

Medico-Legal Applications of Multiplex STR System to Show Allel Frequencies of D16S 539, D13S317, and D7S820 in Iraqis.

Hussein Mahmood Gazi* , Mohammed Shafik Tawfeek**, Nabeel Ghazi Hashim***, Hayder Hadi Lazim****

ABSTRACT:

BACKGROUND:

Disputed paternity is one of the most important medico-legal problems in Iraq. In addition to identification of unknown corpses. Paternity cases are resolved by doing ABO typing and HLA serotyping and both are less accurate than DNA typing. To our knowledge this is the first study of such type in Iraq.

OBJECTIVE:

To show the medico-legal importance of determination of allel frequencies in Iraqi population.

SUBJECTS AND METHODS:

Whole blood was obtained in EDTA tubes by venepuncture from 38 individuals. The DNA was extracted using the Wizard Genomic DNA Purification Kit and the quantity was estimated by UV-absorbance. The multiplex analysis of D16S539, D7S720, and D13S317 was performed using the Gene Print STR Multiplex system. Amplification was performed in eppendorf thermal cycler. The PCR products were typed by vertical electrophoresis on 0.4 mm thick 7% denaturing polyacrylamide gel and silver staining.

RESULTS:

In our study the DNA fingerprinting test has high accuracy rate.

CONCLUSION:

Since the DNA typing is the most accurate method so it can be routinely used as a paternity test, it is the only test that can meet the increasingly imperative demand to resolve the social and judicial problems involved in paternity suits and other medico-legal problems.

KEY WORDS: medico-legal, multiplex str system, allel frequencies.

INTRODUCTION:

One of the most revolutionary advances in identification in recent years is the so-called 'DNA profiling or fingerprinting'. This is a technique in which virtually unique sequences of bases in the DNA strands of chromosomes are used to compare one blood or tissue sample with another, and to investigate genetic relationships.⁽¹⁾

The technique of determining the sequences is extremely complex, relying on cutting the DNA strands at predetermined points by the use of restriction enzymes. The fragments of DNA are separated using electrophoresis and the different

fragments are then identified using a radioactive probe. Auto-radiography produces a picture of a series of bars of varying density and spacing, which bears a marked resemblance to the 'bar code' used for pricing articles in a supermarket. From the presence of different bars in given positions, comparison may be made with other samples, known or unknown –the classical forensic 'comparison technique'.⁽²⁾

The development of DNA typing resulted from genetic research by scientists in the United States and England: Roy White of Howard Hughes Medical institute at the University of Utah and a British geneticist, Alec Jeffrey, at the University of Leicester.⁽³⁾ Sir Alec Jeffrey invented DNA profiling in the 1980s. He detected differences in numbers of variable number of tandem repeats (VNTRs) among individuals by cutting these regions of DNA with restriction enzymes.⁽⁴⁾

* Department of Pathology and Forensic Medicine/ College of Medicine/ Al-Mustansiriyah University.

**Lecturer / Medical Research Center / College of Medicine / Al-Nahrain University.

*** Department of Pathology and Forensic Medicine/ College of Medicine/ Baghdad University.

****A Specialist of Forensic Medicine / Medico-legal Institute/ Baghdad.

The nomenclature for DNA markers is fairly straightforward. If a marker is part of a gene or falls within a gene, the gene name is used in the designation for example, the short tandem repeat (STR) marker TH01 is from the human tyrosine hydroxylase gene located on the chromosome 11. The '01' portion of TH01 comes from the fact that the repeat in question is located within intron 1 of the tyrosine hydroxylase gene. Sometimes the prefix HUM- is included at the beginning of a locus name to indicate that it is from the human genome. Thus, the STR locus TH01 would be correctly listed as HUMTH01.⁽⁵⁾

