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Research paper

Proficiency testing to detect *Trichinella* larvae in meat in the European Union

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ABSTRACT

According to the Commission Implementing Regulation (EU) 2015/1375 (replacing the Commission Regulation (EC) No 2075/2005), all animals, which are potential carriers of *Trichinella* spp. larvae, should be tested at the slaughterhouse or game-handling establishments according to one of the approved tests. One of the core duties of the European Union Reference Laboratory for Parasites is to organize proficiency testing (PT), as stated in the Commission Regulation (EC) No. 882/2004 of the European Parliament and of the Council. The aim of this work was to evaluate the results of PTs of the digestion method carried out by the National Reference Laboratories for Parasites (NRLPs) over a nine year period (2007–2015). Participating laboratories received a panel of samples consisting in 35 g or 100 g of minced pork or horse meat spiked with *Trichinella spiralis* live larvae. The number of spiked samples varied from 2 to 9 over the years. A negative control was also included in the panel, except during the 2015 PT, when only positive samples were used. The percentage of NRLPs, which passed the PT, increased from 83.3% in 2007 to 100% in 2014. Considering the number of recovered larvae, the heterogeneity in participant's results reduced overtime. The values of the overall mean difference between spiked and recovered larvae decreased during the study period, witnessing a general improvement of NRLPs performance and confirming the effectiveness of PT for a good performance of this test.

1. Introduction

Nematode worms of the genus *Trichinella* are zoonotic parasites circulating among carnivorous and omnivorous animals of all continents but Antarctica (Gottstein et al., 2009). In the European Union, these parasites are widespread in susceptible wild animals of most countries (Pozio et al., 2009), and they are still circulating in backyard and free-ranging pigs of 13 EU countries (Pozio, 2014). Humans acquire *Trichinella* infection by consuming raw or undercooked meat from pigs, horses, wild boars and other game animals (Pozio et al., 2003). According to the Commission Implementing Regulation (EU) 2015/1375 (European Union, 2015) replacing the Commission Regulation (EC) No 2075/2005 (European Community, 2005), carcasses of domestic pigs, horses, wild boar and other farmed and wild animal species intended for human consumption in the EU market, should be systematically tested for *Trichinella* spp. larvae at slaughterhouses or game-handling establishments unless animals are reared under controlled housing conditions (only pigs).

Since the artificial digestion technique does not allow the use of internal controls ensuring the proper execution of the test, laboratory staff shall be properly trained. Moreover, according to the current Commission Implementing Regulation (EU) 2015/1375 (European Union, 2015), a quality control program of the tests used to detect *Trichinella* and a regular assessment of the testing, recording and analysis procedures used in the laboratory have to be planned.

One of the tasks of the European Union Reference Laboratory for Parasites (EURLP) is to organize PTs for National Reference Laboratories for Parasites (NRLPs) of the European Union (EU) to evaluate the NRLP performance during the years. Since 2007, the EURLP organized an annual PT to detect *Trichinella* sp. larvae in meat of

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susceptible animals by an approved digestion method, according to the current EU legislation.

Until 2012, only few international publications on PT were available (Forbes et al., 1998; Vallée et al., 2007), but no international guidelines for the organization of PTs to detect *Trichinella* sp. larvae by artificial digestion existed, and every country had a different approach for PT organization. In 2012, the International Commission on Trichinellosis (ICT) published the Guidelines "Recommendations for Quality Assurance in Proficiency Testing", which represents a landmark for the organization of PTs to test the laboratory performance to detect *Trichinella* sp. larvae in meat samples by artificial digestion (International Commission on Trichinellosis, 2012).

The aim of this work was to evaluate the PT performance to detect *Trichinella* larvae in meat by digestion methods at the European NRLPs over a nine year period.

2. Materials and methods

2.1. Laboratories

The number of laboratories increased from 24 in 2007 to a maximum of 33 in 2014. In addition to NRLPs of the EU member countries, laboratories from Iceland, The former Yugoslav Republic of Macedonia, Montenegro, Norway, Serbia and Switzerland, participated in one or more PTs (Table 1).

2.2. PT organization

From 2007–2011, the PT management was paper-based. Since 2012, the management of the PT was in digital form on the website of the EURLP (https://www.iss.it/site/PT_CRLP/login.aspx). A restricted access to the PT area was provided to each participant by username and password. Each laboratory could select the type (pig or horse) and weight (35 g or 100 g) of meat. The PT result had to be submitted online by each laboratory. After evaluation, the individual PT report for laboratory performance and the final PT report with the results of all participating laboratories could be downloaded from the website.

