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Biomarker-Based Evaluation and Molecular Identification with Phylogenetic Analysis of *Echinococcus granulosus* from Liver Samples of Sheep and Goats in Al-Qadisiyah Province, Iraq

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Abstract Hydatid disease caused by Echinococcus granulosus is an important zoonotic disease for livestock health and economy in endemic areas such as Iraq. The study aimed at molecularly isolating E. granulosus from sheep and goats and use cytokine biomarkers to determine patterns of immune responses in infected hosts. There were 150 liver samples from sheep (n=75) and goats (n=75) from an abattoir located in Al-Diwaniyah city, Iraq. The 18S rRNA gene was amplified by PCR. PCR products were sequenced, and the phylogenetic analyses were done to establish genetic variation and evolutionary relationships. For biomarker analysis, the cytokines IL-4 and IL-10 were measured with ELISA. The results verified the presence of E. granulosus in all the infected samples. Phylogenetic analysis identified genetic sub-groups within local isolates and regional genetic diversity. Cytokine measurements indicated significantly high IL-10 levels in the animals, especially those with more severe infections, which indicates an anti-inflammatory response. IL-4, by contrast, was variable based on host immune modulation. It explains in detail the molecular and immunological nature of E. granulosus disease in sheep and goats. This combination of phylogenetic and biomarker profiling makes region-specific diagnosis and immune test critical. These discoveries will lead to better diagnostic and management methods for hydatid disease in endemic areas for improved veterinary medicine and economic sustainability.

Keywords: Biomarkers, Cytokines, ELISA, PCR, Phylogenetics

Introduction Echinococcus granulosus, also known as the unilocular hydatid, is a tapeworm responsible for echinococcosis. Echinococcosis is a parasitic disease with significant public health implications that predominantly affects humans and animals (1, 2). The life cycle of the parasitic organism was declared in the early 1920s: definitively, it needs a carnivore to host the adult stage, whereas the larval stage grows into an intermediate host. In humans, the larval stage may cause disease as metacestodes or hydatid cysts, for which the clinical aspects are related to localization, size, and growth in the host organism (3). The resistance of the outer layer of the hydatid cyst, called a "germinal layer," allows the elimination of light and nonspecific host immune response, among others. In addition, E. granulosus may be responsible for providing a significant financial impact since it involves livestock animals in the life cycle and the cystic lesions reduce organ and carcass values. Transmission to humans is mainly due to poor hygiene practices, such as close contact with infected canines and eating food contaminated with the eggs (4).

largely based on some cyclic outbreaks of cystic echinococcosis that were characterized in Mediterranean countries. So far, research and control programs have focused on the interaction between humans, carnivores, and rodents in wild or domestic environments, to find new strategies for personal protection and reduction of infection. Up to now, only the observation of a strict worldwide circulation is possible, and the availability of new diagnostic tools for both human and animal hosts can provide more details concerning everyday transmission (5-9).

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The disease is globally distributed and mostly found in regions with pastoral communities depending on livestock agriculture. Livestock species such as cattle, sheep, goats, equids, swine, and camels act as intermediate hosts. The highest prevalence of E. granulosus has been reported from Central Asia and North and East Africa. Hydatid cysts cause liver condemnation, resulting in huge economic losses to livestock owners (10, 11). Many factors contribute to the continuation of active transmission of E. granulosus: social customs around slaughtering, improper waste disposal, poor livestock management, and socio-economic conditions. Male and markedly more female animals harbor active cysts. Effective transmission by predators and intermediate hosts is required for the persistence of the E. granulosus lifecycle. Dogs are the definitive host of E. granulosus, while herbivorous and omnivorous mammals are frequently infected by ingesting Echinococcus eggs in the contaminated environment (12, 13).

Desert and less developed societies in remote underprivileged locations, where discarded dog fecal matter is not properly addressed and living with dogs is common, remain at higher risk of transmission of E. granulosus infection. Intermittent prevalence and incidence of echinococcosis have been reported from previously free areas as well as variable rates of infection among farm community subpopulations. A vast array of risk factors associated with echinococcosis may be directly related to exposure risk, such as proximity to domestic and/or stray dogs, uncontrolled access of wild dogs to slaughter fields, lack of hygiene and handwashing practices, in addition to risk factors related to underlying social, educational, and cultural factors (14).

Humans become intermediate hosts, especially in parts of the world where dogs have a relationship with the household, as well as with human handling of livestock and a lack of appropriate infrastructure to separate the environment where definitive hosts, mainly dogs, roam. The lifecycle of E. granulosus starts and ends in the intestine of canids, while intermediate hosts are principally herbivorous domestic animals or wild mammals, but humans may also be an accidental intermediate host. The main natural intermediate wildlife hosts of E. granulosus are rabbits, rodents, and ungulates (15, 16). The lifespan of the adult worms in the definitive host is 5 to 15 years and is by far longer in wild carnivore hosts. Adult worms may produce thousands of eggs each day (17,18).

