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Designing and modelling of an antigenic protein TES-26 for diagnosis of *Toxocara canis* infection

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Abstract
The impact of zoonotic toxocariosis has been phenomenally increased affecting more than 1 billion people worldwide. Serological investigations are currently practised to achieve satisfactory results in disease diagnosis. However, the usage of native antigen in the diagnostic kits has been proven to be insufficient and difficult to standardize. This leads to a greater scope to identify other potential antigenic excretory-secretory proteins of *Toxocara canis* larvae (TES). In this study, Tc-TES-26 gene of Indian strain has been characterized for the first time. Phylogenetic tree analysis reveals closer similarity to South America and UK strain. The domain information of TES-26 belonged to phosphatidylethanolamine-binding protein which regulates major immune pathways. The analysis on antigenic B cell epitopes reveals 19 promiscuous peptides which could be used as potential biomarkers. Further, the secondary structure and homology model prediction of this protein reveals structural information to understand the binding activity and interaction with host cells.

Keywords: Toxocariasis, TES-26, diagnosis of *T. canis*, antigen, *in silico* modeling

1. Introduction
Toxocariosis is still considered as a neglected helminth disease affecting millions of people world-wide including stray dogs and pets [1, 2]. The most common pathogens causing toxocariosis are *Toxocara canis (T. canis)* and *Toxocara cati* (T. *cati*) affecting dogs and cats respectively. They belong to ascarid nematodes in order Ascaridida, superfamily Ascaridiodea and family Toxocaridae. Humans get infected unintentionally from the embronated eggs or larvae that have been shed in the faeces of infected animals or uncooked paratenic hosts [3, 4]. Subsequently the infective larvae penetrate the human intestinal wall and via circulation migrate to different organs inducing inflammation and other symptoms. Clinically, two well defined manifestations are systemic toxocariasis vis-a-vis visceral larva migrans and ocular toxocariasis which cause visual impairment in children and adults [4, 5].
Initially the diagnostic method of toxocariasis was based on histological examination of *T. canis* larvae or tissue fragments through biopsy of infected samples. Other diagnostic methods also include immunoblot assays [6], ELISAs [7], and indirect fluorescent antibody test [8] etc. Recently, the disease diagnosis is performed through enzyme-linked immunosorbent assay (ELISA) IgG which detect antibodies against *Toxocara* excretory–secretory (TES) antigenic products [9]. The serological tests using these immunological techniques are considered to be the most effective diagnosis approach for laboratory detection of toxocariosis [10]. However, this assay shows disadvantages due to cross-reactivity with ascariasis, strongyloidiasis and nematodes [11, 12]. A better and enhanced diagnostic method has been adapted through the production of highly specific recombinant proteins which shares characteristic antigenic and immunogenic structures with native TES. This recombinant protein technology paved way for unlimited yield, reduced cross reactivity and higher sensitivity and specificity [12].
The antigenic repertoire of TES comprises of multiple glycoproteins secreted by the *Toxocara* larvae that includes about 50 different types of lectins, mucins and other components [13]. The molecular weights of TES glycoprotein fractions range from 15 to 400kDa [14]. Especially the specificity of immunodiagnosis is reported to be profoundly increased by using smaller molecular weight TES fractions like 24 to 35kDa [15].

Amongst the TES family, the notable lectins like C-type lectin (CTL-1) or TES-30 and TES-26 are shown to have remarkable diagnostic value [16]. A scientific study reported that the TES-26 antigen on comparison with cathepsin L-1 showed higher sensitivity in tested *T. canis* infected groups [17].

The multi-epitope peptides of antigenic proteins are sought to be used as biomarkers to improve the accuracy of diagnostic kits and vaccine development [18]. Insilco tools and web servers have enabled to study these immunodominant epitopic regions of a particular antigen to reconstruct multi-epitope domain. In this context, this study focuses on identifying a novel specific antigenic profile of TES-26 through immunoinfomatics and *insilco* tools which could be used for routine serological diagnosis of larval toxocariosis.

