

FIRST SERBIAN EXTERNAL QUALITY ASSESSMENT TO DETECT *TRICHINELLA* LARVAE IN MEAT BY THE MAGNETIC STIRRER METHOD

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Abstract

Introduction. Inter-laboratory comparative testing must be conducted under the direction of the National Reference Laboratory for *Trichinella* in each Member State of the EU with the aim to ensure the quality of the approved digestion method and to evaluate the competence of laboratories in *Trichinella* detection. For the first time in Serbia, the National Reference Laboratory for Trichinellosis (NRLT INEP) organized such external quality assessment (EQA) for the detection of *Trichinella* larvae in meat by the Magnetic Stirrer Method (MSM). The aim of this study was to enable laboratories interested to take part in the EQA and to assess the quality of their MSM performance.

Materials and Methods. The EQA was organized by NRLT INEP according to ISO/IEC 17043:2010. The EQA test panel, which was sent to eight laboratories within Veterinary Institutes, consisted of three 100±5 g minced pork meat balls with identical numbers of larvae. Two meat balls were spiked with five viable *Trichinella spiralis* muscle larvae (L1), and one meat ball was not spiked.

Results and Conclusions. Evaluation made on the basis of qualitative results showed that 100% of participants successfully completed the testing. Quantitative evaluation showed that, on average, only 60% of *Trichinella* larvae were detected among all participants. The main importance of this study is that it enabled laboratories across the country to be compared. This should be efficient stimulus for improvement of analytical performance over time. Therefore, it will be of value if the participants and other official laboratories in Serbia take part annually in EQAs organized at national level. The results obtained in this study could provide useful information to the Ministry of Agriculture, Forestry and Water Management, Veterinary Directorate and be of importance for promoting the one health concept in the country as a whole.

Key words: *Trichinella*, external quality assessment, meat

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INTRODUCTION

Zoonotic parasites of the genus *Trichinella* are widespread in the world. Many domestic and wild animals can be infected by *Trichinella* spp. (Pozio and Murrell, 2006; Pozio and Zarlenga, 2013). Human infection is acquired after consumption of raw or undercooked meat of different animal origin which contains *Trichinella* larvae (Pozio et al., 2003). To protect consumers' health in the European Union, the Commission Regulation (EU) No. 2015/1375 defines specific rules for control of *Trichinella* in meat. Mandatory examination of all slaughtered pigs and other susceptible animal species intended for human consumption is still the main method for combating and controlling trichinellosis in Europe (Riehn et al., 2013). From validation in 1979, and after improvements made over years, the magnetic stirrer method for pooled sample digestion (MSM) was defined as a reference method among those approved for digestion (Regulation No. 2015/1375). Regulation EU 2017/625 requires, among other things, that all the laboratories performing official controls for *Trichinella* participate on a regular basis in inter-laboratory comparative or proficiency testing (PT). It should be conducted under the direction of the National Reference Laboratory for *Trichinella* in each Member State of the EU, with the aim to ensure the quality of MSM and to evaluate the competence and performance of laboratories in *Trichinella* detection. In different EU member states, PTs have already been performed for more than 10 years (Marucci et al., 2009; Riehn et al., 2013; Marucci et al., 2016).

It is in the best interest of the veterinary service in Serbia that each official laboratory for *Trichinella* detection participates not only the international PTs at individual level (for their accreditation needs), but also participates in PTs organized at national level, with the aim to compare with their performance with those of the other laboratories and improve the level of their competence in the field. Hence, for this purpose, in 2017 and for the first time in Serbia, NRLT INEP organized an external quality assessment (EQA) for the detection of *Trichinella* larvae in meat among Veterinary Institute laboratories as a first step toward the above-mentioned final goal.

MATERIALS AND METHODS

Animals and parasite used in EQA. *Trichinella spiralis* (ISS 161) was maintained at NRLT INEP by passage in *Wistar* rats. The animals used complied with all the relevant national regulations and institutional policies for the care and use of animals. This research was approved by the local Institutional Animal Care and Use Committee and by the Veterinary Directorate (Consent No 323-07-11214/2016-05/6 issued on 21.02.2017).

