Detection of *Trichinella* infection in food animals

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**Abstract**

The first part of this review article deals with classical methods used for the detection of *Trichinella* larvae in muscle samples of those animal species which are recognized as traditional sources of trichinellosis for human beings, as well as those species which are important for epidemiological reasons. Special consideration is given to the main applications of these methods (routine slaughter inspection, and epidemiological studies in reservoir animals), and to the major factors that may influence detection methods (sampling site, sample size). Historical, current and future aspects concerning national and EU legislation for *Trichinella* inspection are also presented.

The latter part of this review is directed at serodiagnostic methods for the detection of *Trichinella*-specific antibodies in different animal species. Classical methods of serodiagnosis such as the complement fixation test and immunofluorescence antibody test are reviewed and the characteristics and performance of the ELISA are discussed. Factors dependent upon the animal species being tested or on components of the ELISA test system are considered. This paper also reviews systematic development of the ELISA in relation to improvements in test specificity and sensitivity. Additionally, remarks are made on implementing this test for surveillance and control programs in domestic pigs and wildlife. © 2000 Elsevier Science B.V. All rights reserved.

**Keywords:** *Trichinella* detection; Trichinoscopy; Artificial digestion; Legislation; ELISA

1. Introduction

As early as the 1860s, two scientists, Friedrich Albert Zenker and Rudolf Virchow, provided the essential prerequisites for the official examination of pork for *Trichinella* in Germany (Giese, 1996). The original method using microscopy, and later trichinoscopy, was limited to the investigation of individual animals. The pooled digestion method was introduced in the 1970s and is gradually replacing trichinoscopy. Although the pooled digestion test meets the requirements for more efficient, reliable and cheaper slaughter
inspection, trichinoscopy is still used in some countries. Today, routine slaughter inspection for *Trichinella* by the pooled sample digestion is generally required in all member states of the European Union (EU) for trade between EU countries as well as for pork imported from third world countries, according to Directives 64/433/EEC (European Economic Community, 1964) and 77/96/EEC (European Economic Community, 1976). Methods of direct detection for *Trichinella* are also important for epidemiological studies in wildlife, especially where reservoir host species are concerned.

Besides methods of direct detection, serodiagnostic methods, particularly ELISA, have been developed and improved during the last two decades. Using serology, it has become possible to perform additional *Trichinella* control measures (Directive on Zoonoses 92/117/EEC (European Economic Community, 1992)) to ensure consumer protection. It is generally accepted that current serodiagnostic methods cannot replace classical meat inspection for this zoonotic nematode, but the ELISA can be useful for surveillance programs at the farm level and for epidemiological studies in wildlife (Nöckler et al., 1995; Gamble, 1996b).

According to the “Manual of Standards for Diagnostic Tests and Vaccines” published by the Office International des Epizooties (OIE), France, two main methods are recommended for the diagnosis of trichinellosis: (1) direct detection of first-stage larvae encysted in striated muscle tissue, and (2) indirect detection of infection by tests for specific antibodies (Gamble, 1996a).

2. Methods of direct detection

2.1. Applications of the methods

The direct detection of *Trichinella* larvae in muscle samples is usually done at post-mortem inspection. In order to prevent human trichinellosis in many countries, the examination of muscle samples of pigs and of all other animal species (e.g. horses, wild boars etc.) that may potentially serve as a source of this foodborne infection, is a part of routine slaughter inspection. The detection of larvae is also possible in live animals during ante-mortem inspection by taking muscle biopsies (Kazacos et al., 1986). However, this application is rarely used because of the amount of work involved, the limited sample size that can be collected and the difficulty of obtaining samples from predilection sites such as the diaphragm. Direct detection is useful for epidemiological studies in wildlife, in which indicator animals (e.g. foxes and raccoon dogs) are examined for the presence of this nematode in order to investigate the reservoir competence of the host and to evaluate its importance within the sylvatic and domestic cycles. Indicator animals provide an estimation of the prevalence of *Trichinella* in the environment.

2.2. Factors important for direct detection

Direct methods for the detection of *Trichinella* larvae in muscle samples are designed to provide maximum sensitivity, but have limitations. Methods suitable for routine meat inspection are designed primarily to prevent clinical trichinellosis in humans and do not have the capacity to prevent infection entirely. The efficiency of the direct detection of
Trichinella larvae depends on the specific methods used, the site sampled and the sample size (for detailed information see below).

The correct choice of a suitable diagnostic method is necessary in order to obtain reliable results. Trichinella spiralis, Trichinella nativa, Trichinella britovi, Trichinella nelsoni and Trichinella murrelli induce the formation of a nurse cell in the striated muscles of the host, whereas the non-encapsulating species Trichinella pseudospiralis and Trichinella papuae are characterized by the lack of a capsule around the muscle larva (Murrell et al., 2000). Larvae of non-encapsulating species would be more difficult to detect by trichinoscopy. Therefore, all samples should be examined in conjunction with or by the digestion method because trichinoscopy cannot ensure the detection of all Trichinella species.

