



Full Length Research paper

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

E. Ihsan Al-Saimary^{1*}, N. Maani Al-Shemari¹ and M. A. Mohammed Al-Fayadh²

¹Department of Microbiology, College of Medicine, University of Basrah, Iraq.

²Department of Microbiology, College of Medicine, University of Al-Qadysia, Iraq.

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This research is a study on the molecular level of *Echinococcus granulosus* (larval stage) on, where 7 samples of hydatid cysts were collected from parasite intermediate hosts: Human (liver, spleen, lung) and liver of sheep, goat, cattle and buffaloes. 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 were used during which the genetic variations were revealed among isolated (extracted DNA) hydatid cysts which was collected from human and other intermediate hosts except cows and buffaloes. The results of this study showed the following: 1. One primer (OPA – 01) was able to diagnose sample 1 which represent the isolated DNA of liver hydatid cyst 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. The ability of primer OPE – 07 to determine fingerprinting of DNA sample of Goat liver hydatid cyst; 5. Amplification process to the DNA samples extracted from cows and buffaloes liver hydatid cysts was not completed by using all 10 primers.

Keywords: Hydatidosis, *Echinococcus granulosus*, polymerase chain reaction (PCR).

INTRODUCTION

Cystic echinococcosis is a cosmopolitan, hyper endemic zoonotic disease caused by infection with metacestode (larval stage) of the tape worm *Echinococcus granulosus*. It is one of the most important parasitic diseases in under developed countries, especially rural communities, where man is in close contact with dogs (definitive hosts) and various domestic animals which act as Intermediate hosts (Nepalia et al., 2006). Hydatid cyst is considered to be a major public health problem that can cause severe morbidity in human; as a result, economic losses occur for individual, family and society. In addition, echinococcosis infects wide range of livestock which lead to further economic losses (Taherkhani and Rogan, 2000). Hydatidosis is diagnosed by different ways using X-ray, CT scan, other immunological and serological tests including modern technique polymerase chain

reaction (PCR), which have high sensitivity and specificity in detection of hydatidosis infection. PCR is also used in genotyping *E. granulosus* to facilitate treatment and vaccination. Also, PCR purification of soluble protein of whole parasite body gives 100% protection (Leder and Weller, 2003). PCR diagnostic antigen (EgP-29) cloned from *E. granulosus* and expressed in *Escherichia coli* encode protein gives protection of 96.6% to prevent secondary hydatidosis against different geographical isolates (Bartlett, 2003). Mitochondrial Cytochrome Oxidase sub unit 1(CO 1) and NADH dehydrogenase use PCR to determine the strains and sub strains of *E. granulosus* (G1-G10) to facilitate control (Stefaniak, 1997). Other applications for PCR in the world ensure the environmental source in CE transmission by examination of soil samples using specific primer for sheep strain (G1) and this positive result explains why children suffer from CE without contact with risk factors and why epidemiologic studies have failed to detect an association with dog ownership or contact as a risk factor for

*Corresponding author. E-mail: ihsanalsaimary@yahoo.com.

Table 1. Random primers and their sequences.

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

developing CE (Dowling and Torgersson, 2000). In this study, PCR is used of to determine the genetic variations between samples taken from different intermediate hosts (human, sheep, cow and buffaloes) and the fingerprinting to each sample was examined using different random primers.

MATERIALS AND METHODS

Patients and sampling

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 2009 to February 2010. The cysts were in the liver, lung, ovary and spleen and the samples of hydatid cysts from animal were gotten from slaughterhouses. During the study period, 30 cases of space occupying lesions were admitted. Among these cases, 14 patients were found suffering from liver hydatidosis, 7 patients with lung hydatidosis, 6 female with ovarian hydatidosis and other different organs. The diagnosis of patients was confirmed by serological tests 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). The materials used in this study as followed:

1. Go Taq Green master max, 2x (pH 8.5) promega Co.
2. Nuclease free water promega Co.
3. 100 (bp) DNA ladder promega Co.
4. Blue orange 6x loading dye promega Co.
5. DNA extraction solution consist of 100 µl of Tris – HCl , 10 ml of EDTA and 0.5 ml Tween 20, the volume is filled to 100 ml by adding 89.400 of D. w. All these solutions were sterilized by autoclave and kept in cooling state before usage (Al-Ghezi, 2008).
6. Random primers and their sequences (Table 1) used in this study provided was by Alpha DNA Co.

