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Trichinella spiralis – New method for sample preparation and objective detection of specific antigens using a chemiluminescence immunoassay

Jana Braasch [a], Stefanie Ostermann [a], Monika Mackiewicz [a], Catherine Bardot [b], Caroline Pagneux [b], Viola Borchardt-Lohölter [a,*], Erik Lattwein [a]

[a] Institute for Experimental Immunology, affiliated to EUROIMMUN Medizinische Labordiagnostika AG, Seekamp 31, 23560 Lübeck, Germany
[b] EUROFINS Laboratory in Moulins, Boulevard De Nomazy - BP 1707, 03017 Moulins, France

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ABSTRACT

The parasitic roundworm *Trichinella spiralis* is most commonly transmitted to humans through consumption of raw or undercooked meat of infected pigs or game. To prevent human infection, slaughterhouses perform meat safety surveillance using the gold standard "Magnetic Stirrer Method". We introduce a fast and objective method using automated detection of specific *Trichinella spiralis* antigens by a newly developed immunoassay based on chemiluminescence (ChLIA). Panel A comprised muscle tissue samples from non-infected pigs (n = 37). Panel B comprised muscle tissue samples from non-infected pigs spiked with different amounts of *Trichinella* larvae without collagen capsules (n = 56). Panel C contained muscle tissue samples from experimentally infected pigs including *Trichinella* larvae encapsulated in collagen (n = 32). Each sample was shredded with PBS buffer in a knife mill, destroying *Trichinella* larvae. Following centrifugation, the supernatant (muscle tissue extract containing released excretory and secretory *Trichinella spiralis* antigens) was used for *Trichinella*-specific antigen detection by the new Trichinella ChLIA. The overall accuracy of the Trichinella ChLIA was 97.6 %. The specificity of the Trichinella ChLIA was 100 % (panel A). The sensitivity in samples from experimentally infected pigs was 100 % representing a detection limit of 0.01 larvae per gram. Cross-reactivity with parasites other than *Trichinella spp*. was not observed. This new meat inspection method for the detection of *Trichinella spiralis* antigens presents high specificity and high sensitivity, especially in truly infected samples. In contrast to the gold standard, this new approach to meat safety surveillance does not require longsome digestion or microscopy by trained personnel.

Introduction

Trichinella spiralis is a worldwide- distributed parasitic roundworm (nematode) belonging to the genus *Trichinella*. At present, several species are recognised in the genus, e.g. *T. spiralis, T. nativa, T. britovi, T. pseudospiralis, T. murrelli, T. nelsoni, T. papuae, T. patagoniensis, T.zimbabwensis* (Diaz et al., 2020). All species can develop in mammals, but *T. papuae* and *T. zimbabwensis* also infect some reptile species and *T. pseudospiralis* develops also in birds.

Trichinellosis refers to a worldwide distributed zoonotic infection of humans with the larval and adult stages of primarily *T. spiralis* or other *Trichinella spp*. (Pozio and Darwin Murrell, 2006). The clinical picture of trichinellosis usually begins with a sensation of general discomfort and headache, increasing fever, chills and sometimes diarrhoea and/or abdominal pain. Pyrexia, eyelid or facial oedema and myalgia represent the principal syndrome of the acute stage, which can be complicated by myocarditis, thromboembolic disease and encephalitis. The infective larvae are meat-borne. They are typically found in pork, but also in meat from horses (domestic cycle) as well as from game and wildlife (sylvatic cycle). Trichinellosis is transmitted to humans through consumption of raw or undercooked meat of infected domestic pigs and game, whose skeletal muscles contain *Trichinella* larvae encapsulated in collagen (Despommier, 1998).

To survive for years in the host's muscles, *T. spiralis* manipulates the host immune system with the help of numerous proteins that are secreted into the surrounding tissue. The so-called excretory-secretory proteins (E/S proteins) are predominantly secreted by the stichosome, which is located in the oesophageal wall (Gold et al., 1990). The E/S proteins of *Trichinella spp*. can induce specific host immune responses and are therefore often used as antigens for antibody detection (Bien

* Corresponding author at: EUROIMMUN Medizinische Labordiagnostika AG, Seekamp 31, 23560 Lübeck, Germany.
E-mail address: v.borchardt-lohoelter@euroimmun.de (V. Borchardt-Lohölter).

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et al., 2013; Gamble et al., 1988).

The prevalence of trichinellosis depends on cultural food practices and varies between countries. Nowadays, besides pig, other infection sources of human trichinellosis caused by *Trichinella spp.* are bear, deer, moose and walrus at a global scale and wild boar and feral hog throughout Southeast Asia (Diaz et al., 2020). Trichinellosis is considered to occur only infrequently in many European Union (EU) countries, which may be related to underreporting (Dupouy-Camet et al., 2002; Troiano and Nante, 2019). Moreover, many physicians do not recognise trichinellosis since the symptoms are unspecific and often thought to be due to other diseases. Consequently, available laboratory tests (e.g. ELISA and western blot) are seldom performed (Bruschi et al., 2019; Gnjatovic et al., 2019; Wang et al., 2017). Another reason for the low incidence of trichinellosis is the decrease in prevalence of infection with *Trichinella spp*. in pigs because of controlled housing conditions in commercial swine herds (Murrell, 2016). Human trichinellosis is declining worldwide since effective control has been established through meat inspection (Murrell, 2016).

