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Comparative proteomic analysis of surface proteins of *Trichinella spiralis* muscle larvae and intestinal infective larvae

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ABSTRACT

The critical step for *Trichinella spiralis* infection is that muscle larvae (ML) are activated to intestinal infective larvae (IIL) and invade intestinal epithelium to further develop. The IIL is its first invasive stage, surface proteins are directly exposed to host environment and are crucial for larval invasion and development. In this study, shotgun LC-MS/MS was used to analyze surface protein profiles of ML and IIL. Totally, 41 proteins common to both larvae, and 85 ML biased and 113 IIL biased proteins. Some proteins (e.g., putative scavenger receptor cysteine-rich domain protein and putative onchocystatin) were involved in host-parasite interactions. Gene ontology analysis revealed that proteins involved in generation of precursor metabolites and energy; and nucleobase, nucleoside, nucleotide and nucleic acid metabolic process were enriched in IIL at level 4. Some IIL biased proteins might play important role in larval invasion and development. qPCR results confirmed the high expression of some genes in IIL. Our study provides new insights into larval invasion, host-*Trichinella* interaction and for screening vaccine candidate antigens.

1. Introduction

Trichinella spiralis is a tissue-dwelling parasitic nematode infecting many kinds of carnivores and omnivores, and is the main aetiological agent of trichinellosis. Humans acquired this disease by the ingestion of raw or insufficiently cooked meat containing the *T. spiralis* muscle larvae (ML). *T. spiralis* ML are released in the stomach and activated to "intestinal infective larvae (IIL) by intestinal content or bile after 0.9 h post-infection (hpi) (Campbell, 1983). Then, the IIL invade, occupy and migrate through host's intestinal epithelium cells (IECs) where they molt and develop to adults and reproduce newborn larvae (Liu et al., 2013). Therefore, ML are activated to IIL in intestines and invade intestinal mucosa to further develop is the critical step for establishing *Trichinella* infection in host (Ren et al., 2013). The IIL is the first invasive stage during the process of *Trichinella* infection.

Since the parasite cuticle is directly in contact with the host environment, the cuticle surface of parasitic nematodes is antigenic in many infected hosts (Pritchard et al., 1985). In a number of experimental systems antibodies are produced against surface molecules and mediate antibody dependent cell mediated cytotoxic reactions. *T. spiralis* surface proteins include the cuticle proteins themselves and the excretory–secretory (ES) proteins which were incorporated on the cuticle (Ortega-Pierres et al., 1984; Pritchard et al., 1985). They are directly exposed to the host's immune system, are the main target antigens which induce the immune responses, and may play an important role in the invasion, development and immune escape process of *T. spiralis* larvae.

In recent years, proteomics has been a powerful technique for detection and identification of specific proteins from different species of the genus *Trichinella* or different samples of *T. spiralis* (Robinson et al., 2007). Many studies have focused on the ES and somatic proteins with the aim of identifying key molecules correlated with the development process of the *T. spiralis* larvae (Bien et al., 2012; Robinson and Connolly, 2005). Previous proteomics studies showed that microenvironmental factors influence protein secretion by *T.spiralis* L1 in vitro and correlated this with infectivity to mice (Bolas-Fernandez et al., 2009). However, up to now, the comparative proteomic analysis of surface proteins of *T. spiralis* ML and IIL has not been reported.

In the present study, we utilized the shotgun LC-MS/MS approach in combination with bioinformatics analysis to identify

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and characterize the surface protein expression profiles of *T. spiralis* ML and IIL. These data are expected to provide valuable hints for uncovering the molecular mechanism of the invasion of IECs by *T. spiralis* larvae and better understanding the interaction between parasite and host.

2. Materials and methods

2.1. Parasite and experiment animals

T. spiralis isolate (ISS534) used in this study was collected from domestic pigs in Nanyang, Henan Province, China. Specific pathogen-free (SPF) six-week-old male Kunming mice were obtained from the Experimental Animal Center of Henan Province (Zhengzhou, China). All animal procedures reported herein were reviewed and approved by the Life Science Ethics Committee of Zhengzhou University (Permission No. SYXK 2011-0001)

2.2. Collection of ML and IIL

T. spiralis ML was recovered from infected mice at 42 days post infection (dpi) by artificial digestion with 1% pepsin (1:3,000) and 1% hydrochloric acid (Gamble et al., 2000; Li et al., 2010). After recovery, a part of ML were directly pooled for extracting surface proteins, the other part were orally inoculated into 50 mice, with 5000 ML per mouse (Ren et al., 2013). The infected mice were euthanized at 6 h post infection, the small intestine was opened longitudinally, and washed three times in ice-cold normal saline, then cut into 2 cm long fragments with sharp scissors and cultured in normal saline at 37 °C for 2.5 h. Then, IIL larvae released from small intestine to normal saline were collected by Baermann's method as previously described (Gamble et al., 2000).

