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RESEARCH ARTICLE

High Genetic Variability in Full-length *cox*2 and *nad*6 Genes of *Echinococcus granulosus* sensu stricto and *Echinococcus ortleppi* Recovered from Cattle

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ABSTRACT

Echinococcus granulosus (G1/G3) and *E. ortleppi* (G5) are prevalent worldwide. Numerous studies have been conducted to characterize these parasites and utilized mainly *cox*1, *nad*1, and *nad*5 genes. However, there is a lack of comprehensive global data on genetic diversity in *E. granulosus* and *E. ortleppi* based on *cox*2 and *nad*6 genes. The current pilot research work was executed to look into the genetic variation of the *E. granulosus* (G1/G3) and *E. ortleppi* (G5) populations in Pakistan using *cox*2 and *nad*6 and infer phylogenetic relationships with other *E. granulosus* sensu lato populations. Followed by cyst collection from slaughtered cattle in Pakistan and DNA extraction, the full mitochondrial cytochrome oxidase 2 and NADH dehydrogenase subunit 6 were amplified. After confirmation of the identity of isolates through BLAST analysis, population indices pertaining to neutrality and diversity were calculated using the DNASp program. MJNs were constructed using PopArt for intraspecific data investigation. Nucleotide sequences (n=17) analyzed in this investigation were matched with GenBank reference sequences and *Echinococcus* genotypes G1 (n=5) and G3 (n=7) were discovered. Five sequences were identified as *E. ortleppi* (G5). For *E. granulosus* (G1/G3), a high haplotype diversity (Hd=1.00) was found for *cox*2 (582 bp), *nad*6 (456 bp), and concatenated *cox*2-*nad*6 (1038 bp) gene sequences. The overall value of Tajima's D for the entire population was negative and insignificant. Regarding *E. ortleppi*, *cox*2–*nad*6 π and Hd were 0.07290 and 1, respectively with insignificant Fu's Fs. A dataset of 40 *cox*2 and 35 *nad*6 sequences was utilized to form a median-joining network to compare the geographical kinship between the Pakistani isolates and sequences reported elsewhere. The analysis of concatenated *cox*2-*nad*6 obtained in this study revealed a high haplotype diversity (1.00) while low nucleotide diversity (0.08207) was observed. Non-significant values of Tajima's D = -0.54361 and Fu's Fs = 1.303 were observed. High haplotype diversity in the current study shows a significant level of genetic divergence across haplotypes. The amalgamation of low π and high Hd indicates population expansion. We recommend amplifying a greater number of isolates in the future to ascertain the resolution power of *cox*2 and *nad*6 genes in the context of genotype differentiation.

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INTRODUCTION

Animals around the world are susceptible to different types of parasite-borne acute, chronic, and severe diseases. The parasitic disease cystic echinococcosis (CE), which has been ignored, has a global distribution and significant economic and zoonotic consequences (Wang *et al*., 2018). The larval stage metacestodes of the Taeniidae family member *Echinococcus granulosus* sensu lato (G1, G3, G5, G6/7, G8/10 and *E. felidis*) are responsible for CE prevalence throughout the world. The adult tapeworm requires a definitive canid host (commonly a dog), while the parasite's larval stage requires an intermediate host (usually livestock, primarily sheep) to grow as a cyst in internal organs (Otero-Abad and Torgerson 2013; Craig *et al*., 2017). Human actions, such as feeding dogs raw offal containing infectious hydatid cysts following home slaughtering, contribute to the parasite transmission cycle (Larrieu *et al*., 2019).

Echinococcus granulosus (s.l.) is the causative agent of a widespread disease that leads to significant economic losses, amounting to approximately 3 billion USD annually (WHO, 2019), as well as human suffering (Hammad *et al*., 2018). In the veterinary sector, the impact of CE on livestock results in reduced productivity and quality of meat, milk, and wool, lower birth rates, and delayed performance and growth (Bingham *et al*., 2016). These consequences contribute to production losses in affected sheep and goats (Larrieu *et al*., 2019). *Echinococcus granulosus* (G1/G3) is the most prevalent *Echinococcus granulosus* species within the *E. granulosus* (sensu lato) (G1, G3, G5, G6/7, G8/10 and *E. felidis*), mostly found in Asia, Australia, South America and Africa (Deplazes *et al*., 2017; Thompson 2017). CE exhibits higher prevalence in regions where communities rely on animal husbandry and agricultural practices for their livelihoods. Several studies have been conducted to determine the prevalence and molecular characteristics of CE, with reports coming from various Asian countries (Hammad *et al*., 2018; Guo *et al*., 2019; Metwally *et al*., 2018; Nematdoost *et al*., 2021).

