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Cystic and Alveolar Echinococcosis: Molecular survey in Al-Anbar Province

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ABSTRACT

Forty specimens of surgically removed hydatid cyst were obtained from patients in different hospitals in Al-Anbar province. The aim of study was designed to determine the type of common larval stage (hydatid cyst) of echinococosis in Al-Anbar province according to PCR molecular differentiation.The study was carried out during the period from first day of January to the last day of December 2013.The age of patient ranged from (10 – ≥ 65) years old, (19 males and 21 females). A questionnaire paper was performed to each patient which includes information about age, sex,residence,occupation,exposure variable, water source, site of hydatid cyst and case history.From the obtained results, it's revealed that the age group (21 – 30 years) presented high hydatidosis parasitic infestation which was 35% , whereas the age group more than50 years presented low parasitic infection which was 2.5% . 47.5% of patients were males and 52.5% were females.Twenty eight(70%) patients live in rural areas. Also, twenty patients (50%) were exposured to livestock animals, (40%) of patients were farmers, twenty seven patients(68%) were consumed river water, The most common localization was the liver (65%). Purification of DNA was done by QIAamp DNA Mini kit from QIAGEN for cyst. Specific primers were designed for the diagnostic PCR. It amplifies a 133-basepair segment within the newly described repeated unit that amplified a target repeated sequence (EgG1 *Hae* III) newly identified in the genome of the common sheep strain of *E. granulosus*. Primer based on the flanking sequences allowed the PCR amplification of *E. multilocularis*, a fragment of mitochondrial 12S rDNA was amplified by PCR from human genomic DNA by using the cestode-specific primers 60 were used as targets for (PCR) amplification.The total number of the cysts specimens that gave positive results of *E.granulosus* by PCR was 37 (92.5%) patients and the number of specimens that gave negative results was 3(7.5%),while all specimens gave negative result of *E. multilocularis* by using PCR analysis.The present study showed that *E. granulosus* is a major causative of hydatidosis in Al-Anbar Province while *E. multilocularis* is not detected by using PCR approach.

Introduction:

Echinococcosis is an endemic zoonosis characterized by world wide distribution particularly in Mediterranean countries,one of the most serious and life-threatening zoonoses in the world [(1)].

Echinococcosis an important zoonosis, caused by larval stages of tapeworms (cestodes) belonging to the genus Echinococcus (family Taeniidae) [(2)].

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An infection (hydatidosis) was characterised by long term growth of metacestode (hydatid) cysts in the intermediate host, the two major species of medical and public health importance *E. granulosus* and *E. multilocularis*, which cause cystic Echinococcosis and alveolar echinococcosis, respectively, these are both serious life-threatening diseases, the latter especially so, with a high fatality rate and poor prognosis without careful clinical management [(3)].

Echinococcus granulosus is the causative agent of cystic echinococcosis (CE), or cystic hydatid disease(CHD), and *E.multilocularis* in humans causes alveolar echinococcosis(AE), or alveolar hydatid disease(AHD), two other species of the genus

Echinococcus, namely, *E. vogeli* and *E. oligarthrus*, are mainly restricted to sylvatic animals and occur in some are as of Central and South America [4].

Molecular biology researches on parasites and parasitic diseases have evolved significantly, many of the findings led to the development of new tools for the polymerase chain reaction (PCR), in the present, increasing of the diagnostic sensitivity, which can be achieved using PCR, allows to study changes even at cell level, which is more than necessary in the study of parasites, PCR had an important role in the discoveries made in the systematics and epidemiology of parasites, the PCR amplification is characterized by a high sensitivity and specificity [5].

The aims of the study are:

1- To determine the type of larval stage (hydatid cyst) of echinococosis according to PCR molecular differentiation

2- To study some factors in relation to type larval stage of parasite

Materials & Methods:

A total of 40 specimens of surgically removed hydatid cyst were obtained from patients operated in Al-Falluja teaching Hospital,Al-Ramadi teaching Hospital,Al-Rashed private Hospital,Heet general Hospital. during the period from January 2013 to December 2013. The age of those patients ranged from ten to 65 years with different sexes 19 males and 21 females were male.

