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Original article

Comparison of different techniques and stains for direct diagnosis of *Trichinella spiralis*

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ABSTRACT

Background: The aim of *Trichinella* inspection is to reliably detect larvae in meat at levels which are capable of causing human trichinellosis. Trichinoscopy is a rapid, cost effective but is less sensitive in detection of low muscle larvae (ML) numbers. Artificial digestion is more convenient, and flexible, but may destroy immature larvae**.** Baermann technique may enhance the efficiency of diagnosis. Giemsa and Leishman stains provide identical contrasting coloration as Hematoxylin and Eosin (H-E) with the both advantages of that they can be used for staining non-histological sections and rapid preparation. **Objectives:** The primary aim of the study was to compare the efficiency of digestion and Baermann techniques for detection of ML in muscles. Secondary aim was detection of ML by Giemsa and Leishman stain and their comparison with routinely used stain H-E. **Methods:**Muscles from 36 mice orally infected with *Trichinella spiralis* encysted larvae were examined. Larvae from 1 g samples of different muscles were recovered by digestion and Baermann techniques after different dose of infection. Muscle samples were prepared for staining with Giemsa, Leishman and H-E. **Results:** Number of larvae recovered by Baermann was higher than that recovered by digestion method (P >0.5). Leishman stain was the best stain for rapid detection of ML after 30 min followed in validity by Giemsa then H-E. **Conclusions:** Baermann concentration technique showed higher sensitivity than digestion method. Leishman stain is superior to Giemsa stain as it takes less time. Both stains are considered good alternative to H-E for rapid and easy diagnosis of trichinellosis in post-mortem animals.

Introduction

Nematodes of genus *Trichinella* are a lethal zoonotic agent and one of the common parasites worldwide [1]. They have been detected in the muscle tissue of a broad range of mammalian species from all continents with exception of Antarctica [2]. Among the currently recognized species of *Trichinella*, *T. spiralis* is regarded as the most important agent of human trichinosis [3] and it has been ranked third among zoonotic parasites assessed for their importance as foodborne pathogens [4]. Eating raw or undercooked meat containing infective *Trichinella* larvae is the main source of infection to human. Moreover, infection is generally associated with a complex symptomatology and sometimes mortality among untreated patients [5]. In order to

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preserve public safety, it should be assured that pork does not have pathogenic *Trichinella* larvae [1]. There are actually many protocols for detecting *Trichinella* in muscle specimens, include direct approaches like trichinoscopy, artificial digestion and muscle compression, and indirect (serological) procedures like ELISA [6]. Since the approach serves efficiency and reliability, it is regularly used to evaluate the existence of encysted larvae of *Trichinella*. Trichinoscopy is currently implemented in any laboratory field areas. However according to Forbes et al. [7] is less sensitive than digestion assay in detection of low ML levels. Artificial digestion is a more convenient method because of its flexibility and cost effectiveness. Therefore, it has been the tool of preference for regular carcasses inspections in several countries. Accordingly, inspection by digestion has been recommended by the European Union, the International Commission on Trichinellosis (ICT) and the World Organization for Animal Health (OIE) [8]. However, if larvae are immature, there is possibility for their destruction by digestion process [9]. Actually, the simplest way of parasitological diagnosis is compression of muscle biopsy between two slides and its examination microscopically. But under certain circumstances, this method is not sensitive enough to detect larvae especially when larval density is low or when the sample is examined before the larvae have coiled and become capsulated. In this case there is a risk of confusing the larvae with the muscle fibers making larval detection more difficult [10].

Serology testing using an excretory-secretory (ES) antigen was shown to be an accurate method for detecting both ante-and post-mortem infections. Another research also showed that there is a 4–7 weeks lag in between times when larvae become infectious for a new host and the development of positive serology [11]. This is being caused by a pause in *Trichinella* antibodies production [6]. A modern, non-microscopic process for the identification of *Trichinella* in fresh meat has been recently created. The *Trichinella* Antigen Test (Trichin-L) detects *Trichinella* antigen using monoclonal antibodies and allows the results to be assessed objectively through agglutination. The performance of the process differs according to the meat product being studied and was lower in sensitivity compared to the digestion approach [12]. The Baermann technique is a conventional parasitological method used mainly to separate nematode larvae from fecal and soil material. The method is focused on the efficient movement or migration of larvae into warm water (≥37 C) through a gauze pad. This approach is valuable for diagnosing early infection as *T.spiralis* pre-encapsulated larvae has a warm water movement feature [13].

