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GENOTYPING OF FORENSIC DNA SAMPLE, DETERMINING ABO BLOOD GROUP

[a]Qasim M. Ali Al-AMeri , [b]Haider Kamil Zaidan, Ali H. Al-Saadi ,[c]Mufeed J.Ewadh
[a]University of Babylon-College of Pharmacy, Hilla, IRAQ
[b]University of Babylon-College of Science, Hilla, IRAQ
[c]University of Babylon-College of Medicine, Hilla, IRAQ

ABSTRACT
The DNA obtained from crime scene is an important tool to investigate and collect a huge amount of data related to the suspects, one of these important data is blood group. A new approach of ABO genotyping by a polymerase chain reaction and restriction fragment length polymorphism method was used in this study. Instead of amplifying the loci containing the positions of nucleotides 258 and 700 of cDNA of the A transferase separately, we successfully amplified these 2 loci together in one reaction mixture using 2 sets of primers. The amplified DNA products were digested at the same time with restriction enzymes KpnI and AluI. The digested DNA products were then separated by electrophoresis on agarose and polyacrylamide gel. In addition, we evaluated the influence of various amplification parameters (concentration of template DNA, primers, $MgCl_2$, and number of cycles). In particular, high Mg^{2+} concentration (3mM) made effective amplification of this locus without producing any unspecific band. By using that optimized condition for PCR, our study proved to be time saving, more economic, and convenient in interpreting the results.

KEY WORDS: ABO Genotyping, Polymerase Chain Reaction, Restriction Fragment Length Polymorphism.

INTRODUCTION

Genomic DNA was extracted from Forensic sample according to the kit procedure (Bio Basic/ Canada). The kit used Proteinase K and Lysis buffer H was used to extract DNA from forensic sample such as hair, nail, saliva and dried blood. The DNA extraction method used is depend on extraction DNA from epithelial cells that can be collected with each of the forensic sample, while extraction mtDNA from hair shaft could be a useful tool for maternity test.

The PCR master mix used in this study is the HF(High Fidelity) master mix from Bioneer/ South Korea which contain Top DNA polymerase which is able to amplify up to 30kb, also the master mix contain Pyrophosphate (PPi) which has high affinity for Mg^{2+}. By adding PPi to the reaction mixture, the Mg^{2+} ions necessary for normal PCR is bound thus preventing DNA polymerase activity. This PPi-Mg^{2+} binding prevents non-specific before PCR (zero-cycle) product formation. Upon thermal cycling, the pyrophosphatase (PPase) that is also added to the mixture is activated (>70 °C) and hydrolyzes the PPi to 2 phosphate groups and facilitates the release of Mg^{2+}, which is then available for DNA polymerase to use and resume normal activity.

PCR Amplification

TABLE 1: Sequences of Primers Used for PCR Amplification of ABO Gene Locus

Primer	Target DNA fr.	Sequence	Length
ABO-1	199 or 200bp	5´– CACCGTGGAAGGATGTCCTC – 3´	20mer
ABO-2		5´– AATGTCCACAGTCACTCGCC – 3´	20mer
ABO-3	128bp	5´– TGGAGATCCTGACTCCGCTG – 3´	20mer
ABO-4		5´– GTAGAAATCGCCTCGTCCTT – 3´	20mer

The standard method of amplification described for the ABO gene locus was as follows (Lee, *et al* 1992): all the reaction component were collected in 0.2 PCR tube in room temperature because of using a hotstart PCR premix which prevent the zero cycle of DNA polymerase. Primers (1 + 2) were used for amplification of the 200-bp fragment including the 258th nucleotide and (3 + 4) for 128-bp fragment including the 700th nucleotide. The reaction mixture 20 and 50 µl contained 20-40 ng of template DNA, 300 mM of dNTP, 1U Top DNA polymerase (Bioneer-Korea), 10-50 pmole of each primer, and 1x of reaction buffer (10 mM Tris-HC1 pH 9.0 at 25 ^{0}C 30 mM KCl, and 1.5 mM $MgCl_2$). A total of 30-35-40 and 45 cycles of amplification were carried out, but the conditions were varied with each set of primers: primer 1 + 2, 94 ^{0}C denaturation (1 min), 54^{0}C annealing (1 min), 72 ^{0}C extension (1 min); primer 3 + 4, 94 ^{0}C denaturation (2 min), 56 ^{0}C annealing (1min), and 72 ^{0}C extension (1 min) and an additional 72 ^{0}C for (7 min) for final extension. The reaction then incubated at 4 ^{0}C until preparing the gel and loading the sample.

