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Validation of a latex agglutination test for the detection of *Trichinella* infections in pigs

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ABSTRACT

An antigen detection kit (Trichin-L), based on latex agglutination and developed by the Bio-Rad company was validated at five European laboratories. The validation parameters included specificity, sensitivity, robustness and reproducibility. Specificity was evaluated by testing parasite antigens from five non-*Trichinella* parasites in addition to the *Trichinella* genus. To evaluate sensitivity, 10 pork samples spiked with 1, 3, 6 or 15 *Trichinella* larvae were tested in each laboratory. To evaluate the robustness of the test, the solubilized antigens were maintained at room temperature and tested at different times. Reproducibility was assessed in each laboratory using 40, 100 g minced pork samples, each spiked with *Trichinella spiralis*. The use of larval homogenates obtained from the Trichin-L kit as a template for parasite identification at the species level by a multiplex PCR, was also evaluated. The results showed a high specificity and sensitivity where solubilized antigens maintained their stability and reactivity for up to three days. Reproducibility was high, as similar results were obtained in the five laboratories. The larval homogenates obtained using the Trichin-L kit were successfully used in multiplex PCRs to identify *Trichinella* species.

1. Introduction

Zoonotic nematodes of the genus *Trichinella* show a cosmopolitan distribution and affect livestock (pigs and horses) and wild animals (mainly wild pigs and carnivores) (Pozio and Murrell, 2006). In the European Union, it is mandatory to test for the presence of *Trichinella* sp. larvae by artificial digestion in all susceptible animals prior to marketing their meat (European Commission, 2005). Today in the European Union, laboratories performing the digestion test must follow one of the approved methods (European Commission, 2005). According to the European Commission's Directorate General for Health and Consumer Policy (DG SANCO), validation is required prior to adopting any new method or apparatus for digestion. These guidelines entrust such validation to the European Union Reference Laboratory for parasites (EURLP) and four National Reference Laboratories (NRLs) for Trichinella (www.iss.it/binary/crlp/cont/Guidelines_for_the_validation_of_apparatuses_for_the_detection_of_Trichinella_larvae.pdf). On April, 2010, BIO-RAD (Marnes la Coquette, France) contacted the EURLP to request validation of the Trichin-L kit. The procedure is based on the current digestion protocol, but sedimentation

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steps and microscopic diagnosis of the larvae are replaced by antigen detection based on latex agglutination. The aim of the present work was to validate the Trichin-L kit, according to the guidelines approved by the DG SANCO.

2. Materials and methods

2.1. Equipment and consumables

As indicated in the Guidelines, four NRLs should be selected among those which have demonstrated good performance in the most recent proficiency test for the detection of *Trichinella* larvae in muscle samples. The selected NRLs were those of Austria, Belgium, Estonia and Sweden. BIO-RAD provided each laboratory (EURLP and NRLs) the following: a blender, a magnetic stirrer with a thermometer probe, a vacuum pump, a 0.5 l steel funnel, a 10 l plastic tank to collect digestion fluid, a steel sieve, a pestle for 15 ml Falcon tubes, and a rocker. Furthermore, the company provided the Trichin-L kits, containing the following disposable materials and reagents: 300 g of pepsin, a sample diluent, forceps, 20 μm nylon mesh filters, negative and positive controls, latex beads, sticks and latex agglutination cards.

2.2. Tested samples

As prescribed by the Guidelines, a total of 200, 100 g samples of minced pork, prepared according to a previously published protocol (Marucci et al., 2009), were tested (under blind conditions) in the selected laboratories by the Trichin-L kit. Each laboratory tested: (1) 10 negative samples (to evaluate the amount of undigested meat on the sieve and the kit specificity); (2) 10 samples containing 15 *T. spiralis* larvae; (3) 10 samples containing 6 *T. spiralis* larvae; and (4) 10 samples containing 3 *T. spiralis* larvae.

2.3. Specificity

Since worms of the genus *Trichinella* share many antigens with other nematodes, the cross reactivity of the Trichin-L kit was tested against 0.1 and 0.5 mg of crude antigens from the following nematodes: *Ascaris suum*, *Trichuris suis*, *Toxocara cati*, and *Anisakis pegreffii*; as well as 0.1, 0.3 and 0.5 mg of crude antigens from the tissue protozoa *Toxoplasma gondii*. Furthermore, 100 muscle samples of different weights (range 48–102 g; average 72.17 g) from diaphragm pillars from backyard pigs collected at slaughter and that tested negative by the digestion method, were also examined by the Trichin-L kit.

