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Original Article

Identification and Immunological Characterization of Somatic Proteins from Adults of *Toxocara cati* by Proteomics Technique

Nooshinmehr SOLEYMANI [1], Ruth Birner GRUNBERGER [2,3,4,5], *Khalil ABNOUS [6], *Hassan BORJI [1], Faezeh VAHDATI [6]

1. *Department of Pathobiology, Faculty of Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran*
2. *Austrian Center of Industrial Biotechnology, Graz, Austria*
3. *Diagnostic and Research Institute of Pathology, Medical University of Graz, Graz, Austria*
4. *Omics Center Graz, Bio Tech Med-Graz, Graz, Austria*
5. *Institute of Chemical Technologies and Analytics, Vienna, Austria*
6. *Pharmaceutical Research Center, Pharmaceutical Technology Institute, Mashhad University of Medical Sciences, Mashhad, Iran*

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***Correspondence**
Email:
hborji@um.ac.ir
abnouskh@mums.ac.ir

Abstract

Background: *Toxocara cati* is considered as one of the main etiological agents of toxocariasis with global and regional importance. As there is no information on proteomics of *T. cati*, herein, we reported the results obtained by proteomic analysis of somatic proteins extract, using a mass spectrometry (LC–MS/MS) approach.

Methods: Somatic extract fractions were separated by two-dimensional SDS-PAGE and were electro blotted on to PVDF membranes for immunoblot analysis, then collected the immunogenic spots which response of antibodies of the paraten-ic hosts (mice) to the antigens (Mashhad, 2017), and analyzed by LC–MS/MS. The LC-MS/MS data were analyzed by Mascot database, Taxonomy *Toxocara*, and common contaminants, in Omics Center, Biotechnology Medical University of Graz (Austria, 2018).

Result: The protein spots were isolated between 15–140 kDa ranges using 3–10 non-linear IPG strips and Brilliant Blue Coomassie. Ten proteins were character-ized as immunogenic proteins, seven of them were identified and three of them were unknown proteins.

Conclusion: This study provided additional information about the somatic anti-gens of *T. cati*, which can lead to the development of new strategies for novel im-muno-modulators, drug targets, subunit vaccines and immunodiagnostic kits for toxocariasis.

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Introduction

Toxocara cati (Schrank, 1788) is a roundworm of both wild and domestic cats, and one of the causative agents of toxocariasis, which is a global zoonotic parasitic disease and remains a problem throughout the world (1). Humans are infected by ingestion of embryonated eggs, or using via consumption of paratenic hosts, which are a source of third stage larvae (2).

The epidemiological patterns of toxocariasis in stray and pet cats in Iran revealed that the prevalence of infection was 8%-78.8% (3). The third stage larvae have extra luminal migration to the liver, lungs, and other organs, and the larvae cannot develop further and remain as hypobiotic stages in the body tissues, and produce characteristic symptoms known as visceral larva migrans syndrome (VLM)(4,5). Migration of third stage larvae in the eye, damage the retina and evoke granulomatous leading to ocular larva migrans (OLM) (6-8). Other classified forms of toxocariasis are neurological larva migrans (NLM) and covert toxocariasis (CT) (1,9). The diagnosis of toxocariasis is often difficult, and available diagnostic tests are primarily immunological. Clinical signs and symptoms are also helpful in some cases. The tests are available for the immunodiagnosis including enzyme-linked immunosorbent assay (ELISA) and Western blotting (WB) using *T. canis* excretory-secretory production and anti-*Toxocara* antibodies (IgG) were detected by a ELISA commercial kit. (5,10,11). Moreover, cross-reactivity is a matter with other helminths such as Ascaris. In addition, similarities in some antigens of *T. cati* and *T. canis* we may miss some toxocariasis cases (12, 13). Even though, many similarities between the antigens of *T. canis* and *T. cati*. Thus, the characterization of *T. cati* antigens is very valuable for immunological diagnosis of toxocariasis caused by *T. cati*. It even provides important information for the development of a vaccine and study the host-parasite interactions (13).

