

Guidelines for the detection of *Trichinella* larvae at the slaughterhouse in a quality assurance system

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Summary. The European Community Regulation (EC) No. 2075/2005 lays down specific rules on official controls for the detection of *Trichinella* in fresh meat for human consumption, recommending the pooled-sample digestion method as the reference method. 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 international standard, and performing microbiological testing following the EA-04/10:2002 international guideline. Technical requirements for the correct implementation of the method, such as the personnel competence, specific equipments and reagents, validation of the method, reference materials, sampling, quality assurance of results and quality control of performance are provided, pointing out the critical control points for the correct implementation of the digestion method.

Key words: European Union, *Trichinella*, meat inspection, pooled-sample digestion method, quality control.

Riassunto (*Linea guida per la ricerca di larve di Trichinella al macello in un sistema di assicurazione di qualità*). Il Regolamento della Comunità Europea No. 2075/2005 definisce norme specifiche applicabili ai controlli ufficiali volti alla ricerca di larve di *Trichinella* nelle carni destinate al consumo umano, raccomandando come metodo diagnostico di riferimento la digestione artificiale di campioni aggregati. Scopo del presente documento è fornire una guida specifica che consenta ai laboratori accreditati secondo la norma internazionale ISO/IEC 17025:2005 di eseguire correttamente il metodo della digestione artificiale, seguendo altresì le indicazioni fornite dalla linea guida internazionale EA-04/10:2002, applicabile ai laboratori che eseguono analisi microbiologiche. Vengono forniti i requisiti tecnici necessari alla corretta esecuzione del metodo, le competenze del personale, la strumentazione e i reagenti, le modalità di validazione del metodo, il campionamento, l'assicurazione di qualità e il controllo della qualità delle prestazioni, sottolineando altresì i punti critici da tenere sotto controllo per una corretta applicazione del metodo.

Parole chiave: Unione Europea, *Trichinella*, controllo delle carni, metodo di digestione artificiale di campioni aggregati, controllo di qualità.

INTRODUCTION

In order to prevent human trichinellosis due to the consumption of infested meat, the European Community adopted a regulation stating specific rules aimed at the detection of *Trichinella* larvae in fresh meat of susceptible animals [1]. Obligations of competent authorities and of food business operators involve sampling of carcasses, training of personnel performing the test, methods of detection, inspection on *Trichinella*-free holdings, monitoring programmes, and import health requirements.

The pooled-sample digestion method for the detection of *Trichinella* larvae in meat of susceptible animals is recommended for routine use as reference method, and is considered satisfactory if the limit of

detection of one larva per gram of meat is achieved. Equivalent methods, such as the mechanically assisted pooled sample digestion method/sedimentation technique and the automatic digestion are also set out, together with the trichinoscopic method which should be used only in exceptional circumstances. The validation of the digestion method on pork and horse meat was performed by an accredited laboratory of Canada [2], while similar or different methods employed for the same purpose were not, thus preventing comparison among them. Confirmed performance equivalency data for different or modified methods, provide a scientifically valid rationale for a reliable test used in different countries for the same purpose, such as meat or herd certification.

The aim of this document is to provide specific guidance to implement an appropriate *Trichinella* digestion method by an accredited laboratory according to the ISO/IEC 17025:2005 international standard [3]. A laboratory performing the digestion assay, or equivalent methods, in a Quality Assurance System (QAS), uses techniques related to microbiology. The international guideline EA-04/10:2002 [4] provides an additional guidance on the interpretation of ISO/IEC 17025 for those laboratories performing microbiological testing, thus a “*Trichinella* laboratory” should apply this document for those points fitting its area of competence, even though ISO/IEC 17025 remains the authoritative document.

PERSONNEL

Competence requirements

Technician: The pooled-sample digestion method for the detection of *Trichinella* larvae in meat should be performed by skilful 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.

Supervisor or head of laboratory: a degree level in veterinary medicine or biological science and at least 5 years of professional experience, two of which in the specific field should be required for person in charge of supervising an accredited laboratory performing the detection of *Trichinella* larvae. He/she should have demonstrated knowledge of the epidemiology, biology and diagnosis of nematodes of the *Trichinella* genus, of legislative requirements, and specific parasitological techniques as well as basic statistical methods.

