Molecular Characterization and Morphological Identification of Egyptian *Trichinella spirals* Isolates

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Abstract: A microscopically and molecular biological study to identify and characterize the Egyptian *Trichinella spirals* isolates patterns through application specific stain technique and multiplex polymerase chain reaction (Multiplex-PCR) was described. The application of Giemsa technique to stain compressed diaphragm and muscle samples obtained from rats experimentally infected with *Trichinella spirals* is described. Diaphragm samples from rats heavily infected with 20 muscle larvae per gram of body weight (20 ML/gbw) were cut into several pieces and stained with Giemsa, besides, muscle samples were stained with hematoxylin-eosin. The ML was observed under the microscope as blue structures surrounded by non-infected muscle cells, which appeared with a pink coloration; similar contrast was observed in both diaphragm pieces and muscle samples. Muscle larvae of all experimentally infected rats were analyzed by a multiplex polymerase chain reaction (Multiplex-PCR) to examine the patterns of isolates of *Trichinella spirals*. The muscle larvae of *Trichinella* isolates showed a 385 bp band patterns.
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1. Introduction

Genus *Trichinella*, includes several species infecting mammals, birds, and reptiles. These nematodes alternate enteric and skeletal muscle stages during their life cycle. *T. spirals* is a relatively small nematode, adult female being 1.4-4.0 mm, adult male 1.4-1.8 mm, and muscle stage larvae approximately 1 mm long (Appleyard *et al*.,1999, Appleton *et al*.,2001 and Soad *et al.,*2010).

Trichinella infection results in formation of a capsule in infected muscles. The capsule is a residence of the parasite which is composed of the nurse cell and fibrous wall. The process of nurse cell formation is complex and includes infected muscle cell response (de-differentiation, cell cycle reentry and arrest) and satellite cell responses (activation, proliferation and differentiation). Some events that occur during the nurse cell formation are analogous to those occurring during muscle cell regeneration/repair (Zhiliang *et al.,* 2008). The life cycle of this nematode begins with the enteric phase of infection, when a person or an animal eats contaminated meat containing first-stage muscle larvae. Digestive juices from the stomach (pepsin and hydrochloric acid) dissolve the capsule-like cyst and release the larvae, penetrating into epithelial cells of the small intestine (Bandi *et al*., 1993). Shortly thereafter, the larvae molt 4 times (10 through 28 hr post-oral ingestion [POI]), and mature into adults that mate (30-34 hr POI). Female worms can produce 500-1,500 newborn larvae (immature L1) during a lifespan, prior to expulsion by the host immune system. The migratory phase of infection begins when these newborn larvae are passed into tissues, enter the lymphatic system, and then enter the general circulation at the thoracic duct. These larvae are distributed widely throughout tissues by circulation, and eventually make their way through blood capillaries into muscle fibers, so initiating the muscle phase of infection. Once in muscle fibers, they encysted, undergo development, become infective within 15 days, and remain for months to years.

Trichinella spirals evidence several biological differences that include host specificity, phenotypic characteristics induced in infected host cells, and interactions with the host immune system. Additionally, each of these parasites must migrate extracellular through the tissues of the host in order to infect the various host cells they inhabit (Bolas and Wakelin, 1990). The mechanisms involved in this migration remain to be elucidated. Furthermore, the role of the parasite in regulating changes in these host cells remains unknown. Nowadays, there are several procedures to search for *Trichinella* in muscle samples, including direct methods, such as trichinoscopy or muscle compression. The major use of these methods has been for post-mortem detection. Since the method represents facilities in performance and economy, it is routinely used to investigate the presence of *Trichinella* muscle larvae (ML), although its diagnostic sensitivity values depends on the experience of the operator or mistakes in the sample examination procedure (Vignau *et* al., 1997).

