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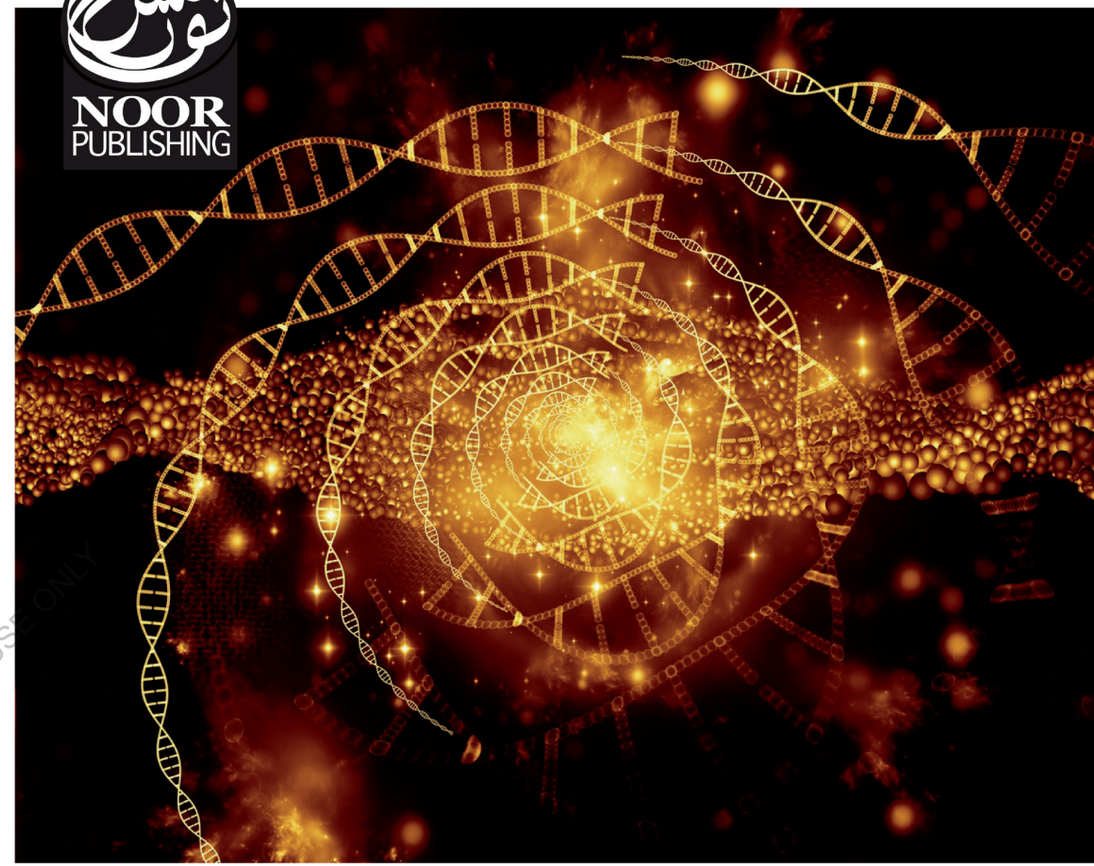

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Molecular characterization of antigens extracted from hydatid cysts of human and other intermediate hosts with immunological study

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My deep thanks to my Father, Mother, Brothers, Sisters . My deep thanks also to my wife for their great support throughout my work and study .

Summary

A study of larval stage of *E. granulosus* according to molecular and immunological levels were carried out in the present study, where 7 samples of hydatid cysts were collected from intermediate hosts " Human (Liver, Spleen, Lung) and liver of Sheep, Goat, Cattle and Buffaloes" . Also, 30 patients infected with hydatid cyst surgically confirmed diagnosis including in this study for immunological tested from various Baghdad hospitals .

The study showed that the incidence of hydatidosis in females was higher than that of the males . The highest age distribution of hydatidosis patients was between (12 – 40) years . The percentage of liver hydatidosis was higher (64.66%) than any other organ .

A - Molecular study : DNA was extracted from germinal layer cells of hydatid cysts which were isolated shortly or preserved for various periods in 70% ethanol .

Genetic analysis of isolated DNA from hydatid cysts collected from human and animals was done by Polymerase Chain Reaction (PCR) to determine genetic variation depending on Random Amplified Polymorphic DNA . In the present study 10 primers have been used, during which the genetic variations were revealed among isolated (extracted DNA) of hydatid cysts which was collected from human and other intermediate hosts .

The current results of this study have shown the following :

1 - It was found one primer (OPA – 01) was able to diagnose sample numbered 1 which represent the isolated DNA of liver hydatid cyst which was obtained from human at age group 10 – 20 years old .

2 - The ability of primer OPC – 10 to determine fingerprinting of DNA sample of Sheep liver hydatid cyst .

3 - The ability of primer OPC – 05 to determine fingerprinting of DNA sample of human spleen hydatid cyst which was obtained from human at age group 30 – 40 years old .

4 - The ability of primer OPE – 07 to determine fingerprinting of DNA sample of Goat liver hydatid cyst .

5 – Amplification process to the DNA samples which extracted from Cows and Buffaloes liver hydatid cysts wasn't completed by using all 10 primers .

B – immunological study :

The study of concentrations of IgG, IgM, C3 and C4 were carried out by radial immuno diffusion assay (RIDA) and the results of determination concentration of IgG, IgM, C3 and C4 were significant increasing in IgG concentration in males compared with females and highly increasing in males at age group 10-20 years old .There was significant difference in IgM concentration, significant increasing in females specialy at fourth age group comparative with significant decrease in IgM concentration in males at different ages . Also, there was significant increasing in C3 and C4 concentration in females compared with males .

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LIST OF ABBREVIATIONS

ELISA	Enzyme Linked immuno sorbent assay
PCR	Polymerase Chain Reaction
DNA	Deoxy nucleic acid
WHO	World Health Organization
CE	Cystic echinococcosis
CMI	Cell mediated cytotoxicity
IgG	Immunoglobulin-G
IgM	Immunoglobulin-M
IgA	Immunoglobulin-A
IgE	Immunoglobulin-E
C3	Complement-3
C4	Complement-4
Il-4	Interlukin-4
ADCC	Antibody dependant cell mediated cytotoxicity
SDS-PAGE	Sodium dodecyle sulphate – poly acryl amide gell electrophorosis
MRI	Magnetic resonance imaging
PAIR	Penetration, Aspiration, Injection, Re aspiration
CO1	Cytochrome oxidase sub unit 1
NADH	Nicotine amide adenine dinucleotide hydrogenase
EDTA	Ethyline diamine tetra acetic acid
Taq	Thermous aquatiqus
m.w.	Molecular weight
G ≡ C	Guanine ≡ cytosine
A=T	Adenine = Thyamine
con.	Concentration
m.	Mean
s.e.m	Standard error of mean

Chapter one
Introduction & lieratures review

Introduction:

Cystic echinococcosis is a cosmopolitan , hyper endemic zoonotic disease caused by infection with metacestode (larval stage) of the tape worm *Echinococcus granulosus* . Its one of the most important parasitic disease in under developed countries specially rural communities, where man enclose contact with the dogs (Definitive hosts) and various domestic animals which act as Intermediate hosts (Nepalia *et al.,*2006) . 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 , added Echinococcosis infects wide range of livestock which lead to further economic losses (Taherkhani and Rogan, 2000) .

Development of cyst is slow and variable . It depends on number of factors including species , strain of parasite , species of host , degree of infection (Permin and Hansen, 2002) . Other factors have also been suspected specially age , sex and immunological status of the host (McManus *et al.,* 2003) .

Healthy importance of Echinococcosis due to affect many organs in human and animals as Liver (most frequent site 50%-70%), Lung (25%)

Spleen , Ovary , Bone and difficulty treated organs as Brain , Blood vessels and Vertebral column (Heath *et al. ,* 2003) .

So, development of Hydatid cyst cause pressure necrosis to adjacent tissues and organs causing lose of vital organs their functions (Schipper *et al.*, 2000) . Further complications due to secondary bacterial infections and anaphylactic shock resulted from rupture of cyst during surgical interference which may lead to sudden death (Morar and Feldman, 2003).

The Hydatid cyst always start as fluid – filled cyst like structure (Type I) which may proceed to type II lesion if daughter cysts and / or matrix develop . Type II lesion becomes hyper mature and due to starvation the cyst die and become mummified , inert calcified type III lesion (Ito *et al.,* 2003) .

In some countries the control programs lead to a marked decrease in incidence of the disease (Eckert *et al.*, 2001) . There is some evidence that the spreading of disease because of lack meat inspection , poor dogs management and inappropriate legislation (Arbabi and Hooshyar, 2006) .

The epidemiology and control of hydatidosis is often consider to be a veterinary matter since controlling parasites in animals can regulate the disease (Abbasi *et al.,* 2003) . However collaboration between veterinarian and public health workers is essential for successful control of hydatidosis (Abdul Wadood, 2005) .

The preferred and effective treatment of cystic echinococcosis is surgical excision , and good chemotherapy is lacking and some time offers alternative , specially for inoperable cysts and patients at high surgical risk (Nayyef and Rissan, 2007) . Two Benzimedazole compounds (Mebendazole and Albendazole) have been tested clinically for use in the chemotherapy of hydatidosis , varying from stabilization to complete disappearance of Hydatid lesion (Al-Joboori, 2005) .

The host immune system may be exposed to varying antigen types or concentrations and the immune response divided into preencystment and

post encystment represented by inducing of humoral and cellular response (Torgerson and Heath, 2003) . Therefore, there is an ability to use vaccines to control on this disease as cyst fluid, cyst membrane or protoscolices (Ammori *et al.,* 2002 and Al-Nasiri, 2006), BCG vaccine (Anadol *et al.,* 2001) .

Hydatid cyst diagnosed by many ways as x-rays , CT scan , ultra sound waves other serological test as ELISA (Campos-Bueno *et al. ,* 2000). In addition to modern technique polymerase chain reaction (PCR) which is discovered by Kary mullis and Fallona , 1986 . This technique word wide in spreading due to speed , efficacy , more easily and high sensitivity so that used for testing the genetic variation in the DNA of *Echinococcus granulosus* strains which become observed after electrophoreses in gelatinous field (Arcari *et al.,* 2003) .

1 - 2 Literatures review :

1-2-1 Historical review :

Hydatid disease , unicystic or unilocular or Hydatid disease , (William *et al. ,* 2000) **;** Is a zoonotic word wide parasitic disease that cause serious public health problem in certain parts of the word (Aribas *et al.,* 2002) . At different years many researchers explain the history of hydatid disease (Aygum *et al.,* 2001 **;** Biava *et al.,* 2001 and Bickel *et al.,* 2001) .

in which cystic " The first description of the disease was in" Talmud Hippocrates, " water bladder "lesions in killed animals were recognized as Galen and Al-Rhaze were also familiar with the disease under the "Watery balloon" and " Liquid tumor " " Water filled liver "descriptions respectively (Rausch, 1997 and Wang *et al.,* 1997) .

In the 17th century , the Italian physician Francesco Redi (1684) recognized the animal origin of Hydatid cyst . The adult worm in the dog's intestine was discovered by Hartman, 1695 (Brunette and Filice, 2001) .

Hunter (1773) describe the Hydatid cysts as smooth, spherical, fluid filled structure (Bouree, 2001) .

Goeze (1782) demonstrate the protoscolices as granules formed on the inner surface of cyst and found similarity between these protoscolices and *Taenia* scolex so that named *Socialis granulosus* and *Taenia visceralis* (Smego *et al.,* 2003) .

Batsch (1786) named *Hydatigena granulosus* (Chin, 2000) .

Gemmelin (1790) named *Taenia granulosus* (Chow *et al.,* 2001) .

Rudolphi (1801) the first named the genus of Hydatid with *granulosus* (Ci-Peng *et al.,* 2005) .

Vonsiebold (1825) demonstrate adult worm in intestine of experimentally infected dogs and named *Taenia echinococcus* (Cobb *et al.,* 2003) .

Leukart (1886) the first describe the general appearance and life cycle and named the last scientific name *Echinococcus granulosus* (Craig *et al.,* 2007) .

1 - 2 - 2 Classification of the *E. granulosus* :

The parasite cause Hydatid disease is classified as follow (Karyakarte and Damle, 2004) **:**

Kingdom **:** Animalia
Subkingdom **:** Metazoa
Phylum **:** Platyhelminthes
Class **:** Cestoda
Sub class **:** Euocestoda
Order **:** Cyclophyllidae
Family **:** Taenidae
Sub family **:** Echinococcinea
Genus **:** *Echinococcus*
Species **:** *granulosus*

Biotypes : Northern and European biotypes

The genus *Echinococcus* has four medically important species (Craig *et al.,* 2003) **:**

1. *E. granulosus* (Batsch, 1786) 3. *E. oligarthus* (Diesing, 1863)

2 . *E. multilocularis* (Leukart, 1863) 4 . *E. vogeli* (Rausch and Bernstein, 1872)

Other species (Daeki *et al.,* 2000) :

1. *E. longimanubrius* 2 . *E. cruzi* 3 . *E. ortleppi*

4 . *E. camerani* 5 . *E. fellidis* 6 . *E. lyeaonti*

7. *E. minimum* 8 . *E. patagonieus* 9. *E. intermedius*

Also *E. granulosus* has four genetically different sub strains (Thompson and McManus, 2002 and Pearson *et al. ,* 2002) **:**

1 . *E. granulosus granulosus* 2 . *E. granulosus canadensis*

3 . *E. granulosus equines* 4 . *E. granulosus boeilis*

***E. granulosus* has many genetically identical strains** (Thompson *et al.,* 1995 and McManus, 2002) **:**

Sheep strain , Cattle strain , Buffalo strain , Camel strain Lion strain , Pig strain , Horse strain , Cervid strain , Tasmania sheep strain and Lagomorph strain till now unknown (Rausch, 1995) .

1- 2 - 3 Species of *Echinococcus* :

Among the genus *Echinococcus* there are several species , and the most common one is associated with human disease is *Echinococcus granulosus* and it is the most wildly distributed through out the word (Jiang, 2002) , while *Echinococcus multilocularis* is limited to the north part of the globe (W.H.O , 2003) . It has been reported that *E. granulosus* and *E. multilocularis* are not seen together in the same area (Hakan and Aker, 2001) . One of the most prominent differences between these two species is that *E. granulosus* metacestode grows in a double - walled cyst

by endogenous budding , the outer layer is formed by fibrous tissue from the host and the inner laminated layer is of the parasite.

In contrast, *E. multilocularis* metacestode grows by exogenous budding and has potential to spread to adjacent host - tissues (Ito *et al.* , 2002) .

E. vogeli and *E. oligarthus* are rarely implicated as a cause of this disease and their distribution is limited to the middle east and south America (Eckert and Thompson, 1997) .

