Journal of Animal & Plant Sciences, 31(5): 2021, Page: 1293-1301
ISSN (print): 1018-7081; ISSN (online): 2309-8694
https://doi.org/10.36899/JAPS.2021.5.0330

MOLECULAR AND BIOCHEMICAL CHARACTERIZATION OF *ECHINOCOCCUS* SPP. IN HYDATID CYST FLUID COLLECTED FROM HUMAN AND LIVESTOCK IN NORTHERN KHYBER PAKHTUNKHWA AND GILGIT BALTISTAN

M. Abdullah[1], I. Ali[2], K. S. Haleem[3], A. U. Rehman[1], S. Qayyum[3], Z. Niaz[3], S. Ahmed[3], I. Khan[4], M. N. K. Khattak[5], N. Sultana[6] and I. Tauseef[3*]

[1]Department of Zoology, Hazara University Mansehra, 21300, KPK, Pakistan; [2]Institute of Biotechnology & Genetic Engineering, University of Agriculture, Peshawar, Pakistan; [3]Department of Microbiology, Hazara University Mansehra, 21300, KPK, Pakistan; [4]Department of Microbiology, AUST, Abbottabad; [5]Department of Applied Biology, College of Sciences, University of Sharjah, United Arab Emirates; ; [6]Department of Biochemistry, Hazara University Mansehra, 21300, KPK, Pakistan.
[*]Corresponding Author's E-mail: isfahan@hu.edu.pk

ABSTRACT

The *Echinoccocus* spp. infection results in cystic or alveolar echinococcosis causing billions of dollars loss to the international economy in respect of expensive medical treatments, permanent loss of entire or part of organ, and a substantial decrease in livestock productivity. Therefore, the study was designed with objectives to know existence of the species, their genotypes and biochemical profile of hydatid cyst fluids of *Echinococci* in the samples collected from northern Khyber Pakhtunkhwa and Gilgit Baltistan. Cyst samples (n = 46) were collected from two different sources i.e. livestock and human (n=34 and n=12, respectively). PCR-RFLP based analysis was used to investigate the mitochondrial gene (*rrnL*, large subunit of ribosomal RNA) based species detection and genotyping. Biochemical assay of all hydatid cyst fluid was performed to analyze the concentration of various parameters i.e. uric acid, glucose and triglyceride etc. One-way ANOVA followed by Tukey's post-hoc test was also performed to determine the statistically significant differences among the biochemical parameters. Among all collected cysts, 56.5% (n=26) were positive for *Echinococcus granulosus* while 43.4% (n=20) were detected as *Echinococcus multilocularis*. Genotyping of the positive *E. granulosus* revealed the highest frequency (>90%) of G1–3 genotype while G6 genotype was found at low level (<10%). In case of human, all samples were positive for G1–3 genotype. Biochemical analysis of cyst fluids showed a significant ($P \leq 0.05$) interspecies variation in all tested parameters. In conclusion, *E. granulosus* and *E. multilocularis* are found frequently in northern KP and Gilgit Baltistan. Further, *E. granulosus sensu stricto* (G1-3) and G6 genotypes are responsible for infections in both human and animals. A comprehensive surveillance program in whole northern Pakistan is needed for effective prevention/control of *Echinococcus* species.

Keywords: Cyst, *Echinococcus*, Human, Livestock, Genotypes of *E. granulosus*
Published first online January 21, 2021 Published final August 07, 2021.

INTRODUCTION

Echinococcus, an important cyclophyllid cestode tapeworm of canids and other animals, causes a zoonotic disease known as cystic echinococcosis (CE) (Rojas *et al.* 2014). Among all known species of *Echinococci*, *E. granulosus* and *E. multilocularis* are clinically important and cause cystic echinococcosis or alveolar echinococcosis, respectively (McManus *et al.* 2012; Shanshan *et al.* 2018). Globally, the annual economic losses due to this disease have been estimated as reaching up to 3 billion US dollars (WHO, 2013 and 2017). The worm resides in the digestive tract of the definitive host (domestic/wild canids) whereas the oncosphere produces hydatid cyst in the intermediate hosts (wild or livestock mammals). Human CE, reported worldwide, occurs by accidental ingestion of eggs leading to development of fluid-filled hydatid cysts which multiplicate there asexually (Rokni, 2009). Cyst fluid contains a number of organic and inorganic constituents i.e. protein, lipid, carbohydrates, electrolytes and trace elements which may have a key role in the immunology, metabolism and physiology of cystic echinococcosis (Shanshan *et al.* 2018). Demographic changes and a variation in genotypes of *Echinococcus* results in a change in the level/concentration of these biochemical parameters (Eslami *et al.* 2016).

