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ORIGINAL ARTICLE

Gelatin medium for preserving of *Trichinella* spp. for quality control in laboratories – estimation of larvae viability

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Abstract

Introduction and Objective. Official food control laboratories ensure food safety using reliable, validated methods. Council Regulations (EC) No. 853/2004, 854/2004 and 882/2004 of the European Parliament established hygiene rules the production of food of animal origin, together with requirements for official controls. This leads to detailed requirements for *Trichinella* control set out in Commission Implementing Regulation (EU) 2015/1375 of 10 August 2015. These regulations require the laboratory to participate in proficiency testing (PT) to confirm their competence and improve the quality of testing, and require the PT Organizer to use methods for the preparation and preservation of parasite larvae in order to evaluate and improve detection. Traditional methods of preparing such larvae expose them to rapid degradation, making it necessary to simultaneously isolate the larvae and place them in meatballs to ensure quality.

Materials and method. We developed a technique for preserving of *Trichinella* spp. for quality control such as PT sample preparation. The procedure protects larvae against toxic oxygen activity and bacterial destruction via a gelatin barrier. To estimate the viability of larvae preserved by this method, gelatin capsules with 10 larvae of *T. spiralis* in each were stored (4–8 °C) during 45 days of an experiment. Samples were tested at 2 day intervals (3 samples each day of testing).

Results. In total, 75 samples were tested. Larvae remained alive up to 3 weeks. The number of living larvae diminished after 27 days through day 43, after which no living larvae were observed.

Conclusions. The gelatin medium procedure facilitated easy, high-throughput sample preparation and supported 100% recovery for 3 weeks. The method allows fast, efficient and accurate PT sample preparation.

Key words

Trichinella spp, gelatin medium, proficiency tests

INTRODUCTION

Meat safety and quality determine the placement of meat and meat products on the market. These factors also affect conditions of international trade. Testing meat for zoonotic parasites in the genus *Trichinella* by field laboratories underlies those goals [1].

Nematodes of the genus *Trichinella* cause trichinellosis – a parasitic zoonosis. Humans become infected through the consumption of raw meat containing live *Trichinella* larvae (e.g. meat of wild boar, pigs and horses) [2, 3]. Symptoms depend on the developmental stage of the parasite. Acute infection at the enteral stage manifests by diarrhoea, nausea, vomiting, abdominal pain and fever. Symptoms of the parenteral phase of infection involves, among others, fever, weakness, muscle pain and periorbital oedema. Major complications are cardiovascular (myocarditis and tachycardia), neurological (meningitis or encephalopathy) and respiratory (dyspnea, pneumonia, and obstructive bronchitis) [4, 5]. The complications can sometimes lead to death [2].

Historically, pork has been recognized as a main source of trichinellosis. Obligatory investigation of pork for *Trichinella*, and other activities increasing biosecurity on pig farms, have nearly eradicated this parasite from domestic swine. Thus, in recent years, most cases of human trichinellosis derive from the consumption of infected wild boar meat, hunted illegally and not examined for the presence of *Trichinella* spp. In some countries, human cases continue to result from the consumption of pork derived from backyard pigs, untested or tested by an unreliable compression method [6].

Commission Regulation (EC) No. 2015/1375 of 10 August 2015 laying down specific rules for official controls for *Trichinella* in meat recommends the magnetic stirrer digestion method (MSD) as a reliable and reference method for routine use in laboratories [7]. In accordance with the regulations of

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the hygiene package, i.e. Regulations 853/2004 [8], 2017/625 [9] of the Council (EC) and the European Parliament, a quality assurance system regulates laboratories performing tests for official purposes, and laboratories testing meat for *Trichinella* [10]. According to these regulations, all laboratories involved in the testing of samples for *Trichinella* detection should participate in Proficiency Testing (PT) programmes. PT's, as defined in PN-EN ISO/IEC 17043, enable laboratories to demonstrate their competence to accreditation bodies or other third parties [11]. Such proficiency testing has a significant educational component. Proficiency tests enable laboratories to monitor routine implementation of diagnostic methods, monitor long-term trends, enhancing corrective and preventive actions [12]. The organization of PT's is under the competence of European Reference Laboratory or National Reference Laboratories (NRLs) [13, 14]. The organizers must provide reliable methods for samples preparation. The prepared PT samples are used to check the integrity of the measurements, skills of the technical personnel, and the laboratory's competence in performing tests [13].

