Parasitology International 86 (2022) 102449

Contents lists available at ScienceDirect

Parasitology International

journal homepage: www.elsevier.com/locate/parint

Evaluation of the PrioCHECK™ Trichinella AAD kit to detect *Trichinella spiralis, T. britovi*, and *T. pseudospiralis* larvae in pork using the automated digestion method Trichomatic-35

Walter Basso [a,*], Nelson Marreros [b], Larissa Hofmann [a], Christine Salvisberg [a], Britta Lundström-Stadelmann [a], Caroline F. Frey [a]

[a] Institute of Parasitology, Department of Infectious Diseases and Pathobiology, Vetsuisse-Faculty, University of Bern, Länggassstrasse 122, CH-3012 Bern, Switzerland
[b] School of Agricultural, Forest and Food Sciences HAFL, Bern University of Applied Sciences, Länggasse 85, CH-3052 Zollikofen, Switzerland

ARTICLE INFO

Keywords:
Trichinellosis
Pig
Artificial digestion
Diagnostic
Pepsin
Serin-endopeptidase

ABSTRACT

Trichinellosis is a potentially deadly parasitic zoonosis that is contracted by consuming undercooked infected meat. Reliable detection of infectious *Trichinella* spp. larvae in meat is therefore pivotal to ensure consumer's safety. The recently authorised PrioCHECK™ Trichinella Alternative Artificial Digestion (AAD) test kit appears promising when used with the standard magnetic stirrer method, but evaluation with other apparatus types is lacking.

In this study, the performance of the AAD kit in an adapted Trichomatic-35 (TM35) instrument was evaluated, first, at the Swiss National Reference Laboratory for trichinellosis (NRL); second, in a ring trial involving four Swiss official laboratories. Proficiency pork samples spiked with larvae of *Trichinella spiralis*, *T. britovi*, or *T. pseudospiralis* were tested with the AAD kit and with the reference pepsin-HCl digestion method in TM35 instruments.

At the NRL, both methods yielded identical qualitative and similar quantitative results independently of the *Trichinella* species. In the ring trial, satisfactory results were obtained for 47/50 (94.0%) (AAD) and 62/67 (92.5%) (reference method) of the analysed samples. Technical problems impairing analysis were more frequently observed with the AAD kit ($n = 22$) than with the reference method ($n = 5$) and were mainly (16/22) reported by one of the external labs. When no technical issues were recorded, the performance of both methods was comparable, in agreement with the observations at the NRL; however, these results suggest a need for further training with the kit and standardisation of the adapted TM35 instruments.

1. Introduction

Trichinellosis is a worldwide occurring parasitic zoonosis caused by different *Trichinella* species, which can affect a wide range of hosts such as mammals, birds, or reptiles [1,2]. In Europe, the most commonly detected *Trichinella* species in animal and human outbreaks are *T. spiralis*, *T. britovi* and *T. pseudospiralis* [2–5]. Human infection occurs through the consumption of undercooked meat infected with *Trichinella* spp. larvae. Pork is regarded as the most frequent infection source worldwide, but meat from horse, wild boar, bear, and other game species can also lead to trichinellosis outbreaks [2,5–7]. Although the infection in animals is usually subclinical, human infection can cause a life-threatening disease. Therefore, correct identification of *Trichinella*-infected meat is pivotal to ensure food safety and consumer protection [1,2,8].

In Europe, the artificial digestion with pepsin and hydrochloric acid (HCl) in a magnetic stirrer is the reference method for detection of *Trichinella* larvae in meat [4,9,10]. But this method has some constraints such as the dependency on the availability of pepsin, the risk of infection with living larvae if no caution measures are taken, the handling of hazardous components such as HCl and allergic reactions to pepsin. In recent years, a commercial digestion kit: PrioCHECK™ Trichinella Alternative Artificial Digestion (AAD) Kit (Thermo Fisher Scientific) was authorised in the EU [9] and Switzerland [10] as an equivalent to the reference method for testing pork. This assay uses a recombinant enzyme (serine-endopeptidase of the enzyme group subtilisin) instead of

* Corresponding author.
E-mail address: walter.basso@vetsuisse.unibe.ch (W. Basso).

https://doi.org/10.1016/j.parint.2021.102449
Received 10 June 2021; Received in revised form 10 August 2021; Accepted 30 August 2021
Available online 2 September 2021

naturally extracted pepsin and it requires a higher digestion temperature of 60 °C, which kills the larvae during the digestion process. Additionally, no HCl is added to the AAD kit and the sample is digested at a slightly alkaline pH. The AAD kit seems to be a promising safe alternative to the standard digestion with pepsin-HCL.

Few studies compared the AAD kit with the standard pepsin-HCl digestion method [11–13]. Two of these studies found that both methods performed similarly in detecting *T. spiralis* larvae in pork [11,12], whereas one study reported a lesser efficacy of the AAD kit, particularly in detecting larvae of *T. pseudospiralis* [13]. These results highlight the need for further assessment of the performance of the AAD kit. In all mentioned studies the digestion was performed in a magnetic stirrer according to the instruction manual of the kit as it is stated in the EU Regulation 2015/1375, Annex I, Chapter II [9], but the kit has not yet been evaluated with other equivalent methods.

The automated artificial digestion of meat with pepsin-HCl in the Trichomatic-35 (TM35) instrument (Foss GmbH, Germany, Fig. 1) is considered equivalent to the standard method utilising the magnetic stirrer [9]. This method is also authorised In Switzerland [10] and is widely used by officially recognised laboratories. But given the lack of information about the use of the AAD Kit together with the TM35 instrument, the Swiss National Reference Laboratory for trichinellosis (NRL) carried out an internal evaluation of their performance in detecting *Trichinella* spp. larvae in pork, and a subsequent field trial in which four officially recognised laboratories participated.