The hydatid cyst was transfered in a Petri dish and observed under the stereo microscope in order to identify the presence of protoscoleces,the content of the cyst was aspired with a 10 ml syringe and subsequently was cut a piece of germinal layer, both samples were transfered in a 2 mL tube.

The tube was centrifuged for 3 min at 3.000 x g, and the liquid phase was discarded. deionised water(Milli-Q) was added in the 2 mL tube,the solution was re-suspended by vortex, this step was repeated for 3 times,two hundred mg of the pellet was collected and transfered in a 2 mL tube.

DNA extraction from samples:

The DNA extraction kit was purchased from QIAGEN company. And the DNA was extracted according to(6)with some modifications: 200 µl from protoscolices suspension was pipeted into 1.5 ml eppendorf tube or 25 mg of tissue (germinal layer) was cut into small pieces were placed in a 1.5 ml microcentrifuge tube and 180 µl of Buffer ATL was added 20 µl Proteinase K was added into a 1.5 ml microcentrifuge tube and mixed by vortex and incubated at 56°C until the tissue was completely lysed or placed the sample in a shaking water bath 200 µl Buffer AL was added to the1.5 ml microcentrifuge tube, the 1.5 ml microcentrifuge tube was mixed by pulse-vortexing for 15 second, and incubated at 70°C for 10 min 200 µl ethanol (96–100%)was added to the sample Precipitate to the QIAamp Spin Column, The mixture from previous step was carefully applied (including the precipitate) to the QIAamp Spin Column (in a 2 ml collection tube) and centrifuge at 6000 x *g* (8000 rpm) for 1 min. the QIAamp Spin Column was placed in a clean 2 ml collection tube, and the tube containing the filtrate was discarded. The QIAamp Spin Column was opened and 500 µl Buffer AW1 was added and the QIAamp Spin Column was centrifuged at 6000 x g (8000 rpm) for 1 min.The QIAamp Spin Column was placed in a clean 2 ml collection tube and the collection tube containing the filtrate was discarded .The QIAamp Spin Column was opened and 500 µl Buffer AW2 was added and the QIAamp Spin Column was centrifuged at full speed (20,000 x g;14,000rpm) for 3 min.

The QIAamp Spin Column was placed in a new 2 ml collection tube (not provided) and the collection tube containing the filtrate was discarded, Centrifuge at 20,000 xg (14,000 rpm) for 1 min.The QIAamp Spin Column ws placed in a clean 1.5 ml microcentrifuge tube and the collection tube containing the filtrate was discarded, the QIAamp Spin Column was opened and 200 µl Buffer AE or distilled water was added. the QIAamp Spin Column was incubated at 65°C for 60 min, and then centrifuge at 6000 x g (8000 rpm) for 1 min. a 65°C at 60 min incubation of the QIAamp Spin Column loaded with Buffer AE or water, before centrifugation, generally increases DNA yield. The specific primers were synthesized from BIONEER company, and they were designed on the basis sequence information of the gene repeated unit that amplifies a highly repeated sequence of *E.granulosus* DNA(133bp) according to (7):EgF 1(5 GAA TGC AAG CAG CAG ATG 3) EgR 2(5 GAG ATG AGT GAG AAG GAG TG 3) and *E.multilocularis* DNA(375bp) according to(8) EmF1(5 ATC ACC TTC CCATTC CTA AGC CGT G 3)

EmR2(5 ACT GCA TGC TCA TGC TGC GTC CAT GGG 3)

PCR reaction kit (PCR Premix) that was used for DNA amplification was purchased from (BIO NEER) company. The PCR reaction was carried out in 20 µl solution containing (Top DNA polymerase), 1 U, each dNTP (dATP, dGTP, dCTP, dTTP) 250 µM, 1.5 mM $MgCl_2$, Tris-HCl (pH 9.0), 10 mM, KCl, 30 mM, Template DNA, 5 – 50 ng, Primer, 5 – 10 pmole.

DNA negative control was monitored by assaying twenty negative controls containing distilled water instead of extracted DNA in the amplification reaction.

Agarose gel electrophoresis is a procedure that consist of injecting DNA into agarose gel and then applying an electric current to the gel. As a result, the smaller DNA fragments move faster than the larger fragments through the gel toward the positive electrode. All PCR products of the samples were detected and analyzed after amplification by Agarose gel electrophoresis (Horizontal Gel unit, USA).

