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Effectiveness of two methods for mesocercariae of *Alaria alata* detection in wild boars (*Sus scrofa*)

Zanda Ozoliņa*, Gunita Deksne

Institute of Food Safety, Animal Health and Environment "BIOR", Lejupes 3, Riga LV–1076, Latvia

*Corresponding author, E-mail: zanda.ozolina@bior.lv

Abstract

Over the past decade, frequent detection of trematode *Alaria* sp. mesocercariae in wild boar muscle samples during official *Trichinella* inspection has raised concern as a potential risk of human and animal health. In the present study 60 wild boar muscle samples were examined with two methods, *Alaria* sp. mesocercariae migration technique (AMT) and the official *Trichinella* inspection method (TIM). The results of the present study shows that AMT had 4.86 (95% CI: 2.22-10.99) times higher odds of testing positive than with TIM. The cause of low proportion of positive mesocercariae observation using TIM is mainly associated with inappropriate mesh size of the sieve used in comparison to *Alaria alata* mesocercariae size. In addition, a shortening the AMT procedure time for 30 min is sufficient to obtain only qualitative results for *A. alata* mesocercariae in muscle samples.

Key words: *Alaria alata*, mesocercariae, mesocercariae migration technique, wild boar.
Abbreviations: AMT, *Alaria* sp. mesocercariae migration technique; TIM, *Trichinella* inspection method.

Introduction

Alaria spp. is a trematode with tetraxenous life cycle and is present in all climatic zones (Galaktionov, Dobrovolskij 2003). Genus *Alaria* include seven species worldwide, however *Alaria alata* is the only known species of the genus in Europe (Möhl et al. 2009). This trematode infects muscles of paratenic host (e.g. wild boar and human) and does not form a cyst (Möhl et al. 2009). Mesocercariae is pear-shaped, compressed from ventral side and gibbous from dorsal side and it is 0.3 to 0.7 mm long and 0.141 to 0.282 mm wide (Skrjabin 1960; Möhl et al. 2009).

In early studies, *Alaria* sp. specimens regarding their biology were identified by naked eye or with a magnifying glass, later the compression method was used (Riehn et al. 2010). Nowadays *A. alata* is accidentally found by *Trichinella* spp. inspection method (TIM) with artificial digestion with a magnetic stirrer (Jakšić et al. 2002; Möhl et al. 2009; Riehn et al. 2010).

Using TIM, the prevalence of *A. alata* mesocercariae in wild boars ranged from 1.8 to 91.0% in Croatia and 24.0% in Branderburg, Germany (Jakšić et al. 2002; Möhl et al. 2009). A five year study in France showed prevalence of 0.6% (Portier et al. 2014). In Estonia, from 2009 to 2011, *A. alata* was detected in 52 wild boars in total and an increase of positive findings was observed, four cases 2009 and 24 in 2011 (total investigated sample number not known, Estonian Food and Veterinary Laboratory, unpublished data). In Lithuania, mesocercariae prevalence in wild boars was 40.0 and 30.5% in 2008 and 2011, respectively (Lithuanian Food and Veterinary Laboratory, unpublished data). In Latvia, a similar study with the routine samples from official *Trichinella* inspection from 2010 to 2012 were done and overall in 4088 wild boars analyzed *A. alata* mesocercariae prevalence was 7% (Esīte et al. 2013).

The differences in infection mode, predilection sites and the size of *Trichinella* and *A. alata* suggest that TIM might be not suitable for mesocercariae detection, causing false and inadequate results (Möhl et al. 2009). For several years, a specific and more sensitive detection method for digenetic trematode *A. alata* is available, the *Alaria* sp. mesocercariae migration technique (AMT; Riehn et al. 2010). Still this method is not applied for routine examinations. However, studies which used AMT have greater reliability. Studies in Germany using AMT showed *A. alata* prevalence 11.5% in wild boars and 33.3% in raccoons (Riehn et al. 2012; Rentería-Solís et al. 2013), while during similar study in Austria prevalence in wild boars was 6% (Paulsen et al. 2014).

