MEAT INSPECTION FOR *TRICHINELLA* IN PORK, HORSEMEAT AND GAME WITHIN THE EU: AVAILABLE TECHNOLOGY AND ITS PRESENT IMPLEMENTATION

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A new EU directive relating to meat inspection for *Trichinella*, expected to come into force in 2006, imposes important modifications to current legislation. Nevertheless, several issues need more attention. Optimisation of methods, especially concerning sensitivity and digestibility of the meat to be inspected, along with further simplification of the legislation with regard to the number of techniques accepted, is recommended to guarantee that all member states of the EU will be given tools to perform inspection of consumer meat at the same high level. Additionally, there is a need for guidelines and protocols regarding optimal proficiency testing procedures.

This paper presents an overview of the current methods for *Trichinella* meat inspection and their implementation in the EU, listing advantages and disadvantages for each method, including some suggestions for specific points of improvement.

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Introduction

Pork, horsemeat and game may be infected with muscle larvae of the zoonotic nematode *Trichinella*, which can cause severe disease in humans. Consequently, all countries in the EU perform mandatory official inspection of slaughtered animals intended for export to prevent distribution of infected meat to consumers. *Trichinella* infections have worldwide socioeconomic significance, and are of medical and veterinary concern in France, Germany, Italy, and Spain, but foremost in the east and central European countries [1, 2] where human trichinellosis is reported to be a very important zoonosis. Some of the new EU member states (Latvia, Lithuania and Estonia) as well as some candidate countries (Bulgaria, Romania, Turkey and Croatia) have outbreaks every year (reported by the International Commission on Trichinellosis (ICT) [3]. The costs for inspection of pork in the EU is estimated to €570 million annually [1, 4].

All procedures for *Trichinella* inspection are based on direct detection of the parasite larval stages in muscle tissue, initially (from around 1860s) by direct microscopy of compressed muscle tissue, termed trichinoscopy [5.6.7.8.9,10], later (in the 1970s) by pooled digestion of 1 g muscle tissue from up to 100 pigs, which allowed for significant improvements in sensitivity of the inspection test and were less labour intensive, hence allowing larger numbers of animals to be examined.

In the present EU legislation (Directive 77/96/EEC), seven methods are accepted [TABLE 1]: six digestion methods and trichinoscopy. In the anticipated future EU legislation (SANCO/1900/2002 Rev. 8 draft, in force 01-2006), the number of inspection methods has been reduced to four with magnetic stirrer digestion as the reference method to be preferred before three alternative (termed 'equivalent') methods. Trichinoscopy is only allowed as a transitional measure, and meat inspected by this method should be clearly marked. Furthermore, such meat is limited to be sold on the national market and is not acceptable for products where the production process does not kill *Trichinella*. The digestion methods have a theoretical detection limit of down to 1 larva per gram muscle tissue (lpg). However, there are several critical steps, which may compromise the sensitivity of the techniques [11, 12] and many of these are not adequately addressed in the new EU legislation.

Trichinella inspection methods after the present legislation

Below are brief descriptions of the methods allowed for meat inspection according to directive 77/96/EEC, Annex 1, (amended by Council Regulation (EC) No. 807/2003) [TABLE 1], along with some critical points and suggestions for improvements.

Method I: Trichinoscopy

The classical method for detection of *Trichinella* in pork is trichinoscopy (also termed the compressorium technique). Muscle samples from each of the two diaphragm pillars are cut into 7 very small (oat kernel sized) pieces, which are subsequently squeezed between two glass plates and examined under a microscope at 30-40 X magnification for the presence of capsules containing *Trichinella* larvae. The microscopic examination must last at least 3 minutes to ensure adequate time for the finding of larvae. For routine inspection, trichinoscopy is labour intensive, it is not as sensitive as the digestion methods (examines less tissue, 14 oat kernel sized pieces ~0.5 g) and finally does not detect larvae of *T. pseudospiralis* as this species lacks the physical structure (a surrounding collagen capsule) that is detected for other *Trichinella* species 2-4 weeks after infection. Due to the inherent errors of trichinoscopy, this method should no longer be used, hence there are no suggestions for improvements.