EQA concept. The EQA for detecting presence of *Trichinella* larvae in meat was organized by NRLT INEP according to ISO/IEC 17043:2010. This standard defines inter-laboratory comparative testing as evaluation of participants' ability to perform a given analytical procedure against pre-established criteria. A member of NRLT

INEP was previously trained for PT organization by the European Union Reference Laboratory for Parasites (EURLP), Istituto Superiore di Sanita, Rome, Italy (accredited by ACCREDIA as a PT provider in conformity with ISO/IEC 17043:2010). The EQA was announced to two Scientific Veterinary Institutes (SVIs) and ten Veterinary Specialized Institutes (VSIs) in 2017. NRLT INEP offered each participating laboratory the opportunity to assess its performance through a confidential system of sample testing and to determine its ability to perform a given analytical procedure.

The aim of the EQA was for the laboratories to correctly identify positive and negative samples.

The *EQA panel* consisted of three samples with identical numbers of larvae for all participants. Preparation of the three samples was described by Marucci et al. (2016). Briefly, test material consisted of three 100 ± 5 g minced pork meat balls. Two meat balls were spiked with five viable *Trichinella spiralis* muscle larvae (L1), and one meatball was not spiked (negative control). Larvae were obtained by the artificial digestion of *T. spiralis*-infected rat carcasses. Larvae were counted under a stereo-microscope using a watch glass and transferred to the meat balls by rinsing with PBS. To ensure that no larva remained on the glass, it was examined twice under the stereo-microscope and rinsed with PBS. Each meat ball was closed in a plastic bag and sealed under vacuum, followed by a code addition on the envelope. Each envelope containing the meat ball was then stored at 4°C until forwarding the same day. Meat balls were forwarded in a polystyrene box containing ice packs with the aim of maintaining a temperature of 4-15°C during transportation. In the box, ice packs were separated from the meat balls by a cardboard separator to avoid direct contact. The packages were forwarded by courier. To check the sample stability over time, and to estimate the suitability of the packing and forwarding conditions, three meat balls were packed as those forwarded, stored at room temperature, and tested at the NRLT INEP two days after packaging. Each participant laboratory was invited to fill in the enclosed form with the information about package content and its condition of preservation at the moment of opening.

All participants analyzed the three sample meat balls using the MSM for pooled sample digestion (EU 2015/1375).

Results of the evaluation were only qualitative according to EU 2015/1375. The participant had to identify the samples as positive if *Trichinella* larvae were present or negative if larvae were not present. Final evaluation was considered positive if all three samples were properly identified. Quantitative evaluation, based on the number of larvae recovered from each sample, was also included. To compare the results between the different laboratories, the number of recovered larvae was calculated and presented as a percentage of expected number of larvae in the sample (detected/expected x 100). The NRLT INEP provided an EQA report for each participant. To guarantee confidentiality, each participant could access only their own EQA report, and in the overall summary of performance, only laboratory codes were displayed.

RESULTS AND DISCUSSION

Eight laboratories that cover the epizootiological area across the country (two SVIs, located in Novi Sad and Belgrade and six VSIs located in Subotica, Pozarevac, Zajecar, Jagodina, Nis and Kraljevo) agreed to participate in this voluntary EQA (Figure 1). All eight packages were delivered to the laboratories within 24 h. At delivery, the internal temperature of all packages was less than 15°C. Therefore, the optimal time for delivery and cooling conditions for samples were maintained. The time elapsed from the arrival of the package and the start of testing was approximately 2 h for all laboratories.

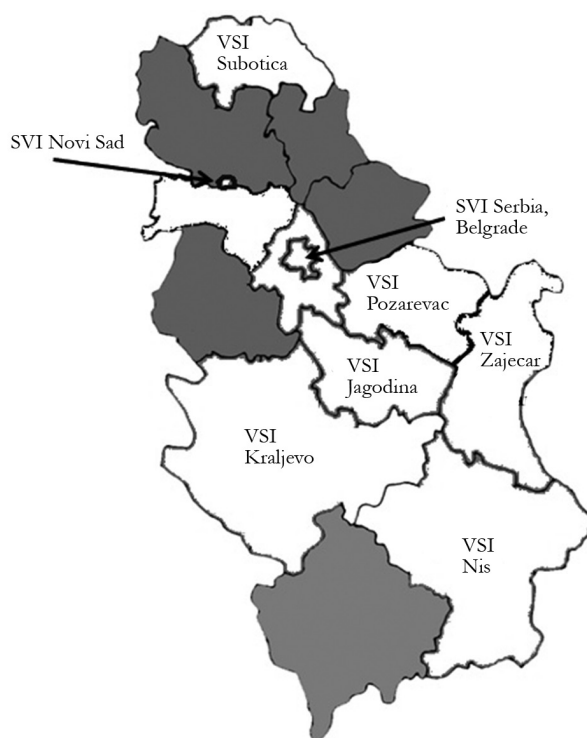


Figure 1. Epizootiological area of Serbia covered by participants in the EQA 2017 for *Trichinella* larvae detection

Legend: White – Epizootiological areas covered by the laboratories that participated in EQA 2017, (the name of each institute corresponds to the name of the city in which it is located). Dark gray – Epizootiological areas not covered by participating laboratories. Light gray – Kosovo, no data. Note: SVI Serbia, located in Belgrade, is a scientific institution covering the whole of Serbia, while SVI Novi Sad is a scientific institution covering Vojvodina province.

