Genotypic Characterization of Buffalo Isolates of *Echinococcus granulosus*

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(Received: 18 February 2015; accepted: 12 April 2015)

Cystic echinoccosis (CE) caused by the larval stage of the cestode *Echinococcus granulosus* is one of the most widespread zoonoses of veterinary and medical importance. Molecular techniques have allowed the identification of 10 different genotypes (G1-G10) of the parasite. Numerous studies have provided evidence that *Echinococcus granulosus* exists as a complex of different strains, that differ in wide variety of criteria that have impact on the epidemiology, pathology and control of cystic hydatid disease (CHD). On sequence analysis of mitochondrial COX1 and NADH genes showed the occurrence of the G1 genotype, the common sheep strain, G2 genotype, the Tasmanian sheep strain, the G3 genotype, the buffalo strain. The present study is an update of *E. granulosus* strains by genotyping a large samples of isolates and checking out genetic differentiation within and among the G1,G2,G3 genotype using additional gene marker, the RRNS gene. Sequencing of RRNS gene revealed fixed nucleotide substitution among G1,G2 and G3 genotypes. This study provides further evidence of the occurrence of the *E.granulosus* G1 and G2 sheep strain.

Key words: *Echinococcus granulosus*; Genotypes; Mitochondrial DNA genes.

Cystic echinoccosis (CE) is a zoonotic parasitic infection of many mammalian species caused by the larvae of *Echinococcus granulosus* which is a small tapeworm. The lifecycle involves, dogs and other canids are typical definitive hosts and ungulates especially cattle, buffalo, sheep, goat, pig and horse are intermediate hosts in which the hydatid cysts occur (Moro and Schantz, 2009). It causes severe economic loss and public health problem to both human beings and livestock in many temperate and tropical areas including India. The most tangible economic effects of this are the loss of offal from food animals. This may result in the entire loss of an infected organ or at least the trimming and down grading of that organ (Irsadullah *et al*., 1989).

The body weight of infected animal will be 1 per cent less than uninfected animals (Torgerson and Macpherson,2011). CE often leads to a decline of health status that in turn translates into serious production losses to livestock industries. Economic losses arise not only from the condemnation of infected viscera, but also from decreases in yield and quality of meat, milk and value, reduced birth rate and fecundity, and delayed performance and growth. Other CE-associated costs that should be considered include the elimination of the infected viscera and dead animals or the potential bans on export of infected animals and their products (Battelli, 2009). Therefore, productivity losses due to CE in different livestock species with CE have been estimated to be in the range of 2.5–20 per cent for reduction in carcass weight, 2.5–12per cent for reduction in milk production, 3–12 per cent for decrease in fecundity, and 0.2 per cent for decrease in hidevalue (Cardona and Carmena, 2013).

The global annual live-stock production loss due to CE is estimated to be US$141, 605, 195 (Budke, 2006). In addition, this disease is of utmost zoonotic importance in human beings and requires
expensive and prolonged medical treatment, often surgical interventions and the mortality may go up to 2–4 percent (Eckert et al., 2001). The global annual monetary loss due to CE in man has accounted for US$ 193, 529, 740 (Budke, 2006). The cases of CE in humans and domesticated animals such as sheep, cattle, buffaloes, pigs and wild animals are being increasingly reported from India (Bhatacharya et al., 2007; Sunil Kumar, 2007) and from different parts of Tamilnadu, India (Parija and Sheela Devi, 1999; Raman and Lalitha John, 2003; Sangaran, 2013 and Jeyathilakan, 2007).

A high degree of intra-specific variation has been detected within E. granulosus and a number of well characterized strains are now recognized. They display significant differences in life cycle patterns and host preferences (Thompson and McManus, 2002; McManus, 2002). To date, molecular genotyping, using mitochondrial DNA (mtDNA) sequences, have identified 10 distinct genetic variants or strains (genotypes G1–G10) within E. granulosus (Bowles and McManus, 1993; Bowles et al., 1994; Zhang et al., 1998; Snabel et al., 2000; McManus, 2002; Lavikainen et al., 2003; McManus and Thompson, 2003; Turcekova et al., 2003; Capuano et al., 2006; Helier et al., 2012). Two former strains (horse and cattle strains) have been recently recognized as distinct species: E. equinus and E. ortleppi, respectively (Thompson and McManus, 2002; Jenkins et al., 2005; Nakao et al., 2006; Naidich et al., 2006; Rinaldi, 2007; Simsek et al., 2011).

