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COMPARISION OF KNOWN INFECTED FRESH AND FROZEN MEAT SAMPLES FOR THE RECOVERY OF *TRICHINELLA* LARVAE USING THE MAGNETIC STIRRER DIGESTION METHOD

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Dedication

This thesis is dedicated to my family,

my husband Van & my son Ha

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List of abbreviations and acronyms

CV	Coefficient of Variation
EC	European Community
EU	European Union
g	Gram
ITM	Institute of Tropical Medicine
l	Liter
lpg	Larvae per gram
mg	Milligram
ml	Milliliter
No	Number
NRLT	National Reference Laboratory for Trichinellosis
^{o}C	Celsius grade
OIE	Office International des Epizooties
PCR	Polymerase Chain Reaction
SD	Standard Deviation
spp.	Species

Keywords

Trichinella spiralis, artificial digestion method, incomplete digestion method, ring test, freezing.

Summary

The artificial digestion method is the routine method for diagnosis of trichinellosis in Belgium. The National Reference Laboratory for Trichinellosis (NRLT) organizes annual ring tests by using proficiency samples to assess the performance of this method in all field laboratories in Belgium. The first part of this work describes the incomplete artificial digestion method for collecting encapsulated *T. spiralis* larvae to prepare proficiency samples. The method proved to be accurate and allowed to prepare samples with exact numbers of larvae. A total of 52 proficiency meat samples were prepared; half of the samples were kept at 4°C until testing, while the other half were stored at -20°C before testing to kill the larvae. The second part of this work describes using the artificial digestion method to compare the recovery of *T. spiralis* larvae from the fresh meat and frozen meat samples. The recovery of larvae from fresh meat samples was higher than frozen meat samples and there were larger variations in recovery of larvae from frozen meat compared to fresh meat samples. It was concluded that frozen meat samples cannot be used for a ring test. The fresh meat samples can be used to organize annual ring trials in Belgium by using the artificial digestion method with magnetic stirrer.

Chapter I: Introduction

Trichinellosis is a world-wide zoonosis caused by nematodes belonging to the genus *Trichinella.* Humans acquire the infection by consuming raw or improperly cooked meat infected with larvae of *Trichinella* species (Zimmer *et al.,* 2008).

Trichinellosis is a parasitic infection of mammals, including humans, birds and reptiles. Human trichinellosis is not notifiable in every country and consequently, information on occurrence is incomplete (Zimmer *et al.,* 2008). *Trichinella* species infection in humans was documented in 55 (27.8%) countries. The average yearly incidence of the disease in humans worldwide is probably close to ten thousand cases with a mortality rate of about 0.2%. Trichinellosis was documented in domestic animals (mainly pigs) of 43 (21.9%) countries and in wildlife of 66 (33.3%) countries (Pozio, 2007). The emergence of trichinellosis is explained by the modifications of consumer habits, importation of meats from countries where trichinellosis is endemic and failure of veterinary control due to human error or to social upheavals (Dupouy-Camet, 2000).

In Europe, more than 1100 cases of human trichinellosis were identified for the year 2004 (Dupouy-Camet, 2006). *Trichinella* spp. infection in humans has been reported in 23 (47.9%) countries, infection in domestic animals in 24 (50.0%) countries and in wildlife in 34 (70.8%) countries (Pozio, 2007). The annual costs for inspection of pork in the EU are estimated at €570 million (Murrell & Pozio, 2000; Webster *et al.,* 2006). Therefore, trichinellosis is considered as a major concern to public health authorities (Dupouy-Camet, 2006).

According to Commission regulations of the European Commission (EC) No 2075/2005, pigs, horses, wild boars and other susceptible animals slaughtered in 27 member states and intended for the EU market should be tested for larvae of *Trichinella* spp. In addition, each member state should implement a monitoring program on *Trichinella* infection in wildlife (Pozio, 2007). Nowadays, horsemeat and pork are important sources of human trichinellosis in the European Union (EU) and there is a requirement to provide safe food for EU and export markets; therefore, the need for suitable and sensitive methods to detect trichinellosis are of crucial importance (Dupouy-Camet, 2006).

In Belgium, the domestic pig and horse populations are virtually free of trichinellosis. Only one outbreak was documented in humans following the consumption of wild boar meat (Fameree *et al.,* 1979; Pozio, 2007). Recently, Schynts *et al.* (2006) detected larvae of *Trichinella* spp. in a wild boar hunted in Southern Belgium. These larvae were identified as *Trichinella britovi*. This was the first report of the identification of *Trichinella* larvae at the species level in Belgium.

Currently, all procedures for *Trichinella* inspection are based on detection of the parasite larval stages by direct examination in muscle tissue, initially by direct trichinoscopic examination and later by artificial digestion method (Webster *et al.,* 2006). According to Commission regulations of EC No 2075/2005, trichinoscopic examination fails to detect non-encapsulated *Trichinella* species infecting domestic and sylvatic animals and humans and is no longer suitable as a detection method for standard use (Official Journal of the European Union, 2005).

A second procedure is known as the digestion method. The artificial digestion method has a higher sensitivity due to the 50 to 100 times higher sample size compared to trichinoscopy (Vignau *et al.,* 1997). According to Commission regulations of EC No 2075/2005, four digestion methods for *Trichinella* can be used, and one of the four methods, the digestion method using a magnetic stirrer, is accepted as the reference method.

Recently, Vallée *et al*. (2007) developed an incomplete artificial digestion method to free encapsulated *Trichinella* larvae from muscle tissue for use in the production of proficiency samples. These samples were used to evaluate the sensitivity of artificial digestion procedures for the diagnosis of animal trichinellosis, and their use in ring trials led to a significant improvement of the test performance in routine laboratories.

The performance of the artificial digestion method with magnetic stirrer depends on both technical expertise and the equipment used, in other words the sensitivity of the digestion method relies on laboratory expertise rather than on the parasite burden and shows that the sensitivity is a valid performance indicator (Vallee *et al.,* 2007).

In order to assess the quality of routine laboratories in Belgium, the National Reference Laboratory for Trichinellosis (NRLT), at the Department of Animal Health of the Institute of Tropical Medicine (ITM), Belgium is mandated to organize an annual ring trial. The existing recommendations about organizing ring trials are all about using fresh samples, with a known number of live larvae. Since *Trichinella* spp. is virtually absent in Belgium, the NRLT prefers not to take any risks for spreading the parasites. Hence, the NRLT would like to use the recommended method, however, with adding a freezing step to effectively kill the larvae before the samples will be dispatched to the laboratories.

Therefore, the objective of the present study was to evaluate the performance of the artificial digestion method with magnetic stirrer for recovery larvae on known infected fresh and frozen meat samples prepared by incomplete digestion method. The hypothesis was that there is no difference in recovery of the number of larvae between fresh and frozen samples.