Nowadays, most outbreaks involve consumption of raw meat of infected game or pigs from small suburban farms and backyards (Diaz et al., 2020). Trichinellosis outbreaks were observed for example in Eastern Europe in the early 1990s and early 2000s (Djordjevic et al., 2003; Kurdova-Mintcheva et al., 2009; Neghina, 2010) as well as in Belgium in 2014 and in France and Serbia in 2017 (Barruet et al., 2020; Messiaen et al., 2016). Reasons were amongst others laxity in veterinary control over meat production for economic reasons and high-risk animal production practices such as feeding of food waste or exposure to carcasses of swine or wildlife (Poizo, 2007). Other countries which have reported rather recent outbreaks are Romania, Argentina, Chile, Mexico, Canada, Russia and India (Chalmers et al., 2020; Murrell, 2016). In the USA, where bear meat is an important source of infection, five outbreaks were reported between 2008 and 2012 (Wilson et al., 2015). Between 2010 and 2013, 1009 cases of human trichinellosis were reported in the EU (Murrell, 2016). Porcine trichinellosis was confirmed also in Henan, China, but its prevalence in indoor- raised pigs decreased from 2010 to 2015 (Cui et al., 2013; Cui and Wang, 2011; Jiang et al., 2016). Accumulating incidences of *Trichinella* spp. in commercially produced pork could result in loss of trust in food safety followed by a decrease in consumption. Further consequences could be abating profitability for farmers and meat processors (Poizo, 2007). Therefore, sensitive detection of infected meat is of great interest to ensure continuous meat safety surveillance.

To prevent human infection in the EU, every year more than 200 million pigs are tested for *Trichinella spp.* (Alban et al., 2011) in slaughterhouses and by Expert services for Veterinary affairs according to EU Regulation (EC No. 2015/1375). These tests detect larval densities of *Trichinella spp*. that constitute a food safety hazard. Direct detection of *Trichinella spp*. larvae in muscle tissue of an animal is limited to post mortem inspection. Adequate sample collection requires prior identification of suitable sampling sites, which differ between animal species. In domestic pigs and wild boars, the main sites for *Trichinella spp.* sampling are the diaphragm pillar and the tongue, whereas in horses, the tongue and the masseter proved to be the most important loci (Nöckler and Kapel, 2007).

The current gold standard for meat inspection is the “Magnetic Stirrer Method” (also named “digestion method”), which involves digestion of the muscle tissue and detection of undigested larvae by microscopy (EC No. 2015/1375 Article 6). A detailed protocol for the digestion method for detection of *Trichinella spp*. muscle larvae in pork has been described previously (Forbes and Gajadhar, 1999). In short, the digestion method involves 100 g of pooled 1 g samples of muscle tissue from hundred pigs. The sample pool is digested using artificial digestive fluid consisting of 0.5 % pepsin and 0.5 % HCl. The digest is stirred for 30 min at 44–46 °C. During this process, the *Trichinella spp.* larvae are released from muscle cells. The digestion fluid is then poured through a sieve which allows the passage of *Trichinella* larvae. Following sedimentation for 30 min, a 40 mL sample is quickly released into a tube. After further 10 min of sedimentation, the supernatant is withdrawn and the remaining 10 mL of sample are examined for the presence of *Trichinella* larvae by either trichinoscope or stereo-microscope (Nöckler et al., 2000). The sensitivity of the digestion method is 100 % for muscle samples with a larval density of three larvae per gram (3 lpg) (Forbes and Gajadhar, 1999). A disadvantage of the digestion method is the time-consuming processing and need for trained personnel for the evaluation via microscope. In large slaughterhouses, however, fast diagnosis is of high relevance, since meat processing must be suspended for the duration of *Trichinella* testing. Moreover, evaluation of the result requires trained and experienced personnel; the staff’s expertise often determines the test’s sensitivity (Riehn et al., 2013). Another testing method is based on the current digestion protocol, but sedimentation steps and microscopic diagnosis of the larvae are replaced by antigen detection based on latex agglutination (Gayda et al., 2016; Interisano et al., 2013).

Here, we introduce a novel preparation method for meat samples involving shredding of the sample instead of digestion. In the next step, specific *T. spiralis* antigens are detected by a newly developed immunoassay based on chemiluminescence (ChLIA). The result of the Trichinella ChLIA is given in concentrations allowing objective evaluation. In this study, we describe the experimental setup, illustrate the analytic steps, present the analytical performance of the Trichinella ChLIA and compare it to the gold standard.

Material and methods

Samples

A total of 215 muscle tissue samples from domestic pigs were used to assess sensitivity, specificity and cross-reactivity of the new Trichinella ChLIA (Table 1).

Panel A. 37 *Trichinella*-negative samples were obtained from a local butcher shop (Krummesse, Germany). These samples consisted of 100 g muscle tissue from the diaphragm pillar of non-infected domestic pigs. The meat had been tested with the digestion method at the local Expert service for Veterinary affairs and Food safety (Mölln, Germany).

Panel B. 32 spiked samples originated from the German Federal Institute for Risk Assessment (BfR) in Berlin, Germany. Each sample consisted of 90 g muscle tissue from the diaphragm of *Trichinella*-negative domestic pigs spiked with 10 g minced meat containing one, three, four, five, ten or fifteen *T. spiralis* larvae. The samples from BfR were frozen and hence contained dead larvae. 24 spiked samples originated from the French Agency for Food, Environmental and Occupational Health & Safety (ANSES) in Maisons-Alfort, France. Each sample of 100 g muscle tissue from the diaphragm pillar of *Trichinella*-negative domestic pigs contained a chunk of gelatine surrounding four living *T. spiralis* larvae. The samples from ANSES were non-frozen. Larvae in these 56 samples are lacking the naturally existing capsule, a collagen structure surrounding the parasite with its excretory and secretory antigens. Therefore, samples in this panel are not identical to samples subjected to meat inspection in slaughterhouses. Instead, these artificially generated samples resemble samples regularly used in proficiency testing.