In this EQA, no laboratory reported false negative or false positive results, so the final evaluation of qualitative results for all eight participating laboratories was positive (100% of the laboratories passed) (Table 1).

Table 1. EQA 2017. Qualitative and quantitative *Trichinella* larvae detection results reported by eight Serbian laboratories using the magnetic stirrer method of digestion

Laboratory code	Number of larvae		Difference	Results	Final Evaluation
	spiked	found			
1	0	0	0	positive	positive
	5	2	3	positive	
	5	4	1	positive	
2	0	0	0	positive	positive
	5	2	3	positive	
	5	4	1	positive	
3	0	0	0	positive	positive
	5	4	1	positive	
	5	5	0	positive	
4	0	0	0	positive	positive
	5	3	2	positive	
	5	3	2	positive	
5	0	0	0	positive	positive
	5	2	3	positive	
	5	3	2	positive	
6	0	0	0	positive	positive
	5	3	2	positive	
	5	1	4	positive	
7	0	0	0	positive	positive
	5	2	3	positive	
	5	2	3	positive	
8	0	0	0	positive	positive
	5	4	1	positive	
	5	4	1	positive	

Data analysis showed the ability of the participating laboratories to qualitatively classify meat samples as either true-positive or true-negative was excellent. Since the specificity of the testing method depends on operator skills to differentiate *Trichinella* larvae from other parasites, the demonstrated skills were very satisfactory. The overall success for all participants of 100% is equivalent to the best achieved (100% in 2012, 2014 and 2017) among EU laboratories in PTs organized by EURLP, 2007-2017. The percentage of National Reference Laboratories for Parasites (NRLPs) in EU member countries which passed PTs during 2007-2017 was between 83.3% and 100% (Marucci et al., 2016; data from EURLP web site). An exception occurred in 2009, when three samples of 100 g, each spiked with one larva, were included in the PT panel, and only 18 of 26 (69.2%) of participant laboratories correctly identified these samples.

Although this confirmed the theoretical sensitivity limit of the artificial digestion procedure (1 larva per gram of meat), this low number of spiked larvae was not applied in the following years.

Quantitatively, none of the participating laboratories succeeded in detecting all larvae in the two spiked meat balls. Results expressed as the percentage of detected in relation to the expected number of larvae are presented in Figure 2. Two out of eight laboratories were very successful (Nos 3 and 8), since their level of L1 detection was 90% and 80%, respectively. In three laboratories (Nos 1, 2 and 4), the level of L1 detection was 60%, one laboratory (No 5) detected 50% of L1, while two laboratories detected 40% of L1 spiked into the meat balls (Nos 6 and 7).

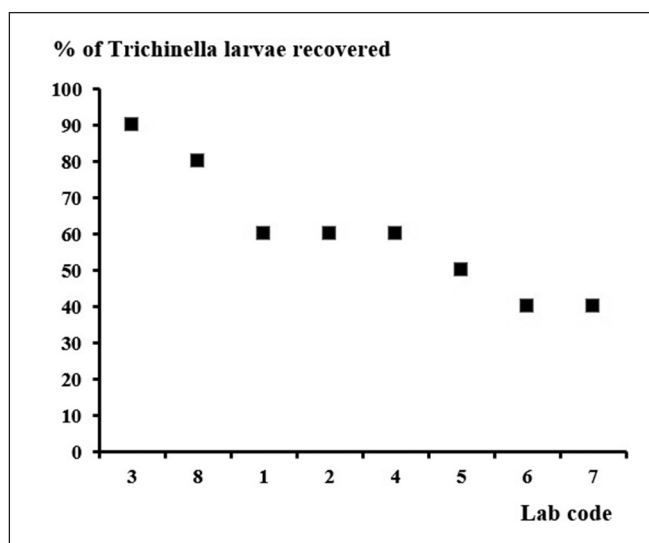


Figure 2. Quantitative results for eight participating laboratories in EQA 2017 in Serbia. Values are expressed as percentage of detected in relation to the total number of *Trichinella* larvae spiked into the meat panel.

