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Molecular and Immunological Characterization and Incidence Rate of Hydatid Cyst Isolated From Liver and Lungs for Human in Kurdistan Region, Iraq

Muslim Abbas Allu*

Nursing Department, Technical Institute of Zakho/Duhok, Ministry of Higher Education and Scientific Research, Kurdistan Region, Iraq.
*Correspondence to: Muslim Abbas Allu (E-mail: muslim.allu@dpu.edu.krd)
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Abstract

Objective: This study aimed to identify characteristics, location, cyst stage, and species/genotypes of *Echinococcus granulosus* s.l. complex in humans from the Kurdistan region, Iraq. Surgical removal of 25 echinococcal cysts from 22 patients in Dohuk and Zakho cities (January 2020 to December 2022) was analyzed.

Methods: Characteristics of 22 surgically confirmed cystic echinococcosis (CE) cases, including anatomical site, cyst fertility, and patient demographics, were examined. Molecular analysis using mitochondrial NAD5 gene confirmed genotypes G1 or G3 of *E. granulosus* s.s., indicating sheep–dog–human transmission.

Results: Liver (78.6% fertile, 21.4% sterile) and lungs (81.8% fertile, 18.2% sterile) were the most common anatomical sites for CE in females (59.1%) compared to males (40.9%). All analyzed samples belonged to genotypes G1 or G3. No statistically significant correlation was found between genotypes and variables such as fertility, location, and cyst stage.

Conclusion: The study underscores the prevalence of genotypes G1 or G3 in the *E. granulosus* s.l. complex, confirming sheep–dog–human transmission in Kurdistan, Iraq. Recommendations include monitoring and control programs in sheep and dog populations and public health education campaigns to reduce the risk of acquiring CE in humans.

Keywords: *Echinococcus granulosus,* G1/G3 genotypes, human cystic echinococcosis, ELISA, immune response, Kurdistan region, Iraq

Introduction

Hydatid cyst (HC) or hydatidosis is a helminthic zoonotic disease that results from infection with the cyst stage of *Echinococcus granulosus*.[1,2] This disease is a chronic disease and highly endemic in most of the world countries including Mediterranean region.[3] The adult parasitic stage (tapeworm) resides Hydatid in the small intestine of dogs and other wild canid definitive hosts, which contaminate the environment with infective eggs contained in their feces. The asexual larva (metacestode) is found in a wide variety of ungulate intermediate hosts, including humans (due to the ingestion of infective eggs) that act as dead-end hosts.[3] CE may develop in any organ or tissue of humans and intermediate hosts, even if most of the infections can be found in the liver and secondarily in the lungs.[4]

Hydatid cystic disease has been known since ancient times and are one of the most serious epidemics that harm humans both in terms of health and economics.[5] According to The World Health Organization (WHO), the Current estimates that cystic echinococcosis results in the loss of 1–3 million disability-adjusted life years (DALYs) annually. Annual costs associated with cystic echinococcosis are estimated to be US$ 3 billion for treating cases and of losses to the livestock industry. The disease causes 19,300 deaths worldwide annually, WHO classifies it among 17 neglected tropical diseases (NTDs).[6]

Approximately 60%–70% of hydatid cysts takes place in the liver and 20%–25% followed in the lungs. Bones, kidneys, spleen, muscles, CNS and behind the eye is the other organs infected by cysts.[7] However, symptoms of hydatid disease can product from the release of antigenic material and secondary immunological reactions that develop following cyst rupture. Fever and acute hypersensitivity reactions ranging from urticarial and wheezing to life-threatening anaphylaxis may be the principal manifestations. While allergic episodes may develop after cyst rupture, fatal anaphylaxis is uncommon.[8] Cystic echinococcosis can be controlled through numerous blocking measures that break the life cycle between the definitive and intermediate hosts; however, the most operative means to resistor hydatid disease in humans and eliminate the consequences of Echinococcus infections in livestock is through the broad range education of people living in endemic regions.[9] Contemporary phylogenetic hypotheses have been largely based on data derived from mitochondrial sequences, as mtDNA provides useful genetic markers, whereas an alternative source of phylogenetic characters from the nuclear genome has not received much attention.[9]

