Veterinary Parasitology 159 (2009) 197–205

Contents lists available at ScienceDirect

Veterinary Parasitology

journal homepage: www.elsevier.com/locate/vetpar

Trichinella diagnostics and control: Mandatory and best practices for ensuring food safety

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ARTICLE INFO

Keywords:
Trichinella
Trichinellosis
Diagnosis
Detection methods
Quality assurance
Control
Biology
Epidemiology
Reference laboratories

ABSTRACT

Because of its role in human disease, there are increasing global requirements for reliable diagnostic and control methods for *Trichinella* in food animals to ensure meat safety and to facilitate trade. Consequently, there is a need for standardization of methods, programs, and best practices used in the control of *Trichinella* and trichinellosis. This review article describes the biology and epidemiology of *Trichinella*, and describes recommended test methods as well as modified and optimized procedures that are used in meat inspection programs. The use of ELISA for monitoring animals for infection in various porcine and equine pre- and post-slaughter programs, including farm or herd certification programs is also discussed. A brief review of the effectiveness of meat processing methods, such as freezing, cooking and preserving is provided. The importance of proper quality assurance and its application in all aspects of a *Trichinella* diagnostic system is emphasized. It includes the use of international quality standards, test validation and standardization, critical control points, laboratory accreditation, certification of analysts and proficiency testing. Also described, are the roles and locations of international and regional reference laboratories for trichinellosis where expert advice and support on research and diagnostics are available.

1. Introduction

Effective *Trichinella* diagnosis and control are essential for ensuring food safety, and facilitating international trade and regulations. Quality assurance is an underlying theme of effective diagnostic and control programs, and requires the use of reliable methods. This paper contains information on the biology of the various *Trichinella* species and genotypes, and internationally recognized diagnostic methods and control programs. The information provided herein is based on a series of expert presentations given at a workshop on *Trichinella* Diagnostics and Control organized in conjunction with the 12th Conference of the International Commission on Trichinellosis (ICT) which was held in September, 2007 in Croatia. Information contained in this paper is consistent with established ICT recommendations, World Organization for Animal Health (OIE) guidelines or best practices in jurisdictions such as

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0304-4017/$ – see front matter.
doi:10.1016/j.vetpar.2008.10.063

the European Union (EU). Further details of the methods and programs discussed in this paper may be obtained from the references provided.

2. Biology and epidemiology

Nematode worms of the genus *Trichinella* are peculiar parasites characterized by a direct life cycle, no exogenous stage, two generations in the same host, and a broad host spectrum involving mammals, birds and reptiles (Pozio, 2007a). The infective stage is a first stage larva (L1) parasitizing the cells of striated muscles, where it can survive for years. When the larva penetrates a muscle cell, the host cell changes its structure to create a nurse cell which, depending on the species of *Trichinella*, may or may not develop a thick collagen capsule (encapsulation). Currently, two main clades are recognized within the genus *Trichinella*: one that encompasses species that encapsulate in host muscle tissue, and a second that includes species that do not encapsulate following muscle cell invasion. The species and genotypes of the first clade infect only mammals (*T. spiralis*, *T. nativa*, *T. britovi*, *T. murrelli*, *T. nelsoni*, and *Trichinella* T6, T8, T9, and T12), whereas of the three species that comprise the second clade, one infects mammals and birds (*T. pseudospiralis*) and two infect mammals and reptiles (*T. papuae* and *T. zimbabwensis)* (Pozio and Darwin Murrell, 2006; Krivokapich et al., 2008). *T. spiralis* and *T. pseudospiralis* have a cosmopolitan distribution; *T. nativa* circulates in arctic and sub-arctic regions of North America, Europe and Asia; *T. britovi* in temperate areas of Europe and Asia, and in northern and western Africa; *T. murrelli* in temperate areas of North America; *T. nelsoni* in eastern Africa; *Trichinella* T6 in arctic and sub-arctic regions of North America; *Trichinella* T8 in southern Africa; *Trichinella* T9 in Japan; and *Trichinella* T12 in Argentina (Pozio and Darwin Murrell, 2006; Krivokapich et al., 2008).

Except for the existence of a capsule and possibly a size difference in one of the non-encapsulating species, all species and genotypes of the genus *Trichinella* are morphologically indistinguishable at all developmental stages; consequently, only biochemical or molecular methods can be used reliably to identify the genotype of the parasite. Many methods have been developed for this purpose; however, today, the most widely used are those based on the polymerase chain reaction (PCR) amplification of a single larva (Zarlenga et al., 1999; Pozio and La Rosa, 2003).

