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Proteomic analysis and immunodetection of antigens from early developmental stages of *Trichinella spiralis*

Rosa Ma. Bermúdez-Cruz[a], R. Fonseca–Liñán[a], Lucia Elhy Grijalva-Contreras[a], Guillermo Mendoza-Hernández[b], M. Guadalupe Ortega-Pierres[a,*]

[a] *Departamento de Genética y Biología Molecular, Centro de Investigación y de Estudios Avanzados del Instituto Politécnico Nacional (CINVESTAV-IPN), Avenida Instituto Politécnico Nacional # 2508, Col. San Pedro Zacatenco, México DF C.P. 07360, Mexico*
[b] *Departamento de Bioquímica, Facultad de Medicina de la UNAM, México D.F., Mexico*

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ABSTRACT

Trichinella spiralis is an ubiquitous parasitic nematode that lives in muscle tissue of many hosts and causes trichinellosis in humans. Numerous efforts have been directed at specific detection of this infection and strategies for its control. TSL-1 and other antigens, mainly from muscle larvae (ML), have been used to induce partial protection in rodents. An improvement in protective immunity may be achieved by using antigens from other parasite stages. Further, identification of other parasite antigens may provide insights into their role in the host-parasite interaction. In this study, *T. spiralis* antigens from early developmental parasite stages, namely ML and pre-adult (PA) obtained at 6 h, 18 h and 30 h post-infection, were identified by proteomic and mass spectrometry analyses. Our findings showed a differential expression of several proteins with molecular weights in the range of 13–224 kDa and pI range of 4.54–9.89. Bioinformatic analyses revealed a wide diversity of functions in the identified proteins, which include structural, antioxidant, actin binding, peptidyl prolyl cis-trans isomerase, motor, hydrolase, ATP binding, magnesium and calcium binding, isomerase and translation elongation factor. This, together with the differential recognition of antigens from these parasite stages by antibodies present in intestinal fluid, in supernatants from intestinal explants, and in serum samples from mice infected with *T. spiralis* or re-infected with this parasite, provides information that may lead to alternatives in the design of vaccines against this parasite or for modulation of immune responses.

1. Introduction

Trichinella spiralis is a parasitic nematode that infects a wide range of carnivores and omnivores, including humans and is the main causative agent of trichinellosis. Infection in humans occurs by the ingestion of raw or not completely cooked meat, mainly pork, that contains *T. spiralis* larvae (Murrell, 2013). There is a need to develop prophylactic methods for *Trichinella* infection in animals as well as improved methods for the early detection of infection.

In this context, *T. spiralis* antigen-group 1 from muscle larvae (ML) known as TSL1 antigens (Ortega-Pierres et al., 1989) and ML excretory-secretory products (Quan et al., 2004) have been reported to induce good protection against *T. spiralis* in animals. Recently, several recombinant proteins and synthesized epitopes from *T. spiralis,* have also been demonstrated to provide partial protection in different animal models (Ortega-Pierres et al., 2015). Nonetheless, it is important to determine if other antigens from early developmental stages of the parasite life cycle, namely ML and pre-adult (PA), could play a major role during infection/establishment, development of larvae to adult worms, immune evasion strategies and/or modulatory effects on host responses. These antigens may provide novel and promising candidates that in conjunction with available adjuvants could induce better protection against the parasite or be used as possible immunomodulatory agents.

Using proteomic and mass spectrometry analyses, ML specific antigens from *T. spiralis*, excretory/secretory proteins (Liu et al., 2016; Robinson and Connolly, 2005; Robinson et al., 2007; Sun et al., 2015a; Wang et al., 2013), surface proteins (Cui et al., 2013; Liu et al., 2015) and somatic proteins have been analyzed (Bien et al., 2015). Likewise, antigens from PA stages have been analyzed using cDNA libraries from 14 h, 20 h and 48 h post-infection (pi) *T. spiralis* stages that were screened with serum from infected pigs (Zocevic et al., 2011). Antigens from the adult stage of *T. spiralis* have also

* Corresponding author.
E-mail address: gortega@cinvestav.mx (M.G. Ortega-Pierres).

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been identified using pig sera from different stages of early infection (Bien et al., 2013; Liu et al., 2013a).

However, earlier PA stage antigens have not yet been fully explored and these may contain key components important for parasite survival, immune recognition or as markers for early detection of *Trichinella* infection.

The aims of this study were to identify differentially expressed antigens in *T. spiralis* from ML and PA (6 h, 18 h and 30 h) parasite stages by proteomic and mass spectrometry analyses. Also, we aimed to determine the recognition of parasite proteins from these stages by antibodies present in the intestinal fluid (IF), supernatants from intestinal "*ex vivo*" explants (SIE) and serum samples (SS) collected early after infection from infected and from re-infected mice. These developmental stages evolve in the intestine of the infected host soon after infection and during parasite molts (between 6 h and 30 h) and these are the stages that are initially exposed to the immune system of the host, especially at the intestinal epithelium. Hence, the immunoreactivity of proteins from these stages was analyzed using IF and SIE as well as in SS collected at day 5 from infected or re-infected animals with *T. spiralis* ML.

