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Molecular Identification of *Entamoeba histolytica* Using RAPD Markers

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ABSTRACT

The aim of this study to isolate and identified of *Entamoeba histolytica* from isolated from patients and study the genetic diversity among these isolates by using RAPD. Collected 20 samples from 7samples were isolated. *Entamoeba histolytica* isolates were characterized depending on its morphological, then extracted DNA from its. RAPD markers randomly banding with sites of genome where the primer NPN-07 achieved discriminative power (19.1) and 43 bands, The dendrogram of RAPD was reverted than isolates number 5 and 7 had the great genetic diversity 0.33361 while the isolates number 5 and 6 had the lowest genetic similarity 0.98521.

INTRODUCTION

Entamoeba histolytica is an anaerobic parasitic protozoan, part of the genus *Entamoeba*. Predominantly infecting humans and other primates, *E. histolytica* is estimated to infect about 50 million people worldwide. Previously, it was thought that 10% of the world population was infected, but these figures predate the recognition that at least 90% of these infections were due to a second species, *E. dispar*. Mammals such as dogs and cats can become infected transiently, but are not thought to contribute significantly to transmission.

The pathogen city of *Entamoeba histolytica* is due to presence of virulence associated genes in the genome which can be divided into four clusters depending on phylogenomic analysis (Nireman *et al*., 2015).One of the diagnostic methods for *Entamoeba histolytica* is Polymerase Chain Reaction (PCR), which can amplify some specific fragment of DNA into millions of copies (McPherson *et al*., 2001). In recent years, different molecular typing techniques have been applied to study the genetic diversity of *Entamoeba sp.* and the possible occurrence of similarity and difference between them, Random Amplified Polymorphic DNA (RAPD) analysis can be performed as a method for study genetic diversity with large number of different strains of microorganisms. It is inexpensive and requires less amount of DNA (Panepinto *et al*., 2010). Moreover, RAPD analysis is technically being commonly used as an indicator for determination the genetic diversity. The aim of the study detection of the unique bands and polymorphism between isolates and comparative study between RAPD and ISSR markers for genetic diversity between different *Entamoeba histolytica* isolates.

MATERIALS AND METHODS

***Entamoeba histolytica* isolates.**

A total of 7 *Entamoeba histolytica* clinical were examined according to their microscopic features, and were sub cultured for using in DNA extraction as show in Table 1.

Table 1: *Entamoeba histolytica* isolates examined during this study

***Entamoeba histolytica* isolates**	**Sources**
*EH*1	stool
*EH*2	stool
*EH*3	stool
*EH*4	stool
*EH*5	stool
*EH*6	stool
*EH*7	stool

Genomic DNA extraction

The DNA was extracted by small-scale method commercial kit (Bionner-Korea) DNA Purity was measured depending on optical density by spectrophotometer. DNA quality was visualized by agars gel electrophoresis with ethidium bromide and visualized under UV light (Sambrook *et al*., 2001)

Molecular Analysis

RAPD assay

Six of RAPD primers were used in this study, the primers was synthase by (Bioneer-Korea) in lyophilized form and dissolved in sterile distilled water to get final conc. of (10pmol/ml) (Hatti *et al*., 2010)The primers and their sequences are listed in (Table 2).

Amplification of genomic DNA was performed with the following master amplification reaction as in Table 3.

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

No.	**Primers name**	**Sequences(´5 -´3)**
1.	NPI - 06	AAGGCGGCAG
2.	NPE-16	GGTGACTGTT
3.	NPN-07	GAGCCCGAG
4.	NPQ-17	GAAGCCCTTG
5.	NPD-20	ACCCGGTCAC
6.	NPL-05	ACGCAGGCAC

Table 3: Amplification reaction contents

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, following table shows in Table 4.

Table 4: RAPD program for PCR Reaction

Steps	**Temperature (°C)**	**Time (min.)**
Initial denaturation	94	5
Denaturaion	95	1
Annealing	40	1
Extension	72	2
Final extension	72	10

Followed by a hold at 4°C (Hatti *et al*., 2010). Each PCR amplification reaction was repeated twice to ensure reproducibility the products analyzed by electrophoresis in 1.5% agars gels with 0.5µl stained ethidium bromide at 5vt/cm for 3hours.