During the past decade in Europe, increased attention has been paid to *A. alata* occurrence in wild boars. However, due to the scarcity of methodology and data predicting the risk of human alariosis is difficult. Although the main source of infection is considered the consumption of an insufficiently cooked wild boar meat, *A. alata* is still not considered to be a zoonotic agent in Europe (Möhl et al. 2009; Riehn et al. 2011a; Riehn et al. 2011b, Riehn et al. 2011c). The aims of present study were to define the level of possible false-negative results using TIM and to improve the AMT to shorten as possible performance time.

Materials and methods

Overall, in the present study samples from 60 wild boars were collected and analyzed during 2014 from the whole territory of Latvia. Hunters were asked voluntarily to submit wild boar muscle samples. Each sample contained 200 to 350 g of different types of muscles (diaphragm, tongue and other skeletal muscles) appropriate for *Trichinella* testing. Only sufficient samples (enough amount of the sample; not frozen before submission to the laboratory; no features of decomposing) were selected for the present study. All samples were stored at 2 °C until examination, which was performed within 24 h.

Artificial digestion method with magnetic stirrer for *Trichinella* sp. detection (Regulation EC No 2075/2005) and *Alaria* sp. migration technique (Riehn et al. 2010) was used to detect *Alaria* sp. mesocercariae presence in wild boar muscle samples.

The protocol of *Trichinella* sp. detection using artificial digestion with magnetic stirrer is described in Annex I, Chapter I of the regulation EC No. 2075/2005 (EC2005). Individual wild boar muscle samples (50 g) were separated from membranes and tendons before the sample preparation. Tap water (46 to 48 °C) was poured into 1 L flask and 8 mL of 25% hydrochloric acid and 5 g pepsin (1:10000 NF) were added. Muscle samples were crushed with a meat grinder until no visible pieces of muscles were present. Previously prepared digestion fluid was added to each ground muscle sample and a 5-cm-long stir bar was added. Glass was placed on the magnetic stirrer and the artificial digestion regime was at 44 to 46 °C, stirred for 30 min. Afterwards, the digestion fluid was drained through a sieve (d =11 cm, mesh size 180 μm) into the sedimentation funnel and the digestion fluid was allowed to stand for 30 min. Then, 40 mL of the digestion fluid was quickly run off into a measuring cylinder and allowed to stand for 10 min. Supernatant (30 mL) was then carefully withdrawn by suction to remove the upper layers and leave a volume of not more than 10 mL. The remaining 10 mL sample of sediment was poured into a Petri dish. Subsequently, the sample was examined by stereo-microscope at a 20 times magnification. In addition to standard protocol, in the present study we analyzed the remaining material in the sieve. The used sieve was carefully washed off with tap water and the resulting solution was collected in a Petri dish. The collected sample was examined by stereo-microscope at a 20 times magnification.

The protocol of *Alaria* sp. mesocercariae migration technique was used as described by Riehn et al. (2010) with some modification. Briefly, the 50 g of different wild boar muscles were selected and separated from the membranes and tendons. A rubber tube attached to a funnel was concluded with a 60 mm clip. Wild boar muscle was chopped with scissors approximately in 5 × 5 mm pieces and placed in a sieve with a mesh size of 0.8 mm. The sieve was placed in a glass funnel, then rinsed with 300 mL of 46 to 48 °C tap water in a way such that the meat was completely submerged in water. The suspension was settled for 30, 60 and 90 min at room temperature. After that, the clip was opened and 40 mL fluid was quickly run off into a measuring cylinder and allowed to stand for 10 min. Supernatant (30 mL) was then carefully withdrawn by suction to remove the upper layers and leave a volume of not more than 10 mL. The remaining 10 mL sample of sediment was poured into a Petri dish. Subsequently, the sample was examined by stereo-microscope at a 20 times magnification.