Methods II and III: Digestion of single or pooled samples with either no mechanical intervention or manual shaking of digestion fluid

These manual methods allow for artificial digestion of pools of minced meat samples (10 grams from each of 10 pigs (method II) and 1 gram from each of 100 pigs (method III). Any *Trichinella* larvae present in the pooled sample are released into the artificial digestion fluid and settle at the bottom of the beaker. For method II, the digestion fluid is left undisturbed for 18-20 h, whereas for method III the fluid must be shaken twice per hour for 4 h. The sediment from the digest is examined for larvae under a stereomicroscope at 20-40 X magnification. Although the methods work on a pool of samples, they are too time consuming and with the inbuilt risk of dead or young larvae being digested along with the muscle tissue resulting in a false

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negative outcome. Since other pooled digestion methods are superior, methods II and III have been omitted from future legislation.

Methods IV and V: Mechanical digestion (with a stomacher blender) of pooled samples followed by either sedimentation or filtration

The pooled sample (100 x 1 g pieces) and the digestion fluid are mixed in a plastic bag placed in the stomacher chamber where it is mechanically agitated for 25 min at 41°C. The fluid is then passed into a sedimentation funnel through a 177 mm sieve with addition of ice and either left to sediment under 1 minute vibration cycles every second minute for 30 min total (method IV), or poured through a 35 mm filter, which will hold back larvae (method V). Larvae have a tendency to adhere to the plastic bag causing a risk for false negative readings. Thus, the sensitivity of this method is less than the theoretical 1 lpg. Because the pooled sample consists of 1 g pieces, there is a risk of undigested residue after digestion for the recommended time (authors' own observations). For the improvement of these methods, the pooled sample may be subjected to blending or mincing prior to digestion, the initial filtration to retain undigested particles could be done with a larger mesh size (355µm) that allows all larvae to pass (see Method VI below), and finally, the adhesion of larvae to the plastic bag could be lessened by flushing the plastic bag twice.

Method VI: Mechanical digestion of pooled samples with magnetic stirrer

Minced or blended meat samples are placed in the digestion fluid for 30 min at 46-48 C° under constant stirring by the use of a magnetic stirrer, and subsequently poured through a sieve into a sedimentation funnel. After a sedimentation period of 30 min, the sediment is removed from below the funnel, and the volume further reduced through more sedimentation steps. The digestion is more complete with this method because the 1 g meat samples are minced. Improved larval recovery can be obtained by changing the filter size from 177µm (180µm) to 355µm (11; authors' own observations).

Method VII: Mechanical digestion with the Trichomatic35

The Trichomatic35 apparatus blends, digests and filters a maximum of 35 pooled 1 g samples in one short process (5-8 min). Digested material is filtered under high pressure and the resulting filter is examined under a stereomicroscope as above. This method is fast with a high sensitivity [13] but a disadvantage might be that the filter requires extra washing procedures to prevent cross contamination between samples [14]. It is therefore recommended to use a new filter for every sample tested. The Trichomatic35 is no longer on the market and once the existing spare parts have been distributed, no more will be available from the manufacturer.

Future legislation and performance of the future recommended techniques

In the future EU legislation (SANCO/1900/2002 Rev. 8 draft, in force 01-2006), the magnetic stirrer method is identified as the reference method and the two versions of the stomacher method and the Trichomatic35 method may be considered equivalent methods if the magnetic stirrer method is not accessible. For routine inspection, trichinoscopy will only be allowed as a national transitional measure, as it does not detect the non-encapsulating species, *T. pseudospiralis*, or young larvae of encapsulated species with incomplete capsule development. Thus, meat inspected by trichinoscopy cannot be sold to other EU countries or exported out of the EU.

Related to the four digestion methods, which remain in the future legislation, there are inherent critical aspects that compromise the sensitivity of the methods and therefore need to be dealt with. These aspects are, for example, related to washing and sieving procedures, the nature of employed materials (plastic versus glass), incubation times, contamination problems, and the condition of the meat to be inspected [11, 15, 16]. Other problems are related, for example, to the technical equipment failure, enzyme failure, and human errors, which all lead to a lack of compliance with protocols [17], reducing the efficiency of the methods.

Table 1

Methods used for meat inspection for *Trichinella* in pork, horsemeat and wild boar in EU (according to current Directive 77/96/EEC)

Method number according to Directive 77/96EEC - Annex 1	Method	Detection limits according to the Directive (larvae/ g)	Disadvantages	Advantages and practical considerations	PIG Grams of meat to be examined (diaphragm)	HORSE Grams of meat to be examined (tongue or masseter)	WILD BOAR Grams of meat to be examined (diaphragm)
I	Trichinoscopy/ compressorium	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 (10g) increases sensitivity	10	10	10
III	Digestion (twice hourly manual shaking)	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 x plastic bags Lower sensitivity than stated in the EU directive	Short digestion (25min)	1	5	1
VI	Magnetic stirrer (constant mechanical treatment)	1-3	Filter size needs adjustment Lower sensitivity than listed in the EU directive	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, easy manageable, Very short digestion time (5-8min)	1	5	1

