IOSR Journal of Pharmacy and Biological Sciences (IOSR-JPBS)
e-ISSN: 2278-3008, p-ISSN:2319-7676. Volume 9, Issue 3 Ver. II (May -Jun. 2014), PP 06-10
www.iosrjournals.org

Genotyping of Echinococcus granulose in Samples of Iraqi Patients

Dr. Maysara S. Khalf [1]; Dr. Mohammad A. Al –Faham [2]; Dr. Lazim H.Al-Taie[3] ; Dr. Hydar Aed Alhussian[3]

[1]*Assi.lecturer College of Medical & Health Technology.*
[2]*Assit .Prof .College of Medicine /Al-Nahrain university*
[3]*Assit. Prof .College of Medicine / Baghdad university.*

Abstract:
Background: Cystic hydatid disease is an important zoonosis, affecting humans and animals and is a significant public health and economic problem throughout the world and Iraq. Since extraction of DNA from the parasite is a primary and crucial step which has a principal effect on PCR results, in the current study five simple methods for DNA extraction from protoscoleces of Echinococcus granulosus were applied and compared with each other. The aim of study was to ascertain the genotype(s) of the parasite responsible for human hydatidosis inIraq.

Patients and Methods: *After collecting hydatid cysts from patients after surgical operation , DNA samples were extracted from (60) hydatid cyst , using five different methods involving the use of glass beads, mechanical grinder, freeze-thaw, boiling and crushing. For all DNA samples extracted, one PCR assay based on amplifying rDNA-ITS1 region was performed and amplicons resolved on 2% agarose gels*
For genotyping of echinoccocus 5 ng template DNA were added into the PCR mix.
And 11 primer pair were used for genotyping .

Result: *Most of the CE patients(60) patients were found to be infected with hydatid cyst of eitherG4 26(43%),G1/G/G3 18(30%), G5 12(20%),G6/G7 4(7%). And the study the association of genotypes and organ ,our results show*
In the liver (20%) G1/G/G3 ,(27%)G4 ,(7%) G5 ,(20%) G6/G7. Lung (7%) G1/G/G3,(10%)G4,Liver and lung (3%) G1/G/G3 andG4 ,finally liver ,lung and spleen (3%) G1/G/G3.

Conclusion: *These findings demonstrate the zoonotic potential of G4 (Horse strain), G1 (sheep strain) and G3 (buffalo strain),G5(Cattle Strain) , (G6) (Camel Strain) ,(G7) (Pig strain) genotypes of E. granulosus as these emerged as predominant genotypes infecting the humans in Iraq.*

Key word: *Echinococcus granulosus , PCR , Germinal layer , genotypes .*

I. Introduction

Hydatidosis/cystic echinococcosis (CE) is a severe zoonosis caused by the larval stages of a cyclophyllidean cestode called Echinococcus granulosus. Two hosts are involved in the completion of the life cycle of E. granulosus. The definitive hosts are carnivores which harbour mature tape worms in the intestine (1,2). The intermediate hosts of E. granulosus include ungulates both domestic and wild animals and humans. The adult worm lives in the small intestine of carnivores (definitive host), and the intermediate larval stage (Hydatid cyst or Hydatid) develops in the internal organs of a wide range of mammalian species such as goats, sheep and cattle, including humans, which acquire the infection through accidental ingestion of the tapeworm eggs (3)

Cystic Echinococcosis is known to be one of the most important parasitic infections in livestock worldwide and one of the most widespread parasitic zoonoses (4,5).

The disease has a worldwide distribution and is endemic in many countries of the Mediterranean basin, North and East Africa, Western and Central Asia, China, South America and Australia (6,7).

However, even if the distribution of Echinococcus granulosus is considered worldwide, it is higher in developing countries, especially in rural communities where there is close contact between dogs and various domestic animals (3).

In some western countries, CE is being considered a remergingzoonosis, due to recent increases in the observed prevalence (8,9).

In humans, CE is the most common presentation and probably accounts for more than
95% of the estimated 2 to 3 million global cases (10,11).

The echinococcosis/hydatidosis has considerable socioeconomic impact in both human and animal health in different countries (12). In humans, after a long latency period, the disease consequences may include;

poor quality of life (disability adjusted life years [DALYS]); costs of medical treatment, lost opportunity for income generation and mortality in some cases(11) while in animals there is reduced productivity and monetary losses due to abattoir condemnations (13,14). The DALYS for human CE was recently estimated to be more than that for onchocerciasis and almost the same as that for Africa trypanosomaisis(15).

