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Genetic Characterization and Strain Differentiation of Echinococcus granulosus Isolates from Ovine Hydatid Cysts Using PCR–RFLP Analysis

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***Abstract*—** Cystic echinococcosis (CE), caused by the larval stage of Echinococcus granulosus, remains a serious zoonotic and public health problem in Iraq and other endemic regions. This study investigated hydatid cysts recovered from sheep slaughtered at an abattoir in Kerbala Province, Iraq, with the aim of characterizing the genetic diversity of local E. granulosus isolates at the molecular level. Of 6,343 sheep examined, hydatid cysts were most frequently recovered from the liver and lungs, and infection rates were further analyzed according to month of slaughter and the age and sex of the animals. The mitochondrial nad1 gene was targeted by conventional PCR for species-level identification, and amplification was achieved for all examined samples, confirming the utility of this marker for detecting E. granulosus. PCR–RFLP analysis of the amplified nad1 fragments using the restriction enzyme HinfI revealed distinct restriction fragment patterns among isolates, indicating genetic polymorphism and suggesting the circulation of more than one genotype, predominantly the G1 genotype.

This study provides the first molecular evidence describing the E. granulosus transmission cycle in Kerbala Province and confirms PCR–RFLP as a practical and reliable tool for strain-level differentiation. Continued molecular surveillance, ideally combined with broader genetic analyses, is recommended to strengthen control and prevention efforts against this parasite

***Keywords* —** Echinococcus granulosus; Hydatid cysts;Kerbala. nad1 gene; PCR–RFLP;Sheep.

INTRODUCTION

Cystic echinococcosis (CE), or hydatid disease, is caused by the larval stage of Echinococcus granulosus sensu lato and affects both humans and a wide range of domestic animals. It remains a significant public health and economic problem in several endemic regions, including parts of `the Middle East, North Africa, South America, and Asia (1,2).The pathogenesis of cystic echinococcosis begins when a definitive host, typically a dog or other canid, ingests hydatid cyst material containing viable protoscoleces, which develop into adult tapeworms in the small intestine and release eggs in the feces. Intermediate hosts, including sheep, cattle, goats, and humans, become infected after ingesting food or water contaminated with these eggs. Once ingested, the oncosphere hatches in the intestine, penetrates the intestinal wall, and disseminates via the bloodstream or lymphatic system, most commonly lodging in the liver and lungs, though other organs such as the spleen, kidneys, brain, and bones may also be affected. Within the host tissue, the oncosphere gradually develops into a fluid-filled hydatid cyst composed of an outer laminated layer and an inner germinal layer, from which protoscoleces and daughter cysts may form. The slow-growing nature of the cyst often results in a prolonged asymptomatic period, with clinical manifestations depending on cyst size, location, and whether rupture or secondary infection occurs. Cyst rupture can lead to anaphylactic reactions or secondary echinococcosis due to the dissemination of protoscoleces, while pressure effects from cyst enlargement may cause organ dysfunction. The severity and progression of pathogenesis are influenced by the genotype and strain of E. granulosus, underscoring the importance of molecular strain identification in understanding disease dynamics and guiding clinical management."

In livestock, CE causes economic losses through the condemnation of infected organs at slaughter, reduced productivity, and impaired reproductive performance, while in humans it typically requires costly surgical and medical treatment (3). Sheep serve as an important intermediate host in the life cycle of E. granulosus. Hydatid cysts developing in sheep tissues typically contain viable protoscoleces, which infect dogs, the principal definitive host, when infected offal is consumed. This continuous cycle between dogs and sheep maintains the parasite in endemic areas and poses a substantial risk to human health (4). Characterizing the genetic variation of E. granulosus is therefore essential for understanding local transmission patterns and improving control strategies. As demonstrated in (5), E. granulosus is not a single, genetically uniform species, but a complex of several genotypes (G1–G10) that differ in host range, pathogenicity, geographic distribution, and zoonotic potential. Among these, the sheep strain (G1) is responsible for the majority of human cystic echinococcosis

cases worldwide (7). Accurate identification and differentiation of these strains is critical for epidemiological surveillance and risk assessment. Because morphological and host-based diagnostic methods cannot reliably distinguish between genotypes owing to their close phenotypic resemblance, molecular techniques have become the standard tool for genetic characterization. Among these, PCR–RFLP is the most widely applied approach, whereby specific DNA regions are amplified and subsequently digested with restriction enzymes; the resulting band patterns reveal genetic variability among isolates.

PCR–RFLP is inexpensive, reproducible, and well suited to the high-throughput processing of large sample sets, making it particularly advantageous in resource-limited laboratory settings; consequently, it has been widely applied in epidemiological studies of E. granulosus (9). Target regions typically comprise mitochondrial genes or ribosomal DNA segments. Applying PCR–RFLP to differentiate E. granulosus strains from ovine hydatid cysts can provide valuable insight into parasite diversity, transmission patterns, and the epidemiology of cystic echinococcosis, information that can subsequently inform the planning of prevention and control programs.