2.2.1. Sample location

After the enteral and migratory phases, larvae of T. spiralis reach the striated muscles where they become infective for a new host as early as 17 days post-infection; larvae prefer sites in muscle tissues that are well supplied with blood. Predilection sites differ among animal species and may be dependent on the specific mobility behavior of the species under investigation (Kapel et al., 1995).

Identification of the predilection sites in an animal species will determine the choice of muscle to be tested for Trichinella larvae. Experimental studies using doses that mimic natural infections, were done in different species to determine typical predilection sites for Trichinella larvae. In domestic pigs, the three main predilection sites for T. spiralis are the diaphragm crus, the tongue and the masseter muscle (Gamble, 1996b; Forbes and Gajadhar, 1999). Comparable results are also available for experimental T. britovi infection in pigs, with neck muscle appearing to be another predilection site in addition to the tongue, the diaphragm and the masseter (Kapel et al., 1998). In horses experimentally infected with T. spiralis, the tongue and masseter were found to be typical predilection sites (Soulé et al., 1989, 1993; Gamble et al., 1996). In a naturally infected horse, most larvae of T. spiralis were detected in different muscles of the head. Of these, the tongue and the masseter proved to be the fourth and the fifth most important predilection sites (Pozio et al., 1998). Results of experimental infections with T. pseudospiralis in poultry (cock-broilers) demonstrated that the muscles of the head (e.g. masseter and the neck) were typical predilection sites (Petrov et al., 1999). Wild boars experimentally infected with T. spiralis, T. nativa, T. britovi, T. pseudospiralis, T. murrelli, Trichinella T6 and T. nelsoni harbored most larvae in the diaphragm and the tongue (Kapel, 2000).

Experimental studies on predilection sites of Trichinella muscle larvae were also done in wildlife. In silver foxes (Vulpes vulpes fulva), experimentally infected with T. spiralis, the diaphragm crus and the forearm musculature proved to be predilection sites suitable for examination (Nöckler and Voigt, 1998). There are comparable results for arctic foxes (Alopex lagopus) experimentally infected with T. nativa (Kapel et al., 1995), red foxes (Vulpes vulpes) naturally infected with trichinae (Cristea, 1996; Maleczewska et al., 1997) and for raccoon dogs (Nyctereutes procyonoides), naturally infected with T. spiralis (Thiess, 1999, pers. commun.).

The order of importance of predilection sites can vary in low infection rates. The tongue appears to be a predominant predilection site in common food animals. This has been demonstrated experimentally in domestic pigs (Gamble, 1996b; Forbes and Gajadhar, 1999;
Table 1
Predilection sites for *Trichinella* larvae in different animal species

<table>
<thead>
<tr>
<th>Animal species</th>
<th>Predilection sites</th>
<th>Aim of examination</th>
</tr>
</thead>
<tbody>
<tr>
<td>Domestic pig</td>
<td>Diaphragm, tongue, masseter</td>
<td>Meat inspection</td>
</tr>
<tr>
<td>Horse</td>
<td>Tongue, masseter</td>
<td>(Domestic meat)</td>
</tr>
<tr>
<td>Wild boar</td>
<td>Forearm, diaphragm</td>
<td>Meat inspection</td>
</tr>
<tr>
<td>Bear</td>
<td>Diaphragm, masseter, tongue</td>
<td>(Game meat)</td>
</tr>
<tr>
<td>Walrus</td>
<td>Tongue</td>
<td></td>
</tr>
<tr>
<td>Fox</td>
<td>Diaphragm, forearm muscles</td>
<td>Epidemiological studies</td>
</tr>
<tr>
<td>Raccoon dog</td>
<td>Diaphragm, forearm muscles</td>
<td>(Reservoir animals)</td>
</tr>
</tbody>
</table>

Serrano et al., 1999) and horses (Gamble et al., 1996; Voigt et al., 1998). Some of the sampling sites recommendations of the International Commission on Trichinellosis are summarized in Table 1.

2.2.2. Sample size

The amount of sample to be used for the detection of *Trichinella* larvae must be chosen to provide an adequate level of sensitivity and an acceptable cost-benefit relationship. It is generally accepted that for routine meat inspection, to prevent clinical trichinellosis in humans, it is necessary to ensure a sensitivity of approximately 1–3 larvae/g (LPG) of tissue taken from the predilection site. Theoretically, a 1 g sample would be enough for the detection of at least 1 LPG of tissue, on condition that there is a homogenous distribution of larvae in the tissue investigated. In practice, this is true for high larval densities, but in cases of low infection rates, larvae are not distributed homogeneously. Using 1 g muscle samples from pigs and horses experimentally infected with *T. spiralis*, the sensitivity of the pooled sample method of artificial digestion was between 3 and 5 LPG, whereas a 5 g sample increased the sensitivity of this method to approximately 1 LPG (Gamble, 1996b, 1998; Gamble et al., 1996; Forbes and Gajadhar, 1999).