However, the taxonomy of E. granulosus is still in a state of flux. Only few studies aimed to the genetic characterization of E. granulosus strains have been carried out in the European Mediterranean region, with the exception of recent investigations in Spain, where the G1 (common sheep strain), the G4 (horse strain) and the G7 (pig strain) genotypes were recorded (Gonzalez et al., 2002; Daniel Mwambete et al., 2004). In the Greek region of Peloponneseus, the G1, the G3 (Indian buffalo strain) and the G7 strains were found to occur (Varcasia et al., 2006). In India, cystic echinococcosis represents one of the most important zoonosis, with relevant economic losses and a significant public health impact. Its occurrence is primarily linked to the sheep–dog cycle.

The highest prevalence values in dogs and livestock were reported from Sardinia (Gabriele et al., 2006; Garippa, 2006; Scala et al., 2006a) and Sicily (Battelli et al., 2002; Giannetto et al., 2004). On the basis of their occurrence in intermediate hosts, mainly sheep and, to a lesser extent, cattle, it has been supposed that the dominant strain occurring in Italy is the common sheep strain (G1 genotype).

The aim of the present paper is to identify the E. granulosus strains occurring in different regions of Chennai, by sequence analysis of three mitochondrial genes (cytochrome c oxidase I gene, NADH dehydrogenase subunit I gene, small subunit ribosomal mitochondrial DNA), which will be of immense value in the control of Cystic echinococcosis (CE).

MATERIALS AND METHODS

Samples and DNA extraction

In total 90 hydatid cysts were collected in buffalo from perambur slaughter house Chennai. Recovered isolates refer to protoscolices were accepted for molecular analysis. DNA was extracted from ethanol preserved or frozen samples. Genomic DNA was extracted using the QIAamp DNA mini kit (Qiagen, Germany) following manufacturer’s instructions. The obtained DNA was stored at -20°C until used for PCR amplification.

PCR amplification

Three target sequence of mitochondrial DNA NADH dehydrogenase (NADH), Cytochrome oxidase (COX 1), and small subunit of ribosomal RNA (RRNS) were amplified according to the following protocol: PCR was performed using 10 µl Amplicon Master Mix (Amplicon, Denmark), 1.0 µl Forward primer (10pm/ µl), 1.0 µl Reverse Primer (10pm/ µl), 1.0 µl DNA Template, 7.0 µl Nuclease free water(Qiagen® Germany) in 20 µl volume of reaction.

Two conserver primers COX1(Forward) 5’TTTTTGCCATCC TGAGGTATAT – 3’ and COX1 (Reverse) 5’TAA CGA CATA ACAT AAT GAAATG-3’ (Bowles et al., 1992) were used to amplify the mtDNA region corresponding to the part of COX1 gene, under following conditions: an initial denaturation step at 94°C for 1 min, followed by 30 cycles at 94°C for 30 s (denaturation), 50°C for 30 s (annealing), 72°C for 30 s (extension), and a final extension step at 72°C for 7 min.
Two conserved primers NADH (Forward) 5’AGGTTTGCC GAT TTC TTG AAG 3’ and NADH (Reverse) 5’ CAA CAG CAT AAA GCG CAA AAA ATAA 3’ (Busi et al., 2007) were used to amplify the mtDNA region corresponding to the part of NADH gene, under following conditions: an initial denaturation step at 94°C for 2 min, followed by 30 cycles at 94°C for 30 s (denaturation), 45°C for 30 s (annealing), 72°C for 40 s (extension), and a final extension step at 72°C for 7 min.

