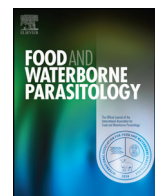




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Validation of a new commercial serine protease artificial digestion assay for the detection of *Trichinella* larvae in pork

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ARTICLE INFO

Article history:

Received 26 October 2017

Received in revised form 29 March 2018

Accepted 6 April 2018

Available online 9 April 2018

Keywords:

Pork

Food safety

PrioCHECK *Trichinella* AAD assay

Pepsin/HCl digestion method

Diagnostic assay

Validation

ABSTRACT

Trichinella is a zoonotic nematode parasite transmitted by the ingestion of raw or under-cooked meat. Control of the parasite is essential to facilitate public health and trade in products from susceptible food animals, including pork and horse meat. The standard method for detecting *Trichinella* muscle larvae uses pepsin enzyme and hydrochloric acid (HCl) in an artificial digestion procedure. A new artificial digestion assay using serine protease was recently developed and commercialized (PrioCHECK™ *Trichinella* AAD) for the detection of *Trichinella* larvae in the muscle of infected animals. The assay uses no hazardous substances such as HCl or pepsin. Activation of the enzyme requires an elevated digestion temperature of 60 °C which kills the parasite and reduces the risk of contaminating the environment with *Trichinella*. Compared to the pepsin/HCl method, digestion time for the PrioCHECK *Trichinella* AAD assay is reduced by a third. A recent study demonstrated these features of the new assay and its suitability for digesting various muscles from domestic and wild animals. To further validate the assay's performance relative to the conventional pepsin/HCl digestion method several comparative studies were conducted using samples from different muscle sites spiked with low levels of encapsulated first stage *Trichinella* larvae (L1). Multiple muscle samples were collected from diaphragm, tongue, masseter, and loin of 3–4 month old pigs. Samples were spiked with 3, 4, 5, or 25 *Trichinella spiralis* L1. A total of 320 meat samples of 100 g each were used to compare the diagnostic proficiency of the PrioCHECK *Trichinella* AAD assay with the pepsin/HCl digestion method. Comparative and validation data produced from these studies showed that both methods are capable of consistently detecting *Trichinella* in 100 g samples which contained as few as 3 L1 or 0.03 larvae per gram of meat. Overall, the PrioCHECK *Trichinella* AAD assay performed satisfactorily according to international guidelines of the World Organization for Animal Health (OIE), European Union (EU) and International Commission on Trichinellosis (ICT) for the detection of *Trichinella* infection in pork.

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1. Introduction

Trichinella is a meat-borne nematode parasite that infects many species of animals. It is prevalent in wild carnivores and omnivores, with potential for spillover between susceptible species of wildlife and livestock (Pozio, 2015). Historically there has been a close association between *Trichinella spiralis* and pigs, but other species of the parasite can also infect porcine hosts (Pozio and

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guidelines of ICT and ISO 17025 (Gajadhar and Forbes, 2002; ICT, 2014). The analysts had many years of experience conducting *Trichinella* testing for routine diagnostics and research on pork, horse meat and meat of wild animals.

The commercial serine digestion assay (PrioCHECK *Trichinella* AAD, Thermo Fisher Scientific) for the detection of *Trichinella* L1 in muscle samples employs a serine protease enzyme at 60 °C (± 2 °C) for 20 min to digest muscle tissue and kill released L1 that are isolated and concentrated in subsequent sedimentation processes and detected by stereo-microscopic examination. The assay was used according to the manufacturer's instructions, with the following exceptions, based primarily on the previous study by Konecni et al., 2017. In the present study, 1000 mL of water was used to rinse the remnants in the digestion beaker (300 mL rinse) and the sieve (700 mL rinse) into the separatory funnel. This resulted in a final volume of 3 L for a primary 30 min sedimentation in the separatory funnel. A 4 L separatory funnel was used to accommodate this 3 L volume. Further details of the assay were followed as recently published (Konecni et al., 2017).

