Molecular appraisal of Indian animal isolates of *Echinococcus granulosus*  

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*Background & objectives:* Cystic echinococcosis (CE) has a wide host range and distinct entities, not only reflected phenotypically but also by genotypic variation. Considering this fact, this study was undertaken to characterize the Indian isolates of *Echinococcus granulosus* to find out difference between Indian cattle, buffalo and sheep isolates on the basis of random amplification of polymorphic DNA (RAPD) PCR and PCR mediated restriction fragment length polymorphism (PCR-RFLP) of internal transcribed spacer gene 1 (*ITS1*).

*Methods:* A total of 22 isolates of *E. granulosus* obtained from Indian cattle, buffalo and sheep (December 2004 - November 2005) were analysed by 26 random primers of 8-10 mers. After isolation of protoscoleces from fertile cyst, DNA was extracted, quantified and amplified by random primers. Internal transcribed spacer gene 1 (*ITS1*) was amplified using specific primer and digested by two restriction enzymes (*Msp1* and *Rsa1*).

*Results:* Of the 26 primers, only two primers (5'ACC TGG ACA C3' and 5'TCA TCC GAG G3') could discriminate cattle, buffalo and sheep isolates collected from eastern part of India. Samples were further analysed by PCR mediated RFLP of internal transcribed spacer gene1 (*ITS1*) using two restriction enzymes (*Msp1* and *Rsa1*). No *ITS1* variants could be detected.

*Interpretation & conclusions:* Our findings showed genotypic variation among Indian animal isolates of *E. granulosus* on the basis of RAPD fingerprinting.

**Key words** Buffalo - cattle - *Echinococcus granulosus* - PCR-RFLP - sheep
Denominated Indian buffalo isolate is generally considered as the most significant host for sustaining the life cycle\textsuperscript{4,6}. From eastern part of India, many host species including man\textsuperscript{7} have been identified who support the intermediate stage of this parasitic flatworm. But comparative profile of cattle, buffalo and sheep isolates of \textit{E. granulosus} has not been studied.

We undertook this study to evaluate the comparative profile of cattle, buffalo and sheep isolates of \textit{E. granulosus} on the basis of random amplification of polymorphic DNA-PCR (RAPD-PCR) and polymerase chain reaction based restriction fragment length polymorphism (PCR-RFLP) to identify internal transcribed spacer gene1 (\textit{ITS1}) variant.

**Material & Methods**

\textit{Collection of parasite:} During the period of study (December 2004 - November 2005), the parasitic cysts of \textit{E. granulosus} were collected from slaughterhouses, which are under jurisdiction of Kolkata Municipality Corporation, Kolkata, West Bengal, India. One fertile cyst was considered as an isolate. Fertile cyst of \textit{E. granulosus} was identified on the basis of presence of protoscoleces.

A total of 22 isolates were collected. Ten samples were from buffalo and ten were from sheep. Only two samples were from cattle. During the collection of cysts source of animals were also enquired from the butchers (Table).

Protoscoleces were isolated from the fertile cysts. In brief, hydatid fluid was withdrawn from the cyst, cyst wall was excised and protoscoleces were collected by sterilized needle and syringe. The isolated protoscoleces were washed three times by phosphate buffer saline (PBS) (pH 7.2) and preserved in 70 per cent alcohol (v/v), as described earlier\textsuperscript{8}.

\textit{Isolation of genomic DNA:} Total genomic DNA from parasitic material was isolated using Q-BIO gene kit, USA. In brief, the parasitic cells were suspended in cell suspension buffer, 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) and isolated DNA was dissolved in nuclease free distilled water. Isolated DNA was stored at -20°C till further use. Host DNA was extracted from the blood samples from respective host species following standard methodology\textsuperscript{9}.

\textit{DNA amplification:} A total of 26 RAPD-primers were used for characterization of \textit{E. granulosus} isolates without prior published information.