Panel C. 32 frozen *Trichinella*-positive muscle tissue samples were obtained from BfR. The number of *T. spiralis* larvae (one or three) in these samples was determined using microscopy. Each sample contained 99 g muscle tissue from the diaphragm pillar of *Trichinella*-negative domestic pigs spiked with 1 g muscle tissue sample from experimentally infected pigs including one or three *T. spiralis* larvae encapsulated in collagen. These samples are very similar to samples from naturally infected pigs, which are subjected to meat inspection in slaughterhouses, but with the important difference that the larvae are dead.

Panel D. Additional 60 samples spiked with different *Trichinella* species were used. Each sample contained 100 g muscle tissue from the diaphragm pillar of non-infected pigs spiked with a defined number of

Table 1
List of *Trichinella*-positive and *Trichinella*-negative muscle tissue samples summarised by panel. Panel A comprises samples from non-infected pigs. Panel B consists of samples from non-infected pigs spiked with different amounts of *Trichinella* larvae without collagen capsules. Panel C contains muscle tissue samples from experimentally infected pigs including *Trichinella* larvae encapsulated in collagen. Panel D contains samples spiked with several *Trichinella* species. Panel E contains samples spiked with different nematode and protozoan antigens.

Panel	N samples in panel	N samples in subgroup	N larvae in 100 g sample	Origin
A	37	37	0	Local butcher shop
		8	1	BfR
		6	3	BfR
B	56	24	4	ANSES
		6	5	BfR
		6	10	BfR
		6	15	BfR
C	32	22	1	BfR
		10	3	
D	60	3	1 (*T. spiralis*)	EURLP
		3	3 (*T. spiralis*)	
		6	1 (*T. britovi*)	
		3	3 (*T. britovi*)	
		3	15 (*T. britovi*)	
		6	1 (*T. nativa*)	
		3	3 (*T. nativa*)	
		3	15 (*T. nativa*)	
		3	1 (*T. papuae*)	
		4	3 (*T. papuae*)	
		3	15 (*T. papuae*)	
		4	1 (*T. pseudospiralis*)	
		6	3 (*T. pseudospiralis*)	
		3	1 (*T. zimbabwensis*)	
		4	3 (*T. zimbabwensis*)	
		3	15 (*T. zimbabwensis*)	
E	30		1 mg lysate (*T. suis*)	various
		6	1 mg lysate (*A. suum*)	
		6	1 mg lysate (*T. gondii*)	
		6	1 mg lysate (*S. papillosus*)	
		6	1 mg lysate (*T. cati*)	

larvae from *T. spiralis* (n = 6), *T. britovi* (n = 12), *T. nativa* (n = 6), *T. papuae* (n = 10), *T. pseudospiralis* (n = 10) or *T. zimbabwensis* (n = 10). The frozen larvae originated from the European Union Reference Laboratory for Parasites (EURLP) in Rome, Italy.

Panel E. Since pigs can be hosts for different types of parasites, cross reactions in the use of the Trichinella ChLIA should be excluded. To assess the extent of cross reactivity, 30 samples with nematode and protozoan antigens were tested with the Trichinella ChLIA. This selection represents the most prevalent parasites in pigs. Each sample consisted of 100 g muscle tissue from the diaphragm pillar of non-infected domestic pigs spiked with 1 mg crude antigen from *Trichuris suis* (n = 6), *Ascaris suum* (n = 6), *Toxoplasma gondii* (n = 6), *Strongyloides papillosus* (n = 6) or *Toxocara cati* (n = 6).

Sample preparation method

Each sample (100 *g*) was cooled to 2–8 °C and placed into the precooled stainless steel grinding beaker of a high-quality knife mill (Grindomix GM 200, Retsch, Germany). 200 mL precooled PBS buffer were added to the sample material. The material was grinded at 10,000 rpm for 5 min. We verified that both the chosen knife mill and the duration of shredding lead to successful destruction of the capsules of *Trichinella* larvae and release of antigens (Supplementary Fig. 1). Next, 2 x 2 mL of the sample were withdrawn from the grinding beaker. Sample A is intended for possibly required species determination by PCR and was frozen at −20 °C. Sample B was centrifuged at 20,000 x g at 4 °C for 10 min. Afterwards, the supernatant (mean amount: 1 mL) was transferred into a new reaction vessel. The supernatant corresponds to the tissue extract sample containing released excretory and secretory *Trichinella* antigens that was used as sample material for the detection of *Trichinella*-specific antigens (Fig. 1). The tissue extract sample (minimum 200 µL) was stored at +2 °C to +8 °C until it was loaded into the chemiluminescence instrument. The total time for sample preparation takes approximately 20 min.

