# Genetic Variations of *Echinococcus granulosus* Isolated from Sheep and Cows by Using Fingerprint DNA Method in Iraq

**Zaman A.A. Ibrahim *,[1] , Salwa S.Muhsin[**] and Farhan A. Risan [***]**

[*] Department of Basic Medical Sciences, College of Nursing, University of Baghdad, Baghdad, Iraq,
[**]Department of X RAY, Institute of Medical technology, Foundation of Technical Education, Baghdad, Iraq,
[***]Department of Medical Pathological Technology , College of Health and Medical Technology ,Baghdad , Iraq.

## Abstract

The fingerprinting DNA method which depends on the unique pattern in this study was employed to detect the hydatid cyst of *Echinococcus granulosus* and to determine the genetic variation among their strains in different intermediate hosts (cows and sheep). The unique pattern represents the number of amplified bands and their molecular weights with specialized sequences to one sample which different from the other samples. Five hydatitd cysts samples from cows and sheep were collected, genetic analysis for isolated DNA was done using PCR technique and Random Amplified Polymorphic DNA reaction(RAPD) depending on (4) random primers, and the results showed:

**1**-Ability of OPF-13 primer to find fingerprinting for DNA of hydatid cysts collected from cows with (3) unique patterns and from sheep with (5) unique patterns.

**2-** Ability of OPF-06 primer to found (4) unique patterns for cows and (2) unique patterns for sheep.

**3**-Ability of OPF-19 primer to find fingerprinting for DNA of hydatid cysts collected from cows (5) unique patterns and (4) unique patterns for sheep.

**4**-OPF-16 was unable to find the finger prints of DNA isolated from cows and sheep

**Keywords : Hydatid cyst. OPF Primer .PCR. RAPD.**

**الخلاصة**

ان طريقة البصمة الوراثية Fingerprinting DNA التي تعتمد على النسق المتميز Unique pattern قد استخدمت في هذه الدراسة لتشخيص طفيلي الأكياس ألعدريه ومعرفة الاختلافات الوراثية في مضائفه الوسطية المختلفة "الأغنام والأبقار" . أذ يمثل النسق المتميز عدد الحزم الناتجة مع أوزانها الجزيئية الخاصة وألتتابع ألخاص بالعينة والتي تميزها عن بقية العينات قيد الدراسة والتي كانت تشتمل على خمسة عينات لكل من الأكياس العدريه ألمعزوله من الأغنام والأبقار . وبعد تحليل العينات باستخدام تقانة الـ RAPD بالاعتماد على 4 بادئات عشوائية. أظهرت النتائج ما يلي –

١- تم إيجاد بصمة وراثية للعينات الخمسة لدنا الأكياس العدريه المعزولة من الأغنام والأبقار باستخدام البادئ OPF-6 وبتنسيق متميز unique pattern للعينات جميعا.

٢- قدرة البادئ OPF -13 على التوصل إلى إيجاد بصمة وراثية لدنا عينات الأكياس العدريه المعزولة من الأغنام وبتنسيق متميز بعدد خمسة انساق و(٣) أنساق للأبقار.

٣- قدرة البادئ OPF -19 على إيجاد بصمة وراثية لدنا عينات الأكياس العدريه المعزولة من المضائف الوسطية الأبقار وبنسق متميز بعدد (٥) و (٤) أنساق للأغنام

٤- عدم قدرة البادئ OPF -16 على إيجاد بصمة وراثية لدنا عينات الأكياس العدريه المعزولة من المضائف الوسطية (الأغنام والأبقار) المستخدمة في الدراسة.

## Introduction

Hydatidosis is a most serious zoonosis disease caused by larval stage hydatid cysts Of tapeworms *Echinococcus granulosus* which effect human and wide rang of livestock [1,2] and the ultimate growth of the cyst depends on location inside the host, so in some areas of the body they are unable to expand freely whereas in other most growth results in serious impairments to the function of vital structures or even in death[3]. These parasites live for long times in the hostile medium of the host and their struggle for life gave them various strategies that allow them to feed, reproduce and evade the immune system and immunosurveillance and promote chronic infection [4] .Many methods were used for treatment of this disease by (surgically, chemotherapy, physically…etc), surgical remain the primary choice and method, others still ineffective or complementary) for controlling and inhibition of the disease distribution [5], and many advanced laboratory methods were used for the diagnosis of this parasite in human and animals [6].

