



## Molecular Characterization of Antigens Extracted From Hydatid Cysts of Human and Other Intermediate Hosts

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### ABSTRACT

*This research includes a study of E. granulosus (larval stage) on the molecular level, 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 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 hoists except Cows and Buffaloes .*

*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 cysts .*

*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.*

### 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 diseases in under developed countries specially rural communities, where man in close contact with the dogs (Definitive hosts) and various domestic animals which act as Intermediate hosts [1]. 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 [2]. Hydatidosis is diagnosed by different ways as 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 added to that used in genotyping of *E. granulosus* to facilitate treatment and vaccination . Also, by using Polymerase chain reaction (PCR) purification soluble protein of whole parasite body that's give 100 % protection [3] . By using PCR diagnostic antigen (EgP-29) cloned from *E. granulosus* and expressed in *E.coli* encode protein give protection 96.6 % to prevent secondary hydatidosis against different geographical isolates [4] . Mitochondrial Cytochrome Oxidase sub unit 1(CO 1) and NADH dehydrogenase by using PCR determine the strains of *E. granulosus* (G1-G10) and sub strains also to facilitate controlling [5] . 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 [6], in this study using of PCR to determine the genetic variations between samples taken from different intermediate hosts

(Human, Sheep, Cow and Buffaloes) and known the fingerprinting to each sample by using different random primers .

## MATERIALS AND METHOD

### Patients & 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 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 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) .

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 which consist of :  
100 µl of Tris – HCL , 10 ml of EDTA and 0.5 ml Tween 20 then complete the volume to 100 ml by adding 89.400 of D.w . All these solutions sterilized by autoclave and kept in cooling state to be used [7].
6. Random primers and their sequences (Table-1) used in this study provided by Alpha DNA Co. :

**Table 1**

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'

### Isolation of germinal layer of hydatid cysts

Germinal layer of hydatid cyst from human will be taken after surgical operation, from different animals after slaughtering . Both of them taken to laboratory by clean containers, sterilizing of outer

surface by 70% ethanol then discarding of hydatid fluid, germinal layer would be taken and kept in ethanol 70 % for different periods .

### DNA Extraction [8]

By adding 250  $\mu$ l of sample (Thawed germinal layer by ultra sound sonicator high speed / 10 min. [9] in specific Eppendorf tubes (1.5 ml) containing 1000  $\mu$ l of PBS after that centrifugation at 12000 rpm. /10 min., the supernatant was get away and the sediment was remaining , repeat that three times .

- Added to the remaining sediment 500  $\mu$ l from extraction solution and 6  $\mu$ l of Proteinase – K .
- Incubate all tubes in water bath (37°C) until the next day .
- After that inhibit the action of Proteinase - K by rising the degree of water bath to 100 °C (Boiling degree) for 5 min. then kept the samples in freezing by adding 50  $\mu$ l of (TE) buffer until to be used in

### PCR reaction .

#### Gel electrophoresis of DNA :

- Preparation of Agarose gel at concentration 1% by dissolving 0.5 gm of Agarose in 50 ml TBE buffer (1x) then heating.
- Add Ethidium bromide stain solution 1  $\mu$ l /50ml Agarose .
- 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 mixed extracted DNA with bromo phenol blue in the ratio of 3:1 loaded in the wells of the Agarose gel.
- The tray placed into electrophoresis chamber, the chamber was filled with electrophoresis buffer TBE (1x) until cover the surface of the gel.
- Add Ethidium bromide stain solution 1  $\mu$ l 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 transport of gel into U.V Trans-illuminator
- Either 50  $\mu$ l of TE buffer added to crude DNA to kept in freezing for long periods or used directly in PCR technique as following procedure :
  - \*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 should be taken with separate clean tips under a septic condition .
  - \*Added 5  $\mu$ l of DNA sample to PCR tube , 2  $\mu$ l of primer then 12.5  $\mu$ l of Green master mix and 5.5  $\mu$ l of Nuclease free water to complete the volume to 25  $\mu$ l .
  - \*All tubes were closed, spin the mixture for 5 second by light vortex, The PCR tubes were transferred to preheated Thermocycler .

### PCR Program

3 major steps in PCR, (30-35) cycles each comprising this is done on automated Thermocycler :

1. Denaturation at 94°C ( 60 second ) : The double strand helix melts to become single stranded DNA .
2. Annealing at 45-65°C for (60 second) : 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 second) : Taq DNA polymerase synthesize a new DNA strand complimentary to the DNA template by adding dNTPs in the 5' → 3' direction, temperature differ according to DNA length [10]. Table 2.