Genetic diversity in *E. granulosus* species has been reported since long and it is suspected that this interspecies variation may influence the infectivity and various other characteristics i.e. morphology, specificity of host and epidemiology (Carmena and Cardona, 2014). Mitochondrial DNA sequence analysis has identified ten heterogeneous groups (G1-G10) of variants in *E. granulosus*. Among them, three genotypes, the G1-G3 (G1-G2 found in sheep while G-3 in buffalo), are

grouped in the species *E. granulosus sensu stricto* while other genotypes are *E. equinus* which is found in horses (G4), *E. ortleppi* in cattle (G5), *E. Canadensis* (G6–10 found in camels, pigs and cervids, respectively) and the lion strain (*E. felidis*) (Nakao *et al.* 2007; Rojas *et al.* 2014; Amer *et al.* 2015).

Frequent screening for detection of species and knowledge of circulating genetic variants of *E. granulosus* from a range of hosts in a selected area could be a useful strategy for not only in understanding the disease dynamics but also for designing effective diagnosis, control, prevention measures and treatment (Shariatzadeh *et al.* 2015; Carmena and Cardona, 2014; Wahlers *et al.* 2012). Molecular based approaches are considered as a reliable tool to identify and differentiate the circulating *Echinococcus* spp. Among these, Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) analysis is of paramount importance for genotyping with higher accuracy to confirm the distinctiveness among *Echinococcus* spp. based on sequence-specific endonucleases (Hanifian *et al.* 2013; Ali *et al.* 2015). Nucleotide alterations in the genetic variants of *E. granulosus* populations can readily be detected by this highly verified molecular diagnostic procedure enabling the molecular discernment of genotypes (Dousti *et al.* 2013).

Lack of awareness and meager sterile circumstances regarding the parasite life cycle have made the conditions favorable for the persistence of the infection in many regions of Pakistan (Latif *et al.* 2010; Khan *et al.* 2018). It is believed that *Echinococcus* species may have been introduced into Pakistan from China and Iran (Harandi *et al.* 2002; Xiao *et al.* 2005; Xiao, *et al.* 2006; Parsa *et al.* 2012). Being the closest region to China, having suitable environmental conditions and pastures for traditional rearing of domestic animals, the emergence of echinococcosis is very likely in northern Khyber Pakhtunkhwa and Gilgit Baltistan regions. Further, as the echinococcosis prevalence is linked with the economic dependence on livestock, the people of northern area are continuously exposed to *Echinococcus* species because they mainly rely on livestock for daily food requirements (Latif *et al.* 2010). Hence, the current study was designed to straight the loop and was aimed to determine the frequency of *Echinococcus* spp. and distribution of sub types of *E. granulosus* in livestock and human residing in the areas under investigation.

MATERIALS AND METHODS

Experimental subjects and sample collection: A total of 46 hydatid cysts samples (Sheep (n=15); goats (n=12); buffalo (n=7) and humans (n=12) were collected from the northern areas of KP (Chitral, Swat, Mingora and Mansehra) and Gilgit Baltistan. The inclusion criterion was "all patients/animals with one or more than one hydatid cysts and belonging to the selected areas". Exclusion criteria were the "human/animals found negative for the presence of cysts or not belonging to the study areas".

In case of humans, the cysts were removed surgically from infected patients while the cysts from animals i.e. buffaloes, goat and sheep were taken from the slaughterhouses. Briefly, for the collection animal's cyst, different abattoirs of northern Khyber-Pakhtunkhwa and Gilgit Baltistan were visited once a week and the samples from liver, lungs or other visceral regions were collected. The level of damage to viscera was also considered for each animal. Similarly, for human cysts collection, the samples were obtained from surgery units of different northern area hospitals and/or from those northern area's patients who were brought to Lady Reading Hospital Peshawar (LRH) for admission/treatment (Ethical Approval No: F. No.73/ HU/ORIC/ IBC/ 2015/05). The collected cyst samples were transferred into sterile capped containers having approximately one volume (v/v) of 70% ethanol and stored at 4°C until further processing.

