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# Molecular characterization of sheep and human isolates of *Echinococcus granulosus* from temperate region of India

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Cystic Echinococcosis (CE) is a zoonotic infection of serious public health concern, caused by larval forms (metacestodes) of the tapeworm *Echinococcus granulosus*. Echinococcosis has carnivores as definitive hosts, and the herbivores and omnivores as intermediate hosts. Humans are infected accidently and are not a part of the natural life cycle of parasite. Echinococcosis is diagnosed by different ways using X-ray, CT scan, immunological and serological tests including modern diagnostic technique, i.e. polymerase chain reaction (PCR), which is very sensitive and specific in detecting echinococcosis infection (Beigh *et al.* 2017). Further, PCR has also been used in genotyping of *E. granulosus* to facilitate treatment and vaccination. PCR-based technique, have been used widely for strain characterization within *E. granulosus* (Arbabi *et al.* 2017). To the date, ten distinct genetic types (G1-G10) of *E. granulosus* sensulato (s.l.) have been characterized (Bowles and Mc Manus 1993a, Hassan *et al.* 2016, Snabel *et al.* 2016, Hammada *et al.* 2018). RFLP (Restriction fragment length polymorphism), is a perfect technique by which *Echinococcus* are identified on the basis of sequence and size of the nuclear genomic region rDNA ITS 1. It is also important technique for genotyping of *Echinococcus* spp (Bowles and McManus 1993b, Arbabi *et al.* 2017).

*E. granulosus* sensu stricto (G1-3) has the widest global distribution (McManus *et al.* 2012). Different strains of the *E. granulosus* (i.e. G1, G2, G3 and G5) are observed in animals from Western parts of India (Bhattacharya *et al.* 2008, Pednekar *et al.* 2009) and G1 and G3 strains infect the livestock of North India (Singh *et al.* 2012). The myriad of biologic variety in *E. granulosus* influences the lifecycle patterns, antigenicity, pathology, transmission dynamics and their sensitivity to drugs (Carmena and Cardona 2014, Carlos and Tamara 2016). The early diagnosis of *Echinococcus* species might be of importance for the prevention and control measures, diagnostic assays and drug therapy (McManus 2010).

Enough studies have not been carried out on the molecular and genetic variations of *E. granulosis* in Kashmir valley. Therefore the present study was designed to find out the genotypes of *E. granulosus* currently infecting Sheep and humans in Kashmir valley, using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) and to estimate the genetic variability within the strains by sequencing rDNA-ITS1 gene.

*Sample collection:* The present study was conducted from the year 2013–2016 on locally reared sheep, including both slaughtered and naturally dead cases in different regions of Kashmir valley and hydatid cyst fluid collected from human beings operated for hydatidosis in SMHS-Hospital, Srinagar. A total of 2100 sheep were screened. 87 isolates were collected, 85 from sheep and 2 isolates from human beings.

*Collection of parasite:* Fertile cysts of *E. granulosus* were recognized on the basis of Protoscolices presence. Protoscolices were isolated from the fertile cysts. Prior to DNA extraction, Protoscoleces were washed almost three times using distilled water and preserved in 70% alcohol and then stored in refrigerator until used.

*DNA extraction:* DNA was isolated from ethanol preserved, frozen or fresh samples using standard phenol/chloroform extraction method and ethanol precipitation (Sambrook *et al.* 1989).

*PCR amplification and Restriction fragment length polymorphism (RFLP-PCR)*: The RFLP-PCR was performed as described by Bowles and McManus (1993c) in the rDNA-ITS1 region of the parasite.

*Nucleotide sequencing:* DNA derived from individual hydatid cysts was subjected to sequencing by the primers employed in the PCR. The purified PCR product was sequenced in Macrogen Inc. Lab. (Geumcheon-gu, Seoul, Korea). Multiple sequence alignment was done using the MUSCLE (v3.8.31) configured for highest accuracy (MUSCLE with default settings). Data obtained were compared with the NCBI nucleotide gene bank (National Center for Biotechnology Information; www.ncbi.nlm.nih.gov/BLAST/).