Chapter II: Literature review

2.1. Generalities

Trichinella spp. occurs worldwide, and has formerly been classified as a List B disease by the World Organization for Animal Health (OIE) (OIE, 2000).

Trichinella was discovered in 1835 by James Paget and Richard Owen in the muscles of human cadavers in London and by Joseph Leidy in 1846 in the muscles of swine in Philadelphia (Murrell *et al.,* 2000). Trichinellosis has been regularly reported during the past two centuries and this parasitic disease is emerging or re-emerging in some parts of the world explained by modifications of consumer habits, re-forestation in Europe and increase of wild game (Dupouy-Camet, 2000).

With the expansion of global trading, there is a real potential for outbreaks of trichinellosis in virtually any country due to consumption of infested meat. In order to prevent human trichinellosis, the EU specifies testing of meats to qualify these products for importation into member countries. However, there has been an increase of trichinellosis in the EU from the consumption of imported meat. The ineffectiveness of testing is most likely due to inadequate quality assurance (Gajadhar & Gamble, 2000) so that the accuracy of the method used for diagnosis of trichinellosis in the laboratory system should be evaluated.

2.2. Taxonomy

According to the traditional classification, the genus belongs to the phylum Nematoda, roundworms, class Adenophorea, order Trichocephalida, and family Trichinellidea (Soulsby, 1982).

Up to date, *Trichinella* genus comprises eight species, *T. spiralis, T. nativa, T. britovi, T. murrelli, T. nelsoni, T. pseudospiralis, T. papuae* and *T. zimbabwensisi* and three genotypes, *Trichinella T6, T8* and *T9* are recognized in this genus. Two main clades are recognized in the genus *Trichinella*: the first include *T. spiralis, T. nativa, T. britovi, T. murrelli, T. nelsoni* that are encapsulated in host muscle tissue and the second comprise non-encapsulated species, including *T. pseudospiralis, T. papuae* and *T. zimbabwensis* (Murrell *et al.,* 2000; Pozio, 2007).

2.3. Morphological features

The adult male of *Trichinella* spp. is 1.4 to 1.6 mm long and the adult female 3 to 4 mm. The body is slender and the oesophageal portion is not longer than the posterior part. In the uterus of the female, the eggs measures 30 to 40 µm and contain fully developed embryos (Soulsby, 1982).

Trichinella infection is most easily identified by the presence of coiled larvae in striated muscle. The cysts are lemon shaped 0.3-0.8 x 0.2-0.4 mm in size and often transparent. The muscle larvae are 0.6 to 1.0 mm long (Murrell *et al.,* 2000; Taylor MA *et al.,* 2007).

2.4. Freeze resistance

According to Commission regulations of EC No 2075/2005, freezing meat under specified conditions can kill any parasites present; however, certain *Trichinella* species are resistant when freezing is carried out using the recommended temperature and time combination.

Freeze resistance of *Trichinella* spp. is a potential concern, as larvae might remain infective in meat following commercial freezing treatments. Fifty years ago, all *Trichinella* larvae were considered to be *T. spiralis.* Larvae that were found in the muscles of animals living in arctic and subarctic regions were found to be able to survive freezing for months or even years. Nowadays, it is known that only muscle larvae of *T. nativa*, its related genotype *Trichinella T6* and *T. britovi* are able to survive extended periods of freezing in the muscles of some of their natural hosts (Pozio *et al.,* 2006a), and freezing of meat is a good preventive measure to prevent disease where *T. nativa* is absent (Dupouy-Camet, 2000).

However, some studies demonstrated that *T. spiralis, T. britovi* and *T. pseudospiralis* present a high freeze tolerance in horse meat. Therefore, in future EU regulations, meat from wild boars, game or horse cannot be certified by freezing, but pork meat can be certified after freezing. The freezing can be an expensive alternative to inspection by digestion but may be desirable when the pork meat would be sold frozen anyway (Kapel, 2005).

2.5. Life cycle

Trichinella spp. have a direct life cycle, which completes all stages of development in one host. The parasitic cycle can be divided into two phases: a gastrointestinal (enteral) phase and a muscular (parental) phase (Dupouy-Camet *et al.,* 2002).

Gastrointestinal phase: When ingested, muscle larvae are freed from the cyst by digestion in the stomach and then enter tissues of the small intestine, where they undergo development to the adult stage. After mating in the intestine, females shed newborn larvae that leave the intestine and migrate through the host circulatory system (Capo & Despommier, 1996; Dupouy-Camet *et al.,* 2002).

Muscular phase: The larvae released in the gastrointestinal mucosa migrate to the blood vessels, and then they spread through the body until reaching their final location (i.e. the cells of the striated skeletal muscles) (Capo & Despommier, 1996; Dupouy-Camet *et al.,* 2002).

2.6. Clinical signs

Migration of larvae causes the typical symptoms and signs of the disease (Ribicich *et al.,* 2007) and these clinical signs and symptoms depend on parasitic factors such as the species involved, the number of infective larvae ingested, and host factors such as sex, age, and immune status (Avi Shimony *et al.,* 2007)

Gastrointestinal phase: Clinical signs of the enteral phase are diarrhea and nausea, related to the penetration of the intestinal mucosa. The early stage of the enteral phase with severe infection is associated with upper abdominal pain, diarrhea or constipation, vomiting, malaise, and low-grade fever, all of which can vary in severity and last only a few days. This clinical presentation is characteristic of many enteral disorders (e.g., food poisoning or uncomplicated indigestion), and thus it is easily misdiagnosed (Capo & Despommier, 1996; Kociecka, 2000).

Parenteral phase: Symptoms correlate with decrease of gastrointestinal symptoms. All clinical signs are either directly or indirectly due to the indiscriminate penetration of tissues by the migrating newborn larva. These are high fever, weakness, muscle pain, paralysis, photophobia, skin rash; oedema of the face, swelling of eyelids and the feet. Endocarditis, myocarditis, and even cardiac failure in fatal cases are attributed to the damaging effects of the migratory phase of the infection (Capo & Despommier, 1996; Kociecka, 2000).

2.7. Geographical distribution

The occurrence of trichinellosis in humans is strictly related to cultural food practices, including the consumption of raw or undercooked meat of different animal origin. In some countries, trichinellosis occurs only among ethnic minorities and tourists, because the native inhabitants do not consume uncooked meat or meat of some animal species (Pozio, 2007).

Trichinella spp. infection was documented in domestic animals (mainly pigs) of 43 countries (21.9%) and in wildlife of 66 countries (33.3%) (table 1) (Pozio, 2007). Trichinellosis is endemic in some areas in the world because the information on the occurrence of trichinellosis in domestic animals and/or wildlife is underreported, lack of the knowledge of the disease, sanitary regulations, and adequate veterinary infrastructure (Gajadhar & Gamble, 2000; Pozio, 2007).