The International Commission on Trichinellosis (ICT) provides specific recommendation for Quality Assurance (QA) for organizers of Proficiency Testing [15]. This document includes minimum requirements for producing proficiency test samples capable of supporting accurate assessment of diagnostic test performance. A reliable technique to produce PT samples must enable placement of known numbers of *Trichinella* larvae in meat samples [16].

While preparing the PT samples, it is very important to provide the best possible environment for the larvae to extend their lifespan and ensure that routine investigators in field laboratories can detect living (moving) *Trichinella* larvae. Anerobic metabolism favours survival of the parasite in putrefying flesh [17]; therefore, limiting oxygen exposure can enhance the survival of *Trichinella* larvae in PT samples. To our knowledge, only a few methods relating to proficiency sample production, with a known number of larvae, have been described to date [13, 16, 18].

The National Reference Laboratory in Poland oversees over 700 field laboratories engaged in routine testing of meat for *Trichinella* spp. Preparing PT samples for such large number of laboratories requires a lot of time, and none of the previously described methods worked well when such a large number of samples were needed [19, 20]. Therefore, we developed an improved medium for storing and transporting *Trichinella* larvae useful for quality control purposes. Validation of the process yielded a patent application (236775; Różycki M, Bilska-Zając E, Kochanowski M, Cencek T, Karamon J, Dąbrowska J, Zdybel J, Sroka J). For preparation of the above-mentioned medium, the type of gelatin was chosen based on features such as its density and stability. In addition, 4 types of liquids were used to dissolve the tested gelatins (water, double distilled water, distilled water with antibiotics, peptone water). Based on the experiments performed, gelatin type A acid process with pH 3.8 – 5.0 and hardness of 300 Bloom and peptone water proved to be the best combination.

This article describes the developed method for the preparation of gelatin capsules with a known number of *Trichnella spiralis* larvae useful for quality control purposes, such as proficiency testing. The aim of this study was to estimate viability of *T. spiralis* larvae placed in the patented gelatin capsules and tested under the conditions of routine sample digestion.

MATERIALS AND METHOD

Method of PT samples preparation using gelatin capsules with live *T. spiralis*

Preparation of gelatin capsules with live T. spiralis larvae. The method of preparing the patented medium consists of preparing a substrate based on gelatin from pig skins obtained by the type A acid process with pH 3.8 – 5.0 and hardness of 300 Bloom. The gelatin is dissolved at 55–65 °C in buffered peptone water to form a solution with a gelatin content of 7 – 9%. The liquid medium is brought to a temperature of 45° and poured over a Petri dish to a height of 6 – 8 mm and left under sterile conditions to solidify. In the substrate prepared this way, wells with a diameter of 6 mm are cut using the end of pipette tips with a smooth edge (Fig. 1). The cut, cylindrical fragments of the substrate are removed, leaving empty wells, after which 100µl of liquid substrate at 37 °C is poured into each well. After this step, the plate is left at 4 °C (±2) until the added substrate has completely solidified.

