STUDY OF OPTIMUM CONDITIONS IN DETECTION OF ABO GENOTYPE BY SIMULTANEOUS PCR-RFLP METHOD

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Abstract

Extracted of DNA from blood samples, and amplification two separate segments of the glycosyl transferases gene containing nucleotide 261 in exon 6 and nucleotide 703 in exon 7 of ABO gene locus were amplified . ABO locus gene was amplified by using two sets of specific primers for ABO locus as a whole. The first set includes the primer pair (1 and 2) that was used for the amplification of 200 bp DNA fragment, which contains the nucleotide 261. The second set includes the primer pair (3 and 4) that was used to amplify a 128 bp DNA fragment, which contains the nucleotide703. Nucleotides 261 and 703 were used to distinguish A , B, O alleles by restriction enzyme digestion of the PCR products. The amplified DNA products were then run on 2% agarose gel .Two types of restriction enzyme were used. The first was *KpnI* which determined the deletion at nucleotide 261(G→ -) of O allele, while the second one was *AluI* which determined the substitution in nucleotide 703 (G → A) that detect B allele which can be recognized from A allele (*AluI* digest the DNA of individual with type B allele). Digested DNA fragments were run on 6% low melting agarose. The thirty samples show 14 (A)blood group, 13 of them are Heterozygous AO and one is Homozygous AA . The 14 samples are blood group B, 13 of them are Heterozygous BO and one of them is Homozygous BB. One sample for AB, O blood groups this type have only Homozygous phase and each gene are dominant in AB type so called co-dominant. In O type the gene is recessive and expressed only in Homozygous type only whereas the A and B genes are dominant over O.

دراسة الظروف المثلى لتشخيص انماط الوراثية لمجاميع الدم ABO باستخدام طريقة PCR-RFLP

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الخلاصه

عزلت المادة الوراثية بطريقتين ,الطريقة اليدوية باستخدام عدة العزل وكانت ذات تركيز عالي نسبيا (10 – 5 ميكروغرام) لكنها بنقاوة دون المثالية (3.3 – 0.9). ضبط تركيز المادة الوراثية بما يلائم طريقة تفاعل التضاعف التسلسلي (PCR). تم مضاعفة قطعتان من جين ABO, القطعة الاولى في الاكسون 6 تتضمن النيوكليوتيد 261 , والقطعة الثانية في الاكسون 7 والمتضمنة النيوكليوتيد 703 (جين ABO يتكون من 7 اكسونات و 6 انترونات) باستخدام تسلسلات من بادىء البلمرة (Primers)مخصصة لمضاعفة قطعتان تمثل جين ABO بجميع اليلاته. المجموعة الاول يتضمن زوج البوادئ (1و2) التي نتج من مضاعفة القطعة الاولى 200 زوج قاعد والذي يتضمن النيوكليوتيدات 261 بينما نتج عن المجموعة الثانية من زوج

البوادئ (2 و3) مضاعفة القطعة 128 زوج قاعدة والمتضمنة النيوكليوتيدات 703 . فصلت نواتج PCR على 2% من هلام بأستخدام النيوكليوتيدات 261 و 703 لتميز اليلات مجاميع الدم A,B,O بواسطة أنزيمات التقييد. أستخدمت نوعين من أنزيمات التقييد الاول *KpnI* الذي يحدد الطفرة الوراثية (الحذف) الموجود عند النيوكليوتيد 261 بينما الانزيم الثاني *AluI* فيحدد الطفرة الوراثية (الاستبدال) الموجودة في النيوكليوتيد 703 , تم ترحيل ناتج عملية الهضم بانزيمات التقييد على 6%من هلام الاكاروز واطىء الانصهار لمشاهدة جميع القطع الناتجة من عملية الهضم ومقارنتها مع الحامض النووي القياسي السلمي.