Statistical analyses were conducted using the statistical package Bar chart, Pi chart, Chi-square, Cross tab, Statistical program SPSS eighteen edition and excel 2007 To determine if there was a significant difference between infected patients. Chi-square tests on proportions were carried out. In all cases, a P-value < 0.05 was taken to indicate significance [8].

Results & discussion:

Hydatid cyst consider as major public health problem that can cause severe morbidity in human, as a result economic losses occur for individual, family and society, hydatidosis infects wide range of livestock which lead to further economic losses (9). The pathogenecity of hydatidosis heavily depends on the extent and severity of infection, and the organ on which it is located (10) The liver and the lungs are the most commonly affected, although other organs can also be involved (11) . A total of 40 specimens of surgically removed hydatid cyst were obtained from patients operated in different hospitals in Al-Anbar province. From the obtained results, it's revealed that the age group (21 – 30 years) was presented high hydatidosis parasitic infestation which was 35% , whereas, the age group more than50 years was presented low parasitic infection which was 2.5% . There are 47.5% of patients were males and 52.5% were females, twenty eight patients were lived in rural area (70%); The most common localization organ. The total number of cysts specimens that gave positive results of *E.granulosus* by PCR was 37 (92.5%) patients and the number of specimens that gave negative results was 3(7.5%),while all specimens gave negative result of *E. multilocularis* by using PCR analysis, The sheep strain (G1 genotype) of *E. granulosus* is the most widely distributed strain around the world. It has been found to be dominant strain both in human and animals([12]). Also our results were agreed with a previous study by (13) that sheep strain the most worldwide also predominant in Kurdistan-Iraq and it was mostly responsible of human hydatid disease in Kurdistan(13).

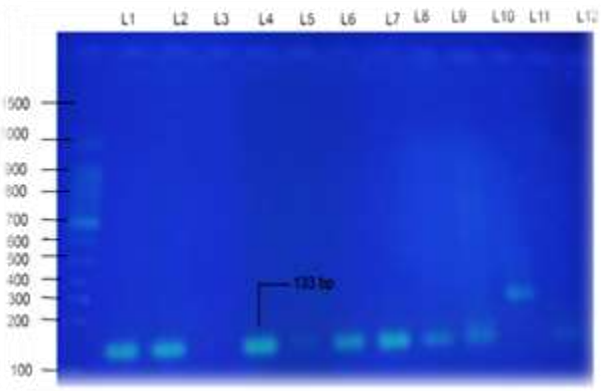

Figure (1): Amplification of *E.granulosus* DNA from the cyst of the infected human. Lane-1, molecular weight marker (100 bp ladder), Lanes (2,3,5,6,7,8,9,10,12) positive samples at 133bp in PCR.

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 addition,the size and localisation of the cysts may be different among the genotypes which may be relevant regarding examination of the organs with naked eye for the cysts. Also the prepatent periods may vary between genotypes which can be important for the programs based on regular antiparasitic treatment of the terminal host, before the parasite produces eggs(14). the genotypes are also important regarding the host specificity and life cycle of the E. granulosus. Although, G1 genotype strains may also infect other intermediate host such as cattle, goats, dog,fertility rate of the cysts in these animals low is or it dosen't produce fertile cysts at all(15).

A search for repeated sequences in the *E.granulosus* sheep strain (G1) was initiated since it is considered the most common strain, and the main source for human infection [16].

A tandemly repeated DNA sequences make useful targets for diagnosis by PCR due to their high abundance, and potential for high degree of specificity[17]

Primers based on the EgG1 Hae III repeated sequence cloned from the sheep strain also amplified DNA from protoscoleces from horse, camel, cattle, or goat hydatid cysts, albeit at a lower sensitivity, and showed different banding patterns [(17)].

In areas where both *E. granulosus* and *E. multilocularis* are coendemic,it is of utmost importance to identify the parasite to the species level, as well as to differentiate them from Taenia spp. Highly specific and sensitive copro-PCR assays have been developed for the detection of *E. multilocularis*, and have been used for screening of foxes in epidemiologic surveys [(18)], In some areas, AE is regarded as an emerging or reemerging disease , the major risk factors are the increasing prevalence among foxes along with rising fox populations and their spread into urban areas(16).