The global distribution of the various species of *Trichinella*, coupled with the wide range of hosts and various cultural eating habits involving raw or undercooked meat, are the main factors which favor human infections in both industrialized and non-industrialized countries. Human trichinellosis has been documented in 55 (27.8%) countries around the world (Pozio, 2007b). In several of these countries, however, trichinellosis is virtually non-existent among resident populations as they do not consume uncooked meat or meat of domestic or feral pigs or wild boar.

Trichinella infection has been documented in domestic animals (mainly pigs) and in wildlife of 43 (21.9%) and 66 (33.3%) countries, respectively (Pozio, 2007b). Although information on the occurrence of *Trichinella* in animals is lacking for 92 countries, it is likely that the parasite is present in at least wild animals in many of these areas.

In some regions of the world, political and economic turmoil, revolutions, and wars have contributed significantly to an increase in the prevalence of *Trichinella* in domestic animals and reservoir hosts, and have been accompanied by an increase in the number of trichinellosis cases in the human population (Gajadhar and Gamble, 2000). This is likely in response to collapses in veterinary infrastructure and services and in some cases to reductions in protein resources which may force select populations to consume a broader spectrum of animals including those known to be reservoirs of *Trichinella*, such as wild boar. For example, in the 1990s, a dramatic increase in infections in domestic animals was observed in Eastern Europe resulting in a concomitant rise in human infections and the commercial export of *Trichinella*-infected meat (Djordjevic et al., 2003; Cuperlovic et al., 2005; Blaga et al., 2007). It has also been suggested that the increase in protein-based diets in countries such as China and Romania has resulted in a broader consumption of pork and a concomitant increase in human trichinellosis (Liu and Boireau, 2002; Blaga et al., 2007).

3. Post-slaughter detection

3.1. Sample selection

For programs which are intended to ensure public health, the detection of *Trichinella* larvae is limited to *post-mortem* inspection of pigs, wild boars, horses, walruses, bears and other animals which are consumed by humans. A detection sensitivity of approximately 1–3 larvae per gram (LPG) is usually achieved in many routine meat inspection programs. Direct detection methods are also used in surveillance programs, where indicator animals such as foxes, racoon, dogs or wild boar are examined to assess prevalence of infection in wildlife reservoirs and the risk of introduction into production animals. The sensitivity of methods used for the detection of *Trichinella* larvae in muscle samples should be optimized, and is dependent on the muscle selected for sampling, the sample size, the specific method used, and the related quality assurance measures employed (Gamble et al., 2000; Nöckler and Kapel, 2007).

Muscle samples to be tested for *Trichinella* infection should be collected from sites of predilection for the species being tested, and include diaphragm pillars, tongue and masseter muscle in pigs, tongue and masseter muscle in horses, and forearm muscle and diaphragm pillars in wild boars. If *Trichinella* predilection sites are not available or unknown for the species to be tested, the tongue is recommended, with the diaphragm as an alternate (Gamble et al., 2000). An advantage of testing diaphragm is its ease of digestion.

The amount of sample used for the detection of *Trichinella* larvae must be selected to provide a sufficient level of sensitivity and an acceptable cost-benefit relationship for the required purpose. For routine slaughter

inspection of pig carcasses, using the pooled sample digestion method, a minimum of a 1 g sample of muscle from a predilection site is examined. A 1 g sample size allows for the detection of ≥3 LPG, a 3 g sample size ≥1.5 LPG, and a 5 g sample size ≥1 LPG of tissue (Gamble, 1996; Forbes and Gajadhar, 1999). In high risk situations, such as in *Trichinella* endemic regions when testing carcasses of wild boars, horses or meat from game animal species, a 5 g sample size should be used in order to adequately increase the sensitivity of the detection method. If the muscles from predilection sites are not available for inspection, larger amounts of skeletal muscle from the carcass (up to 100 g samples) should be tested in order to achieve adequate sensitivity. For epidemiological surveys of wild animals, the sample size should be adjusted considering that the mean intensity of larvae in muscles of wild carnivores is typically less than five LPG. Therefore, these test samples should be a minimum of 5 g, and preferably 10 g per carcass (Gamble et al., 2000).