Isolation of germinal layer of hydatid cysts

Germinal layer of hydatid cyst from human was taken during surgical operation, and from different animals after slaughtering. The samples were taken to laboratory in clean containers, outer surface was sterilized using 70% ethanol and the hydatid fluid was Discarded. Germinal layer was kept in 70% ethanol at different

periods.

DNA extraction (Vicidomini, 2007)

By adding 250 µl of sample (thawed germinal layer by ultra sound sonicator high speed/10 min (Welch et al., 1990)) in specific Eppendorf tubes (1.5 ml) containing 1000 µl of PBS after centrifugation at 12000 rpm/10 min, the supernatant was removed leaving the sediment, this was repeated three times. 500 µl from extraction solution and 6 µl of proteinase – K were added to the remaining sediment. All tubes were incubated in water path (37°C) until the next day. After that, Proteinase - K action was inhibited by rising the degree of water path to 100°C (boiling degree) for 5 min and then samples were kept frozen by adding 50 µl of (TE) buffer until to be used in PCR reaction.

Gel electrophoresis of DNA

Agarose gel was prepared at concentration 1% by dissolving 0.5 gm of Agarose in 50 ml TBE buffer (1x) and then heating. Ethidium bromide stain solution 1 µl /50 ml Agarose was added. The heated Agarose solution was poured into the gel tray and allowed to cool at room temperature for 30 min. The comb was carefully removed from Agarose and mixed extracted DNA 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 it covered the surface of the gel. Ethidium bromide stain solution 1 µl was added to the electrophoresis chamber. Electrical current was connected to the electrophoresis chamber, while cathode was connected to the side of samples, at voltage (65 V) for 45 min. Finally, the gel was transported into U.V Trans -illuminator. 50 µl of TE buffer could be either added to crude DNA to keep it frozen for long periods or used directly in PCR technique in the following procedure: PCR kit (Green master mix, Primers, Nuclease free water, extracted DNA) and the constituents were put in ice container. New PCR tubes (0.5 ml) were labeled with number of sample for amplification reaction (located in ice). To avoid contamination, all solutions should be taken with separate clean tips under a septic condition. 5 µl of DNA sample, 2 µl of primer, 12.5 µl of Green master mix and 5.5 µl of Nuclease free water were added to the PCR tube to complete the volume to 25 µl. All tubes were closed, and the mixture was spun for 5 second by light vortex. The PCR tubes were transferred to preheated Thermocycler.

PCR program

The following steps in PCR (30 - 35) cycles, were done in an automated Thermocycler:

1. Denaturation at 94°C (60 s): The double strand helix melted to become single stranded DNA.
2. Annealing at 45 - 65°C for (60 s): Binding of primers to DNA strand, this temperature depend on type, length and G-C content of primer.
3. Extension at 72°C for (60 s): Taq DNA polymerase synthesized a new DNA strand complimentary to the DNA template by adding dNTPs in the direction, temperature differ according to DNA length (Weigand et al., 1993) (Table 2).

Gel electrophoresis was then made to all PCR tubes as in case of DNA extraction except 1 gm (instead of 0.5 mg) of Agarose which was dissolve in 50 ml of TBE (1x). These steps were applied in gel electrophoresis of extracted DNA and the results were observed by

Table 2. Steps of PCR technique (Yang, 2005).

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

ultraviolet light.

RESULTS AND DISCUSSION

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 and also from Sheep, Goats, Cattle and Buffaloes tested by (10) ten primers provided by Operon technologies Co. These include 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 correspond to other previous tests (Williams et al., 1990; Al- Rubaie, 2005; 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 cystic hydatid disease in intermediate hosts and possibly get the fingerprinting to these samples. Results obtained from using these primers in (RAPD) reactions led to difference in their production of amplified bands which differ in number and its molecular weights resulted from differences in complementary loci on the genome of each sample. This reflects the genetic variance between these samples and this finding is well documented by Bart et al. (2006), Busi et al. (2007).