Two conserved primers RRNS (Forward) 5’ TTA AGA TATATG TGG TAC AGG ATT AGA TACCC 3’ and RRNS (Reverse) 5’ AACCGAGGGTG ACGGGCGGTGTGTACC 3’ (Busi et al., 2007) were used to amplify the mtDNA region corresponding to the part of RRNS gene, under following conditions: an initial denaturation step at 94°C for 2 min, followed by 30 cycles at 94°C for 30 s (denaturation), 55°C for 30 s (annealing), 72°C for 40 s (extension), and a final extension step at 72°C for 7 min. PCR products were detected on a 1.5% ethidium bromide stained low melting agarose gel then bands were cut from the gel and the amplified DNAs were purified by a QIAquick Gel Extraction Kit (Qiagen).

**Sequence Analysis**

Sequencing of COX1, NADH and RRNS amplicons was carried out through amnion sequence services, Bengaluru. DNA sequence comparison was achieved using Gen Bank (http://www.ncbi.nlm.nih.gov) with the BLAST system to find the best region of similarity between sequences. Multiple alignment of sequence information was done using Clustal W parameter considering default gap opening, gap extension penalty and transition weight. The extent of variation among detected mitochondrial genotype was estimated by pair wise comparisons of nucleotide sequence with available sequence information from public data base.

**Phylogenetic analysis**

Phylogenies were constructed by maximum parsimony method using homogenous pattern among lineages and tested by bootstrap with 1000 replicates using MEGA version.

**RESULTS**

**PCR Amplification**

PCR Amplification on COX1, NADH, RRNS gene showed amplicon size of 444bp (Fig-1), 323bp (Fig-2), 370bp (Fig-3).

**Analysis of COX1 gene**

Nine buffalo isolates of *Echinococcus granulosus* were sequenced for COX1 gene. The COX1 gene sequence of different genotypes (G1, G2, G3, G4, G5, G6, G7, G8, G10) available in the GenBank was used along with the sequence of COX1 gene in the present study to do the multiple sequence alignments. By multiple sequence alignment of COX1 gene nucleotide of *E. granulosus*, the nine isolates of the study showed 99% homology with G1 (common sheep strain DQ062857 from Italy), G2 (Tasmanian sheep strain)

![Fig. 1. PCR Amplification of COX1 gene](image1)

![Fig. 2. PCR Amplification of NADH gene](image2)
The present study to do the multiple sequence alignment of COX1 gene in comparison with available sequences for other genotypes of *Echinococcus granulosus* was used along with the sequence of NADH gene in the present study to do the multiple sequence alignment of COX1 gene in comparison with available sequences for other genotypes of *E. granulosus*.

**Fig. 3. PCR Amplification of RRNS gene**

**Fig. 4.** Multiple alignment of COX1 gene in comparison with available sequences for other genotypes of *E. granulosus*.
alignments. By multiple alignment, eight isolates showed 100 per cent homology with G1 (common sheep strain AJ237632), G2 (Tasmanian sheep strain AJ237633) and G3 (buffalo strain AJ237634) as was observed from Poland as per the GenBank accession (Fig-6). Phylogenetic analysis revealed that these eight isolates clade with G1, G2 and G3 genotypes (Fig-7).

**Analysis of RRNS**

Eight buffalo isolates of *E. granulosis* were sequenced for RRNS gene. The RRNS gene sequence of different genotypes (G1, G5, G6, G7) available in the GenBank was used along with the sequence of RRNS gene in the present study to do the multiple sequence alignments. Using multiple sequence of alignment eight isolates showed 100 per cent homology with (G1 sheep strain AY462127) as observed in Switzerland as per the GenBank accession (Fig-8). Phylogenetic analysis revealed that eight isolates clade with G1 genotype (Fig-9).
DISCUSSION

The results obtained indicate the prominent circulation of three strains of *E. granulosus* in Chennai, the common sheep strain (G1 genotype), Tasmanian sheep strain (G2 genotype) and the Indian buffalo strain (G3 genotype). Isolates possessing G2 genotypes were detected in buffaloes from Kolkata by Bhattacharya *et al.*, 2007. The analysis of the obtained cox1 sequences confirms the discrimination of the G1, G2, G3 genotypes as two distinct haplogroups, each composed of several variant haplotypes. In the haplogroup corresponding to the G1 genotype, the majority of isolates corresponded to the common haplotype described for the common sheep strain, whereas several G1 isolates from Chennai showed distinct haplotypes with respect to the sequence chosen as reference.