In order to improve the sensitivity of direct diagnosis of several other parasitic diseases, contrast stains are usually used. The staining properties of the ML as well as the nurse cell (NC), has been widely described from histological sections stained with haematoxylin-eosin (H-E) technique (Matsuo *et al*., 2000; Boonmars *et al*., 2004).Trichinosis is generally diagnosed by detecting stained larvae in biopsied muscle and/or by the detection of antibody in the serological test without species differentiation. Recently, rapid and sensitive genotyping tools for *Trichinella* have been developed. Also studies on the differentiation of genotypes and species of *Trichinella* have been performed successfully by several investigators (Gasser *et al*., 1998; Appleyard *et al*., 1999; Wu *et al*., 1999; Zarlenga *et al*., 1999).

Polymerase chain reaction (PCR)-based methods are popular technique recently (Chambers *et al.,* 1986; Soule *et al*., 1993; Minchella *et al*., 1994; Zarlenga *et al*., 1999). It has been reported that PCR-RFLP (Polymerase chain reaction-restriction fragment length polymorphism) analysis of the rDNA repeat can readily identify and distinguish a species or strain from others (Boyd *et al*., 1989; Zarlenga and Dame, 1992; Gasser and Monti, 1997).

Here we have used the Multiplex PCR repeated analysis of rDNA and examined the patterns of isolates of *Trichinella spirals.* In this study, we performed a microscopically and molecular biological study to identify and characterize the Egyptian *Trichinella spirals* isolates patterns.

2. Material and Methods

2.1. Parasite

Worms of *Trichinella* isolates were isolated from a domestic pig and maintained by numerous passage in laboratory rats (6-week-old, 250g, maintained in Laboratory Animals house, Faculty of Science, Al-Azhar University and the study was supported by Medical Research centre, Ain-Shams, University) were orally infected with 20 ± 0.5 ML per gram of body weight (ML/gbw, approximately 5,000 ML per rat, equivalent to a massive infection) of the *T. spiralis* strain. Six weeks later, animals were sacrificed and diaphragms dissected out; besides, muscle samples of thigh. Additional infected rat were sacrificed and digested in 1% pepsin-HCl at 37ºC. After through several washing in distilled water, muscle larvae were collected under microscope avoiding contamination of host tissue debris and frozen at -70ºC until DNA isolation.

2.2. Giemsa Technique

The diaphragms and the muscle samples were cut into 3-4 mm pieces and compressed between 2 glass slides, then submitted to Giemsa staining. For stain, compressed sample pieces were separated from glass slides, fixed with FAA fixative solution (v/v formaldehyde-acetic-alcohol, 10: 40: 50) during 4 hr at room temperature and transferred to a Petri dish containing 50% ethyl alcohol. Samples were immersed in 10 ml of Giemsa solution diluted 1 : 6 in 0.01 M, pH 7.2 phosphate buffer solution during 45 min at room temperature with slow constant stirring. Afterwards, samples were individually transferred to acidic alcohol (0.02 N HCl in 50% ethyl alcohol) solution during about 45 sec, and then dehydrated with graded alcohol series (30%, 50%, 70% and 100%) lasting 2-3 min in each solution and applying gentle stirring. During this step, samples were also de-stained; a visual inspection was carried out for each sample. Samples were then incubated (5 min each) in a mixture (v/v) of absolute ethyl alcohol and xylene, and finally in absolute xylene. After then, permanent slides were prepared as usual. Microscopic observations were carried out at 10 × and 40 × magnifications. Total NC and ML were counted. The remaining rat diaphragm with larvae was fixed with 10% formaldehyde solution during 24 hr. Afterwards, sample was dehydrated, paraffin embedded, and 3 µm thin slides were cut with microtome, stained with H-E, dehydrated and mounted as (Matsuo *et al.,* 2000; Boonmars *et al.,* 2004).