Pakistan, as an agriculturally reliant country, relies substantially on the livestock sector, which accounts for 60.03% of agriculture and contributes significantly to the national economy, accounting for 11.53% of GDP (Economic Survey of Pakistan, 2022). A recent livestock census reported a population of at least 30.1 million sheep, 51.5 million cattle, and 42.4 million buffalo in the country (Economic Survey of Pakistan, 2022). Mitochondrial DNA markers like *cox*1, *nad*1, and *nad*5 genes have been used to examine the genetic structure of the parasite, however, there is a deficiency of comprehensive global data on genetic information in *E. granulosus* based on complete mitochondrial *cox*2 and *nad*6 genes.

As a result of the scarcity of data on *cox*2 and *nad*6 gene-based genetic information of *Echinococcus* species, the current work was undertaken as a preliminary approach to bridge this research gap and acquire better insights about the molecular characterization of *E. granulosus and E. ortleppi*. Our goal was to determine the population structure of Pakistani isolates of *E. granulosus* using complete *cox*2 and *nad*6 genes.

MATERIALS AND METHODS

Study location, examination of visceral organs, and cyst identification: The study was carried out in Pakistan, which borders Iran, Afghanistan, China, and India. The Main Urban Slaughterhouse of Sialkot was selected. With a moist subtropical environment and four distinct seasons, Sialkot is the thirteenth-most populated city in Pakistan. The slaughterhouse was visited every day during the whole cyst collection period of three months from January to March 2020 except on Tuesday & Wednesday because of the holiday for abattoirs in Punjab. To find *Echinococcus granulosus* cysts on liver, lungs and other visceral organs throughout this time, a total of 1926 bovines were inspected after slaughter. Following the recommendations of the World Organization for Animal Health, the lungs and liver of both slaughtered small and large ruminants were carefully examined to look for cysts. The collected cysts were then moved to the Department of Clinical Medicine and Surgery, UAF for additional processing, under cold storage.

DNA extraction, PCR and sequencing: Phosphate-buffered saline was used to thoroughly clean hydatid cysts after they had been thoroughly cleaned with 75% ethanol. The cystic fluid was discarded since none of the cysts were fertile. Cut portions of germinal coverings were subjected to DNA extraction. For each isolate, the complete *cox*2 and *nad*6 genes were amplified. Table 1 provides details about the primers engaged and the reactional circumstances. The PCR was carried out in 50 µl final volume. 5µl of amplicons were analyzed on a 1.5% agarose gel and then viewed under a trans-illuminator. The remaining amplicons were sent to China for sequencing.

Molecular data analysis: Incorrectly read nucleotides were checked by using Unipro UGENE software (Okonechnikov *et al*., 2012) and were BLAST searched. DnaSP version 6 was used to evaluate the diversity and neutrality indices (Tajima, 1989; Fu, 1997). To visually represent the relations among haplotypes, sequences were converted to the Nexus format (Maddison *et al*., 1997) and generated a haplotype network using the PopArt application (Population Analysis with Reticulate Trees) (Leigh and Bryant, 2015). Using PopART software for intraspecific data investigation, a median-joining network (Bandelt *et al*., 1999) was constructed using the mitochondrial *cox*2 and *nad*6 gene sequences obtained in the present study and those downloaded from the NCBI GenBank database.

RESULTS

There were only 18 cysts discovered throughout this time and all were associated with cattle (located in the liver & lungs). The *cox*2 and *nad*6 genes were amplified, yielding PCR bands of 725 base pairs and 600 base pairs, respectively. All 17 isolates analyzed matched with GenBank reference sequences. *Echinococcus granulosus* genotypes G1 (n = 5), and G3 (n = 7) were obtained and *E. ortleppi* (G5) was observed in five isolates.

Table 1: Primers sequences and PCR reaction conditions to amplify full-length *cox*2 and *nad*6 genes.

Gene	Primer sequence (5'-3')	Conditions					Amplicon length	Reference
		Initial denaturation	Denaturation	Annealing	Extension	Final Extension		
			35 cycles					
*cox*2	Forward TGAGGTAAGTCGTAACAAGG	95°C for 3 min	95°C for 10 sec	50°C for 45 sec	72°C for 45 sec	72°C for 10 min	725 bp	Hu *et al*., 2015
	Reverse ATCTACAGCACGAAAAGCC							
*nad*6	Forward TTTCGTGCTGTAGATGGT	95°C for 3 min	95°C for 10 sec	58°C for 20 sec	72°C for 20 sec	72°C for 5 min	600 bp	Zhan *et al*., 2019
	Reverse CACAGATTTCAAAGGGTT							

Table 2: Population indices for *Echinococcus granulosus* (G1/G3).

Index	*cox*2 (582 bp)			*nad*6 (456 bp)			*cox*2-*nad*6 (1038 bp)		
	G1	G3	Overall	G1	G3	Overall	G1	G3	Overall
Number of isolates	5	7	12	2	5	7	2	5	7
Number of mutations	40	58	84	49	155	172	59	205	230
Parsimony informative sites	1	16	28	0	59	81	0	67	93
Number of haplotypes	5	7	12	2	5	7	2	5	7
Haplotype diversity (Hd)	1	1.000	1.000	1.000	1.000	1.000	1.000	1	1.000
Nucleotide diversity (π	0.02732	0.03338	0.03213	0.10746	0.15943	0.14662	0.05684	0.09027	0.08207
Tajima's D	-1.29250*	-1.03955	-1.52350	----	-0.17409	-0.27964	----	-0.36432	-0.54361
Fu's Fs	0.248	-0.505	-2.917	3.892	1.936	1.033	4.078	2.200	1.303

------ Four or more sequences are needed to compute Tajima's value.