Host- larval stage	Ruminants, pig, Horse, man	*E. multilocularis*	*E. vogeli*	*E. oligarthus*
		Rodents, insectivore	Rodents, man	Coyote
Host - adult stage	Dog , wolf, Hyena,Dingo	Dog , red fox Arctic fox	Bush dog	American lion , Tiger
Length of proglottid	2 – 7 mm	1.2 - 3.7 mm	3.9 - 5.6 mm	1.9 - 2.9 mm
Size of hooks large small	31- 49 µm 22 - 39 µm	28 - 34 µm 23 - 31 µm	49 - 57 µm 30 - 47 µm	43 - 50 µm 25 - 45 µm
No. of mature prog.	3	4 - 5	3	3
No. of testes	25 - 80	16 - 30	50 - 67	15 - 45

Position of genital pore	Near Posterior end of segment	Near middle of segment	Posterior end of segment	Anterior end of segment

Table (1-1) explain the main differences between *Echinococcus* species (Dalimie *et al.,* 2001 and Rausch and D' Alessandro, 2002) .

1- 2 - 4 Larval stage (Hydatid cyst) :

The eggs of the parasite can't mature into adult worms without first passing through the larval stage , and since this cannot take place in the definitive hosts , the eggs must find and enter an intermediate host . Man is an accidental or incidental intermediate host in nature, exposure take place through ingestion and very doubtfully, through inhalation of eggs (Dalimie *et al.,* 2002) .

The cyst is composed of the following components :

- The outer layer (peri cyst) :

Derived from the host and represent the response of the host to the parasite (Pawlowski *et al*., 2001), It consists of compressed host cells , fibroblasts , giant cells and eosinophils which together form a rigid protective layer a few millimeter thick , this layer prevent passage of parasitic secretions which affect the immune system , thus prevent passage of mononuclear cells into cyst . (Siles-Lucas and Gottstein, 2001 and Rajaii, 2005) .

- Laminated layer :

A cellular , elastic , strong , rigid , with 2 mm in thickness , most studies indicate that this layer derived from parasite not from host (Vedat *et al*., 2001 and Dalton and Mulcahy, 2002) consisted from mixture of microfibrial and unknown chemical structure dense granules (Erkilic *et al*., 2004) . This layer protect the parasite from host immune system (Pelaez *et al*., 2000) . Many evidences and studies improved that the embryo begin with laminated layer formation after 2 weeks from egg hatching in vitro

this time is correspond with host specific immune response to be appear (Kotpal, 1996) .

- Germinal layer :

Thin, translucent, consisting from one layer of cells (Engin *et al*., 2000), regulate and control the permeability of wall permitting to some necessary ions to enter to cyst regulating the osmotic pressure inside the cyst finally, this layer consider source to laminated layer and protoscoleces formation (Eckert *et al. ,* 1995) .

- Broad capsule :

Vesicular structures grow from germinal by endogenous budding , its diameter about (250-500) micron having protoscolices, may detach into cyst fluid forming Hydatid sand (Hashemitabar *et al*., 2008) .

- Daughter cysts :

Formed by endogenous budding from germinal layer may be genetically non identical with the mother cyst due to mutation , chemical agents , radiation or immunological influences (Simsek, 2005). May detach and form Hydatid sand (Eckert and Deplazes, 2004), or may form newly cysts in other sites after cyst rupture and protoscolices escaping (Harraga *et al*., 2003) .

- Protoscolices :

There are seven steps for Protoscolecies formation (Galindo *et al.,* 2002), cellular buds formed by a clustering of cells emerge from the germinal layer of Hydatid cysts . The buds elongate and the cells at their bases seem to diminish in number .

Very early on a furrow appears in elongated buds, delimiting anterior (scolex)and caudal (body) regions . Hooks are the first fully differentiated structures formed at the apical region of the nascent scolex . In amore advanced stage , the scolex shows circular projections and depressions that develop into suckers . Acone can later be seen at the center of the hooks , the body is expanded and a structured neck is evident between the scolex and the body . During Protoscolecies development this parasite form remains attached to germenative layer through a stalk . When fully differentiated, the stalk is cutoff and the infective Protoscolecies are now free in Hydatid fluid .

- Cyst fluid :

Colorless – yellowish fluid , pH (6.7 – 7.2), freezing degree (-53Ċ) (Dawood *et al.,* 1995) . This antigenic fluid can be used in serological tests to detect hydatid disease , these antigenicity increase in case of fertile cyst (Arwar *et al.,* 1995).

Biochemical analysis to cyst fluid explain that is consist of Protein , Glucose , Uric acid , Urea , Triglyceride , Fatty acids , Phospholipids, Ca, Na, K, Mg, (Thompson and Lymbery, 1995) , Cholesterol is found in cyst fluid passing through membranes (Duets *et al.,* 2000) . ß-alanine has been reported in pig hydatid cyst (Diker *et al.,* 2008). Thermolabile lipoprotein (antigen A) , Thermostable lipoprotein (antigen B) are be detected in cyst fluid (Dowling *et al.,* 2000) . Albumin , Nacl , Naso4 , some enzymes as Lipase , Protease , Amylase , Oxidase , Phosphatase , are reported (Dyab *et al.,* 2005) . Ammonia, billirubin , Creatinine also reported (Deplazes *et al.,* 1999) . Globulins specially IgG which can be diffuse through cyst membranes (Eckert *et al.,* 2002) .

Inorganic elements are found and play important role in nutrition , Co-factors in metabolic processes and can interfere with host immunological defense mechanisms (El-Mahdi *et al.,* 2004) , these elements Zn, Fe , Cu , Sr , Cd , Cr , Co, Co, is not found in cyst fluid of sheep lungs but Zn, is

found in large quantity which has important role in immunological processes , metabolism and Co-factors to many enzymes . Fe and Cu have importance in respiratory process , Ni, also found in cyst fluid and may be attach with RNA (Erzurumlu *et al.,* 2000 ; Erdogan *et al.,* 2002 and Erkan *et al.,* 2004) . In general, concentration of elements and ions is higher in fluid than cyst membranes (Singh and Gibikote, 2001) . Highly significant differences of inorganic elements in cyst fluid in different hosts and in same host at different organs (Schantz *et al.,* 1995) .

The biochemical constituents variety of hydatid fluid reflect strain variation in different intermediate hosts (Rigano *et al.,* 1996 and Gadea *et al.,* 2000), Glucose , creatinine and Ca is higher in camel cyst fluid while uric acid is higher in man cyst fluid (Gemmell *et al.,* 2001), urea is higher in renal cyst fluid while bile compounds are higher in liver cyst fluid (Filice *et al.,* 2000 and Forzan *et al.,* 2006) .

1 - 2 - 5 Life cycle :

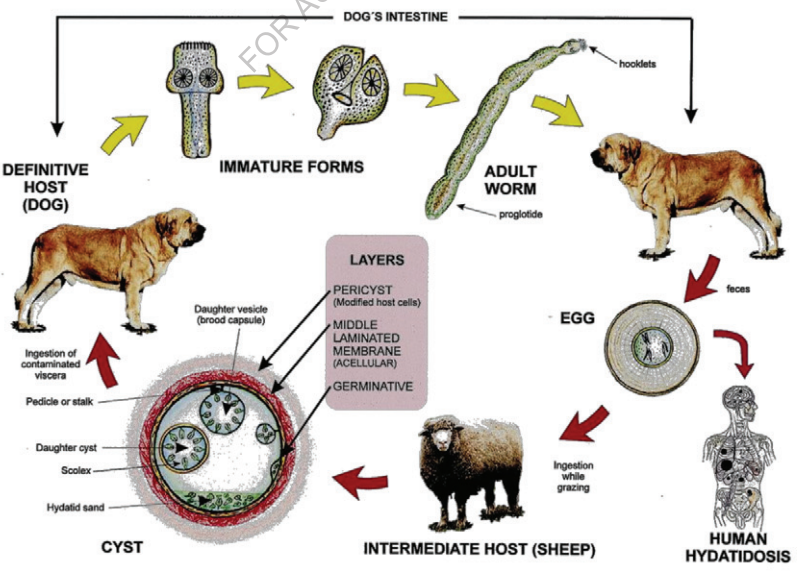

Fig. (2-1) life cycle of *E. granulosus* (Khuroo, 2002)

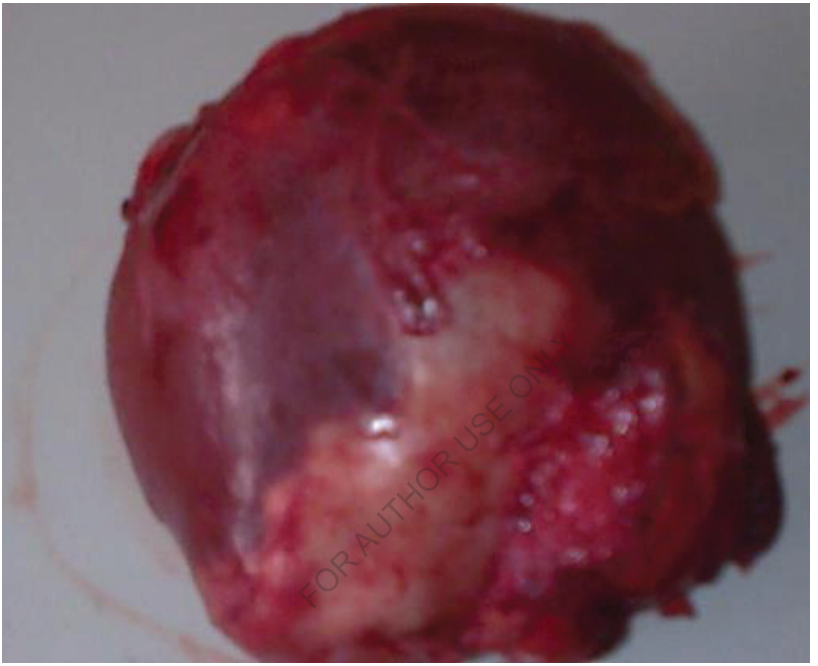

Fig.(2-2)Spleen with hydatid cyst(woman 45 years Baghdad teaching H. 2009 under supervision of Surgeon Dr. Mahmoud Al- Majed)

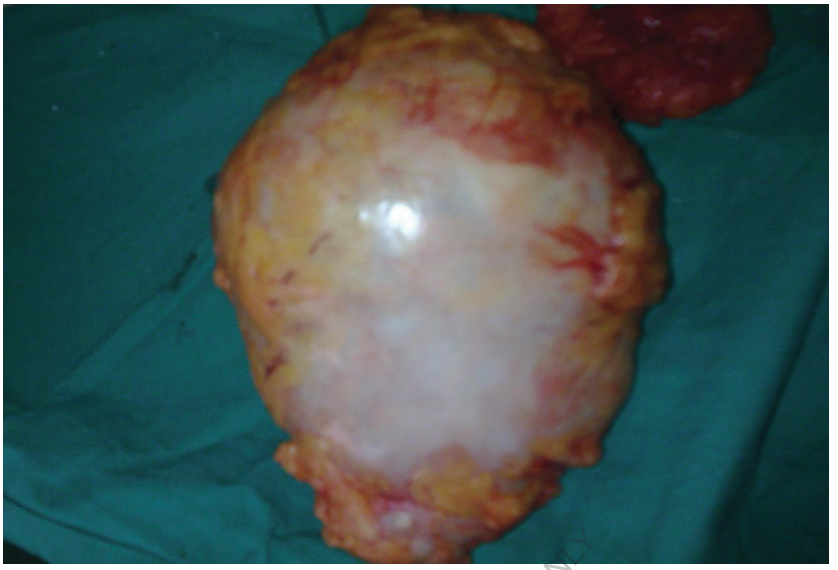

Fig.(2-3)peritoneal hydatid cyst (man 65 years, Baghdad teaching H.,

2009 under supervision of Surgeon Dr. Sabah Al-Janabi)

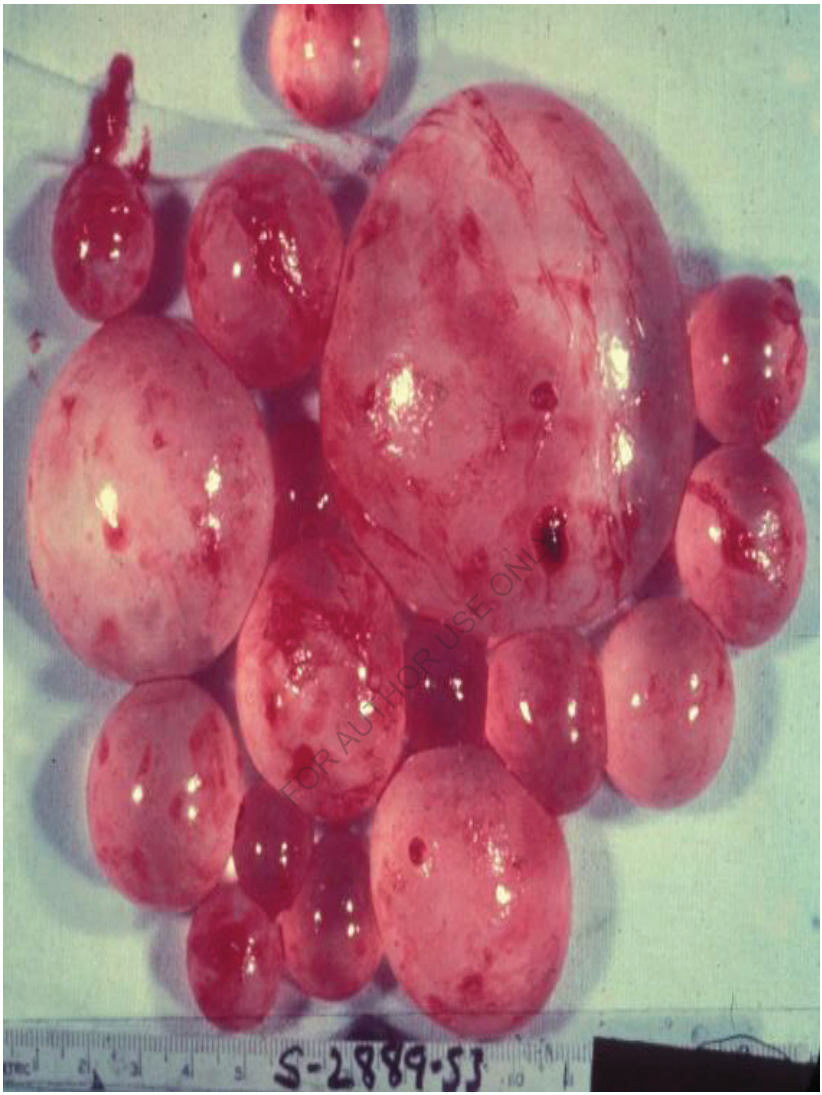

Fig.(2-4) Liver hydatid cysts (Man 57years old, Baghdad teaching H., 2009 under supervision of Surgeon Dr. Suzan Al-Tamimi)

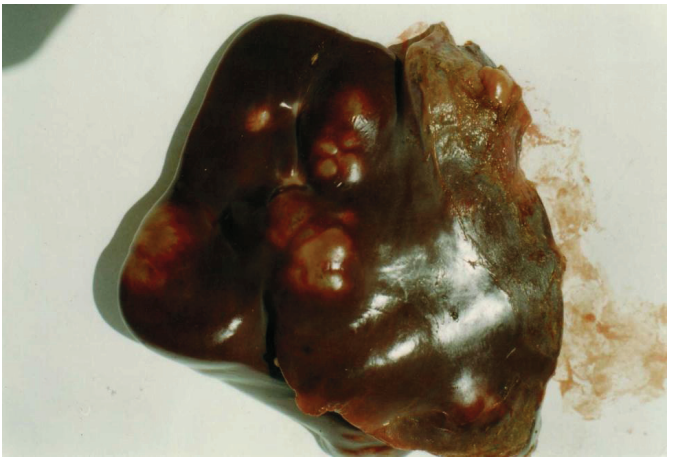

Fig. (2-5) Liver hydatid cyst (Sheep 1.5 year)

