ORIGINAL ARTICLE

CHARACTERIZATION OF PREVALENT ECHINOCOCCUS GRANULOSUS GENOTYPES THROUGH MODIFIED PCR-RFLP TECHNIQUE

Nosheen Basharat[1], Sajid Hamid Ansari[2], Jadoon Khan[1,3], Amina Gul[4], Ijaz Ali[1]

Department of Biosciences, COMSATS University, Islamabad - Pakistan.

Department of Surgery, DG Khan Medical College, Punjab - Pakistan.

Department of Microbiology, Quaid-i-Azam University, Islamabad - Pakistan.

Department of Pathology, Khyber Medical College and Teaching Hospital, Peshawar - Pakistan

ABSTRACT

Objective: We aimed to investigate the genetic diversity of *echinococcus granulosus* using a modified Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) based assay in the infected population.

Materials and methods A total of 18 human hydatid cyst samples were collected from various hospitals of Southern Punjab and Islamabad Capital Territory region of Pakistan. Extracted DNA was used for PCR amplification of mitochondrial NADH dehydrogenase subunit 1 (Nad1) gene followed by sequencing and phylogenetic analysis using Molecular Evolutionary Genetics Analysis (MEGA) Software. The entire sequences were fed into NEBcutter V2.0 to select a single restriction enzyme followed by invitro confirmation through PCR-RFLP.

Results: Amplification on the Nad1 gene was observed in 100% of the samples processed. The Basic Local Alignment Tool (BLAST) and phylogenetic tree analysis revealed 83.3% *E. granulosus* (G1-G3 genotypes), 11.1% *E. multilocularis* and 5.6% *E. Canadensis* (G6 genotype). The use of the *BfaI* enzyme in PCR-RFLP analysis revealed that all of the 18 samples were assigned consecutive genotypes as observed in the sequencing.

Conclusion:

The current study concluded that the *BfaI* enzyme could be used for the genotypic analysis of echinococcosis in developing and frequently affected countries. It will be a cost-effective and easy technique compared to sequencing, which will aid in developing novel therapeutic and control strategies for the parasite.

Key words: *echinococcus granulosus,* Polymerase Chain Reaction, Restriction Fragment Length Polymorphism

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INTRODUCTION

Cystic echinococcosis (CE) is an important socio-economic zoonotic infection caused by canid tapeworm Echinococcus granulosus. The parasite requires two hosts for its life cycle completion: a definitive host (canid) and an intermediate host like (herbivores) and humans. The intermediate hosts get infected through the fecal-oral route by ingesting the parasite eggs in the feces of the definitive host. The eggs hatch to release oncospheres (hexacanth embryos), which migrate to blood vessels through the intestinal wall and infect various organs like the lung and liver, developing fluid-filled cysts (often 3~10 cm in diameter). Human cystic echinococcosis is a chronic infection with an asymptomatic phase (months to years) followed by acute clinical signs like pain or swelling due to the cyst pressure exerted on surrounding parenchymal tissues. [1-3] The clinical manifestation depends upon the size, number, affected organ, and localization of the cysts. The disease can be life-threatening when cysts rupture and their contents (fluid, protoscoleces, and brood capsules) spill into the peritoneal cavity, resulting in anaphylactic shock and establishment of secondary CE. Due to its zoonotic nature, CE can affect millions of humans and livestock population responsible for huge financial and health losses due to the condemnation of infected livers and lungs from livestock animals and also difficult to diagnose and treat in humans. [4, 5]

Cystic echinococcosis is reported from the human and livestock population and is endemic in various parts