Table I: Number of countries with documented *Trichinella* spp. infections in animals and human

Continent (no. of countries)	Number of countries with infection rate (%)		
	Domestic animals	Wildlife	Human
Africa (53)	3 (5.7)	13 (24.5)	7* (13.2)
America (37)	5 (13.5)	3 (8.1)	5 (13.5)
Asia (45)	9 (20.0)	14 (31.1)	18 (40.0)
Europe (48)	24 (50.0)	34 (70.8)	23 (47.9)
Pacific (15)	2 (13.3)	2 (13.3)	2 (13.3)
Total (198)	43 (21.9)	66 (33.3)	55 (27.8)

*: In four of these seven countries, trichinellosis was documented only in ethnic minorities and tourists

2.8. Epidemiology

Trichinella spp. are maintained as having either a domestic or a sylvatic life cycle. The sylvatic cycle occurs in wildlife without contact with human habitation, whilst the domestic cycle acts in human settlements and involves domestic animals like pigs and horses (Pozio, 2000). Humans can become infected from both domestic and sylvatic cycles of *Trichinella* (Soulsby, 1982; Macpherson, 2005).

Human behavior can markedly influence the transmission patterns of *Trichinella*. The increased consumption of game meat, the increased number of farms breeding wild animals for sport hunting, and the increased number of wild boar populations represent increased risk. This has resulted in increased opportunity for the transmission of *T. spiralis* from the sylvatic to the domestic habitat. Related risk factors are the increasing numbers of hunters, and their tendency to leave game carcasses in the field (Murrell & Pozio, 2000).

The spread of infected meat in the environment by humans seems to be important for *Trichinella* infection in rats. The role of the rat in the epidemiology of *Trichinella* species continues to be debated as either a reservoir or an accidental host. It is now accepted that infected rats represent an offshoot of the domestic cycle, being recipients of infection from that cycle (Pozio & Zarlenga, 2005).

2.9. Diagnostic methods

2.9.1. Direct detection

2.9.1.1. Trichinoscopy (Microscopic examination)

The traditional diagnostic method is trichinoscopy, also known as the "compression" method. The main advantage is that it is a simple method. The disadvantages of trichinoscopy methods are the time and labour required (OIE, 2000). In addition, this method has a low sensitivity (approximately 43.4%) (Beck *et al.,* 2005b). According to Commission regulations of EC No 2075/2005, trichinoscopic method fails to detect non-encapsulated *Trichinella* spp. infecting domestic and sylvatic animals and humans and is no longer suitable as a detection method for standard use.

The use of the trichinoscopy method for *Trichinella* control in the EU is not recommended (Webster *et al.,* 2006), but will be tolerated only as a transitional measure to the digestion technique. The trichinoscopic method should only be used under exceptional circumstances for the examination of a small number of animals slaughtered. Although this laborious, costly and insensitive method has gradually been replaced by artificial digestion in industrialized slaughterhouses, it is still the method of choice in many laboratories in Central and East Europe, and in small slaughterhouses in Western Europe (Kapel, 2005).

2.9.1.2. Artificial digestion

The International Commission on Trichinellosis (ICT) provides general recommendations on the artificial digestion method (Gamble *et al.,* 2000), and the OIE recommends the artificial digestion method with magnetic stirrer. The method is widely used on pooled samples and can be employed in a variety of circumstances with a minimum of equipment (OIE, 2000).

Theoretically, sensitivity for testing a 1-g sample with the artificial digestion is an infection level of 1 lpg (lpg: larvae per gram), but the actual sensitivity is closer to 3-5 lpg (Forbes & Gajadhar, 1999; Nockler *et al.,* 2000). Comparison of the artificial digestion method with the trichinoscopy method revealed that with the former method it was 3.2 times more likely to detect infected tissues than the with the latter with both methods tested with 1 gram samples (Forbes *et al.,* 2003).

In the previous EU legislation (Directive 77/96/EEC), six digestion methods for inspection of meat samples were accepted (table 2) (Webster *et al.,* 2006). Up to date, the number of inspection methods has been reduced to four with the magnetic stirrer digestion as the reference method to be preferred over the three alternative methods (Official Journal of the European Union, 2005; Webster *et al.,* 2006). The digestion methods have a theoretical detection limit of down to 1 larva per gram muscle tissue (lpg). However, there are several steps, which may compromise the sensitivity of the techniques and many of these are not adequately addressed in the new EU legislation (Webster *et al.,* 2006).

Table II: Methods used for meat inspection for *Trichinella* spp. in the EU

Method number	Method	Detection limits (larvae/ g)	Disadvantages	Advantages and practical considerations	Pigs Grams of meat to be examined	Horse Grams of meat to be examined	Wild boar Grams of meat to be examined
I	Trichinoscopy	3-5	Laborious, low sensitivity. Does not detect *T. pseudospiralis*	Rapid method if only few samples	0.5	Not allowed	0.5
II	Digestion (no mechanical intervention)	0.1-0.3	Long digestion time (18-20h). Risk of digestion of dead larvae	Pooled samples Large sample size (10 grams)	10	10	10
III	Digestion (manual shaking of fluid digestion)	1-3	Long digestion time (4h) small sample size (1g)	Pooled samples	1	5	1
IV	Stomacher (constant mechanical treatment) and sedimentation	1-3	Larvae may adhere to plastic bag	Short digestion time (25min)	1	5	1
V	Stomacher (constant mechanical treatment) and filtration	1-3	Larvae may adhere to 2 plastic bags.	Short digestion time (25min)	1	5	1
VI	Magnetic stirrer (constant mechanical treatment)	1-3	Filter size needs adjustment	Short digestion time (30min/100g)	1	5	1
VII	Trichomatic 35 blender	1-3	The device is out of production Maximum 35 samples	Pooled samples Very short diges-tion time (5-8min)	1	5	1

Up to date, related to the artificial digestion methods, there are inherent critical aspects that compromise the sensitivity of the methods and therefore need to be dealt with. These aspects are related to washing and sieving procedures, the nature of employed materials, incubation times, contamination, and the condition of the meat to be inspected. Other problems are technical equipment failure, enzyme failure, and human errors, which all lead to a lack of compliance with protocols, reducing the efficiency of the artificial methods (Webster *et al.,* 2006).

2.9.1.3. PCR

PCR (Polymerase Chain Reaction) has been used for diagnosis and for distinguishing up to species level. These advances are of great importance for epidemiological and phylogenetic studies (Mitreva & Jasmer, 2006).

PCR method can be used to detect larvae in the musculature of infected animals (Zarlenga *et al.,* 2001). Unfortunately, due to high cost and difficult implementation PCR is not a practical method for routine testing of food animals (OIE, 2000).