[1]Corresponding author E – mail - : zamanbrahim@yahoo.com
Received : 17 / 3 / 2009
Accepted : 27/ 10 /2009

## Aim of the study

The experimental studies at molecular level till now absent or hardly very rare about this parasite(adult and larval stage) in Iraq , so this study was carried out using DNA technique depending on Polymerase Chain Reaction(PCR) method and strictly indicator Random Amplified Polymorphic DNA(RAPD) to find out the fingerprinting which used to specify the sequences characterize the DNA which isolated from cows and sheep because first of all ,the high sensitivity of this technique to determine the genetic variations between all animals hydatid cysts isolates, and secondly easy and perfect method to be used[7].

## Materials and Methods.

***Samples Collection.***

Genetic materials DNA had been isolated from hydatid cysts specimens of cows and sheep which collected from different slaughterhouses in north, middle, and south of Iraq during the period from June 2005 - June 2006

***Design of experiments.***

The DNA isolation was done as follow.

- One $cm^3$ specimens from tissues germinal layer of hydatid cyst were isolate from cows and sheep & preserved into 70% ethanol with700µL of proteinase buffer was added {Method was cited by [8] and minor modified by [9].}
- The tissues had been cut by small and sharp scissor into small pieces, and then 35µL of proteinase –K enzyme solution has been added and incubated for 24 hours at 55°C in the incubator,
- Ten µL RNAase enzyme solution has been added and incubated at 37°C for 1hour, then the same volume of (Chloroform, phenol, isomil alcohol) solution was added and mixed by vortex For 1 minute and centrifuged at 12000 rpm for 15 minutes.
- The upper layer pulled out and transferred to a clean, dry, tube, this process had been repeated many times till the middle white layer disappeared, then o.6 ml volume of

Isopropanol was added and then left at room temperature for 1hour and centrifuged at 1400 rpm for 10 minutes.

- The supernatants then removed and the precipitants washed with (70%, 95%) ethanol respectively, the tube left open to complete drying from ethanol, then the DNA

Dissolved by 150 µL Tris-EDTA (TE) with pH 8 and incubated for 2 hours at 65° C to complete dissolving.

- Then whole solution was kept at deep freeze (–20°C).
- After the DNA characterization by measuring DNA concentration and estimation of its Purity [10, 11] using Spectrophotometer with wave length 260nm, RAPD was done according to [12] method which includes the following steps:

**1-** The master mixture solution was prepared by mixing the following constituents

In sterile plain tubes with following concentrations for each constituent:

| constituents | Volume of each sample | Final concentration | Sheep (5) samples | Cows (5) samples |
|---|---|---|---|---|
| Distilled water | 17.7 | - | 354 | 424.8 |
| Buffered solution x 10 | 2.5 | 1 X | 50 | 60 |
| dNTPs | 0.5 | 200 µMole | 10 | 12 |
| Primer | 2 | Pmol/R | 40 | 80 |
| Polymerase enzyme | 0.3 µL | 1.5µL | 6 | 7.2 |

**2-** The constituents mixed well by vortex for 30 seconds and centrifuged for 30 seconds to precipitate all solution drops that suspended on tube wall.

**3-** Each tube which contained cows and sheep samples had been distributed in very sterile 0.5 ml Eppendorf tube labeled with names of 5 hydatid cysts isolates of the study.

**4-** Two µL from each DNA sample of both sheep and cows after performing several dilutions for the concentrated samples by D.W to reach the final required concentration starting the RAPD reaction Of DNA chain which ranged between 10-50ngm / each 1 µL of DNA sample and the final volume became 25µL [13].