The amplification reactions were carried out in 25μl volume containing 500mM KCl, 100 mM Tris-HCl (pH 9.0), 1% Triton X-100, 4 mM MgCl\textsubscript{2}, 100 μM dNTPs each (Genei, Bangalore), 15-20 ng of primer (Bio Basic Inc., Canada), 25 ng of DNA and 1.5 units of Taq DNA polymerase (Genei, Bangalore). For data analysis, RAPD assays were performed in duplicate in two different thermal cyclers, i.e., Eppendorf Master cycler personal (Eppendorf, West Germany) and Gene Amp\textsuperscript{®} PCR system 9700 (Applied Biosystem, USA) to assess the reliability of the test. PCR reaction was carried out in 40 cycles, 94°C for 1 min, 36°C for 1 min and 72°C for 2 min followed by a final extension of 10 min. PCR products were analysed after electrophoresis in 1.5 per cent (w/v) agarose gel (Genei, Bangalore) and visualized in ethidium bromide (SRL, Mumbai).

\textit{Analysis of data:} RAPD bands were assessed for presence or absence, and only fragments that were reproducible in at least two reactions, were included for analysis. Positional homology of amplified fragments were assumed, and only polymorphic bands were considered in analysis. Similarity coefficients (S) between isolates were calculated using the formula \[ S = \frac{2N_{xy}}{N_x + N_y}, \] where \( N_x \) and \( N_y \) are the number of fragments amplified in isolates X and Y, respectively. \( N_{xy} \) is the number of bands shared by two isolates\textsuperscript{10}.

<table>
<thead>
<tr>
<th>Host</th>
<th>No. of isolates (code)</th>
<th>Provincial origin (if any)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cattle</td>
<td>02 (CM-1, CM-2)</td>
<td>District: Murshidabad, West Bengal</td>
</tr>
<tr>
<td>Buffalo</td>
<td>03 (BB-1, BB-2, BB-3)</td>
<td>Bihar, West Bengal</td>
</tr>
<tr>
<td>Buffalo</td>
<td>04 (BS-1, BS-2, BS-3, BS-4)</td>
<td>District: 24 Parganas (S), West Bengal</td>
</tr>
<tr>
<td>Buffalo</td>
<td>03 (BM-1, BM-2, BM-3)</td>
<td>District: Murshidabad, West Bengal</td>
</tr>
<tr>
<td>Sheep</td>
<td>10 (1,2,3,4,5,6,7,8,9,10)</td>
<td>Not known because the animals belonged to nomads</td>
</tr>
</tbody>
</table>

Both the provinces are situated in eastern part of India.
PCR-mediated RFLP: Amplification of *ITS1* gene was done using primers described earlier\(^1\). The primer design was: Forward: 5' GTC GTA ACA AGG TTT CCG TA3', and Reverse: 5' TCT AGA TGC GTT CGA A (G/A) TGT CGA TG3'.

The amplicons were purified from gel using clean Genei kit (Genei, Bangalore). PCR products derived from *E. granulosus* were digested with *MspI* and *RsaI* (10 U) using buffers recommended by the manufacturer ((Bio Basic Inc., Canada). Restriction fragments were separated by gel electrophoresis through 2 per cent TBE agarose gel (Genei, Bangalore).

**Results**

Amplicons were also produced with vertebrate DNAs but they did not interfere with the banding patterns produced by samples of parasites.

Of the 26 primers, two primers OPI-01 and OPI-15 could only discriminate isolates of *E. granulosus* collected from cattle, buffalo and sheep. (Figs 1 & 2).

Analysis of data revealed that, by OPI-01 two cattle isolates (CM-1 & CM-2) and two sheep isolates (S-1 & S-10) could be clustered in one group. Two buffalo isolates (BB-1 & BB-3) showed different RAPD profile from that of cattle and sheep isolates. RAPD pattern of both the buffalo isolates were indistinguishable from one another. This result can be appreciated on the basis of number and intensity of bands. This has been revealed that, the bands generated by RAPD fingerprinting in two buffalo isolates were faint (Fig. 1 lanes 5 & 6), in comparison to cattle (Fig. 1, lanes 2 & 3) and sheep isolates (Fig. 1, lanes 8 & 9). Therefore, cattle, buffalo and sheep isolates can be differentiated into two clusters, i.e., cattle-sheep and buffalo cluster. Similarity coefficient in between two clusters was 0.80.