2. Material and methods

2.1. Parasite recovery

ML were recovered from Sprague Dawley rats that were previously infected for at least two months with *T. spiralis* ML strain (MSUS/MEX/91 CM). After euthanasia using CO_2, ML were collected by digestion of rat muscle in 1% pepsin-HCl for 2 h at 42 °C. To obtain parasites from early developmental stages, 20 Sprague Dawley rats were infected with 10 000 ML each. Rats were sacrificed under anesthesia using isoflurane (Janssen et al., 2004) at 6, 18 and 30 h pi, parasites were recovered from the intestines as previously described (Dennis et al., 1970), washed with PBS buffer, and counted. All animals were treated according to general guidelines established by the Ethics Committee of Cinvestav IPN.

2.2. Preparation of soluble somatic extracts

Recovered parasites, including ML and 6 h, 18 h and 30 h PA, were placed in one ml of 20 mM Tris-HCl buffer pH 7.2, supplemented with protease inhibitors and sonicated (50 cycles of 15 s, and lapse times of 10 s, at 0 °C). Parasite lysates were centrifuged for 1 h a 10,000*g* at 4 °C. Protein concentration in the soluble somatic extracts (SSE) was determined by the Bradford method (Bradford, 1976).

2.3. Two dimensional (2-D) gel and image analysis

In these assays, 100 μg of protein from the SSE from each stage were mixed with rehydration buffer (7 M urea/2 M thiourea/2% CHAPS/65 mM DTT/2% ampholytes 0.002% bromophenol blue) in a total volume of 125 μl. The sample was loaded onto 7 cm pH 3–10 immobilized linear gradient (IPG) strips (Amersham, USA) and left over-night for swelling. The next day proteins were separated by isoelectric focusing (IEF) using a Protean IEF Cell (Bio-Rad, USA). IEF was performed at 20 °C with the current limited to 50 mA/strip and the following voltage program: S1: 500 V for 1 h; S2: linear ramp to 800 V, 1000 Vh; S3: linear ramp to 8000 V, 11300 Vh; S4: 8000 V, 4400 Vh, for a total of 17 kVh. Following IEF, the IPG strips were placed in equilibration buffer (6 M urea, 30% glycerol, 50 mM Tris–HCl pH 8.8, 2% SDS) with 1% dithiothreitol for 15 min, then in equilibration buffer containing 2.5% iodoacetamide for another 15 min. The second dimension separation was performed on 12% SDS-PAGE using a Mini Protean cell (Bio-Rad, USA). Proteins were resolved at a constant current of 70 V/gel for 12–16 h until bromophenol blue reached the bottom. After 2-D electrophoresis was completed, proteins were stained with Coomassie blue R-250. Gels were scanned and Melanie software (Biorad USA) version 7.0 was used for analysis. Differentially expressed proteins were identified and selected spots were excised for spectrometric analysis. Three replicates were performed for each parasite stage. The stained 2-D gels were scanned with LabScan software on Image scanner and spots detection and matching were made with the aid of Melanie 7.0 software. Only protein spots that were reproducibly obtained in three independent experiments were selected for further spectrometric analysis, as follows: proteins differentially expressed from (a) ML stage when compared to 6 h, 18 h and 30 h PA stages; (b) 6 h PA stage when compared to 18 h and 30 h PA stages; (c) 18 h PA stage when compared to 30 h PA stage.

2.4. Mass spectrometry analysis

The selected spots (32) were excised from the Coomassie blue R-250 stained-gels, placed in tubes and destained, reduced and digested overnight at 37 °C with porcine trypsin (Promega, Madison, WI). The peptides were extracted and analyzed by spectrometry in a 3200 Q TRAPR hybrid tandem mass spectrometer (Applied Biosystems/MDS SciexR, Concord, ON, Canada), equipped with a nanoelectrospray ion source (NanoSpray II) and a Micro Ion Spray II head as described elsewhere (Xolalpa et al., 2007). The instrument was coupled to a nanoAcquity Ultra Performance LC system (Waters Corporation R, Milford, MA, USA). Spectra were acquired and analyzed as previously described (Paz-Maldonado et al., 2013). The database used for searching was NCBI-nr and protein identification was carried out from the MS/MS spectra data sets using the available online pMASCOT search algorithm (Version 1.6b9, Matrix Science, London UK) (Koenig et al., 2008). Mass tolerances of 0.5 Da for the precursor and 0.3 Da for the fragment ion masses were set, with the taxonomy set to all species. Carbamidomethylcysteine was considered a fixed modification and one missed cleavage for trypsin was allowed. A protein "hit" was accepted as a valid identification when at least two MS/MS spectra matched at the 95% level of confidence ($p < 0.05$).

2.5. Infection of mice with T. spiralis

Animals used in this study were BALB/c mice that were either intragastrically infected with 500 ML or that were infected using this parasite infection schedule and then re-infected after two months with 200 ML of *T. spiralis* strain (MSUS/MEX/91 CM). Non-infected BALB/c mice were used as controls. The groups (five mice each) analyzed were as follows: non-infected control animals, mice that were infected with *T. spiralis* and animals that were infected and re-infected with *T. spiralis.*

2.6. Collection of intestinal fluid, supernatants from intestinal explants and serum samples from normal control and experimental mice

Five days after-infection, mice from the control and experimental groups were anesthetized with isoflurane (Janssen et al., 2004). Blood samples were collected by cardiac puncture and blood allowed to clot to obtain serum samples. Animals were then euthanized by cervical dislocation and the small intestine removed to obtain intestinal fluid and for explant cultures. For intestinal fluid collection, the abdomen was opened, the small intestine was separated from the mesentery, clamped at both ends, removed and rinsed in cold PBS with complete protease inhibitor cocktail (Roche). The lumen of the small intestine was filled with 2 ml of PBS and the distal 4 cm segment was severed to allow for release

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