أظهرت طريقة التحري الجزيئي PCR-RFLP للتحري عن صنف الدم ABO ان من مجموع 14 نموذج صنف دمهم A كان 13 منهم ذوي طرز وراثية هجينة AO وواحد فقط ذو طرز وراثي متجانس AA , بينما كان 14نموذج صنفهم B , 13 منهم ذوي طرز وراثية هجينة BO وواحد فقط ذو طرز وراثي متجانس BB. نموذج واحد فقط لكل من صنف الدم AB ,O حيث كانوا متجانسي الطرز الوراثية , يعتبر صنف AB ذات سيادة مشاركة لأن كلا الاليلين يشفرون لصفاتهم على سطح كرية الدم الحمراء أما بالنسبة للصنف O فهو ذات اليلات متنحية لاتظهر الا في حالة Homozygous وهي متنحية أمام الاليلات Aو B . خلصت الدراسة الى ان طريقة PCR-RFLP هي سريعة ,عملية ,قليلة التكلفة وسهلة في قراءة النتائج. ممكن ان تستغل في الطب العدلي الجنائي , في فحص البنوة وبعض حالات نقل الدم الدوري في مصرف الدم.

Introduction

The polymerase chain reaction (PCR) is a technique used to multiply single piece of DNA many times. PCR has proven to be an extremely powerful tool in the field of molecular biology (1). DNA polymorphisms are identified as RFLPs, have proved to be an invaluable tool in the study of the human genome, not least in providing markers for gene mapping and linkage analysis (2).Since the PCR is a very sensitive method is it essential to take precaution to avoid contamination which would lead to false-postive reaction .To prevent contamination and to secure the quality in a laboratory working materials ,laboratory areas or reagents should be monitored regularly for DNA or amplificates.

The overall efficiency and specificity of PCR is determined by many factors, the quality of the DNA template, the design of the primers and the PCR condition such as MgCl2 concentration, DNA polymerase and buffer condition .All are particularly important.

Several new strategies have been employed to detect ABO genotype and other genetic markers by PCR and DNA typing. Restriction fragment length polymorphism (RFLP) method has taken advantage of altered restriction enzyme recognition sites caused by nucleotide substitution in the ABO locus of the A , B, or O alleles .This method used to differentiate between B and O alleles by PCR-RFLP (3 , 4, 5, 6). Because the molecular and genetic basis for the ABO blood types has been determined many reports on PCR based ABO genotyping have been published (4, 7, 8, 9, 10, 11) but the procedures described are cumbersome, time consuming , and inconvenient in interpreting the results .Here new approach procedure to reduce these disadvantages by simultaneous PCR-RFLP and use two restriction enzymes simultaneously, proved to be time saving, more economic, and convenient in interpreting the results.

Aims of the study:-To modify and find optimal conditions to standardized rapid genotyping ABO methods.

Materials and Methods

Samples Collection

Peripheral venous blood from (30) individuals appear as healthy.

DNA extraction

isolation of genomic DNA using automated BioRobot EZI .

Primers Selection.

Primer sets which were selected for PCR analysis of ABO locus are shown in Table (1) (12,4).

Genomic DNA was amplified by using two sets of primers each one for specific locus

Table 1: Oligonucleotide Primer Sequences used for PCR amplification of ABO locus allele.

Primer	Primer Sequences	Synthssis No	Length	Melting Temperature	Temperature Annealing
O gene	F 5- CACCGTGGAAGGATGTCCTC-3	281878	20	64C°	58C°
	R 5- AATGTCCACAGTCACTCGCC -3	281879	20	62C°	58C°
A and B gene	F 5- TGGAGATCCTGACTCCGCTG-3	281880	20	64C°	58C°
	R 5- GTAGAAATCGCCTCGTCCTT-3	281881	20	60C°	58C°

Analysis of PCR Products

The PCR products and the ladder marker were examined by gel electrophoresis .1 µL of loading buffer plus 5 µL of the amplified product were loaded on 2 % agarose gel stained with ethidium bromide and run at 50 volt for approximately 3 hours (until bromophenol blue reached the end of gel). Bands were visualized on UV transiluminator and then photographed by using photo documentation system.The presence of bands of 200 bp and 128 bp was observed for O , B and A , genotypes compared with DNA ladder.

Restriction Enzymes

DNA substrates commonly used for restriction enzyme digestion include DNA Lambda DNA(48.5 kb) is a linear DNA .The amplified products were checked by electrophoresing in 6% low melting agarose gel in 1X TBE buffer at 50 voltage for approximately 3.5 houres and dircect visualization of the products with ethidium bromide under UV light. Then add 10 µLof each amplified products (which contained 200 and 128 bp fragments) together with 10 U/µ L each of restication enzymes *Kpn*I and *Alu*I were put hnto the same tube and incubated at 37c for 90 mints**.** Transfer(20 µL) of mixture were mixed with 4 µL loading buffer then applied to the wells of the 6% low gelling /melting gel and electric power was adjusted at 50 voltage for approximately 3.5 houres (or until bromophenol blue reached the end of gel). Then the gel was examined in the gel photo documention system and the picture was saved in computer and then printed.