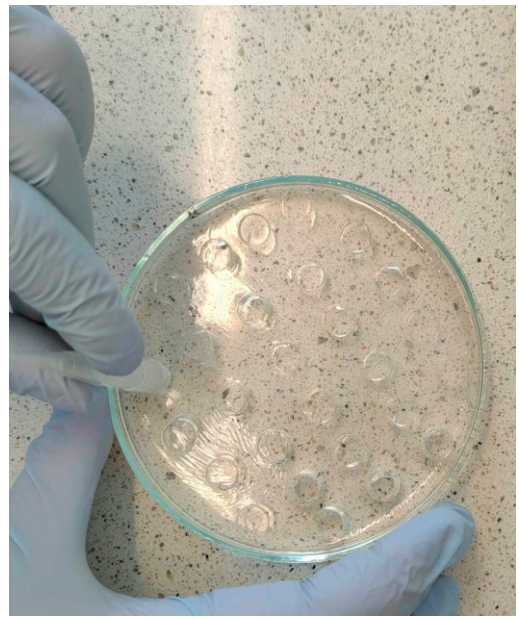

Figure 1. Cutting cylindrical gelatin wells using a 1,000 µl tip

Placing live larvae in prepared medium. The next step is to suspend live *Trichinella* spp. larvae in the prepared transport medium. Live *Trichinella* spp. larvae are obtained from naturally-infected muscle tissue, most often from wild boar (depending on availability of the infected muscle). The larvae from the muscle tissue are isolated using the Magnetic Stirrer Method (MSD), as described in the ISO 18743 Standard [21]. Some of larvae are subjected to extract DNA to perform multiplex PCR for species identification [22]. The rest of the larvae are assessed using a stereomicroscope for their viability and condition. Live *Trichinella* larvae are placed into a new Petri dish with a fresh 0.9% NaCl solution.

Subsequently, a single living larva is picked up with a 1-10 µl pipette and transferred to a transport medium plate for the first well (Fig. 2). These operations are carried out under the control of a stereomicroscope, so that the laboratory staff are sure that the larvae is placed in the medium. This step is repeated for the scheduled/defined number of samples. The same procedure is followed when preparing gelatin capsules containing up to 5 larvae. In such case, multiple larvae are

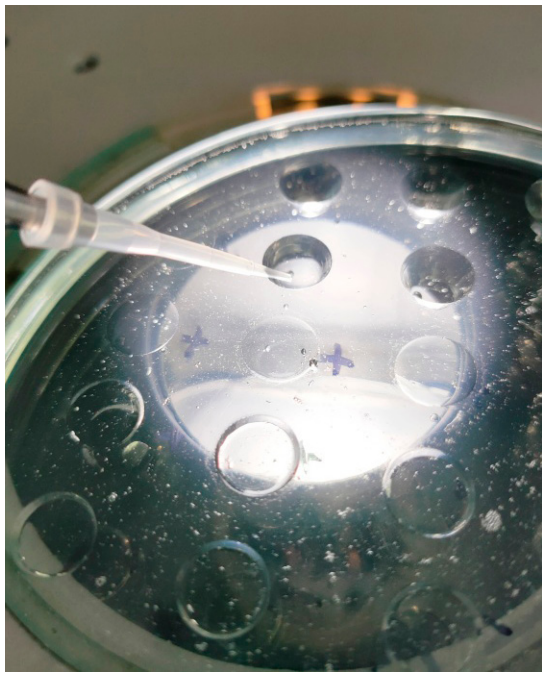

Figure 2. Placing Trichinella larvae into a gelatinous well

transferred by pipette under the control of a stereomicroscope and placed into another petri dish with transport medium. *Trichinella* larvae are protected by then covering them with 200–300 µl of medium at 37 °C to each well with larvae. These gelatin capsules can be stored for about 3 weeks at 4–8 °C, verified in Polish patent 236775.

Quality control of gelatin capsules containing live *Trichinella* larvae. The quality control of the prepared gelatin capsules and the number of larvae placed in each capsule is assessed by 2 independent laboratory technicians. They inspect each petri dish with prepared wells containing a known number of *Trichinella* larvae under a stereomicroscope. Capsules in which at least one of laboratory technicians did not find the larvae or found a different number larvae than expected, are discarded. Only capsules that have passed the quality control process are used to prepare samples for proficiency testing (Fig. 3).

