Cystic echinococcus spp. rflp, is a perfect

Cystic Echinococcosis (CE) is a zoonotic infection of serious public healthconcern, caused by larval forms (metacestodes) of the tapewormEchinococcus granulosus. Echinococcosis has carnivores as definitive hostsand the herbivores and omnivores as intermediate hosts.

Humans are infectedaccidently and are not a part of the natural life cycle of parasite. An important factor in the epidemiology of Echinococcus granulosus is thefertility of hydatid cyst, whereas in case of humans it is a critical step forthe progress of formation of secondary echinococcosis (Oudni et al. 2006). E. granulosus has various strains throughout the world (Bowles and McManus1993a) thus varying the epidemiology, pathology and control of the Echinococcosis (Thompson and Lymbery1988). Some strains are less infective than others for humans (Eckert andThompson 1997). Varioustechniques are used in detection of echinococcosis.

PCR-based techniques areessential assets for strain characterization within E. granulosus. To thedate, ten distinct genetic types (G1-G10) of E.

granulosus sensulato(s. l.) have been characterized (Bowles and Mc Manus 1993a; Bowles et al. 1994; Nakaoet al. 2007). Echinococcus granulosus and Echinococcus multilocularisexist as different ITS1 sequence variants which represent as many as fourevolutionary lineages: (i) a sheep strain of E.

granulosus, (ii) cervid and camel E. granulosus ITS1variants (iii) ITS1 variants of horse, bovine and camel strains of E. granulosus (iv) ITS1 variantsincluding E. granulosus strainsand E. multilocularis (VanHerwerden et al. 2000). RFLP (Restriction fragment length polymorphism) is anaccurate molecular approach to confirm the differences among the Echinococcusspp.

RFLP, is a perfect technique by which Echinococcus areidentified on the basis of sequence and size of the nuclear genomic region rDNAITS 1 (Bowles and McManus, 1993b).

It is also important technique for genotypingof Echinococcus spp. (Bowles etal. 1992; Bowles and McManus1993a). E. granulosus sensu stricto(G1-3) has the widest global distribution (McManus et al.

2012; Snabel et al. 2009; Utuka et al. 2008). G1 leads to maximum of hydatidosis cases inhumans (Moro and Schantz 2009). Different strains of the E. granulosus(i.

e. G1, G2, G3 and G5) are observed in animals from Western parts of India (Bhattacharyaet al. 2008; Pednekar et al. 2009) and G1 and G3 strains infect the North India(Singh et al.

2012). Recent studies have reported the genotypes and the size ofhydatid cysts are related, patients infected with G7 genotype show smallerliver cysts than G1 genotype infected ones (Schneider et al. 2010). The myriadof biologic variety in E. granulosusinfluences the lifecycle patterns, antigenicity, pathology, transmissiondynamics and their sensitivity to drugs (Carmena and Cardona 2014).

The earlydiagnosis of Echinococcus speciesmight be of importance for the prevention and control measures, diagnosticassays and drug therapy (Thompson and McManus 2002; McManus 2010). Less data is available for themolecular and genetic variations of E. granulosis. This study can open up new avenues forthe identification and determination of strains

infective to the humans andhelp in determination of their pathogenic behavior in domestic ruminants inKashmir valley.

The aim of present study was to find out the genotypes of E. granulosus currently infecting Sheep and humans in Kashmir valley, usingpolymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP)and to estimate the genetic variability within the strains by sequencing rDNA-ITS1 gene. Materialsand methodsSamplecollectionThe present study wasconducted from the year 2013-2016 on locally reared sheep, including bothslaughtered and naturally dead cases in different regions of Kashmir valley andhydatid cyst fluid collected from human beings operated for hydatidosis inSMHS-Hospital, Srinagar.

A total of 2100 sheep were screened. 87 isolates werecollected, 85 from sheep and 2 isolates from human beings. Collection of parasite: Fertile cysts of E. granulosus wererecognized on the basis of Protoscolece presence.