In our study, we selectively varied the amplification parameters as follows (only one parameter was varied in each step):

1- Template DNA from10 ng up to 100 ng.
2- Primer concentration from 10-50 pmole.
3- Mg^{2+} at a final concentration from 1.5-3 mM.

Simultaneous Amplification

We performed PCR amplification in a total volume of 50 µl containing the parameters found to be optimal and each reaction mixture contained all 4 primers. This simultaneous amplification (Multiplex PCR) for both O and B allele specific fragments was carried out using a DNA Thermal Cycler (GTC- Cleaver Scientific/UK) according to the following reaction cycles: after initial denaturation at 94 ^{0}C for 2 min, 30 cycles of 94 ^{0}C denaturation (1 min), 56 ^{0}C primer annealing (1 min) and 72 ^{0}C chain extension (1 min, 30 s), with a final 72~ extension of 5 min, and 4 ^{0}C hold for reaction stop.

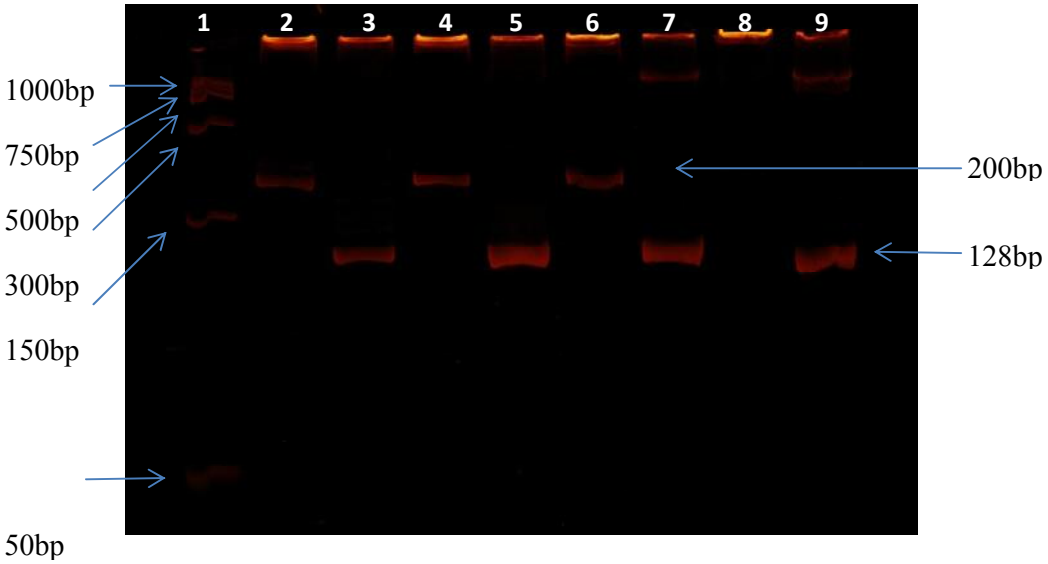
RESULTS AND DISCUSSION

The results presented in this study shows an important and accurate method for ABO genotyping, in figure one which shows the extracted DNA by a spin column method (Fig. 1), this method improve both high yield and purity which is differ from other methods for extracting DNA from blood sample (Dauphin, et al 2009).

Lane 1: DNA extracted from blood stain.
Lane 2: DNA extracted from hair.
Lane 3: DNA extracted from nail.
Lane 4: DNA extracted from saliva
Lane 5: DNA extracted from dried blood

FIGURE 1: The crude DNA extracted by One-Tube Hair DNA Extraction Kit(Bio Basic/ Canada) which show a clear bands. The DNA was run on 2% agarose, 0.5X TBE buffer, 70v, and 20mA for 2 hours. Visualized with Ethidum Bromide. Each well loaded with 10µl of DNA.

According to Yamamoto *et al* 1990, and Clausen *et al* 1994, there are 4 consistent nucleotide substitutions of A transferase leading to amino acid changes (residues 176, 235, 266, and 268) to form B transferase, B allelic cDNA. Formation of O allelic cDNA is due to the deletion of nucleotide position 258, which causes the loss of transferase activity. In the present study, we applied the PCR-RFLP method for the analysis of nucleotide positions 258 and 700 of cDNA from A transferase in a healthy blood donor to determine the ABO genotyping. Two regions of the ABO glycosyltransferase gene were amplified, each containing a diagnostic restriction enzyme site (Fig. 2 A and B).



