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Comparative studies between four chemicals for long-term preservation of *Trichinella spiralis* larvae's

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Abstract

A comparative evaluation of four conservation chemicals (96% ethanol, 1% sodium benzoate, 10% formalin and glycerol), used to preserve *Trichinella spiralis* larvae's over 120 days, was performed. The results obtained after conservation with 96% ethanol, 1% sodium benzoate and glycerol showed no statistically significant differences in both the shape and the diameter in coiled *Trichinella spiralis* larvae's over the tested period. Additional advantage of glycerol as conservation liquid toward formalin is a lack of toxicity.

Keywords: Glycerol, Larvae saving, *Trichinella*.

Introduction

Trichinosis are serious challenge for public health with epidemiological situation, global trade of meat and hunting tourism. According to the current practice, diagnosis of trichinosis needs some time to type the *Trichinella* spp., to keep the evidence for diagnosis, to referee some cases, to take part in collaborative research, emerging forensic cases, and for comparative study about degree of invasion. The necessity for a reference opinion in cases of trichinellosis arises from cases such as outbreaks in Lithuania (Perevoščikovs *et al.*, 2005), the outbreak among hunters in the USA (European Commission: Data of Agricultural Statistics), the trichinellosis epidemic in Laos (Barenes *et al.*, 2008), and the extensive international trade allowing the global distribution of this zoonosis with infected meat (UK/EU Pig Statistics, 2009).

According to European requirements, the positive or doubtful results from parasite samples have to be kept in 90% ethyl alcohol for conservation and identification at species level at the Community or national reference laboratory (EC (European Commission), Regulation No. 2075/2005). But in this European regulation there is no detailed description for specific methods for long-keeping and conservation of detected larvae. The goal was to save intact larvae from digestion method, as the digestion may lead the larva to be destroyed within 60 minutes. Thus our purpose was to compare four methods for long-term preservation of *Trichinella spiralis* larvae, received during digestive diagnostic procedures.

Materials and Methods

The reference method for detection of *Trichinella* larvae was performed by artificial digestion of 1 year-old mice meat delivered by Department of Veterinary Microbiology, Infectious and Parasitic Diseases, VMF,

Trakia University, Stara Zagora, Bulgaria. At the age of 1-2 months, they ate meat from other previously infected mice with high quantity of *Trichinella spiralis* larvae.

Reference magnetic stirrer method for pooled sample digestion for detection of *Trichinella* larvae was used according to EC requirements (EC, Regulation No. 2075/2005). It is based on digestion of muscle in artificial gastric juice and investigation of the digest for the presence of *Trichinella*. The artificial gastric juice is prepared from 25% hydrochloric acid and pepsin with activity 1:10 000 NF (NSF=National Standard Formulary; American Laboratories, Omaha, NE, USA). Mice meat with high quantity of *Trichinella spiralis* larvae was transferred to the center of a meatball, consisted of 102±2 g of minced pork from the diaphragm pillars free of fat and fascia. In this study, when *Trichinella*-positive samples were found, one part of the meat juice was removed and observed over 60 min. It was then found out that Trichinellae were gradually and completely digested by the artificial gastric juice for that time period. This is also confirmed by EC regulations stating that samples, subject to analysis, should be run within 30 min (EC, Regulation No. 2075/2005).

After detection of *Trichinella* larvae, they were immediately processed by the method for conservation and long-term preservation: centrifugation of digestion fluid containing Trichinellae in 10-ml conical bottom tubes at 1000 rpm for 5 min, followed by removal of supernatant until 0.5 cm³ of it remained. Then, 9 ml conservation liquid or glycerol was added. The mixture was centrifuged again at 1000 rpm for 5 min, supernatant was discarded and the remaining 0.5 ml was resuspended with 4 cm³ conservation solution. Thus, conserved *Trichinella* larvae were left at room

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Table 1. Medium diameters (μm) of spiral coils of *Trichinella spiralis* at different conditions of chemical conservation (p - ¹⁻¹²).

Statistical parameters	Chemicals used for conservation			
	96% ethanol, n=10	1% sodium benzoate, n=10	10% formalin, n=10	Glycerol, n=10
30 th day				
X	9,9 ^{1;2}	10,7 ^{1;3}	11,75 ^{2;3}	11,2 ^{10;12}
(\pm) std. dev.	1,1	1,41	0,95	0,9055
Min	8	8	11	10
Max	12	13	13	13
60 th day				
X	10 ^{4;5}	10,7 ^{4;6}	11,9 ^{5;6}	11,1 ^{10;11}
(\pm) std. dev.	0,9428	1,4181	0,7378	0,7057
Min	9	8	11	10
Max	12	13	13	12
90 th day				
X	9,9 ^{7;8}	10,1 ^{7;9}	11,6 ^{8;9}	11,7 ^{11;12}
(\pm) std. dev.	1,1	2,02	1,5	0,7810
Min	8	6	10	11
Max	12	12	15	13

¹0.1760; ²0.0126; ³0.2031; ⁴0.2103; ⁵<0.0001; ⁶0.0289; ⁷0.8210; ⁸0.009; ⁹0.0764; ¹⁰0.7861; ¹¹0.0882; ¹²0.2026.



Fig. 1. *Trichinella spiralis* larvae conserved in glycerol.

temperature, and could be observed in a Petri dish on a trichinoscope or under a microscope. When the conservation liquid in the Petri dish diminished, 10 ml fresh conservation liquid is periodically (at 14-day intervals), added.

A comparative evaluation of *Trichinella* larvae conservation was carried out with 96% ethyl alcohol (M=46, 07 g/mol, ALKALOID AD, Skopje,

R.Makedonia), 10% formalin (Formaldehyde solution 4%, buffered, pH 6.9, Merck Millipore), 1% Sodium benzoate (Sodium benzoate purified (500gm), Merck Millipore), and Glycerol (Merck Millipore) as conservation liquids. During the comparative tests the diameter of spiral coils of *Trichinella* was determined by means of eyepiece micrometer (X7) and objective 5. The diameter of coiled *Trichinella* (n=10) was presented in μm . Statistical analyses of data were performed by t-test (StatmostTM for Windows), with significance level at $p < 0.05$. Results were presented in means (X), standards deviations (Std.Dev.), minimum (Min) and maximum (Max) values (Table 1).

Results and Discussion

The results obtained after conservation with 96% ethanol, 1% sodium benzoate and glycerol showed no statistically significant differences in both the shape and the diameter in coiled *Trichinella spiralis* larvae over the tested period (Table 1). An advantage of glycerol as conservation liquid over the other chemicals is the lack of toxicity and materially influence over the quantity of glycerol in tested period. The shape of *Trichinella* larvae for all conservation liquids used, was similar to the shape of digits 6 or 8, or spirals of various shapes were exhibited (Fig. 1).

In relation to our studies for preservation of *Trichinella*, it should be said that there are some comparative studies only with regard to larvae growth in muscles (Despommier *et al.*, 1975) and on the reproductive potential and infectivity of *Trichinella spiralis* larvae after various means of storage (drying, freezing, salting) (Medina-Lerena *et al.*, 2009).

In future, histological analysis on larval sections based on classical hematoxylin-eosin staining combined with either optical or electronic microcopy analysis must be carried out for a better evaluation of the conservation properties of the chemicals. In additions, further tests to identify which preservation method preserves the larvae to allow a reliable identification using molecular methods such as Polymerase Chain Reaction (PCR) must be performed.

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