Interestingly, in spite having the same score of 40% recovery of larvae spiked in the sample panel, those two laboratories (Nos 6 and 7) differed in the number of larvae recovered from individual samples (No 7 achieved 40% recovery for each sample, while No 6 achieved 60% for one sample and 20% for the other). Therefore, laboratory No 6 was likely close to producing a false negative result. Marucci et al. (2016) indicated that the Z-score could not be used for quantitative evaluation of a single sample containing less than six larvae and, therefore, the detection of at least two larvae was considered acceptable for samples spiked with four or five larvae. If this criterion is taken into consideration, laboratory No 7 would meet the requirements (by finding two larvae, 40%) while laboratory No. 6 would not. In spite of the fact that the aim of this EQA, which was to correctly identify positive and negative samples, was

successfully accomplished, the finding of only one larva in one single sample should initiate an in-house investigation and continuous monitoring of the critical control points for the MSM and the individual analyst's performance. The overall success of MSM performance, on average, was 60% of the muscle larvae recovery.

Quantitative results for *Trichinella* larvae detection (40%-90% of larvae were detected, depending on participating laboratory) were in line with expectations. It was previously shown that laboratories participating in EU-level PTs usually produce lower muscle larvae counts compared to the actual number of larvae spiked in meat (Marucci et al., 2009; Marucci et al., 2016). Our results are also in line with those obtained from the first German PTs, in which 60% of the larvae were recovered by the participants (Riehn et al., 2013). Also, results presented by the same authors indicated that participation in consecutive PTs positively affected laboratory accuracy, and heterogeneity in participants' results reduced over time (it was considered as relatively good performance if there was >80% larvae recovery and no false negative or false positive results). In relation to this, our current results should motivate the laboratories to improve their MSM performances over time. We suggest the final goal that all laboratories should aim to achieve could be set to >40% larvae recovery from an individual sample.

It is important to pay attention to the critical control points (CCPs) for MSM to improve laboratory performance (Djordjevic et al., 2013; ICT guidelines). The detection of smaller numbers of larvae than were spiked in this EQA could be explained by errors related to CCPs, assuming that in each step, 2-3% of the larvae are lost (Riehn et al., 2013).

Previously, five Serbian VSI laboratories participated in *Trichinella* detection PTs organized by different European providers. However, disadvantages of the international PTs were the long transport duration, very high prices for participation and inability of these laboratories to compare their performances with each other (personal communication). NRLT INEP participated (2009-2017) in nine PTs organized by EURLP and successfully passed them all (including when each sample was spiked with one larva; data from EURLP web site). NRLT INEP was recognized by EURLP as a member of the NRLP Network in EU from 2008 and is one of three NRLP outside the EU. Comparison of NRLT INEP results have, over time, clearly demonstrated good laboratory performance and sensitivity of the MSM used. In the current study, the criterion for positive evaluation was the correct detection of *Trichinella*, regardless of the number of larvae detected, i.e., even if only one larva was found in a positive sample, the evaluation was positive. This criterion was based on the test sensitivity. The number of spiked larvae per sample was in agreement with EURLP, which decreased the number of larvae spiked in each meat ball from 50 in 2007 to 3-5 after 2015 (Marucci et al., 2016), as well as with data presented by Forbes et al. (1998), who showed that, even if one larva/g can be detected by the digestion assay in a pool of 100g, a limit of three larvae in samples is more appropriate. For these reasons, NRLT INEP spiked each sample with five larvae. The results of this

first Serbian EQA demonstrate the competence of NRLT INEP to conduct EQA in accordance with the rules envisaged for PT providers, as well as the competence of the participating laboratories to perform the testing.

Serbia is categorized as highly endemic for *T. spiralis*, a parasite which continues to circulate among backyard and free-ranging pigs. Those animals contact the parasite via its sylvatic cycle, and so such pigs are frequent sources of small, family trichinellosis outbreaks. Also, in this century, some trichinellosis cases in Serbia occurred as a consequence of game meat consumption (Pavic et al., in press). Over the years, though, pork has been the most frequent source of human trichinellosis in the country (Sofronic-Milosavljevic et al., 2013). However, the rate of swine infection in Serbia has gradually decreased in this century from 0.14% to 0.005% (NRLT INEP data). The significant reduction of *Trichinella* infection in domestic pigs was achieved not only due to improvements in socio-economic conditions in the country but also due to increased effectiveness of control measures and public education conducted in the field. In Serbia, testing for *Trichinella* larvae in meat is regularly performed by SVIs, VSIs, Veterinary stations, clinics and slaughterhouses. Hence, official laboratories for *Trichinella* detection have probably significantly contributed to the *Trichinella* reduction in the country.