Appropriate management at slaughterhouses to standardize and make hygienic the handling of infected animal viscera is important, as well as potential risk interventions aimed at educating the operators of breeding activities in the abattoir and communicating this risk to local institutional subjects. Public health governance, to prevent human infection, should therefore be based on legislative measures that prioritize the eradication of canine transmission by improving the control of free-running dogs in rural areas (19, 20).

Serological tests, diagnostic tests based on imaging, and surgical and post-mortem findings are useful to diagnose echinococcal infections; however, standardized tests are not available. A variety of serologic tests are available for the immunodiagnosis of echinococcosis, such as indirect hemagglutination, immunoelectrophoresis, and ELISA, which presented a range of sensitivity from 42.0% to 100% and a specificity from 91.4% to 100% (21-23).

Research efforts should aim at improving serological, immunological, and molecular diagnostic techniques, estimating the real burden of the SP, and developing tools that allow for the rapid diagnosis of children who are asymptomatically infected. While some serological tests are used as routine diagnostic tools, they have been less successful in detecting early E. granulosus infections (24-26).

The study aimed at molecularly isolating E. granulosus from sheep and goats and use cytokine biomarkers to determine patterns of immune responses in infected hosts.

Materials and methods

Ethical approval

The study was conducted based on the Ethical Approval issued by the College of Veterinary Medicine, University of Al-Qadisiyah, Iraq (1111 on Oct 22, 2023).

Samples collection

From sheep (n=75) and goats (n=75) slaughtered in an abattoir in Al-Diwaniyah City, Iraq between September 2022 and May 2023, 150 liver samples were collected. They picked animals of all ages, sexes and ailments. Tests were taken at aseptic temperature immediately after slaughter and brought iced on board to the College of Veterinary Medicine, University of Al-Qadisiyah, where they were analyzed.

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In total, we recorded the age, sex and clinical status of all the specimens, to be sure there were strong correlations between the infection state and biological data.

Molecular identification

DNA Extraction

Genomic DNA was extracted from the liver tissue samples using the EasyPure® Genomic DNA Kit (Geneaid, Taiwan) according to the instructions. It was lysis of tissue, digestion of protein by Proteinase K, and binding of nucleic acids to silica membranes in the presence of high-salt salts. Purity and concentration of the extracted DNA were measured on a NanoDrop spectrophotometer, and absorbance ratios of 260/280 nm were determined for DNA quality to downstream use.

Polymerase chain reaction

The test included the amplification of the 18S rRNA gene, a well-known molecular marker for Echinococcus granulosus, by PCR and specific primers. These primers were curated from reference sequences in GenBank (Forward: GGTTTATTGGATCGTGCCC; Reverse: CTGTAACAATTATCCAGAGTC). In each 25 µL PCR reaction, 2 µL DNA template, 1 µL of each primer (10 M), 12.5 µL of AccuPower® PCR PreMix (Bioneer, Korea) and 8.5 µL of nuclease-free water were used. The PCR conditions were first denaturation at 94°C for 5 minutes, 35 cycles of denaturation at 94°C for 1 minute, annealing at 52°C for 1 min, extension at 72°C for 2 minutes, and extension at 72°C for 10 minutes. Prominent products were represented on a 1.5% agarose gel stained with ethidium bromide and recorded in the ultraviolet light.

Sequencing and phylogenetic analyses

Positive PCR products were cleansed by the Geneaid Gel Extraction Kit and sent for sequencing to Macrogen Inc., South Korea. Those sequences were BLAST'd and compared with reference sequences in the NCBI GenBank database. The phylogenetic analyses included using MEGA software to analyze genetic variation and evolutionary distance between isolates. To validate the evolutionary clusters, neighbor-joining trees were built using bootstrap.

Serological analysis

To assess for biomarkers, IL-4 and IL-10 cytokines were measured with an ELISA kit. Standard curves were created by serial dilutions of standard concentrations and OD was measured at 450 nm by a microplate reader. Concentrations from the samples were averaged out. The cytokine concentrations were measured.

Statistical analysis

The data were processed using GraphPad Prism software to analyze them statistically. Comparison of cytokine levels between groups was done using one-way ANOVA and post hoc test, where p-value less than 0.05 was considered statistically significant.

Results

This study's molecular and biomarker analyses were informative about the genetic variety of E. granulosus and the immune response it causes in sheep and goats. PCR amplification of the 18S rRNA gene confirmed that the parasite is highly prevalent in the 150 liver samples we had tested (100 per cent of liver samples were positive for E. granulosus) (figure 1).