The method used for comparative purposes was the traditional magnetic stirrer pepsin/HCl artificial digestion method that is widely used in many countries for the detection of L1. A solution consisting of 1% liquid pepsin (2800–3500 FC units/mg, American Laboratories Inc., Omaha, NE, USA) and 1% HCl at 44 °C (± 3 °C) for 30 min was used for digestion. Isolation and concentration of L1 were conducted by sequential sedimentation in an apparatus with double separatory funnels as previously described (Forbes and Gajadhar, 1999; OIE, 2017). The main differences between the pepsin/HCl digestion method and the PrioCHECK *Trichinella* AAD assay are listed in Table 2.

The final sediment collected from each of the two methods was transferred into a gridded Petri dish and left undisturbed for at least 1 min to allow settling of larvae to the bottom. A stereo-microscope at 10 \times or 16 \times magnification was used to systematically examine the entire gridded dish for the presence of *Trichinella* larvae. If necessary, a higher magnification was used to differentiate L1 from artifacts (Gajadhar et al., 2009; ICT, 2014). During the study, 10 spiked samples had to be replaced from the original source and retested due to inadvertent freezing during storage or spillage from a malfunctioning stop cock on a separatory funnel.

2.4. Statistical analysis

Mean percent larval recoveries for each spike level and muscle site were obtained by dividing the total number of larvae detected for each set of samples by the number of samples. Standard error of the mean was calculated for each sample set. Statistical significance was determined using one way ANOVA and the Tukey's multiple comparison post-hoc test; a *P*-value < 0.05 was deemed significant. All statistical analyses were performed using Prism version 4 software.

3. Results

3.1. Overall results

Both *Trichinella* testing procedures used in this study performed satisfactorily and without any problems. Occasionally, an additional clarification step was required in both methods to remove debris from the sediment that might obscure the detection of larvae during microscopic examination; this requirement was mostly for the PrioCHECK *Trichinella* AAD assay (data not shown). Motile as well as non-motile larvae were recovered by the pepsin/HCl method whereas only non-motile L1 were recovered by the PrioCHECK *Trichinella* AAD assay (data not shown).

Both methods detected *Trichinella* larvae in all spiked samples, regardless of the number of L1 used for spiking or the muscle site (Figs. 1–4). None of the samples tested by either assay yielded larval recoveries >100% of the spiked numbers. The numbers of larvae recovered by the pepsin/HCl method were usually slightly higher, although there was no statistically significant difference between assays within spike level or muscle site (Figs. 1, 2, and 4). For both methods, the minimum mean recovery rates by spike number or muscle site were always >80%, except for the 75.0% and 70.0% recoveries by the PrioCHECK *Trichinella* AAD assay from diaphragm samples spiked with 4 and 5 L1, respectively. When results were combined by muscle sites or spike level, samples spiked with 25 L1, and samples of masseter yielded the highest mean recoveries of 96.0% and 96.6%, respectively, by the pepsin/HCl digestion method (Fig. 1a, b). The PrioCHECK *Trichinella* AAD assay had a marginally higher mean percent recovery from samples containing 3 L1 and samples of tongue, whereas the mean recoveries by the pepsin/HCl digestion method were higher for all other sample sites and spike levels (Fig. 1a, b).

Table 2

Basic differences and similarities between the standard pepsin/HCl digestion method and the serine assay PrioCHECK *Trichinella* AAD, as used in this study for the detection of *Trichinella* larvae in meat samples.