Contrast stains are generally used to enhance the efficiency of immediate diagnosis of many parasitic diseases, including trichinellosis. Histological examination is typically done using H-E. However, histological examination has the disadvantage of requiring the examination of multiple samples [14]. Alcohol-based stains, such as Giemsa or Leishman, are most frequently used in probably qualified research labs with the accessibility of well-trained personnel [15]. In this context, Ramírez-Melgar et al. [16] reported that nurse cells in mice diaphragms could be readily detected by naked eye as blue dots in Giemsa stained compressed diaphragm samples, as the Giemsa stain has the same comparing coloration as the H-E with the advantage that it may be used in non-histological preparations[17]. Another study conducted on fresh and formalin preserved specimens using Giemsa and Leishman stains described that ML and nurse cells are purplish blue structures that compare with the pinkish color of uninfected muscle tissue [14]. The purpose of our research was to evaluate the efficacy of artificial digestion versus Baermann's methods for better detection of the ML in experimentally infected mice in different muscles. Also, to compare between Giemsa, Leishman and standard H-E stains in term of rapid diagnosis of trichinellosis.

Material and methods

Type of study

This descriptive study was conducted in Medical Parasitology Department, Faculty of Medicine, Zagazig University, Egypt during the period from January 2019 to May 2019.

Infection of animals

Trichinella spiralis strain was obtained from laboratory bred infected albino mice from Medical Parasitology Department, Faculty of Medicine, Tanta University, Egypt. Infective larvae were prepared from infected pork muscles by mixing the muscles in digestive fluid for 12 h (20% concentrated HCl and 20% pepsin) [18]. Mice were prevented from food 12 h before infection, then were given infective larvae through oral route using a tuberculin syringe with blunt nozzle.

Experimental design

Female Swiss albino mice (age =5-8 weeks) weighing 20–25 g, were purchased from Animal House, Faculty of Medicine, Zagazig University, Egypt. Mice were kept on standard commercial pelleted diet with free accessible water all through the study in the Animal House**.** Thirty-six infected female Swiss albino mice included in this study were divided into two main groups of 18 mice each. Digestion method was used in Group 1; and Baermann technique in. Group 2. Each group was subdivided into three subgroups of 6 mice each (groups 1a,1b,1c; 2a,2b,2c). Each subgroup in the two major groups received a different dose of infective larvae. Mice from subgroups 1a, and 2a were infected orally with 250 *T. spiralis* ML; mice from 1b and 2b were infected orally with 500 *T. spiralis* ML; mice from 1c and 2c were infected orally with 750 *T. spiralis* ML. Animals were anaesthetized using isoflurane, the animal inhalation anesthetic agent of choice, before submission to painful procedures. They were sacrificed 45th day post infection.

Collection of larvae

- **Digestion method**

Digestion method was done according to John and Petri, [19]. Briefly, each mouse was dissected and 1g from tongue, diaphragm, abdominal wall and thigh muscle were digested in 1% pepsin and 1% concentrated HCl in 200 ml distilled water. The mixture was incubated at 37°C for two hours under continuous agitation using an electric stirrer. The digested product was passed through a 50-mesh/inch sieve to remove the coarse particles. Encysted larvae were collected on a 200-mesh/inch sieve, washed twice with tap water and then suspended in 150 ml of tap water in a conical flask. After a couple of hours, sedimented larvae were counted by 40x magnification of an Olympus microscope.