2.9.2. Indirect detection (Serology)

Indirect (serological) test methods are at present not recommended as a substitute for direct (pooled sample digestion) methods of individual carcasses at slaughter (Kapel, 2005). They cannot replace the direct methods because they fail to detect early stages of infections. Seroconversion occurs not before 3 to 4 weeks post infection (Nockler *et al.,* 2000; Kapel, 2005; Mitreva & Jasmer, 2006).

The ELISA (enzyme-linked immunosorbent assays) is a specific and robust method, which is recommended for surveillance programs that monitor for transmission of *Trichinella* (Mitreva & Jasmer, 2006). Serum samples and meat fluid can be applied for testing by using excretory and secretory (E/S) antigen (Moller *et al.,* 2005; Moskwa *et al.,* 2006) with the sensitivity of this method ranging from 93 to 99 % (Beck *et al.,* 2005a; Bien, 2006) .

Several other serodiagnostic methods have been applied in detecting trichinellosis. These include immunofluorescence antibody test, western blot analysis, complement fixation test, and hemagglutination test (Nockler *et al.,* 2000).

2.10. Situation of trichinellosis in the EU

Trichinella species still occur in some countries of the EU. The four species of *Trichinella* found in EU are *T. spiralis* (cosmopolitan), *T. britovi* (in wildlife from mountainous areas), T. nativa (in wildlife from artic and sub-artic regions) and *T. pseudospiralis* (a cosmopolitan non encapsulating species) (Dupouy-Camet, 2006).

Trichinellosis remains a serious problem in EU countries. Thirty years ago, horse meat has been identified as the main source of human trichinellosis in the EU with more than 3350 cases reported in 14 outbreaks (Dupouy-Camet, 2006). Other outbreaks of pork-related trichinellosis involved patients in Italy (Pozio *et al.,* 2006b), Germany (Littman *et al.,* 2006), Spain (Rodriguez de las *et al.,* 2004) and small outbreak due to wild boar in France (Gari-Toussaint *et al.,* 2005). In addition, the prevalence of *Trichinella* infection in humans,

domestic pigs and in wildlife is high in Serbia, Croatia, Romania and Bulgaria, where the disease has re-emerged in recent years (Dupouy-Camet, 2006; Boireau, 2006). Recently, infected foxes were been found in Ireland, although this country was considered to be *Trichinella* free (Rafter *et al.,* 2005).

2.11. Trends and challenges of trichinellosis control in EU

The EU countries are searching for new ways to certify meat free of *Trichinella*; however, with the expansion of the EU and increasing population, the acceptance of a unilateral method is complicated by the variability of pig and human trichinellosis among EU countries. The demand for changes in the present EU legislation has been supported most heavily by the meat industry in order to reduce the cost for inspection (Kapel, 2005).

Although the risk of *Trichinella* transmission has been eliminated in confined industrialized production, a single finding of *Trichinella* in animals raised in other management systems will presumably have a major negative impact on the export of animals from the same region, irrespective of relative risk assessment. The increasing interest in organic farming may bring drawbacks and new aspects to the control of *Trichinella*. EU with its new endemic member countries will meet challenges inside the union (Kapel, 2005). Therefore, the methods used in each laboratory will need to be validated (Gamble et al., 2000). Quality assurance requirements in laboratory analysis will impact to diagnose trichinellosis for inspection meat. In some countries, obligatory proficiency testing of laboratories already exists (Pozio *et al.,* 2004).

2.12. Situation in Belgium

In Belgium, *Trichinella* infection has not been detected in domestic animals and one outbreak was documented in humans following the consumption of pork from a wild boar (Fameree *et al.,* 1979). In wildlife, *Trichinella* larvae were found in 6.7% of wild boars, 2.2% of muskrats, 6.5% of brown rats and 11.1% of black rats (Fameree *et al.,* 1981).

In November 2004, *Trichinella* larvae were detected in a wild boar hunted in Namur province (Southern Belgium). These larvae were identified as *T. britovi*. This is the first report of the identification of *Trichinella* larvae at the species level in Belgium (Schynts *et al.,* 2006).

The Belgian NRLT has been accredited since 2004. The NRLT is the supervisor laboratory of field laboratories, 30 laboratories of them belong to a slaughterhouse, and 5 laboratories are independent laboratories. In 2007, these laboratories have investigated for trichinellosis about 11.5 million pigs, 10,000 horses and 13,700 wild boars. Since last year, a first national compulsory ring test was organized by the NRLT. This is the first step to quality assurance. By the end of 2009 all field laboratories for *Trichinella* diagnosis will have to be accredited. Like in other EU countries, at present in Belgium there is no quality assurance system that evaluates the diagnostic performance of artificial digestion assays in routine laboratories against fixed sensitivity criteria.

Chapter III: Materials and methods

3.1. Materials

3.1.1. Propagation of *T. spiralis* in the rat

The parasite reference strain Ts.RIVM (*Trichinella spiralis*, Rijksinstituut voor Volksgezondheid en Milieu, Netherlands) was used in this study. It has been maintained in Wistar male rats since 1999 in the Department of Animal Health - ITM, Belgium.

The propagation of *T. spiralis* in the rat was carried out by Department of Animal Health - ITM, Belgium. For propagation of *T. spiralis,* 2.10g of masseter muscle and lower forelimb muscle containing approximately 1290 larvae was fed to a Wistar male rat that had been starved previously for 24 hours. This rat was killed nine weeks post-inoculation for sample collection.

3.1.2. Experimental samples

A total of 52 proficiency samples were prepared and subsequently divided into two groups, respectively for fresh meat and frozen meat groups. According to the guidelines of the Community Reference Laboratory for Parasites (2006), for the preparation of proficiency samples, each group should include one negative sample and samples containing 3, 5, 7, 10 and 20 encapsulated larvae (table III). One group of 26 meat samples was kept at 4°C until testing, and another group of 26 meat samples was frozen at -20^{o}C for at least 3 weeks before testing.

Table III: Experimental samples

Proficiency samples	Number of larvae added in each sample					
	Negative control	3 larvae	5 larvae	7 larvae	10 larvae	20 larvae
Fresh meat samples (n=26)	1	5	5	5	5	5
Frozen meat samples (n=26)	1	5	5	5	5	5

Note: n = samples

3.2. Methods

3.2.1. The incomplete artificial digestion method

3.2.1.1. The incomplete artificial digestion method for collecting encapsulated larvae

The incomplete artificial digestion method for collecting encapsulated *T. spiralis* larvae was conducted by the method described by Vallée *et al*. 2007.

In brief, after killing a rat infected with *T. spiralis* larvae, the muscle tissue was homogenized in a blender and 6.25 ± 0.05g of homogenized muscle tissue was collected for each sample. Next, 125 ml of tap water was taken at 45 ± 1°C in a glass beaker of 500 ml. Then 1.09 ml of hydrochloric acid (HCL) 25%, 25 mg of pepsin (1: 10,000 NF) and 6.25 ± 0.05g of homogenized muscle tissue were added.