Protoscoleces were isolatedfrom the fertile cysts. Prior to DNA extraction, Protoscoleces were washedalmost three times using distilled water and preserved in 70 % alcohol and thenstored in refrigerator until used. DNA extraction DNA was isolated from ethanol preserved, frozen orfresh samples using standard phenol/chloroform extraction method and ethanolprecipitation (Sambrook et al. 1989). Approximately 100 ? l of packed protoscolices was suspended in 400? lTissue lysis buffer (0.

1M NaCl, 0. 01M EDTA, 0. 1M Tris- HCl, 1% SDS). ProteinaseK (Fermentase) was added to each tube containing samples plus 400 ? l lysisbuffer and incubated at 56º C for one hour to remove excess proteins.

Vortexthese tubes for a second to mix DNA pellets in the solution. 400 ? lphenolchloroform-isoamylalcohol (Sigma) was added and centrifuged at 10000 rpmfor 10 min. The supernatant was shifted to another tube and chloroform addedwith shaking and spinning at 10000 rpm for 10 minutes. An equal volume ofIso-Propanol (Sigma) and 0.

1 ml sodium acetate (Merck, Germany) (3M, pH 5. 2)were added to supernatant, and kept at -20 ºC overnight. Next, it was spun for15 min in 15000 rpm and the sediment was rinsed by 300 ? l 70% ethanol. Afterspinning for 10 min in 10000 rpm twice and removing ethanol, pellet wasdissolved in 30 ? l nucleus free water, and kept at -20 º C for PCR process.

PCR amplification The PCR amplification was performed as described byBowles and McManus (1993c) in the rDNA-ITS1 region of the parasite using thefollowing primer pairs (BD1 and 4S): (BD1: 5´ GTC GTA ACA AGG TTT CCG TA 3´), (4S: 5´ ?- TCT AGA TGC GTT CGA TGT CGA TG 3´). The PCR was carried out in a 25 ? lreaction mixture containing: 10x Buffer (fermentas) 2. 5? l, dNTPS (10 mM)(fermentas) 0. 75? l, MgCl2 (25mM) 1. 5? l, BD1-F (12. 5 pmol) 0. 5? l, 4S -R (12.

5pmol) 0. 5? l, Taq polymerase (fermentas) 0. 3? l, Distilled water 16. 95? l, Template DNA 2. 0? l.

The PCR conditions were: Primary denaturingstep at 95°C for 5 minutes, Denaturing step at 95°C for 30 sec, Annealing stepat 50°C for 30 sec, Extension step at 72°C for 5 minutes x 30 times, Finalextension at 72°C for 5 minutes, Hold at 4°C. Aftercompletion of PCR, amplified products were

confirmed and analyzed on Agarosegels (1%) and stained with ethidium bromide. Any nonspecific reaction ordifference in size of band was observed by running the 100bp DNA ladder(Fermentas) along with PCR product. Restriction fragment lengthpolymorphism-PCR (RFLP-PCR) The PCR products were digested by 4-base cutting restrictionendonucleases Rsa 1, Alu, Msp 1 and Taq1 (10 U) using buffersrecommended by the manufacturer (ThermoFischer), which were effective on different regions of ITS1; in definedheat and time. Alu 1 =(5′ AG ? CT 3′), 37°, 6h Rsa 1 =(5′ GT ? AC 3′), 37°, 6h Taq 1 =(5′ T ? CGA 3′), 65°, 6h Msp 1 = (5′ C ? CGG 3′), 37°, 6h 7.

5 ? l PCR product wasused, the total volume was increased to 25 ? l (NFW= 14 ? l, Buffer = 2. 5 ? l andEnzyme= 1 ? l) for digestion. The sizes of the restricted products were assessedby electrophoresis in 2% (w/v) TBE agarose gel, stained with 0.

5? g/ml ethidiumbromide. Nucleotide sequencing DNAderived from individual hydatid cysts was subjected to sequencing by theprimers employed in the PCR. The purified PCR product was sequenced in MacrogenInc.

Lab. (Geumcheon-gu, Seoul, Korea). Multiple sequence alignment was doneusing the MUSCLE (v3. 8.

31) configured for highest accuracy (MUSCLE with defaultsettings). Data obtained were compared with the NCBI nucleotide gene bank(National Center for Biotechnology Information; www. ncbi. nlm. nih. gov/BLAST/).