Detection of Trichinella-specific antigens in larvae

To verify that *Trichinella*-specific antigens would be detected by the two antibodies used in the Trichinella ChLIA, indirect immunofluorescence tests (IIFT) were performed. For the IIFT, frozen sections of *T. spiralis* muscle larvae and encapsulated larvae in muscle tissue were placed at EUROIMMUN BIOCHIP-Mosaics. The incubation was carried out according to the Schistosoma mansoni IIFT incubation scheme (EUROIMMUN). Anti- *Trichinella spiralis* 18H1 and B7 (IgG) antibodies were generated by hybridoma technology (Appleton et al., 1988; Köhler and Milstein, 1975) and Phage Display (Smith, 1985), respectively, and incubated. The epitope of the 18H1 antibody is a tyvelose-containing tri- and tetra-antennary N- glycan, which is unique for Trichinella (Appleton et al., 1988; Ellis et al., 1997; Reason et al., 1994; Wisnewski et al., 1993). 18H1 binds to tyvelose(3,6-dideoxy-D- arabinohexose) on both secreted and surface glycoproteins (McVay et al., 2000). Tyvelose-bearing antigens are called TSL-1 (Denkers et al., 1990; Takahashi, 1997). TSL-1 antigens are produced in the granules of the stichocytes in the stichosome of larvae (Ortega-Pierres et al., 1996; Romarís et al., 2002). These antigens are the modulators for the host immune system and show a high immunogenicity due to the hydrophobic oligosaccharide chains with repetitive tyvelose and fucose (Ellis et al., 1997). Denkers et al. used immunoblotting to show that TSL-1 antigens migrate between 43 and 68 kDa under reduced conditions (Denkers et al., 1990). Immunoblotting analysis with anti-tyvelose mAbs has demonstrated that TSL-1 antigens include at least six different glycoproteins, ranging from 40 to 105 kDa (Arasu et al., 1995; Zarlenga and Gamble, 1990). We replicated these results for both 18H1 and B7 antibodies (data not shown). Based on our earlier experiments like Western Blot analysis and indirect immunofluorescence tests, it seems that the 18H1 and B7 antibodies bind to a very similar epitope. For immunofluorescence testing, the antibody-containing cell culture supernatants were diluted 1:120 and used with a volume of 30 µL for incubation of tissue sections. The antibodies were visualized with FITC-labelled conjugate under the EUROStar III Plus microscope (excitation filter: 450−490 nm, beam splitter: 510 nm, long pass cut-off filter: 515 nm).

Trichinella-specific antigen detection using ChLIA

Processing of the new Trichinella chemiluminescence immunoassay (ChLIA, from EUROIMMUN Medizinische Labordiagnostika AG, Lübeck, Germany) was fully automated with the random access chemiluminescence analysis instrument SuperFlex (PerkinElmer, Inc., USA). The ChLIA uses magnetic particles coated with capture antibodies (anti-*Trichinella spiralis* 18H1 antibody of class IgG) as solid phase (Fig. 1). The capture antibody-coated magnetic particles and the conjugate are incubated for 30 min with sample material (tissue extract), calibrator or quality control. The total time for antigen detection including pipetting, washing and chemiluminescence detection is approximately 45 min. The conjugate consists of acridinium- labelled anti-*Trichinella spiralis* B7 antibodies of class IgG (detection antibodies). During the incubation, the *T. spiralis* antigen from calibrator, control or sample material is bound by the capture antibody as well as the detection antibody. After five washing

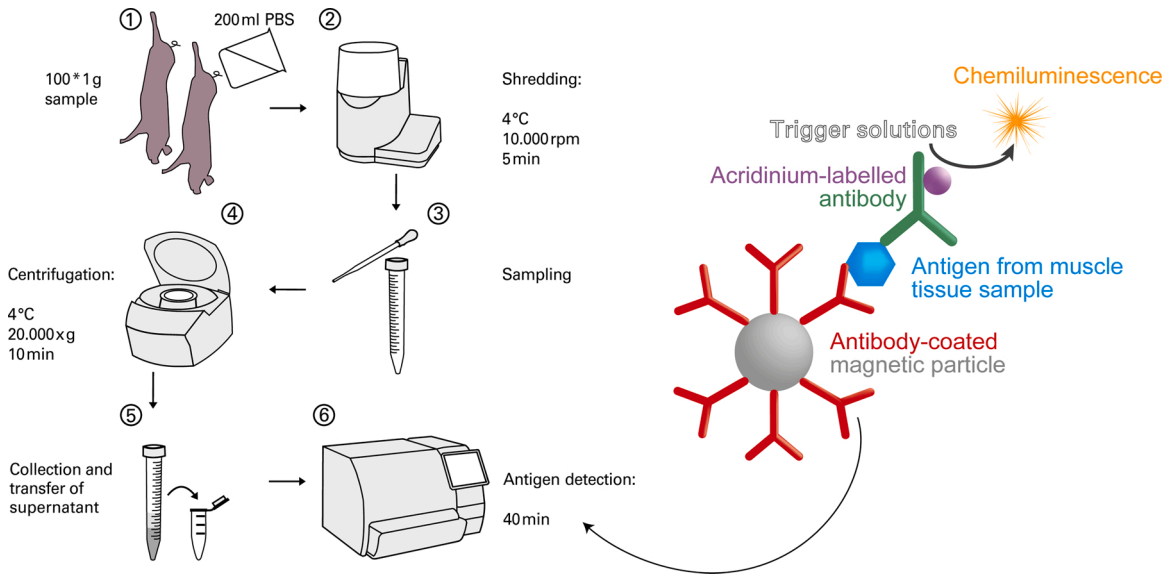

Fig. 1. Preparation method involving automated detection of specific *T. spiralis* antigens by a new chemiluminescence immunoassay.

steps, a trigger solution is added to induce a chemiluminescence reaction. The resulting light signal is given in relative light units (RLU). Within the given measurement range, the concentration is proportional to the amount of the bound analyte. The quantification of the concentration (ng/mL) is calculated automatically based on a lot-specific standard curve. The lot-specific upper threshold value of the reference range (cut-off) for non-infected animals recommended by EUROIMMUN is 1.7 ng/mL. Samples with an antigen concentration ≥1.7 ng/mL were considered *Trichinella*-positive, whereas those with an antigen concentration <1.7 ng/mL were considered *Trichinella*-negative. Antigen concentrations ≥1.7 ng/mL correspond to ≥300 RLU.

Analysis

All 215 samples were tested with the Trichinella ChLIA (Supplementary Material Table 6). Performance of the Trichinella ChLIA was evaluated and compared between panels. The detection limit was defined as the number of *Trichinella* larvae in 100 g pooled pork samples whose specific antigens were reliably detected by the new method. A two-sample *t*-test was used to compare RLU values of samples containing one larva between panel B and panel C.