2.3. Isolation of genomic DNA

Genomic DNA from isolates was purified as described by Rodriguez *et al.* (1996). The parasites were homogenized under liquid nitrogen with lysis buffer (50 mM Tris-HCl buffer pH 8.0, 50 mM ethylene diamine tetra-acetic acid (EDTA), 100 mM NaCl, containing 0.5% sodium dodecyl sulfate (SDS)). Then the homogenate was incubated with 100 µg/ml proteinase K (Sigma, USA) for 3 hours at 37℃. After an equal volume of phenol-chloroform extraction, the aqueous phase was digested with 150 µg/ml RNase (Sigma) for 30 minutes at 37ºC. Additional phenol-chloroform extraction and chloroform extraction was performed subsequently. Total genomic DNA was precipitated by ethanol extraction and the DNA was resuspended in sterilized water. DNA concentration was determined by standard procedures.

2.4. PCR amplification

Polymerase chain reaction (PCR) was carried out using SB2 primers, which are specific for *Trichinella spirals* (forward 5'-CTCCACTTACGCAATGCACG-3' and reverse 5'-ACACCAAACGGCAACTGCTA-3') (Wu *et al.,* 1997). Genomic DNA was amplified in a solution containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl,

5mM $MgCl_2$, 0.2 mM of each dNTP and 2.5 units Taq polymerase (TaKaRa, Shiga, Japan). The following cycling conditions were used: 95^oC, 30 sec (denaturation); 58^oC, 1 min (annealing); 72^oC, 1 min (extension) for 35 cycles on a thermocycler (MJ research). The PCR products were checked on agarose gel with ethidium bromide staining (Amersham, Buckinghamshire, England) as described in manual.

3. Results

By Giemsa staining**,** the ML and nurse cells itself were observed under the microscope as blue structures surrounded by non-infected muscle cells, which appeared with a pink coloration; similar contrast was observed in both diaphragm pieces and muscle samples (Figs. 1 & 2).The presence of multiple ML in a single NC was clearly identified in histological slides (Fig. 3).The ML from all the rats infected with *Trichinella* isolates showed a 385 bp band (Fig. 4).

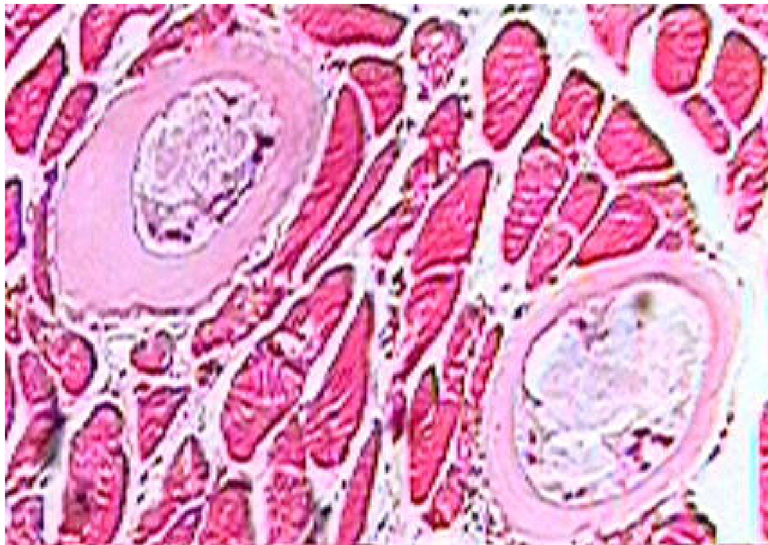

Figure 1. *Trichinella spirals* larvae encysted in striated muscle (H & E stain).