For routine slaughter inspection of pig carcasses and game meats, using the pooled sample digestion method, a minimum of a 1 g sample of tissue from a predilection site is recommended. For the same purposes, a minimum of 0.5 g sample and preferably more may be used for the inspection of individual pig carcasses by trichinoscopy (Gamble et al., 2000). Particularly in endemic areas, a 5 g sample should be used for digestion in order to adequately increase the sensitivity of the detection method. There was no significant difference between 3 and 5 g samples in detecting infection in pig tissue containing 1.0–1.9 LPG, therefore, 3 g samples might be considered the minimal sample size under these conditions (Forbes and Gajadhar, 1999). To ensure high sensitivity, horse meat is examined with the pooled sample digestion method using 5 g or preferably 10 g samples. If the muscles from predilection sites are not available for inspection, carcasses should be tested using larger amounts (up to 100 g samples) in order to achieve adequate sensitivity (Gamble et al., 2000).

Concerning epidemiological studies in reservoir animals, the sample size should be adjusted upward to achieve a sensitivity of less than 1 LPG. Low larval densities occur in the muscle tissues of wild carnivores infected with *Trichinella*. For this reason, the samples to be tested in such studies should have a weight of at least 5 g or more. Current
Table 2
Current recommended sample weightsa for *Trichinella* examination of different animal species

<table>
<thead>
<tr>
<th>Animal species</th>
<th>Sample weight</th>
<th>Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Domestic pig</td>
<td>1 g sample</td>
<td>Pooled sample digestion</td>
</tr>
<tr>
<td></td>
<td>(0.5 g sample)</td>
<td>(Trichinoscopy)</td>
</tr>
<tr>
<td>Horse</td>
<td>5 g sample</td>
<td>Pooled sample digestion</td>
</tr>
<tr>
<td>Game meat (e.g. wild boar, bear)</td>
<td>1 g sample</td>
<td>Pooled sample digestion</td>
</tr>
<tr>
<td>Reservoir animals (e.g. fox, raccoon dog)</td>
<td>Minimum 5 g sample</td>
<td>Pooled sample digestion</td>
</tr>
</tbody>
</table>

a Sample weights of 5 g are required to ensure detection at an infection level of 1 LPG in tissue.

recommendations for sample size are summarized in Table 2, but the limitations imposed on sensitivity by small samples should be considered when referring to this table.

2.3. Main characteristics and performance of direct methods of detection

Trichinoscopy is a simple but tedious method for the inspection of individual carcasses and requiring much time and labor. In contrast, the pooled sample digestion method allows testing of up to about 100 carcasses at the same time. The digestion method requires more technical equipment than trichinoscopy, but is cheaper and has become the method of choice for routine slaughter inspection in most industrialized countries. Because of enhanced sensitivity of digestion tests the use of trichinoscopy as a standard method of control in the EU is discouraged.

In recent years, various ring trials and experimental studies using domestic pigs, wild boars and horses experimentally infected with different *Trichinella* species have been conducted to evaluate the reliability of the official detection methods. Results demonstrated that methods accepted by the EU or recommended by the OIE, respectively, are suitable for examining pork, as well as wild boar and horse meat for muscle larvae, and should be adequate for preventing clinical trichinellosis in humans (Gamble, 1996b, 1998; Gamble et al., 1996; van Knapen et al., 1996; Voigt et al., 1998).

To ensure that tests are performed properly, all laboratories that conduct routine slaughter inspection should introduce and maintain a suitable quality control system. This includes system audits, documentation, training and regular checks of technical staff for their ability to accurately detect *Trichinella* in spiked muscle samples, e.g. four times per year (Gamble et al., 2000). The preparation and use of proficiency panels for quality assurance in *Trichinella* pooled sample tests has been described (Forbes et al., 1998). A well documented quality assurance system (QAS) that meets international standards will soon be essential for any test used in domestic or international trade. Complete data for the validation of a digestion test for pigs and horses are available (Forbes and Gajadhar, 1999).

2.3.1. Trichinoscopy

According to the OIE “Manual of Standards for Diagnostic Tests and Vaccines”, 28 small pieces of muscle of about 2 mm × 10 mm (size of an oat grain), with a total weight of about 0.5 g, should be taken from prescribed predilection sites (Gamble, 1996a). The muscle pieces are compressed between two glass plates until they become translucent,
then examined individually for *Trichinella* larvae, using a trichinoscope or a conventional stereo-microscope (15–40× magnification).