Each PCR amplification reaction was repeated twice to ensure reproducibility the products analyzed by electrophoresis in 1.5% agars gels with stained ethidium bromide 0.5µl at 5vt/cm for 2hour.

Data analysis

Estimation of molecular weight

Computer software Photo-Capture M.W. program was used to determine molecular weight based on comparing the RAPD-PCR and ISSR-PCR products depending on molecular weight of bands and number bands of a 2000bp DNA ladder Bioneer (which consist 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 word (Bibi *et al*., 2010). Only major bands consistently amplified were scored. Polymorphism of each primer was calculated based on the following formula:

Polymorphism % = (Np/Nt)×100

Where Np= the number of polymorphic bands of random primer, Nt= the total number of bands of the same primer.

Efficiency and discriminatory power of each primer calculated according to the formula below:

Efficiency =number of polymorphic bands to each primer / total number of bands to the same primer.

Discriminating power= number of polymorphic band to each primer /total number of polymorphic band to all primer X100 %.

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 demonstrated that some primers generate several bands, while other generates only a few bands. A total of six RAPD primers were used for study the genetic differences between seven *Entamoeba histolytica* isolates, amplified 341 bands,126 bands were polymorphic, with average of (3-43) polymorphic bands, that NPD-20 produce 3 polymorphic bands only, were NPN-07 can be produce 43 polymorphic bands with average range size (100-2000) bp, (Fig. 1). Some isolates could be distinguished from all other isolates with selection of these primers, for instance NPN-07 primers can be produce higher discrimination power 19.1 bands only, while NPL-05 gave 2 unique bands patterns (Table 5).

Table 5: Distinct characteristics of RAPD primers including in the study: primers name, total number of bands, number of polymorphic bands, number of unique bands, percentage of polymorphism, primer efficiency and discrimination value.

NO.	Name of primer	Total number of main bands	Number of unique bands	Number of polymorphic bands	Polymorphism (%)	Primer efficiency	Discrimination Power (%)
1	NPI-06	9	0	18	39.1	0.12	8
2	NPE-16	12	1	35	50	0.50	15.5
3	NPQ-17	9	0	5	8.4	0.089	2.22
4	NPD-20	10	6	3	3.00	0.077	1.33
5	NPL-05	12	2	42	92	0.55	18.6
6	NPN-07	12	0	43	84.3	0.14	19.1

