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Molecular identification of *Echinococcus granulosus*using ISSR and RAPD markers

Suzan Adil Rashid Al-Naqeeb[1], Hanaa N. Abdullah[2], Maysara samer khalaf [3]

Kirkuk technical Institute/Kirkuk, Northern Technical University/Iraq
Health and Medical Technical College /Baghdad, Middle Technical University/Iraq[2 3]

Abstract

In this study, RAPD and ISSR markers have been used to detect the genetic variability, identify the genetic dimension and find the genetic fingerprint of 10 isolates of *Echinococcus granulosus*. The results of SIX primers used in RAPD experiments showed that there were 239 band or 239 bp amplified bands, (90) of them were considered as main bands, and (149) were polymorphic bands. The highest number of bands was (30) in the isolate TR6, while the less number of bands was in the isolate TR7. The results of RAPD experiments also demonstrated that the lowest genetic diversity [0.13005] was between the isolates TR3 and TR9, and the highest genetic diversity [0.55941] was between the isolates TR5 and TR8. Using the SIX primers in the ISSR experiment showed (192) bands, in the isolates of *Echinococcus granulosus* , two of these primer showed monomorphic bands, and six primers showed monomorphic and polymorphic bands, while one showed only polymorphic bands among *Echinococcus granulosus* isolates, and the highest number of bands was (24) in the *TR5* and the less number of bands (16) was in the isolates TR3 and TR8. DNA fingerprint was found in the TR1, TR5, and TR3 isolates. The ISSR markers showed that the lowest genetic polymorphism was [0.05561] between the TR2 and TR7 isolates, and the largest genetic distance was [0.40501] between the TR4 and TR8 isolates. ISSR markers produced high rate of polymorphism depending upon polymorphic rate. The ISSR technique can produce high levels of unique bands in comparison with other markers.

Key words:- *Echinococcus granulosus* , genetic diversity, ISSR, RAPD

INTRODUCTION.

Echinococcus granulosus (EG) is one of the most common larval stages that cause cystic echinococcosis in humans and domestic animals. Cystic echinococcosis is considered as a critical global public health and economic loss problem [1,2]. Several reports of cystic echinococcosis in Iraq have been described in animals and humans [3,4,5]. *E. granulosus* is transmitted predominantly in synanthropic cycles where dogs act as definitive hosts. The highest fertility of the worm is shown in sheep, and it is primarily transmitted in a dog-sheep cycle [1,6]. Based on the genetic diversity related to nucleotide sequencing of the mitochondrial cytochrome c oxidase subunit1 (cox1), and NADH dehydrogenase ssubunit1 (NADH1), ten distinct genotypes of EG designated as (G1- G10) were globally described [5,7,8]. *Echinococcus granulosus* was classified into *E. granulosus sensu strict* (G1-G3), *E. equines* (G4), *E. orteppi* (G5) and *E. canadensis* (G6-G10)[2,8,9]. This study aimed to detect the genetic diversity between *E. granulosus* isolates and study the comparison between RAPD and ISSR markers .

METHODS

A total of (42) *E. granulosus* hydatid cyst/ germinal layers have been collected in this study. The exposed surfaces of the cysts have been cleaned with 95% ethanol. The germinal layers have been isolated from liver hydatid cysts of different intermediate hosts including (7) human cysts, which were obtain by surgical operation from two hospitals in Kirkuk city, (17) sheep cysts, (5) goat cysts and (11) cow cysts have been taken from Kirkuk province slaughterhouse. The germinal layers have been kept in 70% ethanol until use [9,10].

Extraction and quantification of DNA

The DNA was extracted by small-scale method commercial kit [Bionner-Korea] DNA Purity was measured depending on optical density by spectrophotometer. Agar gel electrophoresis with ethidium bromide was used to visualize DNA quality by agars and under the UV light [6].

Molecular Analysis

RAPD assay

Six of RAPD primers were used in this study. The primers were synthesized by [Bioneer-Korea] in lyophilized forms and dissolved in sterile distilled water to obtain the final concentration of (10pmol/ µl) [7]. The primers and their sequences are listed in (table-1).