Proteomics is an efficient, rapid and sensitive technique for the identification of helminth somatic and (ES) proteins using two-dimensional gel electrophoresis in show within gel digestion of spots and analysis with mass spectrometry (LC-MS/MS) (14-15). Over the last two decades several studies to identify and characterize immunogenic proteins have been carried out, especially with the aim to identify the candidates for diagnostic-kits for and vaccine in parasites (16- 18). The present study is the first, to the best of our knowledge other nematodes, such as; *Ascaris suum* (18), *Heligmosomoides polygyrus* (19), *Ancylostoma caninum* (20), *Strongyloides stercoralis* (21), *S. ratti* (22), *Trichinella spiralis* (23), *T. canis* (24, 25) have been studied.

Thus, in view of the above, and the lack of data about the proteome of *T. cati*, the purpose of this study was to identify the immunogenic proteins in the adult *T. cati* whole proteins using two-dimensional electrophoresis (2-DE) and LC-MS/MS techniques.

Materials and Methods

Parasites

T. cati adult worms were collected from naturally infected stray cats, after routine deworming using anthelminthic treatment.

All experimental protocols were revised and approved by the guideline of Animal Welfare Committee of Ferdowsi University of Mashhad (permit number: 40329).

Preparation of somatic products

Five mature worms were washed three times with pre-warmed phosphate-buffered saline (PBS), pH 7.2 and stored at -80 °C until use. For preparation of adult crude extracts, *T. cati* were homogenized and then sonicated, which was performed in ice. To eliminate contami-

nating substances, the sample was suspended in cold pre-treatment solution (2 M thiourea, 7 M urea, 200 mM DTT, 2% CHAPS, 2% IPG buffer pH 3–10, ampholytes (pH 3–10, Bio-Rad), 1 mM phenyl methyl sulfonyl fluoride (PMSF) as a protease inhibitor, BioRad) and stored in at 4 ºC for two hours. Homogenated samples were sonicated for 40 s on ice using a probe sonicator (UP100H, Germany), subsequently centrifuged at 10,000 g for 10 min. The supernatant was dialyzed against PBS. Protein concentration was measured by Bradford assay (Bio-Rad Protein Assay Kit).

Immunization and serum samples

BALB/c mice were immunized with *T. cati* eggs at a dose of 300 ova/mouse given orally by gastric gage. At the end of the treatment period (21 days), anesthesia was performed and 2 mL blood sample were collected in a tube without anticoagulant for subsequent serum collection. The serum was separated after centrifugation at 1,800 g for 5 min and stored at −20 ºC until use. The negative serum was obtained from the mice just before infection of the mice.

Bi-dimensional electrophoresis

Isoelectric focusing was performed in 7 cm immobilized pH gradient IPG strips (Bio-Rad) with linear and non-linear pH ranges 3–10, and actively rehydrated with 100 µL of rehydration buffer 20% ampholyte (pH 3–10, Bio-Rad), 2% (w/v) 3-cholamidopropyl dimethylammonio 1-propanesulfonate (CHAPS) 0.3% (w/v) DL-Dithiothreitol (DTT), 2 M thiourea, 6 M urea, containing 100µg Protein using the Protean IEF Cell (Bio-Rad, USA) and bromophenol blue. The IPG strips were rehydrated by in gel rehydration at 50 V, for 12 h. Then, the IPG strips were subjected to isoelectric focusing using the following conditions: 200 V for 1 h; 500 V for 1 h; 1000 V for 1 h and 8000 V for 6 h until 50,000 Vh were reached. Then, the strips were reduced in the equilibration buffer for 10 min (37.5 mM Tris–HCl, pH 8.8, 6 M urea, 30% (v/v) glycerol, 2%(w/v) SDS and 1% (w/v) DTT) followed by a second equilibration in the same equilibration buffer containing 2.5% (w/v) iodoacetamide instead of DTT for 10 min. The strip was then positioned on the top of a homogenous 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) and embedded in 1% agarose stacking gel. Electrophoresis was carried out for 60 min at 25 mA at 10 °C. After separation, proteins were visualized by staining for 1 h with Colloidal Coomassie Blue G250 (Sigma). After the staining process, the gels were washed briefly with deionized water to eliminate excess dye. Stained gels were digitally scanned on image scanner III (Epson, Japan). Non-stained gels were kept for blotting (26).