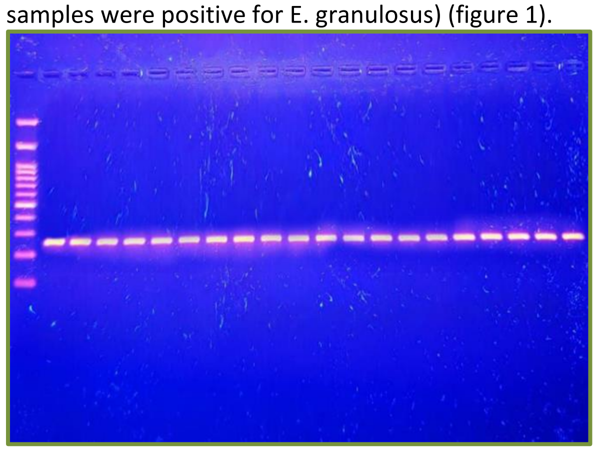

Figure 1: Image of agarose gel electrophoresis of the 18S rRNA gene from Echinococcus granulosus isolated from livers of sheep and goats, M: ladder, PCR products at 225bp.

The PCR products (the corresponding expected amplicon size of 225 base pairs) were sequenced, and phylogenetic analysis showed genetic subgroups among local isolates. These isolates were organized into clades, which meant there was genetic diversity between samples. The tree of life showed an overlap between the local isolates and some global genotypes, suggesting regional variation and evolutionary potential of E. granulosus in Iraq (Figure 2).

Evaluation of biomarkers using ELISA added yet more information about host immune responses to E. granulosus infection. Infected animals had much higher IL-10 concentrations than non-infected controls, with most elevated levels in those with worse infections. This discovery supports IL-10 as an anti-inflammatory cytokine that probably works to regulate the host immune system to inhibit parasitic overexposure to tissue damage. IL-4, on the other hand, was widely varied between samples. Some patients had high levels of IL-4 – a sign of Th2-mediated immunity, but others had low levels, which suggested that immune regulation could vary with host species, age and health (Figure 3).

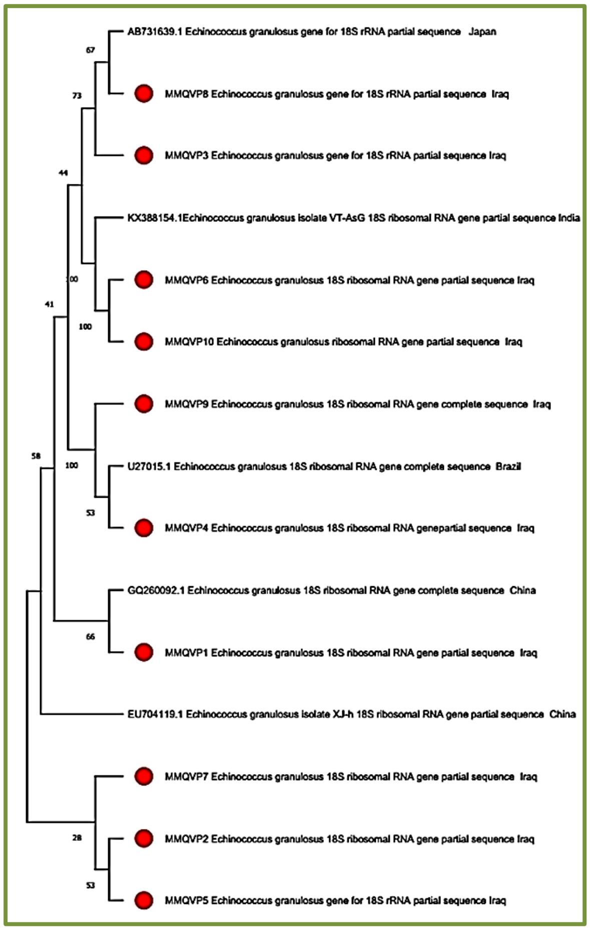

Figure 2: Phylogenetic tree of Echinococcus granulosus isolated from livers of sheep and goats.

Discussion

This study on E. granulosus in sheep and goats from Al-Qadisiyah Province, Iraq sheds new light on the genetic variation and host immune responses of cystic echinococcosis (CE). With molecular and biomarker studies, this work joins and builds on previous work from around the world with important similarities and differences. The fact that E. granulosus was found in this study, detected by PCR in 100% of liver samples, shows how widespread the parasite is across endemic areas. Bosco et al (27) found high CE in sheep (62.9%) and goats (20.9%) throughout the European Mediterranean region, with organ preference being mainly for the liver in sheep and the lungs in goats. Similarly, Aziz et al. reported an epidemic incidence of hydatid cysts in sheep and goats in Sulaimani Province, Kurdistan Region, Iraq, particularly in goat males (28). These results confirm the parasite's persistence in herds, and its commercial and public health costs.