2.4. Sensitivity

To verify that the Trichin-L kit was able to detect larvae belonging to different *Trichinella* species, meat ball samples were spiked with 1, 3 or 5 *Trichinella* larvae (five replicates for each number of larvae), belonging to each of eight species of *Trichinella* (*T. spiralis*, *T. nativa*, *T. britovi*, *T. murrelli*, *T. nelsoni*, *T. pseudospiralis*, *T. papuae* and *T. zimbabwensis*). Furthermore, to check the sensitivity of the Trichin-L kit to detect *Trichinella* sp. larvae in undigested muscle tissues, 10 pieces of mouse muscle (average 177 mg, range 125–232 mg), infected with 1–4 larvae of *T. spiralis*, were tested without prior digestion, directly by the Latex-Agglutination in the Trichin-L kit.

2.5. Robustness

To evaluate the *Trichinella* antigen stability over time, Trichin-L kit filters with the larva homogenate were left in 15 ml Falcon tubes with sample diluent at room temperature for different periods of time (from 4 up to 96 h) and then 50 μl aliquots were tested using the Trichin-L kit.

To determine if the Trichin-L kit can correctly identify the presence of *Trichinella* sp. larvae in the presence of excess antigens, 5000 *T. spiralis* live larvae were spiked on one filter and tested, in triplicate, using the Trichin-L kit protocol.

2.6. Species identification

The ability to determine the *Trichinella* species by PCR using the residual larval homogenate present on the filter of the Trichin-L kit was investigated. Briefly, 1, 3, 5, 10 or 20 larvae were added to the center of filtration membranes using a micropipette under a stereomicroscope. The samples were treated according to the Trichin-L kit protocol and first tested by latex agglutination. Then, 10 μl of the homogenized sample were used for the multiplex PCR reaction (Pozio and La Rosa, 2010). Because the PCR failed, presumably because of the chaotropic nature of the sample diluent in the Trichin-L kit, this experiment was repeated by replacing the buffer with PBS. To remove any trace of the buffer and to concentrate the DNA before PCR, 450 μl of the homogenized sample (i.e., the residual liquid after testing by the latex agglutination) were first subjected to DNA purification (QIAmp DNA MiniKit, Qiagen, MD, USA).

3. Results and discussion

A total of 200 pork samples were tested in the five laboratories. Furthermore, in two NRLs and at the EURLP, eight additional *Trichinella*-negative samples were tested in each of the three labs. Thus, a total of 150 *Trichinella*-positive samples and 74 *Trichinella*-negative samples were tested. The average undigested material on the sieve was 1.7 g (range 0.0–<5.0). All but one of the *Trichinella*-positive samples tested positive; the one which tested negative was due to a technical mistake (Table 1). Out of the 50 negative samples, 37 (74%) tested negative, 10 (20%) tested doubtful in three labs, and three (6%) samples tested positive in one laboratory. Since 26% of negative controls tested doubtful (20%) or positive (6%) and these results were obtained in two out of the five labs, eight additional negative samples were tested in these two NRLs and at the EURLP. In one NRL, the first three negative samples tested positive, the fourth sample tested doubtful, whereas the other four samples tested negative. In the 2nd NRL and in the EURLP, all the eight samples tested negative. The investigation carried out at the NRL with false positive or doubtful results showed that, after digestion, detergent traces present on the apparatus after washing were the cause of the false

Table 1
Latex agglutination results of 150 *Trichinella*-positive samples and 174 *Trichinella*-negative samples tested in five European laboratories by the Trichin-L Antigen Test kit of BIO-RAD. Each sample contained 100 g of minced pork, if not differently reported.

Laboratory code	No. of samples	No. of larvae in each sample	Latex agglutination results (positive/doubtful/negative)
1	10	3	10/0/0
	10	6	10/0/0
	10	15	10/0/0
	18	0	0/0/10
	100[a]	0	0/0/100
2	10	3	10/0/0
	10	6	10/0/0
	10	15	10/0/0
	10	0	0/0/10
3	10	3	10/0/0
	10	6	10/0/0
	10	15	9/0/1[b]
	18	0	6/6/6
4	10	3	10/0/0
	10	6	10/0/0
	10	15	10/0/0
	18	0	0/4/14
5	10	3	10/0/0
	10	6	10/0/0
	10	15	10/0/0
	10	0	0/1/9

[a] Muscles samples from diaphragm pillars with an average weight of 72.17 g (range 48–102 g) and collected from backyard pigs at slaughter and tested negative by the digestion method.
[b] A positive sample tested negative due to a technical mistake.

positive results. In fact, when the digestion apparatus was not washed with detergent, but rather thoroughly rinsed with hot water, no more false positive reactions were observed (data not shown).