At least two published studies have demonstrated that the sensitivity of the recommended methods is lower than stated [11, 12]. Forbes and Gajadhar [18] documented a higher sensitivity of the magnetic stirrer method when compared with trichinoscopy. In early studies forming the basis for recommendation of the stomacher method, larval recovery as low as 79% was reported [9]. A recent comparative testing at the Danish Institute for Food and Veterinary Research (Maddox-Hyttel et al, unpublished data) indicates that the sensitivity of the magnetic stirrer is lower than required by the legislation, and importantly, both the sensitivity and reproducibility of the stomacher methods are considerably lower as compared to the magnetic stirrer method. Thus in the test, the recovery of larvae spiked into ground meat, varied from as little as 34-40% using the stomacher method (V) to an average of 63% (range 18-86%) or 85% (range 74-100%) using the magnetic stirrer method with filter mesh size of 177µm (recommended in the present and future EU legislation) or 355µm (recommended by Gamble [11]), respectively. *Trichinella* larvae from the meat were obviously lost at various steps of the procedures and these steps need to be identified and corrected through optimisation measures to ensure reliable detection methods.

The sensitivity is also related both to the amount of meat and the type of muscle tissue used for inspection [12,19,20,21], and increasing the sample size would improve any detection method [12,18,22]. The detection limit for the artificial digestion is reported to be approximately 1 lpg, if at least 5g of muscle sample per animal is digested [23]. However, according to the legislation, the recommended amount of tissue for pork allows sampling of down to 1g/pig and, as a consequence, the detection may be only 3-5 lpg rather than 1 lpg as stated. The inspection methods are intended to have a detection limit to prevent clinical trichinellosis. There are, however, only estimates [24] and no reliable data on the actual margins of such a limit. Consequently, the detection limit should be as low as possible.

The efficacy of the above digestion tests when used on meat from horses, wild boar, and other animals, is relatively unexplored although important because digestibility varies considerably both between muscle types and animal species. Some muscle groups from horses are readily digested within 30 min (diaphragm, tenderloin, fillet, and rump), whereas others need up to 2-3 times as long (masseter, tongue and leg muscles) [25].

Table 2

Available information on meat inspection (pork) for *Trichinella* in EU: No. of national / local laboratories using the different direct detection methods

Country	*Trichinella* meat inspection level in the country	Approximate number of pigs inspected	I	II	III	IV / V	VI	VII
Austria		5.3 million	1885	37	5	3	56	
Belgium	Majority of meat produced	10.4 million					+	
Czech Republic				2	5	16	20	
Cyprus		357 633	1				4	
Denmark	All for export	23 million (99% of total slaughtered)				10	21	
Estonia	100%	430 509	78			1	5	
Finland	100%	2.2 million	67		2		30	
France	1.1%*	271 100					+	+
Germany	100%	43.3 million	+				+	+
Greece	25%	431 000	93	2	4			
Hungary			+		+		+	
Ireland	All for export	1.3 million (~50% of total slaughtered)					9	
Italy	50%	11 million	+				+	+
Latvia	100%	419 105	65				12	
Lithuania	100%	1.0 million	+				+	
Luxembourg		390						
Malta								
Poland		13 million	+				+	
Portugal	100%		9				12	
Slovakia	100%	1.1 million	+				+	
Slovenia	100%	440 385	11				18	
Spain		33.5 million	1122		1	18	268	10
Sweden	100%	3.4 million	14			1	21	1
The Netherlands	100%	13.9 million					7	
United Kingdom	13%	1.2 million				5	9	

Official numbers and information primarily provided via DG SANCO (R Dwinger) from 2002, 2003 or 2004. Additional information has been provided by participants in the TrichiNet network. For countries with only blank fields under methods and/or blank fields in the first two columns; information has not been provided

I: Trichinoscopy (compressorium)

II: Digestion (single samples)

III: Digestion (pooled samples)

IV or V: Stomacher (sedimentation or filtration)

VI: Magnetic stirrer

VII: Trichomatic35

+: the sign + is employed where the method is in use in the country concerned but the number of laboratories is not available

* Due to demands from import countries, France has begun annually routine examination of several millions of pigs

These requirements for longer digestion time according to muscle type of different hosts have not been addressed in the new legislation and hence, the recommended digestion times may lead to an incomplete digestion of several grams of tissue, depending on the choice of muscle. Consequently, it is imperative that the sensitivity of each method should be listed in detail for different muscle types and animal species.