Table 3: Population indices for *Echinococcus ortleppi* (G5) population.

Index	cox2 (582 bp)	*nad*6 (456 bp)	*cox*2-*nad*6 (1038 bp)
Number of isolates	5	3	3
Number of mutations	22	102	120
Parsimony informative sites	6	0	0
Number of haplotypes	5	3	3
Haplotype diversity (Hd)	1.000	1.000	1.000
Nucleotide diversity (π	0.01632	0.14035	0.07290
Tajima's D	-0.74434	------	----
Fu's Fs	-0.407	3.050	3.219

------ Four or more sequences are needed to compute Tajima's value.

Echinococcus granulosus (s.s.) sequences revealed 230 mutations (*cox*2 = 84, and *nad*6 = 172) and 93 parsimony informative sites (*cox*2 = 28, and *nad*6 =81) (Table 2). The *cox*2 (582 bp), *nad*6 (456 bp), and *cox*2-*nad*6 (1038 bp) gene sequences showed high haplotype diversity (Hd=1.00).

The sequences generated in this study were deposited under the accession numbers OR251026-OR251037 (*cox*2) and OR251038-OR251044 (*nad*6), respectively.

For *cox*2-*nad*6 gene sequences in *E. granulosus* (G1/G3), a low nucleotide and a high haplotype diversity were observed as follows: *cox*2 (Hd = 1, π = 0.03213) and *nad*6 (Hd = 1, π =0.14662), whereas *cox*2-*nad*6 Hd and π were 1 and 0.08207, respectively. The values of Tajima's D were all negative and negligible.

Regarding *E. ortleppi* isolates, 5 and 3 unique haplotypes were detected for the *cox*2 and *nad*6 genes, respectively, whereas a concatenation of both gene sequences (*cox*2-*nad*6, 1038 bp) revealed 3 distinct haplotypes. Overall *cox*2-*nad*6 nucleotide and haplotype diversities were 0.07290 and 1, with insignificant Fu's Fs (3.219) (Table 3). The sequences of the *E. ortleppi cox*2 and *nad*6 genes acquired in this investigation were posted in the GenBank database as OR251038-OR251049 and OR251050-OR251052.

A dataset of 40 *cox*2 and 35 *nad*6 sequences was utilized to form a median-joining network to compare the geographical kinship between Pakistani isolates and those from other parts of the world. In this study's analysis of concatenated *cox*2-*nad*6, 7 haplotypes were observed with a high haplotype diversity (1.00) and a low nucleotide diversity (0.08207). Non-significant Tajima's D (-0.54361) and Fu's Fs (1.303) were found. Hap-1 and Hap-17 were discovered to represent the central haplotypes for G1 and G3, respectively, for *cox*2 gene sequences (Fig. 1). The haplotypes Hap-3 and Hap-2 were the core haplotypes for G1 and G3, respectively, for the *nad*6 gene (Fig. 2). The median-joining network (MJN) of *E. ortleppi cox*2 and *nad*6 gene sequences is mentioned in Fig. 3 and 4, respectively.

DISCUSSION

Infectious illnesses, particularly parasitic ones, are one of the major health concerns in both humans and animals, resulting in severe health effects and economic losses (Alberfkani *et al*., 2022; Mahmood *et al*., 2022). Diseases caused by parasites result in large economic losses due to reduced production and illness (Javed and Alkheraije, 2023; Quratulain *et al*., 2023). Infection with *E. granulosus* has zoonotic effects and remains a substantial concern to livestock productivity. A greater understanding of genetic diversity is important in many disciplines of study, like epidemiology and diagnosis.

There are numerous studies using mitochondrial and nuclear fragments such as *cox*1, *nad*1, *nad*5, *atp*6, *cyt*b, 16S rRNA and 12S rRNA genes to explore the population structure of *Echinococcus* species (Ma *et al*., 2008; Ma *et al*., 2012; Wang *et al*., 2015; Yan *et al*., 2013; Wang *et al*., 2016). Bowles *et al*. released the first reports on *E. granulosus*' evolutionary history in 1992-1993. The phylogeny was based on mitochondrial DNA genes *cox*1 and *nad*1, as well as ribosomal DNA PCR-RFLP patterns (Bowles *et al*., 1992; Bowles and McManus 1993a, Bowles and McManus 1993b). However, varied-length markers are not suited for comparing genetic architecture in different regions, and diverse gene fragments may not be true representative, as each fragment on a gene is distinct in terms of conservation properties. As far as we know, this study is the maiden effort to characterize *E. granulosus* employing concatenated complete *cox*2 and *nad*6 genes since the discriminatory strength of a group of gene targets thumps that of a single gene approach for CE genotyping (Nikmanesh *et al*., 2017).