The majority of AE are a result of environmental contamination with infected fox faeces and subsequent transmission to humans,the red fox (Vulpes vulpes) is likely to be responsible for most of the environmental contamination with E. multilocularis eggs(19)

The negative results of *E. multilocularis* in present study due to the patients in rural area are not exposed or contamination with infected fox faeces or close contact with red fox.

The negative results of *E.granulosus* by using PCR may be using different primer to detect hydatid cyst of human., which represent DNA of human spleen hydatid cyst, gave positive result only by using primer (OPC – 05) and did not amplify by using other primers, This suggests that the hydatid cyst strain which effect spleen differ from other strains which effect liver and lung in human, meanwhile we saw many similarities in the molecular weights to the amplified bands in samples of liver and lung in human, that may explain the relationship between the effected strain of hydatid cyst as reported by [(20)] .

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دودة الاكياس المائية الحويصلية والمتكيسة : مسح جزيئي في محافظة الانبار

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الخلاصة:

أجريت الدراسة الحاليه على 40 عينه من نماذج الاكياس المائيه المعزوله جراحيا تم الحصول عليها من المرضى في مستشفيات مختلفه من محافظة الانبار. الهدف من الدراسه الحاليه لتحديد النوع الشائع لمرض الاكياس المائيه في محافظة الانبار باستخدام تقنية كوثرة الحامض النووي. الدراسه الحاليه نفذت اثناء الفتره من اليوم الاول لشهر كانون الثاني الى اخر يوم من شهر كانون الاول 2013 .تراوحت اعمار المرضى مابين 10 – 65 سنه (19 ذكور و21 اناث). عملت ورقة استبيان خاصه لكل مريض توضح العمر والجنس والسكن والوظيفه ومتغيرات التعرض ومصدر المياه المستهلكه للشرب وموقع الكيس المائي بجسم المريض وتاريخ الحاله المرضيه. اظهرت نتائج الدراسه ان الفئه العمريه مابين (21 – 30) سنه مثلت اعلى نسبة اصابه (35%) ، بينما الفئه العمريه الاكثر من خمسين عاما مثلت اقل نسبة اصابه (2.5%)، اظهرت الدراسه بان النساء اكثر عرضه للاصابه بنسبة (52.5%) بينما كانت نسبة الرجال (47.5%). كذلك تركزت الاصابه في المناطق الريفيه اذ بلغت نسبة الاصابه (70 %) وكانت نسبة المرضى الذين يمتلكون حيوانات اليفه (50 %) .تبين ان اعلى نسبة اصابه من المرضى هم من الفلاحين اذ كانت النسبه (40 %) منهم 68 % يستهلكون ماء النهر مباشره .وجد ان اعلى نسبه اصابه كانت في الكبد (65%) .اجريت عملية تنقية الحامض النووي لعينات الاكياس المائية باستخدم طريقة Qiagen صممت بوادئ خاصه للتشخيص بطريقة الكوثرة وذلك بتضخيم قطعه (133زوج قاعده) ضمن الوحده المتكرره(EgG1HaeIII) لتمييز موروث الخراف الشائعG1 لدودة الاكياس المائيه نوع *Echinococcus granulosus*. وضعت قطعه من بادئ (mitochondrial 12S rDNA) لتضخيم الحامض النووي لدودة الاكياس المائيه نوع *Echinococcus multilocularis* . العدد الكلي للنماذج التي أعطت نتائج ايجابيه لدودة الاكياس المائيه نوع *E. granulosus* باسستخدام تقنية كوثرة الحامض النووي كان 37 (92.5 %) وعدد النماذج التي اعطت نتائج سلبيه كانت 3 (7.5 %) بينما اعطت كل النماذج نتيجه سلبيه لدودة الاكياس المائيه نوع*Echinococcus multilocularis* باستخدام تقنية كوثرة الحامض النووي . اظهرت الدراسه الحاليه باستخدام تقنية الكوثره للحامض النووي ان النوع الشائع لمرض الاكياس المائيه في محافظة الانبار هو النوع الكيسي بينما لم يتم اكتشاف اي حاله للنوع الحويصلي.