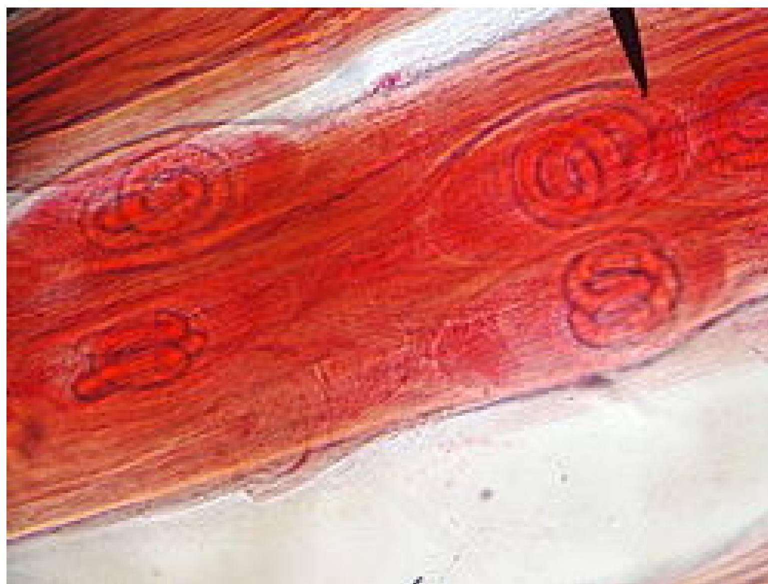

Figure 2. Compressed diaphragm tissue of an experimentally infected rat stained with Giemsa, showing *Trichinella spiralis* muscle larvae inside a single nurse cell. Scale bar = 0.3 mm.

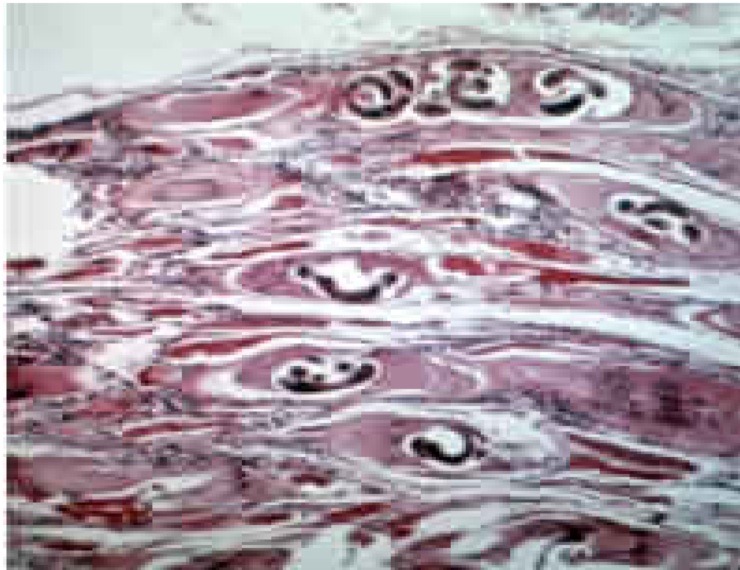

Figure 3. Histological section stained with hematoxylin-eosin technique of one nurse cell containing 2 muscle larvae. Diaphragm sample was obtained from one heavily experimentally infected rat. Scale bar is 0.2 mm.

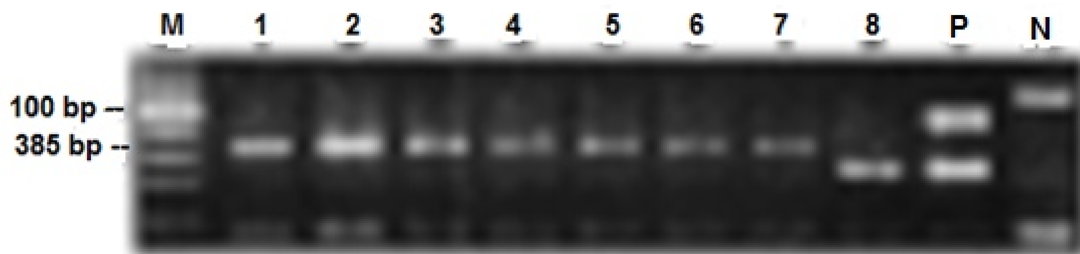

Figure 4. Electrophoretic patterns after multiplex-polymerase chain reaction amplification products of *Trichinella* larvae. Lane M, 100 bp ladder; P (Positive control), N (Negative control). Lanes 1-8, larvae from experimentally infected rats.