Method	Enzyme	Digestion temperature	Digestion time	Digestion volume	Rinse volume added to settling funnel	Larvae recovered
PrioCHECK <i>Trichinella</i> AAD	Serine protease	60 °C (± 2 °C)	20 min	2 L	1 L	Dead
Standard pepsin/HCl digestion	Pepsin protease	44 °C (± 3 °C)	30 min	3 L	1 L	Live/dead

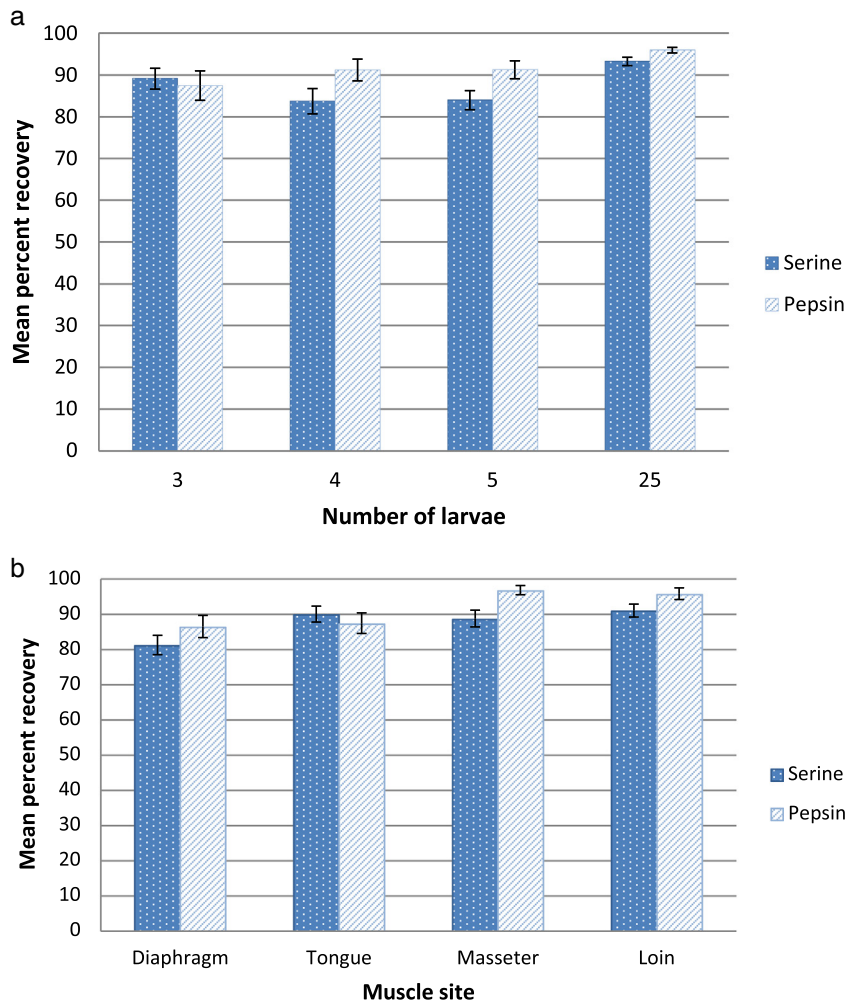


Fig. 1. a. Mean percent larval recovery and standard error, by spike level for digestion by serine compared to pepsin/HCl using combined results obtained from a total of 160 pork samples per method. The samples consisted of diaphragm, tongue, masseter and loin that were each spiked with 3, 4, 5, or 25 encapsulated larvae of *Trichinella spiralis*. b. Mean percent larval recovery and standard error, by muscle site for digestion by serine compared to pepsin/HCl using combined results from 160, 100 g pork samples per method. Samples of diaphragm, tongue, masseter and loin were each spiked with 3, 4, 5, or 25 encapsulated larvae of *T. spiralis*.