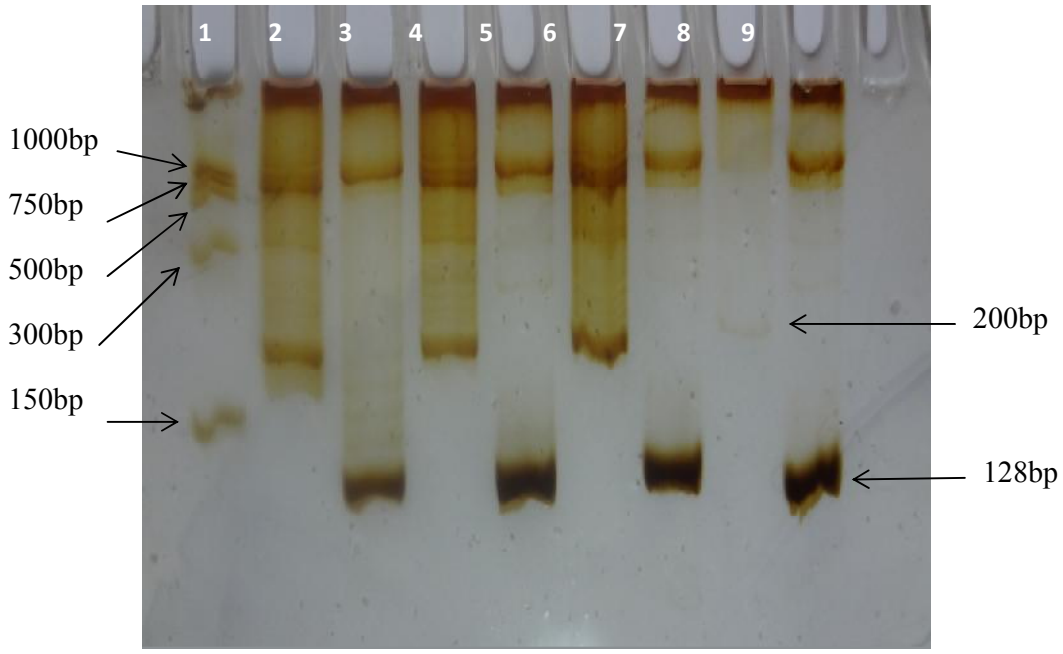


FIGURE 2 A and B: Electrophoretic pattern of amplification products of ABO locus. Primers 1 + 2 amplified a 199- or 200-bp fragment containing the 258th nucleotide, and primers 3 + 4 amplified a 128-bp fragment containing the 700th nucleotide. Unspecific bands were also seen above the 250-bp. Amplified products were electrophoresed in 10% polyacrylamide gel and direct visualization with Ethidium Bromide under UV light (A), or stained with silver stain and visualization under visible light (B).

Lane 1: DNA Ladder (Promega cat# G3161). Lane 2: Amplification product of primer 1+2 for blood group A. Lane 3: Amplification product of primer 3+4 for blood group A. Lane 4: Amplification product of primer 1+2 for blood group B. Lane 5: Amplification product of primer 3+4 for blood group B. Lane 6: Amplification product of primer 1+2 for blood group AB. Lane 7: Amplification product of primer 3+4 for blood group AB. Lane 8: Amplification product of primer 1+2 for blood group O. Lane 9: Amplification product of primer 3+4 for blood group O. Each well loaded with 10µl of DNA.
Primers 1 and 2 amplified a 199- or 200-bp DNA fragment containing the position of nucleotide 258 of cDNA from the ABO locus. If the 258th nucleotide does not exist, the PCR product should be an 199-bp O allele–specific fragment, which will create a KpnI cleavable site on the O allele– specific sequence. But if the 258th nucleotide exists, the fragment should be 200 bp, and there would be no KpnI site. Therefore, if this fragment was completely digested with KpnI, 171- and 28-bp fragments were found and recognized as homozygote OO, and the 171-bp fragment was used as an O allele marker. If the fragment was half digested, 200-, 171-, and 28-bp fragments were found and recognized to be heterozygote AO or BO. If no digestion occurred, no O allele was confirmed and genotypes AA,AB, and BB were possible (Table 1).

TABLE 1: ABO Genotype Interpretation by Restriction Enzyme Digestion of PCR Products.