Furthermore, mtDNA markers are strong contenders for measuring population genetic diversity due to their preserved structure, lack of recombination, and quick evolution. This study employed these two gene sequences to examine the genetic structure of *E. granulosus* (G1/G3)

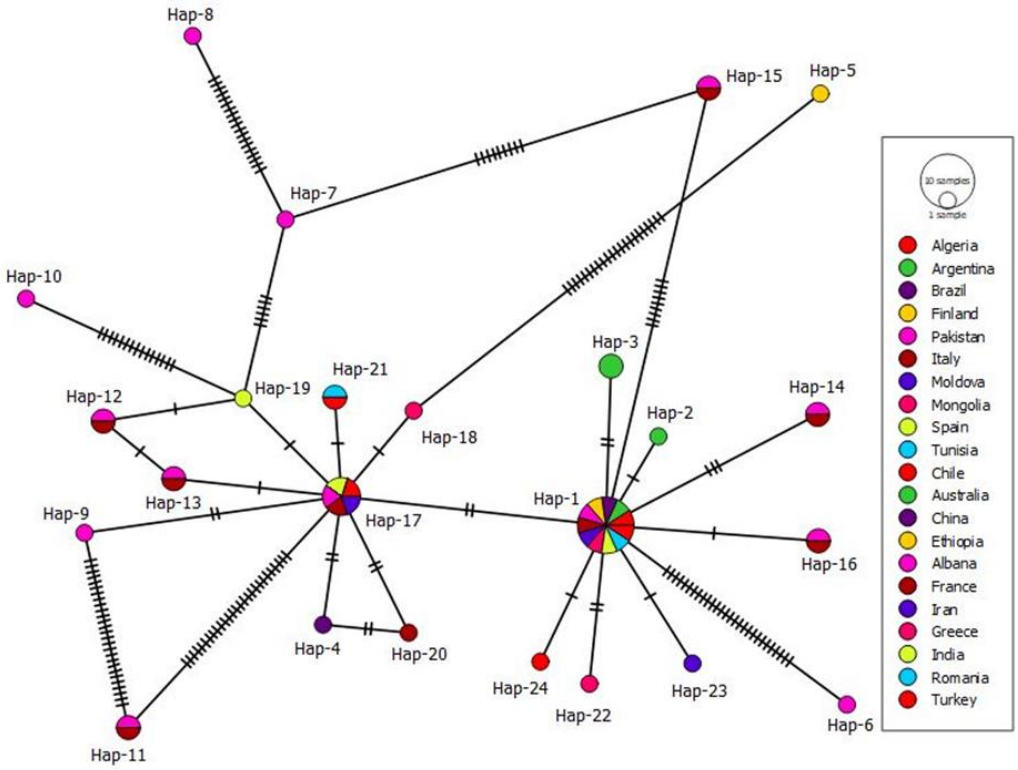

Fig. 1: Complete *cox*2 gene-based MJN of *Echinococcus granulosus* (G1 and G3)

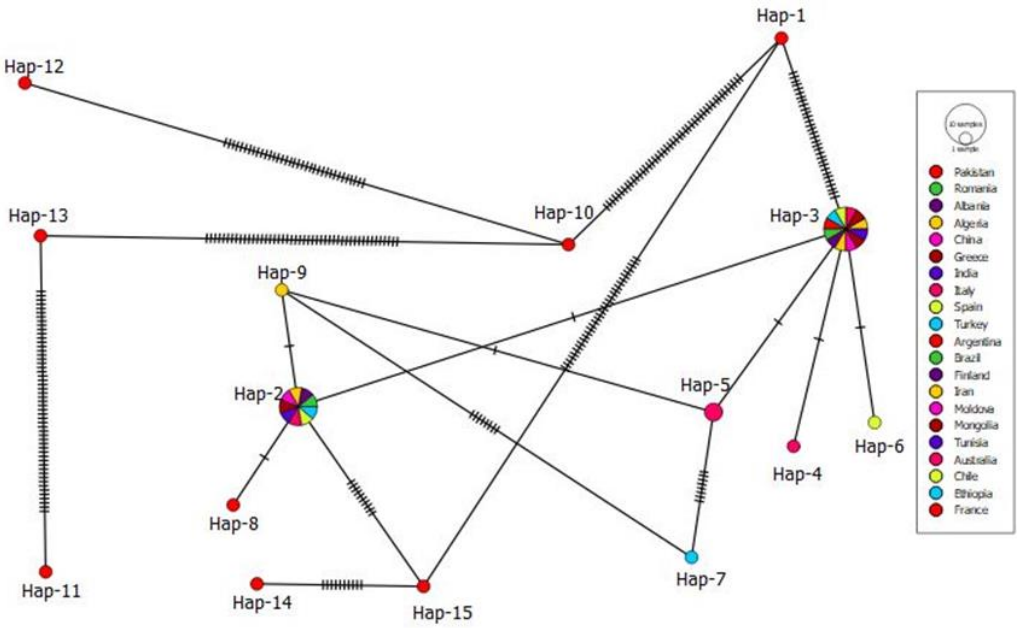

Fig. 2: Complete *nad6* gene based MJN *Echinococcus granulosus* (G1 and G3).