After that Gell electrophoresis is made to all PCR tubes as in case of DNA extraction except 1gm (instead of 0.5 mg) of Agarose dissolve in 50 ml of TBE (1x) and apply all above steps in Gell electrophoresis of extracted DNA after that observe the results by U.V. Light .

## RESULTS

### 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 other previous tests [11-13] . *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 [14,15].

**Table-2:** Explain steps of PCR technique (Yang, 2005)

Steps	Temperature (°C)	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

#### 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 [16,17]. 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 [18] . 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 [19] . 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 fig(1), the results 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, from other hand disappearance of amplified bands in case of samples 3 , 6 , 7 fig. (3).
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 fig. (5).
3. OPA – 03 : There are two amplified bands with m.w 300 - 400 bp appear in samples 1 , 2 , 4 and absence of bands in samples 3 , 5 , 6 , 7 fig. (6).
4. OPA – 13 : There is one amplified band with m.w 600 bp appear in samples 1 , 2 , 4 , 5 . Absence of bands in samples 3 , 6 , 7 fig. (4) .
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 . Absence of bands in samples 3 , 6 , 7 fig. (4).
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 . Absence of bands in samples 6 , 7 fig. (2) .
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 , other amplified band with m.w 1000 bp in sample 4 . Absence of bands in samples 6 , 7 fig. (3).
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 fig. (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. Absence of amplified bands in samples 3, 6, 7 fig. (2).
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, band with m.w 1000 bp in samples 4, 5. Absence of amplified bands in samples 3, 6, 7 fig. (6).

## DISCUSSION

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 different number of amplified bands or fluorescence intensity of band (the last one not considerable due to un exacted conditions) 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 [20]. 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 [21], 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 [22], 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.*, [23]. 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.*, [24]. 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.*, [25]. 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 which effect spleen differ from other strains which effected liver and lung in human and this finding is reported for the first time. While we see 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 [26].

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, [12]; Al-Qadhi, [27] 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*) may be according to geographical distribution of *E. granulosus* isolates, passage infections from other countries or because of 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 [28]. Also these genetic variances may be due to genetic variation in same hydatid cyst with daughter cysts or its Protoscolices which may be resulted from

mutation by physical agents as X- rays, chemical agents as different anthelmintic drugs or any other mutagenic agents lead to alteration in genetic material to the offspring [29].

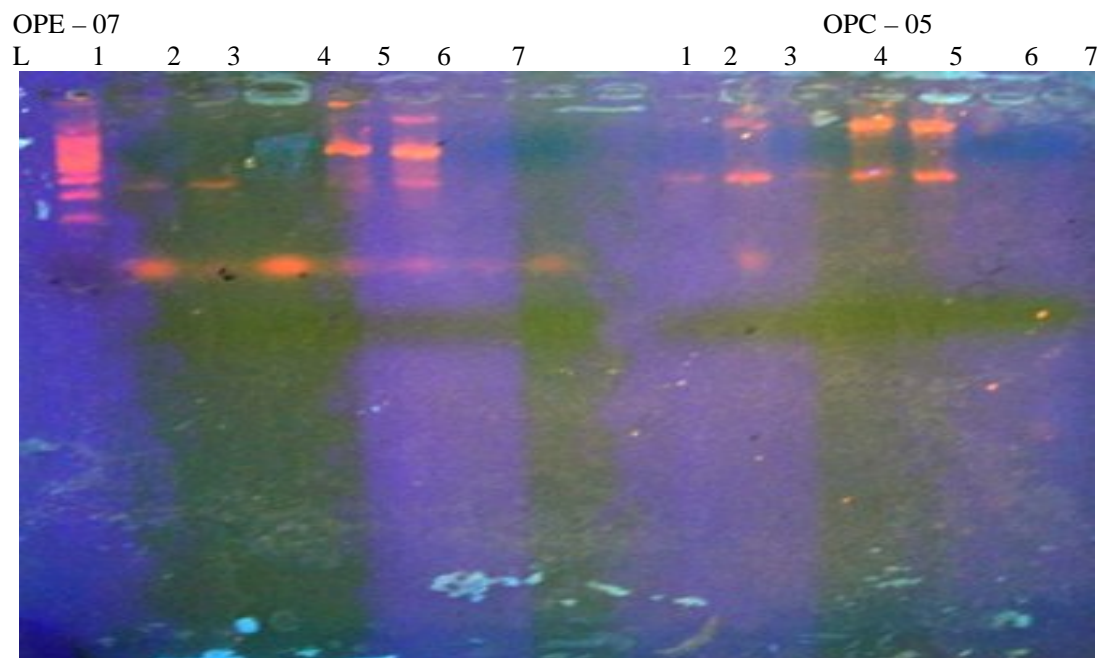
In this study differences in the number of amplified bands through using primers, such as in case of OPA – 01 primer there are 1–5 bands, led to a conclusion that don't found any relationship between primer content of  $G \equiv C$  and this result is in agreement with Ahmed, [30] who explain there isn't any relation ship with primer content of  $G \equiv C$  and disagree with Christofi *et al.*, [31] who explain the efficiency of primer in RAPD increase with increasing of  $G \equiv C$  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  $G \equiv C$  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.