The beaker was placed on a warming magnetic stirrer plate and mixed rapidly. The temperature was monitored with a calibrated thermometer to keep the temperature at 45 ± 1°C. After 17 ± 3 minutes of digestion, 20ml samples were collected from the digestion mixture and were each poured into a 1 liter graduated cylinder containing 6 ml of a NaOH solution (8 mg/ml) and placed on ice. Next, cold water (4 ± 2°C) was added to the cylinder for a final volume of 1 liter and the cylinders were allowed to settle undisturbed for at least 25 minutes. Then 600 ml of supernatant was discarded and replaced with the same volume of cold water (4 ± 2°C).

The suspension was allowed to settle for 25 minutes. Again 950 ml of supernatant was discarded without disturbing the sediment in each tube, and twice 25ml of the remaining 50 ml was transferred in two different falcon tubes and both falcon tubes were added with 25 ml of cold water for a final volume of 50 ml. This suspension was allowed to sediment for at least 15 minutes, followed by discarding of the supernatant to leave 15 ml of the sediment. This final volume of sediment containing encapsulated *T. spiralis* larvae was stored at 4 ± 2°C until their use.

3.2.1.2. Preparation of proficiency samples

The preparation of proficiency samples also was conducted by the method described by Vallée *et al*, 2007.

The encapsulated *T. spiralis* larvae prepared as above were poured into a Petri dish, and microscopically observed at a magnification of X20 or X40 for their collection. If a capsule contained two muscle larvae, it was removed to avoid any supernumerary muscle larvae.

The encapsulated larvae were collected with a micropipette using specific tips (Molecular Bio products - USA), and individually placed on a counting agar disc (3.2%). This step allowed replicate counts to be done to ensure the accuracy of the larvae added and the transfer of the larvae to the meatball without any loss of larvae.

When the number of encapsulated larvae was confirmed, each agar disc was transferred into a 20g meatball prepared from ground fat-free pork as follows: the homogenized pork meat was cut into equal parts, and the agar disc containing encapsulated larvae was placed on one half. The second half of the meatball was placed over the half with the agar disc, and the meat ball was re-formed. The pork meat use to prepare proficiency samples was subject to artificial digestions (two digestions of 100 g, 94.5g of pork meat) to ensure that it was free of *Trichinella* infection.

Proficiency samples for testing were prepared by the addition of 80 g of grinded fat-free pork meat to the pork meatball containing the larvae. The grinded pork meat was manually wrapped around the 20 g pork meatball. The samples were coded and packed in sealed plastic

bags. One group of 26 samples was kept at 4°C until examination, and another group of 26 samples was stored at -20°C for at least 3 weeks before testing.

3.2.2. The artificial digestion method for recovery *T. spiralis* larvae

Adapted from the guidance described by Commission regulation of EC No 2075/2005 and according to the standard operating procedure of the laboratory in the Department of Animal Health - ITM, Belgium, proficiency samples prepared above were analyzed by the artificial digestion method with a magnetic stirrer.

After taking 2.0 liter of tap water at 46 – 48°C in a glass beaker of 3 liter, 16 ± 0.5 ml of hydrochloric acid (HCL) 25%, 10 ± 0.2 g of pepsin (1:10,000 NF), and proficiency sample were added.

The beaker was placed on a warming magnetic stirrer plate for rapid mixing with a stirring rod. The warming magnetic stirrer was adjusted and monitored at a constant temperature of 45 ± 1°C throughout the operation with a calibrated thermometer. The digestion fluid was stirred until the meat particles disappeared (approximately 30 minutes). Next, the digestion fluid was poured through a sieve into the sedimentation funnel for 30 minutes.

A final sample of about 40 ml was poured into a measuring cylinder which was allowed to stand for at least 10 minutes followed by removal of 30 ml of the supernatant fluid. The remaining 10 ml of the sediment was poured into a Petri dish, the measuring cylinder rinsed with 5-10 ml of water, which was added to the Petri dish. Finally, the sample was examined by microscope at 15 to 20 X magnification.

3.2.3. Validation method

The average number of recovered larvae ($\bar{x}$) from fresh and frozen meat samples in each group, coefficient of variation (CV %), and the Standard Deviation (SD) was calculated in Microsoft Office Excel 2007 using the following formulas:

$$\overline{X} = \frac{1}{N} \sum_{i=1}^{N} X_i$$

N is the number of proficiency samples

X_i is larvae recovered on proficiency samples

$\overline{X}$ is mean

Standard deviation (SD) was used to measure of how far apart the recovery from the average of recovered larvae

$$SD = \sqrt{\frac{1}{N-1} \sum_{i=1}^{N} (X_i - \overline{X})^2}$$

N is the number of proficiency samples

X_i is larvae recovered from proficiency samples

$\overline{X}$ is mean

SD is standard deviation

The recovery rate between groups was compared using coefficient of variation (CV), which is calculated by the ratio of the standard deviation to the mean.

$$CV\ (\%) = \frac{SD}{\overline{X}}\ 100$$

SD is standard deviation

$\overline{X}$ is mean

CV is coefficient of variation

Chapter IV: Results

4.1. The incomplete digestion method to collect encapsulated larvae to prepare proficiency samples

An incomplete digestion method was optimized for the recovery of encapsulated muscle larvae from infected rat. The majority of muscle larvae recovered under these conditions was still encapsulated (figure I). *T. spiralis* muscle larvae were clearly visible through the collagen capsule. These encapsulated muscle larvae were collected to prepare proficiency samples as described above. Fresh meat and frozen meat samples were tested within 7 days post preparation, 21 days after freezing, respectively.

To ensure that the pork meat used to make proficiency samples (figure II) did not contain *Trichinella* larvae, two samples of pork meat were processed by the artificial digestion method. The tests were negative.

Figure I: Encapsulated larvae

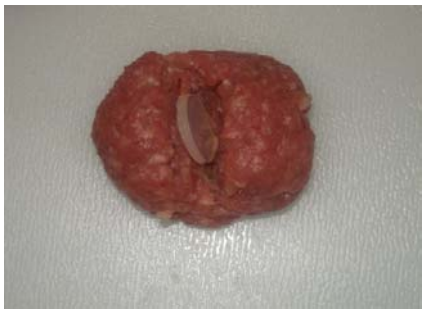


Figure II: Preparation of proficiency samples

4.2. The artificial digestion method for recovery larvae

4.2.1. Recovery of larvae from proficiency samples

Larvae observed in the fresh meat samples were coiled (figure III); while larvae observed in the frozen meat samples were uncoiled (figure IV).