Transmission and maintenance of echinococcosis is dependent on complex interactions of several factors, including environmental, host and pathogen factors. A number of such factors are of local epidemiological significance and the identification of such factors is important in the effective implementation of control strategies In Iraq, echinococcosis has been reported to occur in many parts of the country.(16,17). The "sheep" strain (defined as Gl. on mitochondrial genotypic grounds) is generally considered as the most widespread strain of E. granulosus in the world and the one mainly involved in CE in humans (Thompson and Lymbery, 1990). At least five out of ten strains of Echinococcus granulosus (G1 to G10) are infective to humans in sub- Saharan Africa (Magambo et al., 2006)

The worldwide distribution of CE reveals a geographic heterogeneity of E. granulosus species in many overlapping areas. Some examples are the co existing genotypes E. granulosus s.s. (G1) and E. canadensis (G6) in North African countries [23,30–32], E. granulosus s.s. (G1/G2), E. ortleppi (G5) and E. canadensis (G6/G7) in Argentina [20,33,34] or E. granulosus s.s. (G1), E. canadensis (G6) and E. equinus (G4) in Kyrgystan [35]. In these areas co infections with more than one E. granulosus species/genotype might occur in the intermediate or definitive hosts. In addition, the not yet confirmed hypothesis of an eventual genetic exchange by sexual reproduction between E. granulosus
species/genotypes is still discussed [36).

Extensive research on genetic variation, intermediate host affinities as well as morphological, biological and biochemical differences resulted in a more sophisticated classification of the dog tapeworm E. granulosus into ten genotypes/strains [4–6]: sheep strain (G1), Tasmanian sheep strain (G2), buffalo strain (G3), horse strain (G4), cattle strain (G5), camel strain (G6), pig strain (G7), cervid strain (G8), pig/human strain (G9) and Fenno- Scandian cervid strain (G10). The poorly characterized strain G9 is closely related to E. canadensis (G7) [7] and the existence of G9 as aseparate genotype remains still controversial [8,9].

Different methods for genotyping genetic variants of the E. granulosus complex have been developed so far. Based on PCR amplified sequences of the mitochondrial cytochrome c oxidase subunit 1 (cox1) or the NADH dehydrogenase subunit 1 (nad1),

Genotyping can be performed in a relative time and/or cost intensive way by sequencing [37], RFLP (Restriction FragmentLength Polymorphism) [38,39], fingerprinting [40] or SSCP (Single Strand Conformation Polymorphism) [41]. More recently, pure PCR based methods that simplify the genotyping have been designed. With a consecutive PCR approach a part of the E. granulosus complex (G1, G5, G6/G7) can be genotyped [42] and by applying four parallel PCRs the discrimination between E. multilocularis, E. granulosus s.s. (G1) and an E. ortleppi (G5)/E. canadensis (G6/G7) cluster is possible [30]. Parallel PCR approaches can be combined in a multiplex PCR setup and became rapidly and successfully applied worldwide in many aspects of DNA analyses, especially in the field of molecular diagnosis of infectious diseases such as bacterial [44], viral [45] and fungal [46] infections.

II. Material and Methods

Parasite specimens

This study was conducted in four general teaching hospitals in Baghdad governorate : Baghdad teaching hospital, Al- Shaheed Gazi Al-Hariri hospital, Liver and Digestive disease teaching hospital ,Al-Kadimmiya Teching Hospitals and Ibn –Al- Nafees teaching hospital, the cysts were in the liver, lung, and spleen .

In the period of this study,sixty(60) cases of space occupying lesions have been enrolled . Among these cases, 44patients were found suffering from Liver hydatidosis, 12 patients with lung hydatidosis, 2patients with liver and lung hydatidosis,2 patients with hydatid cyst on spleen,liver and lung . The diagnosis of patients was confirmed by radiological tests such as plain radiography (X-ray), Computed Tomography (C.T. scan), Ultrasound and Magnetic Resonance Imaging (MRI) .

Isolation of germinal layer of hydatid cysts:

Sixty(60) fresh fertile hydatid cysts washed several times with normal saline until the supernatant looked clear. Then, the sediment preserved in 70% ethanol until required.

Germinal layer of hydatid cyst from human will be taken after surgical operation. Samples were taken to laboratory by clean containers, sterilizing of outer surface by 70% ethanol then discarding of hydatid fluid, germinal layer would be taken and kept in ethanol 70 % for different periods .