### 2.3.2. Pooled sample digestion (magnetic stirrer method according to EU Directive 77/96/EEC)

Although there are several published procedures for the pooled digestion procedure for the detection of *Trichinella* in meat (Gamble, 1996a; Forbes and Gajadhar, 1999; Gamble et al., 2000), the magnetic stirrer method described in the EU directive is outlined here. A maximum of 100 g of samples of muscle tissue from prescribed predilection sites of the animals under inspection is pooled. The sample pool is digested using 2 l of artificial digestive fluid consisting of 1% pepsin (1:10.000 US National Formulary), and 1% HCl. The digest is stirred for 30 min at a temperature of 46–48°C in a 3 l glass beaker using a hot plate magnetic stirrer. During this process, the trichinae are released from the muscle. The digestion fluid is then poured through a sieve (mesh size 180 μm), which keeps back any undigested tissues, but allows the passage of *Trichinella* larvae, into a 2 l separatory funnel. Larvae are allowed to settle for 30 min, then a 40 ml sample is quickly released into a 50 ml tube. After a further 10 min of sedimentation to clarify the suspension, 30 ml of supernatant is withdrawn. The remaining 10 ml of sediment is poured into a gridded petri dish. The 50 ml tube is rinsed with 10 ml of tap-water which is added to the petri dish. Subsequently, the sample in the petri dish is examined by trichinoscope or stereo-microscope (15–40× magnification) for the presence of *Trichinella* larvae.

### 2.4. Slaughter inspection: historical aspects, current legislation and future trends

In 1860, the German pathologist Friedrich Albert Zenker discovered the biology and pathogenic role of *T. spiralis* in humans (Giese, 1996). As a result it became possible to detect the sources of epidemics of trichinellosis and to devise specific means to control food-borne infections. Virchow (1864) followed up on these discoveries and was instrumental in introducing inspection for *Trichinella* infection in the domestic pig in Germany. From his many studies, Virchow concluded that trichinellosis was an infection of individual animals, and that a single infected pig could be the cause of infection in hundreds of people. He justifiably demanded that every pig slaughtered for food be inspected individually for trichinae; to use his own words: “If one has slaughterhouses, then nothing is simpler than setting up microscopes there, and preventing the sale of any pork unless there is an official permit certifying the purity of the said animal” (Virchow, 1864).

Various provincial governments, e.g. in Saxony-Gotha, Berlin and Thuringia, followed Virchow’s advice as early as the 1860s and introduced microscopy, and later trichinoscopy, to their pig slaughterhouses. The first common regulation on inspection for *Trichinella* for all of Germany was included in the Imperial Meat Inspection Law in the year 1900. Since 1937, Germany has had mandatory inspection for *Trichinella* even for home slaughter (Hinz, 1991). *Trichinella* inspection was greatly simplified when the pooled sample method of artificial digestion was introduced in 1978, and it became possible to investigate up to 100 pigs in a single procedure.

Trade in each EU Member State is conducted according to specific national regulations, which are currently undergoing a process of harmonization with appropriate EU
legislation. For example, in Germany, the most important regulations are the Meat Hygiene Law (FlHG), the Meat Hygiene Directive (FlHV) and the General Administrative Directive on Official Procedures for Inspection Following the Meat Hygiene Law (VwVFlHG). The Meat Hygiene Law (Bundesrepublik Deutschland, 1993) states that without exception every slaughtered pig and horse is to be investigated for *Trichinella*. This also applies to wild animals destined for human consumption. The FIHV (Bundesrepublik Deutschland, 1997) deals with the appropriate standards for inspection for *Trichinella* infection, as well as with the sampling procedures, while the permitted methods are detailed in the VwVFlHG (Bundesrepublik Deutschland, 1986).

As far as trade within the EU and importation from third world countries is concerned, *Trichinella* inspection in pigs and horses is regulated by Directives 64/433/EEC (European Economic Community, 1964) and 77/96/EEC (European Economic Community, 1976). Whereas, the first Directive prescribes an obligatory inspection for pigs and horses, Directive 77/96/EEC (European Economic Community, 1976) contains rules on sampling as well as on permitted methods for *Trichinella* detection. The inspection of game meat for *Trichinella* is handled separately in Directive 92/45/EEC (European Economic Community, 1992). Alternatively, routine inspection of pork and horse meat is not necessary if meat is frozen according to prescribed procedures laid down in Directive 77/96/EEC (European Economic Community, 1976).

According to EU Directive 77/96/EEC (European Economic Community, 1976), slaughter inspection for *Trichinella* larvae in pigs is prescribed either by trichinoscopy, with the examination of 14 muscle pieces from the diaphragm crus, or by one of the five methods of artificial digestion using 1 g samples from the diaphragm crus, which are pooled to a total weight of 100 g. EU Directive 92/45/EEC (European Economic Community, 1992) prescribes the same procedure for the inspection of game meat, with the difference that samples for trichinoscopy must be taken from each of the following muscles: the masseter, diaphragm crus, forearm, intercostal muscles as well as from the tongue. For the examination of horse carcasses, a 5 g sample taken from the tongue, masseter or diaphragm must be examined using a method of artificial digestion. As a result of outbreaks of human trichinellosis caused by horse meat in 1993 (Ancelle et al., 1998) the sample size was changed from 1 to 5 g samples, according to Directive 94/59/EC, which amends the annexes of Directive 77/96/EEC (European Economic Community, 1976). Results from North America and from an EU ring trial on the detection of low levels of *T. spiralis* in experimentally infected horses have shown that 5 g samples from the tongue should be used for the routine inspection of horse meat (Gamble et al., 1996; Voigt et al., 1998).