Results

Detection of Trichinella-specific antigens in larvae

In Fig. 2 it can be seen that the cut larva from the tissue sections fluoresced strongly after incubation with the two anti- *Trichinella spiralis* antibodies. Individual fluorescence spots were seen on the capsule surface for both antibodies indicating that the antibodies had bound to proteins on the capsule surface (Fig. 2A,B). The 18H1 antibody reacted weakly but positively to all *Trichinella* components (Fig. 2A). In the incubation with the free *Trichinella* muscle larvae, both antibodies showed strong fluorescence on the cuticula (Fig. 2C,D). The inside of the larvae fluoresced with the B7 antibody. The 18H1 antibody reacted strongly both on the cuticula and inside the larva. The epitopes of the anti-*Trichinella spiralis* antibodies were therefore not only exposed on the surface of the intact larvae, but were also on and in the collagen capsule and inside the larva. The tissue sections were cross-sections of the larvae, in which the entire organs such as stichocytes, genitals,

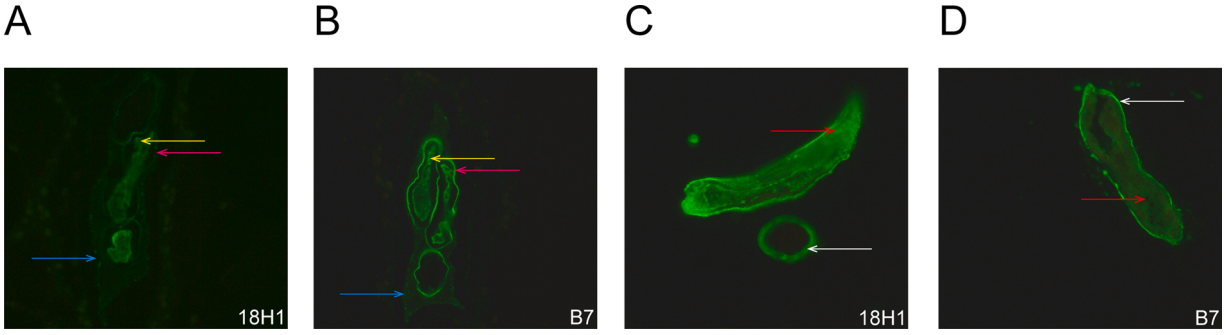

Fig. 2. Detection of *Trichinella*-specific antigens of *T. spiralis* larvae via IIFT. A-B) IIFT with anti-*Trichinella spiralis* 18H1 and B7 IgG antibodies on tissue section of larvae encapsulated in muscle tissue. Yellow arrows: cut larva, magenta arrows: cut collagen capsule, blue arrows: surface of collagen capsule. C-D) IIFT with anti-*Trichinella spiralis* 18H1 and B7 IgG antibodies on tissue section of free muscle larvae. White arrows: cuticula of larva, red arrows: interior of larva (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

Table 2
Results of the Trichinella ChLIA tested with 125 muscle tissue samples from panel A, panel B and panel C. Panel A comprises samples from non-infected pigs (n = 37). Panel B consists of samples from non- infected pigs spiked with different amounts of *Trichinella* larvae without collagen capsules (n = 56). Panel C contains samples from experimentally infected pigs including *Trichinella* larvae encapsulated in collagen (n = 32).

n = 125		Precharacterised samples	
		positive	negative
Trichinella ChLIA	positive	85	0
	negative	3	37

oesophagus and intestine could not be imaged.

Trichinella-specific antigen detection using ChLIA

Detailed results of all samples are listed in Supplementary Material Table 1.

All samples from non-infected pigs (panel A) were negative in the Trichinella ChLIA, indicating a specificity of 100 % (Table 2). The range of RLU values was 103–251 (Fig. 3A).

Of the 56 samples tested from panel B, 53 samples were positive in the Trichinella ChLIA (Supplementary Table 1). Three of the spiked samples in panel B lacking the naturally existing collagen capsule surrounding the *T. spiralis* larva including excretory and secretory antigens were not detected by the Trichinella ChLIA (Table 2). Regarding the number of spiked larvae in panel B, 99 % of 302 larvae were indicated by the Trichinella ChLIA. The analytical sensitivity of the Trichinella ChLIA in 100 g samples of artificially generated pooled reference sample material spiked with 3 *T. spiralis* larvae represents a detection level of 0.03 larvae per gram (lpg) of meat. The Trichinella ChLIA detected *Trichinella*-specific antigens in artificially generated samples spiked with a minimum of three larvae. Among panel B, the ranges of RLU values were 276–338 for one larva, 422–529 for three larvae, 808–1237 for four larvae, 838–962 for five larvae, 1134–1239 for ten larvae, and 1769–1958 for fifteen larvae (Fig. 3B).

We observed that samples from panel B containing four *T. spiralis* larvae had higher and more broadly spread RLU values (mean ± standard deviation = 1020.8 ± 122.7) than samples containing three (481 ± 39.1) or five (917.7 ± 50.9) *T. spiralis* larvae (Fig. 3B). The samples containing four larvae were non-frozen and hence larvae were alive in contrast to the other samples containing dead larvae (Table 1).

All samples including the collagen capsule (panel C) were positive in the Trichinella ChLIA (Table 3). The detection limit of the Trichinella ChLIA is one *T. spiralis* larva in 100 g muscle tissue from experimentally infected pigs (= 0.01 lpg). Among panel C, the ranges of RLU values were 362–531 for one larva and 944–1119 for three larvae (Fig. 3C).

The RLU of samples containing one larva in panel C (n = 22, 425 ± 44.1) were significantly higher ($p < 0.001$, $t(28) = 7.22$) than those in panel B (n = 8, 306.6 ± 22.1) indicating that the Trichinella ChLIA yielded higher RLU values for samples from experimentally infected pigs including the collagen capsule surrounding the *T. spiralis* larva (Fig. 3B,C).

