Veterinary Parasitology 194 (2013) 222–225

Contents lists available at SciVerse ScienceDirect

Veterinary Parasitology

journal homepage: www.elsevier.com/locate/vetpar

Screening of early antigen genes of adult-stage *Trichinella spiralis* using pig serum from different stages of early infection☆

P. Liu[a,1], X.P. Wu[a,1], X. Bai[a,1], X.L. Wang[a,1], L. Yu[a], B. Rosenthal[b], R. Blaga[c], S. Lacour[c], I. Vallee[c], P. Boireau[c], C. Gherman[d], M. Oltean[d], X.N. Zhou[a], F. Wang[a], Y. Zhao[a], M.Y. Liu[a,*]

[a] *Key Laboratory for Zoonosis Research, Ministry of Education, Jilin University; Zoonosis Center of National Institute of Parasitic Diseases, Chinese Center for Disease Control and Prevention, China*
[b] *Animal Parasitic Diseases Laboratory, USDA, Building 1180, Beltsville, MD 20705, USA*
[c] *ANSES, ENVA, UPEC, UMR BIPAR, Laboratory for Animal Health, Maisons-Alfort, France*
[d] *University of Agricultural Science and Veterinary Medicine, Cluj-Napoca, Romania*

ARTICLE INFO

Keywords:
Trichinella spiralis
cDNA library
Immunoscreening
Immunodiagnosis

ABSTRACT

The goal of this work was to identify novel, early antigens present in *Trichinella spiralis*. To this end, a cDNA library generated from 3-day old adult worms (Ad3) was immunologically screened using serum from a pig infected with 20,000 muscle larvae. The serum was obtained from multiple, time course bleeds coinciding with early worm development. Seventeen positive clones were isolated using serum obtained at 20 days post infection (dpi). All clones corresponded to one gene that exhibited high sequence identity with the *T. spiralis* ATP-dependent RNA helicase DDX19B which is involved in parasite growth and development. In addition, nine additional positive clones representing 5 unique genes were identified when the library was screened with 30 dpi serum; four of these five genes displayed high similarity with members of a putative *T. spiralis* serine protease family known to be involved in host invasion and host–parasite interactions. The remaining gene aligned with the *T. spiralis* hypothetical ORF 11.30. The identification of these antigens provides potential candidates for the early diagnosis of trichinellosis and for the development of a vaccine against this parasite.

1. Introduction

Trichinella spiralis is a food-borne, zoonotic parasite that infects a wide variety of mammals, including humans (Webster et al., 2006). In recent years, trichinellosis has become an emerging and re-emerging zoonosis in certain parts of the world, such as Eastern Europe and China. The global importance of trichinellosis in food safety and human health requires the urgent devolvement of useful tools such as sensitive immunodiagnostic tests and vaccines to control parasitic infection (Liu and Boireau, 2002).

The entire life cycle of *T. spiralis* occupies two distinct niches within a single host and involves three major antigenic stages: newborn larvae, muscle larvae (ML), adult worms (Ad). It has been demonstrated that *T. spiralis* expresses complex and stage-specific proteins during

☆ The nucleotide sequences identified in this study (Table 1) have been deposited in the GenBank™, EMBL and DDBJ databases under the Accession Nos. JQ517280 and JQ517281.

* Corresponding author. Tel.: +86 21 64738058; fax: +86 21 64332670.
E-mail addresses: ipdzhouxn@sh163.net (X.N. Zhou), Wangfeng1234cn@yahoo.com.cn (F. Wang), zhaoyingsohu@sohu.com (Y. Zhao), liumy@jlu.edu.cn (M.Y. Liu).

[1] These authors contributed equally to the work.

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http://dx.doi.org/10.1016/j.vetpar.2013.02.001

development. Collectively, these proteins are involved in host invasion, parasite survival, and the generation of a protective immune response. Key antigens, a subset of these stages – specific proteins is relevant to immunodiagnosis and vaccine development (Boireau et al., 1997). Better understanding the roles of these antigens in parasite–host interactions and in the induction of host immune responses would aid in elucidating the mechanism of *T. spiralis* immune escape and in selecting potential candidates for immunodiagnosis and vaccine development.

Currently, the detection of *T. spiralis* antibodies is based on the ML-derived excretory–secretory (ES) antigens. The ES antigens have a higher diagnostic specificity than the crude antigens; however, host antibodies to ES antigens are generally not detectable until 4–5 weeks post infection. As such, they are not useful for detecting early infections (Gottstein et al., 2009; Kapel and Gamble, 2000) which can have consequences for public health. In recent years, a number of encouraging antigens have been isolated from cDNA expression libraries generated from parasites at different stages, and these antigens have been evaluated for the detection of *T. spiralis* antibodies in pigs and other hosts; however, none of these antigens has yielded satisfactory results in detecting early infections. To resolve this problem, it was necessary to isolate novel candidate antigens from the early stages of infection that might allow detection prior to 4–5 weeks.