**Fig. 7.** Phylogenetic analysis of NADH gene nucleotide sequence of *E. granulosus* field isolates of Maximum parsimony method using MEGA6.0 programme

**Fig. 8.** Multiple alignment of RRNS gene in comparision with available sequences for other genotypes of *E. granulosus*
In the present study variation was observed at T56c position (C90T), (G349A). The observation in the present study is in accordance with the result of Busi et al., (2007). The observation on the occurrence of sheep strain in buffalo isolates in the present study correlates with the earlier report by Bowles et al., (1992). The possibility of the sheep strain in buffalo could be due to the life cycle of the tapeworm *E. granulosus* involving dog – sheep. Sheep is a natural intermediate host for the tapeworm because of harbouring more fertile cysts as compared to any other herbivorous intermediate host. From this type of life cycle, the tapeworm eggs could have been transmitted to buffaloes via contaminated environment, which explain the occurrence of G1, G2 genotypes in Chennai, India. It is noteworthy that the same haplotype has been recently recorded in Tunisia (M’rad et al., 2005), in China (Bart et al., 2006a), in Romania (Bart et al., 2006b), in Turkey and in Austria (Obwaller et al., 2004).

This T - C mutation at position 56 is responsible for the substitution of alanine with valine based on the translation to the corresponding peptide sequence by EMBOSS software (Rice et al., 2000). Given that the above microvariant of G1 was found in several parts of the world we interpret its occurrence as a result of retention of an ancestral shared polymorphism between G1 and G2 gene pools rather than independent mutation events, although mitochondrial introgression, even if rare, cannot be excluded. In G3 isolates, intra-strain variation in cox1 was observed only in samples from Chennai, with one major haplotype within the haplogroup, fully corresponding to the available sequence in GenBank, that was observed in 27 isolates and three additional haplotypes being detected in 3–5 isolates. One of the additional haplotypes was affiliated exclusively with the cattle host (observed in three isolates), and displayed two transitions (C = T substitutions) compared with the G3 reference sequence. According to these authors, import/export of sheep may explain the occurrence of the G2 genotype in both India.

The use of three different mitochondrial genes as markers for the identification of the genotypes allows to avoid pitfalls related to the limited usefulness of the NADH marker in distinguishing G1, G2 and G3 genotypes, given the position of one variable site between the G1 and the G2/G3 genotypes at the beginning of the sequence and the identity of nad1 sequences in the G2 and G3 genotypes. A lower rate of interpretable sequences in the NADH gene was previously observed also by Bart et al. (2004). Similarly, the rrnS marker was shown to be useful for the G1 and G3 differentiation. Unfortunately it was not possible to characterize the single G2 isolate for the RRNS gene. The total homogeneity of rrns sequences within G1 facilitates a correct diagnosis.

The occurrence of intra-strain variation may jeopardize the correct identification of hydatid cysts in some cases, thus advocating the need of using a multiple gene approach in the strain determination of *E. granulosus* complex. The present study provides the first characterization of the partial rrns mitochondrial gene towards the identification of the Indian buffalo strain. Obwaller et al. (2004) argued that sequence variation within the genotype cluster G1–G3 can be partially explained by variability within individual isolates (mainly due to heteroplasmy), thus implicating identity of these closely related genotypes questionable.

However, fixed nucleotide differences between G1 and G2 across the rrns and cox1 genes detected in this study substantiate the conserved variation between these close related genotypes. Complementary studies will be undertaken to assess in more detail a possibility of genetic
recombination within the G1–G3 cluster, and to more precisely confirm infectivity of the G3 genotype (even compared with the G1 genotype) for buffaloes as indicated in this report.

ACKNOWLEDGEMENTS

My sincere thanks to all my faculties of Department of Veterinary Parasitology to carry out this work.

REFERENCES