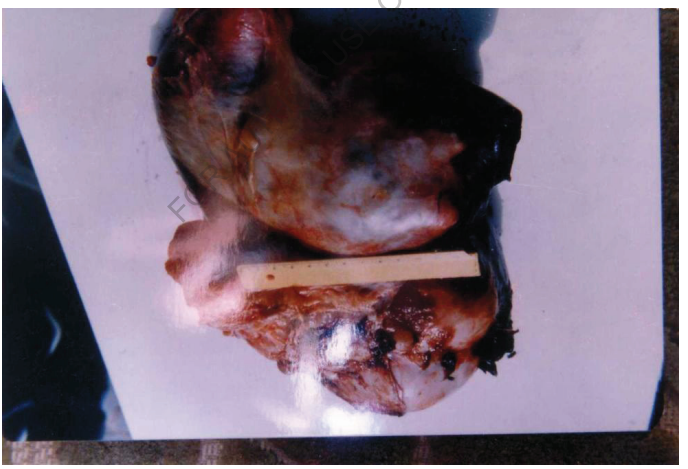

Fig.(2-6) Liver hydatid cyst (cow 2.5 years)

The life cycle of this parasite is a complex and there are two types according to intra specific variation **:** sylvatic cycle (Northern biotype) its spreading in north division of the world (Alaska ,Tundra , Taiga) final hosts are Wolves , Coyotes , Bush Dogs while Reindeer and Moose as intermediate hosts ; the other life cycle type is a pastoral cycle (European biotype) Dogs , Wolves and Foxes as final hosts while Ruminants and some primates as intermediate hosts , man is accidental host in both types of life cycle because it's not participate in the full life cycle (Fotiatis *et al.,* 1999 **;** Romig *et al.*, 1999 and Haag *et al.*, 1999) . The dynamics of transmission of the parasite are determined by the interaction of factors associated with these two hosts and with external environment (Gottstein, 2000) . Intermediate hosts or accidental hosts ingest the embryonated eggs which then hatch in the stomach or small intestine (due to presence of gastric or bile juices) liberating Oncosphere (Gottstein *et al.,* 2001 and WHO, 2001) .

Secretions (Mucopolysaccharidase , Hyaluronidase , Protease) from penetration glands and vigorous burrowing movement facilitate the penetration of intestinal mucosa , also these substances protect the parasite against hosts immune response while developing laminated layer (Gottstein and Reichen, 1996 and Holckman and Heath, 1997) . After that passing into lymphatic or mesenteric venules and is carried by the blood stream to various parts of the body . Most frequently it enters the portal vein and lodges in the liver , reach to the lung and other distant foci within 24 h. after ingestion (The most frequent site of Hydatid cyst in human and sheep in the liver about 75% then lung specially right lung due to transfer of oncosphere through systematic circulation (Shambesh *et al.,*1992) while in cattle infection in the lung more than the liver because of the widening of lymphatic vessels which facilitate passing of oncosphere through the lymphatic vessels to heart then lodge in the lung without passing to liver (Hageman *et al.,* 1999) if not destroyed by phagocytic

cells , it develops into Hydatid cyst (Haddix *et al.,* 1994 and Gonzalez *et al.,* 2002) .

In hatched oncosphere , the microvilli , compressed under the plasma membrane , are uplifted in the activated larva , they increase in number and size and are substituted by short and turnicated microtriches in metacestodes 3 days old , at that time appears the first lamination of the laminated layer that surrounds the metacestodes as an electron dense matrix composed of fine microfibrillated material and remnants of sloughed microvilli . The second lamination appears by the day 6 – 8 and is more electron dense than the first (Rigano *et al.,* 2001), at the end of third week , the young cyst will have attained a diameter of 25µ and the surrounding tissues of the host will exhibit define inflammatory reactions (Hernandez-Pomi *et al.,* 1997) .

Its suggested that secretary vesicles that are elaborated in the perikaryon of the germinal zone and continuously carried to the synsytium via microtubular cytoskeleton , are responsible for the laminated layer formation and are involved in the initial evasion of the immune response of the host (Pedrosa, *et al.,* 2000)

The cyclical production of lamination could be necessary to create layers that can ultimately be sloughed off as the cyst grows and serve to divert the host cellular response to the parasite . The final result is a fully mature metacestodes or Hydatid cyst , and this require five months to develop its morphologic characteristics (Haniloo *et al.,* 2005) . The final host represented by dog and other canine ingest the infected viscera with Hydatid cyst then liberation of protoscolices which reaching to adult worm during 6 – 8 weeks (Hernandez and Nieto, 1994), which is consist of scolex , neck , immature , mature and graved proglottides . The last proglottid contain (500) eggs detach from the body by a process termed Apolysis and releasing to the out side with feces of final hosts , contamination of environment occur and recycle it again (Robert and

Janovy, 2000 and Odev *et al.,* 2000) . Eggs are highly resistant to bad environmental conditions as desiccation without sun light and normal using chlorine in tape water also potassium permanganate (Horton, 1997 and Horton, 2003) . So that, intermediate hosts get infection through ingestion of contaminated materials , inhalation or direct contact with infected dogs specially in children and veterinarian (Idris *et al.,* 1999).

1 - 2 - 6 Types of Hydatid cysts :

There are 4 types of Hydatid cyst (Innis and Gelfand, 1990 **;** Jenkins *et al.,* 1990 and Ferreira *et al. ,* 1998) .

1 - Unilocular cyst **:** One or more vesicular cysts separated from each other by sheaths specific to each one .

2 - Alveolar cyst **:** Characterized by malignant growth formed from large number of acini embedded within affected organ , not surrounded by specific sheath and there is external buds penetrate the surrounding tissue and grow rapidly . The surgical operation is very difficult so that this type is more dangerous (Felleisen and Gottstein, 1994) .

3 - Multi vesicular cyst **:** Large number of adjacent vesicular like cysts connected with other named veterinary echinococcus due to its presence in cattle .

4 - Osseous cyst **:** Growing within bone characterized by irregularity due to presence of solid bony tissues which prevent spherical shape of cyst (Diaz *et al.,* 1995) .

1- 2 - 7 Epidemiology :

1-2-7-1-Epidemiology of hydatid disease in the world :

The disease is endemic in many parts of the world specially middle east (include Iraq), Australia, New Zealand, South America, Central and South Europe . In Bulgaria, Ivanor (1996) observed 244 cases of children infected with hydatid disease during 1980-1994 and noticed that 178 cases were with lung involvement only, while other cases with both lung and liver involvement . He also found that 61.1% were males, 84.8% of whom were with direct or indirect contact with the dogs. In Italy Caremani *et al.,*(1993) calculated the number of the patients with hydatid disease during 2 years and found that 24 cases mostly between 38-45 years were infected . In Africa studies showed that hydatid disease is widely spread but because of the limited studies about this disease, the pictures still unclear however, In Kenya the reported 5 cases four of them in the lung, while the age of the patient was between 35-56 years (Andersen *et al.,* 1997) .

Noorjah, (1987) carried out a large country wide study between 1980 and 1985 of some 3770 patients operated on for hydatid cysts and estimated a prevalence rate of 1.121/100,000 population . The infection rate among females was higher (56.4%) than in males (43.6%) and the age of the patients ranged from 2 – 84 years old, and the highest incidence was found among 20-30 years old . Bastani and Dehadshti,(1995) reviewed the radiological and clinical status of 126 cases of hepatic hydatid disease and attributed 60% of cases to the second and fourth decades of life. Anadol *et al.,* (1998) recorded 376 cases during 20 years (1975-1995). The mean age was 31 years and found 223 cases in males and 153 cases in females, and 222 cases were found in the lungs, while only 56 were seen in the liver. In China, Jun-Jie, (1995) reported that this disease was a big healthy and economic problem and recorded the occurrence of 26065 cases from (1951-1990) where 1/3 of the infections were noticed in children under 15 years . In Kazakhstan Shaikenov *et al.,* (1999) studied the picture of hydatid disease during (1990-1997) and recorded 415 cases of both sexes. In Russia during 15 years 1983-1997 a total of 2863 cases of human

Echinococcus were recorded average of 191 cases per year (Kovalenko *et al.,* 2000) . In Sub-Saharan Africa (Kattan, 2003) , Eastern Africa including Kenya, Tanzania , Uganda and Ethiopia (Irabuena *et al.,* 2000) .

1-2-7-2 The epidemiology of the disease in the Arab countries:

In Oman, Abbas *et al.,* (1996) investigated the presence of antibodies against *E. granulosus* in sera from 306 humans and 390 camels were investigated by indirect haemagglutination assays. Only one of the human and five of the camel sera gave positive reactions. The reactivities of the human sera were confirmed by ELISA. These results, together with isolated reports of hydatid cysts, indicate that *E. granulosus* is endemic in Oman, although with a low prevalence . Amr *et al.,* (1994) estimated that surgical removal of hydatid cyst counted for (0.3%) of all general surgeries and 5% of major general surgical operations. The age of patients ranged from 6-80 years with the highest prevalence among 25-45 years old. Infection rate in females (62.7%) was found higher than in males (37.2%). Infection in the liver and the lung were found to be (61.2%) and (29.8%) respectively. In Saudi Arabia, it was found that most CE infections occurred between 21 to 50 years old with female showing the highest rates 73% and liver as the most common site 82% of infection (Hira *et al,* 1993). In Lebanon, Daher *et al.,* (1996) studied the liver hydatid disease during the period between 1980-1992 and recorded 82 cases of which 39 cases were in males and 43 cases in females. Yaghan *et al.,* (2004) studied CE in Jordan between (1994 - 2003) and found (50%) of patients below 40 years of age and 57% were female and all interviewed patients gave history of contact with dogs and history of ingestion of raw vegetable food . In Saudi Arabia, Hadas-Halpern *et al.*, (2004) found 68 cases during 2 years and the infection in the females was more than males probably because the sheep raising was the jobs of the females in these areas .

In Egypt, the annual incidence of hospital cases was estimated to be less than one/100000 population (Shambesh, 1997) . Andersen, (1994) reported 306 cases in the period between 1976-1986 and (60%) of cases were seen in females who were housewives and students . In Morocco, Craig, (1997) reported the incidence of CE between (1980-1992) and was found to be 3.6-5.5/100/000 persons mostly in rural areas. In Mouritania the annual incidence of *cystic Echinococcosis* was estimated to be 1-2/100,000 for the period 1996-1997 (Beurdeley *et al.,* 1997). Hydatid disease also found in state of Qater, Bahrain, Kuwait (Tor, 2000) . Islamic republic of Iran Adewunmi and Basilin Gappa,(2004) . Levant countries **;** Israel , Palestinian, Syria (Akhmedov, 2004) .

1-2-7-3 Epidemiology of the disease in Iraq :

In Iraq CE is hyper endemic and termed Iraq cancer, the first reported case in 1925 in woman eye by Al-Mukaid, 1925 (Ormeci *et al.,* 2001) .The previous studies have shown a high prevalence of hydatidosis in slaughtered animals in Iraq . Mirani *et al.,* (2000) reported infection rates of 5.9% in sheep, 5.1% in goats, 4.9% in cattle and 20.4% in camels. Abdul-Majeed, (1997) reported infection rate of 9.7% in sheep, 3.1% in goat, 4.3% in cattle and 8.8% in buffaloes . In Al-Tamim CE in sheep , goat and cow 32.6%, 26.3%, 2.9% respectively ; In Diyala 30.8% , 20% , 36.3% respectively ; In Thi-Qar 29.2% , 23.5% , 30.6% respectively (Ali, 1999) . Molan, (1993) reported that the higher infection rate in dogs was closely related to the higher incidence in domestic livestock: 4.5-44% in sheep, 3.1-26.6% in goats, 4.3-13.9% in cattle and 20.4-72% in camels. More recently, Saeed *et al.,* (2000) reported the following prevalence rates for 1991-1998 from northern Iraq : sheep 15% (191/1270), goats: 6.2% (34/550) and cattle 10.9% (33/320) and 49.5% in dogs . Al-Sultan, and Al-Kanary, (2000) reported 105 cases with surgically confirm hydatid disease. The age of the patients was between 7-75 years, and female infected more

than male, liver involved more than other organ (69 cases), lung (14 cases) and the ratio of rural to urban patients was 73/32 . Al-Shammary, (2002), showed a slight preponderance of lung cases (47.4%) over liver cases (42.4%) . The liver is known to be the primary filter for the invasive migration larva, but there is as yet no satisfactory explanation why in some instances the lung also acts as a primary site of infection. Salih *et al.,* (1983) reported the picture of the disease among 410 cases between 1971-1980 infections rates among females were higher (67.3%) than in males (32.7%). Alwan *et al.,*(1990) reported 375 cases of hydatid disease, and the infection rates in liver and lung were found to be 52.5% and 21.4% respectively. In a retrospective study Al-Ubadi, (1996) based on patients admitted to the main hospitals in Baghdad Province, 197 patients (1.22%) of hospital admissions were surgically proven to be hydatid positive during the period from 1995 to the end of 1996. The infection rate was higher in females (63.79%) than in males (36.03%), and the age of the patients ranged between 3 and 70 years. In males the highest incidence was found in the age group of 21-30 years. The organ that was affected more was the liver, followed by the lungs in both sexes of 197 patients, 173 (97.82%) had single organ involved and only (24.18%) showed multiple organs or sites involved by hydatid disease. Another study conducted by Al-Mukhtar (1989), analysed human hydatidosis in Basrah for the period between 1979-1985. The study revealed 386 confirmed cases of hydatid disease (235 females and 151 males) . The common site of lesion was the liver (52.8%), lung (36.8%) and other organs (10.4%) . Mahdi and Benyan (1990) described the clinical manifestation of infection in 58 Iraqi children between 3 and 18 year old and again found the liver to be the primary site (72.2%) . Abdul-Aziz, (1990) studied 124 cases of hydatid cyst in children (65 females and 59 males) below 12 years of age in Mosul from 1980-1989. It was found that the most common site of infection was the liver 56.8% followed by the lung (46.4%) and the brain 7.2% . Falih, (2002)

reported 96 cases of CE in surgical hospital of the Southern Province of Thi-Qar in Iraq . The females showed the highest infection rate 58% . Also the liver was the most common site of infection. Salih and Al-Taie, (1998) studied 313 patients with hydatid disease were admitted to the surgical unit of Mosul hospital between January 1992 and January 1994, the age of the patients between 2-95 years, 260 cases (83%) were treated surgically. The majority 221 (71%) were females. The liver was the site of the cyst in 208 (66.4%). Other sites involved included the lungs in 98 cases (31.3%), spleen 21 cases (6.7%) peritoneal cavity 8 cases (2.5%), brain 7 cases (2.2%), kidneys 4 cases (1.3%), other 5 cases (1.6%). The incidence of the disease was higher in the third and fourth decade. The clinical diagnosis was confirmed mainly by X-Ray, ultrasound and CT scan . Al-Hammo, (1999) reported the result of a survey of human infection with hydatid cysts in patients admitted to the surgical wards in the governmental hospitals in Mosul during the period between 1990-1998. It was found that the infection rate was higher in females (64%) than in males (36%). Females were mostly housewives (47.4%). Incidence of the disease was highest among patients aged between 21-30 years of age. Infected cases were mostly from rural areas (60%). Hydatid cysts were most commonly found in the liver (63.5%), followed by lungs (13.5%), and then other organs. It was also found that 5% of the patients were suffering from multiple infections of hydatid cysts. In a study by Saeed *et al,* (2000) in Arbil Province (North Iraq), where 99 hospital cases were recorded between 1990-1998, the annual average was found to be 12.4 cases and the prevalence of hydatid disease was estimated to be 2 cases per 100,000 populations .