Analysis results of (RAPD)

The results obtained from new studies in numerate and expression of amplified bands were used to determine the genetic varieties on the Agarose gel of obtained samples and know the fingerprinting between them (Carmena et al., 2008; Andresiuk, 2009). Fingerprinting depending on scientific researches in studied genome was represented either by presence of specific band in one sample which are not found in others or presence of unique pattern of bands in one sample different from the others (Dengri et al., 2002). Both genetic variance and fingerprinting depend on presence or non presence of amplification while molecular weight of bands depends on the number of complementary loci to primer's sequences on the template DNA (Dopchiz, 2009). In this study, three

human samples from liver cysts at age groups (10 - 20 , 20 - 30 and 30 - 40 years old) ; Sheep ; Goat ; Cattle and Buffaloes liver hydatid cysts which have numbers 1, 2, 3, 4, 5, 6 and 7 respectively (Figure 1) were used and the results are as follow:

1. - OPA – 01: Many amplified bands differ in molecular weights (m.w) 200 – 1050 bp, 3 bands in sample 1 ; 1 band in sample 2 ; 4 bands in sample 4 and 3 bands in sample 5. On the other hand, there was disappearance of amplified bands in samples 3, 6, 7 (Figure 3).
2. - OPA – 02: Two amplified bands in sample 1 ; 1 band in sample 2 ; 4 bands in samples 4 and 5 with m.w 100 - 800 bp. There was also disappearance of amplified bands in samples 3, 6, 7 (Figure 5).
3. - OPA – 03: There were two amplified bands with m.w 300 - 400 bp which appeared in samples 1, 2, 4 and absence of bands in samples 3, 5, 6, 7 (Figure 6).
4. - OPA – 13: There was one amplified band with m.w 600 bp which appeared in samples 1, 2, 4, 5 and absence of bands in samples 3 , 6 , 7 (Figure 4).
5. - OPB – 12: There was one amplified band with m.w 300 - 400 bp which appeared in samples 1, 2, band with m.w 900 bp in samples 1, 4, 5. There was absence of bands in samples 3, 6, 7 (Figure 4).
6. - OPC – 05: There was one amplified band with m.w 300 bp which appeared in samples 1, 2, 3, 4, 5, band with m.w 900 - 1000 bp in 2, 4, 5. There was absence of bands in samples 6, 7 (Figure 2).
7. - OPC – 10: There was absence of amplified bands in samples 1, 2, 3, 6, 7, band with m.w 500 bp in samples 4 and 5 and other amplified band with m.w 1000 bp in sample 4. There was absence of bands in samples 6, 7 (Figure 3).
8. - OPC – 12: There was one amplified band with m.w 400 bp in samples 1, 2, 4 and absence of amplified bands in samples 3, 5, 6, 7 (Figure 5).
9. - OPE – 07: There was 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 and band with m.w 900 - 1000 bp in sample 5. There was absence of amplified bands in samples 3, 6, 7 (Figure 2).
10. - OPD – 20: There was amplified band with m.w 500 bp in samples 1, 2, 4, 5, band with m.w 300 bp in samples 4, 5, and band with m.w 1000 bp in samples 4, 5. There was absence of amplified bands in samples 3, 6, 7 (Figure 6).



Figure 1. 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, and L: Ladder.