Sample Preparation: Cyst's fluid was taken from all samples and the contents (fluid and germinal layer) were aspirated. Washing with phosphate buffer saline (PBS) was performed by spinning the mixture at 1200 g for 15 minutes. The pellets were then kept at -20°C for DNA extraction and the supernatant were used for biochemical analysis.

DNA Extraction: All the procedures for extraction of DNA were carried out in the Institute of Biotechnology & Genetic Engineering, University of Agriculture, Peshawar, Pakistan. DNA from pellets was extracted using a Genomic DNA extraction kit (Biomatik; manufactured by Geneaid, USA Cat# GEB100) and the instruction manual provided by the manufacturer.

Mitochondrial gene (*rrnL*) amplification for genotyping of *E. granulosus:* A fragment of 570 bp of the mitochondrial gene was amplified using previously reported primers (Xiao *et al.* 2006), Ech-LSU/F (5'-GGTTTATTTGCCCTTTTGCATCATGC-3') and Ech-LSU/R (5'-ATCACGTCAAACCATTCAAACAAGC-3'), manufactured and supplied by Invitrogen (Carlsbad, California). Amplification of gene was performed using two µL template (DNA), one µL of each forward and reverse primer (10 µM), 0.5 µL *Taq* DNA polymerase (Fermentas USA; Cat# EP0401), one µL $MgCl_2$ (1.25 mM final concentration) and 0.5 µL dNTPs (2 mM). The PCR Grade water was used for the adjustment of the final volume up to 20 µL.

The entire amplification reactions were performed in a regular DNA thermal cycler (Bio Rad, Model#T100). The conditions for PCR were first

optimized using a range of annealing and denaturation duration. Following optimized conditions were found suitable and selected for PCR of samples of this study; initial denaturation at 94°C for 5 min, denaturation at 94°C for 40 seconds, primer annealing at 66°C for 60 seconds and extension at 72°C for 60 seconds. The amplification reaction was repeated for 35 cycles followed by a final extension at 72°C for 7 minutes. Negative and positive controls were also set up in every reaction to check the reliability of the reaction. The amplified product (10 µl) was electrophoresed on agarose gel (2%) and visualized/photographed in UV trans-illuminator (UVitec, Cambridge; Model# Essential V6).

PCR-Restriction Fragment Length Polymorphism Analysis for identification of species: For removal of polymerase enzyme, nucleotides, primers and buffer components and avoid hinderance by these components during downstream processing, the cleansing and precipitation of PCR product were performed using ethanol and the resultant pellet was collected and re-suspended in 15 µ1 of PCR grade water. For identification, the purified PCR products of *Echinococcus* species were digested with the *Ssp1* (New England Biolabs, UK; Cat# R0132S) restriction enzyme in a 50 µ1 mixture (Xiao *et al.* 2005; Xiao *et al.* 2006). The concentration of each ingredient was taken as per manufacturer's protocol (10 µ1 PCR product; 1 µ1 restriction enzyme; 5 µ1 buffer and 34 µ1 nuclease free water) and the mixture was incubated for overnight (~ 16 hours) at 37 °C.

Genotyping of *Echinococcus granulosus* through PCR-RFLP: Genotyping was performed through digestion of PCR products of *E. granulosus* with *BglII* (New England Biolabs, UK; Cat# R0144S) restriction enzyme as described previously (Xiao *et al.* 2005; Xiao *et al.* 2006). In accordance with manufacturer's instructions, a 50 µ1 mixture (10 µ1 PCR product; 1 µ1 *BglII* enzyme, 5 µ1 reaction buffer; 34 µ1 nuclease free water) was prepared in a PCR tube and incubated at 37°C for 16 hours. Finally, the digested products along with 50 bp ladders (Gene Ruler, Fermentas; Cat# SM0373), were visualized on 2% of pre-stained (ethidium bromide) agarose gel.

Biochemical analysis of hydatid cyst fluids: The supernatants collected above were spun at 10000 g under low temperature conditions (4°C) for five minutes and analyzed for glucose, urea, uric acid, triglycerides and cholesterol through commercially available diagnostic Kits (ELITech clinical system; France) and the protocol contained within. Similarly, total protein, Creatinine, Calcium, Sodium and Potassium were estimated by Merck diagnostic kits (Merck, Germany).