2. Materials and methods

2.1. Study design

The performance of the AAD kit with the automated TM35 instrument was evaluated for the detection of *Trichinella* spp. larvae in pork samples and compared to the reference digestion method with pepsin-HCl, which was also carried out in a TM35 instrument. In a first step, the AAD kit was compared to the standard method at the NRL for the detection of *T. spiralis*, *T. britovi*, and *T. pseudospiralis* larvae. The detection of *T. spiralis* was repeated with three different kit batches to detect potential differences among AAD kit production lots. In a second step, the AAD kit and pepsin-HCl methods were compared for the detection of *T. spiralis* larvae in a field trial that involved four officially recognised Swiss laboratories (Lab 1–4). These laboratories routinely performed diagnosis of *Trichinella* infection in pork by the reference method using the TM35 instrument.

2.2. Test kits

Three PrioCHECK™ Trichinella AAD test kits (Prod. No 7620030), with Lot No. TA161006B (Kit 1), TA170301B (Kit 2) and TA170109B (Kit 3) were provided by Thermo Fisher Scientific for the internal evaluation at the NRL, and 16 further kits with Lot No TA190611B (Kit 4) were purchased by the NRL for the field trial and sent to the four participating laboratories (4 kits/lab).

Fig. 1. A: Trichomatic instrument (Foss GmbH, Germany); B: reaction chamber; C: water chamber; D: HCl chamber; E: sieve; F: polycarbonate filter on glass for microscopic observation; G: polycarbonate filter with *Trichinella* larva.

2.3. Trichomatic-35 (TM35) instruments

The TM35 instrument with filtration insert (Foss Deutschland GmbH, Hamburg; Patent: DP3314937.2; Type: 10510, CE 95) is an automated digestion system that allows the digestion of 35 g of meat with pepsin-HCl in one single run (Fig. 1). To meet the technical requirements of the AAD kit, the TM35 was adapted as follows: fast rotation time of 2.5 min and digestion temperature of 60 °C. Adapted TM35 instruments were kindly provided by Moritz Gerätereparatur UG (Rellingen, Germany) to all participating laboratories. Pepsin-HCl digestions were performed with standard TM35 instruments (3.5 min fast rotation time, 49 °C digestion temperature). The digestion time was the same for both the standard and the adapted devices, namely eight minutes.

2.4. Proficiency samples

Proficiency samples were prepared with pork purchased from a local supermarket, which tested negative for *Trichinella* by the reference digestion method. Minced pork samples, free of fat and fascia and weighing 35 g, were either spiked with *Trichinella* spp. larvae (positive samples) or left unspiked (negative samples). Larvae of *T. spiralis* and *T. britovi* were recovered from muscle of mice that were experimentally infected with the corresponding *Trichinella* sp. Minute pieces of muscle were squeezed in a compressorium and observed under a stereomicroscope to count the number of encapsulated larvae. The count was double-checked by two experienced technicians and muscle pieces that contained the required number of larvae were transferred into the meat samples. Through this procedure, the larvae were still alive and encapsulated as in natural infections, and they underwent only one digestion. In contrast to larvae of *T. spiralis* and *T. britovi*, larvae of *T. pseudospiralis* are not encapsulated in muscle [2], hence they are easily missed when observed in a compressorium. Given that no exact number of larvae could be determined by this method, pork samples were spiked with 0.1 g of ground muscle from a mouse experimentally infected with *T. pseudospiralis*.

All animal experiments related to this study were authorised by the Cantonal Veterinary Office of Bern, Switzerland (permission no. BE113/17) and complied with the current laws of the country.

2.4.1. Sample sets for the NRL

Six sample sets were prepared to compare the performance of the AAD kit against that of pepsin-HCl in detecting larvae of *T. spiralis* and *T. britovi*. Each set included one negative sample and nine positive samples that were spiked with three, six, or 15 larvae (3 samples/count; total: 10 samples/set; 72 spiked larvae/set). Four sets with samples positive for *T. spiralis* were analysed with one of the AAD Kit 1 to 3 batches or with pepsin-HCl (Table 1). Two sets with samples positives for *T. britovi* were analysed with AAD kit 2 or pepsin-HCl (Table 2).

For *T. pseudospiralis*, two sample sets were prepared and analysed with AAD kit 1 or pepsin-HCl. Each set comprised 15 samples spiked with *T. pseudospiralis* and one negative sample (total: 16 samples/set) (Suppl. Table 1).

2.4.2. Sample sets for the field trial

Two identical sets each containing 10 positive and two negative samples (total: 12 samples/set) were sent to the four participating laboratories at two different time points, four weeks apart (total: four sets/laboratory). Positive samples were spiked with three to 14 *T. spiralis* larvae (Suppl. Tables 2–5).

2.5. Laboratory analyses

2.5.1. Trials at the NRL

Each AAD kit contained three components: Component 1: 20× digestion buffer, Component 2: enzyme solution (ready-to-use) and Component 3: digestion buffer additive (ready-to-use). For digestions

Table 1
Recovery of *T. spiralis* larvae (%, number of damaged larvae and z-score) after digestion of spiked pork samples using three different production batches of PrioCHECK™ Trichinella AAD Kit or pepsin-HCl digestion, using ad-hoc adapted or standard Trichomatic-35 instruments, respectively at the Swiss National Reference Laboratory for Trichinellosis (NRL).