Training

Adequate training for non experienced analysts should include all aspects of the method, including procedure, pre- and post-testing requirements, reporting, *Trichinella* biology and safety. The training should be provided by qualified persons, and the acquired competence should be demonstrated by successful testing of control samples.

ENVIRONMENT

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

VALIDATION OF TEST METHODS

Detection method/s used at the slaughterhouse have to be validated through the provision of objective evidence that the requirements of the detection of larvae of *Trichinella* in muscles have been fulfilled

according to at least one of the methods recognised by the EU legislation [1]. To validate the detection method, the sensitivity, specificity, repeatability, intermediate repeatability, reproducibility, and robustness of the test/s should be evaluated:

- a) the sensitivity should be equal or higher than one larva per 100 grams of tested meat; for this purpose, spiked 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;
- b) 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;
- c) the repeatability should be evaluated at least two times with the same system (technician, apparatuses, working day, etc.);
- d) the intermediate repeatability should be evaluated comparing the results obtained by different technicians, in different days, with different muscle samples collected from different hosts (both domestic and sylvatic);
- e) 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 per gram (LPG) of tissue, 3 samples containing 6-10 LPG and 3 samples containing 11-20 LPG;
- f) 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.

EQUIPMENT

The number of each type of apparatus should be related to the size of the laboratory and the number of samples which should be tested per day.

- a) At least one apparatus for each type should be available for emergency situation. It is recommended that also this supply should be maintained in working order with periodical calibration.
- b) Materials subject to wear and tear (e.g. knife, scissors, tweezers, blender, meat chopper, magnetic stirrer, sieves, glass containers) should be periodically changed, and a stock should be always available.
- c) 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. The personnel working with HCl should be provided with protective masks and gloves.
- d) Consumable materials (both disposables and chemicals) should be stored in appropriate cabinets and a suitable stock should be available.

- e) 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 an accredited calibration service.

REAGENTS

Since the pepsin is a critical material to perform the test, special attention should be paid to store it: the jar with the powder or liquid pepsin should be stored in a closed container in the dark, at a temperature between 4 and 15°C, in a dry environment. In this condition, the enzymatic activity can be considered to be stable for at least one year. Furthermore, since pepsin powder can be allergenic for humans, it should be handled under a chemical hood and the personnel should wear a protective mask. A liquid solution of pepsin can be used as an alternative [5, 6].

REFERENCE STANDARD AND REFERENCE MATERIAL

Each laboratory should have easy access to the following reference materials:

- a) *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 its turn, is easier digestible than that from wildlife;
- b) *Trichinella* larvae after digestion preserved in ethyl alcohol. At least larvae showing two shapes should be stored in ethyl alcohol: 1. those with a coil shape; and 2. those with a comma shape;
- c) figures and pictures showing different shapes of *Trichinella* larvae in muscles after digestion, should be available at the laboratory.

MEAT SAMPLING

- a) *Animal identification.*

Each slaughtered animal should be unequivocally identifiable as well as each piece of its carcass and the identification code should allow tracing back the origin of the animal up to its farm 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.

- b) *Trace back procedures to carcass of origin.*

When an animal is slaughtered, the carcass or its parts should be unequivocally identifiable with a unique code to allow the trace back pro-

cedures 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.

- c) *Sample acceptance/rejection criteria.*

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) [1];
- 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.

Sample have to be rejected if:

- the amount of muscle sample is lower than that requested by the current Commission Regulation (EC) [1];
- 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.

- d) *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 [1]. If *Trichinella* predilection sites are not known for the species to be tested, 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:

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

For public health purposes, testing 1 g sample of pig tissue (diaphragm or tongue) has been shown to be effective in reducing the incidence of human trichinellosis in many countries. However, where 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 of 5 g sample) is recommended.

- e) *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 ground, and blended to facilitate digestion. Insufficient blending will result in poor digestion,

while too much blending could disrupt larvae in the muscle. Blending should be continued 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.