ResultsIn the present study, the region ITS1-PCR and linkedITS1-PCR-RFLP were used to characterize genotypes of E. granulosus DNAisolated 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 7 of human originwas amplified with BD1 / 4S primers (Bowles and McManus1993b). The length of amplifiedfragment for all isolated samples with sheep origin was 1000bps (Fig.

1) andwas between 1000bps and 1100bps in human origin samples (Fig. 2). However, noamplification was observed in the negative controls (Figs. 1 and 2).

The PCR product obtained was subsequently digestedwith four restriction enzyme   (Rsa1, Alu, Msp 1 and Taq 1). Rsa 1, Alu 1, Msp 1 yielded identical   fragments, 300 and 700bp in sheep (Fig. 3) and 325 and 700 bps in humans (Fig. 4). TaqI restrictionenzyme had no effect on PCR product and after digestion intact 1000bps fragmentwas seen (Figs. 3 and 4). Boththe patterns obtained in sheep and humans were identical to common sheep strainof   `E granulosus. Sequencing and phylogenetic analysisThe ITS1 gene fragmentsof hydatid cyst were sequenced.

GenBank (http://www. ncbi. nlm.

nih. gov/) was searched forsimilar sequences (Bowles et al. 1995; Van Herwerden et al. 2000; Bhattacharyaet al. 2007; Huttner et al.

2009 and Khademvatana et al. 2013) withthe BLAST program and a significant homology was detected with E. granulosussequences. All of the isolates examined (GenBank accession nos.

KY129666, KY129667, KY129668, KY129669 and KY129670) were identified as corresponding to the sheepstrain (G1) of E. granulosus and no other genotypes were detected. The phylogenetic tree wasreconstructed using the maximum likelihood method implemented in the PhyMLprogram (v3. 1/3. 0 aL RT) (Fig.

5). Discussion The extensivevariety of E. granulosus (complex) influences its life cycle patterns, host specificity, transmission dynamics, antigenicity, pathology andsensitivity to chemotherapeutic agents (Thompson and Lymbery 1988). Bowles et al. (1995) sequenced threenucleotide data sets (CO1, ND1 and ITS1) in order to delineate relationshipsamong strains and species of the genus Echinococcus.

A highly specificidentification of E. granulosus strains requires approaches for its DNAcharacterization (Utuka et al. 2008). Extensive literature on the molecularbiological methods have been reported to discriminate Echinococcus strains(McManus 2006).

They provided evidence that E. granulosus was nota monophyletic taxon and strains within this species fall into groups whichmight merit recognition as separate species. Until now no report has beenpublished on the strain characteristics of E. granulosus in Kashmirvalley. In this study, PCR-RFLP analysis and ITS1 gene sequencing was done forthe characterization of E.

granulosus in sheep and human beings of Kashmir valley. rDNA-ITS1 fragment was amplified with BD1 /4S primers. The length of amplified fragment for all isolated samples withsheep origin was 1000bp and with human origin was between 1000bps and 1100bpscharacteristics of the sheep strain. Similar results were reported byother workers (Bowles and McManus 1993b; Ahmadi and Dalimi 2006; Bhattacharyaet al.

2008; Shahnazi et al. 2011; Gholami et al. 2012; Ery? ld? z and Sakru 2012; Khademvatana et al. 2013; Hanifian et al. 2013; Dousti etal. 2013; Hashemi Tabar et al.

2015). In contrast, Madawy et al. (2011) reportedthat PCR amplification of ITS1 gene ofhydatid cysts from sheep and cattle showed similar pattern of PCR product ofall isolates with amplified DNA band of the same molecular size at 1115bp. The amplified products of ITS1-PCR with BD1 and 4S primers were 1. 0 and 1. 1 kb, in Mexico from pigs (Villalobos et al.

2007). Vahedi et al. (2014) reported that size of amplicon for ITS1-PCR in caseof humans in Azebaijan province was 900 bp.