2. Materials and Methods

Live adult *T. canis* female worms (n=230) were collected after deworming the infected non-descript puppies and washed three times in normal saline solution. Later, each female worm was kept in a Petri dish containing normal saline and dissected using sterile scalpel at the anterior region of the uterus to release the eggs. The collected eggs were transferred to 2 percent formal saline in glass petri dish and incubated for 28 days at room temperature (28º to 30 ºC) with periodical aeration to induce embryonation. The egg suspension after the incubation period was washed four times with sterile phosphate buffered saline (1X PBS). The hatching of larvae from the embryonated eggs was done according to the procedure of Alcantara-Neves *et al,* (2008) with minor modifications [19]. The larvae sediment was transferred to RPMI 1640 medium supplemented with L-glutamine and sodium bicarbonate (Sigma-Aldrich Co, USA) and incubated overnight at 37 ºC. The emerged larvae were filtered through 20µm pore size polystyrene membrane and the number of larvae in each micro centrifuge tube was recorded. About 10 times the volume of RNA stabilization agent RNAlater®, (Qiagen, Germany) was added per tube and stored at 4 ºC for 2 hours.

Total RNA was extracted from *T. canis* larvae using RNeasy® Mini kit (Qiagen, Germany) according to the manufacturer's protocol. Briefly, the larvae stored in 4 ºC were centrifuged for 10 min at 16,000 × g. The pelleted larvae was washed with 1x PBS to remove the remaining RNA later solution. Later, the lysis buffer was added and the suspension was passed multiple times through 20-guage (0.9 mm) needle attached to 1mL plastic sterile syringe to obtain homogeneous lysate. To the lysate, ethanol was added and the mixture was applied to RNeasy mini spin column. The total RNA bound to the column membrane was eluted with RNase-free water. The concentration and purity of the eluted RNA was measured by NanoPhotometer™ (Eppendorf, Germany) and stored at -80 ºC.

iScript™ cDNA synthesis kit (Bio-rad, USA) was used for cDNA synthesis from the total RNA according to manufactures instructions. Primers for gene encoding TES-26 were designed based on the article Mohamad *et al*., (2009) [20]. The primers with the following specific sequence were used for amplification. Tc-TES-26-F: (forward) 5'<CGCGCTCGAGCACCATGTCAGTTGTACACA>3' and Tc-TES-26-R: (reverse) 5'<GCCGCCATGGTTAGGCCTGCGATCGATAGA>3' sequences were custom synthesized from Eurofins genomics India Pvt. Ltd. The polymerase chain reaction (PCR) was carried out in 20µl reaction volumes with 10µl of Amplicon master mix, 1µl of gene specific forward and reverse primer (20 pmol/ µL each), 2µl of cDNA template and 6µl of nuclease free water. The amplification was carried out with the following conditions, initial denaturation at 95 ºC for 2 min; 40 cycles of denaturing at 95 ºC for 30sec, template-primer annealing at 60 ºC for 30sec, extension at 68 ºC for 2 min; and final extension at 68 ºC for 5 min. The confirmation of the amplified product was analyzed by 1.5 percent agarose gel stained with Ethidium bromide in a horizontal electrophoresis unit (Bio-rad, USA). The amplified PCR product with gene specific primers was given to Eurofins genomics India Pvt. Ltd for Sanger sequencing.

The acquired nucleotide sequence of TES-26 was analyzed through Blastn tool for sequence similarity. This sequence was submitted to NCBI Genbank database (Accession ID: OR610858) through BankIT software. The protein sequence and coding domain sequence (CDS) were identified through ExPASy translate tool. The transmembrane structure of TES-26 was predicted by TMHMM Server v.2.0 (https://www.cbs.dtu.dk/services/TMHMM/). The FASTA sequence of protein sequence was given and three regions like outside, transmembrane and inside regions were studied [21]. To study the signal peptide regions of this antigenic protein, SignalP 4.1 Server (https://www.cbs.dtu.dk/services/SignalP/) was used. This method employs artificial neural network to predict signal peptides and cleavage sites [22].