- **Baermann's method**:

Trichinella larvae in 1g minced meat samples of the same previously mentioned muscles were directly collected by Baermann's method [20**]**. Briefly, a 2-L funnel is set up in a ring stand, with a piece of rubber tubing connected with its stem and a clamp closing the tube. A 1 mm mesh sieve was put with 3-5 layers of cotton gauze. The funnel was filled with pre-heated (42°C) water to a level just covering the gauze, and muscle samples were placed in center of large cheese cloth and tie with a rubber band a pouch were placed at the center of the gauze. Pre-heated (42˚C) water was applied to the funnel again and the tubes were sealed. The device was placed on a stand at room temperature, but the rubber tubing was submerged in a steady water bath at 42 ˚C. Within a few hours, 45 ml of water was taken from the tip of the funnel via the tubing through a 50 ml centrifuge tube and permitted to settle. After another 45 minutes, 35 ml of the supernatant clear fluid of the tube was aspirated and larvae in the remaining 10 ml of turbid water were counted under 40 x magnification of an Olympus microscope.

Histopathological assessment

- **Hematoxylin & Eosin (H&E)**

Samples of infected muscles (3-4 mm) were fixed in 10% formol-saline. Dehydration was performed in ascending grades of alcohol. Xylol was used as a cleansing agent. Impregnation was done in pure soft paraffin for 2 hours at 55°C then in hard paraffin. Three 5 µ thick sections from each sample were processed and stained with H-E, dehydrated, mounted, and examined by 10 × and 40 × magnifications [21].

- **Giemsa stain**

Muscle samples were cut and fixed as mentioned above. Samples were immersed in 10 ml of Giemsa solution as previously described [16]. Then, permanent slides were prepared as usual. Microscopic observations were carried out at 10 × and 40 × magnifications

- **Leishman stain**

Muscle samples were cut and fixed as mentioned above. Samples were immersed in 10 ml of Leishman solution as previously described [14]. Then, permanent slides were prepared as usual. Microscopic observations were carried out at 10 × and 40× magnifications.

Ethical statement

The experiments were done following the ARRIVE guidelines and in accordance with the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines, EU Directive 2010/63/EU for animal experiments or the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978). The experiment was approved by Institutional Animal Care and Use Committee-Zagazig University (IACUC-ZU), approval no. 4297 on 4th February 2018.

Statistical analysis

Data analyses were done with SPSS for Windows version 16.0 (SPSS Inc.). The number of ML recovered from different muscles was compared by Kruskal-Wallis H test. To compare the detection rates of ML by digestion and Baermann's methods, The Chi-square test was used. The level of significance used was 5% ($P < 0.05$).

Results

Digestion method

The distribution of larvae in the muscles of different mice, as estimated by the digestion method, was listed in **table (1)**. Mean number of encysted larvae counted from diaphragm of infected mice from 3 subgroups (1a, 1b, 1c) representing different infective doses was 64.5±35.9. While the mean number of encysted larvae counted from tongue was 37.5±25.1. Furthermore, the mean numbers of encysted larvae counted from thigh and abdominal wall muscles were 21±8.9 and 16±7.5 respectively. The concentration of larvae was greatest in the diaphragm and then in the tongue. By digestion methods; the least concentration of larvae was counted in abdominal wall muscles of infected mice. For each experiment 5 slides were counted.

Baermann's method

The distribution of larvae in the muscles of different mice, as described by the Baermann technique, was recorded in **table (2).** Mean numbers of encysted larvae counted from diaphragms and tongues of infected mice from 3 subgroups (2a,2b,2c) were 91.3±37.1 and 56.3±25.3 respectively. Furthermore, the mean numbers of encysted larvae counted from thigh and abdominal wall muscles were 23.7±9.9 and 22.3±7.5 respectively. The concentration of larvae was greatest in the diaphragm and then in the tongue. By Baermann technique, the lowest concentration of larvae was counted in abdominal wall muscles of infected mice. The H statistic of the number of ML recovered in from different muscles as compared by Kruskal-Wallis H test was 17.345 and *P* value was significant at 0.0006. Detection rates of ML by digestion and Baermann's methods compared by Chi-square test was not significant (*P* value 0.90).

Histopathological assessment:

- **Hematoxylin & Eosin (figures 1-A, B, C)**

Muscles examination of mice with *T. spiralis* infection revealed coiled larvae surrounded with well-formed capsule representing the nurse cells. Nurse cells were surrounded with an intense cellular infiltrate (**figure 1A)**. Non-infected muscle fibers stained pink in color in contrast to purple stained ML **(figure 1B)**. Transformed nurse cell into fibrous capsule appeared as dark purple basophilic staining of muscle cells **(figure 1C)**.