Results and Discussion

DNA Extraction

Genomic DNA was extracted from blood samples by using automated BioRobot EZ1 gives slightly lower yield of DNA (4 - 8 µg) and high purity (1.6 – 2.2). The DNA was checked on agarose gel electrophoresis before doing PCR to see the purity, and integrity Figure (1).

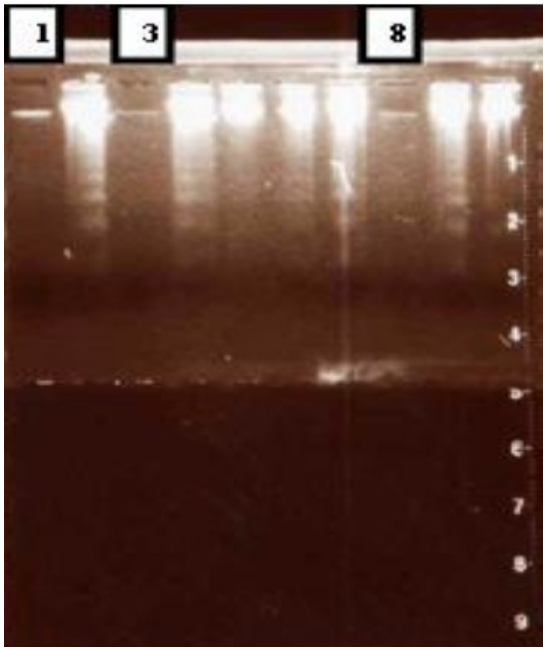


Figure 1: Electrophoresis of Human DNA Samples to Check the Purity and Integrity of DNA. (2% agaroose gel at 50 voltages for approximately 3 hours).

(Integrity of samples on lane 1, 3 and 8 are better than other samples).

The method for PCR-RFLP is very sensitive and needs pure DNA with slightly high concentration (4, 12, 13, 14, and 15). Automated BioRobot EZ1 method was depended for DNA

extraction because it gives high DNA purity and integrity Figure (1).

PCR Amplification

According to the condition of the first trial, the PCR technique failed Figure (2). Many reasons may lead to this result the concentration of DNA is high but low purity(manual DNA extraction), the amount of Taq polymerase is little, primer is also low amount, so the researcher here tried to find a new PCR optimaztion condition according to PCR calculation optimization studies(16,17, 18, 19, 20) .

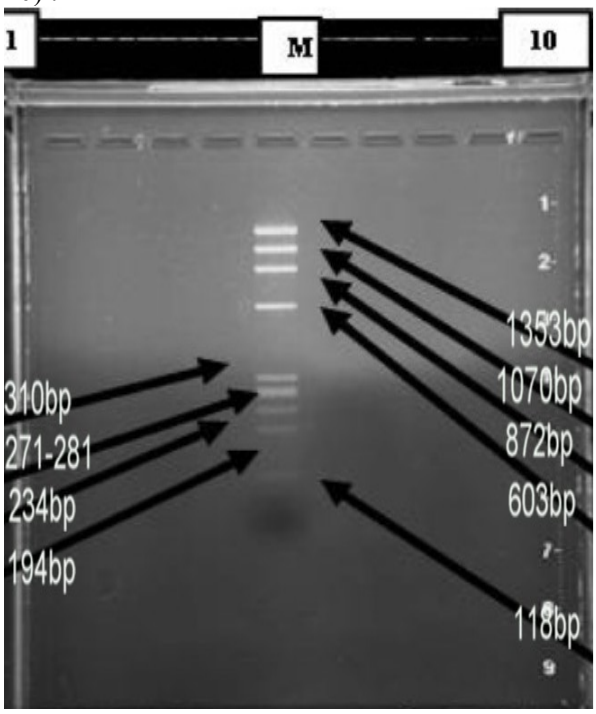


Figure 2: PCR Amplification According to First Trial PCR Condition. (2% agarose gel at 50 voltages for approximately 3 hours).