By OPI-15 primer two cattle (CM-1 & CM-2) and one buffalo isolates (BS-1) showed similar profile as this was noted on the basis of single scorable band of 1 kb. The number of scorable bands were same for one buffalo (BS-4) and two sheep isolates (S-3 & S-4) (Fig. 2). Similarity coefficient between BS1/CM1/CM2 and BS4/S3/S4 were 16.66.

Molecular analysis by PCR-RFLP of *ITS1* of 22 fertile cysts (2 from cattle, 10 from buffalo and 10 from

![Fig. 1.](image1) Ethidium bromide stained agarose (1.5%) gel showing RAPD products using DNA from *E. granulosus* by OPI-01 primer. Lane 1: 1 kb DNA ladder (top to bottom arrows: 2 kb, 1 kb, 750 bp, 500 bp, 250 bp), Lanes 2 & 3: CM1 & CM2, Lane 4: Cattle host DNA, Lanes 5 & 6: BB1 & BB3, Lane 7: Buffalo host DNA, Lanes 8 & 9: S1 & S10, Lane 10: Sheep host DNA, Lane 11: Larval stage of *Taenia hydatigena*, Lane 12: 100 bp DNA ladder (top to bottom arrows: 1000 bp, 700 bp, 500 bp, 300 bp, 200 bp, 100 bp).

![Fig. 2.](image2) Ethidium bromide stained agarose (1.5%) gel showing RAPD products using DNA from *E. granulosus* by OPI-15 primer. Lane 1: 1 kb DNA ladder (top to bottom arrows: 3 kb, 2 kb, 1 kb, 750 bp, 500 bp, 250 bp), Lanes 2 & 3: CM1 & CM2, Lane 4: Cattle host DNA, Lanes 5 & 6: BS1 & BS4, Lane 7: Buffalo host DNA, Lanes 8 & 9: S3 & S4, Lane 10: Sheep host DNA, Lane 11: Larval stage of *Taenia hydatigena*, Lane 12: 100 bp DNA ladder (top to bottom arrows: 1000 bp, 700 bp, 500 bp, 300 bp, 200 bp, 100 bp).
comparison. By OPI-01 primer the most pronounced feature of DNA fingerprints was the variation in the intensity of the bands. The more intense band in cattle and sheep isolates was probably due to priming within the repeated sequence in cattle and sheep isolates, which may be absent in buffalo isolates. This might be indicative of presence of unique genotype in buffalo. In the past, Indian buffalo isolates have been characterized on the basis of morphology of adult worms\(^4\), protein profile\(^{12}\) and RAPD fingerprinting\(^{13}\). Because buffalo is generally considered the significant intermediate hosts for sustaining the life cycle which could be partly explained by the older age (12-16 yr) at which they are slaughtered and differences in strain biology of the region\(^{14}\). Thompson and Lymbery\(^{15}\) suggested that the forms in buffaloes might be the same as that of cattle strain of \(E.\ granulosus\). Molecular genetic data have confirmed the occurrence of the cattle strains in buffaloes from India but has also revealed that the buffaloes may harbour the sheep strain and a unique genotype not seen in other intermediate host species\(^{11,16-18}\). By OPI-15 primer two cattle isolates were indistinguishable from one buffalo isolate. Further analysis revealed that by the same primer one buffalo isolate was having 100 per cent similarity with two sheep isolates. Moreover, single scorable band was observed in three isolates after use of OPI-15 primer. Therefore, this primer may be recommended to have good discriminatory power because the same may be used for RAPD- sequence confirmed amplified region (RAPD-SCAR) analysis.

Although RAPD based on PCR is a relatively fast and simple technique for detection of intraspecific variants\(^{19}\) but the data may be influenced by the quality of template and cycling speed\(^{20}\). Hence this is recommended that the RAPD analysis should be compared with one or more DNA techniques.

During the present study, the results of RAPD fingerprinting were further tested by ITS1-RFLP. As such, no ITS-I variant could be detected. The result is indicative of absence of ITS-I variant which could not be discriminated by using these two restriction enzymes. Different profile by RAPD fingerprinting may be due to amplification of other genomic region.

**References**


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