3.2. Digestion methods

Artificial digestion methods allow for the examination of a pool of at least 1 g muscle samples weighing up to 100 g total, and thus can be used to test up to 100 carcasses per assay (EC, 2005; OIE, 2008a). Compared to trichinoscopy, digestion is a more sensitive, efficient, reliable and cost effective method, particularly in non-endemic countries. Consequently, it has become the method of choice for routine slaughter inspection in most industrialized countries. Among the various digestion methods (e.g. stomacher method and Trichomatic 35®) in use, the magnetic stirrer method is the most widely recognized and used, and is recommended by various authorities as the gold standard (OIE, 2008a; Webster et al., 2006). However, in endemic countries, the pooling of samples for digestion may not be feasible because of the likelihood of the need to re-test each sample individually in order to identify infected carcasses. If pooled or individual sample digestion is not feasible in endemic regions and it is necessary to use other *Trichinella* detection methods such as trichinoscopy, adequate cooking or processing methods capable of killing muscle larvae should be employed to compensate for the lower sensitivity of detection and increased food safety risk.

Individual or pooled samples are digested using 2 l of artificial digestive fluid (20 ml for each gram of meat) consisting of 1% pepsin (1:10,000 US National Formulary) and 1% hydrochloric acid (HCl). The digest is stirred for 30 min or longer at 44–46 °C in a 3 l glass beaker using a heated magnetic stirrer plate or a magnetic stirrer plate in an incubator chamber. When digestion is completed, the digestion fluid is strained (mesh size 180 μm) into a 2 l separatory funnel to remove any undigestable material, and any released *Trichinella* larvae in the fluid are allowed to settle for 30 min. Following settling, a 40 ml sample of the sediment is quickly released from the funnel (using the funnel stopcock) into a 50 ml tube. After a further 10 min of sedimentation in the tube, 30 ml of supernatant is removed and the remaining 10 ml of sediment is poured into a gridded petri dish. The 50 ml tube is rinsed with 10 ml of water and added to the gridded petri dish. The sample in the petri dish is then examined by either trichinoscope or stereo-microscope (15–40× magnification) for the presence of *Trichinella* larvae.

A unique validated version of the magnetic stirrer method described above is available for use on pork and horsemeat (Forbes and Gajadhar, 1999; OIE, 2008a). It was specifically designed to monitor critical control points and to minimize technical errors (Gamble et al., 2000; Gajadhar and Forbes, 2002).

3.3. Trichinoscopy

Trichinoscopy is a simple method that can be employed in any laboratory or field situation where ordinary light microscopy (stereomicroscope) is available. In the trichinoscope or compressorium method, small pieces of oat-grain sized muscle samples (28 pieces correspond to about 1 g of muscle sample) are compressed between two glass plates or slides until they become translucent, and are examined individually for *in situ* *Trichinella* larvae, using a trichinoscope or a dissecting stereo-microscope at 15–40× magnification (Gamble et al., 2000). Trichinoscopy is a laborious and time-consuming method for the inspection of individual carcasses. It has lower sensitivity than digestion methods (Forbes et al., 2003) and larvae of *T. pseudospiralis*, *T. papuae* and *T. zimbabwensis* are difficult to detect because they are not contained within a thick collagen capsule. Because of these limitations, trichinoscopy is not recommended by the ICT, OIE or EU for the routine examination of food- and game-animals intended for human consumption. Nevertheless, trichinoscopy can be a valuable tool in studies requiring rapid preliminary results where positive findings are meaningful, but negative results are of limited value.

3.4. Optimizing of detection methods

The main aim of *Trichinella* inspection is to reliably detect larvae in meat at levels which are capable of causing human trichinellosis. Due to a wide range of regulations governing inspection for *Trichinella* in food animals, there is an urgent need for the optimization and harmonization of detection methods. While trichinoscopy and various versions of the digestion method have been widely applied, specific methods coupled with adequate quality assurance measures have only recently been endorsed internationally (Nöckler et al., 2000; Vallée et al., 2007).

Compared to other digestion methods such as the stomacher method, the on-filter isolation technique, and the Trichomatic®, the magnetic stirrer method has the best performance and has been designated the reference method for EU member states (EC, 2005). However, due to non-uniform larval distribution and technical limitations, the sensitivity of this method is limited to ≥3 LPG when examining the prescribed 1 g of meat (Forbes and Gajadhar, 1999). Further optimization of critical control points may result in improvements to the sensitivity of this assay. Some of the specific steps in digestion which may improve test performance are discussed below.

Table 1
Serological tests for detection of *Trichinella* antibodies.