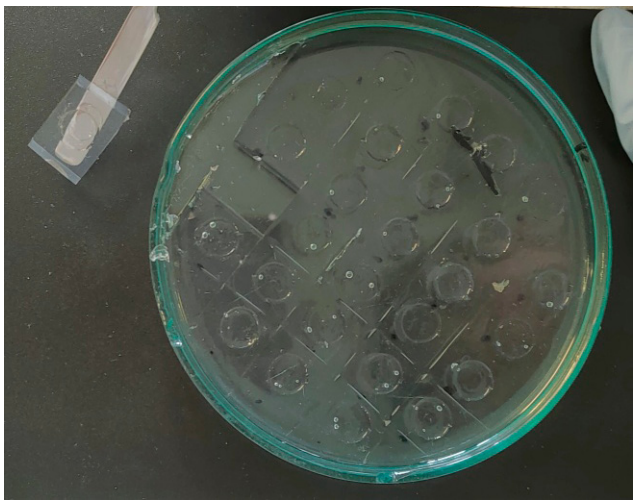

Figure 3. Cutting of gelatinous fields with Trichinella spp. larvae and transfer of fixed larvae from the plate

Matrix preparation. The matrix for PT samples is usually pork meat that does not contain *Trichinella* larvae. The muscle tissue most often comes from young fattening pigs from the longest back muscle (*Musculus longissimus*). In the case of laboratories testing horse meat for *Trichinella*, horse muscle tissue is used as a matrix. In each case, the matrix is stripped of fascia and fat and then mixed in meat grinders. Until the matrix is contaminated with *Trichinella* larvae, it is stored at 4 °C (±2). Just before adding the parasites, the matrix is weighed, 50 g per sample. The prepared meat balls are placed in a suitable sample containers.

Contamination of samples. Gelatin capsules that have passed the quality control are cut out with a fragment of medium to squares of about 1cm x 1 cm and placed inside the 50 g ball of meat matrix (Fig. 4).

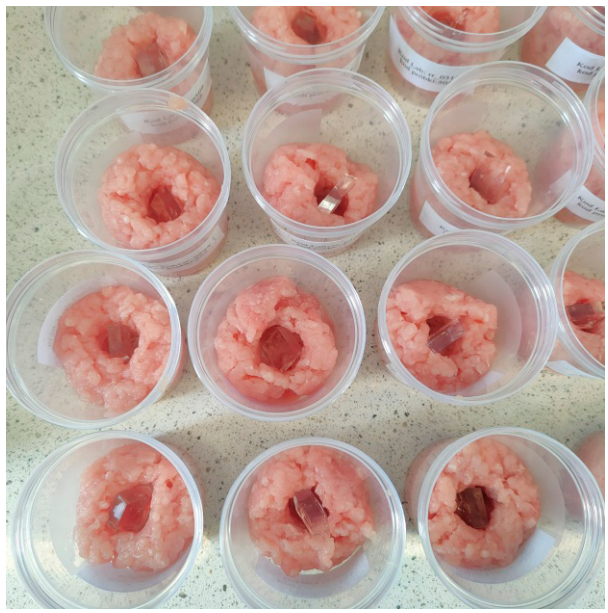

Figure 4. Samples of meat matrix with gelatin capsule

Estimation of *T. spiralis* larvae viability in prepared gelatin capsules under conditions of routine sample digestion process

For this experiment, 75 gelatin capsules each containing 10 live *T. spiralis* larvae were prepared. Capsules were refrigerated (4 – 8 °C) for up to 45 days. In order to accurately assess the viability of the larvae under the conditions of actual sample digestion used during routine meat testing for *Trichinella* (ISO 18743 Standard) [21], the prepared gelatin capsules were placed in pork meat and tested by MSD. Subsequent samples were examined at 2 day interval, 3 samples (n=3) per day of investigation. In total, 75 samples were tested. The number and viability of *T. spiralis* larvae placed in the medium were checked under a stereomicroscope.

RESULTS

The results for the viability of *T. spiralis* placed in gelatin capsules stored for up to 45 days at 4 °C are shown in Table 1.

