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Molecular differentiation of cryptic stage of *Echinococcus granulosus* and *Taenia* species from faecal and environmental samples

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ABSTRACT

Objective: To differentiate cryptic stage of *Echinococcus granulosus(E. granulosus)* and *Taenia* by PCR-RFLP and sequence information of amplicon. **Methods:** DNA were isolated from metacestodes stage of *Taenia* and *E. granulosus* using DNA isolation kit (Q-BIOgene kit, USA), the amplified and purified DNA product was then cloned and sent for sequencing. The generating sequence information was used for amplicons identification. **Results:** Out of 112 faecal and environmental samples, 16 exhibited positive result. The product size of amplicon positive for *E. granulosus* was 310 bp; whereas, for *Taenia* spp. sizes varied from 379 to 388 bp. Restriction profile of actin II with Csp61 also differed *Taenia* spp. and *E. granulosus*. **Conclusions:** The result of the study indicated that, the primers were useful to differentiate cryptic stage of the two genera which is yet to be reported earlier.

1. Introduction

Taenia solium (T. solium) and *Echinococcus granulosus (E. granulosus)* are well known parasites of medical and economic importance. *T. solium* is severely pathogenic to man when the larval stage develops in the central nervous system. The disease is not only important in developing countries but also in areas of non endemicity affecting immigrants, tourists[1-3]. Like *T. solium* cysticercosis, hydatidosis caused by *E. granulosus* is responsible for formation of cysts, mainly in the liver and lungs, cause severe pathological effects in intermediate hosts. *Taenia hydatigena(T. hydatigena)* is harboured by sheep and goats. Semi domesticated black rats (*Rattus rattus*) serve as the intermediate host of *Taenia teaniaeformis (T. teaniaeformis)*. For *T. solium* and *E. granulosus* man and dogs serve as the definitive host of the parasite, respectively. During 80's abdominal hydatidosis in man has been reported from southern part of India[4], operated cases of pulmonary hydatid cysts has been documented from eastern India[5] and records of consecutive cases of human hydatidosis has been reported from northern India[6]. It has been documented in the literature that, neurocysticercosis (NCC) is identified as the single most common cause of community acquired active epilepsy; 26.3% to 53.8% active epilepsy cases in the developing world including India and Latin America are due to NCC[7]. Metacestode stage can be easily differentiated on the basis of their gross morphological characters either as *Cysticercus* (for *T. solium* and *T. hydatigena*), strobilocercus (*T. taeniaeformis*) and hydatid (*Echinococcus*). But the eggs of *E. granulosus* are morphologically indistinguishable to those of other tapeworms of the genus *Taenia*. Therefore, the most immediate priority is to distinguish egg of the parasite in faecal and environmental samples. Like other parasitic infection, intra vitam diagnosis of taeniid tapeworms can not be achieved by microscopical detection of worm eggs in faecal samples by routine serological methods. For that reason copro-DNA test may be used as alternative approach because parasite DNA are excreted with eggs, and proglottids or parasite cells can be detected from faeces after amplification by PCR[8]. Six different actin isoforms have been identified by aminoacid sequences. Moreover, actin is the most abundant protein in eukaryotic cells and actins are highly conserved during evolution[9,10]. Two actin related sequences (EgactI and Egact II) separated by a 4 kb region[11]. The Egact I contained no intron but an intron of 591 bp was observed in the Egact II. Intron sequences are specifically known because intron is closely related to the evolution of eukaryotic genomes which has been established as intron-early and late theories[12,13]. Therefore, to characterize cryptic interspecific variants of *E. granulosus*, analysis of intron of actin II has been taken into consideration from abroad[14,15]and India[16,17]. Considering

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established theories mentioned above, this communication reports for the first time differentiation of cryptic stage of *E. granulosus* and *Taenia* available in India which was further supported by PCR-RFLP and sequence information of amplicon.

2. Material and methods

2.1. Collection of metacestodes

Metacestode stage of *T. solium* (*Cysticercus cellulosae*) was collected from skeletal muscle of pigs. Cysts of *T. hydatigena* were collected from naturally infected sheep. Strobilocercus form of metacestode was collected from liver of semi domesticated black rats (*Rattus rattus*). Protoscoleces of *E. granulosus* was collected from the lungs of naturally infected buffalo. The metacestodes were identified and stored at -20 ℃ till further use.