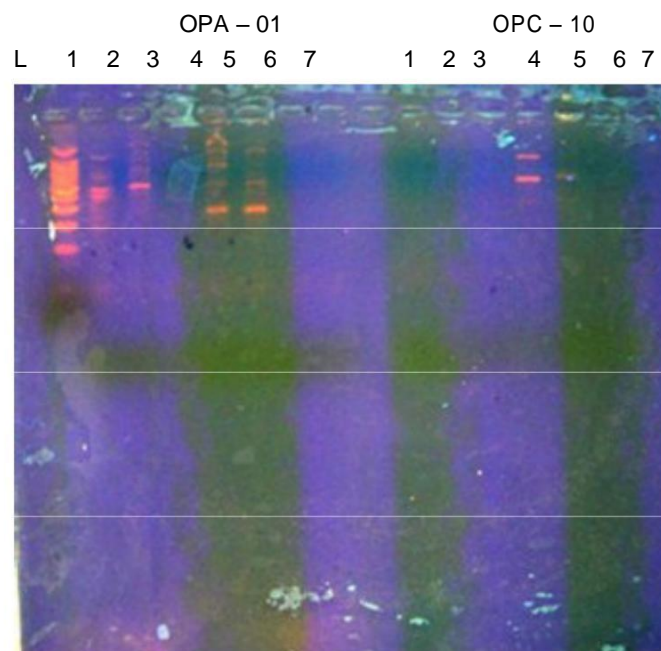


Figure 3. Electrophoresis to the PCR end products by using OPA-01 and OPC-10. 1: Human liver hydatid cyst at age group (10 - 20) years old; 2: Human lung hydatid cyst at age group (20 - 30) years old; 3: Human spleen hydatid cyst at age group (30 - 40) years old; 4: Sheep liver hydatid cyst; 5: Goat liver hydatid cyst; 6: Cow liver hydatid cyst; 7: Buffaloes liver hydatid cyst, and L: Ladder.

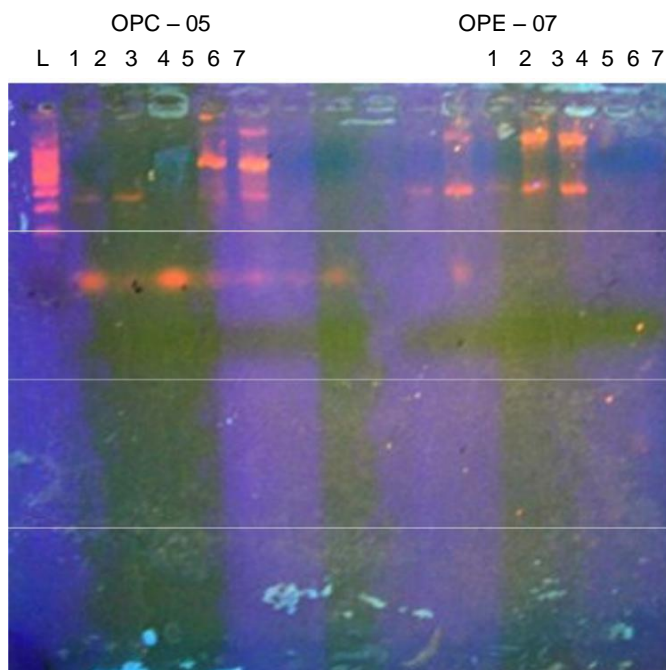


Figure 2. Electrophoresis to the end products by using OPE-07 and OPC-05. 1: Human liver hydatid cyst at age group (10 - 20) years old; 2: Human lung hydatid cyst at age group (20 - 30) years old; 3: Human spleen hydatid cyst at age group (30 - 40) years old; 4: Sheep liver hydatid cyst; 5: Goat liver hydatid cyst; 6: Cow liver hydatid cyst; 7: Buffaloes liver hydatid cyst, and L: Ladder.