4. Discussion

Trichinella spp. is intracellular parasites of vertebrates, in which the entire life cycle is confined to the host. These parasites alternate between enteric and muscle phases of infection, and several species have been shown to induce mortality and morbidity in humans. The parasites establish remarkable intracellular interactions with different host cell types when adapting to these diverse habitats (intestinal mucosal epithelial cells and striated skeletal muscle cells) (Wu *et al*., & Zarlenga *et al*., 1999 and Pozio (2005) A wide range of changes are induced in host cells as the result of infection with *Trichinella* spp., but these have been poorly characterized with regard to the relevant mechanisms and their significance to parasite survival. Furthermore, the role of the parasite in regulating these host cell changes remains largely unknown. Giemsa stain procedure can be applied to muscle samples to contrast the presence of *Trichinella*. The NC, containing the ML, could be easily visualized as bluish-stained oval structures, while non-infected muscle cells were pinkly stained; same contrast has been observed in histological sections stained with H-E (Boonmars *et al*., 2004; Matsuo *et al*., 2000).

Such color contrast can be obtained, when samples have an acidic treatment just after the staining step, otherwise, the colorant excess is not removed during the dehydration process, and therefore, the contrast is lost. At present, regarding multiple larvae in one NC, a simple relationship of one ML in one NC is widely accepted (Despommier, 1998). However, we found 4.4% of NC containing inside more than 2 ML in heavy infections (20 ML/gbw), but a normal rate (1: 1 ML/NC) in lightly infected animals (1 ML/gbw). Since a previous finding reported by Perez and Luengo (1969), where 9 ML inside of a single NC were detected in a muscle

biopsy from a massive infected pig, it is suggested that this phenomenon could be related to the intensity of infection, i. e., more than one new borne larvae invades the myocyte at the same or nearly time. To our knowledge, there is no information regarding the possible fusion of 2 or more NC during the infection process (Despommier, 1998).

The multiple invasion hypothesis mentioned before seems to be more acceptable, suggesting that this observation is more than a simple laboratory finding, but a real and non-studied biological phenomenon. According to the new taxonomic scheme, nematodes belonging to the genus *Trichinella* are divided into 7 valid species, *T. spiralis*, *T. nativa*, *T. britovi*, *T. pseudospiralis*, *T. nelsoni*, *T. murrelli* and *T. papuae* (Murrell and Pozio, 2000). Among them, *T. spiralis* has the widest geographical distribution and the largest range of host species. The majority of human infections are caused by this species (Capo and Despommier, 1996; Murrell and Pozio, 2000). However, since the various genotypes or species of *Trichinella* are distributed worldwide, *Trichinella* isolates should be examined in detail. In this study, we have identified and characterised *Trichinella* isolates infecting human. The genus *Trichinella* has a broad geographical background and host range, and different isolates can often display distinct biological characteristics. Variability within the genus *Trichinella* has been reported in terms of morphology, infectivity, virulence, antigenicity, and enzyme polymorphism. During the last years, diversity has been analysed using parasitological, biological, morphological, immunological and biochemical (allozyme electrophoretic) parameters (Flockhart *et al.*, 1982; Pozio, 1987; Bolas-Fernandez and Wakelin, 1990).

DNA technology has also provided useful approaches to reveal genetic difference within the genus. Polymerase chain reaction (PCR)-based methods are popular technique recently (Chambers *et al.,* 1986; Soule *et al.,* 1993; Minchella *et al.*, 1994; Zarlenga *et al.*, 1999). It has been reported that PCR-RFLP (Polymerase chain reaction-restriction fragment length polymorphism) analysis of the rDNA repeat can readily distinguish a species or strain from others (Boyd *et al.,* 1989; Zarlenga and Dame, 1992; Gasser Monti, 1997; Nagano *et al*., 1999 and Hye *et al*., 2001). Various molecular and biochemical methods including RFLP (Wu *et al*., 1999), RAPD (random amplified polymorphic DNA; Bandi *et al.*, 1993), SSCP (single-stranded conformational polymorphism; Gasser and Monti, 1997) and isoenzyme assay, etc. (La Rosa *et al*., 1982). These methods were also applied to the classification of many subspecies and isolates obtained from geographically distinct areas or different hosts. Further PCR-based molecular studies of the genes and sequences must be performed for proper molecular characterization and identification of species of *Trichinella*.

