Molecular cloning and characterization of arginine kinase gene of *Toxocara canis*

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Abstract *Toxocara canis* is an important gastrointestinal nematode of dogs and also a causative agent of visceral larva migrans in humans. Arginine kinase (AK) gene is one of the important biomolecule of phosphagen kinase of *T. canis* which is emerging as an exciting novel diagnostic target in toxocarosis. The present study was carried out to clone and characterize AK gene of *T. canis* for future utilization as a diagnostic molecule. Total RNA was extracted from intact adult worms and reverse transcription was done with oligo dT primers to obtain complementary DNA (cDNA). Polymerase chain reaction (PCR) was carried out using cDNA as template with specific primers which amplified a product of 1,202 bp. The amplicon was cloned into pDrive cloning vector and clone was confirmed by colony PCR and restriction endonuclease analysis. Sequence analysis of the gene showed 99.8 and 77.9 % homology with the published AK gene of *T. canis* (EF015466.1) and *Ascaris suum* respectively. Structural

analysis shown that the mature AK protein consist of 400 amino acids with a molecular wt of 45360.73 Da. Further expression studies are required for producing the recombinant protein for its evaluation in the diagnosis of *T. canis* infection in humans as well as in adult dogs.

Keywords *Toxocara canis* · Arginine kinase · Cloning

Introduction

Toxocara canis is one of the most common parasitic helminth worm of dogs which continues to stimulate both public concern and heightened scientific interest. According to WHO (2004), the more classical zoonotic infections such as toxocarosis is presently regarded as ‘emerging disease’. It is a widespread gastrointestinal nematode parasite of dogs and also a causative agent of zoonotic disease in humans (MagnaVal et al. 2001; Pawlowski 2001; Despommier 2003).

For *T. canis*, over 4,000 clones from several complementary DNA (cDNAs) have been sequenced and grouped into 1,466 clusters according to sequence identity. The majority of these sequences are derived from adult worm cDNA chosen for the purposes of phylogenetics and drug target discovery (Parkinson et al. 2004). As the examination of stool has no role in the evaluation of toxocarosis in humans as well as in adult dogs, finding toxocara larvae within a patient is the only definitive diagnosis, however biopsies to look for second stage larvae in humans are generally not very effective and therefore, cannot be relied (Bachmeyer et al. 2003).

One of the important phosphagen kinases of *T. canis* is arginine kinase (AK), catalyzing the reversible transfer of

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phosphate from MgATP to arginine yielding phosphoarginine and MgADP, maintaining the normal cellular processes such as contractility, motility and membrane transport (Meyer et al. 1984). Arginine kinase is emerging as an exciting novel diagnostic target in *Toxocara* infection and has recently been isolated and characterized Wickramasinghe et al. (2007). As this enzyme is not present in mammals, it might be a useful diagnostic marker for VLM.

Not so much work has been done in India, except some sporadic reports on incidence studies. So, molecular approaches, such as exploration of AK gene may elucidate many unknown areas of the specified disease and for the purposes of phylogenetics and drug target discovery. The present study is warranted on molecular cloning of the diagnostic molecule, AK gene of *T. canis* for generating a recombinant protein for its possible exploitation in immunodiagnostic studies against toxocarosis.

Materials and methods

Parasite

The adult *T. canis* worms were collected from naturally infected pups after deworming with a dose of piperazine citrate @ 220 mg/kg of body weight. The expelled worms were collected in petridishes. The adult worms were identified, washed three times with 0.9 % NaCl and frozen in 70 % ethanol at -40°C until used for RNA isolation.

