Cloning and sequencing of beta-tubulin and internal transcribed spacer-2 (ITS-2) of *Eimeria tenella* isolate from India

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Abstract Beta-tubulin is an important multifunctional protein of eukaryotes abundant in the cytoskeleton and responsible for the formation of tubulin, structures responsible for cell morphology and which aid in motility and intracellular transportation. It has been used as a genotypic marker for studying the evolutionary history and phylogenetic relationships between eukaryotic organisms. Internal transcribed spacers of the ribosomal RNA genes have been widely used for typing inter-species and intra-species variation. An Indian isolate of *Eimeria tenella* was genotyped following the cloning and sequencing of beta-tubulin and internal transcribed spacer-2 (ITS-2) and compared with other reference isolates of *E. tenella*. The β -tubulin has 99 % intra-species similarity at the gene level and the functional homology of the protein is very high with more than 95 % amino-acid similarity across the Apicomplexa. The ITS-2 sequence had a similar pattern of nucleotide base arrangement with 99 % homology to Houghton and Nippon strains of *E. tenella*.

Keywords *Eimeria tenella* · Beta tubulin · ITS-2 · Genotyping · India

Introduction

Poultry coccidiosis is a disease of immense economic importance to the poultry industry, worldwide. The disease is caused by seven well known species of the genus *Eimeria*, which vary in their pathogenicity and hence the severity of disease. *Eimeria tenella*, the causative agent of caecal coccidiosis is one of the two most pathogenic species, the other being *Eimeria necatrix*. Vaccines and anti-coccidial drugs are available in the world market for control of coccidiosis and the economic burden of chemotherapy further worsened by cases of fast developing anti-coccidial resistance, has paved the way to search for new drug targets. Anti-coccidial vaccines are an important measure for immunoprophylactic control of chicken coccidiosis, but have limitations as they are live vaccines composed of oocysts of *Eimeria* species derived from local isolates/strains.

The poultry industry, along with the associated axillary/supporting industries make the largest contribution to the economy within the livestock sector in India. Studies have been carried out in India on chemotherapeutic resistance against anticoccidials (Panda et al. 1973; Gill and Bajwa 1979; Yadav and Gupta 2001; Gautam and Gupta 2004). Genotyping studies are an essential approach for development of suitable vaccines and drug trials and currently very little information is available on genetic diversity within *Eimeria* species (Beck et al. 2009). Internal transcribed spacer-1 (ITS-1) based genotyping studies for species identification and intra-species variation have been carried out in India (Bhaskaran et al. 2010; Kumar 2012).

Genetic characterization of strains of parasite species from a geographical area should include housekeeping genes, as well as genes related to the host-parasite relationship. Beta-tubulin an important nucleotide binding protein

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forms the conspicuous constituent of the eukaryotic cytoskeletal protein. It is highly conserved across several genera of algae, fungi and protists (Barabona et al. 1988; Conner et al. 1989; Gurr et al. 1988; Trivinos-Lagos et al. 1993). The β -tubulin is a polypeptide monomer of microtubules, the other component being α -tubulin. The polymerization and de-polymerization of β -tubulin monomers is a dynamic process contributing to cell locomotion (flagellar and ciliary motility), intracellular transport and cell morphology. Zhu and McDougald (1995) localized the protein in the cytoskeleton, apical complex and sub-pellicular region of the asexual stages (sporozoites and merozoites) of *E. tenella* by confocal microscopy. Beta-tubulin serves as an important drug target for many nematodes, fungi, moulds, parasitic protozoa, but has not been explored against *Eimeria*. Despite this it is an important factor for genotyping studies.

Internal transcribed spacers (ITS) are regions of unknown function located between the 18S, 5.8S and 28S ribosomal RNA (rRNA) genes located on the same stretch of DNA within the eukaryotic chromosome. Sequencing of ITS is an important tool for understanding congeneric populations, within genus homology (inter-species differences), differences between highly related genera and intra-species differences. ITS has been used in species specific detection of parasites. Kawahara et al. (2008) used real time PCR to detect five important *Eimeria* species of domestic chicken. ITS-2 based species status study of *Otodectes cynotis* from different host species and geographical areas was carried out by Lohse et al. (2002).