The present study aimed to genetically characterize and differentiate Echinococcus granulosus strains isolated from ovine hydatid cysts using PCR–RFLP analysis, with the objective of identifying the predominant circulating genotype and improving understanding of the epidemiology of cystic echinococcosis in the study area.

MATERIALS AND MTHODS

Study Area and Sample Collection

Kerbala Province is located in central Iraq and is characterized by a hot, dry climate, with ambient temperatures ranging from +5°C to +50°C. Sheep samples were collected from Kerbala Provinceabattoirs in Kerbala Province during 2024–2025, and hydatid cysts were obtained

, and hydatid cysts were obtained from the liver and lungs of slaughtered animals. Cyst fluid was collected following standard methodologies (10,11). Following aspiration, cysts were examined microscopically to assess protoscolex viability. Protoscoleces obtained from fertile cysts were washed one to two times in sterile 0.9% sodium chloride solution and preserved in 70% ethanol for subsequent molecular analysis.(6)

DNA Extraction and PCR Amplification

Tissue samples were cut into small pieces using a sterile spatula and homogenized. Genomic DNA was then extracted using a commercial extraction kit, either the Qiagen DNeasy Blood & Tissue Kit or the Thermo Fisher Scientific Genomic DNA Extraction Kit, according to the manufacturer's instructions. Using sterile scalpels, small pieces (20–25 mg) of liver or lung tissue were placed into sterile microcentrifuge tubes, and lysis was achieved with proteinase K and lysis buffer at 56°C for 1–3 hours to ensure complete tissue digestion. The resulting lysate was vortexed to ensure homogenization, then mixed with 96–100% ethanol and loaded onto the kit's spin column. The column was washed sequentially with Buffer AW1 and Buffer AW2, with centrifugation and discarding of the flow-through after each wash step. DNA was eluted from the column using elution buffer, incubated briefly at room temperature, and recovered by centrifugation. Purified DNA was stored at −20°C for short-term use and at −80°C for long-term storage.

DNA Quantification and PCR Amplification DNA concentration was measured for all samples using a Nano Drop spectrophotometer (Thermo Scientific, USA) at 260 nm absorbance (A260). Genotyping targeted the nad1 gene region using conventional PCR with the primer pair NADF (5′-TATTAAAAATATTGAGTTTGCGTC-3′) and NADR (5′-TCTTGAAGTTAACAGCATCACGA-3′). Each 25 µL reaction contained 12.5 µL of Master Mix (Ampliqon, Denmark), 3 µL of extracted DNA, 10 pmol of each primer, and 6.5 µL of nuclease-free water.

Amplification consisted of an initial denaturation step at 95°C for 5 minutes, followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 45 seconds, and extension at 72°C for 45 seconds, with a final extension step at 72°C for 5 minutes. Reactions were carried out using an automated thermocycler (Flex Cycler, Analytik Jena, Germany). PCR products were resolved on a 1.5% agarose gel and visualized under UV light.

PCR–RFLP Analysis of the nad1 Gene

Purified nad1 PCR products were digested with the restriction endonuclease HinfI (Promega, USA) for 1–3 hours according to the manufacturer's instructions. The resulting restriction fragments were separated on a 2% agarose gel, stained with ethidium bromide, and visualized under UV light. Fragment molecular weights were estimated against a DNA size standard using a standard curve generated in Microsoft Excel, and polymorphisms were scored based on the presence or absence of specific bands.

RESULT AND DISCUSSION

A total of 6,343 sheep were examined at an abattoir in Kerbala Province for the presence of hydatid cysts. The majority of cysts were recovered from the liver and lungs, with lower frequencies observed in the kidney and mesentery. Cyst distribution and infection rates varied according to the month of slaughter and the age and sex of the examined animals, as summarized in Tables 1to3. Molecular characterization of the recovered isolates was subsequently performed using PCR amplification and PCR–RFLP analysis of the nad1 gene.

Table 1. Monthly Distribution of Hydatid Cyst Infections in Sheep

Month	Total	Liver	Lung	Kidney	Mesentery	Meat
October	746	262	160	88	35	80
November	770	319	193	71	22	67
December	773	237	120	69	21	81
January	547	217	105	77	26	51
February	690	248	115	98	44	56

Table 2. Distribution of Hydatid Cyst Infections in Sheep According to Age Groups

Age	Total	Liver	Lung	Kidney	Mesentery	Meat
1–2 Years	1100	456	243	162	43	113
2–3 Years	1230	501	277	114	40	98

3–4 Years	988	477	261	137	37	104

Table 3. Distribution of Hydatid Cyst Infections in Sheep According to Sex

Gender	Total	Liver	Lung	Kidney	Mesentery	Meat
Male	809	384	325	187	53	146
Female	5534	2217	1015	574	173	489
Total	6343	2601	1340	761	226	635