Isolation of total RNA of *Toxocara canis*

Total RNA from adult *T. canis* was extracted using Trizol reagent following the manufacturer's recommendations. 1 ml of Trizol reagent was added to 100 mg of the worm. This was subsequently lysed by repetitive pipetting. The mixture was incubated at 30°C for 5 min for complete dissociation of nucleoprotein complexes. This was vigorously shaken for 15 s after adding 0.2 ml of chloroform and then centrifuged at $12,000\times g$ for 15 min at 4°C which permitted separation of the phases into lower organic phase and upper aqueous phase. The aqueous phase was transferred to a fresh tube from which the RNA was precipitated by keeping the tube at room temperature for 10 min following addition of 0.5 ml of isopropyl alcohol. Then the content was centrifuged at $12,000\times g$ for 10 min at 4°C . The supernatant was discarded and the RNA pellet was washed with 1 ml of 75 % ethanol prepared using 0.01 % diethylpyrocarbonate (DEPC) treated water. The sample was mixed by vortexing and centrifuged at $7,500\times g$ for 5 min at 4°C . Finally RNA pellet air dried and dissolved in RNase free water and stored at -40°C until further use.

Synthesis of complimentary DNA by reverse transcription

cDNA was synthesized from total RNA isolated from the adult *T. canis* using oligodT primer following the Sambrook et al. (1989). The cDNA thus synthesized was preserved at -20°C till further use.

Polymerase chain reaction (PCR) based amplification of AK

The entire open reading frame (ORF) of AK gene of *T. canis* was amplified using the forward primer-5'-ATGGCATTTCTCAAGAACCAG-3' and reverse primer-5'-CTATTTTGTGTGTTCTCCAG-the PCR reaction was carried out in a standard 25 ml reaction with initial denaturation of DNA strands at 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 30 s, primer annealing at 52°C for 45 s and strand elongation at 72°C for 45 s. Thereafter one cycle of final extension of the strand was given at 72°C for 10 min. The PCR amplicons were analyzed by 1.25 % agarose gel in a submarine horizontal electrophoresis unit (Genei, Bangalore).

Molecular cloning and characterization of AK

The amplified AK gene of *T. canis* was purified using gel extraction kit following manufacturer's protocol. There after competent *Escherichia coli* DH5 α cells were prepared following the standard calcium chloride treatment method (Sambrook et al. 1989). Ligation reaction for cloning of AK gene into pDrive cloning vector as well as transformation of DH5 α cells was carried out following standard protocol. The positive clones were identified by blue white colony screening. Further confirmation was done by restriction analysis of the plasmid DNA isolated from the white colonies with *Hind*III, *Bam*HI and *Eco*RI restriction enzymes following the standard protocol (Sambrook et al. 1989). The restriction digestion reaction was carried out at 37°C for 4 h. The digested product was visualized in the agarose gel electrophoresis. A subculture of positive clone harboring the desired AK gene was custom DNA sequenced from the Department of Biochemistry, Delhi University, South campus. The sequence information received was analyzed using DNA Star and Gene Tool software. Structure of the protein was predicted using EXPASY and SWISS PROT software.

Results

RT-PCR amplification of AK gene of *Toxocara canis*

The coding sequence of AK gene of *T. canis* was retrieved from GenBank (Accession No. EF015466.1). The entire

ORF of AK gene was amplified and specificity and size of the amplified product was checked by 1 % agarose gel electrophoresis and a single band of 1,202 bp was resolved (Fig. 1). Purification of this amplified product was achieved by using commercial gel extraction kit (Qiagen, Germany). The eluted product was re-checked by electrophoresis on 1.25 % agarose gel.

Construction of recombinant plasmid and cloning of AK gene of *Toxocara canis*

The purified 1,202 bp AK product was used for ligation in a T/A cloning vector to facilitate sequencing and characterization. For this, pDrive T/A cloning vector, having an multiple cloning sites (MCS) incorporated into a LacZ α peptide coding region was chosen for easy selection of recombinant clones.

Confirmation of recombinant plasmid

Colony PCR

The recombinant colonies were grown in LB broth and PCR was carried out using the specific primers for amplification of AK gene. The recombinant clones showed amplification of 1,202 bp size.