The present study has been carried out as a part of multiple locus sequence typing (MLST) of *Eimeria tenella* Indian isolate 1. Beta-tubulin and ITS-2 of Indian isolate 1 of *E. tenella* were partially sequenced, characterized, annotated and compared with the standard reference laboratory strains of *E. tenella*, as well as with other apicomplexan parasites at the level of translated polypeptide.

Materials and methods

DNA extraction

The parasite oocysts used in the study were derived from Indian isolate 1 of *Eimeria tenella* (origin Kaithal district,

Haryana). *E. tenella* oocysts, 1.5×10^5 , were mixed with equal volume of previously autoclaved 0.3–0.5 mm glass beads (Sigma, USA) and a small volume of lysis buffer supplied by the manufacturer (Qiagen) and smashed by vigorous vortexing in order to release the sporocysts and sporozoites. Vortexing was done till 80 % of the oocysts were ruptured and sporozoites were released. QIAmp stool DNA minikit (Qiagen, Germany) was used for extraction of DNA as per the manufacturer's protocol, excepting that the purified DNA was eluted in 100 μ l volume of elution buffer.

PCR and cloning

Eimeria tenella beta-tubulin gene and ITS-2 (both partial fragments) were amplified from genomic DNA using specific primers (Table 1). Amplifications were carried out under thermal cycling conditions of initial denaturation at 95 °C for 3 min, 35 cycles of denaturation at 95 °C for 30 s, annealing at 56 °C for 30 s, extension at 72 °C for 2/1 min(s) and final extension at 72 °C for 15 min in a S1000 thermal cycler (Biorad, USA). The reaction mixture of 25 μ l volume consisted of 2 μ l DNA, 2.5 μ l of 10 $\times$ High Fidelity enzyme mix buffer with 1.5 mM-MgCl₂, 0.5 μ l of 25 mM MgCl₂ (to obtain a final concentration of 2 mM MgCl₂), 0.5 U of High Fidelity enzyme mix (Thermo Scientific, USA), 0.5 μ l 10 mM dNTP mix, 1 μ l each of forward and reverse primers (400 nM) and nuclease free water to make up the volume. Electrophoresis of PCR products was carried out at constant voltage of 5 V/cm in a Biorad electrophoresis assembly, in 1 % agarose gel in 1 $\times$ Tris acetate EDTA (TAE) buffer. Products were purified from other reactants in the PCR mixture using Minelute PCR purification kit (Qiagen, Germany).

Purified PCR products were quantified and ligated into TA cloning vector, pTZ57R/T (provided with the InsTA cloning kit, Thermo Scientific, USA). The ligation mixture of 20 μ l volume containing PCR product 8 μ l (β -tubulin)/ 5 μ l (ITS-2), vector 1 μ l, T4 DNA ligase 1 μ l (5 Weiss U/ μ l), 5 $\times$ ligase buffer 4 μ l and nuclease free water 6 μ l, was incubated overnight at 4 °C.

Each ligation mixture was used to transform *Escherichia coli* DH5 α cells using the chemicals supplied with the InsTA cloning kit and the transformed cells were poured on to ampicillin and X-gal added Luria–Bertani agar (LB

Table 1 Details of primers used for amplification and cloning of *E. tenella* beta-tubulin