The overall accuracy of the Trichinella ChLIA regarding the results from all tested samples from pigs (panel A, B, C) was 97.6 % (95 % confidence interval (CI): 93.2–99.5 %, (Table 2). Furthermore, the results revealed a positive predictive value of 100 %, a negative predictive value of 92.5 % (95 % CI: 80.2–97.4) as well as a sensitivity of 96.6 % (95 % CI: 90.4–99.3 %) and a specificity of 100 % (95 % CI: 90.5–100 %) of the Trichinella ChLIA. Considering only samples from experimentally infected pigs (panel C) the sensitivity of the Trichinella ChLIA was 100 % (95 % CI: 89.1–100 %, Table 3).

All samples from panel D were positive in the Trichinella ChLIA. Hence, the Trichinella ChLIA detected samples spiked with larvae from different *Trichinella* species with a sensitivity of 100 % (Table 4).

All samples from panel E were negative in the Trichinella ChLIA showing that the Trichinella ChLIA did not cross-react with the different nematode and protozoan antigens (Table 5).

Discussion

The present study introduces a novel preparation method for meat samples, accompanied by automated detection of specific *T. spiralis* antigens with a newly developed immunoassay based on chemiluminescence. On the basis of 215 tested samples, the new approach for the detection of *T. spiralis* antigens yielded high specificity, high sensitivity and an overall accuracy of 97.6 %, while cross-reactivity with other parasites was not observed. The lowest theoretically achievable detection level, namely one *T. spiralis* muscle larva in 100 g pooled sample, was achieved by the new Trichinella ChLIA method. Evidence from this study suggests that meat inspection can be accelerated, simplified and standardised by combining the novel sample preparation method with automated antigen detection.

Detection of Trichinella-negative samples

All precharacterised samples from non-infected pigs were negative in the Trichinella ChLIA, indicating a specificity of 100 %.

The outcome of the Trichinella ChLIA was in perfect agreement with the precharacterisation by the digestion method.

Detection of Trichinella-positive samples

Regarding validity of the Trichinella ChLIA, 85 of 88 *Trichinella*-positive samples were identified correctly. Three samples that were generated artificially by spiking muscle tissue samples with one *T. spiralis* larva (panel B) were not detected by the Trichinella ChLIA. The detection limit of the Trichinella ChLIA is three larvae in 100 g artificially generated reference sample material. A possible explanation for this might be that excretory and secretory antigens are missing in these samples because they lack the naturally existing collagen capsule surrounding the *T. spiralis* larvae that includes the excretory and secretory antigens. In contrast, *T. spiralis* larvae in samples from experimentally infected pigs have a collagen capsule and thus contain more *Trichinella*-specific excretory and secretory antigens. Consequently, more antigens can be detected by the ChLIA (panel C).

The most important observation was that the Trichinella ChLIA detected antigens of one *T. spiralis* larva in 100 g sample material (= 0.01 larvae per gram) from experimentally infected pigs. When evaluating the performance of the Trichinella ChLIA based on the experimentally infected samples in panel C only, the sensitivity was 100 %. Since these samples from experimentally infected pigs resemble samples subjected to meat inspection in slaughterhouses, one can assume a comparably high sensitivity of the assay when testing muscle tissue from pigs with natural *T. spiralis* infection. The negative predictive value was 92.5 % based on the samples in panels A, B and C indicating that if the Trichinella ChLIA yielded a negative result, the sample is almost certainly free of *Trichinella* larvae. Results of the Trichinella ChLIA depicted in Fig. 3B-C revealed an approximately linear increase of RLU value with increasing number of *T. spiralis* larvae in the sample. The Trichinella ChLIA yielded larger RLU values in samples with one larva from experimentally infected pigs than in samples spiked with one larva. This evidence suggests that the Trichinella ChLIA not only indicates absence or presence of larval antigens, but also provides an estimation of the amount of larval antigens allowing inference of number of contained larvae. Generally, the size of *T. spiralis* larvae and hence their antigen content is subject to natural variation, which could lead to variability in the detection accuracy of the immunoassay. Nevertheless, the ChLIA method could detect 100 % of the