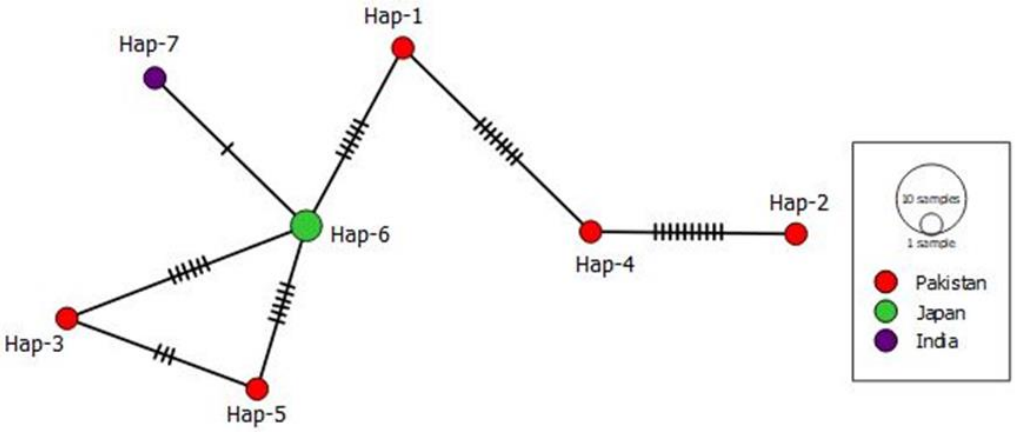

Fig. 3: Complete *cox*2 gene MJN of *Echinococcus ortleppi* (G5).

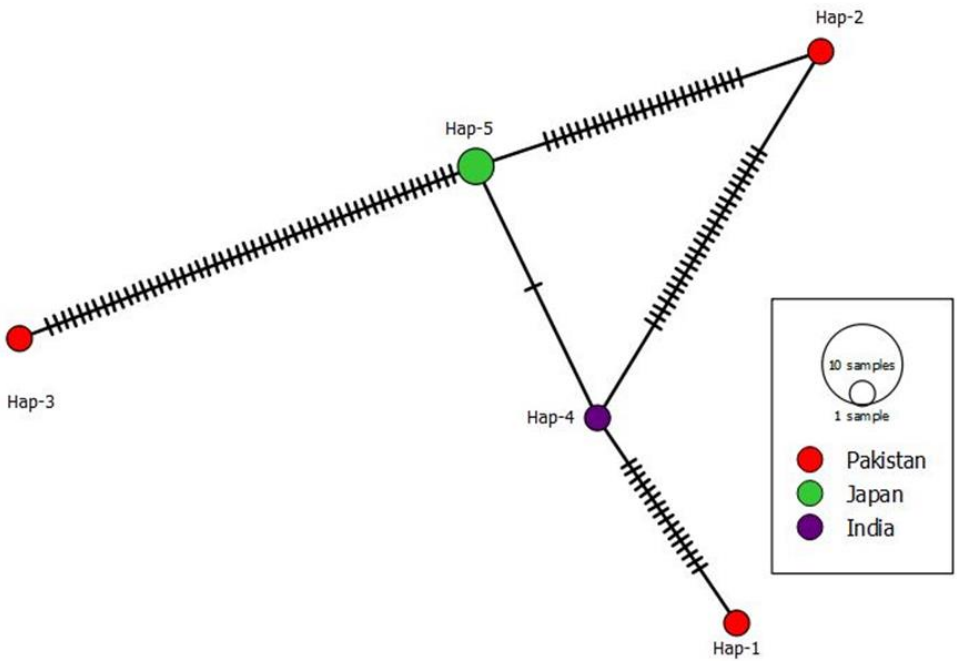

Fig. 4: Complete *nad6* gene-based MJN of *Echinococcus ortleppi* (G5).

and *E. ortleppi* (G5) in Pakistan. Previous research has shown that *E. granulosus* G1 is the most common genotype in the Asian, African, and South American continents (Li *et al*., 2008; Xiao *et al*., 2003; Zhang *et al*., 1998; Beyhan and Umur 2011; de la Rue *et al*., 2011; Andresiuk *et al*., 2013; Boufana *et al*., 2014). In contrast to the previous results, we found G3 to be the most common genotype in this investigation (G3 7/17 (41.17%); G1 5/17 (29.41%)). The G5 genotype has been documented in humans from many Asian, European, and South American countries (Romig *et al*., 2015; Grenouillet *et al*., 2014; Sharma *et al*., 2013; Shi *et al*., 2019; Ebrahimipour *et al*., 2017; Thapa *et al*., 2017; Zhang *et al*., 2000), indicating that it is a significant genotype from a public health standpoint. In this study, we found 5 isolates of *E. ortleppi* G5 (5/17; 29.41)