Restriction Enzyme Digestion	Possible Genotypes Predicted By	
	KpnI	AluI
Complete digestion	OO	BB
Half digestion	AO, BO	AB, BO
No digestion	AA, AB, BB	AA, AO, OO

Primers 3 and 4 amplified a 128-bp fragment containing the position of nucleotide 700 of cDNA from the ABO locus. If the 700th nucleotide is A, this fragment should be B allele specific, and there would be a AluI cleavable site on the B allele–specific sequence. Therefore, if this fragment was completely digested with AluI, 88- and 40-bp fragments were found and recognized as homozygote BB, as shown in Table 1, and the 88-bp fragment was used as a B allele marker. If the fragment was half digested, 128-, 88-, and 40-bp fragments were found to be heterozygote AB or BO. If there was no digestion with AluI, no B allele was confirmed and genotypes AA, AO, and OO were possible. If neither 200- nor 128-bp fragments could be digested, homozygote A allele was determined. Figure 2 shows the electrophoresis patterns of

digested DNA from individuals with different ABO genotypes.

By examining the digested patterns of these 2 fragments, ABO genotypes were easily determined, as shown in (Table 2).

TABLE 2: Pattern of Restriction Fragments Visualized After Digestion of the PCR Product

ABO Genotype	AluI	KpnI
OO	171, 28bp	128bp
AA	200bp	128bp
AO	200, 171, 28bp	128bp
BB	200bp	88, 40bp
BO	200, 171, 28bp	128, 88, 40bp
AB	200bp	128, 88, 40bp

The following parameters were found to be optimal for amplification in 50 µl reaction mixture: 25 ng of template DNA; 1 unit of Taq DNA polymerase (Promega, USA); 10 pmol of each primer; 3.0 mM MgC12; and 30 reaction cycles. Within a certain range, a change in the above parameters had little or no effect, but beyond this range, fading/disappearance of bands or very strong bands occurred and was associated with either increasing or decreasing concentration (and reaction cycles) of the parameters tested. Generally, failure of amplification was associated with low concentrations (template DNA less than 5 ng, MgC12 less than 1.5 mM), but we found that a very high concentration of template DNA (over 125 ng) also led to failure.

The digestion results presented in (Fig. 3), shows the restriction patterns predicted by both enzyme KpnI and AluI (Ciesielka, *et al* 1994).

Also the use of agarose gel electrophoresis rather than polyacrylamide gel electrophoresis is rapid and more costly efficient procedure(Ciesielka, *et al* 1994).

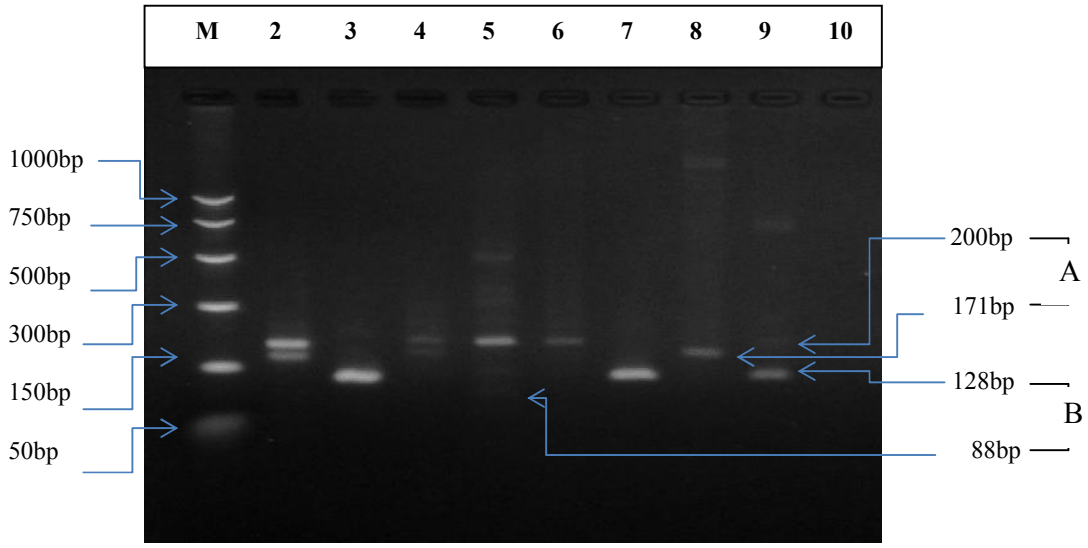


Fig. 3: RFLP pattern of ABO genotypes from blood samples. Restricted fragments were electrophoresed in 2% agarose gel, 0.5X TBE 70V, 20mA for 3 hours and visualized by UV light after Ethidium Bromide staining. A: Digested patterns of 199- or 200-bp fragments. B: Digested patterns of 128-bp fragments. M: 50 base-pair ladder; Lane 2,3: A0; Lane 4,5: BO; Lane 6,7: AB; Lane 8,9: OO. Lane 2,4,6,8 consists of KpnI digest and lane 3,5,7,9 consists of AluI digests).

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