Method	Antigen	Sensitivity	Specificity	Reference
ELISA	Crude antigen	99% (human, pigs)	60% (human)	Andiva et al. (2002)
ELISA	ES antigen	98% (pigs, horses) 99% (human)	98% (pigs, horses) 91–96% (human)	OIE (2008a), Møller et al. (2005), and Yepez-Mulia et al. (1999) Gómez-Morales et al. (2008)
ELISA	Beta tyvelose	<98% (pigs) <98% (horses)	>99% (pigs)	Gamble et al. (1997) and Pozio et al. (2002)
Western blot	Crude antigen	98% (human)	98% (human)	Yera et al. (2003)
Western blot	ES antigen	Unavailable	98% (horses)	Yepez-Mulia et al. (1999) and Pozio et al. (2002)

Regarding the blending of meat prior to digestion, published and unpublished studies differ in their recommendations, varying from 3 to 5 s up to 20 or more seconds at full speed. The intent of blending is to allow for the efficient action of pepsin on meat surfaces, so that the time of blending should be adjusted to maximize digestion efficiency, based on the blending equipment being used. Similarly, the volume and concentration of digestion fluid used in the assay should be sufficient to allow for complete digestion of the sample. The ratio of digestion fluid to meat or the volume specified for 100 g sample size should be maintained when testing smaller amounts of meat. Recent studies and observations have shown that the use of a fluid pepsin formulation or granular pepsin instead of conventional pepsin powder may be advantageous for handling and, more importantly, for a safe working environment by reducing the risk for occupational asthmatic reaction in analysts (Maddox-Hyttel et al., 2007).

Following digestion, the suspension is poured through a sieve into a separatory funnel. The sieve mesh size has been set at 180 μm; however, in some studies, using a mesh size of 350 μm provided for greater larval recovery, particularly when dead or vigorously motile larvae were present in the warm fluid. The sedimentation step may be modified to include a wash step for clarification of the sediment, by using a double separatory funnel technique. This modified digestion method which uses a magnetic stirrer in an incubator chamber for digestion is at least as effective as the EU reference method (Gajadhar and Forbes, 2002).

As apparent from the foregoing discussion, some questions remain regarding method optimization. This should be incentive to try to optimize, standardize, validate, and harmonise *Trichinella* detection techniques such that all authorities use the most sensitive and reliable method for detection of *Trichinella* larvae in meat for ensuring food safety.

3.5. Serological detection

Serological tests allow for the detection of *Trichinella*-specific antibodies, and are commonly performed on serum or tissue fluids collected pre- or post-slaughter. While serological methods for detection of *Trichinella* infection are currently unsuitable for the purpose of meat inspection, there are important applications for surveillance of infection and epidemiological investigations in animal populations (Gamble et al., 2004). The enzyme-linked immunosorbent assay (ELISA) is rapid and is the most commonly used serological assay performed on pigs and wild boar; it can be easily standardized and automated for large-scale testing.

Testing by ELISA has an advantage of increased sensitivity over digestion methods for detecting *Trichinella* infection in lightly infected animals; this sensitivity is particularly useful for detecting ongoing transmission at the farm. Infections as low as one larva per 100 g of tissue have been detected by ELISA (Gamble et al., 1983). The sensitivity and specificity of ELISA are largely dependent on the quality of the antigen used in the test, but also on the procedure and host species (see Table 1). Antigens that are specifically secreted from the stichocyte cells of living L1 larvae and bear the TSL-1 carbohydrate epitope are recognized by antibodies in *Trichinella*-infected animals. These antigens are found in all *Trichinella*, and thus can be used to detect infections with any *Trichinella* species or genotype (Kapel and Gamble, 2000; Gamble et al., 2004). The excretory–secretory (ES) products released from muscle larvae contain these antigens and are commonly used for *Trichinella* ELISA. Because the sensitivity and specificity are greatly affected by the quality of the antigen used in the assay, specific guidelines for the generation of ES antigens and the performance of the assay have been established (Gamble et al., 2004; OIE, 2008a). It is possible that new antigens will be identified which improve the ELISA for detection of *Trichinella* infection. For example, a recombinant antigen derived from newborn larvae may have potential for detecting *Trichinella* infection as early as 15 days post-inoculation (Boireau et al., 2006).

A disadvantage of the ES ELISA for the detection of *Trichinella* infection is the occurrence of a low rate of false-negative results during the early stage of infection. In lightly or moderately infected animals, a time lag occurs between the time when larvae become infective for a new host and positive serology (Gamble et al., 1996, 2004; Nöckler et al., 1995). This slow rate of antibody production indicates that animals infected with lower numbers of larvae might not be detected for several weeks following exposure, even after some muscle larvae have become infective. In an ante-mortem surveillance system, this window of false-negative serology period is not considered relevant. The specificity and sensitivity for pig serology is approximately 98% with ES antigen, thus creating a problem of false positives in low prevalence situations. According to current knowledge, the limitation of serology is more related to the number of false positive reactions than of false negative reactions, consequently this limit does not represent a risk (Gamble et al., 2004).