**5-** The constituents were mixed and left for few minutes in centrifugation to collect the contents of Reaction in the bottom of the tube, then 20-25µL of mineral oil to prevent the evaporation during the duplication process when the temperature reaches up to 94° C[14,15].

**6-** The tubes were transferred to thermocycler to start the duplication reaction according to the the following program:

- One round for 2 minutes at 94° C for primary denaturation of DNA strands .
- Forty duplicated rounds, each round for 2 minutes at 92° C to denatures the matrix ,1 minute at 36° C to bind the primers with DNA matrix, and 2 minutes at 72° C for elongation of the bonded primers ,with last round for final elongation for 10 minutes at 72 °C.

**7-** The tubes were removed from thermocycler, and volume of (23-24) µL from the contents was pulled from under the oil and mixed with 3 µL loaded solution.

**8**-Agarose gel with concentration 1.2% was prepared and electrophoresis by well method was done On the Samples with volumetric indicator formed from DNA treated by (ECO1) enzyme for 4-5 hours.

**9-** The gel was examined after staining with ethidium bromide (1mg/ml) under Ultra - Violet and black and white Polaroid 667 photos were taken to the gel.

**10**- The molecular weights of the duplicating segments were calculated depending on the bands positions with known molecular weights which produced from DNA cutting enzyme (ECO1) only that considered standard volumetric agents.

Four primers were tested.

1- OPF – 13 3' GGCTGCAGAA 5'
2-OPF – 06 3' GGGAATTCGG 5'
3- OPF – 19 3' CCTCTAGACC 5
4- OPF– 16 3' CGAGTACTGG 5'

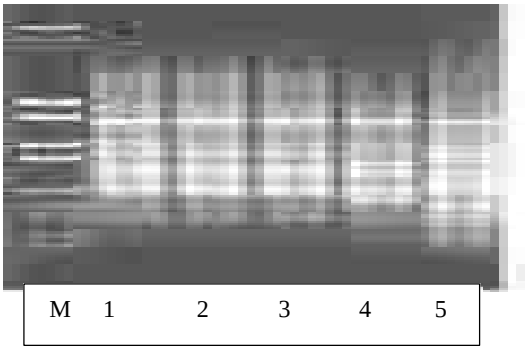
These primers were produced by (Operon Technologies Alameda–A) to be used in final analysis Of RAPD experiments, and the results which appeared in the gel were converted into Binary characters tables by putting number (1) if the band was found and number (0) if the band was not found and the molecular weights were calculated for the duplicated products in comparison with volumetric agent (ECO1) only. In order to characterize each sample for the studied sample, we investigate the finger-prints or the duplicated bands and we determined the organized sequences characterization [14].

## Results and Discussion:

In Iraq the studies about genetic variation between animals infected with hydatid cysts at molecular level almost very rare specially using fingerprint for identification. By using the four primers (OPF 6, 13, 16, 19) the results showed their reactions in RAPD technique that they were different in duplicated bands production and their molecular weights with each hydatid cyst DNA samples taken from cows and sheep.

- The primer OPF-13. This primer was able to find (3) types for unique patterns of DNA which isolated from cows (Figure 1A, Table 1A) and ( 5 ) types for unique patterns of DNA isolated from sheep (Figure 1B, Table 1B), and these results agree with the results conducted by [16] who they said that there are differences in sequences characterizes of duplicated bands and this primer gave a description for genetic material and genetic pattern of DNA isolated from cows and sheep [17].

**Cows A**




**Sheep B**

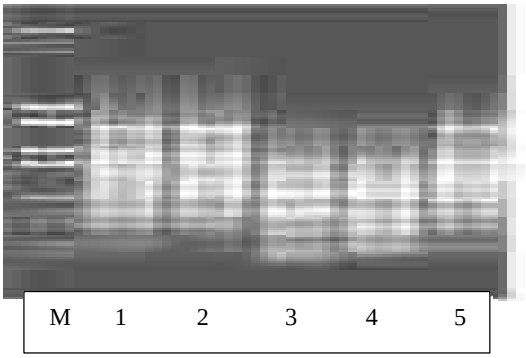



**Figure 1: show the duplication production on agarose gel (1.2%) for hydatid cysts samples isolated from cows (A) and sheep (B) using OPF-13 primer 3.5 hours and with 65 volts .**