Spiked L	AAD Kit 1					AAD Kit 2					AAD Kit 3					Pepsin-HCl				
	Detected L	Recovery %	Damaged L	z-score[a]	Evaluation	Detected L	Recovery (%)	Damaged L	z-score	Evaluation	Detected L	Recovery (%)	Damaged L	z-score	Evaluation	Detected L	Recovery (%)	Damaged L	z-score	Evaluation
3	3	100	1	–	Optimal	3	100	–	–	Optimal	2	66.7	–	–	Optimal	2	66.7	–	–	Optimal
3	3	100	1	–	Optimal	3	100	–	–	Optimal	3	100	–	–	Optimal	3	100	–	–	Optimal
3	2	66.7	–	–	Optimal	2	66.7	–	–	Optimal	3	100	1	–	Optimal	1	33.3	–	–	Optimal
6	6	100	2	0.0	Optimal	4	66.7	–	−2.0	Optimal	6	100	–	0.0	Optimal	6	100	1	0.0	Optimal
6	5	83.3	–	−1.0	Optimal	5	83.3	–	−1.0	Optimal	6	100	1	0.0	Optimal	6	100	–	0.0	Optimal
6	4	66.7	1	−2.0	Optimal	6	100	1	0.0	Optimal	5	83.3	–	−1.0	Optimal	6	100	–	0.0	Optimal
15	15	100	1	0.0	Optimal	14	93.3	1	−0.7	Optimal	15	100	–	0.0	Optimal	13	83.9	–	−1.3	Optimal
15	5	33.3	–	−6.7	Un-satisfactory	12	80	–	−2.0	Optimal	15	100	–	0.0	Optimal	14	93.3	–	−0.7	Optimal
15	15	100	–	0.0	Optimal	15	100	1	0.0	Optimal	14	93.3	–	−0.7	Optimal	15	100	1	0.0	Optimal
0	0	0	–	–	Optimal	0	0	–	–	Optimal	0	0	–	–	Optimal	0	0	–	–	Optimal
$n = 72$	$n = 58$	(mean)	$n = 6$			$n = 64$	(mean)	$n = 3$			$n = 69$	(mean)	$n = 2$			$n = 66$	(mean)	$n = 2$		
100%	80.6%	83.3%				88.9%	87.8%				**95.8%**	**93.7%**				**91.7%**	**86.4%**			

[a] z-scores between −3 and +3 were considered satisfactory. If the z-score was between −2 and +2, the result was considered optimal. If it was outside this range but still between −3 and + 3, the result was considered acceptable, and for more extreme z-scores, the result was considered unsatisfactory. For samples with three spiked larvae, no z-score was calculated, and the results were considered optimal when at least one larva was detected.

Table 2
Recovery of *T. britovi* larvae (%, number of damaged larvae and z-score) after digestion of spiked pork samples using the PrioCHECK™ Trichinella AAD Kit or pepsin-HCl digestion, using ad-hoc adapted or standard Trichomatic-35 instruments, respectively, at the Swiss National Reference Laboratory for Trichinellosis (NRL).

Spiked L	AAD Kit 2					Pepsin-HCl				
	Detected L	Recovery %	Damaged L	z-score[a]	Evaluation	Detected L	Recovery (%)	Damaged L	z-score	Evaluation
3	3	100	–		Optimal	3	100	–		Optimal
3	3	100	–		Optimal	3	100	–		Optimal
3	2	66.7	–		Optimal	3	100	–		Optimal
6	6	100	–	0.0	Optimal	6	100	–	−2.0	Optimal
6	5	83.3	1	−1.0	Optimal	6	100	–	−1.0	Optimal
6	5	83.3	–	−1.0	Optimal	5	83.3	–	0.0	Optimal
15	12	80	–	−2.0	Optimal	15	100	–	−0.7	Optimal
15	11	73.3	–	−2.7	Acceptable	15	100	1	−2.0	Optimal
15	14	93.3	2	−0.7	Optimal	12	80	–	0.0	Optimal
0	0	0	–		Optimal	0	0	–		Optimal
$n = 72$	$n = 61$	(mean)	$n = 3$			$n = 68$	(mean)	$n = 1$		
100%	84.7%	**86.7%**				**94.4%**	**95.9%**			

[a] *Z*-scores between −3 and +3 were considered satisfactory. If the z-score was between −2 and +2, the result was considered optimal. If it was outside this range but still between −3 and +3, the result was considered acceptable, and for more extreme z-scores, the result was considered unsatisfactory. For samples with three spiked larvae, no z-score was calculated, and the results were considered optimal when at least one larva was detected.

with the AAD kit in the adapted TM35 instruments, each pork sample was digested using a solution containing 30 ml of Component 1; 30 ml of Component 2 and 250 µl of Component 3, diluted in 510 ml of tap water. From this solution, 380 ml were poured in the water chamber, 30 ml in the HCl chamber and the remaining 160 ml directly in the reaction chamber with the meat sample (Fig. 1). The proportions of the kit components in the present study were based on previous experiments performed at the NRL, in which the digestibility and the number of recovered larvae of pork samples spiked with known numbers of *T. britovi* were assessed under varying proportions of the kit components (Data not shown).

For digestions with pepsin-HCl, 30 ml 8.5% HCl, 7 g Pepsin (30,000 iE/G, 2000 FIP-U/G Opopharma Handels GmbH, Germany) and 400 ml tap water were used for each sample, as indicated by the manufacturer and in accordance with the EC regulation 2015/1375 [9].

The digestion was carried out with the 8 min. Program, at either 49 °C (standard) or 60 °C (adapted device). The final filtration step was performed using transparent polycarbonate membrane filters with a diameter of 50 mm and a pore size of 14 µm (Sterlitech Corporation, Kent, US). After each digestion, the obtained larvae were counted using a stereomicroscope and photographed for documentation (Fig. 2). The analyses were performed blindly by three experienced technicians. The performance of the digestions, the quantity of undigested material remaining in the sieve as well as any technical problems that may have occurred during the procedure were recorded.

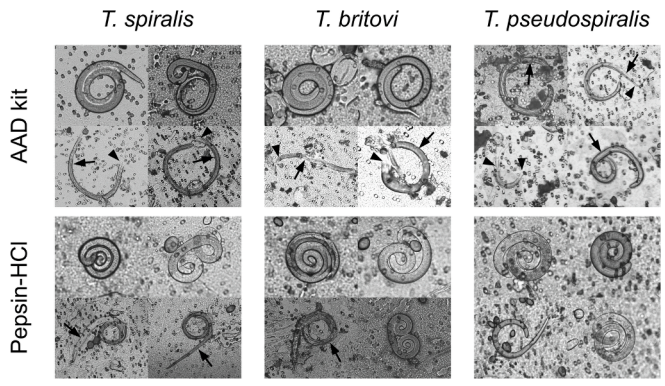

Fig. 2. *Trichinella* spp. larvae recovered on polycarbonate filters after digestion with pepsin-HCl or AAD kit using Trichomatic instruments. Observed non-damaged and damaged larvae (shown with arrows/arrow heads), especially the loss of differentiation of internal structures (arrows) and broken ends (arrowheads).