3.2. Recovery of larvae according to spike level and tissue type in samples

The highest and most consistent mean rates of recovery (>90%) were obtained by both the PrioCHECK *Trichinella* AAD assay and the pepsin/HCl digestion method from all four muscle sites that were spiked at the 25 L1 level (Fig. 2a, b). Samples from the four muscles spiked with 3 L1 consistently yielded $\geq 86\%$ recovery by the PrioCHECK *Trichinella* AAD assay, while the recovery by the pepsin/HCl method was lower (>80%) and more variable for tongue and diaphragm samples that were spiked at the same level. The percent recoveries by both methods for samples containing 4 or 5 L1 were generally reduced and more variable (Fig. 2a, b). Within each spike level, the diaphragm yielded the lowest mean percentage of L1 for the PrioCHECK *Trichinella* AAD, while with the pepsin/HCl method it was either tongue or diaphragm. However, the only mean values below 80% were the 70% and 75% recoveries obtained using the PrioCHECK *Trichinella* AAD for diaphragm samples containing 4 or 5 L1, respectively (Figs. 2a, 4a). For each method, these observed differences amongst muscle sites within spike level or spike levels within each muscle site were not statistically significant. The mean percent recovery of larvae by the PrioCHECK *Trichinella* AAD assay and the pepsin/HCl method according to muscle sites, and for the four spike levels, are shown in Fig. 4a and b, respectively.

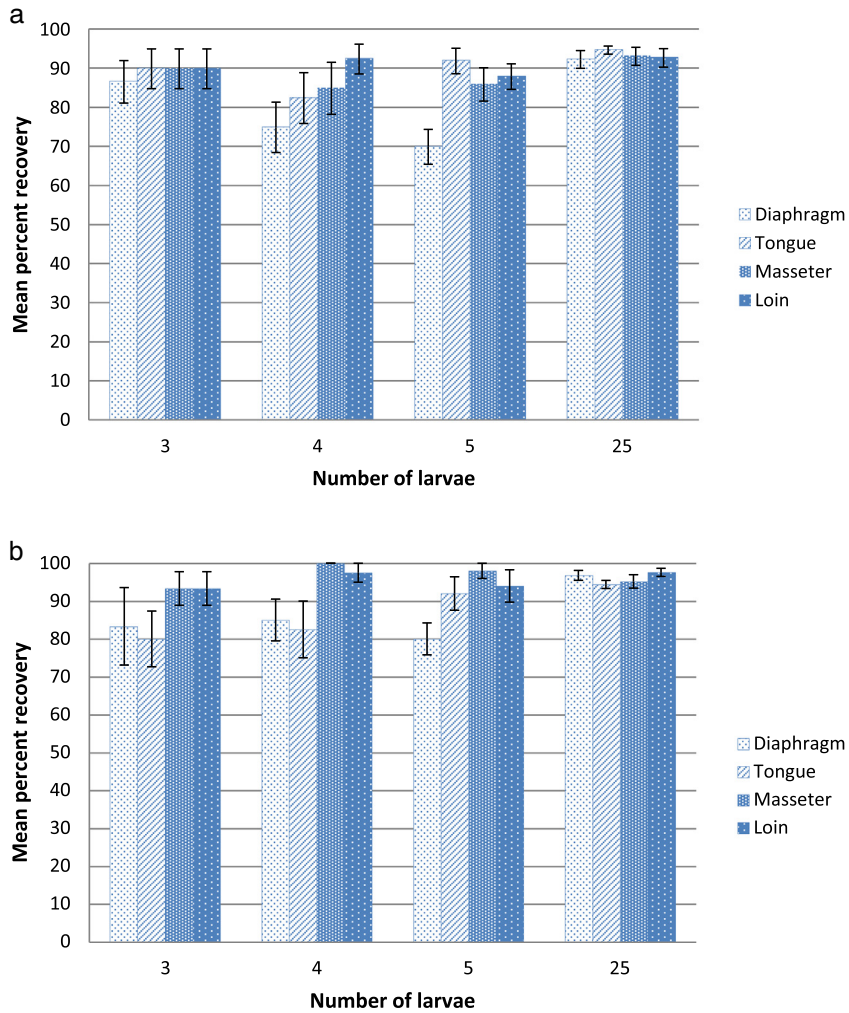


Fig. 2. a. Results of the serine digestion PrioCHECK Trichinella AAD assay showing mean percentage and standard error of larval recovery by spike level from samples of pork diaphragm, tongue, masseter and loin that were spiked with 3, 4, 5 or 25 *T. spiralis* larvae. b. Results of pepsin/HCl digestion method showing mean percentage and standard error of larval recovery by spike level from samples of pork diaphragm, tongue, masseter and loin that were spiked with 3, 4, 5 or 25 *T. spiralis* larvae.