2.2. Isolation of DNA from metacestode stage

DNA samples were isolated from metacestodes stage of *Taenia* and *E. granulosus* using DNA isolation kit (Q-BIOgene kit, USA). DNA isolation was carried out according to manufacturer's protocol. In brief, tissue suspension was homogenized in 200 μL cell suspension solution. The homogenate was further digested by proteinase K after addition of RNAse. After digestion, salt out mixture was added and supernatant was collected after centrifugation. DNA was precipitated after addition of absolute alcohol (v/v). Isolated DNA was dissolved in nuclease free distilled water (Genei, Bangalore). DNA samples were stored at -20 ℃ till further use.

2.3. Amplification of DNA sample, purification and cloning of PCR product

Genomic DNA was used for amplification of DNA samples using the primers described earlier[14]. The design of primer was: Forward 5′GTC TTC CCC TCT ATC GTG GG3′ and Reverse 5′ CTA ATG AAA TTA GTG CTT GTG CGC 3′. In brief, PCR was performed in Gene Amp® PCR system 9700 (Applied Biosystems, USA). A final volume of 25 μL containing DNA (not quantified) , 20 μM of each dNTP (Genei, Bangalore), 10×PCR buffer (Genei, Bangalore), 2.5 mM $MgCl_2$ (Genei, Bangalore) , 10 pmol of each gene specific primers (IDT, USA) and 1 unit of Taq DNA polymerase (Fermentas, USA) were used in reaction. The PCR reactions were started with an initial denaturation step followed by 35 cycles, 95 ℃ for 45 sec, 53 ℃ for 45 sec followed by 72 ℃ for 90 sec with a final extension of 72 ℃ for 10 min. Amplified product was purified using QIAquick Gel extraction kit (QIAGEN, Germany). Further purified product was cloned using InsT/A clone™ product cloning kit (Fermentas, USA) using *E. coli* DH 5 α as primary cloning host. Confirmed clone was sent for sequencing to DNA sequencing facility UDSC (DBT supported, Department of Biochemistry, University of Delhi, South Campus). DNA sequencing was carried out in cloned DNA by universal primers (M13F and M13R) using cycle sequencing (3730 system, Applied Biosystems, USA).

2.4. Evaluation of field sample

A total of 31 canine faecal samples were collected from public parks and 21 samples were collected from in and around slaughter house. Soil samples were collected from in and around pig slaughter house (11), viscinity of cattle and buffalo slaughter house (35) and public park (13). Approximately 20 g of faecal and soil samples were concentrated by centrifugation and floatation was done by zinc chloride solution[18]. The same primer described earlier was used for screening of faecal and environmental samples.

2.5. Confirmation of amplicon

The amplicons were confirmed by generating sequence information as described earlier.

2.6. Differentiation of E. granulosus and Taenia by PCR-RFLP

Location of restriction cuts of amplified product on the basis of sequence information were identified for Csp61 using Primer 5.0 (Premier Biosoft International, USA). In brief, a total volume of 100 μL of PCR product was purified from gel using QIAquick PCR amplification kit (QIAGEN, USA). The elute volume was restricted to 30 μL. Restriction digestion was conducted in 10 μL volume which comprised of gel purified PCR product (6 μL) , 10 × Tango buffer (μL) (Fermentas, USA), and nuclease free distilled water (2 μL) (Genei, Bangalore). The mixture was incubated at 37 ℃ for 6 h and digested product was separated using 2.5% agarose gel.

3. Results

Amplicons exhibited differences in product size for *E. granulosus*, *T. taeniaeformis*, *T. solium* and *T. hydatigena* (Figure 1). The products after sequencing were shown to have the span between 310 to 388 bp. The sequence information was further registered at the Gen Bank database under accession numbers EF179177, EF187446, EF187447, EF192916 for *E. granulosus*, *T. solium*, *T. taeniaeformis* and *T. hydatigena*, respectively. There was no sequence variation among *T. solium* (379 bp) (EF187447), *T. taeniaeformis* (380 bp) (EF 187446) and *T. hydatigena* (388 bp) (EF 192916). However, deletion mutation was found at 21 and 360 positions. Further comparison of data with *E. granulosus* of 310 bp fragment (EF 179177) with *T. solium*, *T. taeniaeformis* and *T. hydatigena* revealed dissimilarity between 31.05 to 31.31 per cent. The details of nucleotide sequence variation and gap due to probable deletion mutation has been depicted in Figure 2. Restriction profile of amplicons of *E. granulosus* and *Taenia* exhibited restriction fragments of different sizes. Because restriction sites were different for *E. granulosus* and *Taenia* which could be deduced on the basis of different restriction sites of Csp61. The details of fragment sizes along with their restriction sites have been explained in Figure 3. Out of 31 canine samples collected from public parks, 3 were positive for taeniid eggs, and 2 of the 3 positive samples were confirmed as *T. hydatigena* based on size of amplicon, restriction profile (Figure 1&2) and sequence information (EF192196). Further investigation on the basis of 22 faecal samples collected from in and around cattle and buffalo slaughter house, 12 samples were positive for teaniid like eggs by microscopy. Out of twelve samples, amplified product of 4 samples was indistinguishable from the product size of *E. granulosus* (Figure 1).