Larval viability was entirely maintained for 21 days (Table 1). On the 23rd day, no movement was observed for

Table 1. Larvae viability under conditions of routine sample digestion. Each of the prepared samples for this experiment contained 10 live *T.spiralis* larvae embedded in a gelatin capsule

Day of experiment	No. of detected larvae in tested samples (alive/dead/missing)			% of alive larvae recovery	Additional remarks	
	Sample No.				Weak larvae movement[1]	Mould on the medium surface[1]
	1	2	3			
D1 –D19	10/0/0	10/0/0	10/0/0	100	-	-
D21	10/0/0	10/0/0	10/0/0	100	+	-
D23	9/1/0	10/0/0	10/0/0	96.7	+	-
D25	10/0/0	10/0/0	10/0/0	100	+	-
D27	9/1/0	10/0/0	9/1/0	93.3	+	+
D29	8/2/0	10/0/0	9/1/0	90	+	++
D31	8/2/0	9/1/0	8/2/0	83.3	+	++
D33	6/2/2	7/2/1	8/1/1	70	+	++
D35	5/0/5	3/2/5	6/1/3	46.7	++	++
D37	3/2/5	5/2/3	3/2/5	36.7	-	++
D39	0/2/8	2/4/4	3/1/6	16.7	-	++
D41	1/0/9	1/0/9	0/1/9	6.7	-	+++
D43	0/0/10	0/0/10	0/0/10	0	-	+++
D45	0/0/10	0/0/10	0/0/10	0	-	+++

[1] Assessment of the intensity of mould appearing on the surface on the gelatin medium and weak larvae movement

one larva in one sample. A clear reduction in viability was observed after day 27, when mould first began to appear on the surface of the medium. Live larvae decreased through day 43, after which no live *T. spiralis* were observed. Day 21th was the first when some larvae seemed less active, with weak movements. Decreasing activity was observed in samples tested through day 35. The first day when a larva could not be observed (live or dead) was day 33. By days 43–45, none of the larvae were observed.

Based on above results the shelf life of gelatin capsules with alive *T. spiralis* larvae was assessed as 21 days with 100% of viability of used larvae.

DISCUSSION

Taking part and obtaining good results in proficiency tests is one of the key elements of confirming the competence of a laboratory. Therefore, PT organizers hold a key responsibility to prepare adequately stable samples for PT. According to recent ICT recommendations, samples for proficiency testing should contain a certain number of *Trichinella* larvae, known to the organizer, but not to PT participants [15]. The ICT also recommends recreating the entire methodological process as much as possible by using PT samples that are most similar to those used in routine testing, and therefore containing live *Trichinella* larvae [15, 23]. Here, we present a novel method for preserving living *T. spiralis* larvae for quality control purposes, such as PT sample preparation. The novelty of this technique lies in using gelatin medium, dissolved in peptone water, which prolongs the lifespan of *Trichinella* larvae.

Gelatin is a mixture of polypeptides obtained by partial hydrolysis of collagen contained in pig skins, after acid treatment, washing, filtration, ion exchange and sterilization. Gelatin has remarkable surface-protective properties, making it widely used to protect foods during their shelf life, protecting foods from drying out, exposure to light and exposure to oxygen. Peptone water consists of sodium chloride, 5.0 g, glucose 10.0 g, monopotassium orthophosphate 1.6 g, dipotassium orthophosphate anhydrous 3.6 g and casein peptone (10.0 g) – polypeptides formed during the enzymatic breakdown of proteins. These characteristics of the medium find application in our methodology because it extends the life of larvae by protecting them from the harmful effects of environment outside a muscle cell.

The method of direct placing the *Trichinella* spp. larvae in the meat matrix under a stereomicroscope is not accurate because the larvae may stick to the surface of micropipette tips which renders it impossible to verify the actual number of *Trichinella* added to the meat ball [16]. This inconvenience necessitated the development of a different procedure of preparing the proficiency tests samples. So far, only 3 methods of preparing samples for PT have been published for laboratories testing meat for *Trichinella* larvae, which fulfill ITC recommendations.

Forbes et al. (1998) presented a method of placing cysts of *Trichinella* (naturally encapsulated larvae) in agar blocks. However, the viability of *Trichinella* spp. larvae trapped in capsules cannot be assessed using this method. Dead larvae are more difficult to detect because they lack movement and because their lighter weight may prevent or delay settling. With typically small numbers of larvae added to contaminated samples, this compromises the precision of PT tests and leads to false negatives [16].

The second procedure of preparing the samples for *Trichinella* proficiency tests was described by Vallée et al. (2007) who used encysted larvae placed directly in a meat balls [18]. In our experience, this approach frequently produced cysts containing dead larvae or cysts containing more than one larva. As with the previous method, the use of cysts and possible dead larvae may negatively impact proficiency test results.