National regulations of EU member states may differ from EU Directives depending on the current epidemiological status in domestic pigs and consumer habits (i.e. consumption of raw meat). Some countries (e.g. The Netherlands and France) do not require *Trichinella* inspection of each pig intended for intra-country consumption, but must follow the EU Directive if pork is produced for trade within the EU community. In comparison to these countries, German regulations for intra-country consumption are similar to EU Directives. The differences that still exist are being overcome by the gradual harmonisation of national and EU legislation. Presently, for pigs on the national level, 1 g samples are prescribed for the inspection of pooled samples by artificial digestion, while 56 muscle pieces from the diaphragm must be examined when applying trichinoscopy. Sampling of game meat must
include an additional 28 pieces from the forearm muscle for trichinoscopy or otherwise 0.5 g from the same muscle for artificial digestion. The legislation on slaughter inspection for *Trichinella* larvae in horses is equivalent to the mentioned EU Directive. Most eastern European countries (e.g. Poland, Lithuania, Croatia, Russia) require routine *Trichinella* inspection of pigs on the national level, with the difference that trichinoscopy is still the main method of choice. In US 5 g samples from pigs in a 100 g pool are examined, which results in a significantly increased test sensitivity (Gamble, 1996b). However, use of this minimum sample size is not mandated by law and is not widely practiced.

The instructions for trichinoscopy and the different methods of artificial digestion are described in EU Directive 77/96/EEC (European Economic Community, 1976) in Annex 1, Point I or Points II–IV, respectively. In Germany, the most common methods used for routine slaughter inspection are based on artificial digestion. These are the magnetic stirrer method for pooled samples and a filter isolation technique. Trichinoscopy is used only on a very limited basis.

Future trends in *Trichinella* control include modern pig production systems that are certified for *Trichinella*-free production. This would mean that the required classical slaughter inspection could be abandoned for pigs raised in these certified industrialized production systems. In this regard, an EU working group has elaborated proposals for this alternative form of *Trichinella* control in domestic pigs (Pozio, 1998). Although the definitions for its main components, e.g. “non-endemic area”, “indicator animals” and “microbiological barrier” are already defined, the prerequisites for the certification and its maintenance as well as control measures are still under consideration. A similar program is under development in US (Gamble, 1999, pers. commun.).

3. Serodiagnostic methods

3.1. Applications and characteristics of the methods

Serodiagnostic methods are used mainly for ante-mortem and post-mortem examination of blood or serum samples for *Trichinella*-specific antibodies, and under some conditions may have a higher sensitivity than methods of direct detection. Other uses include in vivo studies on immune responses in long-term infection and surveillance of live caught wild animals. Because serodiagnostic methods allow testing of samples from living pigs as well as of samples obtained post-mortem, they may be useful for establishing *Trichinella*-free areas and reducing restrictions in international animal trade. Results from experimental studies indicate that tissue fluids (meat juice) from slaughtered pigs or from hunted or other dead animals (e.g. wild boars) may be suitable for serologic examinations using ELISA (Gamble and Patrascu, 1996; Kapel et al., 1998).

3.1.1. Conventional serodiagnostic methods

The immunofluorescence antibody test (IFAT), western blot analysis (WBA), complement fixation test (CFT) and haemagglutination test (HAT) are examples of conventional serodiagnostic methods that are labor intensive and cannot be used in an automated system. As a result, these methods are more expensive in comparison to the enzyme-linked
immunosorbent assay (ELISA) and are preferentially used in human medicine for the ex-
mination of individual samples.

The IFAT and HAT achieve a higher degree of sensitivity than the CFT. Because all
tests are based on the use of somatic antigens from *Trichinella* larvae, cross-reactions may
occur with filariae (i.e. *Onchocerca* spp.) and *Schistosoma mansoni* that were detected by
IFAT in human beings (Saathoff et al., 1978). In investigations of immunofluorescence
cross reactions against *T. spiralis* by WBA, epitopes shared with heat shock proteins were
observed in serum samples from patients with autoimmune diseases (Robert et al., 1996).
Specific antibodies in experimentally infected mice were detected 14 days post-infection.
In comparison to ELISA, the IFAT proved to be less sensitive (Ruitenberg et al., 1975).