Table [1]: The names and sequences of the primers used in this study.

No.	Primers name	Sequences[´5 -´3]
	OPI - 06	AAGGCGGCAG
2.	OPE-16	GGTGACTGTT
3.	OPN-07	GAGCCCGAG
4.	OPQ-17	GAAGCCCTTG
5.	OPD-20	ACCCGGTCAC
6.	OPL-05	ACGCAGGCAC

The following master mix was applied to perform the genomic DNA amplification:

Materials	Final concentration	Volume for 1 tube
PCR pre mix	1x	5µI
Deionised D.W	——	11µI
Primer[10pmol/	10pmol /µl	2µI
DNA template	100ng	2µI

RAPD – PCR premix [final reaction volume = 20 µI].No. of cycles = 40 cycles between initial denaturation and final extension. The following table shows the RAPD program:

Steps	Temperature [°C]	Time [min.]
Initial denaturation	(94)	(5)
Denaturation	(94)	(1)
Annealing	(63)	(1)
Extension	(72)	(2)
Final extension	(72)	(10)

Followed by a hold at 4°C[8], each PCR amplification reaction was repeated twice to ensure reproducibility of the products analyzed by electrophoresis in 1.5% agarose gel with 0.5µl stained ethidium bromide at 5vt/cm for 3 hr).

ISSR assay

Six of ISSR primers provided by [Bioneer – Korea] were used in lyophilized form and dissolved in sterile distilled water to obtain the final concentration of [10pmol/ µl] [10].Recommended by provider, the primers tested in this study are listed in table 2:

Table [2]: The names and sequences of the primers used in this study

No.	Primers Name	Sequence[´5-´3]
1.	$ISSR_2$	GACAGACAGACAGACA
2.	$ISSR_6$	AGAGAGAGAGAGAGAGAGC
3.	$ISSR_7$	AGAGAGAGAGAGAGAGAGT
4.	$ISSR_8$	CTCTCTCTCTCTCTCTCTA
5.	$ISSR_9$	CTCTCTCTCTCTCTCTCTG
6.	$ISSR_{10}$	CTCTCTCTCTCTCTCTCTT

Following master amplification reaction:

Material	Final Concentration	Volume of 1 tube
PCR Premix	1x	5µI
Deionised D.W.	—	17 µI
Primer[10pmol/ **µl**]	10pmol/ µl	2µI
DNA template	100ng	1µI

The final concentration was performed in a 25µl volume. The following program was used in PCR program for ISSR assay: No. of cycles = 40 between initial denaturation and final extension. The ISSR program is shown in the following table :

Steps	Temperature[°C]	Time [min.]
Initial denaturation	94	5
Denaturation	94	1
Annealing	50	1
Extension	72	1
Final extension	72	10

Each PCR amplification reaction has been repeated twice to ensure the reproducibility of products analyzed by electrophoresis in (1.5%) agarose gel with 0.5µl stained ethidium bromide at 5vt/cm for 2 hours .

Estimation of molecular weight

Computer software Photo-Capture M.W. program was used to determine molecular weight based on comparing between the RAPD-PCR and ISSR-PCR products and depending upon the molecular weight and number of bands of the 2000 bp DNA ladder Bioneer [which consists of 13 bands from 100 to 2000 bp].

Estimation of polymorphism, efficiency and discriminatory power

Data generated for molecular weight RAPD and ISSR markers result bands were a score for each bands on the molecular size [1 for present, 0 for absence] the commercial soft ware[9]. Only consistently major amplified bands have been scored . According to the following equation, the polymorphism of each primer has been calculated:

Polymorphism % = [Np / Nt] × [100]

Where (Np) represents the polymorphic band number of random primers, (Nt) represents total number of bands of the same primer. The equation below has been applied to calculate the efficiency and discriminatory power of each primer:

- **Efficiency = The number of polymorphic bands to each primer / Total number of bands of the same primer.**
- **Discriminating power = The number of polymorphic band to each primer / Total number of polymorphic band of all primer X (100%)**

Primer efficiency ranged between [0-1]. Discrimination power of each primer.