Correspondence
Dr Amina Gul
Assistant Professor
Department of Pathology MTI KMC/KTH
Peshawar - Pakistan
Email: dr.aminagul@gmail.com
Cell: +92-333-1971474
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of Pakistan. [6-9] Globally, 10 distinct genotypes (G1-G10) of E. granulosus sensu lato have been defined based on mitochondrial DNA studies among various countries like Africa, Asia, Australia, Europe, South America, and the USA. [10-18] The genetic characterization of E. granulosus plays a central role in understanding transmission patterns of the parasites between definitive hosts and intermediate mammalian hosts/humans as well as diagnosis and control of CE. [19-21] Various DNA-based tools, particularly PCR and sequencing of nuclear and mitochondrial gene regions, have been applied to the genetic classification of echinococcus species and genotypes. [22-24] The PCR-RFLP-based genotypic technique revealed various species of CE. However, there is a need for a novel restriction enzyme that can be simultaneously used to discriminate various species of echinococcus granulosus. Since genotypic identification of echinococcus species is imperative for devising strategies for its control, the current study used a single restriction enzyme BfaI for genotypic characterization followed by verification with currently recognized genotypes through sequencing and phylogenetic analysis.

MATERIALS AND METHODS

The current study was performed from September 2018 to September 2019 at Molecular Virology Laboratory (MVL), Department of Biosciences, COMSATS University Islamabad. This study was reviewed and approved by the Ethical Approval Committee of COMSATS University, Islamabad, under Reference No. CUI-Reg/Notif. 2255/19/2661. A total of 18 hydatid cyst samples removed via surgery were collected from various hospitals of District Dera Ghazi Khan and Rajanpur and transferred to MVL for processing. The cyst contents (fluid and protoscoleces) were aspirated aseptically into sterile test tubes (McManus and Symth, 1978), followed by mild spinning by centrifugation at 3000 rpm for l0 minutes at room temperature. The supernatant was discarded. The residue was taken by Pasture pipette and placed on a microscopic slide and examined under the microscope (40X) for the presence of protoscoleces.

NUCLEIC ACID EXTRACTION

Total nucleic acid was extracted from Cyst fluid (protoscoleces) and the germinal layer using DNeasy Blood and Fluid Kit (Qiagen, Hilden, Germany) and phenol-chloroform method as described earlier. Extracted DNA concentration was determined using a NanoDrop spectrophotometer (Thermo Scientific, USA) and then stored at -20C until further used for PCR amplification.

MOLECULAR CHARACTERIZATION

Molecular characterization of E. granulosus was performed using mitochondrial Nad1 gene fragment as described earlier (Kim et al., 2017). PCR amplified products were separated on 2% agarose gel electrophoresis, pre-stained with ethidium bromide, followed by UV transilluminator visualization according to their fragment size. Sequencing and phylogenetic analyses: PCR products were sequenced in both directions using Forward primer (Nad1-F[A(G/A)(A/T) TTCGTAAGGG(G/C)CCTAATA) and a Reverse primer Nad1-R ((A/T)CC(A/T)CTAAC(T/C) AATTCACTTTC) (Macrogen Inc., Seoul, South Korea) and read by Chromas software (Technelysium Pty Ltd., Queensland, Australia). The sequences were aligned and assembled using DNASIS MAX (version 3.0; Hitachi, Yokohama, Japan) and BLAST searched (http://blast.ncbi.nlm.nih.gov) in the GenBank database. Our sequences were aligned with reference sequences for each genotype in MEGA software (www.megasoftware.net). A phylogenetic tree was created using Maximum Likelihood algorithms with evolutionary distances calculated by the Kimura-2 parameter method and a bootstrap value of 1000. Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP): All sequences were fed into NEBcutter online server (http://nc2.neb.com/NEBcutter2/), and the BfaI restriction enzyme was selected for in-vitro analysis. The entire PCR product of the Nad1 gene was digested through the BfaI enzyme (Biolabs Inc) for 3-4 hours at 37^{0}C followed by 2% gel electrophoresis and visualization on a UV transilluminator.

RESULTS

Molecular Characterization of Echinococcosis

The extracted genomic DNA of the entire 18 hydatid cyst samples were amplified through Echinococcal mitochondrial gene Nad 1 giving a 530bp amplified band (Figure 1) Sequencing and Phylogenetic Analysis: Sequencing and phylogenetic analysis of the entire samples showed 83.3% (15/18) E. granulosus senso stricto (G1-G3 genotype) followed by E. multilocularis 11.1% (2/18) and 5.6% (1/18) E. canadensis (G6 genotype).