DNA markers that fall outside of gene regions may be designated by their chromosomal position. The STR loci D5S818 and DYS19 are examples of markers that are not found within gene regions. In these cases the 'D' stands for DNA. The next character refers to the chromosome number, 5 for chromosome 5 and Y for chromosome Y. The 'S' refers to the fact that the DNA marker is a single copy sequence. The final number indicates the order in which the marker was discovered and categorized for a particular chromosome. Sequential numbers are used to give uniqueness to each identified DNA marker. Thus for the DNA marker D16S539:

D: DNA

16: chromosome 16

S: single copy sequence

539: 539th locus described on chromosome 16.⁽⁵⁾

White developed a technique in 1980 that 'revolutionized modern biology', according to Harlen Levy, a former federal prosecutor who traced the development of DNA identification in his book (And the blood cried out). White discovered that when a DNA molecule was cut, certain repetitive patterns could be identified. These strands of DNA had no known purpose, but they could be used to help locate specific genes. As Levy explains, the technique was called restriction fragment length polymorphism (RFLP) because a restrictive enzyme was used to cut DNA into various fragments and the differences, or polymorphisms, then analyzed.⁽³⁾

Genetic fingerprinting is widely used by forensic scientists to identify individual sources of evidence from crime scenes. DNA testing became commonly available in 1987, a year that marked legal and forensic history when an accused murderer cleared by DNA testing for the first time.⁽⁶⁾

FBI and police labs around the USA have begun to use DNA fingerprints to link suspect to biological evidence – blood or semen stains, or item of clothing – found at the scene of a crime. Another important use of DNA fingerprints in the court system is to establish paternity.⁽⁷⁾

The first of the national DNA databases involving STR data was formed in the UK in 1995 in the first 5 years; it has demonstrated how effective such a venture can be in the detection of the perpetrator of the crimes. By the end of 1999, the database had entries of personal profiles totally well over 700,000 and it is, therefore, not surprising that there were around 700 matches achieved each week.⁽⁸⁾

In the last years, STR profiling has gained a central role in the forensic identification and paternity testing. Using STR markers in the genetic investigation of the representative groups of human population.⁽⁹⁾

The high variability of the STRs and their relative simplicity when used as polymorphic markers makes them useful tools for population genetics and forensic identification purposes.⁽¹⁰⁾

This study is aimed to show the medico-legal importance of determination of allele frequencies in Iraqi population.

SUBJECTS AND METHODS:

This study was conducted in the Medico-legal institute of Baghdad, Research Medical Center and Microbiology Department in the College of Medicine in Al-Nahrain University. Thirty eight individuals (14 families) were chosen randomly in the Department of Clinical Forensic Medicine in the Medico-legal Institute of Baghdad. One milliliter of blood was taken from each subject and an anticoagulant was added. The anticoagulant blood samples were stored at 2-8 °C for 1-4 days. The DNA was extracted using the Wizard Genomic DNA Purification Kit System (Promega Corporation, Madison, WI). This kit is based on a four – step process. The first step in the purification procedure was the lysis of the cells and the nuclei for isolation of DNA from white blood cells. This step involved lysis of the red blood cells in the Cell Lysis Solution, followed by lysis of white blood cells and their nuclei in the Nuclei Lysis Solution. The cellular proteins were then removed by a salt precipitation step, which precipitated the proteins but left the high molecular weight genomic DNA in solution, finally the genomic DNA was concentrated and desalted by isopropanol precipitation. Wizard Genomic DNA purification kit for laboratory use contains sufficient reagents for 100 isolations of genomic DNA from 300 microliters of whole blood samples, and that include:

Cell lysis solution, nuclei lysis solution, protein precipitation solution, DNA rehydration solution, RNase solution, and 1 protocol, in addition to the materials to be supplied by the user which include: Sterile 1.5 ml microcentrifuge tubes, isopropanol, 70% ethanol (in room temperature).

Isolation of genomic DNA from whole blood includes 15 steps starting from the addition of 900 microliters of cell lysis solution to a sterile 1.5 ml microcentrifuge tube for the 300 microliters sample volume until the isolation of DNA and storage of it at 2-8 C.