RESULTS AND DISCUSSION

RAPD-PCR analysis

Based on RAPD assay the data developed from the PCR analysis revealed that some primers generate many bands, while others generate only a few bands. A total of six RAPD primers were used for study the genetic differences between seven *Echinococcus granulosus* isolates, amplified 341(in abstract 239 band) bands,126 bands were polymorphic, with average of [3-43]polymorphic bands, that OPD-20 produce 3 polymorphic bands only ,were OPN-07 can produce 43 polymorphic bands with average range size [100-2000]bp see figure 1. Some isolates can be differentiated from other isolates with the selection of these primers, for instance OPN-07 primers can produce higher discrimination power (19.1 bands only), while OPL-05 gave 2 unique band patterns as shown in table 4.

Table [5]: Genetic distance values among *Echinococcus granulosus* isolates calculated in accordance with [Nei & Lei, 1979].

	1	2	3	4	5	6	7	8	9	10
1	**0.00000**									
2	**0.20273**	**0.00000**						,		
3	**0.21052**	**0.30133**	**0.00000**							
4	**0.15394**	**0.28768**	**0.19011**	**0.00000**						
5	**0.30809**	**0.34484**	**0.30133**	**0.28768**	**0.00000**					
6	**0.21899**	**0.19858**	**0.25039**	**0.15413**	**0.15413**	**0.00000**				
7	**0.39925**	**0.31430**	**0.27080**	**0.37884**	**0.31430**	**0.20273**	**0.00000**			
8	**0.38566**	**0.42588**	**0.38237**	**0.42588**	***0.55941***	**0.31430**	**0.33472**	**0.00000**		
9	**0.24275**	**0.32770**	***0.13005***	**0.17355**	**0.22234**	**0.19415**	**0.29370**	**0.34811**	**0.00000**	
10	**0.32175**	**0.24418**	**0.31845**	**0.30133**	**0.42650**	**0.30168**	**0.27080**	**0.32175**	**0.28420**	0.00000