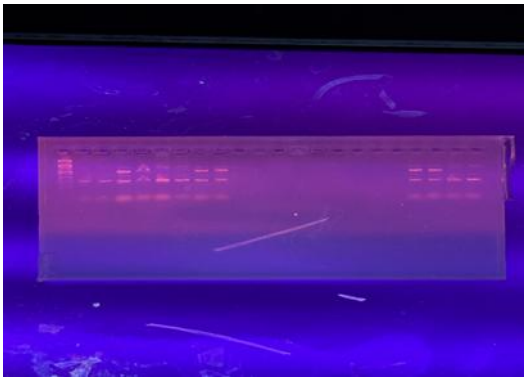

Figure 1. Agarose gel electrophoresis of PCR–RFLP products showing restriction fragment patterns of Echinococcus granulosus isolates from ovine hydatid cysts.

Cystic echinococcosis caused by Echinococcus granulosus is a major zoonotic disease with considerable economic and public health impact in Iraq and other endemic countries. This study characterized isolates recovered from ovine hydatid cysts in Kerbala Province at the molecular level using PCR and PCR–RFLP targeting the mitochondrial nad1 gene. Molecular tools of this kind are increasingly used to elucidate the epidemiology, transmission dynamics, and genetic diversity of this parasite.

Successful amplification of the nad1 gene in all examined samples confirmed that PCR-based detection is a reliable and sensitive method for identifying E. granulosus DNA in material obtained from hydatid cysts. Mitochondrial markers are preferred for this purpose because their relatively high mutation rate allows effective differentiation between closely related genotypes (12). The primers used in this study appeared to amplify the target region specifically, with little evidence of off-target binding, supporting the diagnostic accuracy of the assay. This finding is consistent with previous work showing that PCR-based detection outperforms conventional microscopy, which cannot resolve genotype-level differences (13).

The restriction fragment patterns generated by HinfI digestion revealed genetic polymorphism among the examined isolates, indicating that more than one genotype or strain of E. granulosus circulates within the study area. Similar diversity has been repeatedly reported in other endemic settings, likely reflecting differences in host specificity, pathogenicity, and transmission cycles (14). This pattern is broadly consistent with findings from molecular studies conducted in the Middle East and neighboring regions (15). The predominance of the G1 genotype, commonly referred to as the sheep strain, is consistent with the agricultural profile of Kerbala Province, particularly its extensive sheep farming and the close proximity between livestock and dogs, the definitive host of the parasite. Studies examining the E. granulosus life cycle in the region similarly report G1 as the most widespread genotype, responsible for the majority of human infections (16). The gel electrophoresis profiles obtained in this study correspond to the observed restriction fragment variability; differences in band size (amplicon length) reflect differences in restriction site location within the amplified DNA, which in turn indicate variation in nucleotide sequence among isolates within this relatively small genomic region, consistent with genetic diversity among local E. granulosus populations.

Beyond diagnosis, molecular identification methods offer advantages over traditional culture-based approaches by providing insight into parasite evolution and informing the design of targeted control measures. Because strains may differ in infectivity, host preference, and treatment response, understanding the distribution of circulating genotypes can help guide more effective interventions. The association between specific zoonotic pathogen strains in livestock and human infection is of particular epidemiological importance (17).

It should be noted that this study has certain limitations. Although analysis of a single mitochondrial gene (nad1) provides useful information, it does not fully capture the genetic diversity of the parasite population. (18,19,20).

CONCLUSION

This study demonstrates that PCR–RFLP analysis of the nad1 gene provides an effective method for the detection and differentiation of Echinococcus granulosus genotypes in ovine hydatid cysts. The genetic divergence observed among parasite populations in this endemic area underscores the importance of ongoing molecular monitoring as part of an effective prevention and control strategy.

Recommendations

Based on these findings, several recommendations can be made. Continuous surveillance of the genetic diversity and circulation of Echinococcus granulosus in Kerbala Province is needed, extending as far as possible to definitive hosts such as dogs. The use of nuclear markers for genotyping, or sequencing of the entire mitochondrial genome, would provide greater resolution and reduce the risk of overlooking rarer strains. Closer collaboration between veterinary and medical authorities is recommended for abattoir inspection, ensuring the safe disposal of infected offal and effective control of stray dog populations, measures that would directly reduce transmission from sheep to dogs. Finally, educating livestock producers and abattoir workers about the zoonotic risks associated with hydatid cysts would contribute to controlling cystic echinococcosis and reducing human exposure.Future studies employing multilocus sequencing or whole mitochondrial genome analysis would offer greater genotype resolution. Findings could also be strengthened by including additional samples from other host species or a wider geographic area.

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N/A

Conflict of Interest

The authors declare no conflict of interest.

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