Restriction enzyme analysis

The cloned AK gene was confirmed by restriction enzyme analysis with *Hind*III, *Bam*HI and *Eco*RI restriction enzymes. After digestion with *Hind*III, linearized AK-recombinant pDrive vector was obtained and with *Bam*HI, digested recombinant pDrive plasmid vector showing release of 700 bp AK insert was obtained. By digestion with *Eco*RI restriction enzyme, 1,202 bp AK gene insert

was released, based on the analyzed result of published sequence of AK gene of *T. canis* using DNA STAR and GENE TOOL software.

Sequence analysis of AK gene of *Toxocara canis*

The clones having the correct orientation of AK gene of *T. canis* were selected and custom sequenced for nucleotides using M13 forward and reverse primers by Sanger's dideoxy method and the gene size was confirmed to be 1,202 bp length. Protein composition of *T. canis* AK protein was predicted by DNA STAR, EXPASY TOOL and SWISS PROT software. Nucleotide sequence alignment of the AK gene of *T. canis* Izatnagar isolate (Accession No. JQ768328.1) with published *T. canis* isolate (Accession No. EF015466.1) showed 99.8 % alignment, with changes at 3 nucleotides at 363, 699 and 1032th bp. Deduced amino acid sequence alignment of AK gene of *T. canis* Izatnagar isolate (Accession No. JQ768328.1) with published *T. canis* isolate (Accession No. EF015466.1), showed one

Table 1 AK protein composition

Molecular weight	45360.73 Da
Amino acids	400
Strongly basic (+) amino acids	54 (K,R)
Strongly acidic (−) amino acids	51 (D,E)
Hydrophobic amino acids	137 (A,I,L,F,W,V)
Polar amino acids	
Isoelectric point	
Charge at pH 7.0	98 (N,C,Q,S,T,Y)
%A+T	8.304
%C+G	47.13
Base count	341 a 257 c 310 g 295 t

Fig. 1 Amplification of AK gene of *T. canis*. Lane M: 100 bp plus DNA ladder. Lanes 1 and 2: AK gene

		Percent Identity								
Divergence		1	2	3	4	5	6	7		
	1	■	99.8	77.9	54.7	53.1	48.2	46.7	1	<i>T. canis</i> (Izatnagar)
	2	0.2	■	77.8	54.9	53.4	48.1	46.6	2	<i>T. canis</i> EF015466.1
	3	23.9	24.0	■	54.8	52.4	48.1	46.9	3	<i>A. suum</i> AK gene
	4	54.6	54.2	54.1	■	71.9	56.7	52.0	4	<i>T. cruzi</i> AK gene
	5	52.4	51.9	54.2	30.9	■	60.2	54.8	5	<i>T. brucei</i> , AK1 gene
	6	59.4	59.4	60.6	50.5	45.1	■	77.1	6	<i>C. felis</i> AK1
	7	60.3	60.3	60.6	56.3	53.0	22.5	■	7	<i>C. felis</i> AK2
		1	2	3	4	5	6	7		

Fig. 2 Sequence pair wise distances of AK (nucleotide sequence)

		Percent Identity							Divergence	
		1	2	3	4	5	6	7		
1		■	99.5	88.8	58.9	57.1	52.2	50.1	1	<i>T. canis</i> (Izatnagar)
2	0.3	■	89.0	58.9	57.1	52.2	50.1	2		<i>T. canis</i> EF015466.1
3	11.9	11.6	■	60.6	57.4	53.9	52.5	3		<i>A. suum</i> AK
4	51.5	51.5	48.3	■	81.5	68.0	64.2	4		<i>T. cruzi</i> AK
5	54.0	54.0	53.4	21.0	■	67.1	63.3	5		<i>T. brucei</i> AK1
6	63.1	63.1	59.4	38.6	40.0	■	88.2	6		<i>C. felis</i> AK1
7	68.4	68.4	62.9	45.3	46.3	12.2	■	7		<i>C. felis</i> AK2
		1	2	3	4	5	6	7		

Fig. 3 Sequence pair wise distances of AK (deduced amino acid sequence)

amino acid change at 344th position from cysteine to serine. Protein composition of *T. canis* AK was predicted by DNA STAR, EXPASY TOOL and SWISS PROT software (Table 1).