The IFAT is probably the most frequently used conventional serodiagnostic method and
requires the use of animals in order to produce encysted trichinae (first-stage larvae) in
muscle tissue. Cryostat sections are prepared from the muscle tissue of experimentally
infected laboratory mice or rats. After incubation with the diluted serum sample (e.g. from
1:10 to 1:640), cryostat sections are incubated with species and immunoglobulin specific
conjugates labelled with fluorescent substances. Then, the cuticle of the larva and internal
structures are examined for fluorescent antigen–antibody reactions using a fluorescence
microscope (Ruitenberg et al., 1975; Robert et al., 1996).

### 3.1.2. ELISA

In comparison to the CFT, HAT, WBA and IFAT, the ELISA is easy to conduct, can
be automated and detects infection levels as low as one larva/100 g of tissue (Gamble,
1996a). The ELISA method is recommended for herd surveillance programs and is useful for
detecting ongoing transmission of *Trichinella* at the farm level (Gamble, 1996b). However,
the ELISA may fail to detect infected pigs during both the early and the very late stages
of infection (see below). It is for this reason that this serological method cannot be used to
replace digestion testing for the detection of *Trichinella* larvae at slaughter inspection, but
it can be recommended for practical use in herd surveillance in pigs (Nöckler et al., 1995;
Gamble, 1996b).

### 3.2. Important factors for the detection of specific antibodies

The suitability of a serological detection method depends on specific factors of the test
system and characteristics of host immunity. The test antigen is considered to be an important
factor for the specificity of the test result. In the case of somatic antigens, such as crude worm
extracts, cross-reactions with antigens of other nematodes may cause false-positive results
(Gamble et al., 1983). Excretory–secretory (E/S) antigens, which are metabolic products
collected by in vitro culture of muscle larvae are more specific. Another important factor
in the test is the quality of the blood or serum sample. Samples of poor quality resulting
from extensive hemolysis or microbial contamination, especially in the case of samples
from game or wild carnivores, may significantly decrease the specificity and sensitivity of
the test system (Dedek, 1992).

There are host-specific factors that may influence the evaluation of test results. Differences
in the individual immune response, the presence of maternal antibodies and immu-
nodeficiency syndromes compromise interpretation of test results (Nöckler et al., 1995).
Table 3
Correlation between time of seroconversion and infection dose (T. spiralis) in pigs (Smith and Snowdon, 1989; Gamble, 1996b), in horses (Gamble et al., 1996) and in silver foxes (Nöckler and Voigt, 1998)

<table>
<thead>
<tr>
<th>Animal species</th>
<th>Infection dose (no. of larvae per animal)</th>
<th>LPG</th>
<th>Time of seroconversion, post-infection (weeks)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pig</td>
<td>100</td>
<td>1.62–6.50&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5–7</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>18.4–48.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4–5</td>
</tr>
<tr>
<td></td>
<td>2500</td>
<td>87.6–99.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>8000</td>
<td>12.1–81.4&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>64,000</td>
<td>221.4–466.6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.5–3</td>
</tr>
<tr>
<td>Horse</td>
<td>1000</td>
<td>0.10–0.26&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3–4</td>
</tr>
<tr>
<td></td>
<td>4000</td>
<td>0.39–7.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3–7</td>
</tr>
<tr>
<td></td>
<td>10,000</td>
<td>6.6–60.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3–4</td>
</tr>
<tr>
<td></td>
<td>40,000</td>
<td>484–1060&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2–3</td>
</tr>
<tr>
<td>Silver fox</td>
<td>500</td>
<td>7.4–14.7&lt;sup&gt;d&lt;/sup&gt;</td>
<td>4–6</td>
</tr>
<tr>
<td></td>
<td>2000</td>
<td>4.7–66.9&lt;sup&gt;d&lt;/sup&gt;</td>
<td>2</td>
</tr>
</tbody>
</table>

<sup>a</sup> Of tongue.
<sup>b</sup> Mean of tongue, masseter, diaphragm, intercostal, psoas and rectus abdominis.
<sup>c</sup> Of masseter.
<sup>d</sup> Of diaphragm.

Additionally, a low rate of false-negative results, due to a delayed antibody response may occur in pigs infected with sylvatic Trichinella species (Gamble et al., 1983). In pigs experimentally inoculated with T. nativa, T. murrelli and Trichinella T6, decreasing antibody levels were probably caused by the disappearance of muscle larvae whereas constantly high antibody levels in pigs infected with T. spiralis, T. britovi and T. nelsoni likely reflect the constant antigen stimulation of muscle larvae (Kapel and Gamble, 2000). The reliability of the test is affected by the time of seroconversion and the persistence of antibodies in the host, and these two important factors are explained in more detail below.