Many Iraqi researchers study hydatid diseases with different aspects from which : Therapeutical studies as Al-Khuzaei, G.H. (2005) ; Sida, L.A. (2005) ; Jamil, G.Y. (2006) ; Huda, R.S. (2006) ; Al-Chalabi, F.A.

(2007) . Immunological studies as Al-Qadhi, B.N. (2005) ; Mahmoud, A.M. (2006) ; Al-Nasiri, F.H. (2006) ; Muhand, H.(2006) ; Al-Sa´ad, A.A.(2007) ; Ahlam, (2009) . Epidemiological studies as Al-Jeboori, S.H.(2005) ; AbdulWadood, E. (2005) ; Yacoub *etal.,*(2006) ; Nayyef, A. and Rissan, H.(2007) . Molecular studies as Al-Rubaie, S.S. (2005) ; Al-Ghezi, Z.S.(2008) and other studies having general information about hydatid cyst as Maisam, B.N. (2005) and Al-Ubaidi, N.H. (2005) .

1 - 2 - 8 Immune response to Hydatid cyst :

The immunological relationship between the host and parasite is a series of interactions between the immune host reaction against the parasite and the inhibition of host defences by the latter (Aksoy and Inci, 2004) .

The immune response to Hydatid disease has been divided conceptually into pre encystment and post encystment phases (Al-Mufti and Mahmood, 2002), these are differentiated by the formation of laminated layer around the Hydatid cyst which occur between 2 - 4 weeks post infection in the intermediate hosts including human following ingestion of eggs and release of oncosphere (Zhang *et al. ,* 2003) . Primary infection, very little is known about factors affecting innate susceptibility to infection with *E. granulosus* following ingestion of the infective eggs stage and establishment of primary cyst . host age, sex and physiological status may influence the innate susceptibility or resistance to infection while the immune response is influenced by (Betharia *et al.,* 2002) number, size , Pericystic status (sings of degeneration or not) , location of cyst in liver itself and cyst with or without daughter vesicles (Allen and Maizels, 1996) .

Experimental infections of mice with eggs or oncosphere of *E. granulosus* showed that susceptibility varies with different strains of

mice (Bukte *et al.,* 2004) .After infection, the earliest detectable immunoglobulin G (IgG) response to Hydatid cyst fluid antigens occur after 2-11 weeks in mice and sheep respectively and after 4 weeks in revert monkey (Belding, 1995) . Early infection may be associated with significant cellular inflammatory response , that may cause pathologic changes, since, there is leukocytosis, mainly of eosinophils , lymphocytes and macrophages (Lightowlers *et al.,* 1996) . These changes in many parasitic infections are the primary cause of disease (Kern *et al.,* 2001) .

Neutrophils in association with antibodies can bring about the killing of *E. granulosus* oncosphere , lead to suggesting a possible role for antibody dependent cell mediated cytotoxicity (Abo-Shehada, 1993), so that there is marked activation of CMI at early stage in case of parasitic infection .

In experimentally induced secondary infection in mice , intra peritoneally injected Protoscolieces are surrounded by a considerable cellular infiltration with within 3 days , initially involving activated macrophages and subsequently including nutrophils , eosinophils and lymphocytes (Mwambete *et al.,* 2004). Compared with events occurring during early infection , the immune response to establish cyst has received much more attention . Humoral response requires the coordinate action of T and B lymphocytes mediated by cytokines (Haag *et al.,* 2004) . In human there is frequent occurrence of elevated antibodies levels , particularly of IgG (in acute and chronic stages) , IgM (detected during period of antigenic activity and disappear soon after removal of cyst so that persisting or increasing level of IgM indicate a continued antigenic stimulus caused by presence of additional cysts in the host) , IgE, IgA isotypes (Farrokh, 2001) .

There is significant correlation between levels of total IgG and IL-4 in patients with liver hydatidosis also between total IgE and specific IgE to *E. granulosus*, Also found an activation of the complement system in patients with multiple and larger cysts and those with daughter vesicles (Juma *et al.,* 2000) . In establishment phase also there is cellular infiltration including eosinophils, nutrophils , macrophages and fibrocytes (Franchi e*t al.,* 1999), aged cyst tend to become surrounded by fibrous layer that separate the laminated layer from host tissues (Gottstein and Reichen, 2002) . Eosinophilia and production high level of IgE are the common consequence of infection by helminthes . It has been suggested that the eosinophils has evolved specially as a defense activity against the tissue stage of parasite that are too large to be phagocytosed (Ahmadi, 2004) .

The IgE – dependent mast cell reaction has evolved primarily to localize eosinophils near the parasite and then enhance their anti parasitic functions (Bell, 1996) . Other investigators showed that eosinophils are less phagocytic than nutrophils , but they can kill larval stages of the parasite by both dependant and in dependant mechanisms , their activities being also enhanced by cytokines (Al-Dulaimi *et al.,* 1992) . Killing of helminthes by eosinophils via (ADDCC) is an attractive and widely cited mechanism for resistance to parasitc worms . This mechanism was initially based on in vitro assays in which eosinophils were shown capable of killing wide variety of antibody and / or C- opsonized helminthes (Zworowska, 2000) .

1 – 2 – 9 **Diagnosis of hydatidosis :**

Determining whether the person is infected with Hydatid disease or not is based on the following (Hashemitabar, 2005) **:**

1-An association with dogs or wild canids .

2- Radiological tests including X-rays , ultrasonography , computerized tomography (CT scan) , magnetic resonance imaging (MRI) .

3- Serological and immunological tests **:**

a. ELISA (Lightowlers *et al.,* 2000) .

b. SDS-PAGE (Koul *et al.,* 2000) .

c. Immunoblotting electrophoresis (Onursal *et al. ,* 2001 and Shiranie *et al. ,* 2008) .

d. Complement fixation test (Larrieu *et al.,* 2002 and Macpherson, 2001) .

e. Indirect hemagglutination test (Macpherson *et al.,* 2003) .

f. Intradermal Casoni test (Mandell, 2000 ; Imad and Dandon, 2002 and Mahmoud *et al.,* 2008) .

g. Latex agglutination test (Mario *et al.,* 2006) .

h. Polymerase Chain Reaction (PCR) (Moreno *et al.,* 2004 and Opartrny *et al.,* 2005) .

1 - 2 – 10 Treatment :

1 – 2 – 10 – 1 Surgery : And increasingly chemotherapy (alone or in combination with surgery) and very recently (PAIR) technique are the main forms for treatment of Hydatid disease in human (Parodi *et al.,* 2001 **;** Patrick *et al.,* 2005 and Pierce and Wang, 2007) .

The chief problem lies with recurrence , its estimated that up to 11.3% of patients have recurrences within 5 years of surgery for primary cyst (Pavlov *et al.,* 2004).

1 – 2 – 10 – 2 Chemotherapy :

May be an alternative option in multiple cysts in more than one organ or the patient at high risk from surgery (Ameli and Abbasian, 1995) . A number of anti helminthic drugs have proved to be effective against adult

stage of *E. granulosus* in the final host . The best drug currently available are :

1. Praziquantel , Arecoline HBr (Rahimi *et al.,* 2007 and Rafei *et al.,* 2007) which terminate all juvenile and adult echinococci from dogs at dose 5mg/kg (Righter *et al. ,* 2004) .

2. Benzimedazole carbamates (albendazole and mebendazole) are effective against larval stages , mebendazole has proved to be effective , although the results are variable , some cases are failed treated because of its poor absorption (Sachse and Fery 2008) .

3 . Albendazole may kill protoscoleces within cyst and even reduce the size of cyst , its efficacy reach to 82% due to selective toxicity (Safioleas *et al.,* 2005) .

4 . Praziquantel and isoquline derivatives has recently shown value in treatment and using in combination with Albendazole in some patients is recommended (Saul *et al. ,* 2008) .

Side effects of drugs are head ache , vomition , nausea , itching , abdominal pain and discomfort (Schipper *et al. ,* 2002) .

1 - 2 - 11 Control and preventive measures :

Hydatidosis is major public health problem around the world so that many steps may be have a benefit to diminish its spreading ; Mandatory annual surveillance and treatment for all dogs including sheep dogs , compulsory destruction of all hydatid cysts and infected offal , distribution of an educational pamphlets and covering the animal pits and slaughterhouses to prevent access to those sites by stray and roving dogs . (Tiaoying *et al.,* 2005) . Other studies used modern techniques in genotyping of *E. granulosus* to facilitate treatment and vaccination by using Polymerase chain reaction (PCR) purification soluble protein of whole parasite body that's give 100 % protection after challenge with 2000

protoscolices intraperitoneally (Leder and Weller, 2003) . Fatty acid binding protein (EGDf 1) and Fibrillar protein (EGA31) derived from *E. granulosus* exhibit strong immunogenic properties in dogs (Yacoub *et al.,* 2006) . By using PCR diagnostic antigen (EgP-29) cloned from *E. granulosus* and expressed in *E.coli* encode protein of 238 amino acids having similarity in different isolates after 5 h. at 37Ċ give protection 96.6 % to prevent secondary hydatidosis against different geographical isolates(Bartleltt, 2003) . Mitochondrial Cytochrome Oxidase sub unit 1(CO 1) and NADH dehydrogenase by using of PCR determine the strains of *E. granulosus* (G1-G10) and sub strains also to facilitate controlling (Stefaniak, 1997) . Other applications for PCR in the world ensure the environmental source in CE transmission by examination of soil samples through using specific primer for sheep strain (G1) and this positive result explain why children suffering from CE without contact with risk factors and why epidemiologic studies that have failed to detect an association with dog ownership or contact as a risk factor for developing CE (Dowling and Torgersson, 2000), all these applications which based on using of PCR facilitate the process of study of strains and sub strains of *E. granulosus* then promoting vaccines preparation to control on this fastidious disease .

1 – 2 – 12 Aims of study :

1- Demographical study for factors associated with hydatidosis in human patients (Age, Sex, number, location and size of hydatid cysts) .

2- Determination of genetic variations of *Echinococcus granulosus* strains (larval stage) in human, sheep, goat, cattle and buffaloes according to molecular level by using PCR technique .

3- Determination the concentration of Immunoglobulins (IgG, IgM and components of Complement C3 and C4) in hydatidosis patients by Simple Radial Immuno diffusion Assay (SRIDA) .

4- Known the more susceptible age groups, effect of age and sex on titer of immune elements and age on fertility of hydatid cysts .

5- A study of relationships between various demographical factors with immunological parameters .

Chaper two

Material & methods

2 – 1 Subject selection :

This study was conducted in four general teaching hospitals in Baghdad governorate : Baghdad teaching hospital, Al- Shaheed Adnan teaching hospital, Liver and Digestive disease teaching hospital and Ibn – Al- Nafees teaching hospital, from January – November 2009 , the cysts were in the liver, lung, ovary and spleen . Samples of hydatid cysts from animal origin getting from slaughterhouses . In the period of this study, 30 cases of space occupying lesions have been enrolled . Among these cases, 14 patients were found suffering from Liver hydatidosis, 7 patients with lung hydatidosis, 6 female with ovarian hydatidosis and other in different organs . The diagnosis of patients was confirmed by serological test including Indirect Hemagglutination test (IHA) and radiological tests such as plain radiography (X-ray), Computed Tomography (C.T. scan), Ultrasound and Magnetic Resonance Imaging (MRI) .

2 – 2 Isolation of germinal layer of hydatid cysts :

Germinal layers of hydatid cysts from human were taken after surgical operation, from different animals after slaughtering . Both of them taken to laboratory by clean containers, sterilizing of outer surfaces by 70% ethanol then discarding of hydatid fluid, germinal layers were taken and kept in ethanol 70 % for different periods (Tsimoyiannis *et al.,* 1995).

2 – 3 Blood sample collection :

Blood samples were collected from 30 patients at age range (12-57) years clinically diagnosed and surgically confirmed patients with hydatid disease by vein puncture (Pre operation) , 3ml of venous blood was collected from each individual transferred immediately into plain plastic tubes and the serum was obtained by (cooling centrifuge), centrifugation at 4 Ċ, 4000 rpm /15 min . The serum was dispensed in plastic appendrof

tubes, 0.5 ml in each tube and stored in (-20 Ċ) until used for serological testing .

2 – 4 Patient age groups :

Hydatidosis patients were subdivided into four groups :

a. group 1 between 10 – 20 years .

b. group 2 between 20 – 30 years .

c. group 3 between 30 – 40 years .

d. group 4 > 40 years .

2 - 5 Instruments and Equipments :

The instruments and equipments used in this experiment are listed in table (2-1) below:

Table (2-1) Instruments and Equipments with their remarks

Instrument / Equipment	Manufacturer / state
PCR sprint- Thermal – Cycler – IP20	USA
Ultraviolet transilluminator.	(European)ECX-15.m.
Gel electrophoresis.	(UK)Shandod Scientific CO.
Eppendrof centrifuge.	Hettich EBA 20(Germany).
Micropipettes (different volumes) .	Eppendrof Oxford (USA).
Eppendrof tubes	Sigma(England)
Water distillator	Ogawaseiki (Japan).
Light microscope	Olympus (Japan)
Sensitive balance	GallenKamp (England)
Hot plate with magnetic stirrer	GallenKamp
Incubator	Memmert (Germany)
Electric oven	Memmert
Water bath	Memmert
Vortex mixer	Memmert
autoclave	Stermite (Germany)

Digital camera	Sony (China)
PCR tubes	Eppendorf (USA)
Ultra sound sonicator	Jack ultrasonic / Korea
Cooling centrifuge	Olympus (Japan)

2 – 6 Chemicals and biological materials :

The chemicals and biological materials used in this work are listed in table(2-2) below :

Table (2-2) chemical and biological materials.