Statistical Analysis: The data obtained for determination of biochemical parameters are presented as Mean±SD and were analyzed through one-way ANOVA followed by Tukey's post-hoc test to see the statistical difference between the groups using webpage statpages.info free online calculator.

RESULTS

Observation of hydatid cysts from human and animals: In case of humans, the cysts were surgically removed and collected. Few representative images of hydatid cysts obtained from human liver and ovary are shown in Fig.1 (A-C). In case of animals, the images of infected viscera and hydatid cysts were also obtained. Representative images of both (viscera and cyst) obtained from a buffalo are shown in Fig. 1(D-E). The level and extent of damage caused by hydatid cysts was also observed and shown as Fig.1F.

Confirmation of *Echinococcus* in the samples through PCR: Molecular identification and confirmation of all hydatid cysts was performed using the conventional PCR. Samples from all sources (human and animals) were amplified and their products were run on 2% Agarose gel (Fig. 2). A distinctive fragment of ~ 565 base pair (bp) of the mitochondrial *rrnL* gene was identified for the conformation of presence of *Echinococci*. The confirmatory band was found in positive control and also in all tested samples of this study, hence all hydatid cysts collected in this study were found positive for *Echinococci*.

The figure shows only representative samples on gel after PCR amplification. The size of amplified product (~ 565 bp) was confirmed by comparing with marker DNA. **Lane 1**; amplified DNA from positive control; **(Lane 2-5)**; Ladder DNA **(Lane M)**; Negative control **(Lane NC)**.

PCR-RFLP analysis for differentiation between *E. multilocularis* and *E. granulosus* species of *Echinococci:* The differentiation between both species was performed using *Ssp1* restriction analysis. Digestion of samples and presence of two fragments (295 and 270 bp) represented the *E. multilocularis* while presence of undigested (565 bp) fragment represented the *E. granulosus* (Fig. 3A). Out of 34 animal's samples, 21 (61.7 %) and 13 (38.2 %) were confirmed *E. granulosus* and *E. multilocularis*, respectively (Figure 3b). There was no indication of *Echinococcus oliogarthrus, Echinococcus shiquicus* and *Echinococcus vogeli*, the remaining species of this genus. Likewise, in humans, out of 12 tested samples, five (41.6 %) were *E. granulosus* and seven (58.3 %) were *E. multilocularis* as shown in (Fig. 3B).