2.3. Preparation of surface proteins from ML and IIL

Surface proteins from ML and IIL were prepared with 0.25% hexadecyl trimethyl ammonium Bromide (Sigma, USA) and 2% sodium deoxycholate (Sigma, USA) (Pritchard et al., 1985; Cui et al., 2013). The supernatant was obtained by centrifugation at 4 °C, 11,000 × *g* for 20 min, and dialyzed against deionized water at 4 °C for 2 days. Then the supernatant contained surface proteins were concentrated through Amicon Ultra-3Centrifugal Filter Unit (MW cut off 3 kDa) at 4 °C, 5000 × g. The surface proteins were stored at −80 °C before use. Totally, larval surface protein samples were prepared as three biological replicates, mixed and subjected to the following SDS-PAGE and LC-MS/MS.

2.4. SDS-PAGE

Protein samples (20 μg) were separated by SDS-PAGE on 5% stacking gels and 12% resolving gels (83 × 73 × 1.0 mm) in a mini-vertical electrophoresis system (Bio-Rad Laboratories, USA) (Wang et al., 2011). After electrophoresis, the gel was stained with 0.25% Coomassie brilliant blue (CBB) R-250 (Sigma, USA) for 4 h, and then bleached with the eluate.

2.5. Liquid chromatography and tandem mass spectrometry (LC-MS/MS)

The CBB-stained SDS-PAGE gel lane was manually cut into 7 slices depending on protein molecular weight (MW). Each slice was diced into 1 mm × 1 mm pieces, and then subjected to in-gel tryptic digestion (Wang et al., 2012). After digestion, Peptide mixtures were separated by high-performance liquid chromatography (HPLC) followed by tandem MS analysis.

2.6. Protein identification and annotation

All MS/MS spectra were searched against the NCBI *T. spiralis* protein database (33,133 sequences, 6/10/2014, http://www.ncbi.nlm.nih.gov/) by using SEQUEST algorithm. Multiple peptide identifications were generally returned by SEQUEST for each MS/MS spectrum and for each parention change state. The protein identification criteria used in our study was based on Delta CN (≥0.1) and Xcorr (one charge ≥ 1.9, two charges ≥ 2.2, three charges ≥ 3.75) (Washburn et al., 2001).

2.7. Bioinformatic analysis

MW and isoelectric points (pI) were calculated using the online compute pI/MW tool (http://web.expasy.org/compute_pi/). The online tools (http://www.cbs.dtu.dk/services/TMHMM/) and (http://www.cbs.dtu.dk/services/SignalP/) were used to analyze the presence of a signal peptide and a transmembrane domain. Bioinformatic Analysis InterProscan software (http://www.ebi.ac.uk/Tools/pfa/iprscan/) was used to perform protein sequences searches to identify signatures (Zdobnov and Apweiler, 2001). Then the matched terms were subjected to GO categories using the Web Gene Ontology Annotation Plot (WEGO) (http://wego.genomics.org.cn/cgi-bin/wego/index.pl) (Ye et al., 2006).

2.8. Quantitative real-time PCR (qPCR)

Select six genes from the identified proteins, then further determine the transcription level by qPCR. Total RNAs were extracted from ML and IIL using TRIzol reagent (Takara, Japan) and then reverse-transcribed into cDNA by using a PrimeScript® RT reagent kit with gDNA Eraser (Takara). Using the Primer 5.0 software to designed the gene-specific primers (listed in Table 1). The q-PCR was performed in 7500 fast real-time PCR system (Applied Biosystems, USA) according to the following references (Liu et al., 2013).

2.9. Statistical analysis

A Fisher's exact test calculated by SPSS version 17.0 (SPSS Inc., Chicago, IL) was used to assess significant differences between ML and IIL surface protein samples ($P < 0.05$) based on the number of proteins per GO category in each sample (Zhang et al., 2013). A two-sample t-test calculated was used to assess relative expression levels significant differences between ML and IIL ($P < 0.05$).

3. Results

3.1. Global analysis of surface proteome

Surface proteins from ML and IIL were separated by SDS-PAGE and subjected to shotgun LC-MS/MS analysis (Fig. 1). A total of 287 ML surface proteins and 402 IIL surface proteins were identified by searching the databases of *T. spiralis* in NCBI. Removing redundant sequences, ML and IIL surface protein data were clustered into 126 and 154 unique proteins, of which 82 (65.1%) and 99 (64.3%) proteins were annotated by InterProscan software. A comparison of the identified proteins between ML and IIL was shown in Fig. 2. Overall, 41 common identified proteins constituted 32.5% of total proteins in ML and 26.6% in IIL (Table 2). Of total proteins in different larvae, 85 (67.5%) were ML biased and 113 (73.4%) were IIL biased (Table 3). Out of 41 common identified proteins, 19 (46.3%) proteins possessed a signal peptide and 11 (26.9%) possessed a transmembrane domain. In 113 IIL biased proteins, 23 (20.3%) proteins contained a signal peptide and 24 (21.2%) contained a transmembrane domain.