III. DNA extraction

Germinal layer from Sixty(60)hydatid cyst isolates were selected. For each method, equal volumes of packed germinal layer (about50mg) were washed twice with sterile distilled water to remove ethanol. Then 300 µl lysis buffer (NaCl 0.1M, EDTA 0.01M, Tris- HCl 0.1M, SDS 1%) added to each tube.

-Step two was similar for all methods:30 µg of proteinase K (Roche, Germany) was added to each tube containing samples plus 300 µl lysis buffer and incubated at 56º C for one hour.

Then, 300 µl phenolchloroform- iso amylalcohol was added and centrifuged at 5000 rpm (2000g) for 5 min.

After removing the supernatant to a new tube, chloroform was added prior to shaking and spanning in 5000 rpm for 5 min. Subsequently equal volume of iso- Propanol (Merck, Germany) and 0.1 volume sodium acetate (Merck, Germany) (3M, pH=5.2) were added to the supernatant, and kept at -20 ºC for 20 min. Next, it was spun 12 min in 12000 rpm and the sediment was rinsed by 300 µl 70% ethanol. After spinning 5 min in 5000 rpm (2000g) and removing ethanol, pellet was dissolved in 50 µl deionized water, and stored at -20 º C for PCR process.

mPCR conditions:

The reaction mix for the final mPCR was composed of 100 mM dNTPs and 0.05 units ml21 GoTaq DNA polymerase in 16PCR Buffer (all Promega) and contained the 22 primers specific for 11 targets in the molarities shown in Table 1. For standard genotyping 5 ng template DNA were added into the PCR mix.

Each reaction was performed in single tubes in a volume of 20 ml PCR mix. The cycling conditions were as follows: an initial denaturation step at 94uC for 3 min, 25 cycles (94º C–30 s, 56º C– 30 s, 72ºC–1 min) and a final extension step lasting 5 min at 72uC. 10 ml of the PCRs were separated by electrophoresis in a 2% agarose gel and visualized by red safe staining and subsequent UV excitation. The genotype specific amplicon profile is shown in Figure 1.

IV. Results:

Table (1) : Measurment ofDNA Concentration by Using Scan Drop Methods:

Methods	Without correction	Withcorrection	Unit
DNA detection	1.77	2.71	A
DNA purity	1.33	1.89	
Ratio230/260	32.45	9.88	
Ratio260/230	0.03	0.10	
dsDNA concetration	38.29	135.53	Ng/µl
ssDNA concentration	25.27	89.45	Ng/µl
RNAconcentration	30.63	108.42	Ng/µl

Table (2): Genotypes of E. granulosus in Samples of Iraqi Patients

Genotypes	**Product size/bp**	**Gene marker**	**No.of + cases**	**Percentage**
G1/G/G3	706 bp	Ef1a	16	26.6
G1/G/G3	1001 bp	Cal	2	3.3
G4	124 bp	Cox1	26	43.3
G5	1041 bp	Atp-6	4	6.6
G6/G7	339 bp	Nad1	4	6.6
G6/G7	617 bp	Pold	8	13.3
Total			60	99.7

Table (3): The prevalence of Hydatid cyst Genotypes in the Infected Organs

Organ	Genotypes	Gene marker	Product size/bp	No . of + cases	Percentage (%)
Liver	G1/G/G3	Ef1a	706	10	16.6
	G4	Cox1	124	16	26.6
	G1/G/G3	Cal	1001	2	3.3
	G5	Atp-6	1041	4	6.6
	G6/G7	Pold	617	8	13.3
	G6/G7	Nad1	339	4	6.6
Lung	G1/G/G3	Ef1a	706	4	6.6
	G4	Cox1	124	6	10
Liver+lung	G1/G/G3	Ef1a	706	2	3.3
	G4	Cox1	124	2	3.3
Liver+lung+spleen	G4	Cox1	124	2	3.3
Total				60	99.5%

Table (4): The Genotype Distribution of Hydatid Cyst In The Infected Organs

	G1/G/G3		G4		G5		G6/G7	
	Count	Percent	Count	Percent	Count	Percent	Count	Percent
Liver	12	20%	16	27%	4	7%	12	20%
Lung	4	7%	6	10%	0	0%	0	0%
Liver+lung	2	3%	2	3%	0	0%	0	0%
Liver+lung+splee n	2	3%	0	0%	0	0%	0	0%

V. Discussion:

E chinococcus granulosus and its metacestode in herbivores and humans have been recognized as the most important helminthes zoonoses, with great economic and public health significances in developing countries(1). Hydatidosis is endemic in the entire Mediterranean zone including all Middle Eastern countries (2).