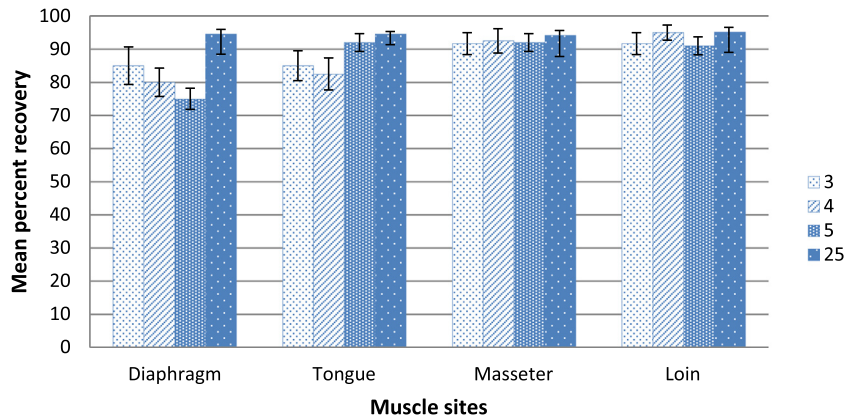


Fig. 3. Mean percentage and standard error of larval recovery by muscle site (diaphragm, tongue, masseter and loin) and spike levels (3, 4, 5 or 25 L1), using combined results of both digestion methods (pepsin/HCl and PrioCHECK Trichinella AAD).