All mesocercariae of *A. alata* were identified by morphological characteristics and counted (Möhl et al. 2009). *A. alata* mesocercariae prevalence in wild boar meat samples was calculated as defined by Bush et al. (1997) and prevalence of both methods was compared using Pearson's chi-square test and Odds Ratio (conditional maximum likelihood estimate of Odds Ratio) calculated using R software.

Results

In the present study 60 wild boar muscle samples were examined both with AMT and TIM. A total of 46 examined samples were found to be infected with *A. alata* mesocercariae with both methods. However, significant (χ^2 = 16.59; $p < 0.01$) difference of mesocercariae prevalence was found between the methods: AMT had 4.86 (95% CI: 2.22–10.99) times higher odds of testing positive than with TIM. The number of counted mesocercariae with AMT ranged from 1 to 61, which equals 0.02 to 1.22 mesocercariae per one gram, while, the number of counted mesocercariae with TIM ranged from 0.02 to 0.56 mesocercariae per one gram. The mean intensity of *A. alata* mesocercariae per sample was observed to be significantly ($p < 0.01$) higher using AMT (Table 1).

Using TIM, trematodes were counted in a Petri dish and, additionally, those remaining in the sieve. In the sieve the number was 2.75 (95% CI: 1.20–6.54) times higher odds of testing positive than following the standard protocol

Table 1. Comparison of *Alaria* sp. mesocercariae migration technique (AMT) and *Trichinella* inspection method (TIM)

Parameter	TIM	AMT
No. of analyzed samples	60	60
No. of infested samples	24	46
Minimal count of mesocercariae	1	1
Maximal count of mesocercariae	28	61
Median	3	5
Intensity	5	9.2
Standard deviation	5.9	14.1
Prevalence	40.0 %	76.7 %
(Clopper-Pearson CI 95%)	(27.6–53.5)	(64.0–86.6)

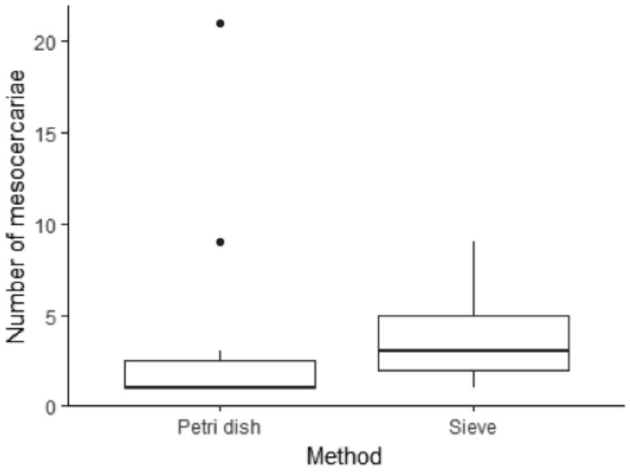

Fig. 1. The number of *A. alata* mesocercariae using standard protocol of *Trichinella* inspection method (TIM) comparing to additional mesocercariae findings using TIM in the sieve.

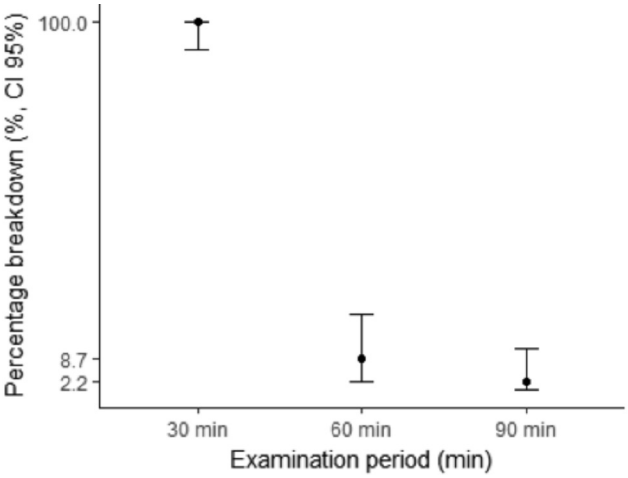

Fig. 2. The percentage of detected *A. alata* mesocercariae in 46 infected wild boar muscle samples using *Alaria* sp. mesocercariae migration technique after examination time of 30, 60 and 90 min.