- **Giemsa stain (figures 2-A, B, C, D, E)**

Muscles of infected mice, stained with Giemsa stain, viable encysted larvae were consistently stained blue to deep blue **(figure 2A)**. Non-infected muscle appeared light blue **(figure 2 E)**. Nurse cell surrounded with chronic inflammatory cell infiltrate as dark blue dots (lymphocytes and plasma cells **(figure 2 C, D, E)**; non-infected muscle stained light blue **(figure 2 E)**; coiled larvae with destroyed capsule **(figure 2 C, E)**. There was essentially no contrast with surrounding tissues as they stained the same color.

- **Leishman stain (figures 3-A, B, C, D, E)**

Muscles of infected mice, stained with Leishman stain, viable encysted larvae consistently stained deep blue with this stain **(figures 3-C, D).** Nurse cells appeared clear **(figure 3-A)**. Also, we did not notice difference in coloration between Giemsa and Leishman stain except for the darker stain in the latter**.**

Table 1. The mean number of microscopic ML/1g in diaphragm, tongue, thigh and abdominal muscle samples collected from experimentally infected mice by digestion method, 1a: subgroup infected with 250 encysted larvae, 1b: infected with 500 encysted larvae, 1c: infected with 750 encysted larvae, (data shows mean number ± S.D) .

Muscle	1a	1b	1c	Mean±SD	Total	n
Diaphragm	59	70	126	64.5±35.9	255	16
Tongue	30	45	79	37.5±25.1	154	16
Thigh muscle	23	19	36	21±8.9	78	16
Abdominal wall muscle	20	12	27	16±7.5	59	16
Total	**132**	**146**	**268**		**546**	

n= number of surviving mice

Table 2. The mean number of microscopic ML/1g in diaphragm, tongue, thigh and abdominal muscle samples collected from experimentally infected mice by Baermann's method, 2a: subgroup infected with 250 encysted larvae, 2b: infected with 500 encysted larvae, 2c: infected with 750 encysted larvae (data shows mean number ± S.D).

Muscle	2a	2b	2c	Mean±SD	Total	n
Diaphragm	67	73	134	91.3±37.1	274	16
Tongue	37	47	85	56.3±25.3	169	16
Thigh muscle	19	17	35	23.7±9.9	81	16
Abdominal wall muscle	22	15	30	22.3±7.5	67	16
Total	**145**	**152**	**284**		**591**	

***Kruskal-Wallis H* Test**	**H statistic = 17.345**	***P*-value = 0.0006** (Significant)**
***Chi-square* Test**	***P* value = 0.90 (Not significant).**	

n= number of surviving mice, **= highly significant, SD= standard deviation

Figure 1 (A, B, C). Section of viable encysted larvae of *T. spiralis* in muscle tissue with intact capsule showing fibrosis and surrounded by marked chronic inflammatory cell infiltrate (lymphocytes and plasma cells) (A, B, C, green arrow). Nurse cells were surrounded with an intense cellular infiltrate (A red triangle); non-infected muscle appeared pink in color (B yellow arrow). Transformation of nurse cell into fibrous capsule appeared as dark purple, basophilic transformation of muscle cells (B, C blue triangle), (The image magnification is 200x scale bar 10 µM, H-E).