The results obtained in this study showed a high degree of genetic discrepancy. Both the genes under study showed the highest haplotype diversity (1.00) while π was higher for the *nad*6 gene (0.14662) than that of the *cox*2 gene (0.03213). In a previous study utilizing a full-length *cox*2 gene to describe genetic diversity, lower Hd (0.667) and nucleotide diversity (0.00148) were obtained (Hu *et al*., 2015) which are in contrast to our results from Pakistan. Upon concatenation of both genes, the observed value of nucleotide diversity was 0.03213. Similar patterns of nucleotide and haplotype diversities were observed for *E. granulosus* (G5) (*cox*2 = 0.01632, *nad*6 = 0.14035; *cox*2-*nad*6 = 0.07290). For *E. granulosus* (G1/G3) negative but insignificant Tajima's D was observed for the whole population while Fu Fs was positively insignificant. Regarding *E. ortleppi* (G5), Tajima's values were not computed as four or more sequences are needed to estimate its value, however, Fu Fs was positive but insignificant for the whole population. Since most of the population structure data consists of *cox*1, *nad*1, *cyt*b, and *nad*5 genes, we were unable to draw a comparative genotypic diversity analysis of findings of the current investigation with other studies from elsewhere.

The current study's Hd divulges a large level of genomic divergence between haplotypes, as demonstrated in the MJNs. According to one study, the amalgamation of low π and high Hd specifies a swift population expansion (Sharma *et al*., 2013). The number of isolates investigated in the current study was less. Thus, to give conclusive remarks, we recommend amplifying a greater number of isolates in the future to ascertain the resolution power of *cox*2 and *nad*6 genes in the context of genotype differentiation.

Conclusions: *Echinococcus* cysts were recovered from slaughtered cattle in Faisalabad, Pakistan, and were identified using mitochondrial *cox*2 and *nad*6 genes in what we believe is the first attempt in Pakistan to detect *Echinococcus granulosus* and *Echinococcus ortleppi* based on these genetic markers. The Pakistani *E. granulosus* (G1/G3) and *E. ortleppi* populations have a significant degree of genetic variety and haplotype diversity. This study provides crucial preliminary data for Pakistan as well as critical baseline data for upcoming studies on the G1, G3, and G5 populations around the world. We urge that in future research, the *cox*2 and *nad*6 genes be amplified. Furthermore, more number of samples should be investigated to assess the genotype differentiation potential of *cox*2 and *nad*6 genes.

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Ethics approval and consent to participate: Not applicable.

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REFERENCES

Alberfkani MI, Albarwary AJS, Jaafar GM, *et al*., 2022. Molecular characterization and phylogenetic analysis of cox1 and ITS-1 gene fragments of *Moniezia* species isolated from sheep. Pak Vet J 42:566-570. http://dx.doi.org/10.29261/pakvetj/2022.073

Andresiuk MV, Gordo FP, Saarma M, *et al*., 2013. *Echinococcus granulosus* genotype G1 dominated in cattle and sheep during 2003-2006 in Buenos Aires province, an endemic area for cystic echinococcosis in Argentina. Acta Trop 127:136–142. https://doi.org/10.1016/j.actatropica.2013.04.008

Bandelt HJ, Forster P and Röhl A, 1999. Median-joining networks for inferring intraspecific phylogenies. Mol Biol Evol 16:37–48. https://doi.org/10.1093/oxfordjournals.molbev.a026036

Beyhan YE and Umur S, 2011. Molecular characterization and prevalence of cystic echinococcosis in slaughtered water buffaloes in Turkey. Vet Parasitol 181:174–179. https://doi.org/10.1016/j.vetpar.2011.04.038

Bingham GM, Larrieu E, Uchiumi L, *et al*., 2016. The Economic Impact of Cystic Echinococcosis in Rio Negro Province, Argentina. Am J Trop Med Hyg 94:615–625. https://doi.org/10.4269/ajtmh.15-0304

Boufana B, Lahmar S, Rebaï W, *et al*., 2014. Genetic variability and haplotypes of *Echinococcus* isolates from Tunisia. Trans Royal Soc Trop Med Hyg 108:706–714. https://doi.org/10.1093/trstmh/tru138

Bowles J, Blair D and McManus DP, 1992. Genetic variants within the genus *Echinococcus* identified by mitochondrial DNA sequencing. Mol Biochem Parasitol 54:165–173. https://doi.org/10.1016/0166-6851(92)90109-w

Bowles J and McManus DP, 1993a. NADH dehydrogenase 1 gene sequences compared for species and strains of the genus *Echinococcus*. Int J Parasitol 23:969–972. https://doi.org/10.1016/0020-7519(93)90065-7