Type of chemicals	Manufacturers name
Bromophenol blue	BDH / UK
Ethidium bromide	BDH
Ethylene Diamine Tetra-Acetic Acid (EDTA)	BDH
Tris-oH	BDH
Boric acid	BDH
Potassium hydroxide	BDH
Ethanol	BDH
Agarose	Promega (USA)
DNA marker	Promega (USA)
Tween 20	Atlas chemical industries Inc. / England
Proteinase - k	Promega (USA)
IgG, IgM, C3 and C4 plates	Bussero, Milano / Italy

2 – 7 Electrophoresis solutions :

These solutions were prepared according to (Vuitton, 2003 ; Nasrieh and Abdul-Hafez, 2004) :

* EDTA buffer (0.5 M) :

This buffer was prepared by dissolving 18.612gm of EDTA in 80 ml distilled water then complete the volume to 100 ml, sterilization by autoclave at (121Ċ / 1.5 Ib for 15 min.), cool it then stored at 4 Ċ until be used .

* Tris –Borate EDTA buffer (TBE buffer, 10x) :

This buffer was prepared by dissolving 3.8 gm Tris-Hcl , 2.7 gm Boric acid and 2 ml EDTA (0.5M) in 50 ml of distill water, the PH was adjusted to 8 , autoclave (121Ċ / 1.5 Ib for 15 min.), cool and stored at 4 Ċ until be use .

* TBE (1X) :

This solution was prepared by mixing 10ml (of stock) TBE- 10x with 90 ml of distilled water , and stored at 4 Ċ until using .

* Tris – EDTA (TE) buffer 1 ml from (1.214 gm of Tris – base in 10 ml of D.w) and 0.2 ml from (1.86 gm of EDTA in 10 ml of D.w) and complete the volume to 100 by adding 98.8 ml of D.w .

* Hydroxyl methyl amino methane – HCL (Tris - base) at concentration 50 mmol. (dissolving of 0.6 gm in 100 ml Dw.).

* Standard Phosphate Buffer saline (PBS) had pH 7.4 .

* Blue / Orange 6X dye : 1.25 gm of Bromophenol blue with 30 ml of glycol in 70 ml of D.w. , the volume become 100 ml , correct the Ph to 8 with NaOH (10 M) then kept at 4 Ċ .

* Ethidium bromide solution (0.5 %) :

This solution was prepared by dissolving 0.25gm of Ethidium bromide stain in sterilized distilled water, stored in sterilized flask, final concentration 5 milligram /milliliter.

2 – 8 Extraction solution of DNA :

100 µl of Tris – HCL , 10 ml of EDTA and 0.5 ml Tween 20 were mixed then the volume was completed to 100 ml by adding 89.400 of

D.w. All these solutions were sterilized by autoclave and kept in cooling state to until to be used .

2 – 9 Agarose gel preparation :

0.5 gm of Agarose was dissolved in 50ml of TBE 1x buffer, the solution was heated to boiling on hot plate and stirrer until all Agarose particles were dissolved, then allowed the solution to cool down at 45 Ċ .

*Other Solutions : Normal Saline : This solution was prepared by dissolving 8.5gm Nacl in 1000 ml distilled water, sterilized by autoclave, stored at 4 Ċ (Richter *et al.,* 2003).

2 – 10 Diagnostic Kits Which include : (Table 2 - 3)

Material	Origin
Go Taq Green master mix, 2x (pH 8.5) 1.25 ml	Promega USA
Nuclease free water 1.25 ml	Promega USA
100 base pair (bp) DNA ladder 250 ml	Promega USA
Blue / Orange 6X loading dye 1.25 ml	Promega USA

2 – 11 Green master mix 2x :

1- bacterially derived Taq DNA polymerase.

2-dNTPs which include : 400 microM of each dATP, dGTP, dCTP, dTTP .

3 – MgCl2 (3M) .

4 - Yellow and blue dyes as loading dye.

2 – 12 Specific primer sequences used for PCR amplification .

These primer were prepared according to information of company by dissolve each primer in 1000 µl of deionized distilled water to obtain stocks in concentration 124.693 picomol / µl of each of the PCR primers.

2 – 13 Random primers and their sequences (Table 2 - 4) used in this study provided by Alpha DNA Co. :

Primer No.	Primer sequence
OPA – 01	3ó CAGGCCCTTC 5ó
OPA – 02	3ó TGCCGAGCTG 5ó
OPA – 03	3ó AGTCAGCCAC 5ó
OPA – 13	3ó CAGCACCCAC 5ó
OPB – 12	3ó CCTTGACGCA 5ó
OPE – 07	3ó AGATGCAGCC 5ó
OPD – 20	3ó ACCCGGTCAC 5ó
OPC – 05	3ó GATGACCGCC 5ó
OPC – 10	3ó TGTCTGGGTG 5ó
OPC – 12	3ó TGTCATCCCC 5ó

2 – 14 DNA extraction : (Vicidomini, 2007)

250 µl of germinal layers were added (sonication of germinal layer by ultra sound sonicator high speed / 10 min. (Welsh and McClelland, (1990)) in specific eppendrof tubes (1.5 ml) containing 1000 µl of PBS after that centrifugation at 12000 rpm. /10 min., the supernatant was get away and the sediment was remaining , this step was repeated three times.

* The remaining sediment was added to 500 µl from extraction solution and 6 µl of Proteinase – K .

* All tubes were incubated in water path (37 Ċ) until the next day .

* After that the action of Proteinase – K was inhibited by rising the degree of water path to 100 Ċ (Boiling degree) for 5 min., the samples were

kepted in freezing by adding 50 µl of (TE) buffer until to be used in PCR reaction .

2 – 15 Gel electrophoresis of DNA :

* Preparation of Agarose gel at concentration 1%, 0.5 gm of Agarose was dissolved in 50 ml TBE buffer (1x) then heated .
* Ethidium bromide stain solution was added to Agarose gel (1 µl :50ml) .
* The heated Agarose solution was poured into the gel tray and allowed to cool at room temperature for 30 minute .
* The comb was carefully removed from Agarose and extracted DNA was mixed with bromo phenol blue in the ratio of 3:1 loaded in the wells of the Agarose gel .
* The tray was placed into electrophoresis chamber, the chamber was filled with electrophoresis buffer TBE (1x) until cover the surface of the gel.
* Ethidium bromide stain solution 1 µl was added to the electrophoresis chamber .
* Electrical current was connected the electrophoresis chamber, cathode was connected to the side of samples, at voltage (65V) for 45 min.
* Finally gel was transported into U.V trans-illminator .
* 50 µl of TE buffer was added to crude DNA and kept in freezing for long periods or used directly in PCR technique as following procedure :

2 – 16 PCR kit (Green master mix, Primers, Nuclease free water , extracted DNA) and these constituents put in ice container :-

*A new PCR tubes (0.5 ml) were labeled with number of sample for amplification reaction (located in ice) **.**
*To avoid contamination, all solutions were taken with separate clean tips under a septic condition .

* 5 µl of DNA sample was added to PCR tube , 2 µl of primer, 12.5 µl of Green master mix and 5.5 µl of Nuclease free water .The volume was completed to 25 µl .

*All tubes were closed, the mixture was spin for 5 second by light vortex, the PCR tubes were transferred to preheated Thermocycler .

2 – 17 PCR program :

3 major steps in PCR, (30-35) cycles each comprising this was done by automated Thermocycler **:**

1 – Denaturation at 94 Ċ (60 second) **:** The double strand helix was melt and became single stranded DNA .

2 – Annealing at 45-65 Ċ for (60 second) **:** Primers were binded to DNA strand , this temperature depend on type, length and G ≡ C content of primer.

3 – Extension **:** At 72 Ċ for (60 second) **:** A new DNA strand complimentary to the DNA template was synthesized by Taq DNA polymerase by adding dNTPs in the 5ó → 3ó direction, temperature differ according to DNA length (Weigand *et al.* , 1993) .

Table (2 - 5) explain steps of PCR technique (Yang, 2005)

Steps	Temperature (Ċ)	Time	No. of cycles
Initial Denaturation	95	5 min.	1
Denaturation	94	30 second	30
Annealing	55	45 second	
Extension	72	1 min.	
Final extension	72	5 min.	1

After that Gell electrophorosis was made to all PCR tubes as in case of DNA extraction except 1gm (instead of 0.5 mg) of Agarose was dissolved in 50 ml of TBE (1x) and all above steps in Gell electrophorosis of

extracted DNA were applied, after that the results were observed by U.V. Light by using U.V trans-illminator .

The end products much affected by many chemical and physical factors as temperature, time, concentrations of reaction mixture (Template DNA , Primers, Taq DNA polymerase, Mg +2 concentration and dNTPs (Seven *et al.,* 2000) .

Taq DNA polymerase is a single strand, poly peptide with M.W 95 kd consider as important factor in PCR reaction characterized by stability to high Denaturation step temperature so that added once only to reaction , this enzyme isolated from Thermophilic Euobacterium named *Thermous aquatiqus* (Salinas *et al.,* 2000) . This enzyme responsible for complementary DNA strand synthesis by adding dNTPs to template at optimum temperature 70 – 75 Ċ , at range 150 nucleotide / 1 second (Eckert *et al.,* 2000 and Thompson, 2001) , recently there are genetically modulated *E. coli* to produce large amount of Taq DNA polymerase (Yi-Cheng *et al.,* 2002) .

2–18 Immunological study

2–18–1 Measurement of immunoglobulins and components of complement concentrations by single radial immunodifusion test (SRID) :

A kits of radial immunodifusion plate (Bussero (Millano) Italy) were used to determine the concentration of immunoglobulins ; IgG and IgM, components of complement C3 and C4 for 30 patients infected with hydatidosis .

Each plate contain monospecific antiserum directed against IgG, IgM, C3 and C4 which was incorporated in an agarose gel layer .The plates were removed from their envelopes and left them to stand at room temperature for few minutes, any condensed water in the wells was evaporated, the wells were filled with 5 µl of sample (patient's serum) and waited for 5 min. to be completely adsorbing, before handling the plates were closed and put in moist champer, after that the plates were incubated from 48-72 hrs. at 37 Ċ (Woollard *et al.,* 2000) . Plates of IgG, C3 and C4 were red after 18 hrs. while IgM plates after 72 hrs., end point of diffusion is indicated by a sharp precipitating ring, which was achieved when incubation time was finished . Readings were done at this time . The diameters of each ring were measured directly by using magnifying lens with micrometers scale . The diameter of the ring was related to antigen concentration and the results were evaluated by using reference standard table (WHO reading, mg/dl) that is packaged with the kit instruction method supplied (Bussero (Millano) Italy .

Plates :

Plate : Agarose gel containing monoclonal antisera IgG .

Plate : Agarose gel containing monoclonal antisera IgM .

Plate : Agarose gel containing monoclonal antisera C3 .

Plate : Agarose gel containing monoclonal antisera C4 .

2 – 19 Statistical analysis :

Statistical program for social sciences (SPSS) ver.14 was used for determining the statistical significance among different variables . Chi square test and differences between two proportions by T- test were used to assess the significance of differences between groups .

P value less than 0.05 ($P < 0.05$) was considered as statistical significant, P value < 0.01 as highly significant and $P < 0.001$ as extremely significant. While P value more than 0.05 $P \geq 0.05$ was considered as statistically not significant .

Chapter three

Results & discussion

3 – 1 Epidemiology of hydatid disease :

Hydatid disease is characterized by cystic space – occupying lesions in the liver , lungs and rarely in other parts of the body (Lone *et al.,* 2002) . All evidences provided ensure that hydatid disease till now major health problem in Iraq in spite of modern equipments available for diagnosis and treatment . The surgically confirmed cases are the only reliable source of data on human hydatidosis , since hydatid infection is a notifiable disease , and its difficult to determine the specific source of infection and its usually impossible to know when the infection was acquired this may be due to the fact that cysts are usually slowly growing and the development of symptoms or the ability to diagnose the conditions may require from 6 months to several years after exposure to the infections (Dziri, 2001) .

3 – 1 – 1 Distribution of disease according to age :

The ages of patients in present study varied between 12 – 57 years, the maximum incidence recorded was among patients between (12 – 40) year was 22 (73.33%) showed in table (3.1) also reported by Yang *et al.,* (2006) while, Al-Sanafi and Farjou, (2001) and Mongha *et al.,* (2008) showed high rate of infection was between (20-30) years . Also in this study showed that cases less than 10 years of age are rare, this may be due to variation of interval times that required for hydatid disease to become clinically manifest .

Table (3 – 1) : Distribution of hydatidosis patients according to age and sex of 30 patients.

Age(years)	Males No.(% Total)	Females No.(% Total)	Total No.(% Total)
10 – 20	4 (40%)	6 (60%)	10 (100%)
20 – 30	1 (11.11%)	8 (88.88%)	9 (100%)
30 – 40	3 (37.5%)	5 (62.5%)	8 (100%)
> 40	0	3 (100%)	3 (100%)
Total	8 (26.66%)	22 (73.33%)	30 (100%)

3 – 1 – 2 Distribution of disease according to sex :

The present study showed that the predominance of hydatidosis was in females 22 (73.33%) than in males 8 (26.66%) table (3 – 2), in rate of infection female : male 2.75 : 1 . Highly infection rate in females is in agreement with most of other studies which have shown a high frequency in females (Abdul-Karim, 2001 and Al-Qadhi, 2005) . The highest risk group in our country specifically and in Arab Gulf region -in general- 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 . Not surprisingly, infection rates among women are shown to be the highest, and children who acquire the disease in early life may not present with symptoms until adulthood

(Nakao, 2007), also Estrogen hormone play an important role in dissolve egg shells and facilitating hatched Oncosphere to penetrate host tissues in females of mice (Brunetti *et al.,* 2005) . However this results doesn't agree with the findings of Torgerson *et al.,* (2003) who observed high rates of infection in males . From this findings we cannot draw a conclusion on human infected with hydatidosis because of sample size doesn't large enough (Safioleas *et al.,* 2005) .

Table (3 – 2) : Sex distribution of 30 patients infected with hydatid disease .

Females	Males	Ratio F : M	Total number
22 (73.33%)	8 (26.66%)	2.75 : 1	30

3 – 1 – 3 Distribution of disease in various organs :

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 (64.66 %) when compared with lung 7 (23.33 %) and ovary 6 (20 %) and other multiple infected organs such as spleen, peritoneum, bone and brain showed in table (3 – 3), generally these proportions approximately in agreement with most of previously recorded data by Ahmadi and Al-Dalimi, (2006) and others .

Table (3 – 3) : Distribution of 30 hydatidosis patients according to site of infection and sex .

Site of infection	Males No. (%+ve)	Females No. (%+ve)	Total No. (%+ve)
Liver	4 (13.33%)	10 (33.34%)	14 (46.67%)
Lung	4 (13.33%)	3 (10%)	7 (23.33)
Ovary	0	6 (20%)	6 (20%)
Multi organs	0	3 (10%)	3 (10%)
Total	8 (26.66%)	22(73.34%)	30(100%)

3 – 1 – 4 Fertility of Hydatid cysts :

Fertile Hydatid cysts are formed in intermediate hosts (human and herbivores) producing protoscolices, the infective form to canines, at their germinal layers . Infertile cysts are also formed, but they are unable to produce protoscolieces . The molecular mechanisms involved in hydatid cysts fertility/infertility are unknown. Nevertheless, previous work has suggested that apoptosis is involved in hydatid cyst infertility and death. On the other hand, fertile hydatid cysts can resist oxidative damage due to reactive oxygen and nitrogen species . On these foundations, we have hypothesized that when oxidative damage of DNA in the germinal layers exceeds the capability of DNA repair mechanisms, apoptosis is triggered and hydatid cysts infertility occurs . Fertility of hydatid cyst is important factor in stimulation of immune response in patients with hydatidosis as observed in case of increasing of IgG and IgM concentrations when there's daughter vesicles within cyst , and this factor directly proportional with advanced ages showed in table (3–4) as we found there was significance difference at level ($P < 0.05$) between 2^{nd} and 4^{th} age groups .