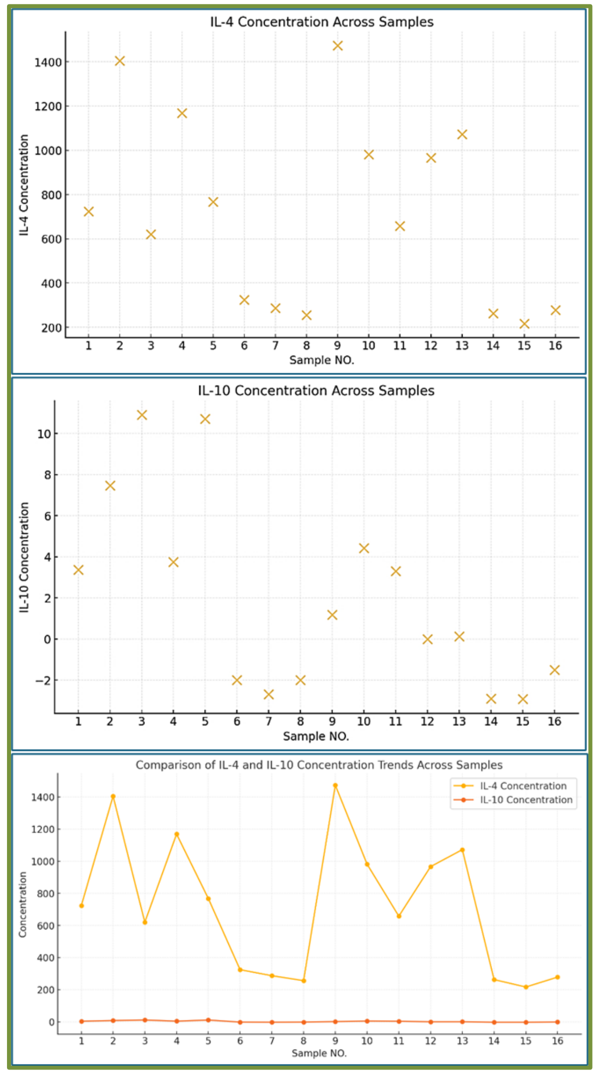

Figure 3: Biomarker levels in sheep and goats affected by Echinococcus granulosus.

Phylogenetic analyses found genetic variation in many Iraqi isolates, with close clustering to genotypes identified elsewhere (Japan, India, and Brazil). Fadakar et al, (29) – G7 genotype in goats in Northeast Iran, and the impact of regional trade and environment on genetic variation. Similarly, Gholami et al. had reported Echinococcus ortleppi (G5) in sheep and goats in Iran, proving yet again CE's gene richness in the Middle East (30). The similarity of Iraqi isolates to Indian (KX388154.1) and Chinese (GQ260092.1) matches that of Moudgil et al who showed large genetic diversity in Indian isolates with widespread hepatic and pulmonary CE (31). These associations point to a regional genetic pool shared by animals moving from place to place.

Cytokine testing showed high IL-10 concentrations in the infected animals, especially the severe cases, which appeared to be an anti-inflammatory response. This observation fits with Antepli Olu et al, (32) who showed that E. granulosus infections cause severe tissue damage, and IL-10 counters over inflammation. It's also notable that the differences in IL-4 expression in this experiment suggest multifactorial immune responses, possibly driven by host species and disease. This echoes Joanny et al. (33) in Lebanon who found difference in cyst fertility and organ tropism between sheep and goats.

This genetic variation in Iraqi isolates matches that around the world. In China, Hua et al. (34), identified G1 and G3 genotypes as most common, with regional differences based on environment and host. Similarly, Laatamna et al. (35), found high genetic variation in Algeria, which points to E. granulosus' general spread and pragmatism. In contrast, Selcuk et al. (36) in Türkiye describes the evolution of Echinococcus canadensis (G6/G7) in goats.

Remarkably, the hepatic involvement was predominant here (which is different from the higher pulmonary involvement reported by Moudgil et al. in India (31). This disparity could be a result of regional differences in parasite tropism or host-pathogen relationships. This high genetic similarity of some Iraqi isolates with other countries and regions suggests the value of transboundary partnerships to address CE.

Other controls of CE have been successful. Poggio et al. in Argentina and Chile showed that One Health-immunization of sheep reduced cyst rates by as much as 75% (37). These results show the promise of these kind of integrated interventions in Iraq and adapted to regional socioeconomic and cultural realities (38-55).

Conclusion

This study describes all the infections of E. granulosus in Iraq using molecular and biomarker evidence to explain the genetic variability of the parasite and immune response dynamics. The results underscore the necessity for regional diagnostics and control plans, based on effective models from other endemic areas. Enabled surveillance and research is essential to reducing the burden of CE and increasing veterinary and public health outcomes around the world.

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Conflict of interest

No conflict of interest was declared by the authors.

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