The B-cell epitopic region of TES-26 was analyzed through tools like IEDB, Bcepred, SVMTrip, Kirloskar, ABCpred and Emini surface accessibility web servers. The tools evaluate epitope specific region through Chou-fausman beta turn, Karplus and Schulz flexibility prediction, Kolaskar and Tongaonkar antigenicity methods [23, 24]. The peptides which included most high score B-cell epitopes having higher antigenic property and corresponding to most *in silico* tool prediction were chosen for further analysis. The protein sequence statistics for constructs were studied using the ExPASy ProtParam server (https://expasy.org/cgi-bin/protpraram) which was used to analyze many aspects like total number of positive and negative residues, hydropathicity (GRAVY), instability index, aliphatic index, length, amino acid distribution, molecular weight and isoelectric point (IEP) [25]. The secondary structure of construct was analyzed through Self-Optimized Prediction method With Alignment (SOPMA) server (https://npsa-prabi ibcp.fr/cgi-bin/secpred_sopma.pl). This tool employs Self-Optimized Prediction method (SOPM) which predicts 69.5% of amino acids in the submitted protein sequence in a three state description of secondary structure like alpha-helix, beta-sheet and coil [26].

The domain sequences of related TES-26 gene sequences (678 bp) were compared through Blastn search tool and retrieved from Genbank database (https://www.ncbi.nlm.nih.gov/). The sequences were aligned by Clustalw method and then the multiple sequence alignment file was analyzed using CLC sequence viewer software version 6.6.2. The conserved and variable regions were noted. The construction of phylogenetic tree with neighbour joining method was performed using MEGA 6 software package and bootstrap was set to 1000 [27]. Further, the three dimensional model of TES-26 gene was constructed by the automated modelling program within the online service SWISS-MODEL. The 3D model was downloaded in.pdb format and overall model quality was accessed through ProSA web server.

3. Results and Discussion

The concentration of total RNA recovered from *T. canis* second stage larvae was estimated to be 15.6 to 20.4 ng/µl with an absorbance purity value of 1.9 (OD260/280). The cDNA was synthesized from the total RNA and its integrity was checked by 1% agarose gel electrophoresis. The amplified product of *T. canis* TES-26 was resolved at 793 bp through agarose gel (Fig. 1), which was the expected band size as reported by Mohamad *et al*., 2009 [20]. The nucleotide sequence of Tc-TES26 gene of Tamil Nadu (T.N) strain from the present study showed 98.39% of sequence similarity with previously published complete cds (KU951900) and 98.12% with U.K isolate (TCU29761) with 94% sequence coverage. The aligned sequence of T.N strain TES-26 from the forward and reverse primer was submitted to NCBI Genbank database and published online with accession ID: OR610858.

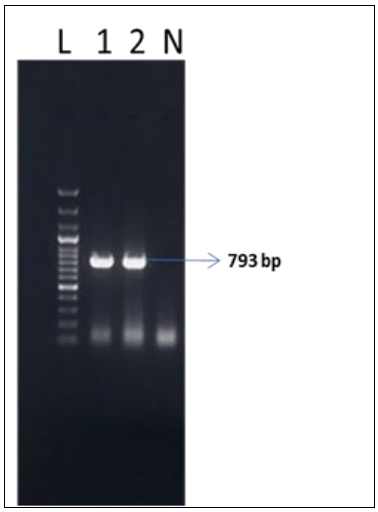


Fig 1: Amplification of TES-26 gene (793 bp) of *T. canis*

The maximum likelihood tree analyzed through phylogenetic analysis revealed that the T.N strain has high similarity with *T. canis* complete cds strains (U29761 and KU951900) and distant similarity with *T. cati* TES26 sequence (MH183032.1). In this tree, *T. cati* sequence played as an out group enabling the sequence cluster between the three *T. canis* sequences as displayed in the Fig.2.