2.5.2. Field trial

Before beginning the trial, each of the four participating laboratories received an adapted TM35 instrument and the AAD kits, together with the instructions on how to perform the analysis as done at the NRL. They were first asked to test additional meat samples from the animals that were routinely tested in each laboratory to detect any technical difficulties in the digestion and to communicate these to the NRL.

The proficiency samples were then tested in parallel with the standard pepsin-HCl or with the AAD kit and the adapted TM35 instrument. The standard digestion method was performed with the reagents and instruments (i.e., pepsin, HCl, standard TM35 devices, etc.) used by the laboratories for their routine activity. The results (i.e., number of detected larvae in each sample) and observations on the digestion performance, technical problems, and date of analysis were documented and returned to the NRL within three weeks after receiving the sample sets.

2.6. Evaluation of the results

In all trials, a qualitative (positive-negative), a semi-quantitative (z-scores) and a quantitative (proportion of recovered larvae) evaluation were carried out. For each sample containing known numbers of spiked *Trichinella* larvae, the percentage of larval recovery and a z-score [14] were calculated as follow: $z = (n_r - n_s) / 0.1 \times n_s$ where $n_s$and n_r are the numbers of spiked and recovered larvae, respectively. Where the number of spiked larvae was low ($n_s \leq 10$) the denominator was increased to $0.166 \times n_s$. *Z*-scores between −3 and + 3 were considered satisfactory. If the z-score was between −2 and + 2, the result was considered optimal. If it was outside this range but still between −3 and + 3, the result was considered acceptable, and for more extreme z-scores, the result was considered unsatisfactory. For samples with three spiked larvae, no z-score was calculated, and the results were considered optimal when at least one larva was detected.

2.7. Statistical analyses

Descriptive statistics and z-scores were calculated using Microsoft Excel software. When assessing the results of samples with known numbers of larvae, the difference in proportions of optimal, acceptable, and usatisfactory results between laboratory assays was tested by Fisher's exact test. To assess the difference in quantitative performance between assays, we modelled the probability of detecting a larva in each proficiency sample against the probability of missing it, using generalised linear models with binomial error distribution and logit link function [15]. When comparing the different AAD kit batches for the detection of *T. spiralis* larvae at the NRL, we used the kit batch numbers

as an explanatory variable. Then, when comparing the results of the AAD kits against pepsin-HCl, we used the assay method as a fixed explanatory variable and the kit batch number as a random variable. When comparing the results of the AAD kit against those of the pepsin-HCl from the field trial, we used the assay method as a fixed explanatory variable, and the participating laboratory as a random variable. The difference in *T. pseudospiralis* larvae number between AAD kit and pepsin-HCl was assessed by two sample *t*-test. The limit of significance was set at $p < 0.05$. Statistical analyses were performed with the R software, version 3.6.2 [16], with additional packages ggplot2 [17] and lme4 [18].

3. Results

3.1. Trials at the NRL

3.1.1. Qualitative and semi-quantitative results

All 84 samples spiked with *T. spiralis, T. britovi,* and *T. pseudospiralis* larvae and all eight negative samples were correctly identified as positive or negative, respectively, by both AAD and pepsin-HCl digestion. All semi-quantitative results were optimal except for two acceptable and one unsatisfactory result that were obtained with different combinations of test, kit, and *Trichinella* species (Tables 1 and 2, Suppl Table 1). Therefore, no significant difference was found between pepsin-HCl and AAD or among AAD kit batches when comparing qualitative and semi-quantitative results at the NRL.

3.1.2. Quantitative results

The total number of recovered *T. spiralis* and *T. britovi* larvae in each trial, the proportion of damaged larvae, and the z-scores for each sample are indicated in Tables 1 and 2. The overall proportions of recovered larvae with AAD kit lots 1, 2 and 3 were: 80.5%, 88.9%, and 95.8% ($n = 72$), respectively. The probability of finding a larva was significantly lower when using Kit 1 compared to using Kit 3 (Fig. 3, Table 3). This difference was mainly due to an outlier result when using Kit 1, where only 5 of the 15 spiked larvae were recovered from one of the samples. When removing that outlier, the differences between kits became nonsignificant (data not shown). By digestion with pepsin-HCl, 91.7% of the spiked larvae were recovered (Table 1). The difference in probability of finding a larva between AAD lots (outlier included) and pepsin-HCl was not statistically significant (Fig. 3, Table 3).

For *T. britovi,* the overall proportions of recovered larvae by digestion with the AAD kit (Kit 2) and pepsin-HCl were 84.7% and 94.4%, respectively (Table 2), but the difference in probability of finding a larva was not statistically significant (Table 3).

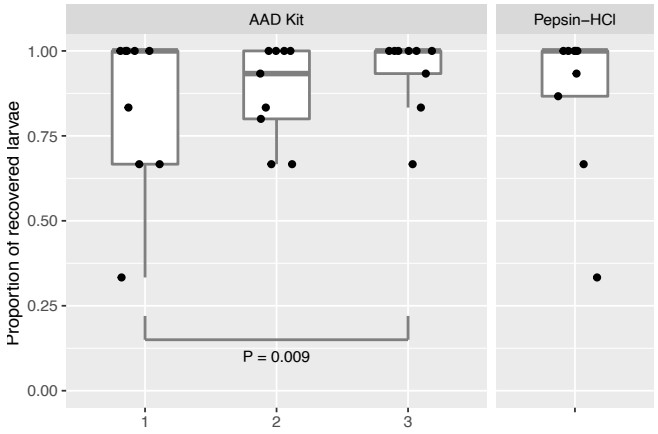

Fig. 3. Proportion of spiked *T. spiralis* larvae recovered from samples analysed at the NRL, depending on the test method (AAD-Trichomatic-35 vs. pepsin-HCl-Trichomatic-35) and AAD-kit batch (1–3). Solid circles show the results of individual samples and summarising boxplots are added in the background. The difference between AAD and pepsin-HCl was not statistically significant (see text).