Homology search of the *T. canis*, AK gene Izatnagar isolate (Accession No. JQ768328.1), at nucleotide level showed a homology of 99.8 %, with the published AK gene (Accession No. EF015466.1), with other parasites it showed a homology ranging from 46.7 to 77.9 % (Fig. 2).

Amino acid analysis of the AK gene of the present isolate revealed 95.5 % homology with *T. canis* AK gene (Accession No. EF015466.1). With other parasites the homology ranged from 50 to 88 % (Fig. 3).

The results of phylogenetic tree constructed from the nucleotide sequences of AKs of different parasites clearly indicated that the AK group is divided into three clusters, the arthropoda, protozoan and nematode AKs. *T. canis* AK (Izatnagar isolate) is a member of the nematode AK cluster forming a branch with *Ascaris suum*. Phylogenetic analysis placed *T. canis* AK (Izatnagar) as a distinct branch within a nematode AK cluster (Fig. 4).

Discussion

The present investigation was aimed to characterize the adult *T. canis* AK gene and its phylogenetic relationship with other known parasites, since no study has been carried out in India on this parasite protein. The results of our study correlated with that of Wickramasinghe et al. (2007), which shows that phylogenetic analysis of our isolate deduced from the nucleotide sequences of AKs indicated that the AK group is divided into three clusters of the arthropoda, protozoan and nematode AKs. *Toxocara canis* AK is a member of the nematode AK cluster forming a branch with *A. suum*. Thus, phylogenetic analysis placed *T. canis* AK (Izatnagar isolate) as a distinct branch within a nematode AK cluster. Likewise, Wickramasinghe et al. (2007) in their study on the phylogenetic analysis of amino acid sequences of AKs with representatives of other phosphagen kinases demonstrated the presence of two major groups: a CK group (CKs, GKs, LKs, and TKs) and an AK group. Their results indicated that the AK group is divided into two sub-groups. The first sub-group comprises the arthropoda, protozoan, and nematode AKs. The second sub-group comprises the molluscan AKs and trematode phosphagen kinases.

Wickramasinghe et al. (2007), determined the cDNA sequence of larval (L2) *T. canis* AK. They also analysed the N-terminus sequence of *T. canis* AK that revealed the presence of a signal targeting peptide, presumably targeting this protein to cytosol or endoplasmic reticulum. An alignment of the deduced amino acid sequence of *T. canis* AK showed 89 % sequence identity with *A. suum* AK, 61 % with *C. elegans*, 62 % with *Trypanosoma cruzi* AK and 61 % with *Limulus polyphemus* AK, which are similar to our results which shows a homology of 88.8 % with deduced amino acid sequence of *A. suum* AK, and 58.9 % with *T. cruzi* AK.

Diagnostic potential of the AK protein of *T. canis* was evaluated by Wickramasinghe et al. (2008). A recombinant AK (45 kDa) was expressed from second-stage larva and

Fig. 4 Phylogenetic tree analysis of AK (nucleotide sequence)

developed into a highly sensitive IgG-ELISA for serodiagnosis of toxocarosis in a mouse model. Since this enzyme is not present in mammals, it might be a useful diagnostic marker for toxocarosis. However, it is necessary to evaluate the specificity of this protein with similar geohelminth infections such as *A. suum*. Research work on these lines will help to provide more reliable diagnostic tools for the detection of cryptic visceral larva migrans in human beings and also to explore accurate diagnostic marker for toxocarosis in future. We undertook a preliminary study on the cloning and characterization of AK gene in *T. canis* and further studies are required for producing the recombinant protein for its evaluation in the diagnosis of *T. canis* infection in human as well as in dogs.

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