### 3.2.1. Time of seroconversion

During the early stages of Trichinella infection there is a “diagnostic window”, during which larvae have become encysted in muscle tissue, but specific antibodies cannot yet be detected in the host animal. In this case, false-negative results may occur when compared with direct tests. The time of seroconversion is correlated to the infection dose (Table 3). Using an E/S-ELISA, pigs experimentally infected with 100, 500 and 2500 T. spiralis larvae per animal seroconverted at 5–7, 4–5 or 4 weeks after infection, respectively. The delayed seroconversion, particularly in lower-level infections, emphasizes that the ELISA cannot replace classical slaughter inspection of individual carcasses, but remains a suitable tool for epidemiological studies (Gamble, 1996b, 1998). With higher infection doses of 8000 and 64,000 larvae per pig, seroconversion occurred earlier, at 2.5–3 weeks after infection (Smith and Snowdon, 1989). Similar results have been obtained in pigs experimentally infected with T. spiralis, T. britovi and T. nativa. In spite of very low recovery rates or no findings of larvae, there was a high antibody response in all pigs, especially in T. nativa infections (Kapel et al., 1998). Horses experimentally infected with 1000, 4000, 10,000 and 40,000 larvae of T. spiralis per animal seroconverted between 2 and 7 weeks post-infection (Gamble...
et al., 1996). However, all horses with worm burdens > 1 LPG were detected serologically by 4 weeks post-infection. Silver foxes (Vulpes vulpes fulva) that were experimentally infected with 500 and 2000 larvae of T. spiralis seroconverted 4–6 and 2 weeks post-infection, respectively (Nöckler and Voigt, 1998).

### 3.2.2. Persistence of antibodies in different host species

Knowledge on the persistence of detectable antibodies and their relation to larval recovery are important data for validation of a serological test, especially if blood samples are taken during late stages of infection. Serum antibodies (e.g., immunoglobulin G with a half-life of 23 days) decline slowly over time after an initial peak. Except in experimental infections, it is impossible to know at what point after infection the sample has been taken. In general, it can be assumed that the blood sample has been taken at a stage of infection following the peak antibody titre.

The persistence of antibodies depends on various factors including activity of antigen stimulation in the host. In experimentally infected pigs, the earliest time after which the antibody titre fell below the ELISA cut-off level was the 80th week post-infection (Nöckler et al., 1995). In other pig sera from long-term investigations, a serum titre was detectable up to 130 weeks post-infection. In practice, if it can be assumed that a slaughter pig with a live weight of 90–100 kg is not older than 25–30 weeks at slaughter, it is unlikely that the results of serological testing of such animals can be influenced by false-negative results due to declining antibody titres (Nöckler et al., 1995). Long-term studies in silver foxes experimentally infected with T. spiralis revealed that specific antibody titres could be detected up to the end of the study at the 76th week post-infection (Nöckler and Voigt, 1998). This period corresponds to the mean life expectancy of the fox in its natural habitat.

The serodiagnosis of trichinellosis in the horse appears to be less reliable than similar tests in pigs. Experimental studies with horses indicated that, unlike other species, anti-Trichinella-IgG persists for a relatively short time. This was confirmed in an EU ring trial with horses, where the earliest decline of antibody titers below the cut-off occurred in week 15, at which time infective larvae were still present in the muscle tissue (Voigt et al., 1998). These results are in agreement with studies which showed that specific antibody titers fell below cut-off levels of the ELISA as soon as 14 weeks post-infection (Soulé et al., 1989, 1993), and that in naturally infected horses, specific circulating antibodies were not detected in spite of the presence of a high worm burden in muscles (Pozio et al., 1997, 1999). This is important because many horses are more than 10 years old at slaughter, thus increasing the likelihood of false-negative results in Trichinella-infected horses, and greatly reducing the usefulness of serologic tests (Voigt et al., 1998). Further long-term studies in horses are needed to investigate correlations between antibody response in various tests and the presence of infective muscle larvae, and to evaluate the negative predictive value of ELISA testing of live horses for export (Gamble et al., 2000).

### 3.3. Performance of ELISA

Amounts of reagents, incubation times, etc., may vary from one ELISA test to another. Nevertheless, all reagents must be tested for optimal concentration in advance in order to obtain reliable test results. In this regard, checkerboard titration of the antigen as well as
of the conjugate and the substrate are recommended. The E/S antigen produced by in vitro cultivation of *Trichinella* muscle larva should follow the protocol by Gamble et al. (1983, 1988) to ensure a high quality of the ELISA and to achieve comparable test results.

The procedure outlined below is for an E/S-ELISA (Nöckler et al., 1995). *Trichinella*-E/S-antigen is diluted in carbonate buffer (0.05 M, pH 9.6) to a concentration selected according to the result of checkerboard titration. Then, each well of the microtiter plate is coated with 50 µl of the *Trichinella*-E/S-antigen and is incubated for 60 min at 37°C. After incubation, microtiter plates are washed three times with phosphate-buffered saline containing 0.05% Tween 20 (PBS-T), pH 7.2. Following each washing, plates are blotted dry.