Immunoblotting

After somatic extract proteins of *T.cati* separated by 2-D electrophoresis, 2-D gels were electro blotted on to polyvinylidene difluoride (PVDF) membranes for immunoblot analysis (BIO-RAD) at 110 V for 75 min using transfer buffer (25 mM Tris, pH 8.3, 192 mM glycine, 20% (v/v) methanol, SDS10%). Blots were blocked with 5% skim milk in Tris buffered saline–Tween 20 (TBST) for two h at room temperature, washing three times with buffer PBS-T containing 0.05% Tween 20, and then incubated with sera from non-infected mice at 1:1000 dilution, for 2 h at room temperature, with shaking . Blots were washed again three times and then incubated with a horseradish peroxidase-labelled anti-mouse IgG (Sigma) at 1:3000 dilution for 1 h at room temperature, with shaking. Immunogenic proteins were recognized with Metal Enhanced DAB Substrate Kit (Thermo Scientific). In order to detect the immunogenic spots with scanner in the gels, immunoblots and their homologous Coomassie-stained gels were aligned to pI and MW and then using UV band software (UVITEC, UK) in order to identify the immunogenic spots in the gels (27).

LC-MS/MS analysis

Two spots from 2-D gel were reduced, alkylated and digested with Promega modified trypsin according to the method of (28, 29). Resulting Peptides were acidified with formic acid (final concentration of 0.1%) and analyzed like the crude extracts with minor modifications. The following gradient, where solvent A is 0.1% formic acid in water and solvent B is acetonitrile containing 0.1% formic acid was used: 0-0.1 min: 2 % B, 150 uL/min; 0.1-18 min: 2% 150uL/min – 300uL/min; 18-100 min 2%-25 % B; 100 -107 min: 25-35 % B, 108 – 118 min: 35 - 95 % B; 118-118.1 min: 95 % - 2%B; 118.1-133 min: 2% B was used.

Database search

The LC-MS/MS data were analyzed by Mascot 2.4 by searching the public Trembl database, taxonomy *Toxocara*, TXID 6264, (13379475 residues, 37973 sequences) and common contaminants. Whole search criteria were used as follows: trypsin, max. Missed cleavage sites: 1; search mode: MS/MS ion search with decoy database search comprised; precursor mass tolerance +/- 10ppm; product mass tolerance +/- 0.8 Da; acceptance parameters for identification: 1 % target FDR (30).

Results

Proteins profiles of the somatic extract of T. cati material (2d-electrophoresis of somatic extracts)

The highest number of spots and lowest streaking were achieved using 3–10 non-linear IPG strips, Brilliant Blue G 250 Coomassie stainings are presented in (Fig. 1) showing the protein spots, which located between 15–140 kDa arrays.

Immunogenic proteins with pH 3-10, and MW higher than 30 KDa, which are identified, by LC-MS/MS (at Omics Center Graz, Bio Tech Med-Graz, Graz, Austria) (Table 1).

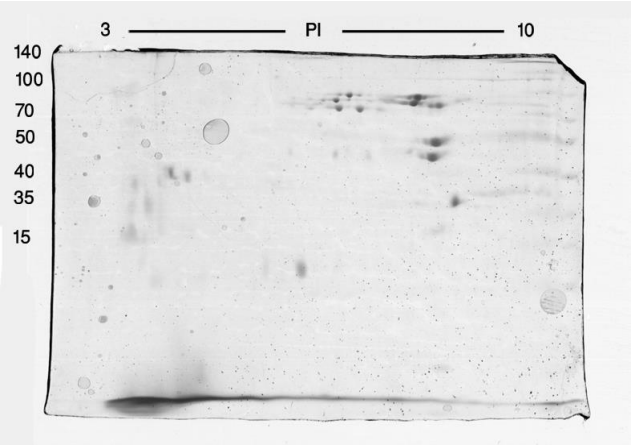

Fig. 1: Somatic proteins of adult *T. cati* by 2D-gel electrophoresis. 7 cm IPG strips with pH 3–10 were used. 10% of polyacrylamide gel in the second dimension was applied

Identification of immunogenic proteins of T.cati

According to the results, when the somatic extract immunoblots were checked with sera from non-infected mice, no spots were observed (figures not presented). In the case of somatic extract, matching the immunoblots with the homologous Coomassie-stained gels allowed us to locate ten spots that contained immunogenic proteins in the 2-D gels (Fig. 2).