Lane 1control negetive.
Lane 2, 3, 4, no PCR products.
Lane M ladder marker .
Lane 6, 7 , 8, 9,10 no PCR products .

Second trial was done and associated with the increase unit of Taq polymerase, concentration of primer and $MgCl_2$ but with reduced DNA concentration.
The amplified products appeared as smears, this means probably DNA samples are too much for PCR reaction and with low purity (21, 20) Figure (3).

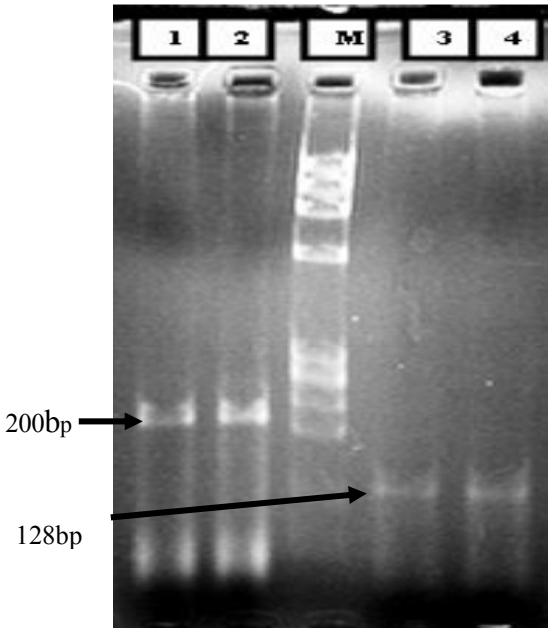
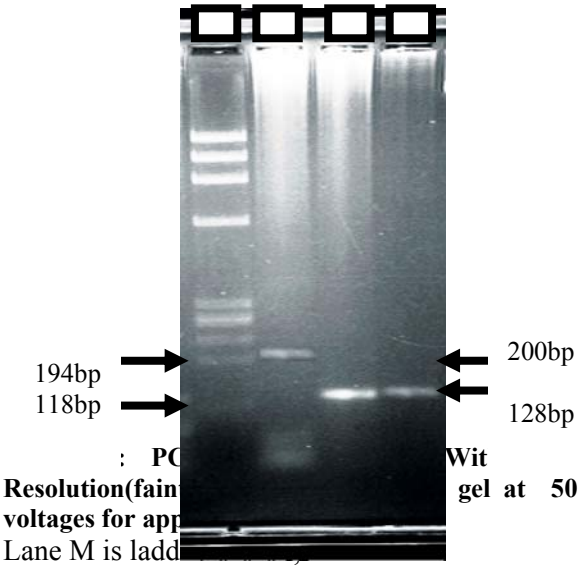


Figure 3: Second Trial PCR Product as Smear. (2% agarose gel at 50 voltages for approximately 3 hours).
Lane 1 and 2 is 200bp bands.
Lane M is ladder marker.
Lane 3 and 4 128bp bands.



: PC Wit
Resolution(fain gel at 50 voltages for app
Lane M is ladd
Lane 1 200bp for O gene,
Lane 2 128bp for A and B genes,
Lane 3 128bp for A and B genes

The main reasons lead to this result is probably inefficient primers concentration or inefficient annealing temperature. The bands appear as sharp and clear Figure (5) in fourth trial optimization as in Table (2).

Table 2: Final PCR Condition Optimization trial.

NO of Trial	DNA Concentration	Taq Unit	Primer Concentration	$MgCl_2$	Annealing Tim	DNA Extraction Method
Fourth Trial	50 ng	5U	30 Picomol	3 mM	58Cº	Instrumental