(Fig. 1). Overall, in 13 samples mesocercariae only in the sieve were found that went undetected by the application of standard protocol of TIM, which corresponds to 21.7% of the samples being false negatives using official *Trichinella* testing method. Only in one sample, mesocercariae were not found in the sieve while two mesocercariae were found in Petri dish.

Using AMT, the samples were examined after 30, 60 and 90 min. In the first 30 min, *A. alata* mesocercariae were found in 46 samples (76.7 %, CI 95% 64.0–86.6) samples (Table 1). In these samples, trematodes were also observed after 60 and 90 min, however only one trematode per each sample was found (Fig. 2.).

Discussion

The present study shows that AMT is significantly better method for *A. alaria* mesocercariae detection compared to the TIM standard procedure. These results can be explained by the different biology of *Alaria* sp. and *Trichinella* sp., specifically different sizes of both parasites. *A. alata* mesocercariae reaches the size of 0.3 to 0.7 mm in length and 0.141 to 0.282 mm in width, while *Trichinella* sp. larvae are 0.775 to 1.050 (average 0.908) mm in length, and 0.026 to 0.042 (average 0.035) mm in maximum width (Skrjabin 1960; Sohn et al. 2000; Möhl et al. 2009). Trematodes do not generate cysts and migrate in host muscle tissue without interruption (Möhl et al. 2009;Riehn et al. 2010). However, a recent study showed that mesocercariae of *A. alata* in amphibian muscle tissues form a cyst-like structure around itself (Patrelle et al. 2015). Temporarily there is no proof that mesocercariae do not leave this cyst and the material of the cyst is unclear.

Mesocercariae prevalence studies in wild boar muscle samples from East Germany showed that false results occurred in 11.5% of the samples when TIM was used (Riehn et al. 2012). In the present study, using standard protocol of TIM together with additional mesocercariae counting in the sieve, we established mesocercariae occurred significantly more frequently than found only in the Petri dish, indicating that routinely we obtain 58.4% of false results originally.

The methods for adult *A. alaria* are approbated, but detection methods for mesocercariae are still being standardized (Riehn et al. 2010). Paulsen et al. (2012) described a modification for AMT (cut musculature dipped in hot water and let stand for 45 min) with the proposed time two times shorter as described previously by Riehn et al. (2010). Also for *A. alata* prevalence studies in frog muscle tissues, a reduced time (30 min) was used (Patrelle et al. 2015). The present study demonstrated that a reduced time (30 min) ensured qualitative results (positive or negative); after 60 and 90 min *A. alata* mesocercariae were found in 5%, which ensured quantitative results. A study in Germany demonstrated that in more than 95% cases when samples were let to stand for only 30 min, there were no significant differences in the mesocercariae count compared with results from samples left to stand for 90 min (Riehn et al. 2010). These results correspond with the results from our study. The study in Germany showed that, only in a few cases, one mesocercariae per muscle sample was found when the sample stand for 60 to 210 min (Riehn et al. 2010).

The present study demonstrated that cause of low mesocercariae observation using TIM was mainly associated with inappropriate mesh size of the sieve used, compared to *A. alata* mesocercariae size. Shortening the AMT procedure time for 30 min was sufficient to obtain qualitative results for *A. alata* mesocercariae in muscle samples. However, our results do not represent prevalence of *A. alata* mesocercariae in the whole territory of Latvia due to deficiency of data.

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