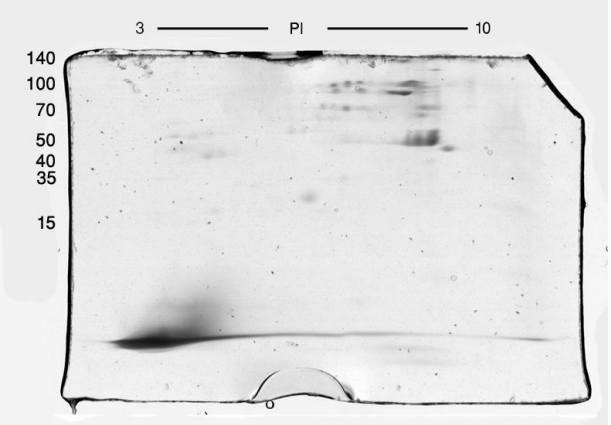

Fig. 2: Identification of somatic antigenic spots recognized by IgG in *T. cati* infected mice by western blotting. A pooled serum of infected mice was used to blot with somatic antigens immobilized in PVDF membrane after 2D-gel electrophoresis which were identified by LC-MS/MS

Table 1: Identification of the immunogenic proteins of the somatic extract of adult *T.cati* by LC-MS/MS (based on Fig. 2). The proteins identified were; actin 41.8 kDa, enolase 47.5 kDa, tropomyosin 31.3 kDa, chaperonin-like HSP-60 85.7 kDa, V-type protein ATP V-type proton ATPase catalytic subunit A 67.8 kDa, heat shock proteins (HSPs) 70 kDa, paramyosin 101 kDa, and 3 of them were uncharacterized proteins and unknown function

Spot No.	Protein	MW(kDa)	Score Mascot	Coverage	Accession No.	Species	Molecular Function
1	Actin	42.4	28321	70.41	A0A0B2VA52	*T.canis* & *A.suum*	ATP binding
2	Enolase	47.5	1336	43.11	A0A0B2VEA6	*T.canis*	Phosphopyruvate hydratase activity
3	Tropomyosin	31.3	11209	37.40	A0A0B2VDB8	*Ascaris lumbercoides*	Unknown function
4	Chaperon like Hsp-60	85.7	3510	46.32	A0A0B2W434	*Ascaris.suum*	Unknown function
5	V-type proton ATPase catalytic subunit A	67.8	1874	51.21	A0A0B2UT46	*T.canis*	Unknown function
6	Uncharacterized protein	101	1749	32.15	A0A183TX44	*T.canis*	-
7	Uncharacterized protein			49.72	A0A183TX44	*T.canis*	-
8	Heat shock protein 70	74.7	1002	26.30	A0A0B2W0B9	*Anisakis pegreffii* & *T.canis* & *Loa loa*	ATP binding
9	paramyosin	101.4	7526	49.14	A0A0B2V6Q8	*T.canis* & *Anisakis simplex*	Motor activity
10	Uncharacterized protein	101.5	10137	58.29	A0A183TX44	*T.canis*	-

Discussion

To our knowledge, this is the first proteomic study carried out on the somatic extract of adult *T. cati*. We separated the somatic proteins of *T. cati* by 2-Dgel electrophoresis, and then they were electro blotted on to PVDF membranes for immunoblot analysis. We collected the immunogenic spots, which bound antibodies from the paratenic hosts (mice) to the antigens. The immunogenic proteins identified in somatic product of adult *T. cati* have been identified were; actin, enolase, tropomyosin, chaperonin-like HSP-60, HSP-70, V-type protein ATPase catalytic domain A, paramyosin, and three of them were unknown proteins.

The first immunogenic protein that was identified in the somatic product of *T. cati* was the structure motor protein actin. Actins play an important role in cell transferring as a part of the cell cytoskeleton. In addition to actin has been identified on the surface of various helminth parasites, including the tegument of *T.canis* (24, 25), *Dicrocelium denderticum* (27), *Schistosoma mansoni* (31), and the intracellular protozoan such as apicomplexan have an actin polymerization-dependent motility for penetration through cell walls (32). Some of human pathogens, such as bacteria disrupt the cytoskeleton of host cells during passive invasion mechanism (33), whereas some helminths, such as *T. cati*, have evolved their own actin

cytoskeletal systems during active invasion mechanism. One of the most abundantly expressed proteins of *T. cati* is actin-binding protein that is secreted into host cells during invasion.