In this study, genes encoding the most abundant, early-stage antigens of *T. spiralis* were investigated by the immunoscreening a 3-day old adult worm (Ad3) cDNA library with pig anti-*T. spiralis* serum obtained at different times following infection. Results from this study will help to improve the serological diagnosis of trichinellosis and facilitate vaccine development.

2. Materials and methods

2.1. Parasite and cDNA libraries

Trichinella spiralis (ISS534) was maintained in female ICR/CD1 mice, and Ad3 were isolated from the small intestines of experimentally infected Wistar rats as previously described (Bai et al., 2012). Total RNA from Ad3 was extracted as previously described (Vayssier et al., 1999), and poly (A) RNA was purified using the Oligotex Direct mRNA the Midi Kit (Qiagen). A lambda ZAP II cDNA library was constructed using the ZAP Express cDNA Synthesis kit and the ZAP Express cDNA Gigapack II Gold Cloning kit (Stratagene), according to the manufacturer's instructions.

2.2. Preparation of antiserum

A domestic pig was experimentally infected per *os* with 20,000 ML of *T. spiralis*. Pre-infection serum was collected as a negative control, and infection sera were collected at 20 and 30 dpi. Cross-reactive antibodies were removed from the serum by absorption with non-recombinant vector lambda ZAP II phage as previously described (Sambrook and Russell, 2001).

2.3. Immunoscreening of cDNA libraries

Approximately 100,000 plaque forming units (pfu) of the Ad3 cDNA library were screened with absorbed antiserum according to conventional methods (Sambrook and Russell, 2001). All positive plaques were rescreened until single plaques could be isolated. After the last screening, the positive plaques expressing the antigens were picked, and PCR was used to amplify the cloned cDNA fragment using T3 and T7 primers. Phagemids were excised with the ExAssist interference-resistant helper phage according to manufacturer's instructions (Stratagene) and propagated in the *E. coli* SOLR strain.

2.4. Sequence analysis

The T3/T7-amplified PCR products were sequenced in both directions on an automated DNA sequencer and analyzed using the BLAST tool for homology searches. SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP/) and SMART (http://www.ebi.ac.uk/Tools/pfa/iprscan/) programs were used to detect signal peptides and to evaluate putative protein family domains, respectively.

3. Results

3.1. Clones identified with 20 dpi pig anti-T. spiralis serum

Seventeen strongly reacting clones were identified using the 20 dpi pig anti-*T. spiralis* serum; however, all clones encoded the same gene or portions thereof. A BLAST analysis revealed high sequence identity between the full-length clone, 20Ad3, and *T. spiralis* ATP-dependent RNA helicase DDX19B which encoded a hydrophobic open reading frame of 67 amino acids. The hydrophobic region at the N-terminus was characterized as a signal peptide, with a putative cleavage site between amino acids 21 and 22.

3.2. Clones identified with 30 dpi pig anti-T. spiralis serum

Nine positive clones were obtained from the library screened with 30 dpi pig anti-*T. spiralis* serum. The BLAST analysis revealed 4 different sequences from 7 clones that exhibited high level identify with members of a putative *T. spiralis* serine protease family (Table 1). The cDNA of clone 30AD3-3 was 1399 bp and contained an ORF of 1287 bp and a 112 bp 3′ untranslated region that included a putative polyadenylation signal (AATAA) and a poly A tail of 17 bp. Analysis of the predicated amino acid sequence of clone 30AD3-3 revealed an N-terminal signal sequence of 18 AA. SMART analysis disclosed a putative trypsin-like serine protease domain between positions 37 and 277. Within this region, conserved residues involved in catalysis, namely His88, Asp142 and Ser233 were identified along with six cysteine residues involved in disulfide bond formation and one potential N-glycosylation site at position 78. Clone 30AD3-5 encoded a protein that is identical to that encoded by hypothetical ORF 11.30 of *T. spiralis* but whose function remains elusive.

Table 1
Clones from the Ad3 cDNA library immunoscreened with pig anti-*T. spiralis* serum at 20 dpi and 30 dpi.