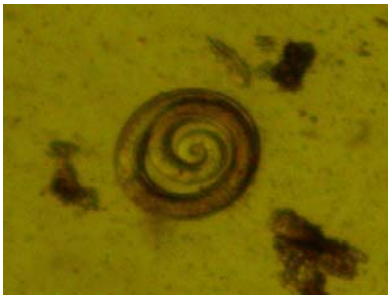


Figure III: Coiled larvae recovered from fresh meat samples

Figure IV: Uncoiled larvae recovered from frozen meat samples

Digestion of negative meat samples, both fresh and frozen, gave negative results, confirming the specificity of the technique. The results of recovered larvae from fresh meat and frozen meat groups containing 3, 5, 7, 10, 20 larvae are described in table IV and figure V, VI, VII, VIII, IX.

Table IV: The results of recovered larvae from proficiency samples

Groups	Number of sample	Number of recovered larvae	
		Fresh meat	Frozen meat
Group 1: 3 larvae	5	3	1
		3	1
		2	1
		3	2
		3	2
Group 2: 5 larvae	5	4	3
		5	3
		5	3
		5	2
		5	3
Group 3: 7 larvae	5	7	1
		7	3
		6	0
		7	5
		6	5
Group 4: 10 larvae	5	5	6
		10	5
		9	7
		10	6
		9	6
Group 5: 20 larvae	5	20	17
		18	5
		18	13
		16	8
		19	17

Figure V: Graph of recovered larvae from fresh and frozen meat samples containing 3 larvae

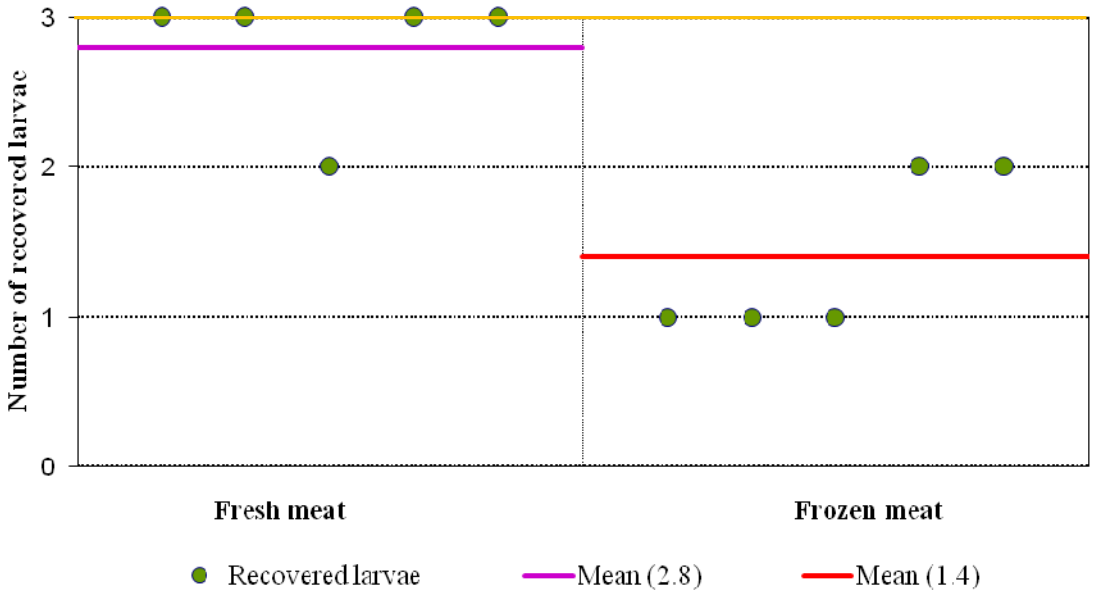


Figure VI: Graph of recovered larvae from fresh and frozen meat samples containing 5 larvae

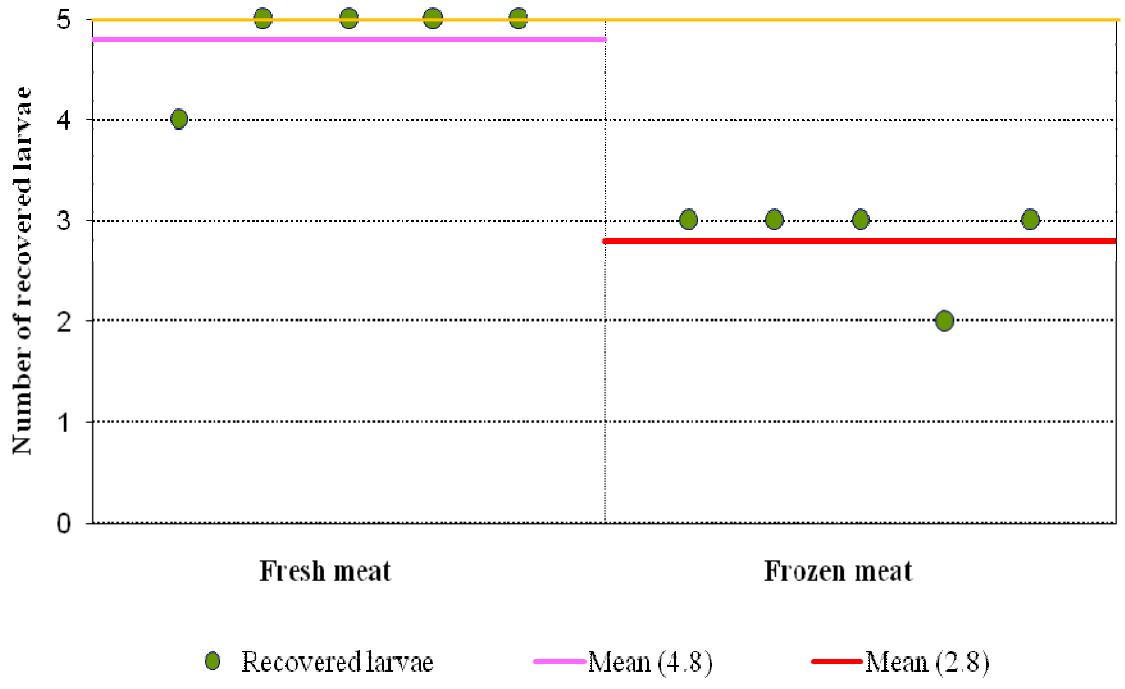


Figure VII: Graph of recovered larvae from fresh and frozen meat samples containing 7 larvae