In Iraq, reports ranging from prevalence rate of 10–96 cases/year among human.[10,11] Duhok Azadi Teaching hospital statistics,[12] to infection rate among animals of 12.3–15.6%, 1.4–6.2%, 1.6%–10.9% among sheep, goats and cattle respectively.[12] On the other hand, much higher prevalence rates have been reported among dogs which ranged from 38–79.1%.[10,13] To date, 10 genotypes (G1-10) within *E. granulosus* have been identified and this categorization follows very closely the pattern of strain variation emerging based on biological characteristics.[14-19] The level of genetic differences between recognized species of Echinococcus are not appreciably greater than those between strains.[20-22] All the genotypes of *E. granulosus* except the dog/horse (G4) and the Finnish cervid (G10) strains have been found to infect humans.[23] Due to the presence of more than one livestock animals in our locality as well as the presence of more than one strain in neighboring countries, such as

in Iran G1, G3 and G6,[24-26] in Turkey G1 and G3[27,28] in Jordan G1 and possibly G4.[29] Therefore, it is necessary to conduct this study in order to identify the *E. granulosus* strain(s) circulating in this Province and in the light of obtained results the. Clinical signs and symptoms of the disease are not specific and depend on the location of the cyst.[30] Diagnosis of the disease relies on paraclinical methods. Imaging techniques are able to identify the cyst in the body, but they are not able to differentiate the cyst from benign or malignant tumors.[31] Moreover, the infection may be associated with other infections such as fungal infection.[32] In these cases, also, differential diagnosis is important. Different immunological methods including Casoni test, complement fixation, indirect hem agglutination, latex agglutination, enzyme-linked immunosorbent assay (ELISA), and immunofluorescent methods have been used for this differential and specific diagnosis.[33] In previously published works, different sensitivity and specificity has been reported for the ELISA test. This variability may be due to the type of antigen and geographic region of the parasite.[34-36]

A feature of infections by helminthes is that polarize the immune response toward TH2 pattern.[37] This response, however, has not been correlated with resistance in all Introduction cases, and its role in the host- parasite interaction remains unknown in many infections.[38] Epidemiological data concerning cystic echinococcosis have shown that most infections do not develop to the disease and it is likely that immune response play a pivotal role in limiting cystic larval development.[39] Very little is known about the factors affecting innate susceptibility to hydatid cyst infection, following ingestion of the infective egg stage and establishment of the cyst.[39,40] In the early stage of the infections, the onchosphere is transported toa host organ such as liver or lung … etc, where it develops into a hydatid cyst. The immature cyst may overcome host defense mechanisms.[41-43] About 8–10 weeks post infection a complex of echinococcal antigens are released from the cyst and stimulate complex immune responses, which include polarized TH2 response balanced with TH1.[44,45]

Numerous studies have provided evidence that *Echinococcus granulosus* exists as a complex of different strains that differ in a wide variety of criteria that impact on the epidemiology, immunology, pathology and control of hydatid disease.[46-49] Cellular and humoral immune responses in humans, in contrast to those in experimentally infected animals, can vary enormously, as evidenced by the different patterns of parasite antigens in different patients and courses of the disease. These disparities are likely related to the strain variation of the parasite and/or genetic differences between host populations.[50,51] In Kurdistan region very little information is known about the immune responses that are induced by the local strain of *Echinococcus granulosus*.

The primary aim of this study was to determine the, location and causative species of the echinococcal cysts isolated from humans in Zakho City, the Duhok governorate using a molecular method validated according to regulation ISO/IEC 17,025 for the identification of species and genotypes belonging to *E. granulosus* s.l. The secondary aim of this study was to search for correlations between the genotype and anatomical location, SHAPE OF echinococcal cysts and the presence of specific potential risk factors for humans. Third aim of this study was undertaken to investigate humoral immune responses that are developed as a result of host defense mechanism against hydatid cysts.

Materials and Methods

Collection of Samples

A total of 22 human lung and liver hydatid cysts were collected from male and female patients during the period from January 2020 to December 2022, from the surgical theaters of 2 hospitals in Zakho and Duhok cities, these districts have 1 million people. According to sex, the rate of infection in females was higher than that of males. According to the anatomical location, the present study revealed that the liver was the predominant site of infection of the parasitic cysts were isolated from there, followed by the lungs.