**Table1: show the calculation method of sequences characterizes using primer OPF-13.**

**A**

| Mw bp | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| 1800 | 1 | 1 | 1 | 0 | 0 |
| 1600 | 1 | 1 | 1 | 0 | 0 |
| 1100 | 1 | 1 | 1 | 1 | 0 |
| 900 | 1 | 1 | 1 | 1 | 1 |
| 790 | 1 | 1 | 1 | 1 | 0 |
| 770 | 0 | 0 | 0 | 1 | 1 |
| 750 | 1 | 1 | 1 | 0 | 1 |
| 730 | 0 | 0 | 0 | 1 | 1 |
| 680 | 1 | 1 | 1 | 1 | 1 |
| 630 | 1 | 1 | 1 | 1 | 1 |
| 540 | 0 | 0 | 0 | 1 | 0 |
| 420 | 0 | 0 | 0 | 0 | 1 |
| 370 | 0 | 0 | 0 | 0 | 1 |
| 360 | 1 | 1 | 1 | 1 | 1 |

**Numbers of unique patterns (3) Isolated from cows.**
**(0) = duplicated bands not exist**
**(1)=Duplicated bands exit**
**Different colors means numbers Of unique patterns.**

**B**

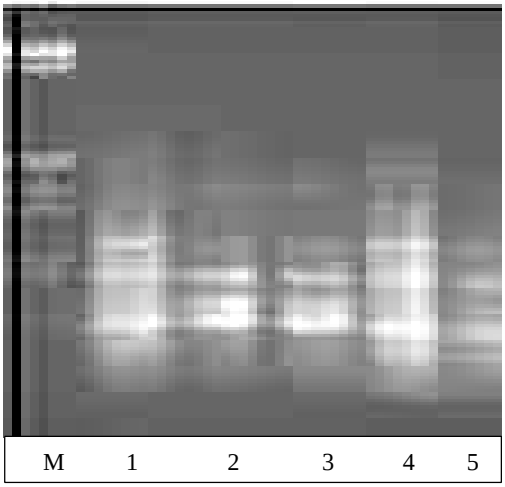
| Mw bp | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| 1800 | 1 | 1 | 0 | 1 | 1 |
| 1600 | 1 | 1 | 0 | 0 | 0 |
| 1100 | 1 | 1 | 0 | 1 | 0 |
| 900 | 1 | 1 | 1 | 1 | 1 |
| 790 | 1 | 0 | 1 | 0 | 1 |
| 770 | 0 | 0 | 0 | 1 | 1 |
| 750 | 0 | 0 | 1 | 1 | 1 |
| 730 | 0 | 0 | 0 | 1 | 1 |
| 680 | 1 | 1 | 1 | 1 | 0 |
| 630 | 1 | 1 | 0 | 1 | 1 |
| 540 | 0 | 0 | 1 | 1 | 0 |
| 420 | 0 | 0 | 1 | 1 | 0 |
| 370 | 0 | 0 | 1 | 1 | 0 |
| 360 | 1 | 1 | 1 | 1 | 1 |

**Number of unique patterns (5) For DNA isolated from sheep.**
**(0) = duplicated bands not exist**
**(1) =Duplicated bands exit**
**Different colors means numbers Of unique patterns.**

***The primer OPF-6.***

This primer was able to found the fingerprint with (4) types of unique patterns for DNA hydatid cysts which isolated from cows (Figure 2A , Table 2A) and ( 2) types for unique patterns of DNA isolated from sheep ( Figure 2B, Table 2B)[18] .