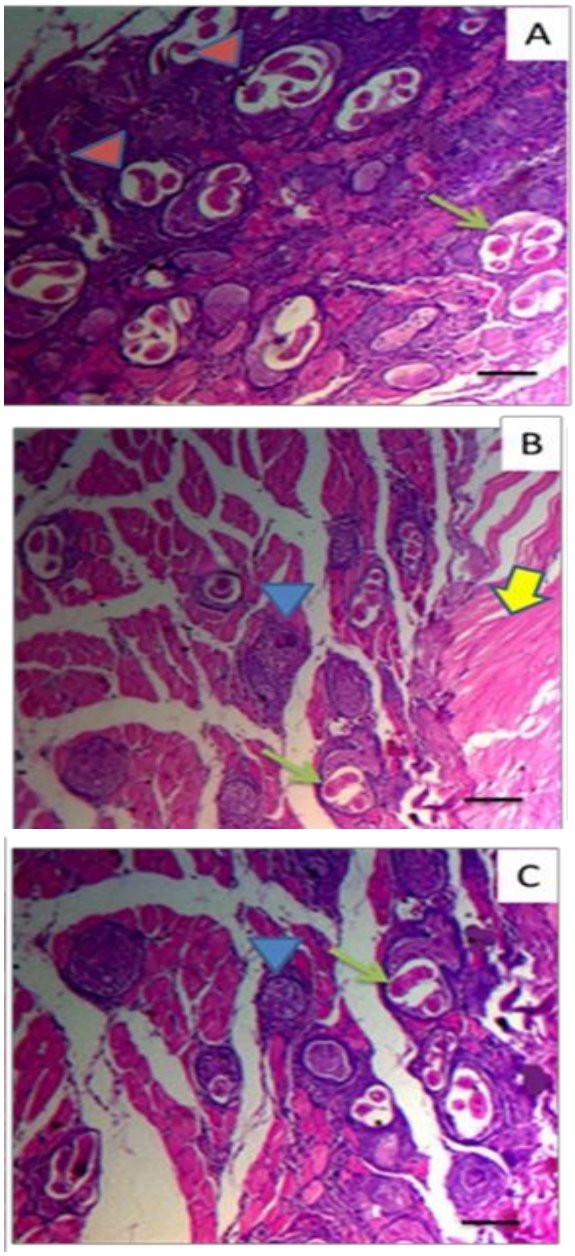


Figure 2 (A, B, C, D, E). Section of viable encysted larvae of *T. spiralis* in muscle tissue of mice, showing encysted larvae inside intact capsule (A, red arrow); nurse cell surrounded with chronic inflammatory cell infiltrate as dark blue dots (orange arrow) (lymphocytes and plasma cells C, D, E); non-infected muscle stained light blue (E, green arrow); coiled larvae with destroyed capsule (C, E). (A, B image magnification is 200x.scale bar 20 µM. C, D, E. The image magnification is 400x.scale bar 50 µM. Giemsa stain)