Bowles J and McManus DP, 1993b. Rapid discrimination of *Echinococcus* species and strains using a polymerase chain reaction-based RFLP method. Mol Biochem Parasitol 57:231–239. https://doi.org/10.1016/0166-6851(93)90199-8

Craig PS, Hegglin D, Lightowlers MW, *et al*., 2017. Echinococcosis: Control and Prevention. Adv Parasitol 96:55–158. https://doi.org/10.1016/bs.apar.2016.09.002

de la Rue ML, Takano K, Brochado JF, *et al*., 2011. Infection of humans and animals with *Echinococcus granulosus* (G1 and G3 strains) and *E. ortleppi* in Southern Brazil. Vet Parasitol 177:97–103. https://doi.org/10.1016/j.vetpar.2010.11.018

Deplazes P, Rinaldi L, Alvarez Rojas CA, *et al*., 2017. Global Distribution of Alveolar and Cystic Echinococcosis. Adv Parasitol 95:315–493. https://doi.org/10.1016/bs.apar.2016.11.001

Ebrahimipour M, Sadjjadi SM, Yousofi Darani H, *et al*., 2017. Molecular Studies on Cystic Echinococcosis of Camel (*Camelus dromedarius*) and Report of *Echinococcus ortleppi* in Iran. Iran J Parasitol 12:323-31.

Fu YX, 1997. Statistical tests of neutrality of mutations against population growth, hitchhiking and background selection. Genetics 147:915–925. https://doi.org/10.1093/genetics/147.2.915

Grenouillet F, Umhang G, Arbez-Gindre F, *et al*., 2014. *Echinococcus ortleppi* infections in humans and cattle, France. Emerg Infect Dis 20:2100–2102. https://doi.org/10.3201/eid2012.140641

Guo B, Zhang Z, Zheng X, *et al*., 2019. Prevalence and Molecular Characterization of *Echinococcus granulosus* Sensu Stricto in Northern Xinjiang, China. Korean J Parasitol 57:153–159. https://doi.org/10.3347/kjp.2019.57.2.153

Hammad SJ, Cavallero S, Milardi GL, *et al*., 2018. Molecular genotyping of *Echinococcus granulosus* in the North of Iraq. Vet Parasitol 249:82–87. https://doi.org/10.1016/j.vetpar.2017.11.010

Hu D, Song X, Wang N, *et al*., 2015. Molecular identification of *Echinococcus granulosus* on the Tibetan Plateau using mitochondrial DNA markers. Genet Mol Res 14:13915–13923. https://doi.org/10.4238/2015.October.29.12

Javed K and Alkheraije KA, 2023. Cryptosporidiosis: a foodborne zoonotic disease of farm animals and humans. Pak Vet J 43:213-223. http://dx.doi.org/10.29261/pakvetj/2023.038

Larrieu E, Gavidia CM and Lightowlers MW, 2019. Control of cystic echinococcosis: Background and prospects. Zoonoses Public Health 66:889–899. https://doi.org/10.1111/zph.12649

Leigh JW and Bryant D, 2015. POPART: full-feature software for haplotype network construction. Methods Ecol Evol 6:1110–1116.

Li T, Ito A, Nakaya K, *et al*., 2008. Species identification of human echinococcosis using histopathology and genotyping in northwestern China. Trans Royal Soc Trop Med Hyg 102:585–590. https://doi.org/10.1016/j.trstmh.2008.02.019

Ma SM, Maillard S, Zhao HL, *et al*., 2008. Assessment of *Echinococcus granulosus* polymorphism in Qinghai province, People's Republic of China. Parasitol Res 102:1201–1206. https://doi.org/10.1007/s00436-008-0894-7

Ma J, Wang H, Lin G, *et al*., 2012. Molecular identification of *Echinococcus* species from eastern and southern Qinghai, China, based on the mitochondrial cox1 gene. Parasitol Res 111:179–184. https://doi.org/10.1007/s00436-012-2815-z

Maddison DR, Swofford DL and Maddison WP, 1997. NEXUS:an extensible file format for systematic information. Syst Biol 46:590–621. https://doi.org/10.1093/sysbio/46.4.590

Mahmood Q, Younus M, Sadiq S, et al., 2022. Prevalence and associated risk factors of cystic echinococcosis in food animals – A neglected and prevailing zoonosis. Pak Vet J 42:59-64. 4. http://dx.doi.org/10.29261/pakvetj/2022.008

Metwally DM, Qassim LE, Al-Turaiki, IM, *et al*., 2018. Gene-based molecular analysis of COX1 in *Echinococcus granulosus* cysts isolated from naturally infected livestock in Riyadh, Saudi Arabia. PloS One 13, e0195016. https://doi.org/10.1371/journal. pone.0195016

Ministry of Finance. Economic Survey of Pakistan (2021–2022). Islamabad, Pakistan:Ministry of Finance, Government of Pakistan.