Examination of Fertility

The isolated parasitic cysts from liver and lung was higher rate of fertile cyst comparative with sterile cyst. The estimation of the viability rate of fertile echinococcal cysts, each cyst fluid was centrifuged for 5 min at 3000 × g rpm. Subsequently, one drop of the precipitate was taken using a sterile pipette and placed onto a clean glass slide together with a drop of 0.1% aqueous eosin solution (v/v), mixed and covered with a cover slip and examined under 40 × magnification. Living protoscoleces did not take up the stain, unlike the dead ones.

Molecular Analysis of Echinococcal Cysts

A total of 25 cyst samples from 22 patients were molecularly analyzed for Echinococcus species identification. 25 cyst samples were analyzed using a Restriction Fragment Length Polymorphism–Polymerase Chain Reaction PCR assay.[52] The first step of the method in conventional PCR amplified a fragment of 444 bp, the cytochrome c oxidase subunit 1 (COXI) mitochondrial gene, while the second step (RFLP) produced two fragments of 235 and 209 bp. All 22 samples were identified as *E. granulosus* s.s. (genotypes G1 and G3), 22 *E. granulosus* s.s. samples were further characterized by the amplification and sequencing of the NADH dehydrogenase subunit 5 (NAD5) mitochondrial gene to differentiate genotypes G1 from G3.[53] Analysis of the informative nucleotide positions within this gene allowed the identification of 14 samples as G1 and 8 as G3. In case of multiple organ involvement, the genotypes were concordant.

Enzyme-Linked Immunosorbent Assay (ELISA)

In this study IgG ELISA was performed to assess humoral immune responses. Antibody responses were analyzed by ELISA with slight modification. Briefly, the wells of polystyrene microplates were coated with 100 µl of 20 µg/ml of hydatid antigens in 0.1 M sodium carbonate/bicarbonate buffer pH 9.2 and incubated overnight at 4°C. After the coating buffer was discarded, the plate was blocked for 60 minutes at 37°C with 1% bovine serum albumin in phosphate buffer saline (PBS), pH 7.4 and washed with PBS -Tween 20 solution (0.05%). The serum samples were diluted 1:200 in PBS- Tween 20 solution containing 0.5% bovine serum albumin. After 90 minutes of incubation at 37°C the plates were washed thrice with PBS- Tween 20 solution. One hundred µl of peroxidase-conjugate anti-human IgG were added to each well and incubated 60 minutes at 37°C. After washing (3 X), a volume of 100 µl of substrate solution was added to each well and incubated for 15 minutes at room temperature (18–22°C). After the last incubation, the reaction was stopped with 100 µl of 1N HCL.

Optical density was measured at 450 nm. The cutoff value of ELISA, which differentiates positive from negative reactions, was established by the mean value of normal serum group plus three standard deviations.

Statistical Analysis

The results were statistically analyzed performed by using Microsoft office Excel 2003 and (SPSS; P values < 0.05) were considered statistically significant.

Results

Table 1 shows the most frequently infected patients were females, 13 (59.1%) in females, while males less frequently encounter the disease, as it's reported in 9 (40.9 %).

The characteristics of the patients enrolled in the study are shown in Table 2. According to sex, the rate of infection in females was higher than that of males, according to the anatomical location, the present study revealed that the liver was the predominant site of infection as 12 case of the parasitic cysts were isolated from there, followed by the lungs at 10 case.

Table 3 Shows that 78.6 % of the isolated parasitic cysts from liver was fertile, and 21.4% was sterile, while of the lungs shows that 81.8% of the isolated parasitic cysts was fertile and 18.2% was sterile.

Nucleotide substitutions of the NAD5 mitochondrial gene, based on the Reference G1 sequence AB786664 are shown in Table 4. A total of 25 cyst samples from 22 patients were molecularly analyzed for Echinococcus species identification. 25 cyst samples were analyzed using a Restriction Fragment Length Polymorphism–Polymerase Chain Reaction PCR assay.[52] Analysis of the informative nucleotide positions within this gene allowed the identification of 14 samples as G1 and 8 as G3. No statistical correlation (P > 0.05) was found in the logistic regression between both genotypes G1 and G3 of *E. granulosus s.s.* and the characteristics of the echinococcal cysts, such as fertility, anatomical location.