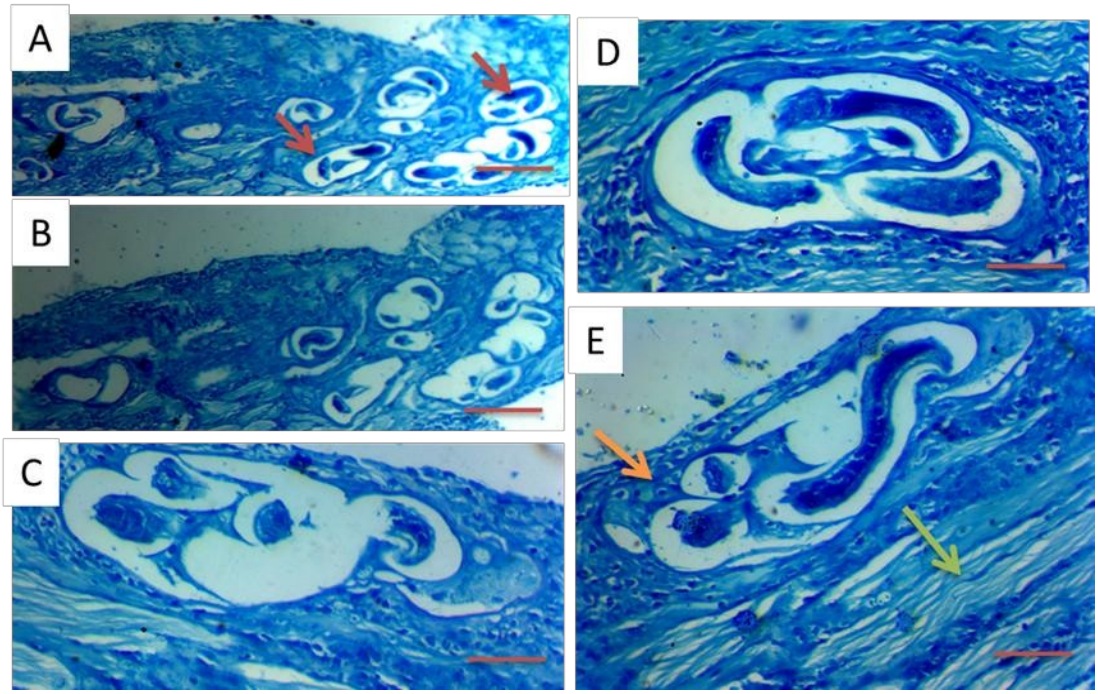
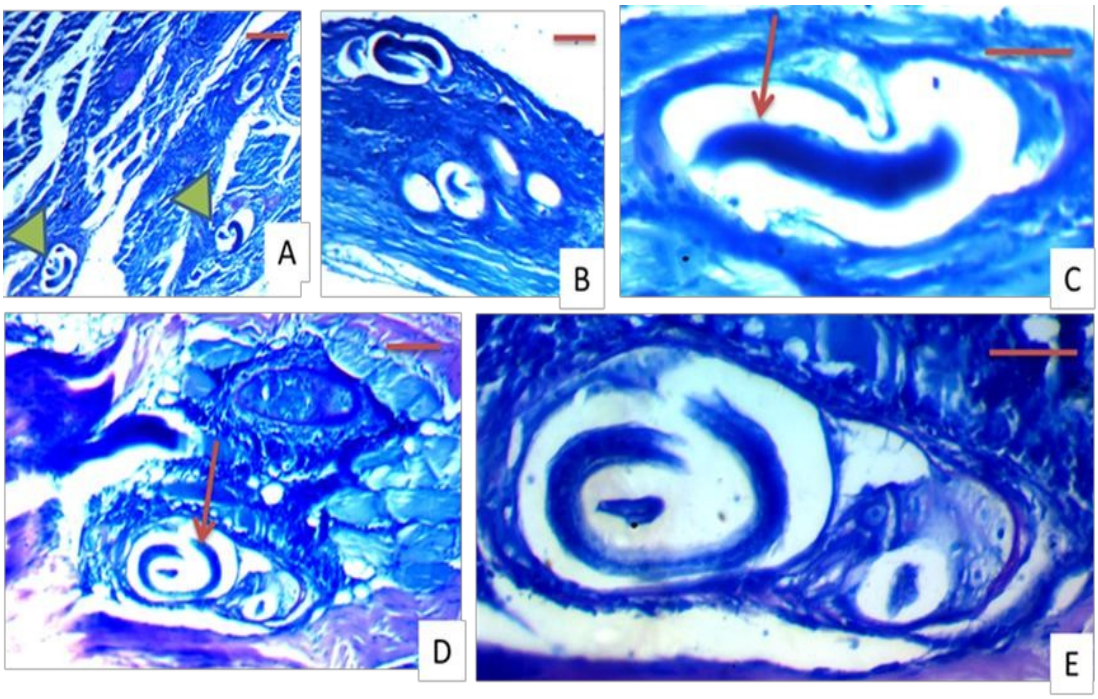


Figure 3 (A, B, C, D, E). Section of viable encysted larvae of *T. spiralis* in muscle tissue, showing clear nurse cells with intact capsule (A, green triangle); coiled larvae (C, D, red arrow) consistently stained deep blue; A, B (image magnification 200x.scale bar 20 µM). C, E (image magnification 400x.scale bar 50 µM). D (image magnification 200x. scale bar 30 µM. Leishman stain**).**



Discussion

Trichinella spiralis is the first described and best-defined member of the Trichinella family and offers useful in vivo models for different pathological, immunological and biomedical studies [22]. In our work, we investigated means of detection of encysted larvae of *T. spiralis* in muscle by two direct methods, artificial digestion and Baermann concentration technique. The idea on which the artificial digestion method is based is the digestion of the muscles with an acidified pepsin solution for the release of *Trichinella* larvae from that tissue, complemented by immediate microscopic examination of the released larvae [7]. Consequently, the artificial digestion method has become the preferred method for the routine check of carcasses of food-animals in many countries and is approved for this role by the ICT, OIE, and EU [23]. The actual value of artificial digestion is its ability to determine one *Trichinella* larva per gram (1/g) of muscle tissue. Due to the irregular distribution of larvae and possible technical difficulties, **Forbes and Gajadhar** [24] estimated the real sensitivity of the process as 3–5/pg while inspecting 1 g of the meat sample. On the other hand, it was feared that this level of muscle digestion sensitivity may give false-negative results in the presence of sufficient larvae to cause infection. Therefore, improvement of detection methods was recommended for ensuring the accuracy of detection of ML, meat security, and public health [25]. Baermann method is regarded as an improvement of the yield by the digestion method. However, there are several factors affecting the sensitivity of this detection method that include the containers used for collection of the larvae, the pore-size of sieve used, and the temperature of the mixture during the sieving process.