The Trichin-L kit did not cross-react with crude antigens of *A. suum*, *T. suis*, *T. cati*, *A. pegreffii* or *T. gondii*. Furthermore, no positive reactions were observed when 100 pork samples, collected at slaughterhouses and previously deemed negative by the digestion method, were tested by the Trichin-L kit. Of the 120 samples containing 1, 3 or 5 larvae belonging to the eight *Trichinella* species, the Trichin-L kit correctly identified 108 (90%) samples as positive. However, the Trichin-L kit failed to recognise 11 samples containing only one larva each (*T. spiralis*, 3; *T. nativa*, 1; *T. britovi*, 1; *T. murrelli*, 2; *T. nelsoni*, 2; *T. pseudospiralis*, 1; and *T. papuae*, 1 sample, respectively) and one sample containing three larvae of *T. pseudospiralis*. All ten pieces of mouse muscles (containing 1–4 larvae) were correctly diagnosed positive by the Trichin-L kit. The Trichin-L kit correctly yielded a positive result for the sample containing about 5000 larvae of *T. spiralis*, thus showing that an excess of antigens does not compromise the kit performance. Evaluation of antigen stability over time showed that the Trichin-L kit correctly identified positive samples stored at room temperature for up to 72 h; samples stored at room temperature for 96 h tested doubtful.

No amplification product was obtained when 10 μl of homogenized samples originating from 1 to 20 larvae were directly used in a multiplex PCR reaction. When the experiment was repeated by replacing the Trichin-L kit's R2 buffer with PBS, PCR amplifications were obtained for samples containing 3, 5, 10, and 20 larvae, but not for samples containing only one larva. When 450 μl of the homogenized sample were treated with a DNA purification kit, all samples (including those containing only one larva) were correctly amplified by multiplex PCR.

According to the Guidelines and the European Commission (2005), the digestion process is considered to be satisfactory if no more than 5% of the starting sample weight (100 g) remains on the sieve. The Trichin-L kit fulfilled this requirement, in that the average amount of undigested material was 1.7 g with a range between 0.0 g and 4.9 g. The Trichin-L kit correctly identified all the samples spiked with 3, 6 and 15 larvae. Some of the negative controls incorrectly tested positive in two laboratories. The laboratory investigation revealed that the detection of false positives was due to the presence of detergent traces on the digestion equipment.

The Trichin-L kit appears to be easy to use (no particular skill or training is required) and shows a very high sensitivity(down to 1 larva) and reproducibility (the same results were obtained in five laboratories). The Trichin-L kit requires less time compared to the current digestion methods and there is no need for personnel skilled in recognizing *Trichinella* larvae. Furthermore, the larval homogenate can be used to identify the *Trichinella* species by multiplex PCR, as requested by the European Commission (2005).

However, the Trichin-L kit is not exempt from weaknesses. In fact, the specificity can be compromised by chemical products, such as trace detergents used to wash the equipment (e.g., Dreft', Procter & Gamble, The Netherlands, containing: 15–30% anionogene surface active substances, 5–15% non-ionogene surface active substances; methylisathiozolinene, phenoxyethanol, and perfumes). The filter crushing and the antigen solubilization procedures could be negatively influenced by the operator's manual dexterity. Furthermore, the digestion protocol suggested by BIO-RAD is not very robust since sample blending must be performed strictly as defined in the kit instructions; indeed, minor changes might impact the assay performances.

In conclusion, the sensitivity of the Trichin-L Antigen Test kit is much greater than the minimum required by the European Commission (2005), and the amount of undigested material is much lower than the accepted limit (5% in weight of the sample). Consequently, the overall conclusion is that the Trichin-L kit meets the requirements for an accurate detection of *Trichinella* larvae in pork samples.

Conflict of interest statement

No financial or personal relationships are maintained with other people or organizations that could inappropriately influence or bias this paper.

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References

European Commission, 2005. Regulation (EC) No. 2075/2005 of the European Parliament and of the Council of 5 December 2005 laying down specific rules on official controls for *Trichinella* in meat. Off. J. ECL 338, 60–82.

Marucci, G., Pezzotti, P., Pozio, E., the Ring Trial Participants, 2009. Ring trial among National Reference Laboratories for parasites to detect *Trichinella spiralis* larvae in pork samples according to the EU directive 2075/2005. Vet. Parasitol. 159, 337–340.

Pozio, E., La Rosa, G., 2010. *Trichinella*. In: Dongyou, L. (Ed.), Molecular Detection of Food Borne Pathogens. Taylor and Francis, CRC Press, Boca Raton, London, New York, pp. 851–863.

Pozio, E., Murrell, K.D., 2006. Systematics and epidemiology of *Trichinella*. Adv. Parasitol. 63, 367–439.