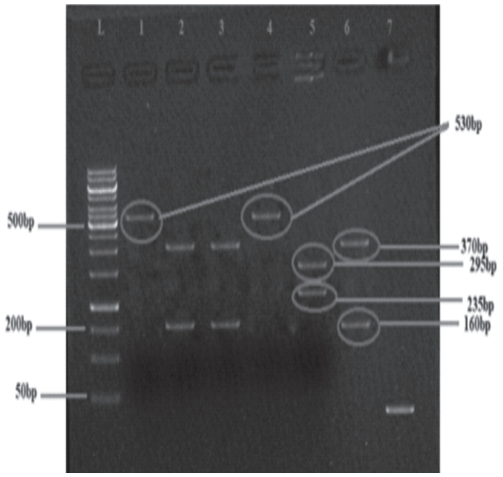

Fig 1: Agarose gel electrophoretogram of PCR product of NAD1 gene and PCR-RFLP of unidentified Echinococcus sp.

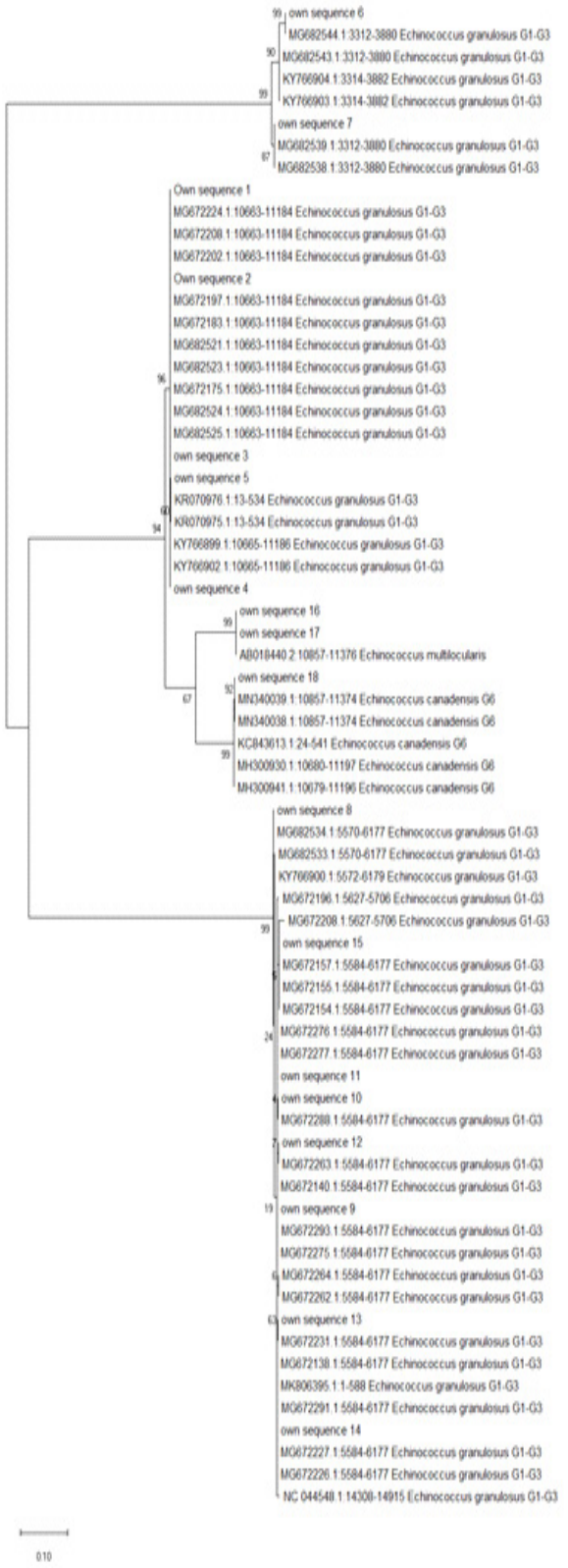

Fig 2:Maximum Likelihood Phylogenetic Tree showing E. granulosus (G1-G3), E. canadensis (G6), and E. multilocularis genotypes based on nucleotide sequences of the NAD1 gene.