Table (3 – 4) explain the effect of age on fertility of hydatid cysts

Type of hydatid cyst	Male				Female				Total
	1	2	3	4	1	2	3	4	
	No. %	No. %	No. %	No. %	No. %	No. %	No. %	No. %	%
Sterile	1 9.09%	0	2 18.18%	1 9.09%	0	6 54.54%	1 9.09%	0	11 36.66%
Fertile	2 10.52%	1 5.26%	0	1 5.26%	1 5.26%	4 21.05%	4 21.05%	6 31.57%	19 63.33%
Total	3 10%	1 3.33%	2 6.66%	2 6.66%	1 3.33%	10 33.33%	5 16.66%	6 20%	30 100%

3 – 2 Results obtained from Random Amplified Polymorphic DNA (RAPD) :

The samples used in (RAPD) are DNA isolated from germinal layer of hydatid cysts of Human at three different age groups also from Sheep , Goats , Cattle and Buffaloes tested by (10) ten primers provided by Operon technologies Co. which are : OPA – 01 , OPA – 02 , OPA – 03 , OPA–13 , OPC – 05 , OPC – 10 , OPC – 12 , OPB – 12 , OPE – 07 , OPD – 20 . The optimum conditions in this experiment corresponding to standardization of other previous studies (Williams *et al.,* 1990 ; Al-Rubaie, 2005 and Bart *et al.,* 2006) . *E. granulosus* exists as a series of genetic variants or strains which differ in a wide variety of criteria that impact on the epidemiology, transmission, pathology and vaccination to control of cystic hydatid disease in intermediate hosts . Also, possibility to get the fingerprinting to these samples . Results obtained from using of these primers in (RAPD) reactions led to that those primers differ in production of amplified bands which differ in number and its molecular weights resulted from differences in complementary loci on the genome of each sample and this reflex the genetic variance between these samples and this finding is well documented by (Bart *et al.,* 2006 ; Busi *et al.,* 2007) .

3 – 2 – 1 Analysis the Results of (RAPD) :

Depending on the results obtained from newly studies in numerate and expression of amplified bands to determine the genetic varieties on the Agarose gel to obtained samples and known the fingerprinting between them (Carmena *et al.,* 2008 ; Andresiuk, 2009) . Fingerprinting depending on scientific researches in studied genome represented either by presence of specific band in one sample and doesn't found in others or presence of unique pattern of bands in one sample differ from others (Dengri *et al.,* 2002) . Both of genetic variance and fingerprinting depend on presence of amplification or not and molecular weight of bands which depend on the

number of complementary loci to primer's sequences on the template DNA (Dopchiz, 2009) . In this study three human samples from liver, lung and spleen cysts at age groups (10 - 20 , 20 - 30 and 30 - 40 years old) respectively ; Sheep ; Goat ; Cattle and Buffaloes liver hydatid cysts which have numbers 1, 2, 3, 4, 5, 6 and 7 respectively and fig. (3–1) explain electrophorosis to end products of extracted DNA .The obtained results after electrophorosis to end products of amplification process by using Thermocycler apparatus as follow :

1 - OPA – 01 : Many amplified bands differ in molecular weights (m.w) 200 – 1050 bp showed in fig.(3–3), 3 bands in sample 1 ; 1 band in sample 2 ; 4 bands in sample 4 and 3 bands in sample 5, from other hand disappearance of amplified bands in case of samples 3 , 6 , 7 .

2 - OPA – 02 : Two amplified bands in sample 1 ; 1 band in sample 2 ; 4 bands in samples 4 , 5 with m.w 100 - 800 bp also disappearance of amplified bands in case of samples 3 , 6 , 7 showed in fig. (3–5) .

3 - OPA – 03 : There are two amplified bands with m.w 300 - 400 bp appear in samples 1 , 2 , 4 showed in fig. (3–6) and absence of bands in samples 3 , 5 , 6 , 7 .

4 - OPA – 13 : There is one amplified band with m.w 600 bp appear in samples 1, 2, 4, 5 showed in fig. (3–4), absence of bands in samples 3, 6, 7.

5 - OPB – 12 : There is one amplified band with m.w 300 - 400 bp appear in samples 1 , 2 , band with m.w 900 bp in samples 1 , 4 , 5 showed in fig. (3–4), absence of bands in samples 3 , 6 , 7 .

6 - OPC – 05 : There is one amplified band with m.w 300 bp appear in samples 1, 2, 3, 4 , 5 , band with m.w 900 - 1000 bp in 2 , 4 , 5 showed in fig. (3–2), absence of bands in samples 6 , 7 .

7 - OPC – 10 : Absence of amplified bands in samples 1, 2, 3, 6 , 7. Band with m.w 500 bp in samples 4 and 5 showed in fig. (3–3), other amplified band with m.w 1000 bp in sample 4 . Absence of bands in samples 6 , 7 .

8 - OPC – 12 : One amplified band with m.w 400 bp in samples 1, 2 , 4. Absence of amplified bands in samples 3, 5, 6 , 7 showed in fig. (3–5) .
9 - OPE – 07 : One amplified band with m.w 200 - 300 bp in samples 1, 2, 4 , 5 , band with m.w 500 bp in samples 4 , 5 , band with m.w 900 - 1000 bp in sample 5 showed in fig. (3–2), absence of amplified bands in samples 3, 6 , 7 .
10 - OPD – 20 : Amplified band with m.w 500 bp in samples 1, 2, 4 , 5 , band with m.w 300 bp in samples 4 , 5 showed in fig. (3–6), band with m.w 1000 bp in samples 4 , 5 . Absence of amplified bands in samples 3 , 6 , 7 .

By using (10) ten primers (OPA-01, OPA-02, OPA-03, OPA-13, OPC-05, OPC-10, OPC-12, OPB-12, OPE-07, OPD-20), the recently results explain wide variety in genetic material (DNA) of tested hydatid cysts samples seen as a various number of amplified bands or fluorescence intensity of bands and their molecular weights . So, the (RAPD) can be applied to differentiate between these samples . The differences between the number of amplified bands may be due to difference in loci to which the primers were bind or number of loci on same genome may be as a result of mutation included in genetic material as deletion, insertion, replacement or inversion of one or more nucleotides of hydatid cyst DNA nucleotide sequences (Lahmar *et al.,* 2007) . Difference in molecular weight which appears through using of these primers this may reflex the differences in the distance between loci on the template DNA of hydatid cysts in different samples with which primer's complimentary nucleotide sequences is binding (Rinaldi *et al.,* 2008), also by using this technique (PCR) we determine the fingerprinting to certain samples as in case of using primer (OPA-01) the amplified band with m.w 400 bp was found in sample 1 only which is represent human liver hydatid cyst at age group 10 – 20 years old and not found in other samples known as marker band so that consider as fingerprinting and can be used to detect this sample by

using this primer and this result also reported by (Lavikainen *et al.,* 2003) , also amplified band with m.w 1000 - 1050 bp in case of sheep liver hydatid cyst doesn't find in other samples so that consider as fingerprinting to facilitate detection of sheep hydatid cyst by using this primer and this result doesn't agree with Mrad *et al.,* (2005). In case of using primer (OPC – 10) marker band with m.w 1000 bp in case of sheep sample and don't appear in other samples so that consider as fingerprinting to detect sheep liver hydatid cyst by using this primer also reported by Kamenetzky *et al.,* (2002) . Also, by using primer (OPE - 7) amplified band with m.w 900 – 1000 bp appear in case of goat liver hydatid cyst only as marker band can consider as fingerprinting specified to this sample also reported by Saarma *et al.,* (2009) . Added to that by using primer (OPC – 05) amplified band with m.w 400 bp appear as marker band which consider fingerprinting to detect spleen hydatid cyst of human . From previous results we can see sample number 3 which represent DNA of human spleen hydatid cyst give positive result only by using primer (OPC – 05) and don't amplify by using other primers that may give a suggestion that the hydatid cyst strain infect spleen differ from other strains that infect liver and lung in human, this finding is reported for the first time . While many similarities showed in the molecular weights to the amplified bands in samples of (1) liver and (2) lung in human that may explain the relationship between the effected strain of hydatid cyst as reported by (Spicher *et al.,* 2008) .

In this study we observed the uncompleted amplification process to the DNA of cattle and buffaloes hydatid cysts samples and didn't see the amplified bands through using different tenth primers due to incompatibility between primers and DNA nucleotides sequences, this may be due to differences in *E. granulosus* strains in this study compared with other previous studies in different our country regions as evidenced by many researchers as Al-Rubaei, (2005) and Al-Qadhi, (2005) in study on cattle and sheep in south, middle, east, and north of Iraq by using different

primers and the results obtained ensure the differences in genetic material of hydatid cysts strains even in same species of intermediate hosts . This may be due to differences in strains and sub strains of adult stage (*E. granulosus*) and may be according to geographical distribution of *E. granulosus* isolates , passage infections from other countries or because occurring of the infection by the final hosts (chiefly stray dogs) which infected with more than one strain and sub strains of *E. granulosus* may be due to getting different food sources (imported freezing meat and viscera as liver) from different world regions in case of infected meat or liver with unobserved hydatid cysts (undiagnosed by veterinarian) and this phenomenon widely spread in Arab-Gulf countries (Saul *et al.,* 2008) .

Also these genetic variances may be due to genetic variation in same hydatid cyst with daughter cysts or its protoscolieces which may be resulted from mutation by physical agents as X- rays, chemical agents as different anthelminthic drugs or any other mutagenic agents lead to alteration in genetic material to the offspring (Dopchiz, 2006) .

In this study differences in the number of amplified bands through using primers, such as in case of OPA – 01 primer there are 1–5 bands, led to a conclusion that don't found any relationship between primer content of G ≡ C and this result is in agreement with Ahmed, (1999) who explain there isn't any relation ship with primer content of G ≡ C and disagree with Christofi *et al.,* (2002) who explain the efficiency of primer in RAPD increase with increasing of G ≡ C ratio due to presence of 3 hydrogen bonds compared with 2 hydrogen bonds between A=T therefore, the binding become more strength between the primer and complementary loci in template DNA and when the number of amplified bands depend on the number of binding loci this lead to increasing in amplified bands. And unobserved relationship between G ≡ C content and the primer's efficiency in this study may be due to the tough binding of primer don't lead to

increase in the number of binding loci which are constant in certain species (Kilani and Al- Hammami, 2002).

Number of these primers as OPA–02, OPA–03, OPA–13, OPB–12, OPC–05, OPC–12 couldn't recognize or detect the fingerprinting of studied samples of DNA and this result disagree with Torgerson *et al.,* (2002) who ensure presence of genetic variances between species and sub species of hydatid cysts by using more advanced technique, but there are different number of amplified bands having the same molecular weight among some of studied samples can be used to resist unfavorable environmental conditions and presence of these bands make to us the RAPD more suitable from other techniques to study other genetic relationships which based on presence of these bands and this result is in agreement with Leder and Weller (2003) .

Other applications for PCR in the world ensure the environmental source in CE transmission by examination of soil samples through using specific primer for sheep strain (G1) and this positive result explain why children suffering from CE without contact with risk factors and why epidemiologic studies that have failed to detect an association with dog ownership or contact as a risk factor for developing CE (Cringoli, 2008) .

1 2 3 4 5 6 7 L

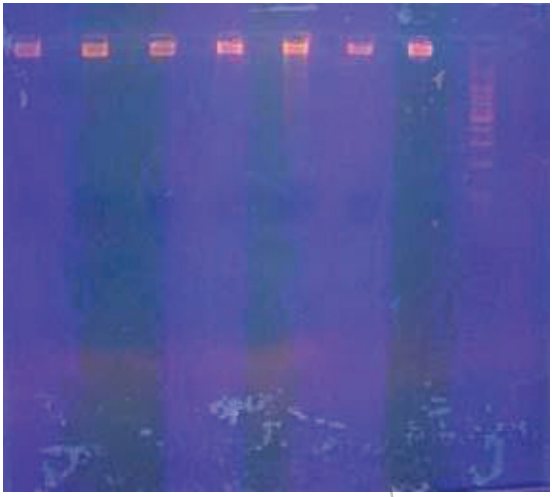

Fig.(3.1) explain Electrophoresis to the end products of DNA extraction to the following samples:

1 : Human liver hydatid cyst at age group (10-20) years old

2 : Human Lung hydatid cyst at age group (20-30) years old

3 : Human Spleen hydatid cyst at age group (30-40) years old

4 : Sheep Liver hydatid cyst .

5 : Goat Liver hydatid cyst .

6 : Cow Liver hydatid cyst .

7 : Buffaloes Liver hydatid cyst .

L: Ladder .

OPC – 05 OPE – 07

L 1 2 3 4 5 6 7 1 2 3 4 5 6 7

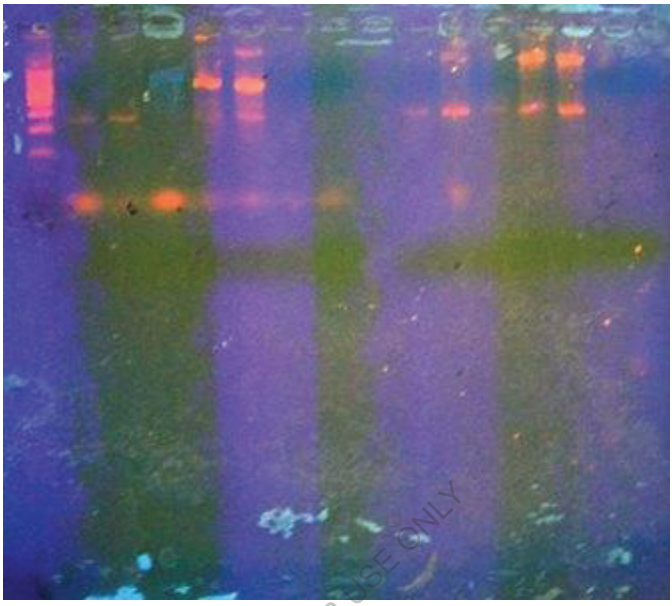

Fig.(3.2)Explain Electrophorosis to the PCR end products by using OPE – 07 and OPC – 05

1 : Human liver hydatid cyst at age group (10-20) years old .

2 : Human Lung hydatid cyst at age group (20-30) years old .

3 : Human Spleen hydatid cyst at age group (30-40) years old .

4 : Sheep Liver hydatid cyst .

5 : Goat Liver hydatid cyst .

6 : Cow Liver hydatid cyst .

7 : Buffaloes Liver hydatid cyst .

L : Ladder .