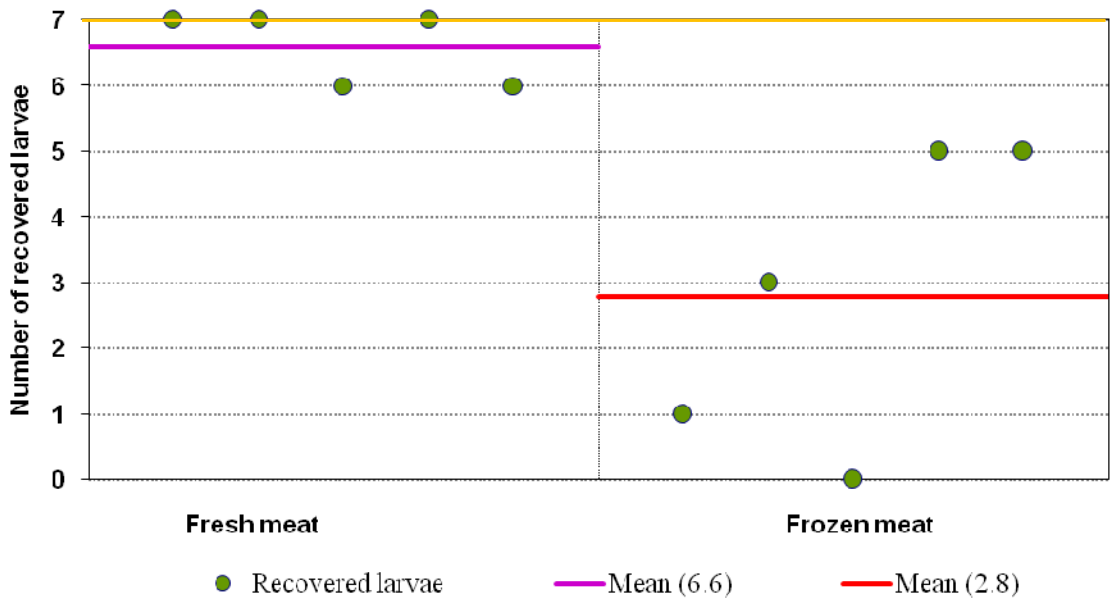


Figure VIII: Graph of recovered larvae from fresh and frozen meat samples containing 10 larvae

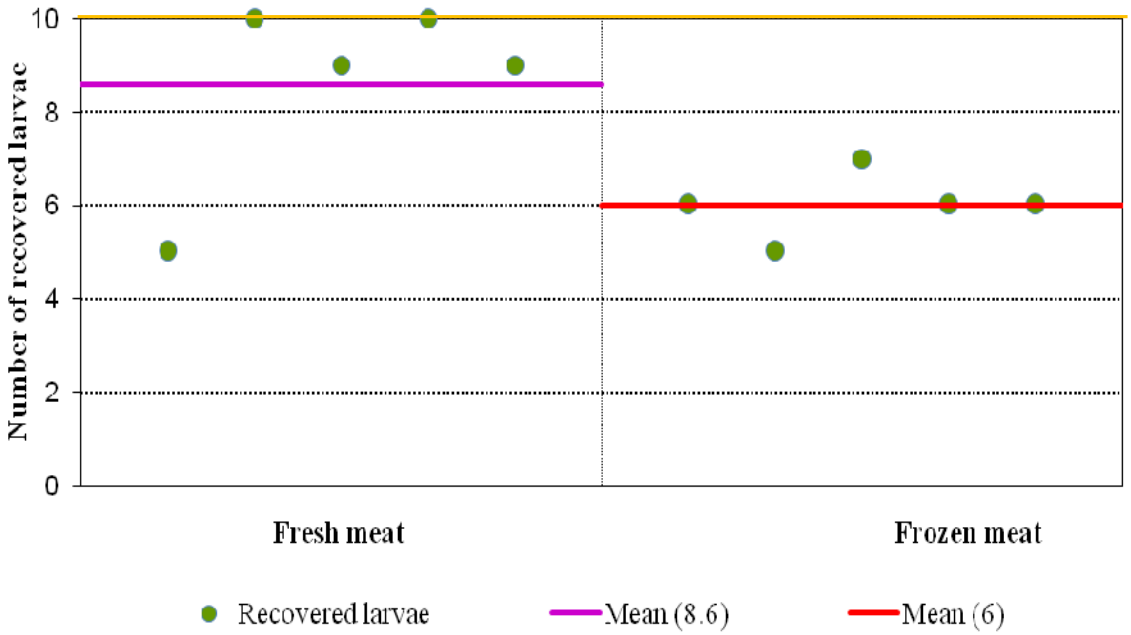
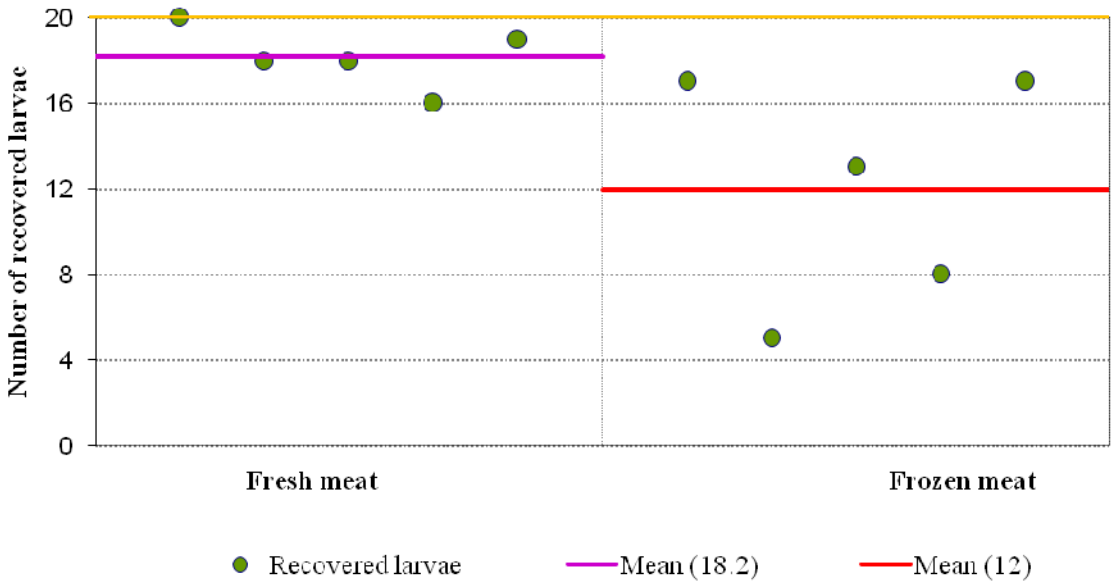


Figure IX: Graph of recovered larvae from fresh and frozen meat samples containing 20 larvae



4.2.2. Comparison of recovered larvae between fresh and frozen meat samples

4.2.2.1. Mean

The results in table V show the mean larval recovery from fresh meat and frozen meat samples. Overall, the mean larval recovery from the fresh meat group was higher than from the frozen meat group.