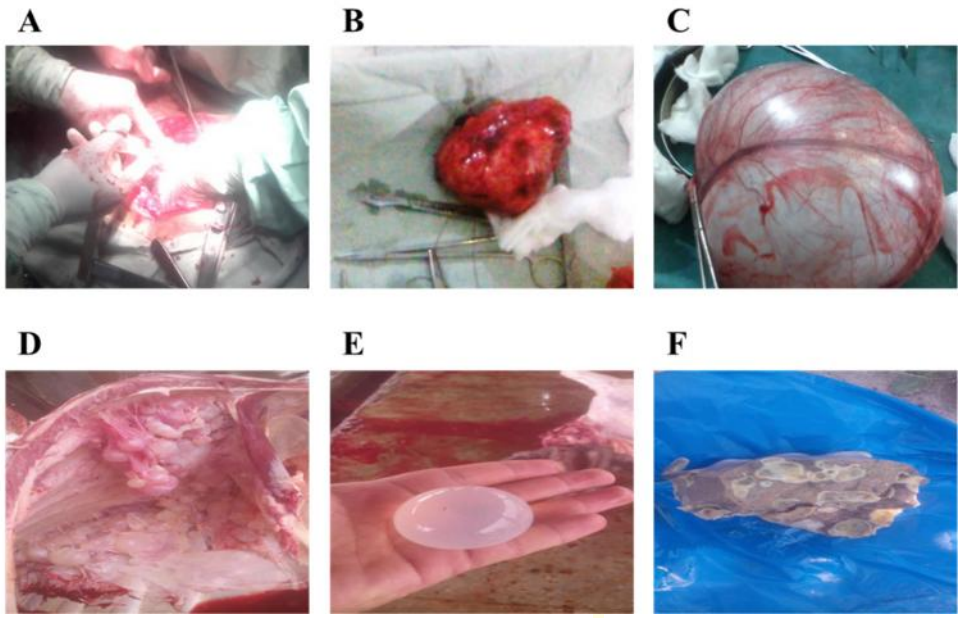

Fig. 1: (A) Intraoperative image of a patient with liver hydatid cyst. **(B)** Surgically removed pre-rinsed hydatid cyst of *Echinococcus granulosus* from humans. **(C)** Huge ovarian hydatid cyst isolated from a 16-year patient. **(D-E)** The infected viscera and hydatid cysts obtained from a buffalo. **(F)** The level of damage to viscera.

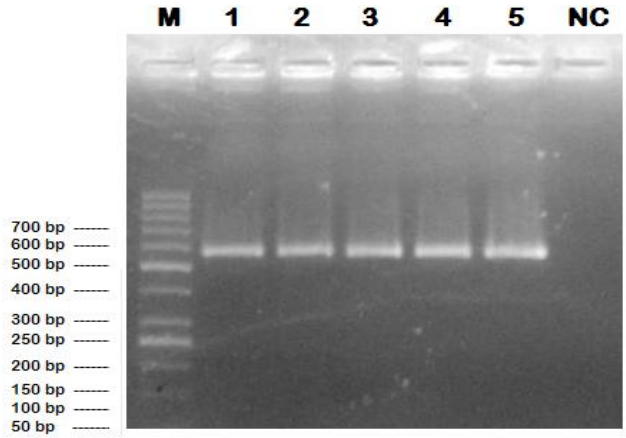

Fig. 2: Amplified fragment of *Echinococcus* on a 2% agarose gel.

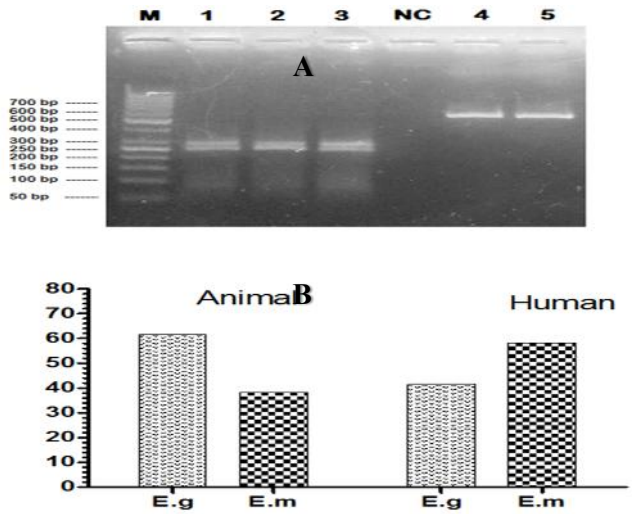

Fig. 3: PCR-RFLP based detection and frequency of *Echinococcus* species in animal and human samples. **(A)** Digested DNA (10 µL) was loaded on 2% pre-stained agarose gel. Presence of digested fragments (295 and 270 bp) confirmed *E. multilocularis* (Lane 1-3) while no digestion (~565 bp) confirmed the presence of *Echinococcus granulosus* (Lane 4-5). Marker/Ladder (Lane M). Negative control (Lane NC). **(B)** Frequency (percent) of *Echinococcus granulosus* and *E. multilocularis* species in animal and human samples. (E.g: *Echinococcus granulosus*; E.m: *E. multilocularis*)

Genotyping of *Echinococcus granulosus* by PCR-RFLP: The PCR amplified products of confirmed positive samples of *E. granulosus* were subjected to another restriction digestion to determine their genotypes. The DNA samples were restricted by using enzyme *BglII* to determine the pattern of digestion. In case of successful digestion, two bands of sizes 158 bp and 407 bp were generated (Fig. 4). Digested samples represented the genotype-6 (lane 1-2) while undigested samples represented genotype 1-3 (G1-3). Out of 21 animal samples, 90.4% (n=19) were found to be *E. granulosus* G1-3 type while only 9.6% were identified as G6 strain. In case of human (n=5), 100% of *E. granulosus* were identified as G1 genotype.

Ten µL DNA amplified from *Echinococcus granulosus* digested with *BglII* was run on 2% agarose gel and the digestion pattern was observed. Lane 1-2 represent genotype G6 generating two bands (158 bp and 407 bp). Marker DNA (Lane M).