OPA – 01 OPC – 10

L 1 2 3 4 5 6 7 1 2 3 4 5 6 7

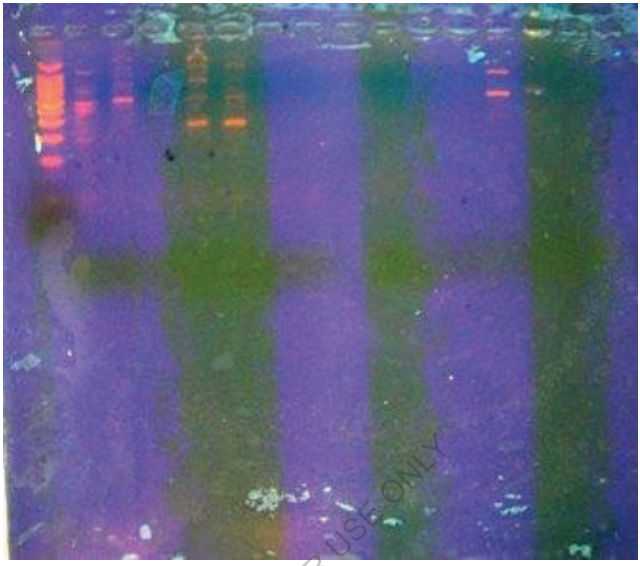

Fig.(3.3)Explain Electrophorosis to the PCR end products by using OPA – 01 and OPC – 10

1 : Human liver hydatid cyst at age group (10-20)years old

2 : Human Lung hydatid cyst at age group (20-30)years old

3 : Human Spleen hydatid cyst age group (30-40)years old

4 : Sheep Liver hydatid cyst

5 : Goat Liver hydatid cyst

6 : Cow Liver hydatid cyst

7 : Buffaloes Liver hydatid cyst

L : Ladder

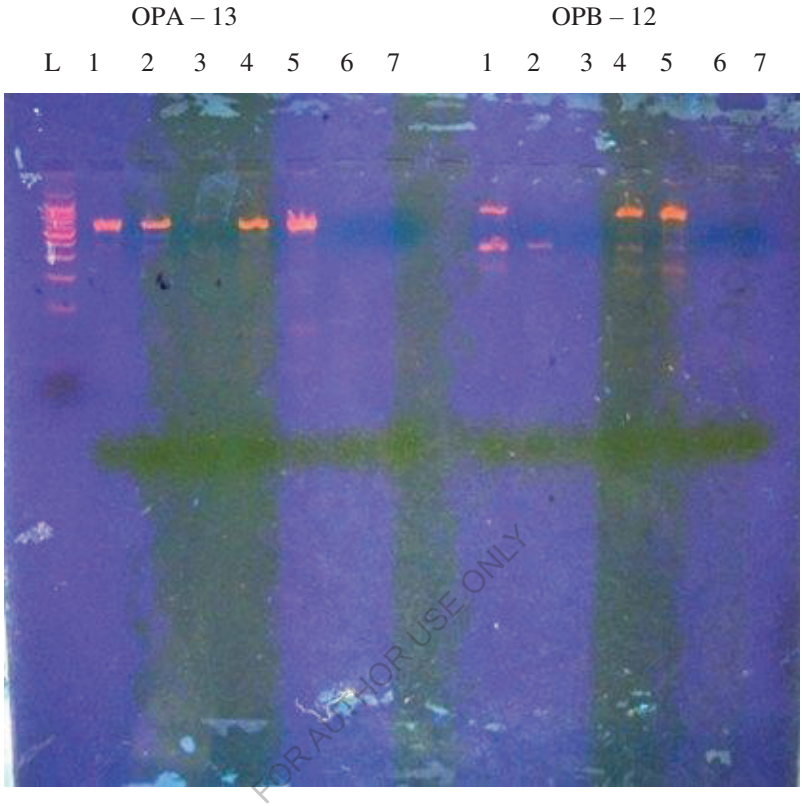

Fig.(3.4)Explain Electrophorosis to the PCR end products by using OPA – 13 and OPB – 12

1 : Human liver hydatid cyst at age group (10-20)years old .

2 : Human Lung hydatid cyst at age group (20-30)years old .

3 : Human Spleen hydatid cyst at age group (30-40)years old .

4 : Sheep Liver hydatid cyst .

5 : Goat Liver hydatid cyst .

6 : Cow Liver hydatid cyst .

7 : Buffaloes Liver hydatid cyst .

L : Ladder .

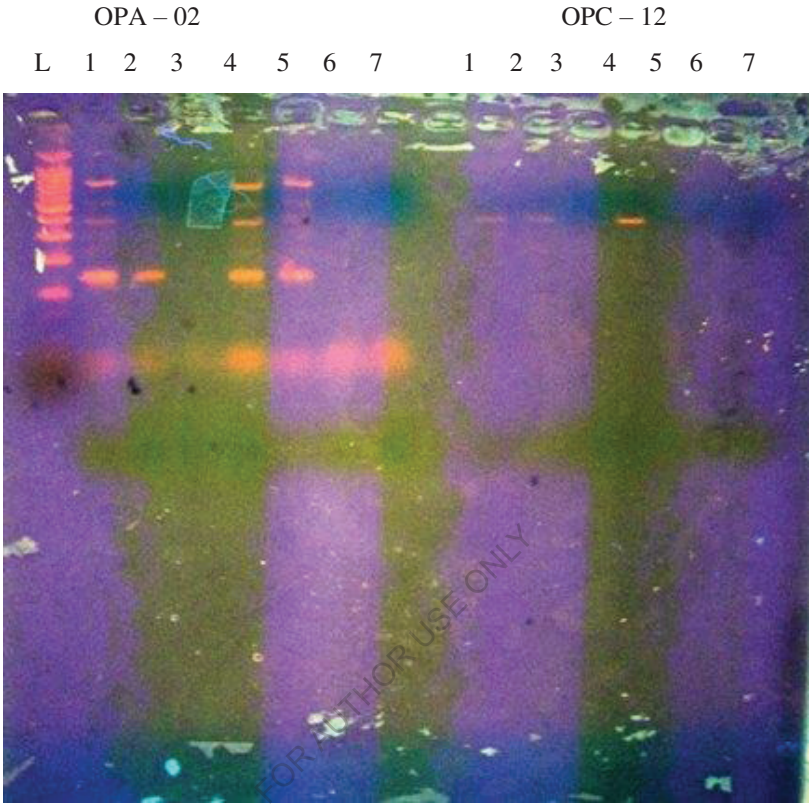

Fig.(3.5)Explain Electrophorosis to the PCR end products by using OPA – 02 and OPC – 12

1 : Human liver hydatid cyst at age group (10-20) years old .

2 : Human Lung hydatid cyst at age group (20-30) years old .

3 : Human Spleen hydatid cyst at age group (30-40) years old .

4 : Sheep Liver hydatid cyst .

5 : Goat Liver hydatid cyst .

6 : Cow Liver hydatid cyst .

7 : Buffaloes Liver hydatid cyst .

L : Ladder .

OPA – 03 OPD – 20

L 1 2 3 4 5 6 7 1 2 3 4 5 6 7

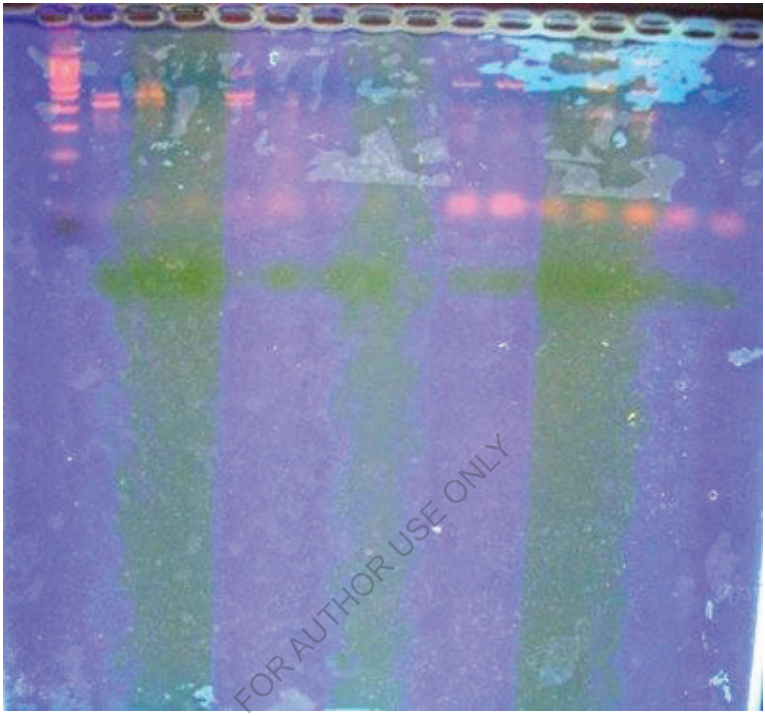

Fig.(3.6)Explain Electrophorosis to the PCR end products by using OPA– 03 and OPD – 20

1 : Human liver hydatid cyst at age group (10-20) years old .

2 : Human Lung hydatid cyst at age group (20-30) years old .

3 : Human Spleen hydatid cyst at age group (30-40) years old .

4 : Sheep Liver hydatid cyst .

5 : Goat Liver hydatid cyst .

6 : Cow Liver hydatid cyst .

7 : Buffaloes Liver hydatid cyst .

L : Ladder .

3 – 3 Immunological study

3–3–1 Measuring of Immunoglobulins IgG, IgM and components of complement C3 and C4 :

Echinococcus infections are among the more dangerous helminthic diseases in human . This disease is usually diagnosed by clinical examinations using different imaging techniques, which are supported by the demonstration of specific serum antibodies . The serological diagnosis is a routine laboratory test depends mainly on the detection of immunoglobulin class G (IgG) antibodies directed against different antigens of *E. granulosus* (Bardonnet *et al.,* 2003) . In this study sera of 30 patients with hydatidosis were taken and the results of analysis of immunoglobulins IgG and IgM and components of complement C3 and C4 concentrations (con.) explained in table (3 – 5) . Statistically highly significant difference ($P < 0.001$) to the IgG con. (compared with normal values in enclosed reference tables which founded in Index) in 1st age group of the males compared with another age groups observed in table (3 – 6) and evidenced by fig. (3 – 7) as we see significant increase in IgG con. among age group between (10 – 20) years and this result supported by Carmena *et al.,* (2006) that found greatly increasing in IgG con. through analysis sera of infected younger ages of males with hydatidosis in study included 560 patients with hydatidosis in case-control study at 2005–2006 . While didn't see any considerable difference between second and third age groups ; also, there isn't any significant difference to the IgG con. among female at all age groups ($P > 0.001$) . Also, there was significant difference at level ($P < 0.05$) to the IgG con. between both sexes at all age groups as explained in table (3 – 7) and evidenced by fig. (3 – 8) which appear important increasing in IgG con. in male compared with females as founded by (Filice *et al.,* 2000) . IgG con. in case of liver hydatidosis in males (m ± s.e.m 147.75±8.35) was more than female liver hydatidosis which is (m ± s.e.m 137.20±5.97) and in case of lung hydatidosis in male

(m ± s.e.m 526±64.96) also more than lung hydatidosis in females (m ± s.e.m 135±29.61) and in case of ovarian hydatid cyst IgG con. which (m ± s.e.m 166±13.13) and these results approximately corresponded with (Junghanass *et al.,* 2008) .

IgM con. also measured in this study which appear significant difference among 2nd age group of males compared with 1st and 3rd groups ($P < 0.001$) table (3 – 8), there was increasing in IgM con. in second age group (20 – 30) years also there was highly increasing in IgM con. in ages between (40 > more) as evidenced by fig. (3 - 9) . There was significant differences ($P < 0.05$) in the IgM con. Between 1st age group in both sexes table (3 – 9), highly increasing in IgM in case of female hydatidosis fig. (3 – 10) as we measured female liver hydatidosis (m ± s.e.m 212.36±21.38), lung hydatidosis (m ± s.e.m 125.30±45.31), in many cases of lung hydatidosis show high levels of IgM which related with recently infections or cysts with many daughter cysts, ovarian hydatidosis (m ± s.e.m 227.90±23.41) in compared with male liver and lung hydatidosis(m ± s.e.m 146.87±50.91)(m ± s.e.m 93.05±20.90) respectively ; other ages don't have significant differences also all female age groups . For instance, few cases of human lung hydatidosis tend to be associated with lower serum antibody levels or not detected in others this also reported by (Unsal *et al.,* 2001) .

The immunological mechanisms underlying undetectable or absent humoral response remain undefined . Among the possible causes of negative serological response are the number , site , integrity and morphology of hydatid cyst , high concentration of circulating immune complexes in hydatid disease , has been documented by previous work (Pavlov *et al.,* 2006) . Thus rendering antibodies unavailable for detection, also the possibility of antigen induced specific immunological tolerance has also been raised . Such complexes in the serum of Hydatid cyst patients may cause false negative reactions in serological tests with

clinically and surgically confirmed disease .This result may be due to the fact, that the immune response in large cyst is weak or completely absent because it has a thick fibrous capsule , which may prevent the release of antigens (Petrov *et al.,* 2001) .

Elements of complement system C3 and C4, there's significant differences at level ($P < 0.05$) in con. of C3 between male 1st age group (10 – 20) years old with 2nd (20 – 30) and 3rd (30 – 40) groups explained in table (3–10) and evidenced by fig. (3 – 11), also there was significant importance at level ($P < 0.05$) to the C3 con. to the 1st group to both sexes table (3–11) . No significance relation to C3 con. among female age groups , increasing in concentration of C3 in female is explained in fig (3–12) . Results obtained in this experiment indicate to significant difference of C4 con. at level ($P < 0.05$) to the 1st and 3rd age groups of males and 1st group to both sexes table (3–12) i.e. there was increasing of C4 in ages between (30 – 40) years old fig. (3–13) and don't observe any significance importance in concentration between female age groups and in case of age sex interference table (3–13), increasing in concentration of C4 in female was explained in fig (3–14) .

Table (3–5) Means of immunoglobulins IgG and IgM and components of complement C3and C4 mg/dl in sera of patients with hydatidosis in different age groups

Age groups / Means	IgG mg/dl	IgM mg/dl	C3 mg/dl	C4 mg/dl
1 (10 – 20)	305	170.62	114.8	19.43
2 (20 – 30)	154.22	193.62	140.3	27.96
3 (30 – 40)	146.5	174.63	148.52	29.41
4 (more than 40)	116	204.3	133.2	21.06

Table (3 – 6) Mean concentration of IgG mg/dl in sera of patients with hydatidosis with statistically comparison of various age groups.

Age (I)	Age (II)	Mean	Standard error	Sig.
1.00	2.00	150.77*	24.41	.000
	3.00	158.50*	25.20	.000
	4.00	189.00*	34.97	.000
2.00	1.00	-150.77*	24.41	.000
	3.00	7.72	25.81	.768
	4.00	38.22	35.42	.292
3.00	1.00	-158.50*	25.20	.000
	2.00	-7.72	25.81	.768
	4.00	-30.50	35.97	.405
4.00	1.00	-189.00*	31.97	.000
	2.00	-38.22	35.42	.292
	3.00	-30.50	35.97	.405

Table (3 – 7) Statistical analysis for relationships between Age and Sex with IgG con. mg/dl of patients with hydatidosis

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	499792.400	6	83298.733	29.504	.000
Intercept	697115.377	1	697115.377	246.912	.000
GENDER	59498.089	1	59498.089	21.074	.000
AGE	200074.083	3	66691.361	23.621	.000
GENDER * AGE	176274.439	2	88137.220	31.217	.000
Error	64936.800	23	2823.339		
Total	1747988.000	30			
Corrected Total	564729.200	29			

Table (3 – 8) Mean concentration of IgM mg/dl in sera of patients with hydatidosis with statistically comparison of various age groups.