Marucci et al. (2016) presented a method in which non-encapsulated larvae are counted on a watch glass, directly placed into meat balls, and the entire sample is vacuum sealed [13]. When applied at the scale needed in Poland, this technique is labour-intensive and difficult because isolating larvae and placing them in meat samples must be hastened to prevent loss of viability owing to oxygen contact.

In Poland, because of the large number of participants to which NRL is obliged to send samples (> 700 participants, ~3000 samples), previously described methods have proven onerous to implement. Therefore, we endeavored to develop a method more appropriate to the needs, one capable of easing quick preparation of a large number of samples containing defined numbers of viable larvae. We aimed to develop the technique in the simplest possible way, using digested already non-encapsulated larvae, while at the same time confirming their viability thanks to their motility. Most importantly, we sought the ability to stably store enumerated larvae before seeding them into meatballs.

The described method employing gelatin capsules protect embedded larvae from environmental factors, prolonging their survival. This gives the PT organizer the opportunity to make a large number of samples in a short period of time. Gelatin substrate preparation takes a maximum of one hour. Transferring larvae to the medium is labour intensive, and depends on the number of larvae transferred. In one day, 200

PT samples can be prepared using this method, about half the time required using the method described by Marucci et al. (2016) [13], assuming that the maximum number of larvae does not exceed 5 in the single sample.

Stability and shelf life are important in preparing samples for PT. Forbes et al. (1998) indicated a shelf life of at least 3 weeks, based only on the recovery of *Trichinella* larvae from prepared PT samples (without taking into account their viability) [16]. Vallee et al. (2007) reported 100% recovery of living larvae up to 9 days post-isolation [18]. Marucci et al. (2016) reported that larvae maintained under vacuum sealed meat remained viable up to 5 days from the date of preparation if stored between 4–15 °C [13]. Our method enables maintenance of complete viability in a defined number of larvae, stored at 4ºC (+/- 2), for up to 21 days. (Gelatin capsules with live *T. spiralis* may perhaps preserve them longer: here, on day 23, one larva appeared to have lost motility; yet by day 25 100% of larvae were classified as alive). Guided by good laboratory practice, we conservatively concluded the shelf life of the capsules to be 21 days. To our knowledge, this is the longest shelf life reported for such specimens. Our observations suggest that using *T. britovi* larvae may allow further prolongation of this interval (unpublished data).

Prolonged maintenance of viable larvae enables the laboratory to prepare meatballs over an period of several days, providing welcome flexibility. As mentioned, submerging *Trichinella* larvae is the most labour-intensive part of PT sample preparation and requires a great deal of concentration. Completing this stage then allows the laboratory enough time for labelling them, shipping them, and testing them in the field laboratories. Providing such a long period of time for the above activities is particularly important for organizers who oversee a large number of field laboratories.

The method described here has been used successfully in our laboratory since 2015 to organize proficiency tests for more than 600 laboratories officially testing meat for *Trichinella* [19, 20]. Here, we showed that this method prolongs the viability of *Trichinella* larvae for 21 days, which benefits preparation of PT samples and quality assurance. These benefits were confirmed by means of a questionnaire issued to sample recipients who were asked whether detected larvae were alive, and by the Organizer (NRL) through internal testing performed after each batch of PT was tested by the participants. The results of these internal tests showed that the submerged larvae were 100% alive after each finished round of proficiency testing (data unpublished).

CONCLUSIONS

A reliable and reproducible method of proficiency samples preparation is an essential part of quality system assurance in *Trichinella* digestion assays. The method for preserving living *Trichinella* larvae in gelatin capsules presented in this article proved to be a certain and accurate technique enabling the storing of larvae for at least 21 days, ensuring their 100% viability. This method has proven to be useful for producing a large amount of PT samples in a short period of time. Additionally, larvae encapsulated in gelatin capsules can be used for many other purposes related to ensure quality assessment, as part of the training for veterinarians examining meat for *Trichinella* larvae.

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