We reported that the number of ML detected by Baermann methods was greater than that detected by digestion methods in all subgroups, but the difference was non-significant (P >0.05). Similarly, **Jiang et al.** [20] recorded a greater load of *T. spiralis* pre-encapsulated larvae (PEL) collected from infected muscle by Baermann's method than by digestion methods. The authors concluded that the Baermann approach is better than the procedure of digestion used to evaluate PEL in muscle samples of low-level infection.

Also, we aimed to demonstrate ML by different stains, using H-E, Giemsa and Leishman stains. Concerning procedure time, Leishman stain (figure 3) took less time for preparation than with Giemsa (figure 2). Both stains were useful in rapid detection of encysted larvae in post-mortem animals, while H-E (figure 1) prepared sections took more time and were technically more difficult than both Leishman and Giemsa. We did not notice a difference in color as both Giemsa and Leishman stained ML and muscle tissues blue except that Leishman stain was darker. Explanations for lack of contrast outlined by **Ramírez-Melgar et al.** [16]; factors as were the effect of alcohol pH used, or if acidification was done prior to staining, and if the residual coloring was not eliminated during the dehydration phase. Additionally, if the time of discoloration extends 45 sec. Support for this concept was provided by reports on the superiority of Leishman stain, due to better demonstration of nuclear chromatin pattern and cytoplasmic color and also because it requires less preparation time than the Giemsa stain [26]. Parallel to these findings, **Sathpathi et al.** [15] concluded that for the diagnosis of *Plasmodium* parasites, the Leishman staining method for thin and thick blood films is a reasonable alternative to Giemsa staining. On the other hand, **Ramírez-Melgar et al.** [16] reported that nurse cells stained blue with Giemsa against the pink stained muscle fibers. Moreover, **Ali and Thabet** [14] stated that both ML and nurse cells stain purplish blue compared to the pinkish color of un-infected muscle fibers in fresh and formalin preserved specimens using Giemsa and Leishman stains.

The distribution load of larvae in the different muscles is related to the public risk posed by these larvae in human consumption. The wide distribution of trichinellosis in carnivorous animals requires optimum precautions be taken by consumers to avoid infection [27]. We found that the concentration of larvae was greatest in the diaphragm followed by the tongue, then the thigh muscle. The lowest concentration of larvae was recorded in abdominal wall muscles of infected mice by both digestion and Baermann methods (P-value 0.0006). Our findings are comparable to **Oliver**, [28] who considered the diaphragm to be the most highly affected muscle in rats. However, another study was done to detect highest concentration of larvae in muscle of experimentally infected pigs, the results showed that the number of larvae retrieved from the tongue, diaphragm, and masseters were significantly greater than the number retrieved from all other tissues examined

[29]. Interestingly, other studies conducted on horses concluded that the diaphragm and tongue yielded the highest *T. spiralis* burdens [30]. Also, **Gamble et al.** [31]reported that the tongue is the initial site of predilection of *T. spiralis* encysted larvae in horses, followed in order by the masseters, neck musculature, trapezius, and diaphragm.

Conclusion

Baermann concentration technique showed higher sensitivity than classical digestion method in detection of ML. Leishman stain is superior to Giemsa stain as it takes less time in revealing the encysted larvae. Both stains are considered a good alternative to H-E for rapid and easy diagnosis of trichinellosis in post-mortem animals.

Author contribution

All authors contributed significantly to this study. Mostafa E designed the study. All authors performed laboratory tests. Mostafa E and Ahmed M performed the data analysis and interpretation. Hassan S prepared the manuscript and processed the data. All authors read and approved the final manuscript.

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