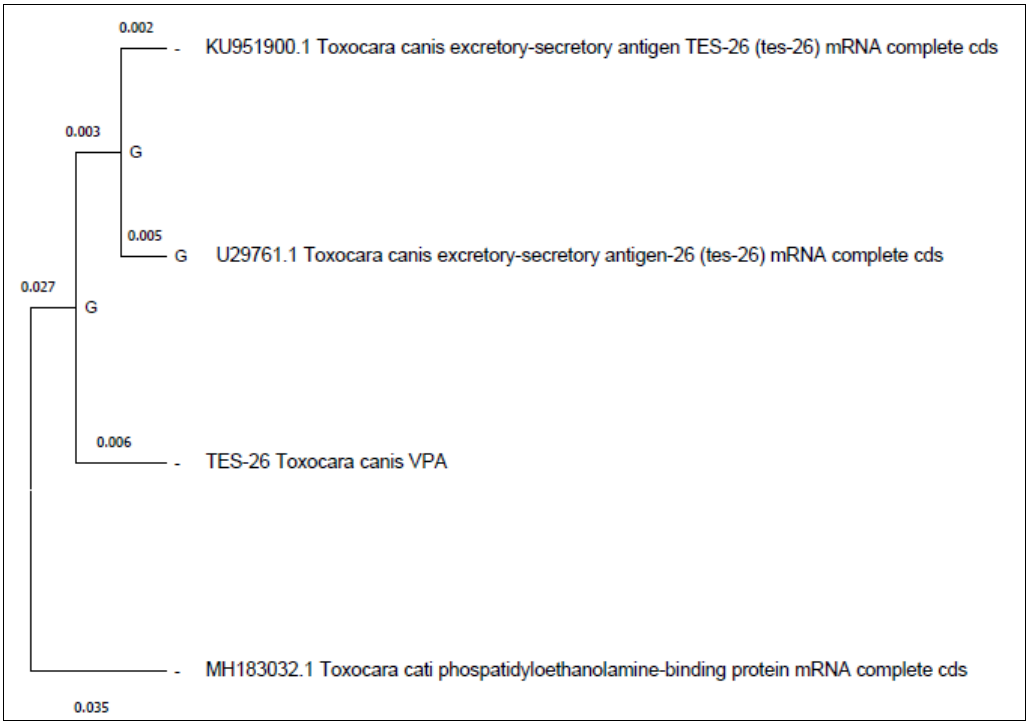
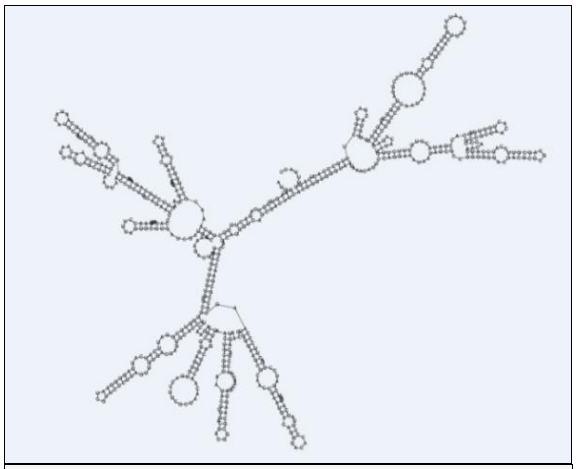


Fig 2: Maximum likelihood distance tree of T.N strain TES-26 gene by MEGA software

Based on InterPro results (Fig. 3), the coding sequence of TES-26 (226 amino acids) belonged to the domain phosphatidylethanolamine-binding protein (PEBP) (Uniprot: PTHR11362). The PEBP majorly involves in lipid binding and also regulates several metabolic pathways including MAP kinase pathway, NF-kappaB pathway etc., [28]. These two are common inflammatory signalling pathways which causes the release of many cytokines like interleukin-6(IL-6), interleukin-8(IL-8), tumor necrosis factor-α (TNF- α) in host cells [29]. TES-26 also retains a hydrophobic motif enabling lipid binding and consists of two ShK/SXC domains. This protein acts as a major constituent in *T. canis* excretory secretory products and represent as an important candidate to interact with host cells.

Fig 3: Inter Pro results of *T. canis* TES-26 CDS sequence

Recently, self optimized prediction method (SOPM) has been described to improve the success rate in the secondary structure prediction of proteins [26]. *T. canis* TES-26 secondary structure by SOPMA server revealed 14.6% as alpha helix, 23.01% as beta strand, 6.19% as beta turns and 56.19% as random coils (Fig.4). These implies that the random coils dominated than the remaining secondary structure elements followed by beta strand, alpha helix and beta turns.



SOPMA :

Alpha helix	(Hh) :	33 is	14.60%
3_{10} helix	(Gg) :	0 is	0.00%
Pi helix	(Ii) :	0 is	0.00%
Beta bridge	(Bb) :	0 is	0.00%
Extended strand	(Ee) :	52 is	23.01%
Beta turn	(Tt) :	14 is	6.19%
Bend region	(Ss) :	0 is	0.00%
Random coil	(Cc) :	127 is	56.19%
Ambiguous states (?)	:	0 is	0.00%
Other states	:	0 is	0.00%

Fig 4: Secondary structure prediction of *T. canis* TES-26 gene

The three dimensional structure (3D) of TES-26 was built based on the template 26 kDa secreted antigen of canine roundworm (Uniprot: P54190) through computational homology model. The T.N strain from this study had 93.81% sequence identity and 0.95 GMQE score with the template. The 3D structural model representation of *T. canis* TES-26 is shown in Fig. 5. The global and per residue model quality has been accessed using QMEAN scoring function [30] and this model was viewed using Swiss-PdbViewer tool (Fig. 5). The predicted model was evaluated to have per-residue model confidence score (pLDDT) as 91.24 which proves that the developed model is relevantly accurate.