For very pure samples (without significant contamination from protein, phenol, free nucleic acids, organic solvents, carbohydrates, etc.), the absorption of ultra-violet (UV) light by the ring structure of purines and pyrimidines can be used to measure the amount of DNA.

In this step we calculated the concentration based on an OD of 1 corresponding to approximately 50 microgram/ ml for double strand DNA: $OD_{260} \times 50 \times 100$ (dilution factor). The OD_{260}/OD_{280} ratio for pure DNA is 1.8. Contamination by protein (which has the absorbance maximum of 280 nm) or phenol will cause the ratio to be significantly lower than these values.⁽¹¹⁾

The co-amplification of the D16S539, D7S820, and D13S317 loci, multiplex system information in table (1) was performed using Gene Print Silver STR III system.

Table 1: Multiplex System Information

STR Locus	Chromoso- mal Location	Allelic Ladder Size Range (bases)	STR Ladder (number of repeats)	K562 Allele Sizes	Repeat Sequence 5'to3'
D16S539	16q24-qter	264-304	5,6,7,8,9,10,11,12, 13.14,15	12,11	AGAT
D7S820	7q11.21-22	215-247	6,7,8,9,10,11,12, 13.14	11,9	AGAT
D13S317	13q22-q31	165-197	7,8,9,10,11,12, 13.14,15	8,8	AGAT

In the amplification thermal cycling the PCR cycling parameters were as recommended by Promega protocol. After the completion of that protocol we stored the samples at -20 °C.

The protocol used for the preparation of denaturing polyacrylamide gel with the dimensions of 21 cm wide X 50 cm high X 0.4 mm thick include 15 steps starting by etching each glass plate on one side in one corner with a marker pen to distinguish the treated sides of the glass plates followed by thoroughly cleaning the shorter and longer glass plates twice with 95 % ethanol and any type of paper towel, and spreading the sigma cote using a circular motion over the entire surface followed by other steps till the polymerization was allowed to proceed for 10-20 minutes. Then we check the polymerization control to be sure that polymerization has occurred.

Polyacrylamide gel electrophoresis includes gel prerun to achieve a gel surface temperature of approximately 50 C, followed by PCR sample preparation and sample loading process which should take no longer than 20 minutes to prevent the gel

from cooling. Then electrophoresis was carried out for 1.5-2 hours on vertical slab gel electrophoresis unit at a constant voltage of 1000 V with a fixed temperature of 50 C. For disposal the plate was immersed and affixed gel in 10% NaOH solution for 1 hour to overnight. This step was ended by keeping all cleaning utensils (sponges) for the longer glass plates separated from those for shorter ones to prevent cross-contamination of the binding solution.

The silver staining system contains sufficient reagents to stain 10 sequencing size gels these include: 500 microliter bind saline, 20 grams silver nitrate (10X 2 g), 60 ml formaldehyde 37% (20X 3ml), 10ml sodium thiosulfate 10 mg/ ml (10X 1ml) and 600 g sodium carbonate (10X 60 g).

Descriptive measures are applied to show the results in tables, photos, and figures.

RESULTS:

A population of 38 individuals was typed. D16S539, D7S720, and D13S317 alleles consisted of variable number of constant repetitive sequence. The distribution of observed allele frequencies for the three loci is summarized in table (2). Figure (1) shows the result of 7 samples.

Table 2: The allele frequencies for 38 Iraqi individuals

D16S539			D7S720			D13S317		
Total samples:38			Total samples:38			Total samples:38		
Allele	Allele frequency	No of Allele observed	Allele	Allele frequency	No of Allele observed	Allele	Allele frequency	No of Allele observed
5	0.000	0	6	0.013	1	7	0.000	0
6	0.000	0	7	0.053	4	8	0.039	3
7	0.000	0	8	0.105	8	9	0.132	10
8	0.039	3	9	0.145	11	10	0.118	9
9	0.171	13	10	0.211	16	11	0.276	21
10	0.132	10	11	0.184	14	12	0.329	25
11	0.316	24	12	0.118	9	13	0.079	6
12	0.250	19	13	0.132	10	14	0.026	2
13	0.066	5	14	0.039	3	15	0.000	0
14	0.026	2	All	1.000	76	All	1.000	76
15	0.000	0						
All	1.000	76						



Figure 1: Gene Print STR multiplex system.