PCR-RFLP ANALYSIS

Upon digestion, through BfaI enzyme PCR amplified product was restricted at two sites resulting in band sizes of 256bp, 108bp, and 166bp. Sequencing of the resultant restriction digested bands showed genotype G1-G3 E. granulosus (83.3%), followed by E. multilocularis 11.1% and E. canadensis (G6) 5.6% confirmed by the phylogenetic tree (Figure 2)

DISCUSSION

Echinococcosis is a neglected, zoonotic public health concern of the modern era infecting both humans and animals. Echinococcosis has a complex etiology attributed to ten diverse species of Echinococcus granulosus Senso stricto (WHO, 2021). Two mitochondrial genes, cytochrome C oxidase (COX) and NADH1 dehydrogenase (NAD1), are generally used for the classification species. [25] Various techniques are used for genotypic isolation of the infective or prevalent Echinococcal species in humans, including PCR-RFLP and amplified gene sequencing followed by phylogenetic analysis. Various species were reported from Pakistan, including E. granulosus. (G1-G3) and E. Canadensis (G6/G7), E. multilocularis, through PCR-RFLP; however, some species like E. equinus (G4) and E. ortleppi (G5) are recently reported through sequencing and subsequent phylogenetic analysis. [6-8]

Most of the genotypic studies rely on sequencing, BLAST, and phylogenetic resemblance; however, besides its accuracy, sensitivity, and specificity, the lack of resources and time consumption constrain the developing world towards old techniques like PCR-RFLP and genotypic specific ELISA. [6, 24] The majority of sequencing data reported 10 different Echinococcus granulosus species (G1 to G10) and E. multilocularus all over the world, including Pakistan from the human and animal population. Similarly, the current study revealed that the majority of the human infection was caused by E. granulosus (G1-G3) followed by E. multilocularis and E. Canadensis (G6). [6-9] E. granulosus sensu stricto (G1-G3) is the most prevalent species in the human population. [26-28] On the broad spectrum, genotype G1 is the most prevalent genotype in the world, which might be due to a wide intermediate host range that facilitates higher circulation in the environment. [28-37] A possible reason describing the prevalence of G1-G3 species in the current study could be the animal trade among the neighboring countries. It thus promotes the genotype distribution from one country to another. [31]

Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) use various restriction enzyme for selected mitochondrial genes (rrnL, large subunit of ribosomal RNA) containing a species-specific SspI restriction site for the differentiation among E. granulosus and E. multilocularis from Pakistan while other reports identify 10 genotypes (G1-G10) by using a variety

of restriction enzymes for COX1 and NAD1 mitochondrial genes however unable to separate G6/7 genotype. Another study used three different restriction enzymes for ITS1 and rDNA and reported two species of echinococcus granulosus. [38] Various reports revealed that there a single restriction enzyme cannot be enough for genotyping through PCR-RFLP. However, a recent report suggested that using the restriction enzyme BfaI might differentiate various echinococcus genotypes effectively. In agreement with findings from earlier reports, the current study used the NAD1 sequence and fed it into NEBcutter online server, which effectively distinguished the reported species through BLAST and phylogenetic analysis. In-vitro confirmation revealed G1-G3, G6, and E. multilocularis by using BfaI restriction enzyme through PCR-RFLP.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The study was approved by the Ethical Approval Committee of COMSATS University, Islamabad under Reference No. CUI-Reg/Notif. 2255/19/2661.

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AUTHOR'S CONTRIBUTION

Following authors have made substantial contributions to the manuscript as under

Basharat N:	Concept/ Idea, Literature, review, Drafting & Final Review
Ansari SH:	Analysis & Interpretation of Data, References
Khan J:	Manuscript Writing, Literature review, Analysis & Interpretation of Data
Gul A:	Concept/idea, Data Collection
Ali L:	Concept/idea, Literature review

Authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.