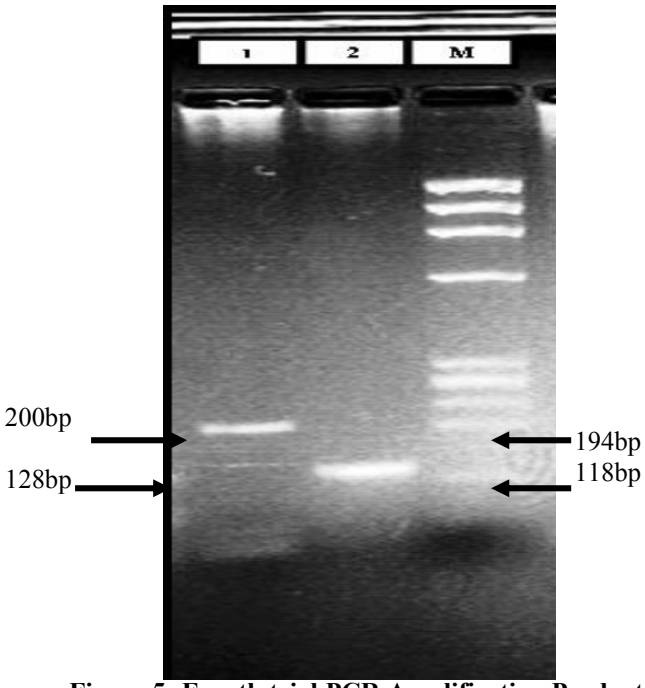


Figure 5: Fourth trial PCR Amplification Product for ABO Loci , 200bp for O gene and 128 bp for A and B genes . (2% agarose gel at 50 voltages for approximately 3 hours).

Lane 1 200 bp for O gene .
Lane 2 128 bp for A and B genes.
Lane M ladder marker.

PCR for ABO loci.

Six samples were selected that represent all types of ABO genotypes Figure (6).

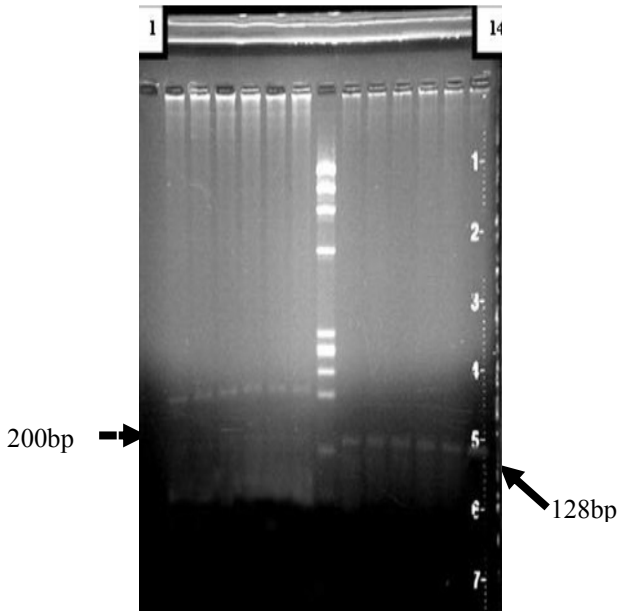


Figure 6: PCR Amplification for ABO Gene Loci by Monoplex PCR , 200 bp for O alleles and 128bp for A and B alleles .(2% agarose gel at 50 voltages for approximately 3 hours).

Lane one control negetive
Lane two 200 bp of AB sample.
Lane three 200 bp of O sample.
Lane four 200 bp of A sample.
Lane five 200 bp of A sample.
Lane six 200 bp of B sample.
Lane seven 200 bp B sample.
Lane eight ladder marker .
Lane nine 128 bp of AB sample.
Lane ten 128 bp of O sample
Lane eleven 128 bp of A sample
Lane twelve 128 bp of A sample.
Lane thirteen 128 bp of B sample
Lane fourteen 128 bp of B sample.

Restriction Enzymes Check up

Before starting digestion of PCR products, activities of restriction enzymes and buffer sterililty were checked by using lambda DNA as standerd DNA which has known number of restriction sites for known enzymes used in this project. The *Kpn I* has 2 sites for restriction in lambda DNA while the *Alu I* has 143 sites in lambda DNA (22) Figure (7) represents restriction enzymes activity and buffer sterility.