Lanes 1-7 show amplification of 2.5 ng of human DNA using the Gene Print STR multiplex system and detected using silver staining. Amplification products using the silver STR III (D16S539, D7S820, D13S317). Multiplex were separated in 7% polyacrylamide denaturing gel. Lanes (L) contain allelic ladders for the respective loci. Numbers to the right of the image indicate the smallest and largest number of repeat units present in corresponding fragments of each allelic ladder. Positive and negative control (+) & (-).

DISCUSSION:

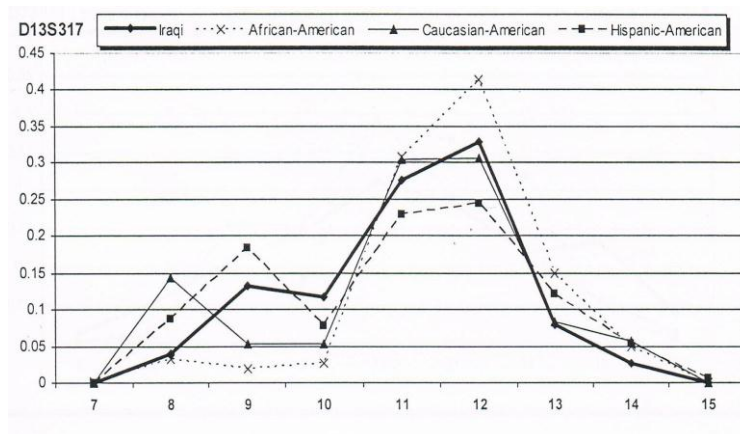
To our knowledge this is the first study on the distribution of STR alleles in Iraq especially from the medico-legal point of view.

The distribution of the observed allele frequencies for the three STRs (D13S317, D7S820, and D16S539) tested in the Iraqi population is compared with the same three STRs of the African-American, the Caucasian-American and the Hispanic-American population.⁽⁵⁾

Our results show that allele's frequency profile for the D13S317 locus in Iraqi population slightly differs from that of the Hispanic-American population and

also the Caucasian-American population. On the other hand there are noticeable differences in allele's frequencies between the Iraqi population and the African-American population, as shown in figure (2):

Allele Frequency

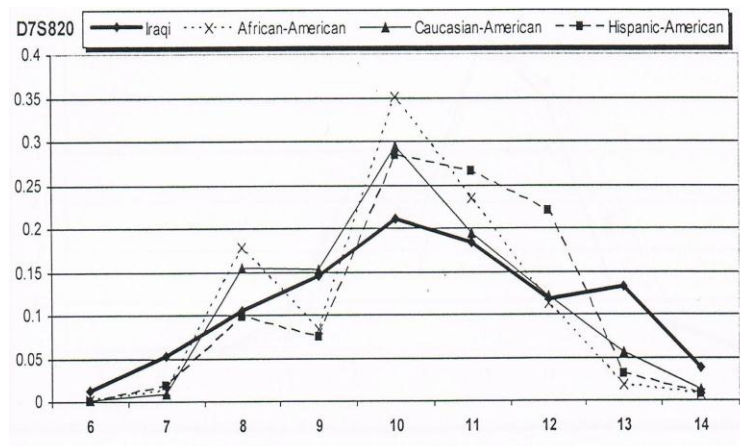


Allele

Figure 2: Allel frequencies profile for the locus D13s317 in Iraqi, African- American, Caucasian- American, and Hispanic- American population.