Thorough comparison of the efficiency of the recommended detection methods (excluding the Trichomatic35, which is no longer produced) is therefore required and the future legislation should include a revised description with correct sensitivity and reproducibility of each muscle type from target animal species. Furthermore, guidelines for proficiency testing are urgently needed to ensure optimal test accuracy and quality of inspection. A recent ring trial among 33 laboratories in Germany [16] only emphasises this need; half the laboratories participating detected false negative or false positive results in between one and six of 10 examined samples. Meat samples for the trial, were prepared as duplicate samples containing between 8 and 71 *T. spiralis* larvae per gram of meat (that is,. a high infection level), or without any larvae (negative controls), and were examined using the magnetic stirrer method. The draft of the future legislation (SANCO/1900/2002 Rev. 8 draft, in force 01-2006) states that the competent authority should ensure that all personnel, who are involved in the examination of samples to detect *Trichinella*, are properly trained, participating in proficiency testing programs and in a regular assessment of the sensitivity and the specificity of the test involved. However, hitherto no protocols or guidelines have been formulated for uniform proficiency testing and quality assurance systems in the EU.

Level of implementation of direct detection techniques in EU

Tables 2, 3 and 4 aim to provide an overview of the rather heterogeneous implementation levels of *Trichinella* inspection in the EU member states. Especially for horsemeat and wild boar meat, data are scarce due to the lack of registration within several countries. Although meat inspection for *Trichinella* is mandatory in the EU, registration and reporting of the number of animals inspected and the methods by which inspection was performed is not required. Comparing the available data on the present inspection methods for a range of EU countries, it is evident that many countries do not have optimal *Trichinella* control. Most countries and laboratories have implemented the magnetic stirrer method at the large slaughterhouses, however all seven methods are reported to be in function in several EU countries and according to the personal experience of the authors, even at the national level, there can be as many variations of the techniques as there are laboratories.

Table 3

Available information on horsemeat inspection for *Trichinella* in EU: No. of national / local laboratories using the different direct detection methods

Country	*Trichinella* meat inspection level in the country	Approximate number of horses inspected	I	II	III	IV / V	VI	VII
Austria		1106						
Belgium		15 628					+	
Czech Republic			1		2	8	9	
Cyprus			1					
Denmark	All for export	1278				1	3	
Estonia		11	+			+	+	
Finland		1323					10	
France	100%	23 623					71	1
Germany	100%	11 295					+	+
Greece								
Hungary			+				+	
Ireland	100%						9	
Italy	100%	50 000					+	+
Latvia	100%							
Lithuania	100%					+	+	
Luxembourg		22						
Malta								
Poland								
Portugal	100%						+	
Slovakia	100%	0-50	+				+	
Slovenia		1415	9				13	
Spain			17	1		6	35	
Sweden	100%	5032					~10	
The Netherlands	100%	2395					7	
United Kingdom						2	2	

Official numbers and information primarily provided via DG SANCO (R Dwinger) from 2002, 2003 or 2004. Additional information has been provided by participants in the TrichiNet network. For countries with only blank fields under methods and/or blank fields in the first two columns; information has not been provided

I: Trichinoscopy (compressorium)

II: Digestion (single samples)

III: Digestion (pooled samples)

IV or V: Stomacher (sedimentation or filtration)

VI: Magnetic stirrer

VII: Trichomatic35

+: The + sign is employed where the method is in use in the country concerned but the number of laboratories is not available

Table 4

Available information on wild boar meat inspection for *Trichinella* in EU: No. of national / local laboratories using the different direct detection methods

Country	*Trichinella* meat inspection level in the country	Approximate number of wild boars inspected	I	II	III	IV / V	VI	VII
Austria								
Belgium		8834					+	
Czech Republic			2		3	4	7	
Cyprus			1					
Denmark	All for export	1141				2	1	
Estonia			+			+	+	
Finland		1221	12				5	
France	1.4%	5000	+				+	+
Germany	100%	370 187	+				+	+
Greece								
Hungary			+				+	
Ireland	-	None	-	-	-	-	-	-
Italy		35 000	+				+	+
Latvia			+				+	
Lithuania	100%	9000	+			+	+	
Luxembourg		1185						
Malta								
Poland	100%	68 000	+				+	
Portugal		2					+	
Slovakia	100%	15 063					+	
Slovenia		2598-3960	11				4	
Spain			567		1		73	4
Sweden	All for the market	6000-7000 (50% of total)	(+)				+	
The Netherlands		1013					7	
United Kingdom								