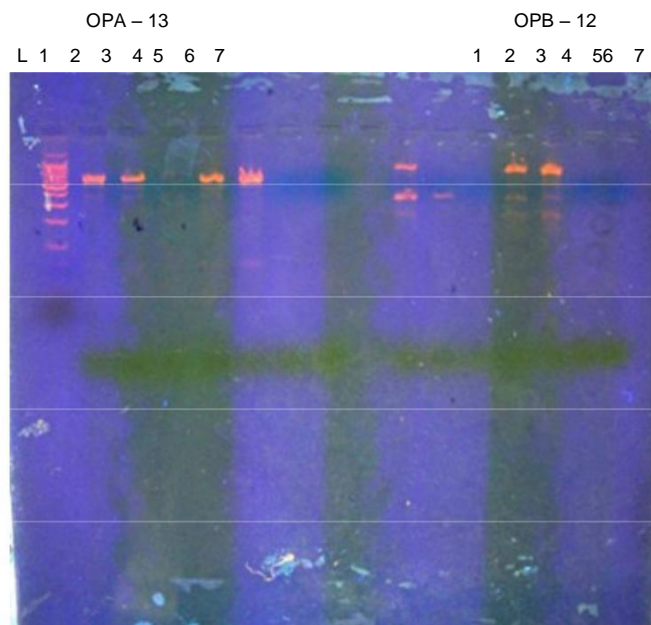


Figure 4. Electrophoresis to the PCR end products by using OPA-13 and OPB-12. 1: Human liver hydatid cyst at age group (10 - 20) years old; 2: Human lung hydatid cyst at age group (20 - 30) years old; 3: Human spleen hydatid cyst at age group (30 - 40) years old; 4: Sheep liver hydatid cyst; 5: Goat liver hydatid cyst; 6: Cow liver hydatid cyst; 7: Buffaloes liver hydatid cyst, and L: Ladder.

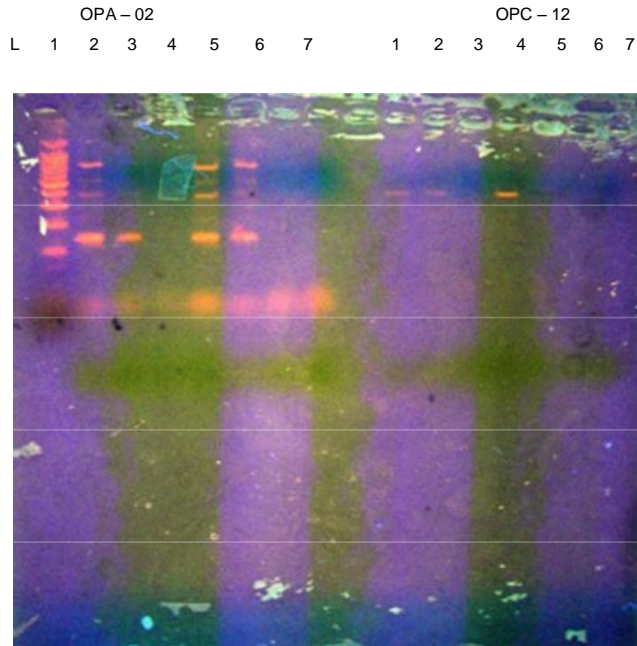


Figure 5. Electrophoresis to the PCR end products by using OPA-02 and OPC-1. 1: Human liver hydatid cyst at age group (10 - 20) years old; 2: Human lung hydatid cyst at age group (20 - 30) years old; 3: Human spleen hydatid cyst at age group (30 - 40) years old; 4: Sheep liver hydatid cyst; 5: Goat liver hydatid cyst; 6: Cow liver hydatid cyst; 7: Buffaloes liver hydatid cyst, and L: Ladder.

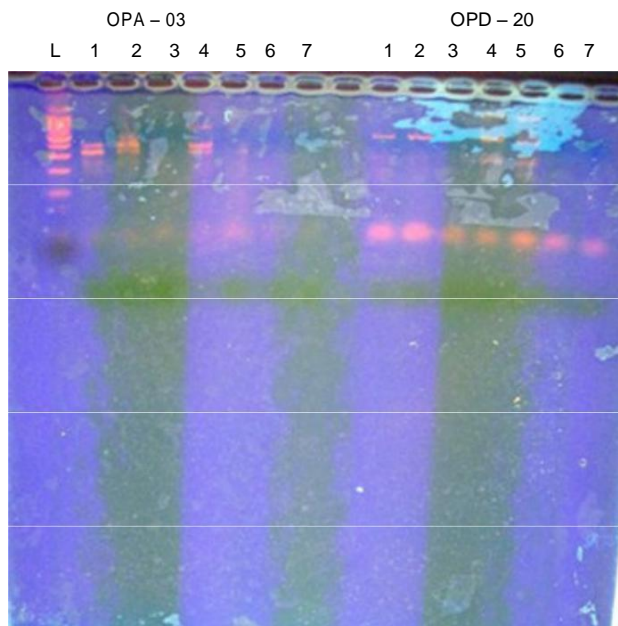


Figure 6. Electrophoresis to the PCR end products by using OPA-03 and OPD-20. 1: Human liver hydatid cyst at age group (10 - 20) years old; 2: Human lung hydatid cyst at age group (20 - 30) years old; 3: Human spleen hydatid cyst at age group (30 - 40) years old; 4: Sheep liver hydatid cyst; 5: Goat liver hydatid cyst; 6: Cow liver hydatid cyst; 7: Buffaloes liver hydatid cyst, and L: Ladder.