In 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.*, [32] 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 [3] .



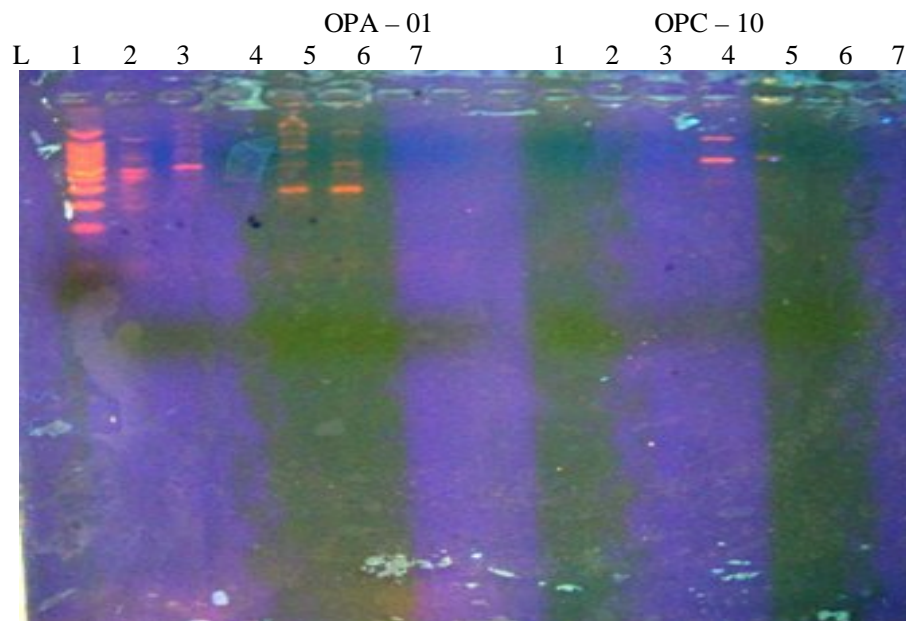
**Fig.(3.1):** Explain Electrophoresis to the end products of DNA extraction to the following samples:

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 .



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

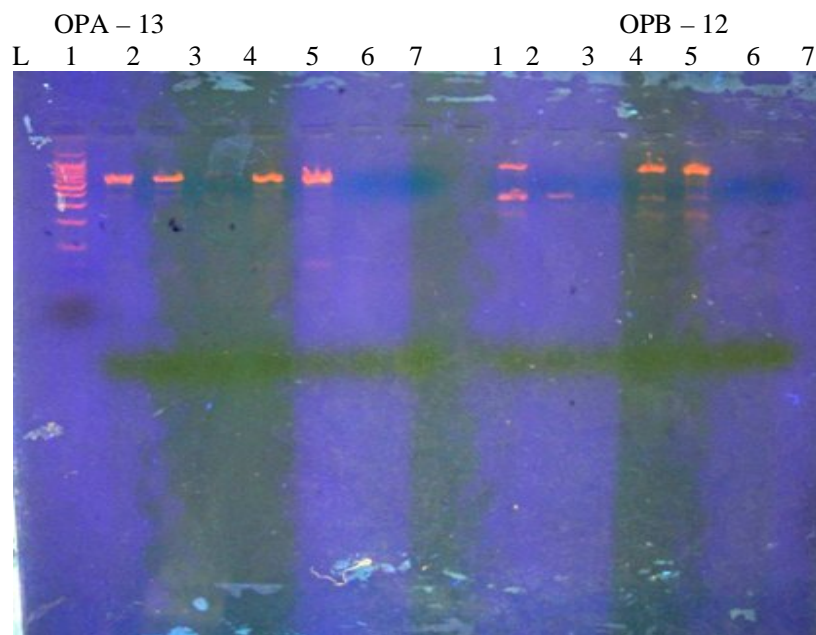
1: Human liver hydatid cyst at age group (10-20) years old. 2 : Human Lung hydatid cyst at age group (20-30) years old.  
 3: Human Spleen hydatid cyst at age group (30-40) years old. 4: Sheep Liver hydatid cyst .  
 5 : Goat Liver hydatid cyst . 6: Cow Liver hydatid cyst .  
 7 : Buffaloes Liver hydatid cyst. L : Ladder .