QUALITY ASSURANCE OF RESULTS/ QUALITY CONTROL OF PERFORMANCE

It is important to demonstrate that the assay is under control because the methods do not have internal biological controls. Consequently, to ensure that the assay system remains reliable, it should be used: 1. a precisely defined protocol; 2. the technical training and certification; 3. documentation and audits; and 4. proficiency sample programs.

a) *Internal quality control.*

Periodic checks on the consistency of test results should be performed by replicate testing and the use of reference material.

b) *External quality control.*

To demonstrate continued proficiency, accredited 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 a minimum of 10 samples, including one negative sample, three samples containing 3-5 larvae per gram of tissue (LPG), three samples containing 6-10 LPG, and three samples containing 11-20 LPG. Acceptable results of testing are:

- a test sensitivity of 90% (with a 95% confidence level) for samples containing 3-5 LPG;
- recovery of a minimum of 75% of total larvae from samples containing 6-10 and 11-20 LPG.

CRITICAL CONTROL POINTS

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

- a) preparation of the solution: combining HCl and water before the addition of pepsin;
- b) sample weighing: the method is designed to test 100 g but it allows up to 115 g to be digested. Trimming and weighing is labour intensive and time consuming; do not try to test 115 samples of exactly 1 g each; the 15 g buffer should be used to allow rapid trimming of samples to a weight of slightly > 1 g;
- c) chopping samples in the blender: the addition of the digest fluid to the blender improves speed and effectiveness of blending;
- d) rinsing blender into digestion beaker: chopped tissues are thoroughly rinsed out of the blender using the digestion fluid;
- e) digestion: during digestion, the spin bar should create a deep whorl on the surface of the digest mixture, and an aluminum foil should be used

to cover the beaker to prevent cross-contamination from accidental splashes;

- f) incubation parameters: 45 ± 2 °C for a time between 30 and 60 min which should be established according to the type of muscle, the animal species and age (see "h" below);
- g) filtering the digestion mixture: ensure that the stopcock of the separatory funnel is closed before filtering;
- h) completion of the procedure (less than 5% of digested muscle on the sieve): the digestibility of muscle samples changes according to the type of muscle, the animal species and the animal age. This 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, clogging the sieve and preventing 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. Therefore, in order to digest the muscle tissue completely, and to avoid the presence of muscle fibres after digestion, the composition of the digestion fluid, the time and the temperature of digestion should vary. Once the established protocol achieves the expected result, the method should be validated;
- i) examination of the sediment: the bottom outside of the reading plate should be etched with grid-lines; this provides reference points to ensure the entire plate is examined; if the fluid in the reading plate is too cloudy, it must be clarified to be properly examined;
- j) microscopic examination: the stereomicroscope must be of high quality; poor quality microscopes usually have a small field of view and poor peripheral focus with the consequence of higher scan time, increased chance of missing larvae, and increased operator fatigue; grid lines should be used to ensure no part of plate is missed; a correct focal plane for reading plates should be ensured;
- k) stability of apparatus: undisturbed settling of the digest for 30 min;
- l) proper use of equipment: materials subject to wear and tear (e.g., knife, scissors, tweezers, blender, meat mincer, magnetic bar, sieves, glass containers) should be periodically changed and a stock should be always available;
- m) remedial measures: a further collection of sediment should be carried out if the first collection is not sufficient;
- n) time sensitive requirement: collected sediment should be allowed to settle undisturbed for at least one min prior to examination;
- o) 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 spiked samples are used to check the sensitivity of the digestion test, only alive larvae should be used, or only muscle tis-

sues with infective larvae from experimentally infected laboratory animals. The use of infective samples should be carried out under strict surveillance by the head of the laboratory. All infected samples should be digested and 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 have been contaminated by the larvae or the infected muscle tissue should be carefully cleaned;

- p) timely completion of test: same day examination of sediment;
- q) adequate equipment: stereomicroscope with $\geq 10\times$ magnification properly maintained;
- r) re-processing of unsatisfactory output: clarification of unreadable sediment by re-suspension of

the sediment and rinsing of container to ensure the complete transfer of larvae;

- s) laboratory worksheet: besides basic requirements of a laboratory worksheet (*i.e.*, sample identification, date of testing, identification of persons testing, reporting and authorizing, etc.), written evidence that critical control points are under control, should be provided.

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