Age (I)	Age (II)	Mean	Standard error	Sig.
1.00	2.00	-23.00	35.37	.522
	3.00	-3.42	36.51	.926
	4.00	-69.01	50.67	.186
2.00	1.00	23.00	35.37	.522
	3.00	19.58	37.40	.606
	4.00	-46.01	51.32	.379
3.00	1.00	3.42	36.51	.926
	2.00	-19.58	37.40	.906
	4.00	-65.60	52.11	.221
4.00	1.00	69.01	50.67	.186
	2.00	46.01	51.32	.379
	3.00	65.60	52.11	.221

Table (3 – 9) Statistical analysis for relationships between Age and Sex with IgM con. mg/dl of patients with hydatidosis

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	65215.658	6	10869.276	1.834	.137
Intercept	575784.534	1	575784.534	97.162	.000
GENDER	16498.731	1	16498.731	2.784	.109
AGE	8271.076	3	2757.025	.465	.709
GENDER * AGE	15327.809	2	7663.905	1.293	.294
Error	136298.149	23	5926.006		
Total	1231967.140	30			
Corrected Total	201513.807	29			

Table (3 – 10) Mean concentration of C3 mg/dl in sera of patients with hydatidosis with statistically comparison of various age groups .

Age (I)	Age (II)	Mean	Standard error	Sig.
1.00	2.00	-25.50	21.28	.243
	3.00	-33.72	21.97	.138
	400	-18.40	30.49	.552
2.00	1.00	25.50	21.28	.243
	3.00	-8.22	22.51	.718
	4.00	7.10	30.88	.820
3.00	1.00	33.72	21.97	.138
	2.00	8.22	22.51	.718
	4.00	15.32	31.36	.630
4.00	1.00	18.40	30.46	.552
	2.00	-7.10	30.88	.820
	3.00	-15.32	31.36	.630

Table (3 – 11) Statistical analysis for relationships between Age and Sex with C3 con. mg/dl of patients with hydatidosis .

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	12140.862	6	2023.477	.943	.484
Intercept	320932.743	1	320932.743	149.535	.000
GENDER	138.064	1	138.064	.064	.802
AGE	8313.293	3	2771.098	1.291	.301
GENDER * AGE	4683.491	2	2341.745	1.091	.353
Error	49362.679	23	2146.203		
Total	594436.950	30			
Corrected Total	61503.542	29			

Table (3 – 12) Mean concentration of C4 mg/dl in sera of patients with hydatidosis with statistically comparison of various age groups .

Age (I)	Age (II)	Mean	Standard error	Sig.
1.00	2.00	-8.97	5.62	.124
	3.00	-10.42	5.80	.086
	4.00	-2.07	8.05	.799
2.00	1.00	8.97	5.62	.124
	3.00	-1.44	5.94	.810
	4.00	6.90	8.15	.406
3.00	1.00	10.42	5.80	.086
	2.00	1.44	5.94	.810
	4.00	8.34	8.28	.324
4.00	1.00	2.07	8.05	.799
	2.00	-6.90	8.15	.406
	3.00	-8.34	8.28	.324

Table (3 – 13) Statistical analysis for relationships between Age and Sex with C4 con. mg/dl of patients with hydatidosis

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	1115.290	6	185.882	1.242	.322
Intercept	8611.966	1	8611.966	57.542	.000
GENDER	342.490	1	342.490	2.288	.144
AGE	640.714	3	213.571	1.427	.261
GENDER * AGE	82.706	2	41.353	.276	.761
Error	3442.253	23	149.663		
Total	22815.810	30			
Corrected Total	4557.543	29			

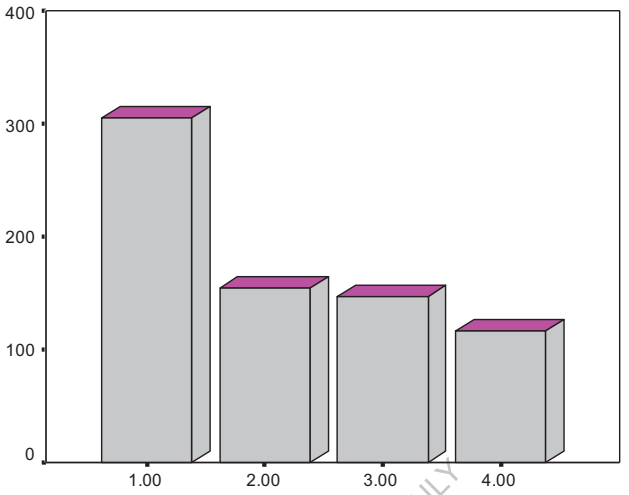

Fig.(3-7) con. of IgG in various age groups of patients with hydatidosis

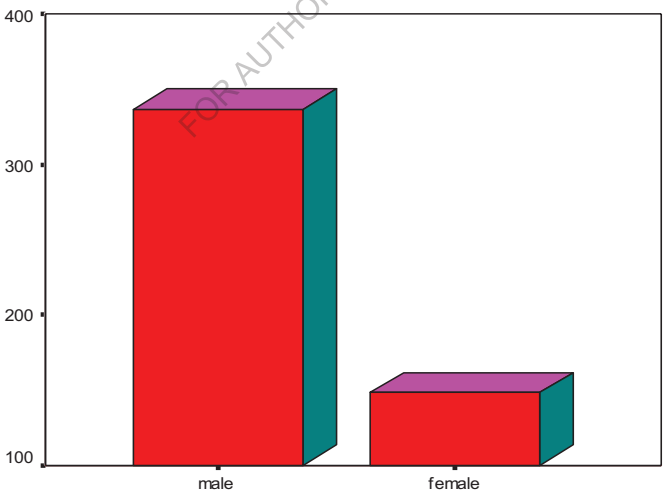

Fig.(3-8) con. of IgG according to sex of patients with hydatidosis

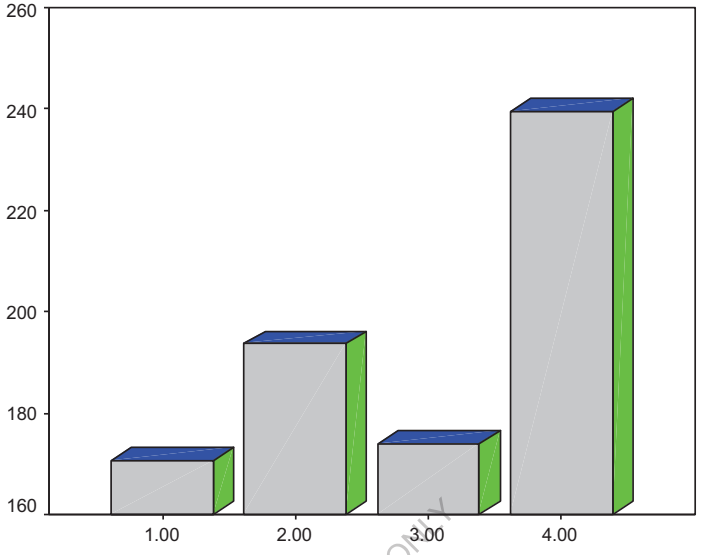

Fig.(3-9) con. of IgM in various age groups of patients with hydatidosis

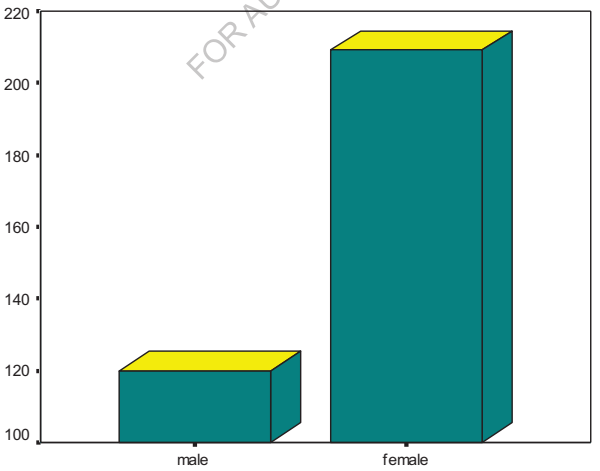

Fig.(3-10) con. of IgM according to sex of patients with hydatidosis

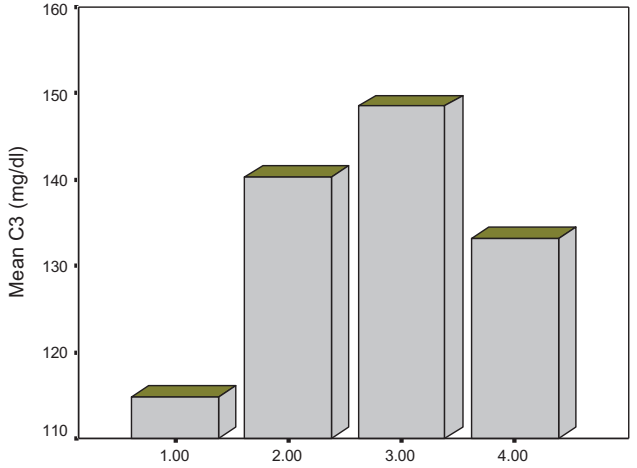

fig.(3-11)con.of C3 in various age groups of patients with hydatidosis

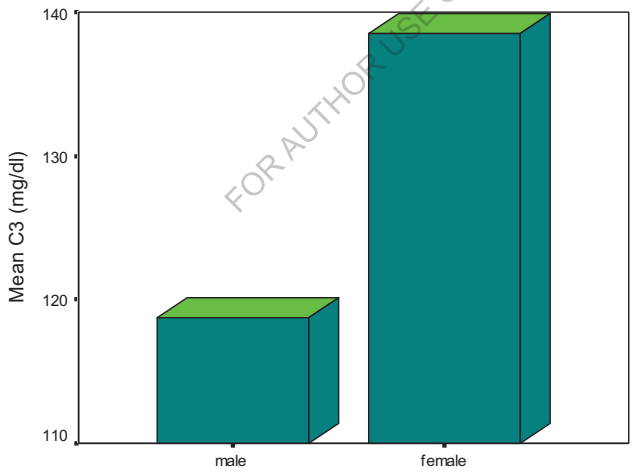

Fig.(3-12) con. of C3 according to sex of patients with hydatidosis

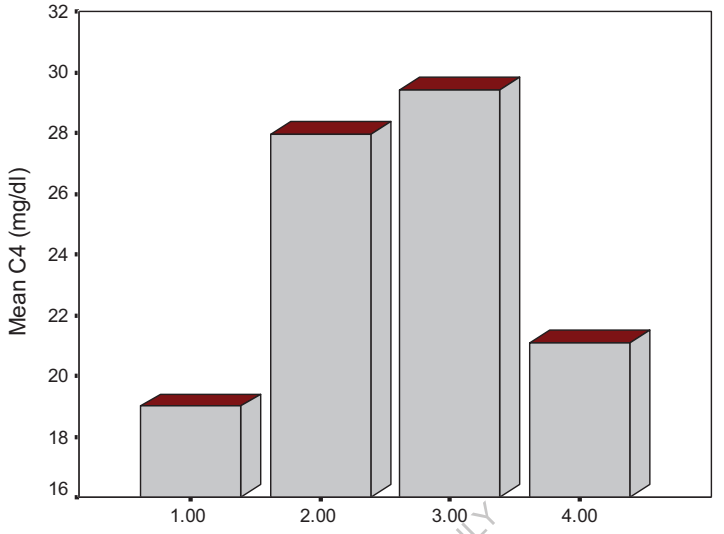

Fig.(3-13)con.of C4 in various age groups of patients with hydatidosis

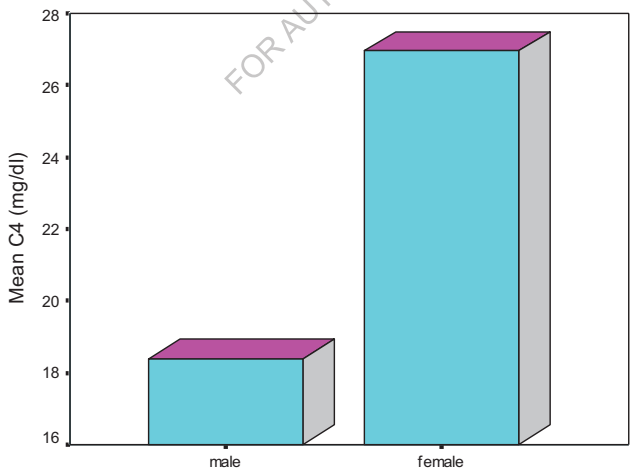

Fig.(3-14) con. of C4 according to sex of patients with hydatidosis

CONCLUSIONS

1. The incidence of the hydatidosis in the females was higher than males . The highest incidence of hydatidosis was in the second and third decade of life . The majority of the studied cases were diagnosed surgically . The number of liver hydatidosis was higher than other sites .
2. RAPD was used successfully in determination the genetic variances between Human, Sheep, Goat , Cattle and Buffaloes samples, this findings was reported as a first time of the similar studies .
3. Determination the fingerprinting of hydatid cysts to human, sheep and goat samples depending on marker bands or unique pattern of bands .
4. By using OPA – 01 primer lead to get on the fingerprinting to detect human liver hydatid cyst .
5. By using OPC – 10 lead to get on the fingerprinting to detect Sheep liver hydatid cyst .
6. By using OPC – 05 lead to get on the fingerprinting to detect human spleen hydatid cyst .
7. By using OPE– 07 lead to get on the fingerprinting to detect Goat liver hydatid cyst .
8. By using all 10 primers the amplification process to the DNA samples of cattle and buffaloes didn't complete .
9. By using OPA – 02, OPA – 03, OPA – 13, OPB – 12, OPC – 12, OPD – 20 primers couldn't detect fingerprinting to samples but they are produce monomorphic bands which are useful in study of other genetic relationships between these samples .
10. There was significant relationship between the age of patients with IgG and IgM concentrations .

11. There was significant relationship between the gender of patients with IgG and IgM concentrations .
12. There was significant relationship between the gender of patients with C3 and C4 concentrations .
13. There was significant relationship between the age of patients and fertility of hydatid cyst. .

RECOMMENDATIONS

1. Further studies by using fingerprinting results for early and rapid detection of this disease in various parts of our country to control and resist this disease .
2. More advanced studies by using other primers and techniques to the cattle and buffaloes DNA to determine the fingerprinting to facilitate detection of hydatid cyst strains .
3. Immunological or serological tests (PCR) should be distributed in hospitals laboratories as an aid for proper diagnosis of the disease.
4. Determination the levels of IgE and other lymphokines and cytokines in hydatidosis patients .
5. Further studies would focus precisely when and how CE antigens modulated the immune system .
6. genotyping of *E. granulosus* in Iraq by using PCR and other advanced techniques .

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