When analysing the samples spiked with *T. pseudospiralis* by either the AAD kit or with pepsin-HCl, a total of 1149 (mean 76.6/sample; range 8–264) and 1253 larvae (mean 83.5/sample; range 22–174) were detected, respectively (Suppl. Table 1, Fig. 4). The difference between methods was not significant ($p = 0.7202$).

3.1.3. Digestibility

The amount of undigested material remaining on the sieve was <0.1 g with the AAD kit in all cases, and < 0.2 g with the pepsin-HCl digestion method. The digestion process was clearly satisfactory according to the guidelines and EC regulation 2015/1375 [9], which stipulates that no more than 5% of the starting sample weight (i.e. 1.75 g of 35 g) should remain on the sieve.

3.1.4. Morphology of recovered larvae

In the assays with pepsin-HCl, the *Trichinella* larvae were generally coiled, and the stichosome was usually visible in all three *Trichinella* species (Fig. 2). In the assays with the PrioCHECK Trichinella AAD™ most of the recovered larvae were also coiled, but the stichosome was often less clearly visible than after digestions with pepsin-HCl. Damaged *T. spiralis* and *T. britovi* larvae (mainly in anterior and/or posterior ends) were more frequently found after testing with AAD than after pepsin-HCl (14/252 and 3/134 larvae respectively, Tables 1 and 2) (Fig. 2). Although not statistically significant, the odds ratio suggested a difference between these methods (OR: 2.56, 95% CI 0.7–14.2; $p = 0.192$). Damaged *T. pseudospiralis* larvae were also observed after digestion with the AAD kit (Fig. 2) but the amount was not quantified.

3.2. Field trial

3.2.1. Technical issues

Two of the eight sample sets were not analysed because of technical issues with the adapted TM35 instruments. Lab 1 reported the deposition of "crystals" on the filter when using the AAD-TM35 method. The presence of these "crystals" increased after using the adapted device for successive samples on the same day and compromised larvae visualisation. Therefore, the lab decided not to complete the analysis of set 2. The "crystals" were interpreted as material released from the inner painted coat of the chamber, probably due to the higher temperature required by the protocol. Lab 4 reported that the adapted TM35 instrument stopped heating before completing the analysis of the first sample set. This Lab received a second adapted TM35 instrument along with the second set of samples, but it reported this second device also stopped working and the second sample set was thus not analysed.

During the analysis of the six remaining sample sets, further results had to be excluded because of technical issues. Lab 1 reported forgetting the pepsin during the analysis of one sample by the standard method. Lab 2 reported the build-up of a "fat layer" on the filter during the analysis of 16 out of 24 samples with the AAD-TM35 method. Lab 3 reported a deficient sealing on three sample bags with the presence of air and loss of meat juice. These samples were still analysed by the AAD-TM35 method but one of these showed an unsatisfactory performance. The latter result was excluded from the evaluation as it could have been due to loss of spiked larvae prior to the analysis. Lab 4 reported filter clogging, overflow, and loss of material with four samples analysed by the standard method and with two samples analysed by the AAD-TM35 method (Suppl. Tables 2–5).

3.2.2. Qualitative and semi-quantitative results

Considering only the analysis of samples for which no technical problems were reported, all labs recognised all positive and negative samples correctly, except for Lab 2, that reported one false positive sample and one false negative sample by the standard method (Suppl. Tables 2–5).

Table 3
Parameter estimates of the AAD-Kit (model a) or of the digestion method (models b to d) on the probability of detecting a larva (PDL) of *Trichinella* sp. spiked in pork samples. The estimates are based on generalised linear models after removing unspiked samples and excluded results. The AAD-kit and the participating laboratory were included as random factor in models b and d, respectively.

Model	*Trichinella* sp.	Trial[a]	N[b]	Factor	Factor level	Estimate	SE	z-Value	*p*-Value
a) PDL ~ kit	*T. spiralis*	NRL	27	Kit	Kit 1	Baseline			
					Kit 2	0.66	0.48	1.37	0.169
					Kit 3	1.71	0.66	2.59	0.009
b) PDL ~ method	*T. spiralis*	NRL	36	Method	AAD	Baseline[c]			
					HCl	0.32	0.68	0.48	0.631
c) PDL ~ method	*T. britovi*	NRL	18	Method	AAD	Baseline			
					HCl	1.12	0.61	1.84	0.066
d) PDL ~ method	*T. spiralis*	Field	97	Method	AAD	Baseline			
					HCl	−0.27	0.20	−1.36	0.172

[a] NRL: trials performed at the NRL; Field: trials performed during the field trial.
[b] Number of samples included in the model calculation.
[c] The baseline of the explanatory is the overall performance of all three AAD-kits; HCl: pepsin-HCl digestion method.

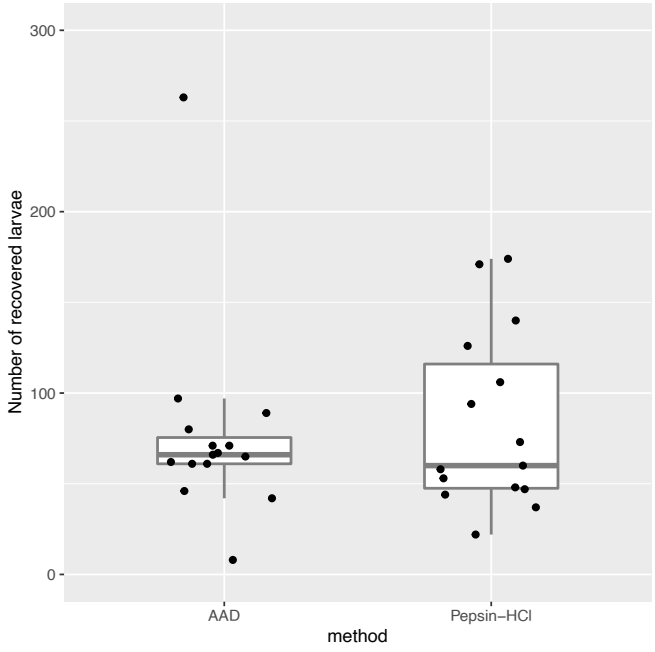

Fig. 4. Number of *T. pseudospiralis* larvae recovered at the Swiss National Reference Laboratory after digestion with the PrioCHECK Trichinella AAD Kit and pepsin-HCl using Trichomatic-35 instruments.
Solid circles show individual spiked samples over summarising boxplots. The difference was not statistically significant. The individual results of the negative samples were not included in the figure.

