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.

**Keywords:** *Trichinella spirals*; ML (Muscle Larva); Giemsa stain; ribosomal DNA technique; Multiplex polymerase chain reaction (Multiplex PCR)

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 spiralis. In this study, we performed a microscopically and molecular biological study to identify and characterize the Egyptian Trichinella spiralis 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 37°C. After through several washing in distilled water, muscle larvae were collected under microscope avoiding contamination of host tissue debris and frozen at -70°C 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°C. After an equal volume of phenol-chloroform extraction, the aqueous phase was digested with 150 µg/ml RNase (Sigma) for 30 minutes at 37°C. 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 spiralis (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, 5
mM MgCl₂, 0.2 mM of each dNTP and 2.5 units Taq polymerase (TaKaRa, Shiga, Japan). The following cycling conditions were used: 95°C, 30 sec (denaturation); 58°C, 1 min (annealing); 72°C, 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 spiralis 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; Zarlenaga 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; Zarlenaga 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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