**Cows A**




**Sheep B**

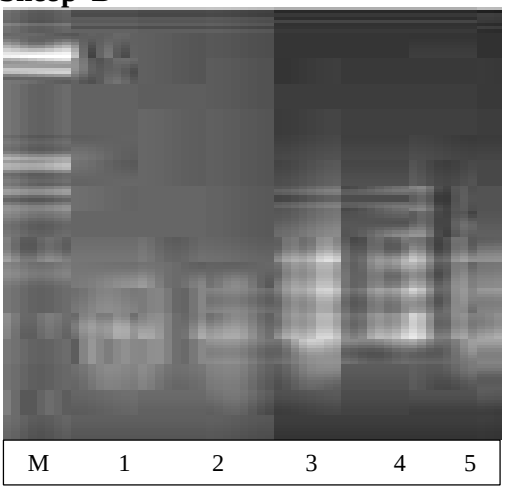



**Figure 2 :show the duplication production on agarose gel (1.2%) for hydatid cysts samples**
**Isolated from cows (A) and sheep (B) using OPF-6 primer for 3.5 hours and with 65volts**

**Table2 : show the calculation method of sequences characterize using primer OPF-6**

**A**

| Mw Bp | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| **1100** | 0 | 1 | 1 | 1 | 0 |
| **1000** | 1 | 0 | 0 | 1 | 0 |
| **990** | 1 | 1 | 1 | 1 | 1 |
| **800** | 1 | 0 | 0 | 0 | 0 |
| **770** | 1 | 1 | 1 | 1 | 1 |
| **650** | 1 | 1 | 1 | 1 | 1 |
| **450** | 1 | 1 | 1 | 1 | 1 |

**Numbers of unique patterns (4) For DNA Isolated from cows.**
**(0) =Duplicated bands not exist**
**(1) =Duplicated bands exist**
**Different colors means number of Unique patterns.**

**B**

| Mw Bp | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| **1100** | 0 | 0 | 1 | 1 | 0 |
| **1000** | 0 | 0 | 1 | 1 | 0 |
| **990** | 0 | 0 | 1 | 1 | 0 |
| **800** | 0 | 0 | 1 | 1 | 0 |
| **770** | 1 | 1 | 1 | 1 | 1 |
| **650** | 1 | 1 | 1 | 1 | 1 |
| **450** | 0 | 0 | 1 | 1 | 0 |

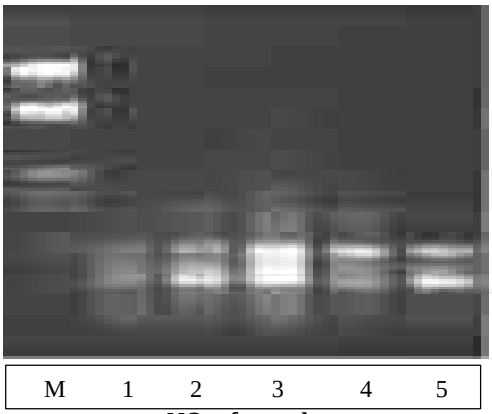
**Numbers of unique patterns (2) For DNA isolated from sheep.**
**(0)=Duplicated bands not exist**
**(1)= Duplicated bands exist**
**Different colors means number of Unique patterns.**

***The Primer OPF -19***

This primer showed (5) unique patterns for DNA hydatid cysts samples of cows (Figure 3A, Table 3A), and (4) Unique patterns for sheep DNA hydatid Cysts samples (Figure 3B, Table3B). It is clear from unique patterns number differences between the samples that the complementary loci for this primer which used in RAPD indicators not evenly distributed between the samples [19], and the appearance of unique pattern specific for cow's hydatid cysts DNA represent fingerprint for these samples differentiated them from other samples by using this primer [20].

From this study, we could diagnosis the DNA of sheep and cows hydatid cysts using RAPD method with banding patterns and their molecular weights, so, by fingerprints the only three primers were able to diagnosis the genetic variations and differences between all hydatid cysts samples [21].