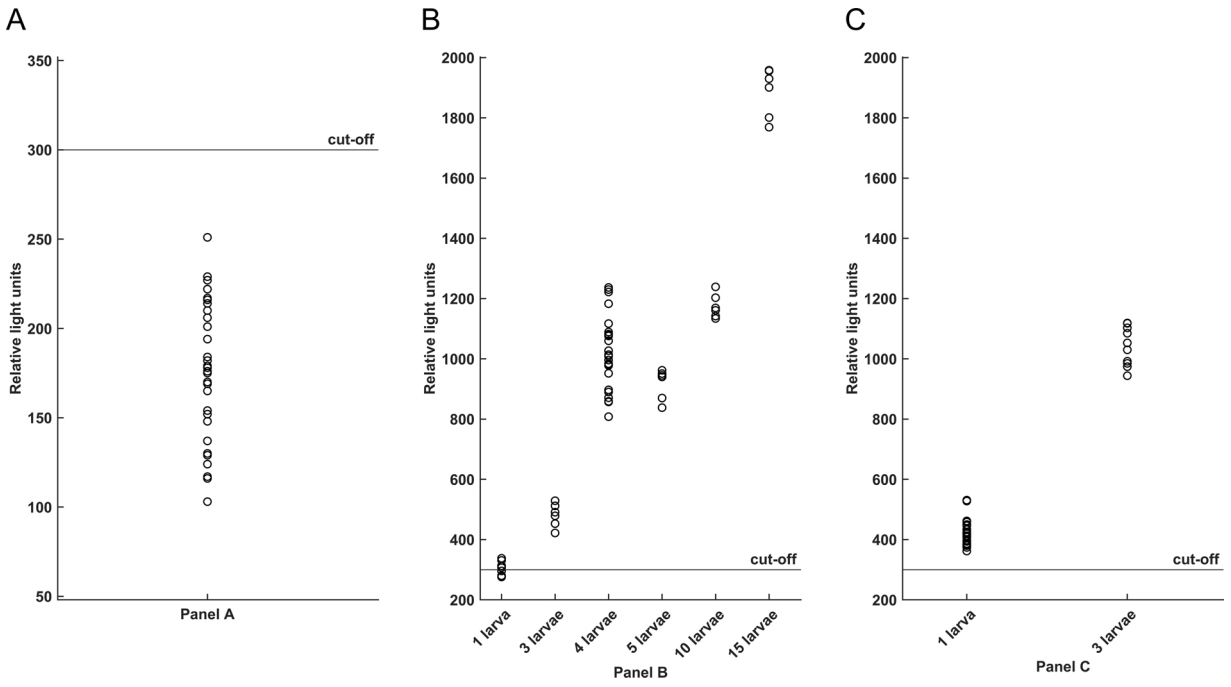

Fig. 3. Results of Trichinella ChLIA. A) Samples from non-infected pigs (n = 37, panel A). B) Samples from non-infected pigs spiked with different amounts of *Trichinella* larvae without collagen capsules (n = 56, panel B). C) Samples from experimentally infected pigs including *Trichinella* larvae encapsulated in collagen (n = 32, panel C). Values above the cut-off at 300 RLU correspond to an antigen concentration ≥1.7 ng/mL and were evaluated as positive.

muscle samples from experimentally infected pigs with a larval density of 0.01 larvae per gram. Surprisingly, samples from panel B containing four *T. spiralis* larvae showed higher and more broadly spread RLU values than samples containing three or five *T. spiralis* larvae (Fig. 3B). An explanation might be that the samples containing four larvae were non-frozen in contrast to the other samples containing frozen larvae (Table 2). Whether the additional gelatine inside the samples from ANSES had an effect on the antigen detection remains a question for future investigations. Taken together, whether the larvae were dead or alive did not influence the correct performance of the Trichinella ChLIA.

Considering samples spiked with larvae from different *Trichinella* species (panel D) the sensitivity of the Trichinella ChLIA was 100 %. Therefore, the new method can be used to detect also other *Trichinella* species in muscle tissue samples of pigs. Furthermore, the Trichinella ChLIA specifically detects antigens of *Trichinella* species and showed no cross reactions with the different nematode and protozoan antigens from panel E, which indicates that a false positive test result is highly unlikely (Table 5). In future investigations, the new method should be tested with muscle tissue samples from pigs infected with even more different *Trichinella* species and bacteria and parasites other than *Trichinella* to fully rule out cross reactions.

In summary, the new sample preparation method combined with the newly developed Trichinella ChLIA reliably detected a single *T. spiralis* larva in 100 g muscle tissue sample from pigs with *Trichinella* infection and therefore its accuracy is comparable to the gold standard.

Table 3
Results of the Trichinella ChLIA tested with 32 muscle tissue samples from experimentally infected pigs including *Trichinella* larvae encapsulated in collagen (panel C).

n = 32		Samples from *Trichinella*- positive pigs
Trichinella ChLIA	positive	32
	negative	0

Table 4
Results of the Trichinella ChLIA tested with 60 muscle tissue samples spiked with larvae from different *Trichinella* species (panel D).

n = 60		Samples with larvae from different *Trichinella* species
Trichinella ChLIA	positive	60
	negative	0

Limitations of the new method

With the proposed method it is not possible to determine the exact number of larvae in a *Trichinella*-positive sample, which is a major limitation. For application in meat inspection, it is solely relevant whether a sample is *Trichinella*-positive or negative, whereas knowing the exact larval burden in a *Trichinella*-positive sample is inconsequential. However, with the new method it is possible to estimate the number of larvae from the RLU value. But such an approach would need to be validated in a large panel comprising samples from experimentally infected pigs.

It was not tested whether the proposed method would function with less than 100 g sample material. We expect that the shredding with the knife mill might not perform properly if the sample weights less than 50 g. This limitation may be a concern for small veterinary laboratories,

Table 5
Results of the Trichinella ChLIA tested with 30 muscle tissue samples spiked with different nematode and protozoan antigens (panel E).

n = 30	Trichinella ChLIA	
	positive	negative
Trichuris suis	0	6
Ascaris suum	0	6
Toxoplasma gondii	0	6
Strongyloides papillosus	0	6
Toxocara cati	0	6

but not for slaughterhouses, where samples are always pooled. Thus, the implementation of the newly proposed method might be most interesting for large slaughterhouses.

Other shredding methods and grinders were tested but did not yield satisfactory results (results not shown).

Samples need to be prepared freshly and can be used for testing within four hours after preparation. If a sample is *Trichinella*-positive during meat inspection, it must be sent to a reference laboratory for species determination. We confirmed (results not shown) that it is possible to successfully determine the *Trichinella* species by PCR using the shredded material if it had been frozen directly after sample preparation (sample A as described in section 2.2).

Samples from experimentally infected species other than pigs were not tested in this study, however, the accuracy of the method was shown for *Trichinella*-negative samples from horses and wild boars (not shown) and we expect that the method performs equally well in *Trichinella*-positive samples from game. A future study will access the performance of the new method based on larger panels including samples from horses and game.

Comparison of the newly developed method and the gold standard

There are a number of important differences between the newly developed meat inspection method and the current gold standard.