Serological responses in pigs persist for at least 6 months after infection with no decline (Nöckler et al.,

2000; Gamble et al., 2004). In horses, however, antibody levels have been reported to decline within a few months following infection, even though infective larvae may persist in the musculature for a least 1 year (Hill et al., 2007). Thus, serological evaluation of *Trichinella* in horses is of limited value (Pozio et al., 1997, 2002; Gamble et al., 2004). Little is known of antibody responses to *Trichinella* infection in game animal species. The standardization and validation of serological assays and the establishment of reference serum banks are required to facilitate improvements and reliability of serological assays for the detection of *Trichinella* infections in animals.

4. Post-harvest processing methods

4.1. Freezing, cooking, preserving

Pork from pigs that have not been tested for *Trichinella* should be processed commercially, or treated by the consumer using methods known to inactivate the parasite. As required by many regulatory authorities, ready-to-eat pork products, such as cold-smoked sausage, must be processed to kill *Trichinella* larvae by heating, cooking, or curing. The regulations require that commercial heating of pork reach an internal temperature of at least 58 °C. The ICT and many national authorities recommend that fresh pork prepared at home be cooked to an internal temperature of 71 °C (160 °F). This recommendation is based on observations that rapid cooking methods, such as the use of microwave ovens, may not heat the pork uniformly or for sufficient time to destroy *Trichinella* larvae. Various jurisdictions, including the EU and USA, have regulations which require that pork be frozen for 20 days at −15 °C (5 °F), 10 days at −23 °C (−10 °F), or 6 days at −29 °C (−20 °F) to kill the larvae, provided that the meat is less than about 15 cm (6 in.) thick (EC, 2005; USDA, 1990). These freezing temperature and time requirements were established for *Trichinella spiralis* in pork; they are not effective for killing *T. nativa* or other freeze-resistant types of *Trichinella* and susceptibility to freezing is not as accurately defined for other species, including *T. britovi* which survive in wild boar meat and pork frozen at −20 °C and −5 °C, respectively, for 3 weeks (Pozio et al., 2006) and in carnivore muscles up to 11 months (Dick and Pozio, 2001) Because highly freeze resistant species *T. nativa* and genotype T6 of *Trichinella* have little or no infectivity for pigs, there is little or no risk in the use of freezing as a method to render pork safe in areas where pigs may be at risk of infection with these *Trichinella*. Curing procedures for most classes of pork products are also specified in legislated regulations (USDA, 1990). However, the effectiveness of curing depends on a closely monitored combination of salt concentration, temperature, and time and for this reason curing is not recommended by the ICT as a method to prevent human exposure to *Trichinella* in pork. In salting procedures, a level of free water below 0.92% in meat products may be adequate to kill *Trichinella* larvae. Although irradiation methods to kill *Trichinella* larvae are approved in some jurisdictions, it may be some time before irradiated pork becomes widely acceptable or available to consumers. The processing methods described above may be considered for use on meat other than pork.

5. Quality assurance

5.1. Quality assurance program for Trichinella diagnostic laboratories

Reliable test results are essential for public health, surveillance and trade. Test results are reliable when laboratories operate within a valid quality assurance (QA) program which includes a validated test method, procedures to confirm laboratory capability, and protocols for documentation, reporting and monitoring (Gajadhar and Forbes, 2002). Key individual system components include a quality manual, a training program, intra- and inter-laboratory proficiency testing programs, protocols, defined and controlled critical control points, and audits (Gamble et al., 2000; Forbes et al., 2005). The use of validated methods is an important component of a QA program and it facilitates the harmonization and mutual recognition of test results from different laboratories and countries. Internationally recognized guidelines for the implementation and use of QA measures are provided by the International Organization for Standardization/International Electrotechnical Commission (ISO/IEC). Testing laboratories with a quality system that meets ISO/IEC 17025 standards can attain official international recognition through accreditation by authorized third party accrediting bodies located in different parts of the world.

Despite increasing requirements for *Trichinella* testing around the world, most detection methods are not adequately defined or validated. Method validation is necessary in order to document that an assay does what it is intended to do. It must be demonstrated to be "fit for purpose". Parameters of an assay which are addressed during the process of validation include diagnostic sensitivity and specificity, reproducibility and repeatability, precision and accuracy, sample suitability and stability, and ruggedness. Method validation is an evolutionary process consisting of several stages and culminates in a course of continuous maintenance and enhancement, including proficiency testing (OIE, 2008b). The use of a validated method within an effective quality program is essential for *Trichinella* diagnostic laboratories to consistently ensure reliable test results.