Biochemical profile of isolated cysts: The biochemical analysis among different animals (sheep, goat, buffalo) and human revealed that glucose concentration varies among cysts of different sources (Table 1).

The glucose level of hydatid cyst fluid of sheep and goats was significantly higher (P ≤0.05) compared to buffalo and humans while it was higher in sheep compared to goats. The total protein contents were significantly higher (*P* ≤0.05) in the cyst fluid of buffalo compared to other sources. Similarly, the estimated level of Urea concentration was significantly higher (*P* ≤0.05) in the cyst fluid of human when compared to others (Table 1). For uric acid, the concentration was significantly higher (*P* ≤0.05) in the *Echinococcus* cyst fluid of human compared to the other hosts. Further, triglycerides in the cyst fluid of buffalo and sheep were found significantly higher (*P* ≤0.05) when compared with the other hosts.

In contrast, the concentration of Cholesterol in human cyst was higher (*P* ≤0.05) than all other sources. For Creatinine, a significantly higher concentration (P ≤0.05) was found in the cyst obtained from sheep compared to others. Creatinine was also statistically higher (P ≤0.05) in goats samples compared to humans. Calcium level in hydatid cyst fluid collected from buffalo was significantly high (*P* ≤0.05) compared to other hosts. The sodium and potassium concentrations of *Echinococcus* cyst fluid were significantly higher (*P* ≤0.05) in sheep compared to rest of the hosts (For detailed comparison, please refer to Table 1).

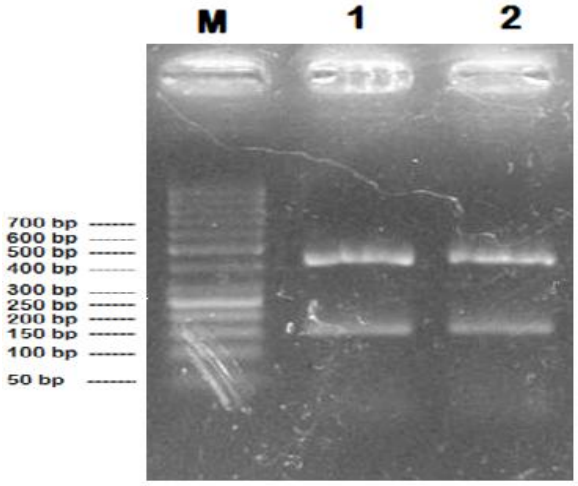

Fig. 4: *BglII* digestion pattern of *E. granulosus* amplified DNA for determination of genotypes.

Table 1: Biochemical parameters of Hydatid cysts fluid from human and animal sources.

Parameter	Host Origin				
	Units	Sheep (n=15)	Goat (n=12)	Buffalo (n=07)	Human(n=12)
Glucose	mmolL^{-1}	2.7±0.74$_{*vsG,B,H}$	2.1±0.18$_{*vs\ S,H}$	1.6±0.34$_{*vs\ S}$	1.2±0.08$_{*vs\ S,G}$
Total Protein	gL^{-1}	0.15±0.01$_{*vs\ B}$	0.073±0.006$_{*vsB,H}$	0.36±0.004$_{*vsS,B,H}$	0.045±0.004$_{*vs\ G,B}$
Urea	mmolL^{-1}	9.2±0.05$_{*vs\ G,B,H}$	8.2±0.1$_{*vs\ S,B,H}$	6.6±0.16$_{*vs\ S,G}$	10±0.5$_{*vs\ S,G,B}$
Uric Acid	mmolL^{-1}	0.61±0.06$_{*vs\ G,B,H}$	0.48±0.093$_{*vs\ S,B,H}$	0.18±0.013$_{*vs\ S,G,H}$	0.83±0.06$_{*vs\ S,G,B}$
Triglyceride	mmolL-1	0.12±0.015$_{*vs\ B}$	0.14±0.025$_{*vs\ B}$	0.77±0.04$_{*vs\ S,G,H}$	0.20±0.25$_{*vs\ B}$
Cholesterol	mmolL^{-1}	0.32±0.4$_{*vs\ H}$	0.16±0.026$_{*vs\ H}$	0.12±0.013$_{*vs\ H}$	0.94±0.03$_{*vs\ S,G,B}$
Creatinine	µmolL^{-1}	49±3.7$_{*vs\ G,B,H}$	42±2.1$_{*vs\ S,H}$	40±1.8$_{*vs\ S}$	37±1.0$_{*vs\ S,G}$
Calcium	mmolL^{-1}	2.5±0.11$_{*vs\ G,B}$	2.8±0.057$_{*vs\ S,B}$	2.9±0.044$_{*vs\ S,G,H}$	1.4±0.043$_{*vs\ B}$
Sodium	mmolL^{-1}	150±4.7$_{*vs\ G,B,H}$	130±2.2$_{*vs\ S,B,H}$	115±4.0$_{*vs\ S,G,H}$	135±5.1$_{*vs\ S,G,B}$
Potassium	mmolL^{-1}	6.4±0.26$_{*vs\ G,B,H}$	5.3±0.34$_{*vs\ S,B}$	3.9±0.42$_{*vs\ S,G,H}$	5.0±0.34$_{*vs\ S,B}$