Out of the total of 59 collected soil samples, 11 were collected from in and around pig slaughter house, and 2 of the 11 were positive for taeniid like eggs, whose amplicons were further comparable with sequence information of *T. solium* (EF187447). Out of 35 faecal samples collected from in and around cattle and buffalo slaughter house, 6 were positive on

the basis of their product size. Three of the 6 positive samples were confirmed to have homologous sequence information of *T. hydatigena* (EF192916) and the rest 3 positive samples were similar to *E. granulosus* (EF179177). Out of 13 soil samples collected from public parks 2 were amplicon indistinguishable from *Taenia*. After sequencing the sequence information was comparable with *T. hydatigena* (EF192196).

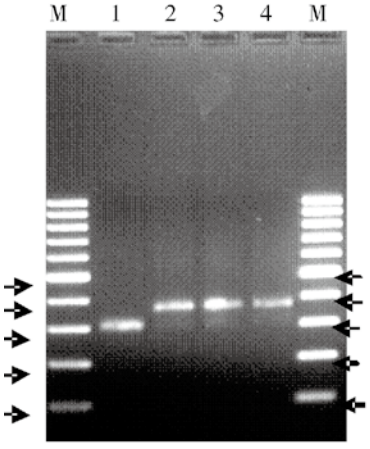

Figure 1. Amplified products of Actin II gene of *E. granulosus* and *Taenia* sps.
M= Molecular weight marker
(arrows from the bottom 100, 200, 300, 400, 500 bp),
Lane 1: Amplicon of *E. granulosus* (310 bp),
Lane 2: Amplicon of *T. teaniaeformis* (380 bp),
Lane 3: Amplicon of *T. solium* (379 bp),
Lane 4: Amplicon of *T. hydatigena* (388 bp)

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T. solium          GTCTTCCCCT CTATCGTGGG -AGCCAGTGA AATCATTTCT
T. taeniaeformis   .......... .......... g?........ ..........
T. hydatigena      .......... .......... -......... ..........
E. granulosus      .......... .......... TC.T.CAA... C....GG.-.

T. solium          CCCTCGAGAG GGAGTCAGAC CCACCTGCTT ACCAAGGTCC
T. taeniaeformis   .......... .......... .......... ..........
T. hydatigena      .......... .......... .......... ..........
E. granulosus      AGT.G..T.. .T..G...-- -----..T.. C---..CCG.

T. solium          ATCAGAAGAA GGCGAAGTTG AAAAAGAACA AAGGGAAAAA
T. taeniaeformis   .......... .......... .......... ..........
T. hydatigena      .......... .......... .......... ..........
E. granulosus      .C.G...CTG .TACC.AC.- ---------- -..T.G.CC.

T. solium          ATTGAAGGTT GTTAAGGCAA ACAGTGCGAA ATCGTCCAAT
T. taeniaeformis   .......... .......... .......... ..........
T. hydatigena      .......... .......... .......... ..........
E. granulosus      ...------.. C.C..AT--. .G..ACA... ..G..TTGC.

T. solium          CCAAAGTCGA ATATGTGGCT TAAGAAGAAA AAGTGAAGTC
T. taeniaeformis   .......... .......... .......... ..........
T. hydatigena      .......... .......... .......... ..........
E. granulosus      .TC.T.CACC .A....---- -.T.GT...G ....C--.G.

T. solium          TTCTTGTCTG TGTACATAAT TCTGTATGAT CAAATCATGA
T. taeniaeformis   .......... .......... .......... ..........
T. hydatigena      .......... .......... .......... ..........
E. granulosus      ..T.CA...A AC..G...G- ---.C..... T.G--TG..G

T. solium          GCCTTTCCCT CCCCCCTTCA ACTTAGATGC TGTCGTGTAT
T. taeniaeformis   .......... .......... .......... ..........
T. hydatigena      .......... .......... .......... ..........
E. granulosus      ---------- ---------. GA.C.AG... .C..T....G

T. solium          GATCGCTATT TTTTTTCATT TCCCTCCTTA AGTGTCTGCC
T. taeniaeformis   .......... .......... .......... ..........
T. hydatigena      .......... .......... .......... ..........
E. granulosus      AG....C..C .GAGGG..G. --.T.T..-- .T.T..GC..