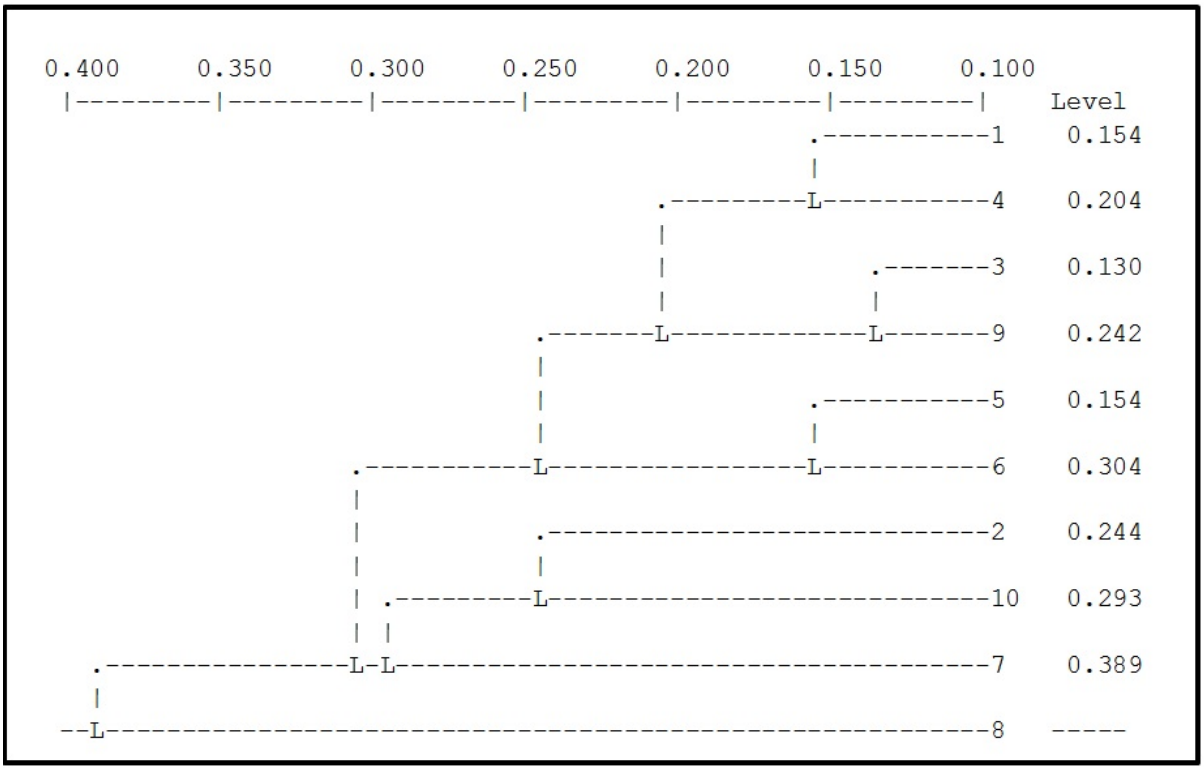

Figure [1]: A dendrogram showing the genetic fingerprint and the relationship between *Echinococcus granulosus* isolates extracted from RAPD data.

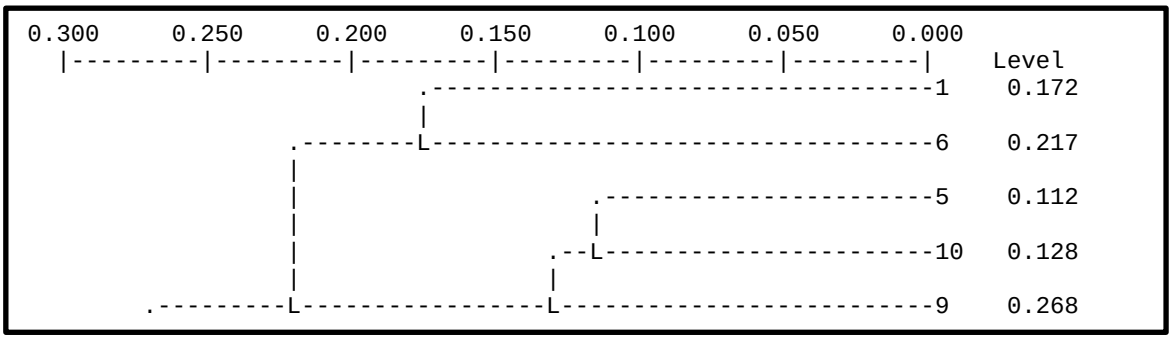

Figure [2]: A dendrogram showing the genetic fingerprint and relationship among *Echinococcus granulosus* isolates extracted from ISSR data.

Table [5]summarized the in formations which can be obtained from RAPD analysis, and from genetic distance the ration genetic diversity among the *Echinococcus granulosus* isolates from 0.9852 to 0.3336.The highest similarity 0.9852 [98.5%] was obtained between isolates numbers [5 and 6] while 0.48562 [48.5%] similarity between isolates numbers (2) and (6). The lowest level of similarity 0.3336 [33.3%] was recorded between the isolates number (5) and (7) [10].

Cluster analysis illustrated genetic relationship among seven of *Echinococcus granulosus* isolates showing two major clusters [figure-1], the first cluster contained two main groups, first group, 5 and 7 isolated in one sub group cluster with low genetic distance 0.3336. These were introduced from environmental sources and isolated number 1 formed separated line due to different in isolate source, while isolate number 2 and 6 formed another sub clusters with genetic distance 0.48652 these isolates introduced from environmental and clinical source, second group contained isolate number 3 only, during clusters analysis showing the levels of genetic relatedness also dendrogram indicates difference between isolates based on source of the isolates, present result appeared multiple difference in isolates of *Echinococcus granulosus* came from two factors including genetic factor and environment factor, also the results indicate that the clinical isolate have greater genetic variability than the environment isolates during gene distance and dendrogram, genetically different may be come from clinical ones on the other hand the clinical isolates of patients constitute one group according to genetic characteristic with the environmental isolates, genetic difference observed in this study come from adept fungi to grow and ability isolates that infected patients to reactive and general more variability in relation to the original strain [11]. Genetic diversity may be attributed to mutation or recombination that occurs in fungal cell into resistance to anti-mycotic treatment or under environmental stress [12]. Environmental and clinical isolates of *Echinococcus granulosus* may be different in genotype consisted of gene involved in transport, regulation of transcription, metabolism of molecular with 1-3 carbon and paroxysm all proteins [13,14].