Of the 67 samples analysed by the standard method, 48 were considered optimal, 14 were acceptable, and 5 were unsatisfactory. Of the 50 samples analysed by the AAD method, the corresponding results were 42, 5, and 3, respectively. The difference in proportions between both methods was not significant ($p = 0.258$). On the other hand, a significant difference in the proportion of semi-quantitative results was found between laboratories (Fig. 5. $p = 0.012$). At least 70% of the results from labs 1, 3, and 4 were optimal, whereas it was only the case for 59.4% (19/32) of the results from lab 2. Also, only labs 1 and 2 reported unsatisfactory results, most of which were reported by lab 2 (6 of 8 unsatisfactory results, Suppl Tables 2–5, Fig. 5).

3.2.3. Quantitative results

The quantitative results are presented in Table 4 and Suppl. Tables 2–5. Considering only the samples for which no technical issues were reported, 282 of the 335 (84.2%) total larvae spiked in samples analysed by AAD were recovered, whereas 340 of the 430 (79.1%) of the

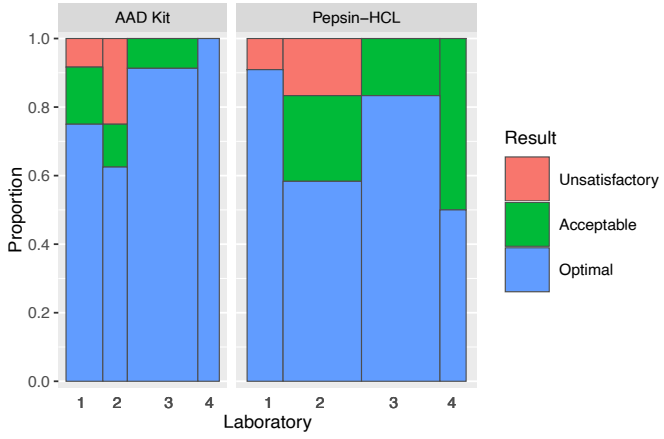

Fig. 5. Mosaic plot showing the proportion of optimal, acceptable, and unsatisfactory results for each laboratory. Laboratory 2 reported higher number of unsatisfactory results (FET: $p = 0.014$).

Table 4
Results of the four laboratories (Lab 1–4) participating in the field trial, considering qualitative results and z-scores of each analysed sample. Only samples for which no technical problems were reported by the laboratories were considered in the analysis.

Lab no.	Method	Satisfactory results[1]	Total number of detected larvae	Mean proportion of recovered larvae /sample [95% CI]	Mean days (range)
1 [1 set]	Pepsin-HCl	90.9% (10/11)	85.9% (61/71)	88.1% [76.3, 99.9]	8.8 (5–13)
	AAD	91.7% (11/12)	82.9% (63/76)	78.6% [63.3, 93.8]	8.5 (5–13)
2 [2 sets]	Pepsin-HCl	83.3% (20/24)	72.0% (118/164)	74.5% [63.5, 85.5]	6.5 (1–12)
	AAD	75.0% (6/8)	77.8% (56/72)	78.1% [65.5, 90.7]	11.6 (5–22)
3 [2 sets]	Pepsin-HCl	100% (24/24)	84.8% (139/164)	81.1% [74.5, 87.7]	4.4 (1–7)
	AAD	100% (23/23)	86.4% (127/147)	86.8% [79.8, 93.8]	4.4 (1–7)
4 [1 set]	Pepsin-HCl	100% (8/8)	74.2% (23/31)	76.0% [60.2, 91.8]	7.5 (3–12)
	AAD	100% (7/7)	90% (36/40)	84.2% [74.4, 94.0]	7.6 (3–12)

[1] Satisfactory results: number of samples which obtained z-scores within the range 3 and − 3 and/or qualitative correct results (samples spiked with 3 L and negative samples)/number of analysed samples; Mean days: mean days passed since receiving the samples until analysis.

total larvae spiked in samples analysed by the standard method were found. This difference was not significant ($p = 0.076$). Also, the difference in probability of finding a larva was not significant between AAD and pepsin-HCl when considering the lab as a random factor (Table 3). Alternatively, no significant effect was found between AAD and pepsin-HCl when the lab was included as fixed effect and an interaction term between lab and assay was added (data not shown).

3.2.4. Digestibility

Labs 1 and 3 reported satisfactory digestion with both methods. Labs 2 and 4 reported technical issues, which could have been related at least to some extent, to a poor digestion. Lab 2 reported building of a "fat layer" on the filter during analysis of both sample sets by the AAD-TM35 method, and Lab 4 reported clogging of the filter and overflow during sample analysis by both methods.

4. Discussion

At the NRL, the PrioCHECK™ Trichinella AAD test kit used with an adapted TM35 instrument was easy to operate and achieved a good digestion of pork samples. The proportions of recovered larvae were comparable to the standard artificial digestion method based on pepsin-HCl, and all positive and negative samples were correctly identified. Based on these results, the PrioCHECK™ Trichinella AAD test kit seems to fulfil the requirements to be used as an alternative method for the detection of *Trichinella* spp. in pork.