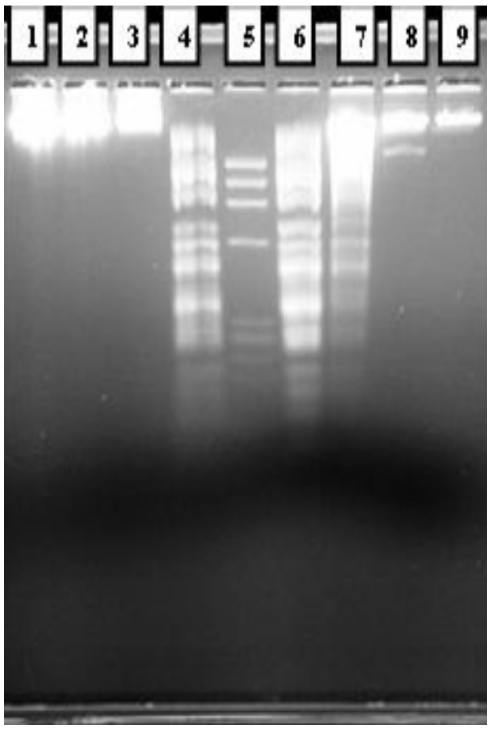


Figure 7: Check Up Restriction Enzymes by Using Lambda DNA as standard. (2% agarose gel at 50 voltages for approximately 3 hours).

Lane one lambda DNA with *Kpn I* buffer 10X .
Lane two lambda DNA with *AluI* buffer 10X .
Lane three lambda DNA with free nuclease water .
Lane four lambda DNA with *Kpn I* and *Alu I* enzymes .
Lane five ladder marker.
Lane six lambda DNA with *Alu I* enzymes only .
Lane seven lambda DNA with *KpnI* and *Alu I* enzymes without free nuclease water.
Lane eight lambda DNA with *Kpn I* enzymes
Lane nine lambda DNA only.
The suitability of buffer for each enzymes was checked , the nil effect of changing buffer of another on the action of enzymes was confirmed as in Figure(8).The concentration of lambda DNA is high in this trial (10 µg = 20 µL).

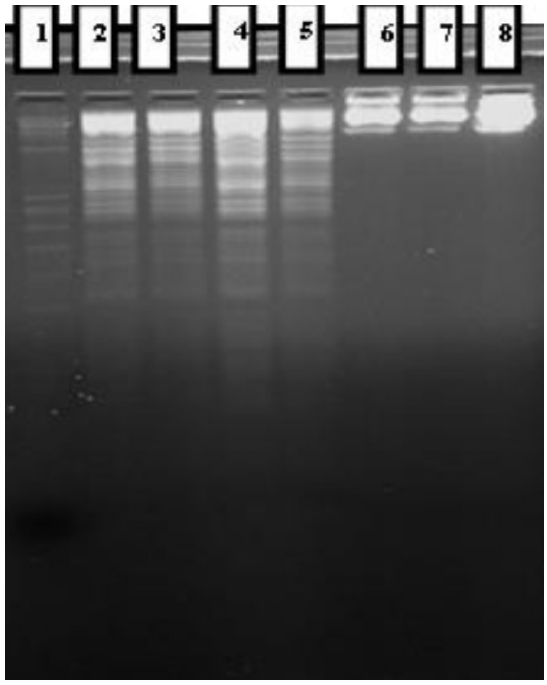


Figure 8: Confirmation Of Change Buffe Has No Effect or Inhibition On The Action Of Enzymes (2% agarose gel at 50 voltages for approximately 3 hours).

Lane one marker ladder.
Lane two lambda DNA with AluI enzyme and its buffer.
Lane three lambda DNA with AluI enzymes and KpnI buffer .
Lane four lambda DNA with AluI and KpnI in AluI buffer .
Lane five lambda DNA with AluI and KpnI in KpnI buffer.
Lane six lambda DNA with KpnI and its buffer.
Lane seven lambda DNA with KpnI and AluI buffer .
Lane eight lambda DNA only as standard.

Restriction Fragment Length Polymorphism

After all the above processes for check up restriction enzymes activity the researcher started to digest the amplified PCR product for ABO loci (200 bp for O alleles and 128 bp for A and B alleles) .This step was very important and complement to PCR . The second step was used to differentiate the alleles type for individuals and its type as homozygous or heterozygous by gel electrophoresis.

In sterilized eppendorff tubes , the amplified product of monoplex PCR of each loci was digested by addition of two restriction enzymes to each tube .Ordinary agarose gel was used in the beginning to detect digestion step , some

desired bands appeared clearly but unfortunately some bands did not appear specially of low moleculer weight like bands of 88bp in Figure (9) and (10).