Regarding sample processing, the new method differs from other methods as it involves mechanical shredding of the muscle tissue as well as any contained larvae instead of digestion. In contrast to the digestion method (Forbes and Gajadhar, 1999), preparation of the sample by the new method does not involve handling of dangerous acids (hydrochloric acid) or free infectious larvae, which improves work safety.

Disadvantages of the digestion method are the use of expensive pepsin (which is extracted from pig stomach), as well as the temperature and time dependence during the digestion. If the digestion is too short or too long or the temperature is too high or too low, muscle tissue, collagen capsule, connective tissue or tendons may not be digested properly, causing false negative results if the larva is not found under the microscope (Riehn et al., 2013). Manual microscopy for determination of the number of larvae requires training and experience, for example because tissue fibres can be confused with larvae (Rossi and Poizo, 2008). Avoiding this, the new method provides an objective and automatable evaluation by the chemiluminescence analyzer. There is no need for personnel skilled in recognising *Trichinella* larvae under the microscope. The importance of objective result assessment becomes obvious considering that in a documented case the personnel performing the test using the digestion method had never seen a *Trichinella* larva before (Marucci et al., 2009).

The major disadvantage of the digestion method is its time-consuming sample preparation. In large slaughterhouses, however, a fast diagnosis is of high relevance, as the meat processing must be suspended for the duration of meat inspection. The total time from sample processing to results takes about 60 min in the newly developed method instead of about 90 min when using the digestion method. The reason for this saving of time is the omission of digestion and sedimentation steps and the overall reduction of working steps in the new method. Including cleaning of the utensils the hands-on time for personnel is about 30 min for the digestion method and about 15 min for the Trichinella ChLIA. Utilising *T. spiralis* antigen detection with the fully automated chemiluminescence analyzer, twelve pooled samples of 100 × 1 g muscle tissue can be processed in parallel, which results in a capacity of testing up to 1200 pigs in 40 min for specific *T. spiralis* antigens. Another advantage of the automation with the chemiluminescence analyzer SuperFlex is its random access functionality, which allows loading of additional samples at any time without stopping the run or waiting until it is finished. However, the implementation of the newly proposed method might be most interesting for large slaughterhouses.

Of note, accurate performance of laboratory detection of *Trichinella* larvae crucially depends on correct execution of the respective method of choice as well as usage of appropriate material and clean utensils (Gajadhar et al., 2019; Gajadhar and Forbes, 2002). As an example, with the digestion method, the use of a small domestic blender with a plastic container instead of a professional blender with a glass container and the use of a partially obstructed sieve have been documented to lead to failure of testing (Marucci et al., 2009).

Outlook

Individual control of carcasses during meat inspection incurs high costs in relation to the large proportion of *Trichinella*-negative cases. Thus, implementation of convenient and cost-efficient methods is desired. The total time from sample processing to results takes about 65 min in the newly developed method instead of about 90 min when using the digestion method. The reason for this saving of time is the omission of digestion and sedimentation steps and the overall reduction of working steps in the new method. Including cleaning of the utensils the hands-on time for personnel is about 30 min for the digestion method and about 15 min for the Trichinella ChLIA. Realistically, the newly proposed method would substantially accelerate meat inspection while adding the advantages of both objective result evaluation and improved work safety.

Nowadays, the demand for organic meat from animals raised with methods that are sustainable and sensitive to animal welfare is increasing (Murrell, 2016). However, free- range pig production involves varying degrees of outdoor exposure, bearing the risk of spillover of *T. spiralis*, (in Europe also *T. britovi* and *T. pseudospiralis*) from wild animal reservoirs (Burke et al., 2008). For example, a higher risk of infection of domestic pigs was confirmed for outdoor farming compared to indoor farming in areas where *Trichinella* is endemic (Nöckler et al., 2004). Therefore, meat safety testing might become even more relevant in future to prevent transmission of trichinellosis to humans.

Evaluation of the proposed method in a quality assessment scheme involving several reference laboratories of the EU constitutes the natural progression of this work and has already been initiated. Future research could examine living encapsulated *T. spiralis* larvae with the new method. We expect that the RLU values would be very high.

Conclusion

Current EU regulations on testing for the presence of *Trichinella* spp. larvae require a detection sensitivity of at least one muscle larva per gram of meat. This study has shown that the introduced sample preparation method combined with the newly developed *Trichinella*-specific antigen detection method using a chemiluminescence immunoassay reliably meets this criterion. Detection of specific *T. spiralis* antigens is a further development and has the major advantage of allowing objective evaluation of larval burden. Furthermore, the fully automated processing of the Trichinella ChLIA on the chemiluminescence analysis instrument allows easy and efficient performance. Altogether, the novel meat inspection method provides a solid alternative to the current gold standard, promising accurate and objective meat safety surveillance.

Declaration of competing interest

JB, SO, MM, VBL and EL are employees of EUROIMMUN Medizinische Labordiagnostika AG. Patent applications IN17208994.8, BR102018076467−5, CA3,027,972, CN109946448A, EP18209802.0, RU2018145047 and US16/225,007 have been filed for the protection of the technology by EUROIMMUN Medizinische Labordiagnostika AG.

CRediT authorship contribution statement

Jana Braasch: Methodology, Validation, Investigation, Writing - review & editing. **Stefanie Ostermann:** Resources, Supervision, Project administration, Writing - review & editing. **Monika Mackiewicz:**

Investigation, Writing - review & editing. **Catherine Bardot:** Resources, Writing - review & editing. **Caroline Pagneux:** Resources, Writing - review & editing. **Viola Borchardt-Lohölter:** Formal analysis, Visualization, Writing - original draft, Writing - review & editing. **Erik Lattwein:** Conceptualization, Supervision, Writing - review & editing.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vpoa.2020.100033.

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