Primers		Sequence	Approximate product size	Annealing temperature used
CIDte21f	Beta-tubulin	CCATGAAATGAAAGCGGGTCAC	2,007 bp	$T_a = 56^\circ\text{C}$
CIDte21r		GGGGTATTCTTCGCGGACTTTG		
CIDte26f	ITS-2	TTTAGTCCATCGCAACCCTTGA	923 bp	
CIDte26r		TCCGAATGAAAGGAGCATACGA		

agar) plate and incubated overnight at 37 °C. From the population of blue-white selection of the colonies on each plate, four white colonies were picked using a bacteriological loop and streaked on a fresh LB agar plate containing 1.2 mg/ml ampicillin and incubated at 37 °C. Colony PCR was performed to confirm the presence of the desired insert. In short the 4–6 white colonies from the LB agar plate were streaked onto a fresh ampicillin added LB agar plate and incubated overnight at 37 °C. PCR reaction mixture of 25 µl was prepared as per the composition mentioned earlier except the template DNA. A single colony from the streak was touched with a sterile tip (10 µl volume) and transferred to the respective PCR reaction mix. The PCR reaction was carried out under the same conditions as above with 30 cycles of amplification.

Plasmid extraction and restriction digestion

Two positive colonies were inoculated into 5 ml of LB broth containing ampicillin and cultured overnight at 37 °C in a shaking water bath. Plasmid extraction was performed using plasmid extraction kit (Invitrogen, USA). Plasmid quantity was estimated by UV spectrophotometry. Restriction double digestion of 500–600 ng recombinant plasmids (beta-tubulin and ITS-2) with restriction enzymes, *EcoRI* and *HindIII* was carried out in a total 20 µl volumes including 1 µl each of the two Fast digest enzymes, 2 µl Fast Digest green buffer and nuclease free water, incubated at 37 °C for 15 min.

Sequencing of the clones

Cultured broth was used for preparation of stab cultures in 2 ml micro centrifuge tubes, containing 1 ml volume of LB agar. The stab cultures of the confirmed recombinant clones were forwarded to the DNA sequencing facility in the Department of Biochemistry, University of Delhi. The sequences received were aligned with the known sequences of beta-tubulin of other *E. tenella* isolates. Multiple alignments were carried out using the Megalign programme of the DNASTar Lasergene software version 6, pairwise alignment report and phylogenetic tree construction was done using Mega 5.1.

Results

DNA extraction, PCR and cloning

400–500 ng genomic DNA was extracted from 10⁵ oocysts, with absorbance ratio 260:280 nm, >1.7. A single PCR product of approximately 2.0 kb was amplified during the reaction (Fig. 1a) with primers CIDte21f and CIDte21r;

Fig. 1 a Beta-tubulin amplicon, 2,006 bp, b *E. tenella* ITS-2 amplicon, 923 bp

Fig. 2 a Beta-tubulin colony PCR b *E. tenella* ITS-2 colony PCR, 923 bp

and a single PCR product of approximately 0.9 kb was amplified with CIDte26f and CIDte26r (Fig. 1b). Colony PCR amplified similar sized products (Fig. 2a, b).

Plasmid extraction and restriction digestion

Plasmid yield from the broth culture of positive recombinant colonies was 70 ng/µl. Restriction digestion of the beta-tubulin recombinant plasmid with *EcoRI* and *HindIII* generated 1,200 bp and 900 bp fragments (Fig. 3). ITS-2 recombinant plasmid on restriction digestion with the same enzyme pair generated 923 bp fragment (figure not shown).

Sequence analysis of beta-tubulin

A 2,006 bp contiguous sequence was generated during the Sanger sequencing and the BLAST search in NCBI gave hits with the *E. tenella* beta-tubulin. The sequence was submitted to the NCBI database via Sequin, after annotation of introns, exons, coding sequence (cds), 5' UTR (un-translated region)

Fig. 3 Characterisation of *E. tenella* beta-tubulin by *EcoRI-HindIII* double digest: release of two products because of the presence of internal *HindIII* restriction site (a) against an uncut plasmid (b)

based on the reference sequence (accession numbers U19609.1 and ETU19609). The detailed annotation of the beta-tubulin sequence is presented in Table 2.

Intraspecies distance relationship was analyzed with CLUSTAL W algorithm of the Megalin programme in Lasergene DNA Star ver 6. Figure 4 depicts 99 % intra-species similarity of beta-tubulin.

The translated coding sequence (predicted amino acid sequence of the cds) when applied to BLAST homology search, provided several hits with >90 % homology, among protozoa. Pairwise alignment with MEGA5.1 programme showed high similarity with other apicomplexans (Table 3; Fig. 5).

