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Trichinella detection: Identification and statistical evaluation of sources of error in the magnetic stirrer method for pooled sample digestion

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ABSTRACT

Proficiency testing (PT) is the use of inter-laboratory comparisons to determine the performance of individual laboratories for specific tests or measurements, and to monitor a laboratory's performance. Participation in proficiency testing provides laboratories with an objective means of assessing and demonstrating the reliability of the data they are producing. To ensure the reliability of *Trichinella* detection and meat hygiene within the European Union and afford optimal protection to the consumer, PT is conducted under the direction of the European National Reference Laboratories for *Trichinella*. Evaluation of data from the national PT showed that lab-internal shortcomings are frequent. These shortcomings are specifically related to: (1) improper sample collection and preparation; (2) incorrect transposition and application of the protocol as laid down in Annex I, Chapter I, Nr. 3 (a–g) of the Commission Regulation (EC) No. 2075/2005; (3) insufficient sedimentation times; and (4) improper equipment.(e.g. Prost and Nowakowski, 1990; Rossi and Pozio, 2008; Forbes and Gajadhar, 1999; Rossi and Pozio, 2008). To test the hypothesis that both method based errors as well as internal lab errors can influence the accuracy and precision of the magnetic stirrer method for pooled sample digestion (MSM), we initiated a study to evaluate the analytical uncertainty of the MSM. Results presented here are based on: (i) data from PT in Germany (2008, 2009, and 2010); (ii) within-lab performance conducting high volumes of MSM; (iii) larval recovery experiments; and (iv) statistical evaluation of data resulting from these procedures. Quantitative data from the PT show that on average only 60% of *Trichinella* larvae were detected. Even laboratories that showed relatively good performance (>80% larva recovery, no false negative or false positive results), frequently reported samples with an unexpectedly low larval count (loss of >2 larvae). In our own laboratory, high numbers of repeated analyses of standards and re-analyses of residual fluids indicated that these outliers could be described by a binomial distribution based on a laboratory-specific *Trichinella*-detection probability. Results of recovery experiments indicate that only a part of the total larval losses can be attributed to lab-internal shortcomings inasmuch as a significant number of L1 could be isolated from the residual and washing fluids.

1. Introduction

In order to monitor and efficiently control trichinellosis in humans and animals, the European Union (EU)

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established a legal framework applicable to each Member State: Directive 2003/99/EC defines the monitoring of zoonoses and zoonotic agents, and Regulation (EC) No. 2075/2005 defines specific rules to control for *Trichinella* in meat. According to these legislations, mandatory examination of all slaughtered pigs and other susceptible animal species is still the main method for combating and controlling trichinellosis in Europe. In this context, the application of suitable and sensitive detection methods is a key factor for ensuring a high level of consumer protection.

Following introduction of the artificial digestion of muscle tissue in the 1970s, a new and better method for the detection of *Trichinella* was established: the magnetic stirrer method for pooled sample digestion (MSM). This procedure was validated in 1979 by Köhler and later introduced into German and EU legislation for *Trichinella* meat inspection. Over the years, numerous research groups (e.g. Forbes and Gajadhar, 1999; Gamble, 1999) evaluated the MSM and other digestion techniques. Because of the benefits of the MSM specifically related to the detection of non-encapsulated *Trichinella* spp., better larval recovery rates, reduced examination times, and lower costs, this method was defined as a reference method when Commission Regulation (EC) No. 2075 was introduced in 2006. However, it was noted that due to non-uniform larval distribution within tissues, and some technical limitations, the sensitivity of this method is limited to 3 larvae per g when examining the prescribed 1 g of meat (Forbes and Gajadhar, 1999). Apart from these methodological limits, lab-internal shortcomings in the implementation of the MSM protocol can also influence the sensitivity of the method. Table 1 gives a detailed overview of many error sources in the different steps and sub-steps of MSM.

To ensure the quality of the MSM and to evaluate the competence of laboratories in *Trichinella* detection, proficiency testing (PT) must be conducted in each Member State of the EU in accordance with Regulation (EC) No. 882/2004 and under the direction of the National Reference Laboratories for *Trichinella*. In Germany, PT has been in place since 2004. The statutory accreditation of all official *Trichinella* laboratories within the scope of Regulation (EC) No. 2075/2005 requires among other things, the regular participation in inter-laboratory testing. As a result, the number of participants has been steadily growing from 33 in 2004 (Nöckler and Reckinger, 2005) to108 in 2010 (Mayer-Scholl et al., 2011). Beginning in 2008 and in order to meet the legal requirements, every participating laboratory is mandated to analyze samples using both qualitative and quantitative methods. Three ranges of tolerance based on the z-score were defined for each sample size; a within tolerance range, an intermediate range (yellow), and an outside tolerance range (red). Outside the tolerance range means effectively that fewer than 50% of L1 were found in small samples (<10 L1) or fewer than 70% in larger samples (≥10 L1). To test the hypothesis that error sources beyond lab-internal errors may also influence the performance of the MSM, we initiated a study to evaluate the total analytical uncertainty of the MSM.

2. Materials and methods

2.1. Data from German proficiency tests

The PT results for the years 2008–2010 (Mayer-Scholl et al., 2009, 2010, 2011) were used in this paper. The qualitative and quantitative results, changes from the preceding years, and specific issues were evaluated. For further reduction of data, we arbitrarily defined a "good lab" by three attributes: (1) no false negative results, (2) no false positive results, and (3) ≥80% detection of L1.

2.2. Evaluation of in-lab performance, recovery experiments, and error analysis

Pig muscle tissues were spiked with a defined number of viable L1 by an independent person in accordance with the methods described by Mayer-Scholl et al. (2011). Artificial digestion of the spiked tissue samples was performed by the MSM according to Annex I, Chapter I of Regulation (EC) No. 2075/2005. In addition, all residual fluids from the sedimentation steps as well as washing fluids from the beakers, the separation funnel, and the sieve were examined under a stereo microscope in all cases where more than 2 L1 were missing and a few cases for which fewer were missing. An assessment of the efficacy of visualization was performed in two steps: first, the use of a stereo microscope (Olympus SZX 12, Olympus Deutschland GmbH, Hamburg, Germany) was compared with a trichinoscope (IX Q2, Erdmann & Grün KG, Wetzlar, Germany) and second, the use of a Petri dish was compared to a larval counting basin using the stereo microscope. Table 2 gives an overview of all experimental conditions and details of the respective experiments.

3. Results

3.1. Analysis of data from German proficiency tests (2008–2010)

The ability of the participating laboratories to qualitatively classify sample material as either true-positive or true-negative was quite good; the overall sensitivity to detect *Trichinella* positive samples was 89% in 2008, 95% in 2009, and 93% in 2010 whereas the overall specificity was 93%, 99% and 97% in 2008, 2009 and 2010, respectively (Mayer-Scholl et al., 2009, 2010, 2011). In contrast, on average only 60% of *Trichinella* larvae were detected by quantitative analysis. In the 2009 German PT for example, only 40 of 87 participants (46%) were able to find at least 35 of 51 (70%) L1 in total and only 13 labs (15%) provided a result that fell within the tolerance range for each positive sample. If criteria for "good labs" (see Section 2) are applied, 15 (17%) laboratories are left, among which 1 lab (7%) had no outliers (L1 loss >2), 12 labs (80%) had 1 outlier, and 2 labs (13%) had 2 outliers. This means that low L1 counts occur regularly even in the hands of experienced laboratories. Data analyses show further, that this error is not predictable and is not uniformly distributed.