Therefore the low melting agarose as alternative for ordinary agarose was used. This type has many properties such as separation of wide range of bands with high resolution, easy in preparation (21). Six samples represent all genotypes were selected and digested after many trials to change the concentration of low melting agarose .It was found that 6% was the most suitable concentration gel to detect small band like 88bp in AB , BO, BB alleles Figure(11).

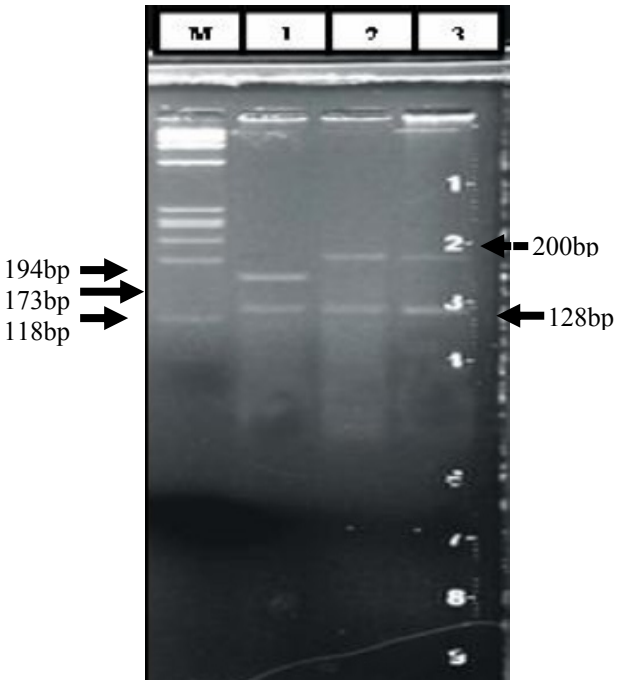


Figure 9: Digestion PCR product of ABO locus on 4% ordinary agarose. (4% ordinary agarose gel at 50 voltages for approximately 3 hours).

Lane M is marker.
Lane 1 O blood group (OO) two bands appear 173bp and 128 bp.
Lane 2 AB blood group (AB) three bands 200bp and 128bp,(88bp does not appear).
Lane 3 A blood group (AA) two bands appear 200 bp and 128bp.

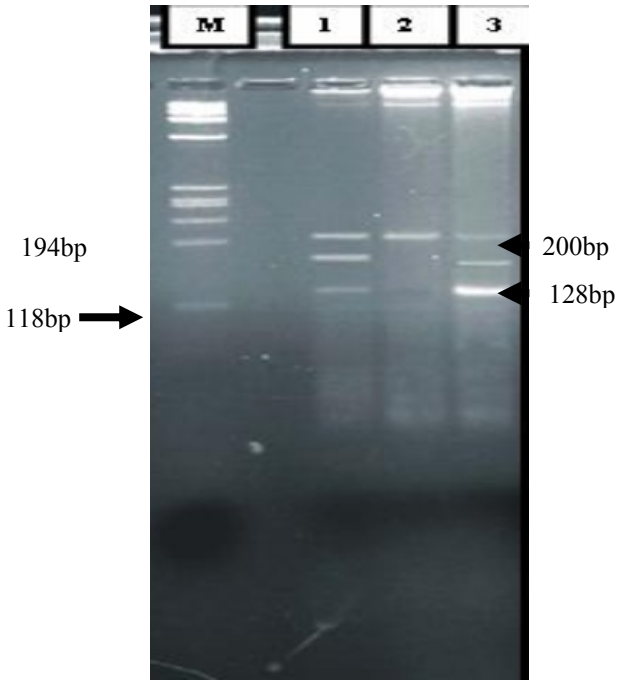


Figure 10: Digestion PCR product of ABO locus on 4% ordinary agarose. (4% ordinary agarose gel at 50 voltages for approximately 3 hours).

Lane M is marker.
Lane one B blood group (BO) four bands 200 ,173 ,128 and (88bp does not appear).
Lane two B blood group (BB) two bands 200 and (88 bp does not appear).
Lane three A blood group (AO) three bands 200, 173 and 128 bp.