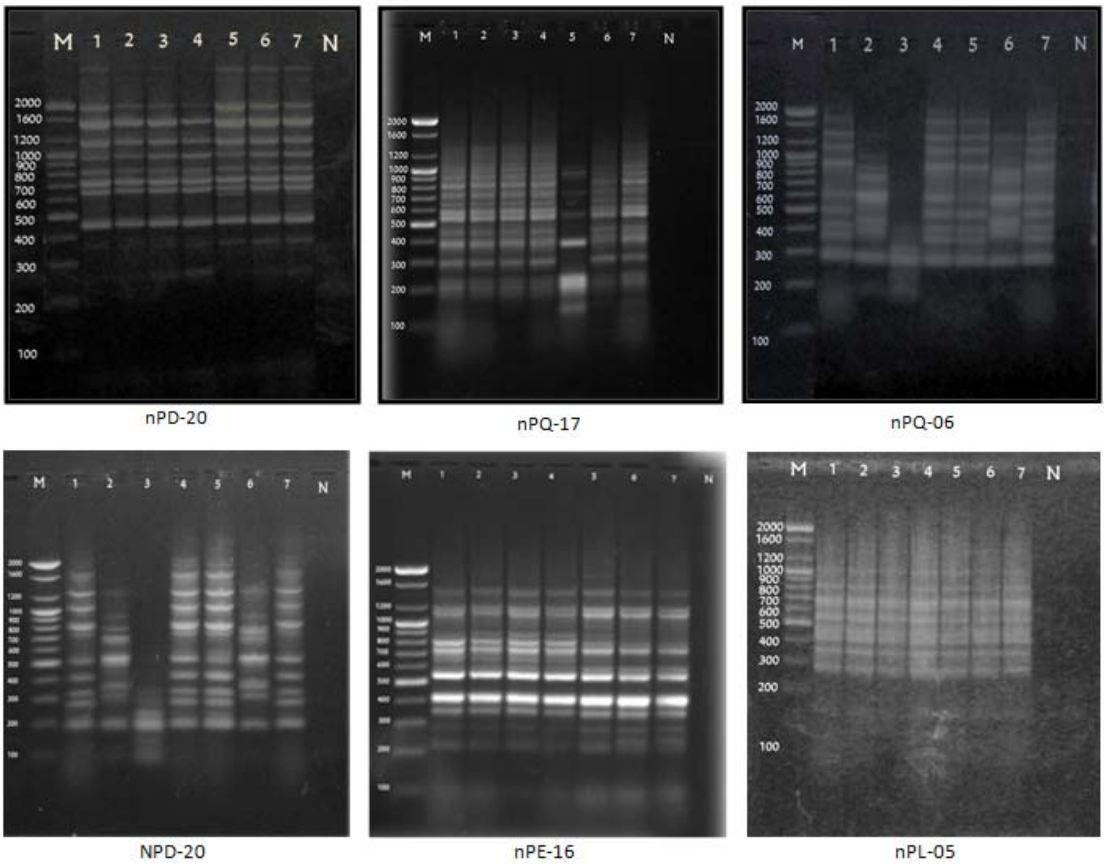

Fig. 1: PCR produced RAPD primer on 1% agars gel electrnphoresis with ethidium bromide, M=1000bp.,N=negative control, Lines= *Entamoeba histolytica* isolates (EH1,EH2, EH3, EH4, EH5, EH6, EH7), (1X TBE, 7Volt, 2hr, 0.5ethidium bromide

Table (6) summarized the informations which can be obtained from RAPD analysis, and from genetic distance the ration genetic diversity among the *Entamoeba histolytica* isolates from 0.9852 to 0.3336.

Table 6: Values of genetic distance among *Entamoeba histolytica* Isolates calculated according to (Nei and Lei, 1979).

	1	2	3	4	5	6	7
1	**0.00000**						
2	**0.69226**	**0.00000**					
3	**0.85701**	**0.69857**	**0.00000**				
4	**0.64981**	**0.97773**	**0.97398**	**0.00000**			
5	**0.10389**	**0.62219**	**0.95737**	**0.45770**	**0.00000**		
6	**0.64708**	**0.48562**	**0.56583**	**0.87972**	**0.98521**	**0.00000**	
7	**0.74012**	**0.69881**	**0.92887**	**0.67972**	**0.33367**	**0.58919**	**0.00000**

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 obtained between isolate number (5 and 7). (Nei *et al*., 1979).

Cluster analysis illustrated genetic relationship among seven of *Entamoeba histolytica* isolates showing two major clusters (Fig. 2), 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 patients, present result appeared multiple difference in isolates of *Entamoeba histolytica*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 (Latge, 2010).

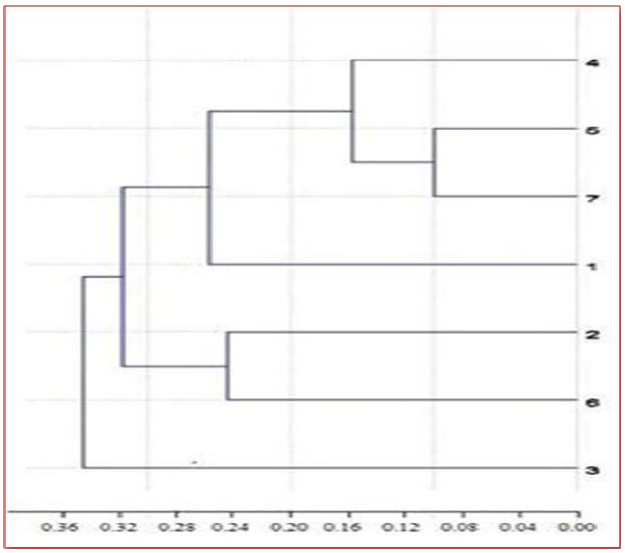

Fig. 2: Dendrogram illustrated genetic fingerprint and relationship between *Entamoeba histolytica* isolates developed from RAPD data.

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