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References

1. Appleton, J. A. and Romaris, F. A. (2001): pivotal role for glycans at the interface between *Trichinella spiralis* and its host. Vet. Parasitol. 101:249–260.
2. Appleyard, G. D.; Zarlenga, D.; Pozio, E. and Gajadhar, A. A. (1999): Differentiation of *Trichinella* genotypes by polymerase chain reaction using sequence-specific primers. J Parasitol. 85:556–559.
3. Bandi, C.; La Rosa, G.; Comincini, S.; Damiani, G. and Pozio, E. (1993): Random amplified polymorphic DNA technique for the identification of *Trichinella* species. J. Parasitol. 107:419–424.
4. Bolas-Fernandez, F. and Wakelin, D.(1990): Infectivity, antigenicity and host responses to isolates of the genus *Trichinella*. J. Parasitol.. 100:491–497.
5. Boonmars, T. Wu, Z.; Nagano, I.; Nakada, T. and Takahashi Y.(2004): Differences and similarities of nurse cells in cysts of *Trichinella spiralis* and *T. pseudospiralis*. J. Helminthol. 78:7–16
6. Bowles, J.; Blair, D.and McManus, D. P. (1994): Molecular genetic characterization of the cervid strain ('northern form') of *Echinococcus granulosus*. J. Parasitol. 109:215–221.
7. Bowles, J. and McManus, D. P. (1993): Rapid discrimination of *Echinococcus* species and strains using a PCR-based method. Mol Biochem. Parasitol. 57:231–239.
8. Boyd, D.; De-Vos, T.; Klassen, G. and Dick, T. (1989): Characterization of the ribosomal DNA from *Trichinella spiralis*. Mol. Biochem. Parasitol. 35:67–72.
9. Capo, V. A and Despommier, D.D. (1996): Clinical aspects of infection with *Trichinella* spp. Clin. Microbiol. Rev. 9:47–54.
10. Capo, V. A.; Despommier, D. D.and Polvere, R. I. (1998): *Trichinella spiralis*: vascular endothelial growth factor is up-regulated within the nurse cell during the early phase of its formation. J. Parasitol. 84:209–214.