Table 1. **The distribution of patients with HC according to sex**

Sex	No. of patients	%
Males	9	40.9
Females	13	59.1
Total	22	100

Table 2. **Showing the number, the host gender and the site of the isolated hydatid cysts from human body**

Host gender	Liver site	Lung site	Total number
Human (male)	5	4	9
Human (female)	7	6	13
Total	12	10	22

Table 3. **The number and fertility rate of human cysts isolated from anatomical location with infected by *E granulosus***

Cyst location	No. of examined cysts	Fertile cysts	(%)	Sterile cysts	(%)
Liver	14	11	78.6	3	21.4
Lung	11	9	81.8	2	18.2
Total	25	20	80	5	20

Table 4. **The number and percentages of G1 and G3. *E granulosus* sensu stric, identified in the current study and their anatomical location**

Genotype	No.	%	NAD5 nucleotide substitutions						Location			
									Liver		Lung	
									No	%	No	%
G1	14	63.6	G	A	C	G	A	G	10	45.5	4	18.1
G3	8	36.4	C	G	T	A	G	A	6	27.3	2	9.1
Total	22	100							16	72.8	6	27.2

Table 5. **Detection of anti-hydatid antibody in 22 patients with surgically confirmed cystic echinococcosis**

Organ involved	No. of cases	No. of positive	%	Cut-off value
Liver	12	11	91.66	
Lung	10	8	80.00	1.27
Total	22	20	100	

Table 5 shows the results of ELISA on the sera from 22 surgically confirmed cystic echinococcosis patients. The highest sensitivity obtained was in patients with liver (91.66%), while patients with lung infestation exhibited the lowest sensitivity (80%).

Discussion

The present study showed that the involvement of the liver was at the highest degree 12 cases and 10 cases in lungs. That is in agreement with most other similar studies, in Iraq[54] showed the liver was the most frequent site of infection involved in 67 and the lung was affected in 21. In Iraq during January 1986 to January 2006, 763 patients underwent surgery for thoracic hydatidosis in teaching hospital in Baghdad, during these 20 years, hepatic hydatid cysts occur in 70% and pulmonary cysts in 20%.[55-57] The same results were found in various parts of the world, a total of 390 surgically confirmed HC cases were found in the records of surgical hospitals of the West Bank of the Palestinian Authority for an 8- year period 1990–1997.

A total of 117 patients were admitted with the diagnosis of HC during the five year study period in Saudi Arabia from December 1999 to December 2004.[58] Liver was the primary site in 93 (79.5%) patients, this result was higher than the result of the current study may be due to the long period of study. The liver acts as the primary filter for the parasite, on the contrary, the lungs acts as the secondary filter but in few studies some researchers found that the lungs were the predominant site of HC in Theqar (Iraq),[59] in Iran,[60,61] in Baghdad (Iraq)[61-65] in Pakistan, in Erbil (Kurdistan region, Iraq).[63] In this aspect, the present results contradict other studies in Iran as they reported higher infection rates in urban populations. Although hydatid disease may be found in any areas, but people who are living in rural area are at higher risk for acquiring the infection and HC is generally considered a rural disease because of its characteristics of transmission, which involves dogs and domestic animals such as sheep, cattle and goats.

A study reported that the number of women which underwent surgery were more often than men, and that housewives had the highest rate of surgery.[66] The high rates of women and housewives might be due to sweeping their yards, where the dust contains *E. granulosus* eggs from dogs in rural areas, and cleaning and eating raw vegetables, urban situations.[66] The differences in studies related to females and males' ratio could be attributed to the difference in socio-economic factors from country to country. Regarding the number of cysts, it was noted that most of the HC cases had single cysts in each of the affected organ followed by multiple cysts. These findings were in accordance with those of other studies which reported that most of the patients were affected with unilocular hydatid cyst.

The present study higher cases were reported in females as compared with males. This agrees with many reports. A retrospective study was undertaken to determine the incidence of HC among patients hospitalized in Kashan during 1993–2000 in Iran. From 85 patients, 47 females (55.3%) and 38 males (44.7%) had hydatid cysts.[67] In another study, it was found that the seropositivity rate of females was higher than that of males 17.2% and 9.4% respectively in prevalence study of HC in Turkey.[68] In northern Iraq, a study of[69] found that the HC is endemic, and that females appear to be at the greatest risk of infection. As previously discussed, the higher prevalence of CE in females in this study might be related to many factors, such as occupation and cultural habits; in addition, females are more in close contact with infection sources, such as soil or vegetables contaminated with viable eggs of *E. granulosus* from dog feces.[70] Nevertheless, being female cannot be excluded as a confounding factor of living in rural contaminated endemic areas, since large cohort studies on CE did not find any statistically significant difference between the male and female prevalence.[71]