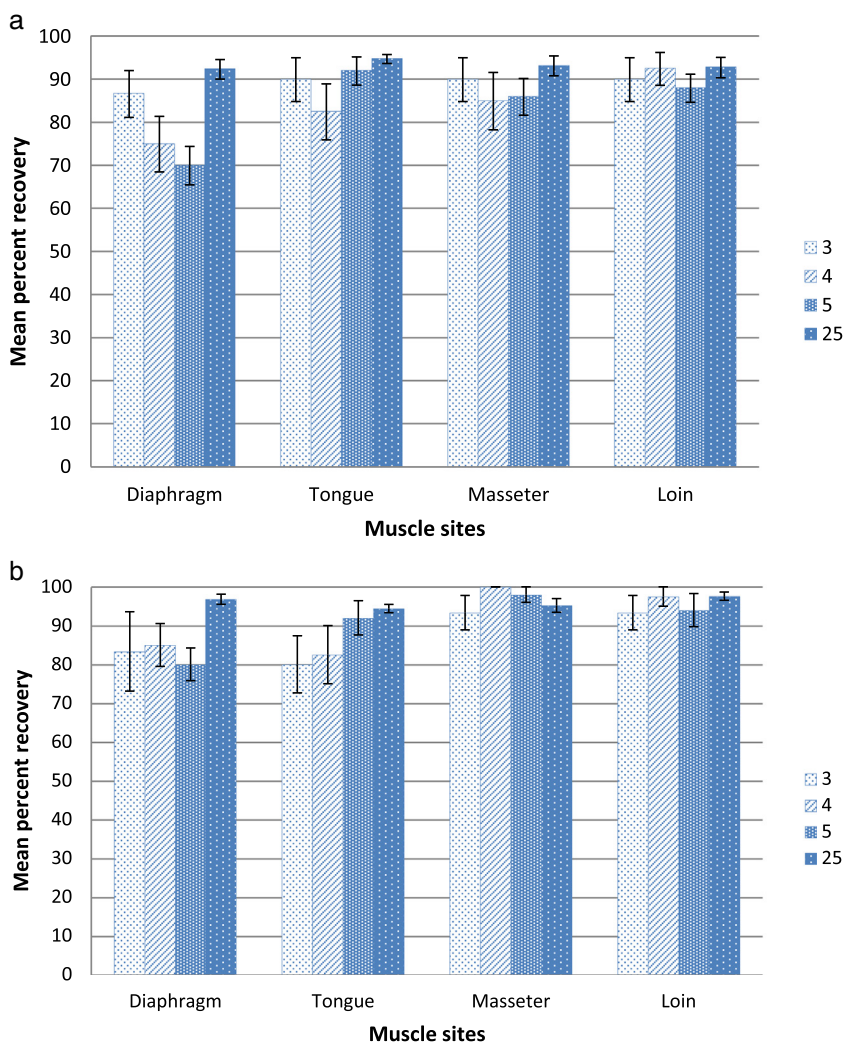


Fig. 4. a. Results of the serine assay PrioCHECK Trichinella AAD showing mean percentage and standard error of larval recovery by muscle site (diaphragm, tongue, masseter and loin) and number of spiked larvae (3, 4, 5, or 25 L1). b. Results of pepsin/HCl method showing mean percentage and standard error of larval recovery by muscle site (diaphragm, tongue, masseter and loin) and number of spiked larvae (3, 4, 5, or 25 L1).

3.3. Recovery of larvae according to combined results of both assays

The combined results of both methods yielded mean percent of larval recoveries that were generally lower and more variable for diaphragm and tongue samples with all levels of spikes except 25 L1 (Fig. 3), although this variability within muscle sites was not statistically significant. Except for diaphragm samples spiked with 5 L1, all muscle sites containing 5 or 25 L1 consistently yielded the highest mean percentages of larval recovery (Fig. 3).

4. Discussion

This report describes an experimental study for the validation of an alternative artificial digestion method to detect *Trichinella* in pork muscle samples. As far as we know, it is the largest such study to date using proficiency samples that are recommended for QA purposes (ICT, 2014). A total of 320 digestion assays were performed on 100 g pork samples derived from four muscle sites, including tongue, diaphragm, and masseter which are well recognized as preferential sites for *Trichinella* infection and for diagnostic sampling. The loin was also used because of ease of acquisition from carcasses and retail markets, and because it is widely used by consumers.

The present study used artificially prepared proficiency samples to ensure precise numbers of larvae in the samples tested, unlike many other validation studies that have used naturally infected meat containing uncontrolled numbers of larvae (Gamble, 1996; Forbes and Gajadhar, 1999; Leclair et al., 2003). Spike levels included 3, 4 and 5 L1 in accordance with the ICT recommendation of 3–5 L1 per proficiency sample for validating digestion methods (ICT, 2014). Also, identification of positive samples

harbouring ≥ 1 larva per gram is generally accepted to demonstrate sufficient diagnostic sensitivity for the lowest larval burdens that may cause clinical disease in humans (Dupouy-Camet and Bruschi, 2007). The individual 100 g samples spiked with 3 L1 in this study represent a detection level (analytical sensitivity) of 0.03 larvae per gram of meat. Detection of 3 or 5 larvae in 100 g samples represents a much higher level of sensitivity than the 1 larva per gram recommended by regulatory and public health authorities for trade and food safety, respectively (ICT, 2016; OIE, 2017). However, it should be noted that the sensitivity of detecting an infected pig (diagnostic sensitivity) depends on the size of the corresponding sample tested, and is therefore lower for digestion assays of 100 g of pooled samples from multiple carcasses.

To further demonstrate the performance of the PrioCHECK Trichinella AAD assay and to validate its diagnostic capability for detecting *Trichinella* in pork, replicate samples were tested by the standard pepsin/HCl digestion method for comparison. Overall, the results of both methods indicated reliable detection of the parasite in every type of muscle tested and at all spike levels. This confirms previous data that the PrioCHECK Trichinella AAD assay adequately digests and isolates larvae from a variety of muscles, including tongue which has a high proportion of connective tissue. Within spike levels, the mean larval recovery between the two methods were not significantly different; the PrioCHECK AAD Trichinella assay had a slightly higher (2%) mean recovery rate for samples with a larval load of 0.03 LPG (spiked with 3 L1), whereas mean recovery rates for pepsin/HCl were slightly higher (3–5%) for samples containing 0.04–0.25 LPG (spiked with 4, 5, or 25 L1).