**Fig.(3.3):** Explain 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 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





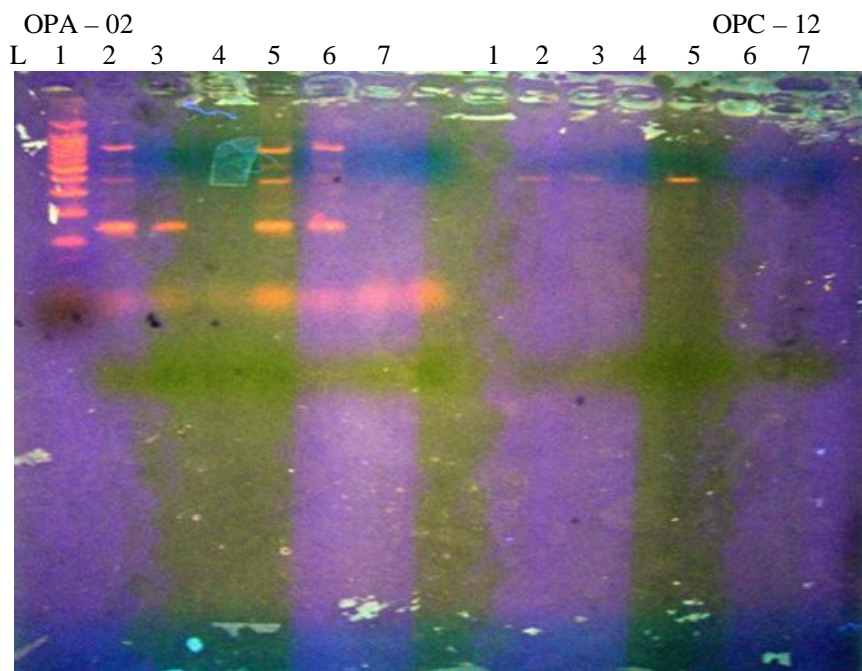
**Fig.(3.4):** Explain Electrophoresis to the PCR end products by using OPA – 13 and OPB – 12

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 .L : Ladder .



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

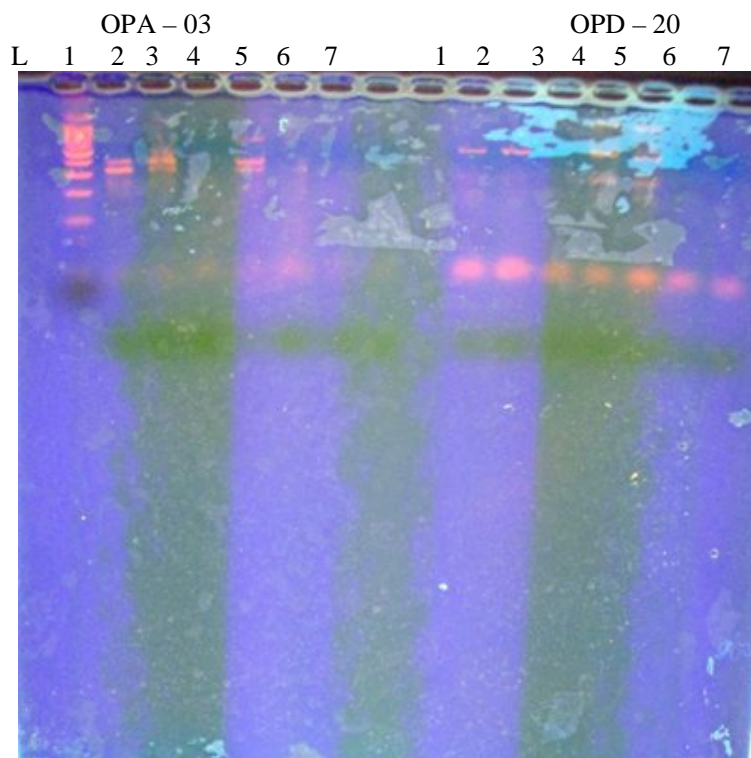
1 : Human liver hydatid cyst at age group (10-20) years old , 2 : Human Lung hydatid cyst at age group (20-30) years old

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 .





**Fig.(3.6):** Explain 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. L : Ladder .

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