**Cows A**




**Sheep B**

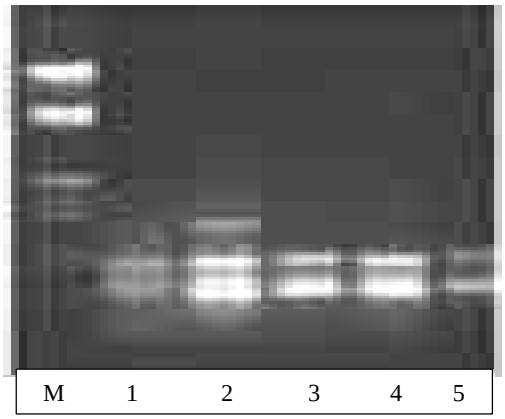



**Figure 3: show the duplication production on agarose gel (1.2%) for hydatid cysts samples Isolated from cows (A) and sheep (B) using OPF-19 primer for 3.5 hours and with 65 volts .**

**Table-3 show the calculation method of sequences characterizes using primer OPF-19.**

**A**

| Mw bp | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| 1050 | 0 | 1 | 1 | 1 | 0 |
| 1000 | 0 | 1 | 1 | 1 | 1 |
| 980 | 0 | 1 | 1 | 0 | 0 |
| 860 | 0 | 0 | 1 | 1 | 0 |
| 750 | 1 | 1 | 1 | 1 | 1 |
| 630 | 0 | 1 | 1 | 0 | 1 |

**Numbers of unique patterns (5) For DNA Isolated from cows.**
**(0) =Duplicated bands not exist**
**(1)= Duplicated bands exist**
**Different colors means numbers of Unique patterns.**

**B**

| Mw bp | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| 1050 | 0 | 1 | 0 | 0 | 0 |
| 1000 | 1 | 1 | 1 | 1 | 1 |
| 980 | 1 | 1 | 1 | 1 | 1 |
| 860 | 0 | 1 | 1 | 1 | 0 |
| 750 | 0 | 1 | 1 | 1 | 1 |
| 630 | 0 | 1 | 1 | 1 | 1 |

**Numbers of unique patterns (4) For DNA isolated from sheep.**
**(0) =Duplicated bands not exist**
**(1)= Duplicated bands exist**
**Different colors means numbers of Unique patterns.**

***The Primer OPF-16***

This primer had no ability to found hydatid cyst DNA and gave them unique patterns by finger prints for cows and sheep, this primer failed to recognize the genetic variations by using RAPD reaction and fingerprints method in the presence of hydatid cysts sample which had no broad genetic background and its high recognition ability failed to show a unique pattern of the studied samples [18].

## Acknowledgements

Sincerely, we must gift our deep thanks to all staff members in all Baghdad–city slaughter- houses for their great help to obtain intact hydatid cyst with our best heart wishes for them. Deep thanks to all members of department of Medical Bio-Technology Institute /University of Baghdad for their great help.

## References

1. McManus, D.P.; Zhang. W.; Li, J. and Bartley P.B. Echinococcosis. Lancet.2003.362: 1295- 1304.
2. Zhang,W.and McManus,D.P. Recent advances in the immunology and diagnosis of Echinococcosis . FEMS Immunol . Med .Microbiol .2006. 47:24-41.
3. John,D.T. and Petri, W.A. Markell and Voge's.Medical Parasitoloy. 9th ed ,Saunders (Elsevier).Philadelphia.2006. Pp. 224- 231.
4. Rigano , R.;Buttari,B .; Profumo , E .; Ortona ,E.; Delunardo , F . ; Margutti ,P .; Mattei ,V .; Teggi , A; Sorice , M . and Siracusano , A. *Echinococcus granulosus* antigen B impairs human dendritic cell differentiation and polarizes immature dendritic cell maturation towards a Th1 and Th2 Cell response . Infect.Immun.2007.75 (4):1667-1678.
5. Ammann,R. and Eckert,J. Clinical diagnosis and treatment of Echinococcosis in Humans.In:*Echinococcus* and Hydatid Disease(Thompson,R.C.A.and Lymbery,A.J.ed): CAB INTERNATIONAL, Wallingford, U.K. 1995. pp. 411-463.
6. McKerrow, J. Parasitic Disease. In: Medical Immunology. (Stites , D. P . ,Terr ,A .I. Parslow,T.G.Ed):9thedition , Middle East Edition.2004. pp: 725-738.
7. Turceková L.; Snábel,V.; D'Amelio, S.; Busi ,M. and Dubinský ,P .Morphological and genetic characterization of *Echinococcus granulosus* in the Slovak Republic. Acta.Trop. 2003.85:223-229.
8. Abraham, K.M.; Longo,N .S. and Hewitt,J.A. Detection of transgenic integrants and homologous recombination in mice by polymerase chain reaction.In: Meltzer, S.J(Ed) Methods in molecular biology.volum (92): PCR in bioanalysis.human press,INC N.J.1998.