In the present study, the region ITS1-PCR and linked

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ITS1-PCR-RFLP were used to characterize genotypes of *E. granulosus.* DNA isolated from hydatid cyst Protoscolices recovered from sheep and human isolates in the Kashmir Province. The rDNA-ITS1 fragment of samples including 85 from sheep and 2 of human origin was amplified with BD1 / 4S primers (Bowles and McManus 1993b). The length of amplified fragment for all isolated samples with sheep origin was 1000 bp and was between 1,000 bp and 1,100 bp in human origin samples. However, no amplification was observed in the negative controls. Similar results were reported by other workers (Bowles and McManus 1993b, Bhattacharya *et al.* 2008, Gholami *et al.* 2012, Hanifian *et al.* 2013, Dousti *et al.* 2013, Hassan *et al.* 2016, Arbabi *et al.* 2017). The PCR product obtained was subsequently digested with four restriction enzyme (*Rsa 1, Alu, Msp 1* and *Taq* 1). *Rsa 1, Alu 1, Msp 1* yielded identical fragments, 300 and 700 bp in sheep and 325 and 700 bp in humans. TaqI restriction enzyme had no effect on PCR product and after digestion intact 1000 bp fragment was seen, which is in accordance with Bowles and McManus (1993b), Gholami *et al.* (2012), Arbabi *et al.* (2017) who reported similar results**.** Molecular analysis by PCR-RFLP of ITS1 of cattle, buffalo and sheep showed similar patterns with *Msp1* and *Rsa1* (Bhattacharya *et al.* 2008). Hanifian *et al.* (2013) reported that *Rsa1* showed two bands approximately 655 bp and 345 bp. *Alu1* yielded 800 bp and 200 bp and *Taq1* had no effect on PCR product. Dousti *et al.* (2013) reported that by digesting amplified ITS1 fragment with *Alu1* restriction enzyme, yielded 200 and 800 bp fragments; with *Rsa*I, 345 and 655 bp fragments but the *Taq*I restriction enzyme had no effect on PCR product. Arbabi *et al.* (2017) using PCR-RFLP of ribosomal ITS1 gene, reported the existence of common strain (G1) from sheep, cattle, humans and goat. The isolates from all the sheep strains (G1) gave similar RFLP patterns despite of being diverse in their hosts or geographical origin. Hassan *et al.* (2016) examined human, sheep and goat ITS1 region of *E. granulosus* isolates by the use of PCR-RFLP and reported that the isolates from the human, sheep and goat samples belong to the same genotype. In India, Pednekar *et al.* (2009) reported four different genotypes of *E. granulosus* namely G1, G2, G3 and G5 genotypes in Maharashtra and Western parts of India. Singh *et al.* (2012) reported 2 genotypes, (G3) and (G1) from Punjab. Similarly, Sharma *et al.* (2013) have reported 3 genotypes of *E. granulosus* to infect the livestock in north India namely G1, G2 and G3 genotypes.

*Sequencing and phylogenetic analysis:* The ITS1 gene fragments of hydatid cyst were sequenced. GenBank (http: //www.ncbi.nlm.nih.gov/) was searched for similar sequences (Bowles *et al.* 1995, Van Herwerden *et al.* 2000, Bhattacharya *et al.* 2007, Huttner *et al.* 2009, Arbabi *et al.* 2017) with the BLAST program and a significant homology was detected with *E. granulosus* sequences. All of the isolates examined (GenBank accession nos. KY129666, KY129667, KY129668, KY129669 and KY129670) were identified as corresponding to the sheep strain (G1) of *E. granulosus* and no other genotypes were detected. The phylogenetic tree was reconstructed using the maximum likelihood method implemented in the PhyML program (v3.1/3.0 aL RT) (Fig. 1).

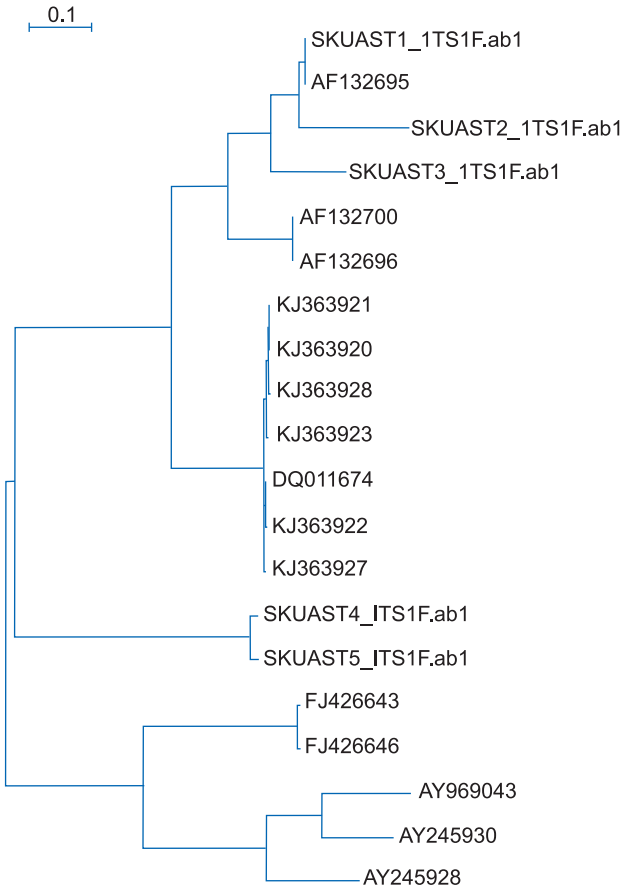


Fig. 1. Genetic relationships of haplotypes, which represent *Echinococcus granulosus* sample from Kashmir valley (SKUAST 1–5), to reference sequences selected from previous studies. The relationships were inferred based on phylogenetic analysis of ITS1 sequence.

In conclusion, the study inferred that G1 strain in sheep in Kashmir valley is a potential zoonotic parasite and its control both in definitive and intermediate host would in a long way help to curb the disease.

## SUMMARY

The aim of the present study was to investigate *Echinococcus granulosus* genotypes in the Kashmir valley. A total of 2,100 sheep and human patients were screened for the presence of hydatidosis. Total 87 isolates were collected, 85 from sheep and 2 isolates from human beings. The rDNA-ITS1 fragment were amplified with BD1 / 4S primers. In addition, fragments of genes coding for ITS1 were sequenced. The length of amplified fragment for all isolated samples with sheep origin was 1,000 bp and with human origin was between 1,000 bp and 1,100 bp. The products on digestion with restriction enzymes *Rsa 1, Alu 1, Msp 1* yielded identical fragments, 300 and 700 bp in sheep and 325 and 700 bp in humans. Intact 1000 bp fragment was observed with *Taq* 1. The molecular findings

reveal that sheep strain (G1) is the predominant genotype in sheep and humans in Kashmir valley.

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