar disc and another meatball will be used to cover the disc. Then, only one meatball of 20g will be formed containing the disc in its inner. These pork meatballs containing Ts capsules will be separately packaged under vacuum, identified with a code and stored at 4°C ± 2°C until their use.

Calibrated meat samples will be prepared by the addition of 80 g of fat-free pork (or horse meat) to each meatball containing the capsules.

These calibrated samples will be then packed again under vacuum and stored at 4°C ± 2°C until their use.

2.3 Sample forwarding

Calibrated samples should be sent by a courier in hermetical plastic bags heat-welded and preserved at 4°C ± 2°C until their arrival in the laboratories.

According to the legislation, the forwarding should be done with a level 2 infectious pathogens within a UN3373 packaging.

Samples should be delivered within 48 hours.

Samples should be tested within 24 hours after their arrival in the laboratory.

3. Reference

Forbes LB, Rajic A, Gajadhar AA (1998). Proficiency samples for quality assurance in *Trichinella* digestion tests. J Food Prot 61, 1396-99.

Vallée I, Macé P, Forbes L, Scandrett B, Durand B, Gajadhar A, Boireau P. (2007). Use of proficiency samples to assess diagnostic laboratories in France performing a *Trichinella* digestion assay. J. Food Prot. 70, 1685-1690.

ANNEX 4

Critical control points and recommendations

The following points are critical for the correct implementation of digestion method:

1. preparation of solution (combining HCl and water before the addition of pepsin);

2. incubation parameters (45±2°C for 30 min);

3. completion of procedure (lack of undigested muscle on sieve). The digestibility of muscle samples change according to the type of muscle, the animal species and its age. It is a crucial aspect for the detection of *Trichinella* larvae in muscles. The presence of undigested muscle fibres can prevent the detection of *Trichinella* larvae in the sediment, can clog the sieve and can prevent the release of larvae from the tissue strongly reducing the sensitivity of the method. In addition, muscle fibres can be confused with nematode larvae by insufficiently skilled technicians. If the digestion is not fine, this means that the procedure was not followed carefully When the established protocol reaches good results, the method should be validated. Then the procedure cannot be changed without a further validation process.

4. stability of apparatus (undisturbed settling of digest for 30 min); for the stomacher method, the recommendation has been to shake the sedimentation chamber/funnel at 1 minute interval to improve the settlement of the larvae towards the bottom of the funnel

5. working order of equipment (unobstructed flow from stopcock);

6. proper use of equipment (rapid release of stopcock); All apparatuses should be of good quality and for professional use, those which are on the market for the housework cannot be used. All requested apparatuses should be maintained in working order, cleaned after each working session and critical apparatuses (e.g. balances, thermostats, thermometers, pipettes) should be periodically calibrated by a certificate calibration service. The number of each type of apparatuses should be related to the size of the laboratory and the number of samples, which should be tested per day. At least one apparatus for each type should be available for an emergency situation. It is recommended that also this supply should be maintained in working order with periodical calibration. Materials subject to wear and tear (e.g. knife, scissors, tweezers, blender, meat mincer, magnetic sitter, sieves, glass containers) should be periodically changed and a stock should always be available.

7. the blender apparatus should be for professional use and should have a timer to establish the proper grinding time which is a critical point of the digestion procedure

8. remedial measure (further collection of sediment if first collection is not sufficient);

9. further remedial measures as necessary (concentration of large volumes of collected sediment);

10. time sensitive requirement (collected sediment allowed to settle undisturbed for at least 1 min prior to examination);

11. quality of output (clarity of sample sediment for examination). It is important to stress that the sedimentation time of live and dead larvae is quite different, consequently, when spike samples are used to check the sensitivity of the digestion test, only live larvae should be used, or only muscle tissues with infective larvae of experimentally infected laboratory animals. The use of infective samples should be carried out under strict surveillance of the head of the laboratory. All infected samples should be digested and

possible residues should be appropriately destroyed by heat (1 min at 60°C in the core of the sample) or freezing. After digestion, live larvae present in the sediment should also be destroyed. All apparatuses and materials which could had been in touch with the larvae or the infected muscle tissue should be carefully cleaned.

12. timely completion of test (reading must be performed within 30 min after the larva recovering, under no circumstances should examination be postponed until the following day);

13. adequate equipment (stereomicroscope with ≥10 X magnification and properly maintained);

14. re-processing of unsatisfactory output (clarification of an un-readable sediment);

15. complete transfer of sample (re-suspension of sediment and rinsing of container to ensure the complete transfer of larvae).

16. decontamination procedures. After the detection of positive samples or after a proficiency test, all apparatuses which were in contact with the infected meat should be washed with tap water and accurately rubbed because *Trichinella* larvae stick very easily on the wall of the apparatuses and the only sterilization procedure is does not remove the larvae. After washing, the apparatuses should be tested with meat samples known to be *Trichinella*-free to test the level of decontamination procedures.

ANNEX 5

References

Commission Regulation (EC) No. 2075/2005. Specific rules on official controls for *Trichinella* in meat. OJ L 338, 22.12.2005, p. 60.

EN ISO/IEC 17025:2005. General requirements for the competence of testing and calibration laboratories. May 2005, p. 28.

EA-04/10:2002. Accreditation for microbiological laboratories.. July 2002, rev. 0, p.26.

Forbes LB, Rajic A, Gajadhar AA. 1998. Proficiency samples for quality assurance in *Trichinella* digestion tests. J. Food Prot. 61, 1396-1399.

Forbes LB, Gajadhar AA. 1999. A validated *Trichinella* digestion assay and an associated sampling and quality assurance system for use in testing pork and horse meat. J. Food Prot. 62, 1308-1313.

Forbes LB, Scandrett WB, Gajadhar AA. 2005. A program to accredit laboratories for reliable testing of pork and horse meat for *Trichinella*. Vet. Parasitol. 132, 173-177.

Vallée I, Macé P, Forbes L, Scandrett B, Durand B, Gajadhar A, Boireau P. (2007). The use of proficiency samples to assess diagnostic laboratories in France performing a *Trichinella* digestion assay. J. Food Prot. (in press).

Web sites

International Organisation for Standardization. www.iso.org/iso/en/ISOOnline.frontpage

European Co-operation for Accreditation. www.european-accreditation.org/

International Laboratory Accreditation Cooperation. www.ilac.org/home.html

homepage-address for the live *Trichinella* larva movie: http://www.kobe-u.ac.jp/parasite/japanese/movie.html

International *Trichinella* Reference Center: http://www.iss.it/site/Trichinella/index.asp

The *Trichinella* page: http://www.trichinella.org/

International Commission on Trichinellosis: http://www.med.unipi.it/ict/welcome.htm