ISSR-PCR analysis

ISSR-PCR method was applied in this study to reveal the genetic diversity among different studied *E. granulosus* isolates in order to search the genetic diversity between *E. granulosus* isolates and study the environmental differences. A total of 178 use full bands were scored from the amplified products with the seven Inter Simple Sequence Repeat [ISSR], 120bands were polymorphic,

with average of [6] polymorphic bands ISSR10, and ISSR6 produce 32 polymorphic bands with average range size [100-2000]bp. [figure-3]. ISSR9 primers can be produce high unique bands can be produce 5 unique bands,[table-5]

From genetic distance, the ratio of genetic similarity among the ten *Echinococcus granulosus* isolates from (0.97868 to 0.1025) were shown in table 5.The highest similarity 0.97868 [99.8%] was obtained between isolates number 1 and 2, while 0.21556 [21.5%] genetic similarity was obtained between isolates number 3 and 6, whereas the lowest level of similarity 0.1025 [10.2%] appeared between isolates number 5 and 7.

Table [6]: Genetic distance values among *Echinococcus granulosus* isolates calculated in accordance with [Nei & Lei, 1979].

1	0.00000									
2	0.40392	0.00000								
3	0.29356	0.09485	0.00000							
4	0.40392	0.12516	0.23795	0.00000						
5	0.22803	0.29758	0.26727	0.23304	0.00000					
6	0.17185	0.24977	0.37361	0.24977	0.17087	0.00000				
7	0.37949	0.05561	0.15046	0.11624	0.22803	0.23639	0.00000			
8	0.29356	0.31799	0.20764	0.40501	0.26727	0.37361	0.29356	0.00000		
9	0.24515	0.32307	0.36687	0.25408	0.13882	0.18453	0.24515	0.22377	0.00000	
10	0.26738	0.27630	0.24599	0.27630	0.11225	0.20675	0.20675	0.18145	0.11754	0.00000

During the dendrogram which was constructed based on [Nei & Lei1979], the genetic distance using UPGMA cluster analysis and depicted genetic relationship among seven *Echinococcus granulosus* isolates showed two major clusters, the first one contained two main distances, these were introduced from patients sources, and isolate number 2 which formed a separated line that came from environmental sources, and the second cluster which contained the isolates number [7,5] with the lowest genetic similarity 0.1025.These isolates introduced from patients and environment sources, [figure-4] group, first group contain isolates number [1,2] with higher genetic similarity 0.9782.

In this study, each of genetic distance based on ISSR and on RAPD markers don't show geographic profiling between isolates. It has been reported that the dendrogram generated by ISSR better with genealogy and the know pedigree of the ISSR than RAPD results. On the another hand, it has been found that the data on RAPD genetic distance have more relationship with the geographic distribution in comparative with ISSR data that based on number of chromosomes, ISSR markers are highly polymorphic and are useful in studies of genetic diversity[15]. Numbers of analysis studies used both ISSR and RAPD technique were found that ISSR produce more information with fewer number primer than number RAPD primer, during among study found a number polymorphic bands was still higher with less number [16]. ISSR less primers means less time, less DNA, less supplied and less samples, RAPD markers don't have the specific target compared to ISSR markers. In fact, ISSR markers are known to be more sensitive than RAPD markers. In this study, it was obvious that the dendrogram based on RAPD markers was not in accordance with the dendrogram based on ISSR markers, thus, both dendrograms were in agreement with the groups of geographic origin, but RAPD markers greatly agrees with these group than ISSR markers. The differences in clustering pattern of genotypes using RAPD and ISSR markers may also be attributed to marker sampling errors and the levels of detected polymorphisms[17].

CONCLUSION

ISSR markers produced high rate of polymorphism depending upon polymorphic rate. The ISSR technique can produce high levesl of unique bands in comparison with other markers. ISSR was less efficient in dendrogram results .

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