Official numbers and information primarily provided via DG SANCO (R Dwinger) from 2002, 2003 or 2004. Additional information has been provided by participants in the TrichiNet network. For countries with only blank fields under methods and/or blank fields in the first two columns; information has not been provided

I: Trichinoscopy (compressorium)

II: Digestion (single samples)

III: Digestion (pooled samples)

IV or V: Stomacher (sedimentation or filtration)

VI: Magnetic stirrer

VII: Trichomatic35

+: The + sign is employed where the method is in use in the country concerned but the number of laboratories is not available

Furthermore, a surprisingly large number of countries still use trichinoscopy and although it is likely that the method is primarily applied to detect *Trichinella* larvae in muscles from wildlife or from a limited number of domestic pigs (single animal examination), the use of this technique represents a major problem. Because of the low sensitivity and inability to detect of *T. pseudospiralis*, this method should be abolished as soon as possible.

Conclusions

In conclusion, there are several indications that the sensitivity of the recommended methods - used in their present form - is effectively lower and more variable than stated in the present legislation and accordingly also in the new EU Commission legislation draft for the future meat inspection procedures. Despite the fact that the new legislation draft requires quality control on the actual procedures, and calls for proficiency testing of *Trichinella* control laboratories, there are presently no guidelines for proper and uniform proficiency testing of the recommended direct detection methods. Thus, the future challenge is to develop and implement a meat inspection system, which is more complete, comprising a fully optimised gold standard method for *Trichinella* detection with reliable sensitivity and in addition provide guidelines for a quality assurance system to ensure uniform meat inspection within the EU. This will ensure a high quality of food and food safety for the consumers, and reinforce export opportunities.

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ORIGINAL ARTICLES

Outbreak report

FIRST GENERAL OUTBREAK OF VEROCYTOTOXIN-PRODUCING *ESCHERICHIA COLI* O157 IN DENMARK

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This report describes the first general outbreak of verocytotoxin-producing *E. coli* (VTEC) in Denmark. Twenty five patients, 18 children and seven adults, with culture-confirmed VTEC O157:H- infection and indistinguishable pulsed-field gel electrophoresis DNA profiles, were identified during a six month period from September 2003 to March 2004. The outbreak strain possessed the virulence genes: *eae*, *vtx*1 and *vtx*2c. All patients but one presented with diarrhoea; none developed haemolytic uraemic syndrome. The outbreak was restricted to Copenhagen and surrounding areas. A case-control study including 11 cases and 55 matched controls revealed an association between VTEC O157:H- infection and shopping in a specific supermarket chain in Copenhagen and surrounding area, matched odds ratio (OR): 8.7 (95% confidence interval (CI): 1.1-71). After exclusion of three assumed secondary cases, only consumption of a particular kind of organic milk from a small dairy was associated with disease OR: 8.7 (95% CI 1.6-48). Environmental and microbiological investigations at the suspected dairy did not confirm the presence of the outbreak strain, but the outbreak stopped once the dairy was closed and thoroughly cleaned.

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Key words: *Escherichia coli* O157; VTEC; outbreak; milk; diarrhoea; Denmark

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Introduction

Verocytotoxin-producing *Escherichia coli* (VTEC) is an important cause of gastroenteritis, in particular in industrialised countries [1,2]. In recent decades, VTEC has caused a number of outbreaks affecting large numbers of people [3,8], including outbreaks associated with both pasteurised and unpasteurised milk [9,12].

VTEC is mandatorily reportable in Denmark both through laboratory based surveillance and clinical notifications from the treating physician. Based on laboratory reports, the incidence has increased from 1.0 per 100 000 population in 1999 (53 cases), to 3.1 per 100 000 in 2004 (168 cases) [13,15]. This trend is most likely due to an increased number of stool specimens examined for diarrhoeagenic *E. coli*, including VTEC. General outbreaks of VTEC gastroenteritis have not previously been seen in Denmark; only sporadic cases or small family clusters of infection have been detected [13].

In late 2003, the Danish VTEC reference laboratory at Statens Serum Institut observed that seven isolates of VTEC O157:H- had identical patterns as judged by pulsed-field gel electrophoresis. The samples were received over a period of four months. In January and February 2004, seven additional isolates were detected, and we initiated an investigation of this first general outbreak of VTEC infection in Denmark. The objectives of the investigation were to characterise the outbreak and, if possible, determine the vehicle.