11. Chambers, A.E.; Almond, N.M.; Knight, M.; Simpson, A. J. G. and Parkhouse, R. M. E. (1986): Repetitive, DNA. as a tool for the identification and comparison of nematode variants: application of *Trichinella* isolates. Mol. Biochem. Parasitol. 21:113–120.
12. Dame, J. B.; Murrell, K.D.; Worley, D.E. and Schad, G. A. (1992): *Trichinella spiralis*: Genetic evidence for synanthropic subspecies in sylvatic hosts. Exp. Parasitol. 64:195–203.
13. Despommier, D. D. (1998): How does *Trichinella spirals* make itself at home? Parasitol Today. 14:318–323.
14. Flockhart, H. A.; Harrison, S. E.; Dobinson, A. R. and James, E. R. (1982): Enzyme polymorphism in *Trichinella*. Trans. Roy. Soc. Trop. Med. Hyg. 76:541–545.
15. Gasser, R. B. and Monti JR. (1997): Identification of parasitic nematodes by PCR-SSCP of ITS-2 rDNA. Molec. and Cell. Probes. 11:201–209
16. Gasser, R. B.; Zhu, X. Q.; Monti, J. R.; Dou, L.; Cai, X. and Pozio, E. (1998): PCR-SSCP of rDNA for the identification of *Trichinella* isolates from mainland China. Mol. Cell. Probes. 2:27–34.
17. Hye, S. K.; Myung S. C. and Kyoung, H. J. (2001): PCR-RFLP patterns of four isolates of *Trichinella* for rDNA ITS1 region. Korean J. Parasitol. 39(1): 43–48.
18. La Rosa, G.; Pozio, E.; Rossi, P. and Murrel, K. D. (1982): Allozyme analysis of *Trichinella* isolates from various host species and geographical regions. J. Parasitol. 78:641–646.
19. 19. Matsuo, A.; Wu, Z.; Nagano, I. and Takahashi, Y. (2000): Five types of nuclei present in the capsule of *Trichinella spirals*. Parasitol. 121:203–210.
20. Minchella, D. J.; Eddings, A. R. and Neel, S.T. (1994): Genetic, phenotypic and behavioral variation in North American sylvatic isolates of *Trchinella*. J. Parasitol. 80:696–704.
21. Murrell, K. D. and (2000): Pozio E. Trichinellosis: the zoonosis that won't go. quietly. Int. J. Parasitol. 30:1339–1349.
22. Nagano, I.; Wu, Z.; Matsuo, A.; Pozio, E. and Takahashi, Y. (1999): Identification of *Trichinella* isolates by polymerase chain reaction-restriction fragment length polymorphism of the mitochondrial cytochrome c-oxidase subunit I gene. Int. J. Parasitol. 29:1113–1120.
23. Pérez, C. and Luengo, J. (1969): Quiste de *Trichinella spiralis* con 9 larvas en su interior. Bol. Chil. Parasitol.
24. Pozio, E. (1987): Isoenzymatic typing of 23 *Trichinella* isolates. Trop Med Parasitol. 38:111–116.
25. Pozio E. (2005): The broad spectrum of Trichinella hosts: From cold- to warm- blooded animals. Veterinary Parasitolo. 132 :3–11.
26. Rodriguez, E.; Nieto, J. and Castillo, J.A. (1996): Garate T. Characterization of Spanish *Trichinella* isolates by random amplified polymorphic DNA (RAPD) J. Helminthol. 70:335–343.
27. Soule, C.; Guillou, J. P.; Dupouy-Camet, J.; Vallet, C. and Pozio E. (1993): Differentiation of *Trichinella* isolates by polymerase chain reaction. Parasitol. Res. 79: 461-465.
28. Soad, E. H.; FaragalLa, M. E. and Nagwa T. (2010): Comparison of Two Antigens for Diagnosis of Trichinellosis in Pigs. World Appl.Sci. J. 8 (4): 457-461.
29. Vignau, M.L.; Del-Valle-Guardis, M.; Risso, M.A. and Eireas, D.F. (1997): Comparision between two methods for diagnosis of trichinellosis: trichinoscopy and artificial digestion. Mem Inst Oswaldo Cruz. 5:585–587.
30. Wu, Z.; Nagano, I. and Fukumoto, S. et al. (1997): Polymerase chain reaction primers to identify *Trichinella spiralis* or *T. pseudospiralis*. Parasitol Int. 46:149– 154.
31. Wu, Z.; Nagano, I.; Pozio, E. and Takahashi, Y. (1999): Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) for the identification of *Trichinella* isolates. Parasitol. 118:211–218.
32. Zarlenga, D. S. and Dame, J.B. (1992): The identification and characterization of a break within the large subunit of ribosomal RNA of *Trichinella spiralis*: comparison of gap sequences within the genus. Mol Biochem Parasitol. 51:281–290.
33. Zarlenga, D. S.; Chute, M. B.; Martin, A. and Kapel, C. M. (1999): A multiplex PCR for unequivocal differentiation of all encapsulated and non-. encapsulated genotypes of *Trichinella*. Int. J. Parasitol. 29:1859–1867.
34. Zhiliang, W.; Sofronic-Milosavljevic L. j; Isao, N. and Yuzo, T. (2008): *Trichinella spiralis*: nurse cell formation with emphasis on analogy to muscle cell repair Parasites & Vectors 1:27.

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