5.2. Test standardization

International standards provide a reference framework and a common technological language, between suppliers and their customers, which facilitate trade and the transfer of technology. According to ISO/IEC, standardization is defined as "the activity of establishing provisions for common and repeated use, aimed at the achievement of the optimum degree of order in a given context", and is achieved through consensus agreements between national delegations representing all the economic stakeholders concerned. They agree on specifications and criteria to be applied consistently in the classification of materials, in the manufacture and supply of products, in testing and

analysis, in terminology and in the provision of services. Major standardization bodies are the International Organization for Standardization (ISO), a network of the national standards institutes of 157 countries, and the European Committee for Standardization (CEN), founded in 1961 by the national standardization bodies in the European Economic Community (EEC) and European Free Trade Association (EFTA) countries. Moreover, international organizations such as World Health Organization (WHO) and OIE provide guidelines and recommendations on production of standards and controls for human and animal health, respectively, as well as international biological reference preparations (OIE, 2008a,b).

Laboratories accredited according to ISO/IEC 17025 for *Trichinella* digestion testing are required to use validated diagnostic methods to confirm that the methods are fit for the intended use. Validation is "the confirmation by examination and the provision of objective evidence that the particular requirements for a specific intended use are fulfilled" and "includes specification of the requirements, determination of the characteristics of the method, a check that the requirements can be fulfilled by using the method and a statement of the validity". The validation process for a new method is complex and time consuming, whereas standard methods need only to be demonstrated to be fit for intended use by the laboratory, using appropriate documentation. Standardization allows for comparison of biological measurements worldwide and ensures the reliability of diagnostic procedures. A standardized test should be thoroughly validated; otherwise, it should have a history of use indicating that it is "fit for purpose". Only under exceptional circumstances, such as lack of availability of alternate test choices, should a standardized test be instituted without supporting evidence of fitness for purpose to ensure food safety.

5.3. Practical control elements of the digestion assay

ISO/IEC 17025 provides QA standards for laboratory assays which address equipment, reagents, critical control points, technical procedures, adherence to the protocol, documentation, reporting, technical training, maintenance of the test system, proficiency samples, record keeping and validation. Although a number of tests are used for the detection of *Trichinella* larvae in muscle tissue, only a few have been extensively validated. The magnetic stirrer method for pooled sample digestion is listed by the ICT, EU and OIE as a recommended method (EC, 2005; OIE, 2008a). Historical information on performance indicates that this method should be reliable if conducted under an appropriate system of quality assurance (Rossi and Pozio, 2008). Another magnetic stirrer method using a double separatory funnel procedure is internationally recognized and accepted by the EU for trade and is listed as an alternative method by the OIE. This assay has been extensively validated for both pork and horsemeat and incorporates a number of modifications specifically designed to reduce or minimize technical error and improve performance and reliability (Forbes and Gajadhar, 1999; Forbes et al., 2008).

The following is an overview of the practical application of quality assurance guidelines to important technical aspects of *Trichinella* digestion assays:

i. *Equipment and reagents*: The equipment and reagents used by various laboratories should either be identical among laboratories or proven to be equivalent to allow for the separation of technical error from equipment or reagent error when assessing proficiency sample results. The use of glassware instead of plasticware, particularly for separatory funnels is recommended in order to avoid problems associated with surface roughness and electrostatic charge.
ii. *Sample acceptance/rejection criteria*: All factors used to assess a sample as fit for testing, such as correct tissue, identification, and condition, must be specified.
iii. *Sample trimming*: Recommended sample weights are based on muscle that is free of fat and other undigestable tissues. These tissues are not known to harbour *Trichinella* larvae and undigested components can compromise larval detection by impeding flow through sieves or reducing sample clarity.
iv. *Addition of HCl to the digest solution*: HCl must be diluted in the appropriate volume of water before adding pepsin in order to prevent degradation of the pepsin prior to use.
v. *Use of the digest solution in blending*: Digest solution should be added to the tissue in the blender to facilitate tissue homogenization and used to rinse residual tissue from the blender into the digestion beaker to prevent larval loss in the blender.
vi. *Digestion temperature and duration*: The temperature and time should be monitored regularly during the digestion process if an incubation chamber is not used, in order to prevent overheating and/or prolonged digestion which could result in the destruction of larvae.
vii. *Filtering the digest fluid following digestion*: The sieve must have adequate pore size and be free of debris prior to its use. Pre-measuring the rinse water is recommended to avoid overflowing the separatory funnel and losing larvae. Only a minimal amount of tissue (undigestable) should remain on the sieve; the presence of digestable tissue indicates incomplete digestion.
viii. *Sedimentation time in the separatory funnel*: The separatory funnel containing the filtered digestion fluid must be left undisturbed for at least 30 min to allow larvae to settle to the bottom.
ix. *Collection of the primary sediment*: The stopcock on the separatory funnel must be fully opened to ensure that no larvae are trapped on the edge of the opening or fail to be rinsed out due to low flow velocity. A collection beaker should be placed beneath the stopcock to collect any fluid that may overflow or splash out of the collection tube. An OIE alternate method eliminates fluid loss at this step by adding a second smaller separatory funnel for direct collection of primary sediment.
x. *Secondary sedimentation*: Ten minutes is necessary for secondary sedimentation or clarification to occur. Any