Another immunogenic protein that reacted with antibodies in sera of infected mice was enolase. Enolase is a metallo enzyme responsible for the catalysis of 2-phosphoglycerate to phosphoenolpyruvate, the penultimate step in glycolysis (34). In spite of its cytosolic function and localization, it has been found in ES of *H. contortus* (35, 36). As well as, enolase has been found to be a constituent of both the somatic and the ES extract of the adult worms that were able to bind host plasminogen, possibly enabling tissue invasion (37). Enolase has been restricted on the surface of nematodes used by pathogens when invading tissues (37-38). It is recommended that the enzyme may function as the pathogenic role during parasite infection as; mechanisms of evasion and migration (27).

In this study, another important protein group identified in the somatic extract of adult *T. cati* were HSP-60, and HSP-70, which are also present in tegument of *T. canis* larval (24, 25). HSP-family is one of the proteins that are highly conserved and present in all cells in the response to stressful conditions. Many members of the HSP family function as chaperones with roles in folding/unfolding of proteins, signaling, assembling multiprotein complexes, transport of proteins into the correct subcellular compartments, cell-cycle control, and protection of cells against apoptosis. Furthermore, extracellular HSPs can regulate antigen-presenting cells of the immune system (39-41). Once more, Heat shock proteins act as protein chaperones and transfer antigenic peptides to the class I and class II molecules of the major histocompatibility complexes (MHC I & II). HSPs are immuno-modulatory molecules that have been recognized in nematodes, and have been implicated in immune modulation and evasion, which are important for parasite survival (39-41) A HSP 60 kDa, was identified in the extract of larval and adult Ascaris (41). Furthermore, the Hsp-70 was also identified in the stress defense mechanisms in trematodes. Therefore, they are valuable candidates of the therapeutic agents in allergic and autoimmune diseases, and for developing vaccines or anthelminthic therapies (41, 42).

Role of HSPs in induction of several inflammatory cytokines and the signaling pathways are yet to be fully cleared. Nevertheless, the recent research have revealed that overexpression of HSP60 encourages the secretion of interleukin 6 (IL-6) via signaling through CD 14, along with the over expression of TNF-α and nitric oxide from human monocytes. Moreover, these proteins has been demonstrated as a main target for T cell and Ab responses and the expression of a variety of interleukins, including IL-12 in chronic inflammation (43, 44).

Additionally, we found paramyosin which is known in muscles of a wide variety of helminths including *T. canis* (24, 25), *S. mansoni (31), S. japonicum* (45, 46), and *Fasciola hepatica* (47). Actually, paramyosin is a myosin-binding protein that has been identified in invertebrate animals. Paramyosin has been identified from tapeworms, more work has been expected at providing reagents appropriate for specific and sensitive immunodiagnostics, and it can be considered as vaccine candidates or targets for anthelminthic therapies (46-50).

In recent years, a large and growing application of 2-D gel electrophoresis is within the field of proteomics. The analysis contains identification, the systematic separation, and quantitation of many proteins concurrently from the somatic extract and the ES product of parasites (51). Proteomics is a specific and unique technique, which is able to discover post-translational and co-translational modifications, which cannot be detected from the genome sequence (52, 53). Recently, hundreds of parasite genomes have been sequenced, but this is only the first step in a long way until we identify proteins that govern host–parasite interactions (54). Two-dimensional electro-

phoresis is used in this field due to its unequaled facility to separate hundreds of proteins that may have roles in immunomodulatory and immune evasion, which may help parasites to live and expand in host body. The characterization of novel immuno-modulators can form the next generation of therapeutics possibilities for inflammatory diseases (55). Furthermore, characterization of immunogenic proteins of adult *T. cati* can open a completely new domain of possibilities for the discovery of vaccines, drug targets and diagnosis kit for toxocariasis. In order to, if we able to characterize all the proteins from a particular parasitic helminth can form the discovery the development of vaccines, and drug targets for diseases, and also the discovery the immunodiagnosis kits.

In this study, characterizing of somatic extract immunogenic proteins of adult *T. cati* play a key role in the host-parasite interactions, and it is valuable discovery of diagnostic kits for toxocariasis caused by *T. cati*.

Conclusion

The results gained in this study deliver information about the somatic antigens, which can be useful for future studies on the detection of *T. cati*, which are useful for diagnosing toxocariasis and the improvement vaccination against toxocariasis.

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Conflict of interest

The authors declare that there is no conflict of interest.

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