Alleles frequencies profile for the locus D7S820 show no observed differences between Iraqi population and the Caucasian-American and also the Hispanic-American population, while the African-

American population shows a bimodal distribution and it remarkably differs from the Iraqi population, as shown in figure (3):

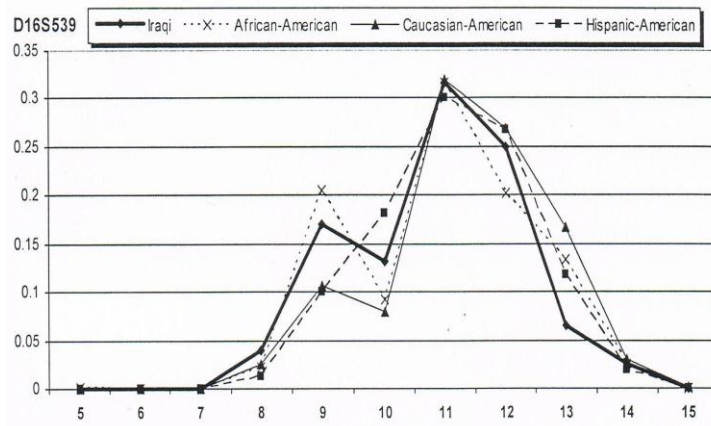


Allele

Figure 3: Allel frequencies profile for the locus D7s820 in Iraqi, African- American, Caucasian- American, and Hispanic- American population.

A bimodal distribution at locus D16S539 was present in the Iraqi, the African-American, and the Caucasian-American with no observed differences, while there were noticeable differences between the

Iraqi population and the Hispanic-American, as shown in figure (4):
Allele Frequency



Allele

Figure 4: Allel frequencies profile for the locus D16s539 in Iraqi, African- American, Caucasian- American, and Hispanic- American population.

Difficulties or drawbacks aroused in our study, those were:

1. Contamination of our DNA samples with proteins and DNA. We prepared a rehydration solution, which is one of four solutions provided to us in the Wizard Genomic DNA purification kit after it had been wasted, in contaminated flasks. This problem had been discovered during the DNA quantitation.
 2. Unstable electricity.
 3. Very slow polymerization of the polyacrylamide gel.
 4. No result could be obtained in 6% denaturing polyacrylamide gel i.e. no DNA could be detected after silver staining.
 5. Gel melting due to high temperature.
- These problems necessitate some modifications in our protocol including:
1. Fortunately the DNA was of plant source so it did not affect our DNA, since we are using human specific primers. In case of proteins which are considered one of the PCR inhibitors, the genomic DNA template was diluted with sterile water and the Taq DNA polymerase

volume had been increased from 0.15 microliter per sample to 0.2 microliter per sample. With this approach some fraction of Taq polymerase binds to the inhibiting molecules (proteins) and removes them from the reaction so that the rest of the Taq can do this job and amplify the DNA template^(11,12,13,14,15)

2. We did not have solutions for this problem and repeated electrical shut down affected the performance of our kit negatively and their performance decrease to 70% or even more.
3. The polymerization process is initiated by the generation of free radicals provided by ammonium persulfate and stabilized by the compound TEMED, so we increased the TEMED from 50 microliter to 100 microliter, and the ammonium persulfate from 500 microliter to 3.5 ml. These modifications had had their effects on the DNA separation i.e. the polyacrylamde pores size.^(11,12,13,14,15)
4. We used 7% denaturing polyacrylamide gel instead of the recommended 6%. This modification had been achieved for two component of the acrylamide solution the first one is the deionized water which had been decreased from 36.25 ml to 34.375 ml and the second component is the 40% acrylamide: bis (19: 1) which had been increased from 11.25 ml to 13.125 ml. The other components of the acrylamide solution remained as they were, i.e. urea 31.5 gm and 10x TBE 3.75 ml.^(11,12,13,14,15)
- 5- The recommended electrical force for each centimeter length ranging between 1.25- 1.625 watt, so for our system which is 50 cm length the electrical force was between 62.5- 80 watt. In our experiments we used 63 watt and the voltage was about 1450-1490 volt so the temperature yeld was very high in addition to that, our

system was not supplied with cooling system and we were working in a hot room. All these factors in turn resulted in melting of the gel and failure of experiments. To solve this problem we^{14,15} used 1000 volt and 40 watt, so the temperature yield was much more less than before and our experiments succeeded.^(11,12,13)