subsequent pipetting, pouring and rinsing steps must be done carefully to avoid loss of larvae. Many of these steps have been eliminated from the OIE alternate method in which the final sediment is added directly to the reading plate (Forbes et al., 2008; Forbes and Gajadhar, 1999).

xi. *Gridlines on the reading plate*: Gridlines are essential reference markers to ensure that no part of the reading plate has been missed during viewing.

xii. *Sedimentation in the reading plate*: The reading plate should sit undisturbed for at least 1 min to allow larvae to settle before microscopic examination.

xiii. *Assessment of the reading plate*: A stereomicroscope with sufficiently high quality optics should be used for the examination of the reading plate.

xiv. *Clarification*: If the sediment in the reading plate cannot be accurately read because the supernatant fluid is too cloudy, or the sediment contains excessive particulate matter, it must be clarified and re-read.

xv. *Documentation*: The laboratory worksheet is an important element of the overall quality assurance system. It provides written evidence of controls in the daily testing process, provides raw data for reports, and is a critical audit document. The basic components include sample tracking information, documentation that the method has been performed correctly by qualified personnel, documentation of problems and irregularities, and a written record of results.

xvi. *Proficiency samples*: Proficiency testing is essential for training and certification of analysts and laboratories and to demonstrate the required levels of sensitivity and effectiveness of testing programs (Gajadhar and Forbes, 2002). Proficiency samples can be produced by adding known numbers of encysted or naked muscle larvae into defined quantities of ground meat (Forbes et al., 1998; Vallée et al., 2007; Marucci et al., 2009). It is essential to mitigate the risk of pre-existing *Trichinella* larvae in the ground meat or meat added for proficiency testing, such as pre-testing 100 g of the originating carcasses tongue or by using ground beef.

6. Control and surveillance

6.1. Control and surveillance in endemic regions

It is clear that *Trichinella* infection in animals is a continuing threat to food safety and prosperity in many countries, especially in resource-poor areas. This may be due, in part, to the absence or suspension of control measures which are routinely employed at the farm, processing and consumer levels (Gajadhar et al., 2006). During the post-war period in the Balkans, *Trichinella* was one of the most serious food-borne pathogens in the region. A dramatic increase in the number of positive pigs was a consequence of breaches of control measures in a number of areas where the prevalence was previously low (Blaga et al., 2007; Cuperlovic et al., 2005; Djordjevic et al., 2003). The following prevalence rates from Croatia clearly demonstrate the high risk for humans with traditional food habits of eating cured pork products. In the period between 1997 and 1999, 600,240 slaughtered pigs were tested for *Trichinella*, and 0.16% of them were found to be positive. The highest prevalence was found in the district of Vukovar where up to 10% of backyard swine herds were infected. About a decade later (2006), the prevalence rate showed a significant decrease whereby only 0.02% of 950,000 pigs tested positive. This decrease was a result of 10 years of government-funded intensive monitoring and control activities. The greatest success within the eradication program was achieved through continuous rodent control at all sites where infected pigs were detected, prompt disposal of infected swine carcasses, and compensation to the owners for condemned pigs.

Today, despite the remaining presence of infected backyard pigs, the goal of eradication of swine trichinellosis in Croatia is attainable. An important step towards the goal is a new National Directive that is aligned with the EU Directive. The new directive includes legislation for a *Trichinella*-free pig certification program. So far, four holdings have enrolled in the program, and have implemented a mandatory *Trichinella* risk-based wildlife monitoring program. Additionally, all backyard pigs serve as sentinels through continuous serological screening for *Trichinella* infection. Anticipated achievements of the eradication program will help to facilitate the national control of *Trichinella* infection in pigs and enhance food safety to levels similar to those of EU countries.

6.2. Farm/herd certification programs in areas of low prevalence

Control of *Trichinella* infection in pork has traditionally been accomplished by inspection of individual carcasses at slaughter or by post-slaughter processing to inactivate parasites. Declines in prevalence of this parasite in domestic swine in developed countries during the last 30 years, coupled with improvements in pork production systems, allow pork safety to be documented at the farm level. The comprehensive control and documentation of farm practices has made it possible for the development of a certification program for *Trichinella*-free farms and herds for implementation in the USA (Pyburn et al., 2005).