When mean larval recoveries between muscle sites were compared for each method, there was no significant difference. However, as shown by the combined results of both digestion methods (Fig. 3), the highest rates of larval recovery were consistently obtained from loin, as well as masseter samples. This was probably due to the lesser amounts of fat and fascia in this muscle site that facilitated more complete digestion, and a commensurate reduction in obstructive non-digestible tissues on the sieve. However, it should be noted that, regardless of muscle site, both digestion methods usually resulted in negligible amounts of non-digestible materials (data not shown) that were well under the 5% acceptable EU-recommended limit (European Commission, 2015). Nevertheless, as noted in this study and previously, additional sediment clarification steps may be required more often for the PrioCHECK Trichinella AAD assay than the pepsin/HCl method (Konecni et al., 2017).

Based on previous studies of naturally infected animals, tongue and diaphragm have generally yielded the highest larval recoveries and are thus recommended as preferred sampling sites for *Trichinella* testing (Gamble et al., 2000; Kapel et al., 2005). However, it is not surprising that these predilection muscles did not consistently yield higher larval recoveries by either method in this study, as all samples were spiked and not naturally infected. The results from the present study which used spiked samples demonstrate that both digestion methods perform comparably on samples from all muscles that are adequately trimmed and processed according to validated procedures.

The extensive data presented in this study validate the performance of the PrioCHECK Trichinella AAD assay for testing fresh pork samples for *Trichinella* according to international guidelines, including those of the EU, ICT and OIE (European Commission, 2015; ICT, 2014; OIE, 2017). Although the rates of larval recovery were comparable to those obtained by the pepsin/HCl method, the PrioCHECK Trichinella AAD assay possesses technical and safety advantages, as well as some potential disadvantages relative to the standard pepsin/HCl method. The digestive enzyme serine is non-hazardous and safe for use by analysts, a third less time is required for the digestion process, and the risk of environmental contamination is mitigated by the elevated digestion temperature which is sufficient to kill any *Trichinella* larvae present (Konecni et al., 2017). Some potential disadvantages which can be mitigated by adequate training include the elevated digestion temperature (+15 °C compared to pepsin/HCl method) requiring skin protection, decreased visibility of non-motile recovered larvae, and the occasional need for an additional clarification step. Nevertheless, these issues may be outweighed by specific conveniences and advantages of the kit over the pepsin/HCl method. The serine assay PrioCHECK Trichinella AAD is currently employed for both large-scale and low volume testing in several regulatory jurisdictions for various purposes including trade, food safety, and production of pigs in *Trichinella* negligible risk compartments.

Conflict of interest

Patrik Buholzer is an employee of Thermo Fisher Scientific. All other authors declare no conflict of interest in this study.

Acknowledgements

The excellent technical assistance provided by Michael Clancy and Cheryl Scheller is greatly appreciated. Initial statistical analysis of the results was kindly provided by Andrew Gajadhar. All proficiency samples used in this study were prepared at the CFIA's Centre for Foodborne and Animal Parasitology, and the PrioCHECK Trichinella AAD Kits were supplied by the manufacturer, Thermo Fisher Scientific.

References

- Alban, L., Petersen, J.V., 2016. Ensuring a negligible risk of *Trichinella* in pig farming from a control perspective. *Vet. Parasitol.* 231, 137–144.
- Dupouy-Camet, J., Bruschi, F., 2007. Management and diagnosis of human trichinellosis. In: Dupouy-Camet, J., Murrell, K.D. (Eds.), *FAO/WHO/OIE Guidelines for the Surveillance, Management, Prevention and Control of Trichinellosis*, 1st ed. FAO/WHO/OIE, Paris, France, pp. 37–68.
- European Commission, 2015. Regulation (EU) 2015/1375. 2015. Laying down specific rules on official controls for *Trichinella* in meat. *Off. J. Eur. Union* 58, L212 (11 August, 2015).