*Indicates significant difference at *P ≤0.05*.

The abbreviation S (sheep), G (goats), B (buffalo) and H (humans). The data are presented as Mean±SD and ANOVA posthoc tukey test is used to determine the significant difference overall and between the different groups.

DISCUSSION

Pakistan is one of the neglected endemic regions for *Echinococci* where majority of population (~96%) have not even heard about the worm (Khan *et al.* 2018). Although few studies in Punjab and Sindh provinces have produced some valuable data, frequency of *Echinococcus* species and the distribution of genotypes in northern Khyber Pakhtunkhwa and Gilgit Baltistan is not well documented. Therefore, an investigation concerning local *Echinococcus* species and their genotypes was required. Hence, the results of this study document the first-hand information on these aspects.

The PCR-RFLP data revealed that the species circulating in human and animals of study areas are either *E. granulosus* or *E. multilocularis*, though *E. granulosus*

was predominant. These findings are in accordance with previous studies conducted locally and worldwide (Muqaddas *et al.* 2019; Guo *et al.* 2019). Another local study which used same primers and enzyme for RFLP based analysis has also reported the abundance of *E. granulosus* in the collected samples (Ali *et al.* 2015). High frequency of this strain suggests its high transfer potential between animals and other hosts (Latif *et al.* 2010).

Analysis of genetic diversity in *E. granulosus* showed that genotype G1-3 is the main causative agent for *Echinococcus* related infections in human and animals of the selected areas. These results are in agreement with various studies conducted in different countries for genotypic analysis of *E. granulosus* and reported G1-3 as the most prevalent source of infection both in animals and human (Euzeby, 1991; Breyer *et al.* 2004; M'rad *et al.* 2005; Romig *et al.* 2006; Varcasia *et al.* 2007; Busi *et al.* 2007; Schneider *et al.* 2008; Pezeshki *et al.* 2013). A score of other genotyping studies based on PCR-RFLP method have also reported the predominance of G1-3 strain in tested population (Tigre *et al.* 2016; Fallahizadeh *et al.* 2019). This validates that in addition to cost-effectiveness, this method is authentic as well. Results of this study are also in good agreement with few studies conducted at local level. Studies conducted in Punjab and Sindh Provinces have reported either exclusive or predominant presence of G1-3 genotype (Ehsan *et al.* 2017; Latif *et al.* 2010; Mehmood *et al.* 2020). Similarly, a local RFLP based study with same primers and restriction enzymes, reported the over-representation of G1-3 in the population (Ali *et al.* 2015). This necessitates the launching of extensive awareness, prevention and control programs.

The exact reason for the predominance of G1-3 genotype in the study area is unclear. One reasonable explanation for higher frequency of G1-3 genotypes in northern areas of Pakistan is that "as the G1-3 genotypes are prevalent in Chinese region sharing boundary with northern Pakistan" (Xiao *et al.* 2006; Guo *et al.* 2019), demographic expansions of the parasite might have resulted due to the movements of carrier hosts. Further, as Iran is a hyperendemic area with G1-3 predominant genotypes and has shared borders, it is quite likely that the spread of G1-3 genotypes may have occurred from this region (Xiao *et al.* 2006; Parsa *et al.* 2012; Khademvatan *et al.* 2013). In addition, the presence of *Echinococcus* infected stray dogs with local migratory tribes, might have further added to the potential of parasitic migration and transmission, as reported elsewhere (Daryani *et al.* 2007).