DISCUSSION

By using ten primers (OPA-01, OPA-02, OPA-03, OPA-13, OPC -05, OPC-10, OPC -12, OPB- 12, OPE-07, OPD-20), the recent results explain wide variety in genetic material (DNA) of tested hydatid cysts samples seen as a different number of amplified bands or fluorescence intensity of band (the last one was not considerable due to un exacted conditions) and their molecular weights. So, RAPD can be applied to differentiate between these samples. The differences between the numbers 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 the use of these primers may reflex the differences in the distance between loci on the template DNA of hydatid cysts in different samples with which primer's complementary nucleotide sequences is bound (Rinaldi et al., 2008). Also by using this technique (PCR), we determined the fingerprinting of certain samples by using primer (OPA-01), the amplified band with m.w 400 bp was found in sample 1 only which represent human liver hydatid cyst at age group 10 – 20 years old and not found in other samples known as marker band so that fingerprinting can be used to detect this sample by using this primer. This result is also reported by Lavikainen et al. (2003). Also amplified band with m.w 1000 - 1050 bp in case of sheep liver hydatid cyst were not found in other samples so that fingerprinting was considered to facilitate detection of sheep hydatid cyst by using this primer and this result does not agree with Mrad et al. (2005). Primer (OPC – 10) marker band with m.w 1000 bp of sheep sample did not appear in other samples, therefore, fingerprinting was considered to detect sheep liver hydatid cyst, and this was also reported by Kamenetzky et al. (2002).

Also, by using primer (OPE - 7) amplified band with m.w 900 – 1000 bp appeared in case of goat liver hydatid cyst only as marker band and fingerprinting can be considered as specified to this sample, and this was also reported by Saarma et al. (2009). Added to that, by using primer (OPC – 05) amplified band with m.w 400 bp appeared as marker band which considered fingerprinting to detect spleen hydatid cyst of human. From previous results we saw that sample 3, which represent DNA of human spleen hydatid cyst, gave positive result only by using primer (OPC – 05) and did not amplify by using other primers. This suggests that the hydatid cyst strain which effect spleen differ from other strains which effect liver and lung in human and this finding is being reported for the first time. Meanwhile we saw many similarities 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 did not see the amplified bands through using the different ten 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 other country regions as evidenced by many researchers as Al-Rubaei, (2005); Al-Qadhi, (2005) in the study on cattle and sheep in south, middle, east, and north of Iraq by using different primers and the results obtained showed 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*) according to geographical distribution of *E. granulosus* isolates, passage of infections from other countries or because of the infection occurring in the final hosts (chiefly stray dogs) which is infected with more than one strain and sub strains of *E. granulosus* as a result of getting food sources (imported freezing meat and viscera as liver) in case of infected meat or liver with unobserved hydatid cysts (undiagnosed by veterinarian) from different world regions and this phenomenon is 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 Protoscolices which may be as a result of mutation by physical agents as X-rays, chemical agents as different anthelmintic drugs or any other mutagenic agents which could 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 there was no relationship found between primer content of G C and this result is in agreement with Ahmed, (1999) and disagrees with Christofi et al. (2002) who explained that the efficiency of primer in RAPD increase with increase of G C ratio due to the presence of 3 hydrogen bonds compared with 2 hydrogen bonds between A=T. Therefore, the binding become stronger between the primer and complementary loci in template DNA and when the number of amplified bands depends on the number of binding loci this leads to increase 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 which did not lead to increase in the number of binding loci which are constant in certain species.

Number of these primers as OPA–02, OPA–03, OPA–13, OPB–12, OPC–05, OPC–12 could not recognize or detect the fingerprinting of studied samples of DNA and this result disagrees with Torgerson et al. (2002) who reported the presence of genetic variances between species and sub species of hydatid cysts by using more advanced technique although different number of amplified bands having the same molecular weight among some of

studied samples can be used to resist unfavorable environmental conditions. The presence of these bands makes RAPD more suitable from other techniques to study other genetic relationships and this result is in agreement with Leder and Weller (2003).

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