T. solium          TAGGTCCTTT AATCAGCTTT GTCTGTGTAC TTGTTTGCGC
T. taeniaeformis   .......... .......... .......... ..........
T. hydatigena      .......... .......... .......... .........-
E. granulosus      .GT.A.---- ..CGTA.C.A T..C.AAATA A.C.......

T. solium          ACAAGCACTA ATTTCATTAG
T. taeniaeformis   .......... ..........
T. hydatigena      .......... ..........
E. granulosus      .......... ..........
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Figure 2. Alignment of sequence information of Actin II of *T. solium*, *T. taeniaeformis*, *T. hydatigena* and *E. granulosus*.
(.) indicates similarity of sequence information. (–) indicates gap.

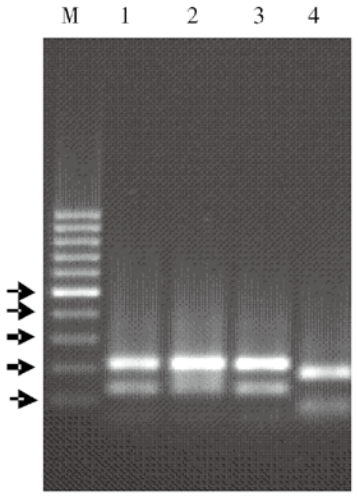

Figure 3. Restriction fragment length polymorphism analysis of Acin II gene amplified product.
M= Molecular weight marker
(arrows from the bottom 100, 200, 300, 400, 500 bp), Lane(1–3)= Taenia sp., Lane 4= *E. granulosus*; Lane1–3: 171 bp (lower band), 176 bp (upper band) , Lane 4: 81 bp (lower band), 184 bp (upper band).

4. Discussion

This is not possible to identify taeniid cestodes on the basis of traditional methods particularly after microscopic examination of eggs. In order to overcome limitations molecular approaches has been explored like PCR–RFLP, single stranded conformation polymorphism (SSCP), amplification of DNA of parasite and base excision sequence scanning thymine–base reader analysis with mitochondrial genes[19–22]. Although immunological methods are available based on primary binding assay to measure antigen or antibody but preparation of worms recovered from experimental animals is impractical for regular use[20]. In the recent past, cytochrome c oxidase (*coxI*) and cytochrome b (cytb) genes were used because their nucleotide sequences have been determined completely. These genes have been used to discriminate *T. saginata* and *T. solium*[20]. Although SSCP may be useful approach without DNA sequencing but the methodology is time consuming[22].

On the contrary, during the present investigation we could easily discriminate *Taenia* and *E. granulosus* on the basis of product size using primer for amplification of actin II of *E. granulosus*. Further, products have been confirmed on the basis of their sequence information and RFLP because on the basis of Csp61 recognition sites *Echinococcus* and *Taenia* could be differentiated. The different restriction sites have been described through figures along with their predicted product sizes deduced *in silico* by primer premier 5.0. Therefore, our *in silico* analysis matched with the bench top observation.

It can be summarized that, PCR based RFLP is not only useful for identification of taeniid specimens but also for the speculation of *Taenia* and *Echinococcus* prevalence in a particular area and for screening environmental samples. Because *T. solium* and *Echinococcus* eggs are infective to man and cause infection. During the present study panel of parasites is less because we could not include other parasites described earlier[23]. Because in earlier study[24] this has been indicated that, *Taenia* egg discriminated by molecular tool may be speculated as *T. hydatigena*, *T. multiceps* or *T. taeniaeformis*. Therefore, present investigation on *Taenia* and *Echinococcus* is useful and novel approach which is yet to be

Table 1
Showing results of copro-DNA and parasitological tests of faecal and environmental samples.

Type of samples		Number of samples(112)	Number positive by microscopy(16)	Number positive by PCR
Faecal samples(53)	Canine faecal sample (from public park)	31	3	2 (confirmed as *Taenia* based on product size and *T. hydatigena* on the basis of sequence information.)
	Canine faecal sample (from " in and around slaughter house")	22	12	4 (confirmed as *Echinococcus* based on product size and sequence information.)
Soil samples (59)	From "in and around slaughter house"	11	2	2 (confirmed as *Taenia* based on product size and *T. solium* on the basis of sequence information.)
	From in and around cattle and buffalo slaughter house	35	6	3 (confirmed as *Taenia* based on product size and *T. solium* on the basis of sequence information.)
	Collected from public parks	13	2	2 (Confirmed as *Taenia* based on product size and *T. hydatigena* on the basis of sequence information.)

reported earlier. Because seroepidemiology using monoclonal antibody directed against *Echinococcus* oncospheres has not been found suitable for epidemiological studies[25].

Conflict of interest statement

We declare that we have no conflict of interest.

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