Sequence analysis of ITS-2

The partial ITS-2 sequence of 923 bp size showed high sequence homology with the other reference strains of *E. tenella*. The similarity analyses by CLUSTALW algorithm of Megalin programme is depicted in Fig. 6. The phylogeny of the isolates were obtained by neighbor joining tree method (N-J method), the India isolate grouped with the Houghton isolate in the same clade. The sequence was submitted to the NCBI gene bank.

		Percent Identity					
		1	2	3	4	5	
Divergence	1		99.9	99.5	99.7	99.9	1
	2	0.1		99.6	99.7	99.9	2
	3	0.3	0.2		99.7	99.6	3
	4	0.3	0.3	0.1		99.7	4
	5	0.1	0.1	0.2	0.3		5
		1	2	3	4	5	
							Et b tubulin India 1.seq
							Et b tubulin Houghton.seq
							Et b tubulin Nippon2.seq
							Et b tubulin Weybridge.seq
							Et b tubulin Wisconsin.seq

Fig. 4 Intraspecies homology: percent similarity between beta-tubulin

Discussion

Zhu and Keithly (1996), first reported the detailed sequence of *E. tenella* β -tubulin from the *E. tenella* Wisconsin strain. They reported a 2,225 bp gene sequence with 1,550 bp open reading frame (orf), a 146 bp 5' untranslated region (UTR), a 315 bp 3' UTR and 146 bases of poly A tail. In the study, a 91 bp 5'UTR was annotated after aligning with the known sequence of β -tubulin from the Houghton strain. Since the gene was partially sequenced, it covers only two introns (the first intron being partial from the 5' end) and two exons. Exon1 is complete and exon2 is partial from the 3' end. The pairwise alignment results revealed no differential variation from the β -tubulin sequence of known strains, namely the Houghton, Wisconsin, Weybridge and Nippon2. The point mutations were present in the introns, one substitution in intron1, second substitution and single deletion in intron2. No change in exon indicates the strict conservation of structure and function of the polypeptide sequence, which is of immense importance for survival of the unicellular organisms.

The orf sequence was GC rich (60 %), similar to *Cryptosporidium parvum* (62 % GC content, Caccio et al. 1997) contrary to the AT rich sequence of another apicomplexan, *Plasmodium falciparum* (Delves et al. 1989). In *E. tenella* the gene contains three intron sequences (Zhu

Table 2 Detailed annotation of *E. tenella* India 1, beta-tubulin

Gene fragment	Location	Size (base pairs)	Remarks
Intron1; intron2	1–894; 1,082–1,622	894; 541	5' end partial
Exon1; exon2	895–1,081; 1,623–2,006	187; 384	3' end partial
5' UTR	895–985	91	
Coding sequence (cds)	986–1,081; 1,623–2,006	96; 384	3' end partial
Point mutations when aligned with reference Houghton strain	Deletion at 955		Point mutation in 5'UTR
	Substitution at 489		Point mutation in intron 1 (A substituted by C)
	Substitution at 1,372		Point mutation in intron 2 (C substituted by G)

Table 3 Translated coding sequence of β -tubulin: relative pairwise distance between Indian isolate1, other apicomplexans and ciliate *Paramecium tetraurelia* with >90 % similarity

Query sequence	Subject sequence	Distance
<i>E. tenella</i> β -tubulin India1	gi21542248 <i>E. tenella</i> β -tubulin	0.0000
<i>E. tenella</i> β -tubulin India1	gi343480759 <i>E. maxima</i> β -tubulin Houghton strain	0.0000
<i>E. tenella</i> β -tubulin India1	gi237845055 <i>Toxoplasma gondi</i> β -tubulin	0.0645
<i>E. tenella</i> β -tubulin India1	gi401410134 <i>Neospora caninum</i> β -tubulin	0.0645
<i>E. tenella</i> β -tubulin India1	gi135491 <i>Plasmodium falciparum</i> β -tubulin	0.0847
<i>E. tenella</i> β -tubulin India1	gi510900607 <i>Babesia equi</i> β -tubulin	0.0780
<i>E. tenella</i> β -tubulin India1	gi417858 <i>Babesia bovis</i> β -tubulin	0.0645
<i>E. tenella</i> β -tubulin India1	gi145495025 <i>Paramecium tetraurelia</i> β -tubulin	0.0513