**9.** Al-Rubbie, S.S. Genetic and morphological study of protoscolece of *Echinococcus granulose* from human, sheep and cows.*Ph.D Thesis*,2005. College of science, University Of Baghdad- Iraq.(In Arabic).

**11.** Sambrook, J., Fritsch, E.F. and Maniatis, T. Chapter 14: *In Vitro* Amplification of DNA by the Polymerase Chain Reaction," In*:* Molecular Cloning: A Laboratory Manual, 2nd Edition, Cold Spring Harbor Laboratory Pres, Cold Spring Harbor, New York.1989 (B).

**12.** Williams, J. G. K.; Kubelik , A .R .; Livak ,K.J.; Rafalski,J.A. and Tingsey,S.V. DNA polymorphisms amplified by arbitrary primers and useful as genetic markers. Nucleic Acids Research.1990.18:6531-6535.

**13.** Udupa,S.; Weigand, F.; Saxena, M.C.and Kahl,G. Genotyping with RAPD and microsatellite markers resolves pathotype diversity in the *Ascochyta* blight pathogen of chickpea. Theoretical.App.Genet .1998; 97: 299-307.

**14.** 14-Scott, J.C. and McManus, D.P. The random amplification of polymorphic DNA can discriminate species and strains of *Echinococcus*. Tropic.Med. parasitol. 1994.45, 1-4

**15.** Weigand, F.; Baum,M. and Udupa,S. M. DNA Molecular Marker Techniques. ICARDA, Aleppo, Syria: 1993. 51(En).

**16.** Siles–Lucas,M .; Feillesen,R.;Cuesta – Bandera ,C.; Gottstein,B. and Eckert,J. Comparative genetic analysis of Swiss and Spanish isolates of *Echinococcus granulosus* by southern hybridization and random amplified polymorphic DNA technique. Appl . parasitol. 1994 .35(2): 107-117.

**10.** Sambrook, J.; Fritsch, E.F. and Maniatis, T. Molecular Cloning: A Laboratory Manual , 2nd Ed, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.1989 (A). 9:16 .

**17.** Scott, J.C.; Stefaniak, J.; Pawlowski,Z.S.and McManus,D.P .Molecular genetic analysis of human cystic hydatid cases from Poland: identification of a new genotipic group (G9) of *Echinococcus granulosus* . Parasitol. 1997.114(Issue 01):37-43.

**18.** Bhattacharya, D.; Bera,A.K.; Bera, B.C.;Maity,A. and Das,S.K. Genotypic characterization of India cattle, buffalo and sheep isolates *of Echinococcus granulosus*. Vet.Parasitol. 2007.143: 371-374.

**19.** Varcasia,A.; Canu ,S .; Lightowlers ,M. W .; Scala , A. and Garippa , G . Molecular characterization of *Echinococcus granulosus* strains in Sardinia. Parasitol.Res.2006. 98(3):273-277.

**20.** Bart, J.M.; Abdukader,M.; Zhang, Y.L.;Lin,R.Y.;Wang,Y.H.;Nakao,M.;Ito,A .;Craig,P.S.;Piarroux, R.;Vuitton ,D.A.and Wen,H. Genotyping of human cystic echinococcosis In Xinjiang, PR China. Parasitol. 2006. 133 (5):571-579.

**21.** Daniel-Mwambete, K.; Ponce-Gordo,F. and Cuesta-Bandera,C. Genetic identification and host range of the Spanish strains of *Echinococcus granulosus*. Acta.Trop.2004. 91(2):87-93