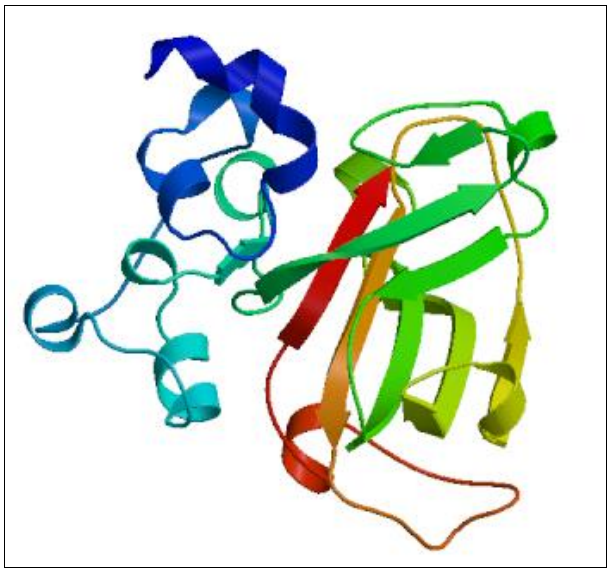


Fig 5: 3D model structure prediction of *T. canis* TES-26 gene by Swiss Model server

The evaluation of model quality was done through the Ramachandran plot analysis (Fig. 6). The Ramachandran plot for the TES-26 modelled protein (Fig. 6) shows the distribution of amino acid backbone confirmations present in the protein structure. Each amino acid residue is shown as a dot in a graph of φ vs. Ψ.

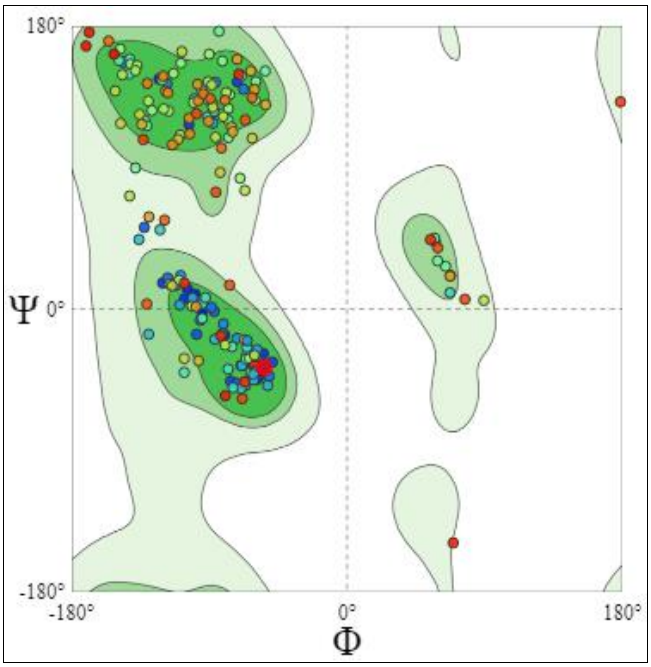


Fig 6: Ramachandran plot evaluation of 3D structure model of *T. canis* TES-26 gene

In this plot (Fig. 6), all the amino acids except for a single arginine residue in sequence position 218 were found to be in the favourable region (95.09%). Merely, 0.45% was predicted to be in the outlier quadrants and 0.53% as rotamers (Fig.7). Based on the spatial arrangement and steric effect of amino residues, this model shows to have energetically favourable protein folding. There is a lack of experimental structural data on *T. canis* TES-26 protein and this 3D structure information would enable us to understand the specific binding of ligands to this antigenic protein.