In the present studyaliquots of amplified ITS1 fragments were digested by 4-base cuttingrestriction endonucleases MspI, RsaI, AluI and TaqI. Two clearly distinguishable patterns wereobtained with Rsa 1, Alu 1, Msp 1, which yielded identical fragments, 300 and 700 bp in sheep and 325and 700 bp in humans which areidentical to sheep strain (G1) of E. granulosus. Whereas, TaqI had no effect on PCRproduct, which is in accordance with Bowles and McManus (1993b); Ery? ld? z and ? akru (2012); Gholami et al.(2012) who reported similar results.

Molecular analysis byPCR-RFLP of ITS1 of cattle, buffalo and sheep showed similar patterns with Msp1 and Rsa. 1 (Bhattacharya et al. 2008). Molecularanalysis of ITS1 gene of cattle and sheep isolates by RFLP showed no variationsand showed similar patterns in all the isolates with Msp1 and Rsa1. Digestionof amplification product of ITSI with MSP1 yielded 661 bp and 406 bp, whileaswith RSA1 yielded 745 bp and 360 bp fragments (Madawy et al.

2011). Similarly, Hanifian et al. (2013) reportedthat Rsa1 showed two bands approximately 655bp and 345bp. Alu1 yielded800bp and 200bp and Taq1 had no effect on PCR product. RsaIrestriction endonucleases showed two different bands, 300 and 600 bps in cattleand sheep. Furthermore, Vahedi et al.

(2014) used restriction endonucleases (RsaI and HpaII), showedtwo different bands, 300 and 600 bps and 200 and 600 bps and reported all thestrains as G1in human. Dousti et al. (2013) reported that by digesting amplified ITS1 fragment with Alu1restriction enzyme yielded 200 and 800 bp fragments; with HpaII, 300and 700 bp fragments; and RsaI, 345 and 655 bp fragments. The TaqIrestriction enzyme had no effect on PCR product. The sequence and size of ITS 1 (nuclear genomic rDNA region)can be used as a reliable genetic marker for easy and rapid identification of Echinococcusisolates using PCR-RFLP analysis (Ahmadi and Dalimi 2006). Bowles andMcManus (1993b) using PCR-RFLP of ribosomal ITS1 gene, examined 149 isolatesfrom various intermediate hosts and reported the existence of different strainsfrom sheep, camel, horse, cattle and pig.

The isolates from all the sheepstrains (G1) gave similar RFLP patterns despite of being diverse in their hostsor geographical origin. Ahmadi and Dalimi (2006) examined human, sheep andcamel ITS1 region of E. granulosus isolatesby the use of PCR-RFLP and reported that the isolates from the human and sheepsamples belong to the same genotype while as the camel isolate belonged to thecompletely different genotype. InIndia, Pednekar et al. (2009) reported four different genotypes of E. granulosus namely G1, G2, G3 and G5 genotypes in Maharashtra and Westernparts of India.

G3 (63%) was predominant followed by the G5 (19. 56%), G1 (13%)and the G2 (4. 34%). Singh et al., (2012) reported 2 genotypes, (G3) and (G1)from Punjab.

Similarly, Sharma et al. (2013a) have reported 3 genotypes of E. granulosus to infect the livestock in north India: G1, G2 and G3 genotypes. The G3 genotype (71. 8%) was found as predominant genotype. The second mostcommon genotype was the G1 genotype in 27. 16 % isolates followed by the G2genotype from livestock (cattle, buffalo, pig and sheep). Sharma et al.

,(2013b) have revealed the zoonotic potential of G1-G3 complex (94%), G5 (3. 1%)and G6 (3. 1%) genotypes of E. granulosus by genotyping of hydatid cystsfrom Cystic Echinococcosis (CE) patients. The ITS1 sequence data obtained in this studyconfirmed RFLP patterns and were identified as corresponding to the G1 strain ofE.

granulosus. The sheep strain, confirmed as G1 at the DNAsequence level, showed remarkable uniformity with isolates from sheep fromgeographically diverse regions as reported previously by other workers (Bowleset al. 1995; Van Herwerden et al. 2000; Bhattacharya etal. 2007; Huttner et al.

2009 and Khademvatana et al. 2013). In conclusion, the study inferred that G1strain in sheep in Kashmir valley is a potential zoonotic parasite and itscontrol both in definitive and intermediate host would in a long way help tocurb the disease.