The overall proportions of recovered larvae of *T. spiralis* and *T. britovi* larvae with each AAD Kit and with pepsin-HCl, from our *in house* trial, were comparable to those reported in a former study that used both the AAD kit and pepsin-HCl in a magnetic stirrer on paired pig diaphragm samples [11]. In the former study, 74% ± 10% of spiked larvae/sample were recovered using the AAD Kit, vs. 90% ± 11% with the reference method, but the differences were not significant. However, the proportion of recovered larvae with the AAD Kit was significantly lower when spiked horse tongue samples were analysed, with 54% ± 25% of spiked larvae vs. 92% ± 10% by pepsin-HCl digestion [11]. A further study compared the standard AAD Kit with the standard pepsin-HCl on pork samples spiked with *T. spiralis* larvae and reported similar qualitative results with both methods, but lower quantitative performance of the AAD kit [13]. As well, low proportions of recovered larvae were reported for *T. pseudospiralis* larvae when tested by standard AAD [13]. When pork samples were spiked with 1 to 10 *T. pseudospiralis* larvae, the authors obtained more false negative results with the AAD kit ($n = 9/15$ samples) than with the pepsin-HCl digestion ($n = 2/15$ samples) [13]. Moreover, the quantitative larval detection of *T. pseudospiralis* larvae was significantly lower than that of *T. spiralis* ($p < 0.0001$) [13]. The same observation was made after comparative analysis of meat from pigs and mice experimentally infected with *T. spiralis* and *T. pseudospiralis*, respectively [13]. In homogenised pig samples, 88.0 ± 6.5 ($n = 5$) *T. spiralis* larvae were recovered by the reference method, vs. 61.0 ± 15.3 ($n = 5$) using the AAD kit ($p = 0.0194$, paired *t*-test). In mouse samples, 440.2 ± 56.5 ($n = 5$) *T. pseudospiralis* larvae were recovered by the reference method and significantly less, i.e. 106.2 ± 49.3 ($n = 5$) using the AAD kit ($p = 0.0001$, paired *t*-test) [13]. The characteristics of the automated detection system used in our trials (i.e. a combination of vacuum and filter for larvae capture) might have accounted for a higher diagnostic sensitivity than in the cited studies [11,13], in which the AAD kit was used in combination with a magnetic stirrer and a sedimentation system for larval recovery. Low proportion of recovered *T. pseudospiralis* larvae after double digestion, first with pepsin and then with AAD [13], suggested that this *Trichinella* species does not withstand that treatment. In contrast, larvae in our study were digested only once, as they were added encapsulated (*T. spiralis* and *T. britovi*), or as homogenised meat samples (*T. pseudospiralis*). The recovery of *T. pseudospiralis* in our study was not statistically different between both methods.

In all analyses performed at the NRL, the observed amount of residual undigested material on the sieve was <0.1 g with the AAD kit and < 0.2 g with the pepsin-HCl digestion method, which both completely fulfilled the criteria of satisfaction of the Guidelines and EC regulation 2015/1375 [9]. Accordingly, in the field study, two labs (Labs 1 and 3) also reported satisfactory digestion with both methods. However, Lab 2 reported a build-up of a "fat layer" on the filter during analysis by the AAD method, and Lab 4 reported clogging of the filter and overflow during sample analysis by both methods. Similar observations were reported in the study from Konecsni et al., 2017 [11], which reported a good digestibility with the AAD kit but also commented that the AAD Kit required a further clarification step more often than the pepsin-HCl method. The authors also mentioned that the foaminess of the AAD Kit's digestion liquid might lead to some technical difficulties during the filtration/ sedimentation steps [11].

The morphological structure of the recovered larvae appeared to be more frequently affected after digestion with the AAD kit, although the difference was not statistically significant. For some individual larvae, the visualisation of the internal structures, characteristic for the genus *Trichinella*, such as the stichosome was not possible. Although the identification of the damaged larvae as "nematode larvae" was assured, in some individual larvae a diagnosis based exclusively on morphological characteristics was difficult and, in some cases, would have been impossible. Nevertheless, the occurrence of damaged larvae was not constant, and in all samples intact larvae were also present. While some species such as *T. nativa* and *Trichinella* genotype T6 are especially resistant to cold temperatures and may resist freezing for long time periods [2], other species such as *T. nelsoni* might be more resistant to warm temperatures. If this differential characteristic could be correlated with a lower or higher resistance to digestion with the AAD kit, which requires a higher temperature (i.e. 60 °C) than the standard method, was not studied here, but may represent an additional aspect to be covered in further studies.

To yield a definitive specification of recovered damaged larvae, the following two solutions can be proposed: (i) retesting of suspicious samples by another officially recognised method such as pepsin-HCl digestion; (ii) performing PCR of the recovered larvae. A PCR-approach has been recently published as proof of concept [11]. In that study, larvae recovered from muscle tissues of two naturally infected bears after artificial digestion with the ADD kit proved suitable for PCR analysis and genotyping, and a diagnosis of infection with *Trichinella* T6 could be achieved. A full validation of the PCR with respect to all *Trichinella* species (including *T. spiralis*, *T. britovi* and *T. pseudospiralis*) after recovery by AAD-TM35 digestion has to date not been reported.

Considering all samples effectively analysed during the field trial (i.e., excluding samples for which technical problems were reported), satisfactory results were obtained for 62/67 (92.5%) samples analysed by the standard method and for 47/50 (94.0%) samples analysed by the AAD method. Technical problems impairing analysis were more frequently reported for samples analysed by the AAD kit ($n = 22$) than for those analysed by the standard method ($n = 5$). However, technical problems with the AAD method were mostly reported by Lab 2 ($n = 16/22$), which did not report any difficulty with the standard method, and by Lab 4, which reported five technical issues with the AAD method and also four issues with the standard method.

Most spiked samples were effectively analysed by all four participating laboratories within two weeks after receipt as was expected, except for one sample set which was analysed by Lab 2 with the AAD kit 21–22 days after receipt (Table 4, Suppl. Table 3). This was the sample set with which the most technical issues were reported. It is possible that compromised sample integrity after three weeks of storage could have been responsible to some extent for these technical issues, mostly reported as "building of a fat layer".

5. Conclusions

The field trial showed that when no technical issues were reported, the performance of the AAD method was comparable to that of the standard method, in agreement with the observations at the NRL. For laboratories relying on the TM35 in their routine diagnostic work, this is a valuable information because it could represent an alternative to pepsin in times of shortage. In this case, a further option would be to use the AAD kit in combination with the magnetic stirrer (as it is already authorised for this use), but most laboratories using TM35 are not equipped for and trained in the magnetic stirrer method. Therefore, this could greatly affect the quality of the analyses, and the switch from pepsin-HCl to the AAD kit while still being able to use the TM35 instrument seems to be a practical option. However, the high number of technical issues reported mainly by one of the participating labs suggests that further training with the adapted devices and the AAD kit and/or standardisation of the TM35 instruments is needed.