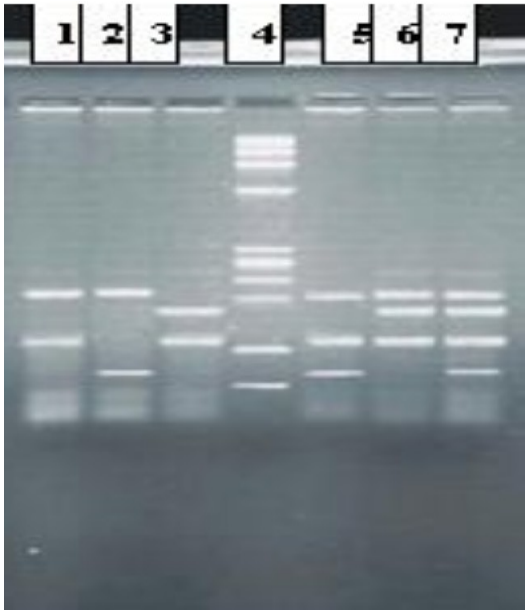


Figure 11: Final Result of PCR-RFLP for Detection Six Common Blood Group. (6% Agarose low gelling /low melting temperation , 50 V for approximately 3,5 hours)

Lane one A blood group phenotype (AA) Homozygous. Two bands appear 200 bp and 128 bp.
Lane two B blood group phenotype (BB) Homozygous. Two bands appear 200 bp and 88 bp.
Lane three O blood group phenotype (OO) Homozygous. Two bands appear 173 bp and 128 bp.
Lane foure ladder marker.
Lane five AB blood group phenotype (AB) Heterozygous .Three band appear 200bp and 128bp and 88pb.
Lane six A blood group phenotype (AO) Heterozygous .Three bands appear 200bp, 173bp and 128bp.
Lane seven B blood group phenotype (BO) Heterozygous. Four bands appear 200bp, 173bp, 128bp and 88 bp .

The molecular genetic basis of the three major alleles of the ABO system in PCR-RFLP method was interpreted in Table (3).
ABO primers (1 + 2) amplified 200 bp fragment (O alleles specific fragment) and required digestion with Kpn I restriction enzyme ,if complete digestion occurred ,the result would be two fragments (173 bp) which appeared clearly and 27 bp was not visible.
In the other hand primers (3 + 4) produced B alleles specific 128 bp fragment and if digested with AluI , 88 fragment appear and 40 bp was not visible and neglected. The bands of PCR-RFLP for ABO system genotyping can be shown and diagramed in Figure (12).
Genomic DNA was analyzed from blood samples of different ABO phenotypes .Thirteen (A) blood group individuals all of which are heterozygous (AO) its common in all population (23, 14).
Therefore the researchers obligated to select the homozygous person by selection of special breeding. All thirteen (B) blood groups are heterozygous. This is common in all populations too, therefore the researchers ought to select Homozygous person by breeding .The draw back of this method is the needs to relativly high quantity of DNA (50ng) in PCR reaction. All the A and B samples showed at least one functional allele ,devoid of the single base deletion .The B samples which can be recognized from A samples by substitution in nucleotide 703.The O sample had the single base deletion at position 261 as common type of mutation. The AB samples showed two functional alleles. All the B and AB samples showed the present of AluI site in B allele (24, 25). In the present study, the PCR –RFLP molecular technique was used for genotyping of the ABO blood groups in thirty samples. The molecular base of thirty samples was analyzed as in table (4).

Table 3: Interpretation of ABO Genotypes by PCR-RFLP Procedure(23).

Genotype	***Kpn*** I digestion (visible bands on gel image)	***Alu***I digestion (visible bands on gel image)
OO	Complete digestion (173 bp)	No digestion (128 bp)
AO	½ digestion (200bp + 173bp)	No digestion (128 bp)
AA	No digestion (200bp)	No digestion (128 bp)
BO	½ digestion (200bp + 173bp)	½ digestion (128 bp + 88bp)
BB	No digestion (200 bp)	Complete digestion (88 bp)
AB	No digestion (200 bp)	½ digestion (128 bp + 88 bp)

Table 4: Moleculer Base (alleles) of Thirty Samples Which Have Been Analyzed According To PCR-RFLP Method.

Samples No	Blood type	Phenotype	Genotype
1 -- 13	A	A	AO
14	A	A	AA
15 -- 27	B	B	BO
28	B	B	BB
29	AB	AB	AB
30	O	O	OO

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