2.3. Preparation of PT samples

Larvae were obtained by artificial digestion of 3–6 month-old female mice infected with *Trichinella spiralis* (isolate code ISS03). The artificial digestion was done using a modified version of the magnetic stirrer protocol (Commission Regulations, 2005; 2015) to obtain live larvae suitable for sample spiking. Briefly, the digestion fluid was prepared by adding 2 ml of 25% hydrochloric acid and 1.25 g of pepsin to 250 ml of tap water preheated at 47 ± 1 °C. The infected mouse was skinned, eviscerated, chopped in a blender with a small amount of digestion fluid, transferred to a beaker containing the remaining volume of digestion fluid and incubated at 45 ± 1 °C for 10 min. The digestion fluid was filtered through a 180 μm sieve and transferred to a 1000 ml conical glass, 750 ml of cold (4 °C) PBS was added and the fluid was kept at room temperature for 10 min for sedimentation. The sediment was collected from the bottom of the conical glass with a glass Pasteur pipette and transferred to a Petri dish. The required number of larvae was counted under a stereo-microscope at 20x magnification using a watch glass, and transferred to an hollow made in the centre of each meat ball by rinsing the watch glass with 200 μl of PBS. The watch glass was then examined under a stereo-microscope, to ensure that no larva remained on it. The whole procedure was followed by a second operator through a monitor connected to the stereo-microscope to guarantee that each sample was spiked with the exact number of larvae.

Meat balls consisted of 100 ± 2 gr or 35 ± 2 gr of minced pork or horse meat, free of fat and fascia. The PT panel consisted of 10 meat ball samples (9 spiked with larvae and 1 negative control) from 2007 to 2010, 5 meat ball samples (4 spiked with larvae and 1 negative control) from 2011 to 2012, and 3 meat ball samples (2 spiked with larvae and 1 negative control) in 2013 and 2014. In 2015, each of the 3 meat balls contained 3 larvae. The maximum number of larvae spiked in each meat ball decreased from 50 in 2007 to 3 in 2015, while the minimum number of larvae spiked in each meat ball was 3 larvae, except three years (2009–2011), when a single larva was spiked in some samples. Each meat ball was vacuum-sealed in a plastic bag and labelled with a numeric code. PT panels were packed in a polystyrene box, which contained ice packs to maintain a temperature of 4–15 °C during transportation. The stability of the samples had been evaluated by ad hoc experiments. Larvae contained in under vacuum sealed meat samples were stored between 4 and 15 °C and remained viable up to 5 days from the date of preparation. Participants were informed not to exceed the prescribed limit of storage time and temperature prior testing.

2.4. Digestion tests

According to the current EU legislation, the reference method for detecting *Trichinella* spp. larvae in meat is the magnetic stirrer method; however, other four equivalent methods could also be used. All the laboratories that participated at the PT, used the magnetic stirrer method, or the mechanically assisted digestion method (Stomacher), or the automatic digestion method (Trichomatic 35®).

2.5. Result analysis

According to the current EU legislation, the result of the artificial digestion method has to be expressed only qualitatively, i.e. as positive if *Trichinella* spp. larvae are found in the sample, or as negative if no larva is found in the sample. The PT was considered passed if the laboratory correctly identified the minimum number of positive samples established by the PT organizer, without taking into account the number of recovered larvae. In the PTs organized from 2007 to 2012, the detection of one false negative was accepted, while from 2013 onwards, all the spiked samples have to be recognized as positive to pass the PT, due to the reduction of the sample number from 5 to 3. Up to 2012, the report of one false positive did not invalidate the PT performance; while from 2013 onwards, a false positive was no longer accepted.

Although the official PT evaluation was made on the basis of qualitative results only, a quantitative evaluation based on the number of larvae recovered from each sample was also included in the report. Such additional information allowed to assess the competence of participating laboratories and to monitor their improvement. The PT evaluated only the laboratory performance as a whole, not the individual analyst's performance.

The Z-score was successfully used for the quantitative evaluation of single samples containing at least 6 larvae up to 2013. After 2013, due to the reduction in the number of spiked larvae, no statistical parameter was applied. Consequently, the results were evaluated according to the following criteria: (i) the detection of at least 2 larvae was considered acceptable for samples spiked with 4–5 larvae; and (ii) the detection of at least 1 larva was considered acceptable for samples spiked with 3 larvae. The maximum acceptable overestimation was 2 larvae for samples spiked with 4–5 larvae, and 1 larva for samples spiked with 3 larvae.

Data were statistically analysed using STATA (StataCorp LP Texas, USA). For each laboratory, the difference between expected and reported number of larvae per sample was estimated and the absolute mean of this difference was calculated over all the PT panel samples. These data allowed the laboratory to monitor