In several years, it may be possible to use small DNA suitcases which will of course be linked to national or may be international database by satellite or phone line. The search will be done almost instantaneously. An eventual match will be known by the investigator before they leave the crime scene.⁽¹⁶⁾

CONCLUSION:

The triplex PCR system has a high combined exclusion chance due to the variety of alleles and well-balanced allelic distribution. Together with high sensitivity, this qualifies the triplex PCR for paternity testing that has a great medico-legal importance. In addition to other medico-legal applications.

Acknowledgements:

For their kind and great comments and advices, the authors of this study acknowledge:

1. Dr. Ban A. Abdul-Majeed the Professor of Molecular Genetics in the department of Pathology and forensic Medicine./ Baghdad Faculty of Medicine./ University of Baghdad./ Assistant of the dean of Baghdad Faculty of Medicine for scientific affairs.

2. Dr. Alaa G. Hussein the Professor of Histopathology and the chairman of department of Pathology and forensic Medicine in the College of Medicine of Al-Nahrain University.

3. Dr. Khulood W. Abood the professor and director of Bio-technology Research Center. In Al-Nahrain University.

REFERENCES:

1. Knight B. Forensic pathology. 3rd ed. London. Arnold, 2004;105.
2. Shepherd R. Simpson's Forensic Medicine. 12th ed. London. Arnold, 2003;52.
3. Bettelheim A, Cooper MH, Jost K, Koch K, and Masci D. DNA databases. The CQ researches 1999;9: 458- 62.
4. Lewis R. DNA profiling began with forensics. Human Genetics-Concepts and Applications. 7th ed. USA. McGraw. Hill, 2007;273.
5. Butler JM. Forensic DNA Typing Biology, Technology, and Genetics of STR Markers. 2nd ed. USA, 2005; 23-24.
6. Robinson KM, Wieland SF, Morgan SL, Lambert SJ. A Prototype DNA profiling project for high school/ college students. Page 4.

7. Betsch DF. DNA Fingerprinting in Human Health and Society. Biotechnology Information Series. November 1994.
8. Martin PD, Shmitter H, Schnider PM. A Brief History of the Formation of DNA Databases in Forensic Science within Europe. Forensic Science International 2001;119: 225-31.
9. Egyed B, Furedi S, Angyal M, etal. Analysis of Eight STR Loci in Two Hungarian Populations. Forensic Science International 2000;113:25-27.
10. Bell B, Nieves P, Abecia E, Martinez JB, Hinojal R, Martinez CA. Population Genetics of the STR Loci HUMCSF1PO, HUMF13A01 and D12S391 in Asturias (Northern Spain). Forensic Science International 2000;113:21-23.
11. Sauer P, Muller M, Kang J. Quantitation of DNA. Qiagen News 1998; 2, 23-26.
12. wizard Genomic DNA Purification Kit. Technical Manual No. 050. Promega Corporation, Madison, USA.
13. Gene-print STR System (Silver Stain Detection). Technical Manual No. D004. Promega Corporation, Madison, USA.
14. Devor EJ. Gel Electrophoresis. Integrated DNA Technologies, 2005;5.
15. Sambrook J, Fritch FF, Maniatus T. Molecular Cloning 1989;1:640-42.
16. Duncan GT, Tarcey ML, Stauffer E. Techniques of DNA Analysis, in ed. of: James SH, Nordby JJ. Forensic science. 2nd ed. Boca Raton. Taylor and Francis, 2005;309.