Fig. 5 Beta-tubulin of *E. tenella*: Phylogenetic evaluation with apicomplexans and *Paramecium tetraurelia* (NJ tree)

Percent Identity							Divergence	
	1	2	3	4	5			
1	■	99.2	99.1	98.7	98.5	1		ET ITS-2 India1.seq
2	0.8	■	98.8	98.6	98.2	2		ET ITS-2 Houghton.seq
3	0.4	0.8	■	98.7	97.9	3		ET ITS-2 Nippon.seq
4	1.3	1.4	0.9	■	98.3	4		ET ITS-2 Weybridge.seq
5	1.2	1.5	1.1	1.2	■	5		ET ITS-2 Wisconsin.seq
	1	2	3	4	5			

Fig. 6 Intraspecies homology: percent similarity between ITS-2

and Keithly 1996) similar to *Toxoplasma gondi* (Nagel and Boothroyd 1988). In *P. falciparum* and *C. parvum* there are two and one intron/s, respectively (Delves et al. 1989 and Caccio et al. 1997). Within Protista, in general, β -tubulin exists as a multigene family, attributable to various cellular functions. Beta tubulin of apicomplexan parasites, in sharp contrast is a single gene product. This is quite similar to ciliates like *Paramecium tetraurelia*. The phylogenetic tree constructed by the neighbor joining method (N-J method) follows the trend of pair wise alignment distances between species. This predicts a strong homology at the structural level within the evolutionary set up. More than 93 %

conserved sequence is in the C-terminal. The ciliate, *P. tetraurelia* β -tubulin is closely related to *E. tenella* and *E. maxima* than any other apicomplexan parasite, compared in this study. Fast et al. (2002) reported cladal clustering of alveolates (dinoflagellates, ciliates and apicomplexans) based on phylogenetic tree of β -tubulin, Hsp70 (heat shock protein 70) and Hsp90. However they claimed that the β -tubulin phylogenetic tree provides rather less conclusive results on the evolutionary aspect of alveolates.

Heijden et al. (2006) used ITS-1 sequence C-profiling as an epidemiological tool to identify a new isolate of *Histomonas meleagridis* from the outbreaks of clinical histomonosis in poultry farms. The isolate was morphologically dissimilar from the other known isolates. ITS-2 was used as a marker to study the genetic heterogeneity within the congeneric species of *Fasciola* of mainland China by Huang et al. (2004). *Encephalitozoon hellem*, microsporidian parasite of human beings have been ITS genotyped and intra-species variants have been confirmed (Haro et al. 2003). ITS-2 sequencing data has immense potential in field epidemiology, for studying the spatial distribution of pathogens and their spread. The Further studies on multiple genes related to antigens and housekeeping factors will help in analysis of the intra-species and interspecies position of Indian isolate 1 of *E. tenella*.