Funding

This study was funded by the Federal Food Safety and Veterinary Office (FSVO), Switzerland.

Declaration of Competing Interest

The authors declare that they have no competing interests.

Acknowledgements

We would like to thank Edoardo Pozio (European Reference Laboratory for Parasites, Istituto Superiore di Sanitá, Rom, Italy) for providing the *T. pseudospiralis* isolate; Thomas Moritz (Moritz Gerätereparatur UG Rellingen, Germany) for providing the adapted Trichomatic-35 Instruments and Patrik Buholzer, Thermo Fisher Scientific for providing the AAD Kits used for the evaluation at the NRL.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.parint.2021.102449.

References

[1] J. Dopouy-Camet, K.D. Murrell (Eds.), Guidelines for the Surveillance, Management, Prevention and Control of Trichinellosis, FAO/WHO/OIE, Paris, 2007 (108 pp.).

[2] B. Gottstein, E. Pozio, K. Nöckler, Epidemiology, diagnosis, treatment, and control of trichinellosis, Clin. Microbiol. Rev. 22 (2009) 127–145, https://doi.org/10.1128/CMR.00026-08.

[3] C.F. Frey, M.E. Schuppers, N. Müller, M.P. Ryser-Degiorgis, B. Gottstein, Assessment of the prevalence of *Trichinella* spp. in red foxes and Eurasian lynxes from Switzerland, Vet. Parasitol. 159 (2009) 295–299, https://doi.org/10.1016/j.vetpar.2008.10.060.

[4] A. Mayer-Scholl, E. Pozio, J. Gayda, N. Thaben, P. Bahn, K. Nöckler, Magnetic stirrer method for the detection of *Trichinella* larvae in muscle samples, JoVE (121) (2017), https://doi.org/10.3791/55354.

[5] A. Rostami, H.R. Gamble, J. Dupouy-Camet, H. Khazan, F. Bruschi, Meat sources of infection for outbreaks of human trichinellosis, Food Microbiol. 64 (2017) 65–71, https://doi.org/10.1016/j.fm.2016.12.012.

[6] K.D. Murrell, E. Pozio, Worldwide occurrence and impact of human trichinellosis, 1986–2009, Emerg. Inf. Dis. 17 (2011) 2194–2202, https://doi.org/10.3201/eid1712.110896.

[7] EFSA and ECDC (European Food Safety Authority and European Centre for Disease Prevention and Control), The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2015, EFSA J. 14 (12) (2016) 4634, 231 pp, https://doi.org/10.2903/j.efsa.2016.4634.

[8] A.A. Gajadhar, E. Pozio, H.R. Gamble, K. Nöckler, C. Maddox-Hyttel, L.B. Forbes, I. Vallée, P. Rossi, A. Marinculić, P. Boireau, *Trichinella* diagnostics and control: mandatory and best practices for ensuring food safety, Vet. Parasitol. 159 (2009) 197–205, https://doi.org/10.1016/j.vetpar.2008.10.063.

[9] European Commission, Commission Implementing Regulation (EU) 2015/1375 of 10 August 2015 Laying Down Specific Rules on Official Controls for *Trichinella* in Meat 212, Off. J. EU. L, 2015, pp. 7–34, 2015, http://data.europa.eu/eli/reg_impl/2015/1375/oj.

[10] FSVO, (Federal Food Safety and Veterinary Office Switzerland) Technische Weisungen über Trichinellenuntersuchung von Schlachttierkörpern und Fleisch von Hausschweinen, Pferden, Wildschweinen, Bären und Nutrias sowie weiteren empfänglichen Wildtierarten, vom 01.06.2017 (ersetzt die Version vom 24.05.2006), 2017.

[11] K. Konecsni, C. Scheller, B. Scandrett, P. Buholzer, A. Gajadhar, Evaluation of the PrioCHECK™ Trichinella AAD Kit for the digestion and recovery of larvae in pork, horse meat and wild meat, Vet. Parasitol. 243 (2017) 267–271, https://doi.org/10.1016/j.vetpar.2017.07.004.

[12] A. Gajadhar, K. Konecsni, B. Scandrett, P. Buholzer, Validation of a new commercial serine protease artificial digestion assay for the detection of *Trichinella* larvae in pork, Food Waterborne Parasitol. 10 (2018) 6–13, https://doi.org/10.1016/j.fawpar.2018.04.001.

[13] F. Franssen, A. Johne, J. van der Giessen, K. Nöckler, A. Mayer-Scholl, Test sensitivity of a commercial serine protease digestion kit for the detection of *Trichinella spiralis* and *Trichinella pseudospiralis* larvae in pig muscle, Food Microbiol. 78 (2019) 99–103, https://doi.org/10.1016/j.fm.2018.10.012.

[14] ISO 13528, Statistical Methods for Use in Proficiency Testing by Interlaboratory Comparison. https://www.iso.org/standard/56125.html, 2015 (accessed 10.04.2021).

[15] J.J. Faraway, Extending the Linear Model with R: Generalized Linear, Mixed Effects and Nonparametric Regression Models, Chapman & Hall/CRC, Boca Raton, FL, 2006 (301 pp.).

[16] R. Core Team, R: A Language and Environment for Statistical Computing, R Foundation for Statistical Computing, Vienna, Austria, 2019. http://www.r-project.org/index.html (accessed 09.04.2021).

[17] H. Wickham, ggplot2: Elegant Graphics for Data Analysis, Springer (Use R!), Cham, 2016, https://doi.org/10.1007/978-3-319-24277-4, 140 pp. (accessed 10.04.2021).

[18] D. Bates, M. Mächler, B. Bolker, S. Walker, Fitting linear mixed-effects models using lme4, J. Stat. Soft. 67 (2015), https://doi.org/10.18637/jss.v067.i01.