- FAO/WHO (Food and Agriculture Organization of the United Nations and World Health Organization), 2014. Multicriteria-based Ranking for Risk Management of Food-borne Parasites. Report of a Joint FAO/WHO Expert Meeting, 3–7 September 2012. FAO Headquarters Rome Italy <http://www.fao.org/3/a-i3649e.pdf>.
- Forbes, L.B., Gajadhar, A., 1999. A validated *Trichinella* digestion assay and associated sampling and quality assurance system for use in testing pork and horsemeat. *J. Food Prot.* 62, 1308–1313.
- Forbes, L.B., Rajic, A., Gajadhar, A.A., 1998. Proficiency samples for quality assurance in *Trichinella* digestion tests. *J. Food Prot.* 61, 1396–1399.
- Gajadhar, A.A., Forbes, L.B., 2002. An internationally recognized quality assurance system for diagnostic parasitology in animal health and food safety, with example data on Trichinellosis. *Vet. Parasitol.* 103, 133–140.
- Gajadhar, A.A., Pozio, E., Gamble, H.R., Nöckler, K., Maddox-Hyttel, C., Forbes, L.B., Vallée, I., Rossi, P., Marinculic, A., Boireau, P., 2009. *Trichinella* diagnostics and control: mandatory and best practices for ensuring food safety. *Vet. Parasitol.* 159, 197–205.
- Gamble, H.R., 1996. Detection of trichinellosis in pigs by artificial digestion and enzyme immunoassay. *J. Food Prot.* 59, 295–298.
- Gamble, H.R., Bessonov, A.S., Cuperlovic, K., Gajadhar, A.A., van Knappen, F., Noeckler, K., Schenone, H., Zhu, X., 2000. International commission on Trichinellosis: recommendations on methods for the control of *Trichinella* in domestic and wild animals intended for human consumption. *Vet. Parasitol.* 93, 393–408.
- Gottstein, B., Pozio, E., Nöckler, K., 2009. Epidemiology, diagnosis, treatment and control of Trichinellosis. *Clin. Microbiol. Rev.* 22, 127–145.
- ICT (International Commission on Trichinellosis), 2014. ICT quality assurance in digestion testing programs for *Trichinella*. <http://www.trichinellosis.org/Guidelines.html>.
- ICT (International Commission on Trichinellosis), 2016. ICT recommendations on post-harvest control of *Trichinella* in food animals. <http://www.trichinellosis.org/Guidelines.html>.
- Kapel, C.M.O., Webster, P., Gamble, H.R., 2005. Muscle distribution of sylvatic and domestic *Trichinella* larvae in production animals and wildlife. *Vet. Parasitol.* 132, 101–105.
- Konecsni, K., Scheller, C., Scandrett, B., Buholzer, P., Gajadhar, A., 2017. Evaluation of the PrioCHECK *Trichinella* AAD kit for the digestion and recovery of larvae in pork, horse meat and wild meat. *Vet. Parasitol.* 243, 267–271.
- Leclair, D., Forbes, L.B., Suppa, S., Gajadhar, A.A., 2003. Evaluation of a digestion assay and determination of sample size and tissue for the reliable detection of *Trichinella* larvae in walrus meat. *J. Vet. Parasitol. Invest.* 15, 188–191.
- OIE (World Organization for Animal Health), 2017. Chapter 2.1.20. Trichinellosis (infection with *Trichinella* spp.). Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2017 <http://www.oie.int/international-standard-setting/terrestrial-manual/access-online/>.
- Pozio, E., 2014. Searching for *Trichinella*. Not all pigs are created equal. *Trends Parasitol.* 30, 4–11.
- Pozio, E., 2015. Foodborne nematodes. In: Gajadhar, A. (Ed.), *Foodborne Parasites: Occurrence and Control in the Food Supply Web*. Elsevier, pp. 165–199.
- Pozio, E., Murrell, D., 2006. Systematics and epidemiology of *Trichinella*. *Adv. Parasitol.* 63, 367–439.