According to this study, the frequency of human fertile cysts was higher than that reported for sterile cysts. This finding highlights the risk of secondary CE infections during surgical procedures, stressing the use of albendazole as an adjuvant either preoperatively or postoperatively, in the study shows that 78.6 % of the isolated parasitic cysts from liver was fertile, and 21.4% was sterile, while of the lungs shows that 81.8% of the isolated parasitic cysts was fertile. and18.2% was sterile. These results are consistent with Salem[72,73] in Mauritania, and Romania and in Baghdad (Iraq).[74] The fertility rate of hydatid cysts coupled with molecular typing studies are important factors in the epidemiological studies as these could provide valuable information on the pathways of transmission.

The identification of *E. granulosus* s.s. in the Kurdistan region is in line with other studies that identified this species as the most prevalent (88.5%) in humans among all molecularly confirmed *E. granulosus* species in worldwide.[75] The subset of cysts analyzed for genotype G1 or G3 distinction revealed the presence of both the genotypes and the predominance of G1 genotype, with no specific correlation with age, gender or occupation. The results of this study are similar to previous studies from the Kurdistan region and other areas of Iraq, showing that the G1 genotype is predominant in humans from Sulaimani province,[76] Dohuk province,[76,77] Kirkuk province,[78] Misan province[79] and Al- Najaf and Al-Diwaniyah provinces.[80] Furthermore, these genotypes were reported in countries neighboring Iraq, such as Turkey,[81] Iran,[82] Jordan[83,84] and Saudi Arabia.[85] These results are also in line with a recent systematic review that highlighted G1 as the main genotype present in Europe.[86,87] Whether genotypes G1 and G3 may be of different grades of infectivity and pathogenicity to humans and animal species is currently unclear and requires further systematic epidemiological studies. Even though the distinction between G1 from G3 represents a useful tool for source attribution in the field of molecular epidemiology, this study did not find any evidence for potential correlations between cyst biological features and these genotypes.

Immunodiagnostic testing for serum antibodies or circulating antigens provides supportive evidence of echinococcosis. An enzyme-linked immunosorbent assay or indirect haemagglutination test are commonly used as initial screen for anti-hydatid antibodies.[88] Moreover, fast detection of the cases in the endemic areas is very important, in order to prevent further spread of the disease to other areas.[89] The presence of inhibitors in the serum and the role of immune complex which interfere with normal antigen-antibody interaction.[90,91]

In the present study the sensitivity of ELISA was also affected by the localization of the cysts. ELISA technique has a sensitivity of 91.66% in detection of liver cysts, while the sensitivity of this test is around 80% in lung cysts.[92] The high number of false negative results may be a result of several factors such as strain variation of the parasite which may change the immune response that impact on the diagnostic results. Liver cysts release more antigenic materials into surrounding host tissues, unlike lung cysts which are usually surrounded by an intact hyaline cyst wall.[93,94] In helminthes infections not only the innate immune system, but also adaptive immune mechanisms appeared to be involved in the defense against the parasite.[95]

Conclusion

This study confirmed that CE is endemic with higher rates in rural areas and among females, and it is caused by *E. granulosus* s.s. in Duhok and Zakho cities, Kurdistan region of Iraq. Based on the present findings, it is necessary to implement monitoring and control programs in sheep and dog populations to decrease the odds of human infections, the prevalence of infection is highest in females more than males. Infection rates of this parasite vary widely throughout Iraq for a variety of reasons, including the presence or absence of definitive and intermediate host most common strains in the Kurdistan Region of Iraq are *E. granulosus* sensu lato (G1–G3).

The PCR technique had a high specify in the detection of *E. granulosus* especially, the occurrence of G1-G3 sensu stricto cluster genotypes in Duhok and Zakho cities, Molecular studies to specify the local strain of *Echinococcus granulosus* in Kurdistan region and investigation of the production of homologous mammalian cytokines by *Echinococcus granulosus* metacestode are recommended. Finally, the current work showed that the local strain of *Echinococcus granulosus* induces humoral immune responses.

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