The highest risk group in the country is specifically in Arab Gulf region -in general- which are women and children. Traditionally, rural women still bear the biggest burden of tending animals – whether breeding, milking, or wool -shearing- and domestic or stray dogs are never faraway. The added chore of women preparing and cooking contaminated food and vegetables with little clean water at hand increases considerably the risk of infection. In many parts of Middle East during springtime, it is common practice together berries and various wild plants which are eaten unwashed and geophagia among children and pregnant women is well known(2).

But the domestic dog as a definitive host for E. granulosus plays an important role in the spread of infection in the Middle East countries via contamination of environment. Sheep, goats, cattle, camels, buffaloes, pigs, and donkeys have been repeatedly found infected with hydatid cysts in Iran, Iraq,Jordan, Lebanon, Syria, Kuwait, and Saudi Arabia (3-11).

PCR was used as a suitable tool for the characterization of E. granulosus strains in different studies. The success in PCR depends on the quantity and quality of the extracted DNA. There are some comparative studies of different DNA extraction methods forganisms, such as fungi, bacteria, viruses and protozoa (14-18).

The liver act as the first filter for larval infection and the lung acts as the second filter. Distribution of infection in different organs showed that the liver was the most frequently involved 44 (73.3%) when compared with lung 12 (20%) and liver and lung 2(3.3%) and other multiple infected organs such asliver ,lung and spleen2(3.3%) Table 3, generally these proportions approximately in agreement with most of previously recorded data by Ahmadi and Al-Dalimi (2006) (28) .

Genotyping of human cases of CE play an important role in the formulation of control strategies for the prevention of transmission of this parasite.

Strain variation in parasites exhibit variation in the onset of egg production, which is a limiting factor in control programs, which employs adult cysticidial treatment of definitive host to break the cycle of transmission [24]. Further it is postulated that the strain variation in parasite may influence host specificity, life-cycle patterns, development rate, transmission dynamics, antigenicity and sensitivity to chemotherapeutic agents. Therefore it may have implications for the development and design of vaccines and diagnostic reagents [4).

Abdulla etal.,2012 reported G1(sheep strain) recorded in Kurdistan Iraq and there is no adequate study on genotyping of echinococcosis in Iraq.

In Iraq , 26(43%) G4, 18(30%) G1/G/G3were found the commonly encounter ered in Iraqi patients while 4(6.6%) G5, and 4(6.6%) G6/G7. Genotype of infecting strain affects the fertility rate of the cysts in the intermediate hosts and thereby the infectivity of strain for the subsequent hosts in the table (4-10), the present findings, study the association between echinococcus genotypes and related organ, the current study shows 26(43%) G4 (Horse Strain), 18(30%) G1/G/G3 (sheep strain), 4(7%) G6/G7 (camel and pig strain), 12(20%) G5 (cattle strain).

Our results in agreement with other studies by (Elizabeth et al., 2010; Abdullah et al., 2012; Sharma et al., 2013). And disagreement with Ergin et al., (2010); Boubaker et al., (2013).

In addition, the association between genotype and various organ, our finding in the table (4-11) included the following criteria.

In the liver involved 12(20%) G1/G/G3 (sheep strain), 16(27%) G4 (horse strain), 4(7%) G6/G7 (camel and pig strain), and 12(20%) G5 (cattle strain). In the lung 2(3%) G4 (horse strain).

Moreover, the relation between genotype and multiple organ infection liver and lung, our results showed 2(3%) G1/G/G3 (sheep strain) 2(3%) G4 (horse strain) in the liver, lung and spleen. Our finding shows 4(7%) G1/G/G3 sheep strain, 6(107%) G4 horse strain, the total genotypes were recorded in the various organ included the following, 18(30%) were G1/G/G3, 26(43%) were G4, 4(7%) were G6/G7 and finally 12(20%) were G5, these results were statistically not significant ($p>0.05$), and in agreement with Sharmal et al.,(2013).

In table (4-14), our finding, were shows the gene maker distribution anhydrate cyst patients genotypes as he follows: 16(26%) were EF1a (796bp) gene, (2,3%) were CaL (1001bp) gene, while 26(43%) Cox1(124bp) gene, 4(6.61) were ATP-6(1041 bp) gene, 4(6.6%) were Nad1, (339bp) gene, 8(13%) were pold (617bp) gene. These finding in agreement with Sharma et al., (2013); and disagreement Ergin et al.,(2010); Khder et al.;(2013).

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