In this program, knowledge of risk factors for exposure of pigs to *Trichinella* was used to develop an objective audit that could be applied to pork production sites. In a pilot study, 461 production site audits were performed by trained veterinary practitioners. The on-farm good production practices audit includes aspects of farm management, bio-security, feed and feed storage, rodent control programs, and general hygiene. Objective measures of these good production practices were obtained through review of production records and an on site inspection. Of the 461 production site audits, 450 audits (97.6%) indicated adherence to good management practices and these sites were granted either entry into the program, or actual program certification. Verification testing of pigs raised on audited sites for freedom from *Trichinella* was subsequently performed using an ELISA test. Serological testing of 11,713 pigs from these sites verified that all swine from certified sites were negative for *Trichinella*. To remain in the program these sites are audited regularly on a schedule established by the *Trichinella* Certification

Program Standards.Uniform standards stating the requirements of this program have been developed, and U.S. federal regulations in support of the program are currently being finalized. This will result in a legislated process for ensuring the quality and safety of animal-derived food products from the farm through to slaughter. A similar program is now available for countries of the EU (EC Regulation 2075/2005).

7. *Trichinella* organizations and reference laboratories

7.1. International Commission on Trichinellosis

The ICT is a world-wide organization of individuals who are interested in aspects of *Trichinella* and trichinellosis. The principal functions of the ICT are: (1) the promotion and facilitation of studies relating to all phases of *Trichinella* infection in animals and humans; (2) hosting of an International Conference on Trichinellosis every 4 years for presentation of reports on all aspects of *Trichinella* and trichinellosis; and (3) organizing or participating in national or international congresses, symposia, colloquia or workshops that focus in whole or in part on *Trichinella* and/or trichinellosis.

7.2. International reference laboratories

The OIE has established two reference laboratories for trichinellosis: one at the Centre for Foodborne and Animal Parasitology, Canadian Food Inspection Agency, Saskatoon, Canada; and another at the Istituto Superiore di Sanità, Rome, Italy. The tasks of the two reference laboratories are to provide OIE Member Countries with access to the best expertise in the field of *Trichinella* infection in animals and humans and animal product safety, the best diagnostic technologies, standard reagents and training systems with the aim of establishing close and productive collaboration between scientists and technicians working in the field.

The International *Trichinella* Reference Centre (ITRC, www.iss.it/site/Trichinella/index.asp) has been established at the Istituto Superiore di Sanità, Rome, Italy. The ITRC is the official reference laboratory for both the ICT (since 1988) and the OIE (since 1992). ITRC was created as a repository for *Trichinella* strains and as a source of materials and information for international research. To date, more than 2000 isolates of human and animal origin from throughout the world have been examined and identified at the species and/or genotype level with 80 isolates now maintained *in vivo*. More than 200 researchers from 45 countries have relied on the ITRC to identify isolates, and to receive reference strains, antigens, DNA, specific molecular probes, epidemiological information, and reference sera from animals and humans.

In Europe, the European Commission has appointed the Community Reference Laboratory for Parasites (CRLP, www.iss.it/crlp/) for a period of 5 years (2006–2011). The CRLP is also located at the Istituto Superiore di Sanità, Rome, Italy. This reference laboratory performs research, diagnosis, surveillance, and control activities in the field of foodborne parasitic zoonoses including trichinellosis.

7.3. National reference laboratories

Around the world, institutions within governments in other countries such as Argentina, Canada, China, and the USA have been designated and funded as national *Trichinella* reference laboratories to provide laboratory services and expertise for the control of *Trichinella* infections in animals and humans. In the EU, each member state has appointed a National Reference Laboratory for Parasites (NRLP). The network of CRLP and NRLPs has established a practical laboratory support system for surveillance of foodborne parasitic zoonoses including *Trichinella*. An annual workshop is organized for the network to discuss needs and establish new strategies in this field.

Individuals or organizations requiring information, reference material or training in the field of *Trichinella* infections in animals or humans are encouraged to contact any of the international reference laboratories, or a national reference laboratory if available.

Acknowledgements

Information in this paper was originally presented by the authors at an ICT training workshop in September 2007 in Croatia with support in part from MED-VET-NET projects (contract Food-CT-2004-506122: Trichinet WP11, and Trichimed WP27).

Conflict of interest

No financial or personal relationships are maintained with other people or organizations that could inappropriately influence or bias this paper.

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