Test sera, as well as the positive reference control serum (titer 1:640) and negative control sera, are diluted in PBS-T at a 1:100 dilution and 50 µl are added to duplicate wells. Two wells are also coated with PBS-T (blank). The microtiter plate is incubated for 30 min at 37°C and then is washed three times with PBS-T. Peroxidase labelled IgG-conjugate (50 µl diluted 1:1200 in PBS-T) are added to each well, followed by incubation for 30 min at 37°C. After incubation, microtiter plates are washed three times with PBS-T and once with distilled water. The substrate ABTS (2,2'-azino-di (3-ethylbenzthiazoline-6-sulfonate)-di-ammonium) (50 µl) with 0.01% hydrogen peroxide (pH 4.8–5.0) are added to each well.

The reaction is stopped after 15–20 min, when the positive control reaches optical density (OD) values of 1.300–1.400, by the addition of 1% sodium dodecyl sulfate. OD values are measured by a photometer (microplate reader) at 405 nm. Mean OD values of samples (each reduced by the mean OD value of the blank) are calculated as a percentage of the mean OD value of the positive control. Samples with values of <8% are negative, those between >8 and <14% are questionable and those >14% are *Trichinella*-positive. Before routine testing of pig sera for *Trichinella* antibodies can be conducted, the negative, questionable and positive cut-off of the ELISA must be established. For this purpose, a group of field sera from *Trichinella*-negative pigs from different farms are examined with the ELISA and the results are evaluated according to the reference standard method. The mean OD value of each negative field serum (reduced by the mean OD value of the blank) is related to the mean value of the positive control (also reduced by the mean OD value of the blank) and is calculated as an ELISA-index (%). The positive control, which is the same as that used in routine testing, has a titer of 1:640 and the ELISA-index is defined as 100%. The calculated ELISA-index of negative field sera is used for the establishment of the cut-off as follows. The reactive cut-off (negative/questionable threshold) is defined as the mean ELISA-index of the negative pig sera plus three times the standard deviation. This reactive cut-off is comparable to the evaluation by Gamble (1996a) who defines OD values three times higher than the mean value of a negative serum pool are classified as questionable. The mean ELISA-index of the negative pig sera plus six times the standard deviation is considered to be positive (questionable/positive threshold). Under these conditions, the ELISA test achieves a test sensitivity and specificity of about 95% (Nöckler et al., 1995). According to Gamble (1996a) the ELISA yielded a sensitivity of nearly 100% and specificity of more than 97% in abattoir testing. The definition of the cut-off may vary from one test system to another. Test sensitivity and specificity can be validated by the two-graph receiver operating characteristic analysis (TG-ROC) which is based on a plot of the test sensitivity and specificity against the threshold (cut-off) value, assuming the latter to be an independent variable (Greiner et al., 1995).
For a proper evaluation and interpretation of test results, an international reference standard should be used. In this regard, an international serum bank is needed which provides a panel of reference sera consisting of *Trichinella*-positive, -negative and -cross-reactive sera intended for the calibration of the various ELISAs against a common standard. For the adjustment of the sensitivity of the test system, the positive control serum should have an antibody activity that lies on the linear portion of the dose/response curve when pre-diluted in negative serum.

3.4. ELISA — historical aspects, current methods and future trends

Since the mid 1970s, several researchers have been working on improving or replacing direct methods of *Trichinella* diagnosis with serological methods based on the indirect ELISA (Ruitenberg et al., 1974). Initially, somatic antigens made of whole body extracts of muscle larvae were used, but the specificity of the ELISA was poor, due to cross-reactions (Ruitenberg et al., 1976; Ruitenberg and van Knappen, 1977; Clinard, 1978; Taylor et al., 1980). Such whole body extracts possess certain epitopes which also occur in other helminths, and lead to an impurity of the antigen, resulting in a less specific antigen, and a greater likelihood of cross-reactions in the ELISA (Gamble and Graham, 1984).

During the 1980s, the specificity of the ELISA was improved by utilizing E/S antigens obtained from metabolites of *Trichinella* muscle larvae incubated in vitro. The antigens originating from larval secretions consist of a group of structurally related glycoproteins with molecular weights of approximately 45–55 kDa (Gamble et al., 1983; Gamble and Graham, 1984). In order to produce the E/S antigens, optimal culture conditions are required, as well as a meticulous isolation of the *Trichinella* metabolites. The incubation period should not exceed 18 h, in order to avoid the specific metabolic E/S antigens from becoming contaminated with non-specific antigens originating from dead or dying *Trichinella* larvae (Gamble et al., 1988). Many authors have reported on the successful use of *Trichinella*-E/S antigens in the indirect ELISA for the detection of specific antibodies in various animal species (Murrell et al., 1986; Smith, 1987; Smith and Snowdon, 1989; Van der Leek et al., 1992; Jakob et al., 1994; Nöckler et al., 1995; Gamble, 1996b; Gamble et al., 1996).

Recently, a synthetic glycan antigen has been developed for use in ELISA. Early results indicate that use of this antigen increases test specificity and sensitivity. The antigen can be mass produced in a chemically pure form and is considered to be a suitable tool for monitoring *Trichinella*-free production units (Gamble et al., 1997).

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References