Another likely explanation for spread is the higher susceptibility of human to G1-3 genotypes than G6 genotype, as proposed by a previous study (Piccoli *et al.* 2013). The third possibility for the low presence of G-6 genotype (and higher frequency of G1-3) may be due to the fact that its transmission is low due to some unidentified factors responsible for the reduction of contamination by this specific genotype. Though the effect of low contamination on prevalence of G-6 has been hypothesized in a number of previous investigations (Ahmadi and Dalimi 2006; Utuk *et al.* 2008; Vural *et al.* 2008; Šnábel *et al.* 2009), a concrete evidence of its low infectivity is still missing.

Echinococcus cyst fluid contains a score of organic and inorganic constituents (Juyi *et al.* 2013). The ingredients in the cyst play a key role in the immunology, metabolism and physiology of cystic echinococcosis (Siracusano *et al.* 2012). This implies that the function and biochemical nature of the specific organ involved might have an effect on the nature of cyst fluid. Some electrolytes of different organs actively diffuse through cyst layers which easily change the biochemical parameters and alter the cyst fertility and expansion (Conchedda *et al.* 2016). Therefore, chemical estimation can provide useful information about *E. granulosus* sub types and identification of host (Sharma *et al.* 2013). The data of biochemical profiles presented significant ($P \leq 0.05$) differences among livestock and human isolates. It was found that Glucose, Creatine, Sodium and Potassium are lower in the cyst fluid of buffalo, goat and human when compared with sheep. This demonstrates that the range of these biochemical profiles is not influenced by the previous hosts. Contrastingly, Triglyceride, Calcium and total protein were found lower in hydatid cyst fluid of sheep, goat and human as compared to buffalo. Though a previous study didn't notice significant variations in the profiles of protein, lipid, cholesterol and glycerides in cysts isolated from liver and lungs of humans and sheep (Sharif *et al.* 2005), a recent study reported that hydatid cyst fluids contained significantly more glucose and Ca^{2+} ($P \leq .05$) as compared to liver simple cysts (Shanshan *et al.* 2018).

The level of Urea, Uric acid and Cholesterol in the *Echinococcus* cyst were lower in sheep, goat and buffalo as compared to human isolates. These results are consistent with a previous investigation which showed higher level of uric acid in human *Echinococcus* cyst when compared with livestock cyst fluid (Juyi *et al.* 2013). A high concentration of uric acid may be a sign of degenerative changes in hydatid cysts of humans. Surprisingly, in the isolates from goat, all biochemical parameters were lower than other animals. Such lower levels of biochemical constituents in goats have also been reported in a study conducted in Iran (Radfar and Iranyar, 2004). In our study, the level of potassium was also comparable to a couple of previous investigations (Izadi and Ajami, 2006). In summary, a significant difference of biochemical profile in *Echinococcus* cyst content of different secondary hosts (human and livestock) was observed. This difference in concentration of *Echinococcus* cyst contents in livestock and humans may

be due to the fact that several genotypes of *E. granulosus* are circulating in Northern, Pakistan.

Conclusion The current results revealed that *E. granulosus* and *E. multilocularis* are the frequently found species of *Echinococci* in northern Khyber Pakhtunkhwa and Gilgit Baltistan regions. At genotypic level, *E. granulosus sensu stricto* (G1) and G6 genotypes are responsible for infection in both human and animal hosts. Further, the identification of species and genotypes of *Echinococcus* on the basis of PCR-RFLP is a functional and viable method. Moreover, biochemical investigations will be valuable for immunological studies, diagnostic tests and may help to confirm different genotypes of *E. granulosus*.

Author contributions MA and IA performed the experiments. SQ, ZN, IK, SA and MNK provided technical expertise in the experimentation and data analysis. NS, AR and KSH participated in biochemical analysis, and preparation/editing of the manuscript. IA and IT designed and supervised the experimental study and prepared the final manuscript.

Ethical Approval Statement The study was approved (F. No.73/HU/ORIC/IBC/2015/05) by the Ethical Committee of Hazara University, Mansehra, Pakistan.

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