Nematdoost K, Ashrafi K, Majidi-Shad B, *et al*., 2021. Genetic Characterization of *Echinococcus granulosus* Sensu Lato in Livestock and Human Isolates from North of Iran Indicates the Presence of *E. ortleppi* in Cattle. Acta Parasitol 66:446–454. https://doi.org/10.1007/s11686-020-00293-0

Nikmanesh B, Mirhendi H, Mahmoudi S, *et al*., 2017. Multilocus sequence analysis of *Echinococcus granulosus* strains isolated from humans and animals in Iran. Exp Parasitol 183:50–55. https://doi.org/10.1016/j.exppara.2017.10.002

Okonechnikov K, Golosova O, Fursov M, *et al*., 2012. Unipro UGENE: a unified bioinformatics toolkit. Bioinformatics 28:1166–1167. https://doi.org/10.1093/bioinformatics/bts091

Otero-Abad B and Torgerson PR, 2013. A systematic review of the epidemiology of echinococcosis in domestic and wild animals. PLoS Negl Trop Dis 7:e2249. https://doi.org/10.1371/journal.pntd.0002249

Quratulain, Akhtar S, Qayyum M, *et al.,* 2023. Coccidiosis: prevalence, epizootiological risk factors, hematological and serum biochemical profile in clinically infected pet dogs. Pak Vet J 43:831-834. http://dx.doi.org/10.29261/pakvetj/2023.113

Romig T, Ebi D and Wassermann M, 2015. Taxonomy and molecular epidemiology of *Echinococcus granulosus* sensu lato. Vet Parasitol 213:76–84. https://doi.org/10.1016/j.vetpar.2015.07.035

Sharma M, Fomda BA, Mazta S, *et al*., 2013. Genetic diversity and population genetic structure analysis of *Echinococcus granulosus* sensu stricto complex based on mitochondrial DNA signature. PloS One 8:e82904. https://doi.org/10.1371/journal. pone.0082904

Shi Y, Wan X, Wang Z, *et al*., 2019. First description of *Echinococcus ortleppi* infection in China. Parasit Vectors 12:398. https://doi.org/10.1186/s13071-019-3653-y

Tajima F, 1989. Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. Genetics 123:585–595. https://doi.org/10.1093/genetics/123.3.585

Thapa NK, Armua-Fernandez MT, Kinzang D, *et al*., 2017. Detection of *Echinococcus granulosus* and *Echinococcus ortleppi* in Bhutan. Parasitol Int 66:139-141. https://doi.org/10.1016/j.parint.2016.1 2.010

Thompson RC, 2017. Biology and Systematics of *Echinococcus*. Adv Parasitol 95:65–109. https://doi.org/10.1016/bs.apar.2016.07.001

Wang X, Liu J, Zuo Q, *et al*., 2018. *Echinococcus multilocularis* and *Echinococcus shiquicus* in a small mammal community on the eastern Tibetan Plateau:host species composition, molecular prevalence, and epidemiological implications. Parasit Vectors 11:302. https://doi.org/10.1186/s13071-018-2873-x

Wang N, Wang J, Hu D, *et al*., 2015. Genetic variability of *Echinococcus granulosus* based on the mitochondrial 16S ribosomal RNA gene. Mitochondrial DNA 26:396–401. https://doi.org/10.3109/19401736.2013.840590

Wang N, Xie Y, Liu T, *et al*., 2016. The complete mitochondrial genome of G3 genotype of *Echinococcus granulosus* (Cestoda: Taeniidae). Mitochondrial DNA Part A 27:1701–1702. https://doi.org/10.3109/19401736.2014.961129

World Health Organization (WHO) 2019. Echinococcosis. Geneva:https:// www.who.int/news-room/fact-sheets/detail/echinococcosis Accessed 27 Dec 2019.

Xiao N, Qiu J, Nakao M, *et al*., 2003. Identification of *Echinococcus* species from a yak in the Qinghai-Tibet plateau region of China. Am J Trop Med Hyg 69:445–446.

Yan N, Nie HM, Jiang ZR, *et al*., 2013. Genetic variability of *Echinococcus granulosus* from the Tibetan plateau inferred by mitochondrial DNA sequences. Vet Parasitol 196:179–183. https://doi.org/10.1016/j.vetpar.2013.02.010

Zhan J, Wang N, Hua R, *et al*., 2019. Simultaneous Detection and Genotyping of Hydatid Cysts in Slaughtered Livestock via a Direct PCR Approach. Iran J Parasitol 14:679–681.

Zhang L, Eslami A, Hosseini SH, *et al*., 1998. Indication of the presence of two distinct strains of *Echinococcus granulosus* in Iran by mitochondrial DNA markers, Am J Trop Med Hyg 59:171–174. https://doi.org/10.4269/ ajtmh.1998.59.171.

Zhang LH, Joshi DD and McManus DP, 2000. Three genotypes of *Echinococcus granulosus* identified in Nepal using mitochondrial DNA markers. Trans Royal Soc Trop Med Hyg 94:258–260. https://doi.org/10.1016/s0035-9203(00)90313-4