Serum	Clone name (identical clones)	Accession No.	Size (bp/aa)	Similar sequence/Accession No.	BLASTX E value
20 dpi	30AD3 (17)	517280	352/67	*T. spiralis* ATP-dependent RNA helicase DDX19B. XP_003376467.1	2e−37
30 dpi	30AD3-1 (3)	–	1304/346	*T. spiralis* putative serine protease L234. ABY60757.1	0.0
	30AD3-2 (1)	–	775/258	*T. spiralis* putative serine protease Zh68. ABY60762.1	0.0
	30AD3-3 (1)	517281	1399/429	*T. spiralis* putative serine protease Lst19. EU263333.1	0.0
	30AD3-4 (3)	–	1170/390	*T. spiralis* putative serine protease Lst40. EU263329.1	0.0
	30AD3-5 (1)	–	785/133	*T. spiralis* hypothetical ORF 11.30. AAB48488	3e−48

4. Discussion

The quality and the specificity of *T. spiralis* antigens are key factors in the development of reliable immunological methods and effective vaccines to control infection. cDNA expression libraries have been very effective tools for the isolation of candidate immunodominant antigens. To date, a number of encouraging antigens have been identified by the immunoscreening of cDNA expression libraries of *T. spiralis* larvae at different developmental stages using the antiserum from different post-infection stages. The identified antigens include glutamic acid-rich antigenic proteins, a serine proteinase, a serine protease inhibitor and other novel candidates. Although some antigens have been tested for early diagnosis or for vaccine development, these immunodominant antigens have thus far been insufficient for these purposes (Nagano et al., 2001; Wu et al., 2009; Zarlenga et al., 2002; Zocevic et al., 2011). The complicated life cycle of *T. spiralis* and the wide variety of stage-specific host immune responses may explain why these antigens have not yielded satisfactory results. To date, most antibodies used for immunoscreening have been obtained from animals with late-stage infections where the titers of antibodies derived from early stage antigens are likely low. Such low titers will invariably result in missed early antigens during the screening process. It has been demonstrated that antigens from the early stages of infection, i.e., adult worms or NBL, stimulate strong immunity (Denham, 1966), are effective in protecting the host (James and Denham, 1975; Wang and Bell, 1987) and are necessary for early diagnosis (Liu and Boireau, 2010). Thus, the primary challenge in developing a sensitive and specific immunodiagnostic test and effective vaccine is the isolation of early antigens of *T. spiralis*.

In this study, we attempted to identify novel antigens with potential for the early immunodiagnosis and immunoprophylaxis of *T. spiralis* by immunoscreening an Ad3 cDNA library using pig antiserum obtained during the early times following infection. Extensive bioinformatics sequence analysis of positive clones obtained using 20 dpi pig anti-*T. spiralis* serum showed that the corresponding sequences were highly similar to that of the *T. spiralis* ATP-dependent RNA helicase DDX19B gene which was also frequently identified in *T. spiralis* expressed sequence tags (ESTs) from Ad3 (Mitreva et al., 2005). ATP-dependent RNA helicase is believed to be important in parasite development and organ maturation and is involved in almost every process of RNA metabolism in cells, such as pre-mRNA splicing, translation, RNA degradation, and ribosome biogenesis (de la Cruz et al., 1999).

In addition, a number of genes encoding serine proteases (a polymorphic multi-gene family) were identified using the 30 dpi anti-serum. Serine proteases are among the most abundant proteases in the nematode excretory–secretory products, and have been linked to parasite development and nutrition, host tissue and cell invasion, larval migration, blood coagulation and fibrinolysis, and the evasion of the host immune response (Dzik, 2006). Based on the relationship of the host immune response and serine protease, they have been suggested as targets for diagnostic reagents (McKerrow, 1989). Data presented herein support such a premise. In addition to a putative diagnostic reagent, serine proteases expressed in AD3 could be involved in parasite nutrition or possibly in evading the host immune response by degrading immunoglobulin G (Ros-Moreno et al., 2000). A cDNA clone with a sequence similar to *T. spiralis* hypothetical ORF 11.30 was also selected by the 30 dpi antiserum, and was also isolated in previous study (Wu et al., 2009). Our results further confirmed that this protein could be a promising antigen for early diagnosis.

In conclusion, we screened a cDNA expression library encoding Ad3 *T. spiralis* antigens using pig serum collected at different times following infection, and in so doing identified several immunodominant antigens. These antigens are presumably exposed to the host immune system during the early stages of infection and can induce strong host humoral immune responses. As such, they may be promising antigens for the development of an early diagnostic tool. The biological functions of these antigens in *T. spiralis*–host interactions and their potential for early diagnosis and protection will be evaluated in future studies.

Conflict of interest statement

None of the authors has any financial or personal relationship with other people or organisations that could inappropriately influence or bias this manuscript.

Acknowledgements

This study was supported by the Ministry of Science and Technology of China (MOST: 2008ZX10004-11 and 2011AA10A215) and the National Natural Science Foundation of China (NSFC 30825033, 31030064, 30972177, 30950110328, 81070311, 31072124, and CPSF 2011M500366).

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