MolProbity Score	1.68
Clash Score (A75 PHE-A223 THR)	5.03
Ramachandran Favoured	93.75%
Ramachandran Outliers A222 PRO	0.45%
Rotamer Outliers A217 VAL	0.53%
C-Beta Deviations A188 PHE	1
Bad Bonds	0 / 1757
Bad Angles	17 / 2411

Fig 7: Molprobity result of *T. canis* TES-26 gene modelled structure

Further, the list of common B-cell epitopes in TES-26 predicted by most of the *in silico* tools are given in Table 1. About 19 common B-cell epitopes in TES-26 protein was found to be commonly predicted by six tools, especially two specific epitopic regions like QPSTPAA and LYNLVVQD was predicted by four tools stating its relevance and significance as potential antigenic markers. The physico-chemical characteristics for each of these 19 peptides were calculated using Expasy's Prot Param server.

Table 1: List of common B cell epitopes predicted by *in silico* tools

S. No	Epitope	No of tools predicted common B cell epitopes	No of amino acids	MW	PI	Instability index	Aliphatic index	GRAVY
1	APSRRV	3	6	684.8	12	200.1	65	0.9
2	VANQPTV	3	7	727.82	5.49	25.31	97.14	0.129
3	EAQPNDRY	2	8	992.01	4.37	13.98	12.5	-2.45
4	QPSTPAA	4	7	670.72	5.52	91.11	28.57	-0.657
5	QKTCGLCAGCG	3	11	1040.24	7.96	9.09	44.55	0.345
6	RGIVPLVVTS	2	10	1040.27	9.75	-5.7	165	1.29
7	HRYVFLVYRQP	2	11	1477.73	9.99	15.03	88.18	-0.445
8	LYNLVVQD	4	8	963.1	3.8	32.83	170	0.525
9	CFTRPISQV	3	9	1050.24	8.25	43.31	75.56	0.322
10	PGNNIAGGTTLAA	2	13	1156.26	5.96	39.46	83.08	0.192
11	AAINSPLLYNLVVQDS	3	16	1716.95	3.8	60.91	146.25	0.988
12	GCGFISRGIVPLVVT	3	15	1517.85	8.25	12.37	136	1.46
13	CRDEANNCAASINL	3	14	1493.63	4.37	7.23	77.14	-0.293
14	QPNDRYTLIMV	2	11	1349.57	5.84	5.17	97.27	-0.382
15	TFEPLVRDRCQKT	2	13	1592.83	7.89	21.94	52.31	-1.008
16	NVQVNCGNTLTT	2	12	1263.39	5.52	-13.67	80.83	-0.15
17	STPAANT	3	7	660.68	5.24	23.96	28.57	-0.529
18	WWVINIPGNN	3	10	1212.37	5.52	33.08	107	-0.11
19	QVANQPT	2	7	756.81	5.52	14.54	55.71	-0.971

The molecular weight of these peptides ranges between 660.68 to 1716.95 Da. In this analysis, theoretical isoelectric point (pI) values ranges between 3.8 to 12. An isoelectric point above 7 indicates positively charged peptide molecule [31]. The detection of isoelectric point would be useful for developing a buffer system to purify peptides by isoelectric focussing method [32].

The instability index is yet another important parameter to analyze the stability of an epitope. A peptide with instability index lesser than 40 is predicted to be stable [33]. On comparison with this threshold, about 15 epitopes were found to be stable and possess antigenic properties (Table. 1). The aliphatic index of predicted nineteen B-cell epitopes showed significant variation between 12 to 170. This aliphatic index implies as a relative factor to increase the thermostability of globular proteins. An additional parameter like the Grand Average of Hydropathy (GRAVY) was also evaluated between the ranges of -2.45 to 1.46; the lower the value of

GRAVY shows the possibility of better interaction of peptide with water [32]. The results from this study highlights that these nineteen promiscuous B-cell epitopes could be further considered as potential vaccine candidates in human toxocariasis.

4. Conclusion

In conclusion, among the many excretory secretory antigens of *T. canis*, the immunogenic potential of TES-26 has been particularly elucidated. The identification of antigenic B-cell epitopes of this protein could be used as primary vaccine candidates towards human toxocariasis infection. Further the domain analysis reveals that this protein belongs to phosphatidylethanolamines which has a characteristic phenomenon in ligand binding and activation of host metabolic pathways. The detection of secondary and tertiary structural information through bioinformatics analysis helps to understand the exact binding site, interacting molecules between TES-26 and host cell.

5. Acknowledgements

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