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References

- Barabona I, Soares H, Cyrne L, Penque D, Denoulet P, Rodrigues PC (1988) Sequence of one α and two β tubulin genes of *Tetrahymena pyriformis*. Structural and functional relationships with the eukaryotic tubulin genes. *J Mol Biol* 202:365–382
- Beck HP, Blake D, Darde ML, Felger I, Pedraza Diaz S, Regidor-Cerrillo J, Gomez-Bautista M, Ortega-Mora ML, Putignani L, Shiels B, Tait A, Weir W (2009) Molecular approaches to diversity of subpopulations of apicomplexan parasites. *Intl J Parasitol* 39:175–189
- Bhaskaran MS, Venkatesan L, Aadimoolam R, Jayagopal HT, Sriraman R (2010) Sequence diversity of internal transcribed spacer-1 (ITS-1) of *Eimeria* infecting chicken and its relevance in species identification from indian field samples. *Parasitol Res* 106:513–521
- Caccio S, LaRosa G, Pozio E (1997) The β -tubulin gene of *Cryptosporidium parvum*. *Mol Biochem Parasitol* 89:307–311
- Conner TW, Thomson MD, Silflow CD (1989) Structure of three β -tubulin genes of unicellular alga. *Polytomelia agilis*. *Gene* 84:345–351
- Delves CJ, Ridley RG, Goman M, Holloway SP, Hyde JE, Scaife JG (1989) Cloning of a β -tubulin gene from *Plasmodium falciparum*. *Mol Microbiol* 3:1511–1519
- Fast NM, Xue L, Bingham S, Keeling PJ (2002) Re-examining alveolate evolution using multiple protein molecular phylogenies. *J Eukaryot Microbiol* 49:30–37
- Gautam RK, Gupta SK (2004) Drug resistance in field isolates of *Eimeria tenella* in poultry. *Ind J Poult Sci* 39:43–48
- Gill BS, Bajwa RS (1979) Drug resistance in field isolates of chicken from Punjab state. *Ind J Parasitol* 3:132–134
- Gurr SJ, Unkles SE, Kaghorn JR (1988) The structure and organization of nuclear genes of filamentous fungi. In: Kinghorn JR (ed) *Gene structure in Eukaryotic microbes*. IRL Press, Oxford, pp 93–139
- Haro M, del Aguila C, Fenoy S, Henriques-Gil N (2003) Intraspecific genotypic variability of microsporidian parasite *Encephalitozoon hellem*. *J Clin Microbiol* 41(9):4166–4171
- Huang WY, He B, Wang CR, Zhu XQ (2004) Characterization of *Fasciola* species from mainland China by ITS-2 ribosomal DNA sequence. *Vet Parasitol* 120:75–83
- Kawahara A, Taira K, Nagai S, Onaga H, Onuma M, Nunoya T (2008) Detection of five avian *Eimeria* species by species-specific real-time polymerase chain reaction assay. *Avian Dis* 52:652–656
- Kumar S (2012) Molecular identification and prevalence of *Eimeria* spp. of chicken. M.V.Sc. thesis submitted in Veterinary Parasitology to the Deemed University, Indian Veterinary research Institute, Bareilly
- Lohse J, Rinder H, Gothe R, Zahler M (2002) Validity of species status of the parasitic mite *Otodectes cynotis*. *Med Vet Entomol* 16:133–138
- Nagel SD, Boothroyd JC (1988) The α and β -tubulins of *Toxoplasma gondii* are encoded by single copy genes containing multiple introns. *Mol Biochem Parasitol* 29:261–273
- Panda NC, Pradhan HK, Mitra A, Parichha SN (1973) Drug resistance of coccidia exposed to a single type of coccidiostat. *Indian Vet J* 50:22–26
- Trivinos-Lagos L, Ohmachi T, Albrightson C, Burns RG, Ennis HL, Chishom RL (1993) The structure and organization of nuclear genes from *Dictyostelium discoideum* are encoded by single genes. *J Cell Sci* 105:903–911
- Van der Heijden MJF, Landman WJM, Greve S, Peek R (2006) Genotyping of *Histomonas meleagridis* isolates based on internal transcribed spacer-1 sequences. *Avian Pathol* 35:330–334
- Yadav A, Gupta SK (2001) Study of resistance against some ionophores in *Eimeria tenella* field isolates. *Vet Parasitol* 102:69–75
- Zhu G, Keithly JS (1996) The beta tubulin gene of *Eimeria tenella*. *Mol Biochem Parasitol* 76:315–319
- Zhu G, McDougald LR (1995) Confocal laser scanning microscopy of β tubulin and α actinin asexual stages of *Eimeria tenella* (Coccidia, Apicomplexa) in vitro. *Arch Protistenk* 145:112–118