Table V: Mean of recovered larvae from proficiency samples

Groups	Number of larvae added in each sample	Mean	
		Fresh meat	Frozen meat
1	3	2.8	1.4
2	5	4.8	2.8
3	7	6.6	2.8
4	10	8.6	6.0
5	20	18.2	12.0

4.2.2.2. Coefficient of variation (CV %)

Table VI shows the coefficient of variation of recovered larvae from fresh meat and frozen meat samples. The results show that the variation of the number of recovered larvae in each group of samples was higher in frozen meat than in fresh meat, except for the group of samples containing 10 larvae.

Table VI: Coefficient of variation of recovered larvae from proficiency samples

Groups	Number of larvae added in each sample	Coefficient of variation (%)	
		Fresh meat	Frozen meat
1	3	15.97	39.12
2	5	9.32	15.97
3	7	8.30	81.44
4	10	24.11	11.79
5	20	8.15	44.88

Chapter V: Discussion and conclusions

At present, the artificial digestion method with magnetic stirrer is commonly considered as the reference method for the detection of encapsulated *T. spiralis* larvae in meat samples (Community Reference Laboratory for Parasite, 2006). In order to evaluate the performance and the repeatability of this method in field laboratories, the NRLT organizes annual ring tests, using proficiency samples. The use of frozen proficiency samples over fresh samples offers the advantage that the risk for contamination with *T. spiralis* larvae in the environment is avoided, since the larvae are killed by the freezing process. The aim of the present study was to compare the recovery of *T. spiralis* larvae from fresh meat and frozen meat proficiency samples by using the artificial digestion method with magnetic stirrer.

The number of proficiency samples of both fresh and frozen meat samples included in the present study is in accordance with the guideline of the Community Reference Laboratory for Parasites (2006). The incomplete artificial digestion method, described by Vallée *et al*. (2007) was used to prepare the proficiency samples, as this method allows preparing samples with an exact number of larvae. In our study, this method allowed collection of larvae that were still encapsulated and clearly visible through the collagen capsule. These result confirmed previous results described by Vallée *et al*. (2007) that the incomplete digestion method is the method of choice to prepare proficiency samples for test evaluation.

The results of the present study clearly indicate that the recovery of larvae using the artificial digestion method with magnetic stirrer is higher from fresh meat samples compared to frozen samples, irrespective of the number of larvae. Furthermore, there was a higher variability in larval recovery in the frozen meat samples compared to the fresh meat samples, making the interpretation of the recovery in frozen meat samples more difficult and more prone to errors. In contrast to the present results, Henriksen *et al*. (1978) indicated that the artificial digestion method using two sieves offered the same recovery rate for *T. spiralis* larvae from frozen samples compared to fresh samples. Jackson et al. (1977), however, also described that recovery of larvae from frozen meat was low compared to the number observed by examination of corresponding unfrozen samples, and that approximately 78% of larvae in frozen samples would be lost, when the artificial digestion method was used. Therefore, this study seems to confirm the lower recovery of larvae from frozen meat samples.

A possible explanation for the lower recovery rate from frozen proficiency samples might be the fact that *T. spiralis* larvae are not freeze resistant (Zimmer *et al.,* 2008). Following freezing for 3 weeks at -20°C, the larvae in the frozen meat samples are dead prior to the detection (Gamble *et al.,* 2000). During the sedimentation step and the subsequent sieving, the dead uncoiled larvae may remain on the sieve within the fatty debris, while the coiled larvae in the fresh meat samples remain alive, and actively migrate through the sieve. Furthermore, it can be explained that dead *T. spiralis* larvae are more fragile and more prone to destruction during the digestion process. In addition, the encapsulated larvae obtained by the partial digestion method and added to meat samples may be more subjected to the detrimental effects of freezing and digestion than larvae that are naturally present in the muscle tissue.

Conclusions

The incomplete digestion method for collection of encapsulated larvae is the method of choice to prepare proficiency samples for evaluation of the entire artificial digestion method.

In contrast to the working hypothesis, there was a substantial difference in the recovery of the number of larvae between fresh meat and frozen meat samples. It can be concluded that frozen meat samples are not appropriate for use in a ring trial. Therefore, it is suggested that ring trials in Belgium should be organized with fresh meat proficiency samples to accurately assess the performances of the field laboratories on the artificial digestion method with stirrer.

Chapter VI: Recommendations

According to the guideline of the Community Reference Laboratory for Parasites (2006), proficiency samples should be preserved at 4 ± 2^0C when dispatched to the field laboratories. The samples should be delivered within 48 hours and tested within 24 hours after arrival in the laboratories.

Referred to as diagnostic specimens in official regulations of OIE (2000), use of live larvae to organize annual ring trials require strict measures to prevent infective larvae to spread in the environment. Fresh meat proficiency samples must be carefully packed with transporting procedures and official permission. The laboratories have to carry out strict decontamination procedures.

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Annexes

Annex 1: Reagents and equipments for incomplete artificial digestion method

1.1. Reagents

- NaOH (Merck® 1174709 A, Germany)
- Pepsin powder (1:10 000 NF, Wontergentstraat 82 8720, Belgium)
- HCL 25% (Merck® KGa A Z 991516, Germany)
- Agar powder (Invitrogen, 30391-023)

1.2. Preparation of solution

1.2.1. NaOH solution 8 mg/ml

- NaOH 800 mg
- Distilled water 100 ml

1.2.2. Agar disc 3.2%

- Agar powder 32 g
- Distilled water 1 little

1.3. Equipments

- Sedimentation funnel
- Specific tip (Molecular Bio products - USA)
- Precision balance Sartorious BP610, d = 0.01 g
- Magnetic stirrers with hot place (Labinco® L32, L33)
- Magnetic bar 3 to 5 cm length
- Mincer (Illico®, Belgium)
- Microscope (Olympus® SZ 40, Germany)
- Petri dish (90 µm diameter, Sterilin® BS 611, UK)
- Beaker with capacity 500 ml
- Measuring cylinder (1000ml)

- Plastic pipette (Sterilin®, UK)
- Thermometer (VWF 021-172)

Annex 2: Reagents and equipments for artificial digestion method

2.1. Reagents

- HCL 25% (Merck® KGa A Z 991516, Germany)
- Pepsin powder (1:10000 NF, Wontergentstraat 82 8720, Belgium)

2.2. Equipments

- Sedimentation funnel
- Precision balance Sartorious® BP610 d = 0.01 g
- Magnetic stirrers with hot place (Labinco® L32, L33)
- Magnetic bar 3 to 5 cm length
- Microscope (Olympus® SZ 40, Germany)
- Petri dish (90 µm diameter, Sterilin® BS 611, UK)
- Sieve (Stainless steel, pore size 180 µm. BS 410-1, UK)
- Beaker with capacity 3000 ml
- Measuring cylinder (50ml)
- Plastic pipette (Sterilin®, UK)
- Thermometer (VWF 021-172)

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