However, this EQA shows there is room for further improvement and training of personnel performing MSM for *Trichinella* detection. This EQA has provided useful information and presented a solid base for further, more extensive, analyses. For more adequate estimation of Serbian laboratories' performances with regard to detection of *Trichinella* in meat, a larger number of laboratories must participate regularly in the inter-laboratory comparisons or PTs organized by NRLT INEP (according to EU directive 625/2017). Until full integration of veterinary and public health efforts (the one health concept) is achieved, *Trichinella* will continue to circulate in Serbia, with a risk of this parasite spreading to domestic and/or wild swine (highly susceptible to this pathogen) in EU countries that border Serbia.

CONCLUSION

All the participant laboratories successfully passed the first Serbian *Trichinella* EQA, with no false positive or false negative results. The results clearly demonstrate the good performance of participating Veterinary Institute laboratories in the detection of *Trichinella* larvae. The main importance of this study is that it enabled, for the first time, laboratories across the country to be compared. Such inter-laboratory comparison should be efficient stimulus for them to improve their analytical performance over time. Therefore, annual participation of these and other laboratories in Serbia in EQAs organized at the national level will be invaluable. The current results should provide useful information to the Ministry of Agriculture, Forestry and Water Management, the Veterinary Directorate and be of importance for promoting the one health concept in the country as a whole.

Conflict of interest statement

None of the authors of this manuscript has declared any conflict of interest.

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PRVA EKSTERNA PROCENA KVALITETA DIJAGNOSTIKE PRISUSTVA LARVI *TRICHINELLA SPIRALIS* U MESU METODOM MAGNETNE MEŠALICE U REPUBLICI SRBIJI

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Kratak sadržaj

Uvod. Eksterna procena kvaliteta (EPK) je međulaboratorijska kontrola koju sprovode Nacionalne referentne laboratorije za trihinelozu u svakoj državi članici EU u cilju osiguranja kvaliteta korišćenja odobrenih metoda digestije i procene sposobnosti laboratorije u otkrivanju larvi *Trichinella*. Nacionalna referentna laboratorija za trihinelozu INEP (NRLT INEP) je prvi put u Srbiji organizovala EPK za otkrivanje larvi trihinelu u mesu korišćenjem metode magnetne mešalice za zbirni uzorak (MMM). Cilj ove studije bio je da se omogući zainteresovanim laboratorijama da učestvuju u EPK kako bi ocenili kvalitet izvodjenja MMM.

Materijal i metode. NRLT INEP je organizovao EPK prema zahtevima standarda ISO/IEC 17043: 2010. Test panel poslat u osam laboratorija se sastojao od po 3 loptice napravljene od 100 ± 5 grama mlevene svinjetine sa identičnim brojem larvi za sve učesnike. U panelu je u dva uzorka bilo dodato po 5 živih mišićnih larvi *Trichinella spiralis* (L1), a jedan uzorak je bio bez larvi (negativna kontrola).

Rezultati i zaključak. Procena izvršena na osnovu kvalitativnih rezultata je pokazala da su svi učesnici uspešno prošli testiranje (100%). Kvantitativna ocena, izražena u procentima, i zasnovana na broju pronađenih larvi u odnosu na ukupni broj postavljenih larvi, iznosila je 60% na nivou svih učesnika EQA. Glavni značaj ove studije zasniva se na činjenici da je po prvi put omogućila poredjenje rezultata dobijenih primenom iste metode za analizu istih uzoraka između laboratorija širom zemlje. Ovakvo dobijeni rezultati bi trebalo da budu efikasan stimulus za poboljšanje izvodjenja metode magnetne mešalice za zbirni uzorak od strane manje uspešnih laboratorija tokom vremena. Zato bi bilo poželjno da ove ali i druge laboratorije u Srbiji učestvuju u narednim EPK ili PT šemama organizovanim na nacionalnom nivou. Dobijeni rezultati bi mogli da pruže korisne informacije Ministarstvu poljoprivrede, šumarstva i vodoprivrede, Upravi za veterinu i da budu od značaja za promociju koncepta “jedno zdravlje za sve” na nivou države.

Ključne reči: *Trichinella*, Eksterna procena kvaliteta, svinjsko meso