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Genetic Analysis of Coagulation Factor IX (FIX) Gene in Sample of Iraqi Patients With Hemophilia B.

Ismail H. Aziz[1] , Ali H. AL-Musawi[2]

[1]Genetic Engineering and Biotechnology Institute,Baghdad University.
[2]General Directorate of Education in Thi-Qar.

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Abstract: Hemophilia B (HB) is an X-linked recessive bleeding disorder caused by mutations in the coagulation factor IX (FIX) gene. Genotyping patients with HB is essential for genetic counseling and provides useful information for patient management. The aim of this study was to characterize the factor IX gene mutations in 20 unrelated Iraqi males hemophilia B patients from Thi-qar provence. This study was carried out in the Institute of Genetic Engineering and Biotechnology –University of Baghdad, during the period from April, 2016 to April, 2017.Their ages ranged from 4 -30 years .Polymerase chain reaction (PCR) and direct sequencing was performed to detect the mutations in patients. Three functionally regions of the gene (exon 4,exon 5 and exon 7)were screened. Twenty four point mutations were identified, including two missense variant (g15340 del G , p.Aspartic acid convert to Methionine acid) and (.22639 *del* A, p. Asparagine convert to Threonine) , two mutations were silent (g. 22632 del A, g. 22635 del A), while the rest of mutations were intronic mutations. These results conclude that mutations related with of haemophilia B disease.

Keywords: hemophilia B, factor IX gene, missense mutation.

Corresponding author: should be addressed (Email: ahabeeb902@yahoo.com)

Introduction:

Haemophilia B or Christmas disease is a recessively inherited X-linked bleeding disorder which results from deficiency of procoagulant factor IX (FIX). FIX deficiency is characterised by prolonged oozing after injuries, tooth extractions, or surgery, renewed bleeding after initial bleeding has stopped, and delayed bleeding (1).

Up to 30% of cases have no prior history of the disease that arises from new mutations. The incidence of hemophilia B is approximately one in 25000-30000 male live births with a nearly similar prevalence all over the world (20). Among people with hemophilia, approximately 80% have hemophilia A, whereas only 20% have hemophilia B (3). Because of the lack of studies on this gene in Iraq and because it is rare, this study was conducted on this gene.

The disease is caused by different types of mutations in the factor IX gene (FIX). The FIX gene, located on the long arm of the X chromosome at Xq27, spans 33.5 kb of DNA and comprises eight exons (4). FIX is synthesized as a single polypeptide chain that undergoes extensive posttranslational modifications including signal peptide cleavage, disulfide bond formation, glycosylation, vitamin K–dependent gamma-carboxylation of glutamic acid residues in the NH_2 terminal region, beta-

hydroxylation, and propeptide cleavage.(5).

This protein is a vitamin K-dependent serine protease and contains six major domains: signal peptide, propeptide, gla domain, two epidermal growth factor-like (EGF-like) domains, activation and catalytic domains (6). Factor IX is synthesized in the liver as a precursor molecule and is secreted into the plasma (7) after further modification.

Unlike haemophilia A, no common repeat mutation has been identified. However, 20- 30% of cases of mild haemophilia B are due to a small number of founder mutations. Mutations are generally sought in affected males, where available, and then confirmed or excluded in female relatives. Where an affected male is not available, initial mutation analysis may be performed on a known obligate carrier female (8).

Based on the activity level of FIX, HB is classified as severe (<1% of normal), moderate (1–5% of normal), or mild (5–40% of normal) (9).The mutations associated with mild, moderate, and severe phenotypes are distributed evenly throughout the F9 gene (10). Currently, more than 1000 unique variants in the F9 gene (the causative hemophilia B) have been identified worldwide, among which 73% are point mutations, 16.3% are deletions and the remainder are insertions, duplications, or combinations of insertions and deletions (indels) (11).

Large deletions (>50 bp) in the F9 (FIX)gene, 90% of which are associated with the severe phenotype, occur in approximately 5% of patients with severe HB and significantly increased risks for developing inhibitors (10);(12);(13).Although approximately 100 types of large deletions of the F9 gene have been reported, only 19 have defined breakpoints (14);(15);(16).It has been proposed that the underlying mechanisms may be non-allelic homologous recombination (NAHR), non-homologous end joining (NHEJ) or microhomology-mediated break-induced reco --mbination (MMBIR) events (15).

Materials and Methods

This study was carried out in the Genetic Engineering and Biotechnology Institute –University of Baghdad ,20 samples were collected from patients with Hemophilia B (HB) in addition to 20 samples healthy control of Genetic diseases center in Thi-Qar province.During the period from April, 2016 to November, 2016.

Subjects:

This study was included twenty Iraqi patients with Haemophilia B disease and twenty healthy sampls control . The subjects were aged between 4 to 30 years. Patients were selected from Genetic diseases center in Thi-Qar province .The data were collected, and recorded according to a questionnaire forma. Venous blood sample 5 ml was collected from each patients and healthy control together with medical staff specialist from Genetic diseases center . Each blood sample was collected in to EDTA tubes for Molecular studies .

Genomic DNA isolation:

Genomic DNA were extract using gsync DNA mini kit 100 prep(Geneaid,Taiwan).PCR was performed using AccuPower® PCR pre Mix(Bioneer,south korea) . The extracted DNA were measured by ND-2000 spectrophotometer (Thermo Scientific Inc., USA). The extracted DNA were stored −20◦C until use.PCR technique were done using specific primers (Table 1).

Table(1): Sequences of primers (F9 Gene) used in this study(17).

Region	Primers	Sequences	Anneling	Band size
Exon 4	F	5-GGCTTCCAGGTCAGTAGTTTTGC-3	60 C	308 bp
	R	5-TTTTCCAGTTTCAACTTGTTTCAGAGG-3		
Exon 5	F	5-AAATGATGCTGTTACTGTCT-3	56 C	229 bp
	R	5-GTTTGTTAAAATGCTGAAGT-3		
Exon 7	F	5-CAAATGTATTATGCAGTAAGAG-3	56 C	218 bp
	R	5-TGTACCAATCATATTAAAGAGC-3		

F:Forward , R: Reverse bp: base pair

PCR Programs:

1- PCR was carried in Veriti™ thermal cycle (Applied Biosystem) using the standard cycle procedure was a 5-minute denaturation at 95 °C for one cycle, then 30 cycles of 30 seconds of denaturation at 95 °C, 30 seconds of annealing 60°C, 30 seconds extension at 72 °C and 5 min for final extension at 72 °C. PCR products and the DNA ladder were used by 2% agarose gel (2g agarose/100 ml 1X TBE buffer) and run at 100 volt for 75 min electrophoresis. 5 µl of DNA ladder was loaded on 2% agarose gel and that used to estimate the molecular size of the PCR products bands. Fig (1,2 and 3),PCR products were then analyzed by sequencing. For exon 4.

2- PCR was carried in Veriti™ thermal cycle ,(Applied Biosystem) using the standard cycle procedure was a 5-minute denaturation at 95 °C for one cycle, then 30 cycles of 30 seconds of denaturation at 95 °C, 30 seconds of annealing 56°C,30 seconds extension at 72 °C and 5 min for final extension at 72 °C. PCR products were then analyzed by sequencing for exon 5 and DNA sequencing.

The PCR products 20 samples of the exon 4 , exon 5 and exon 7 gene primer was sending to Macrogen company (south korea) for sequencing. Sequence analysis was performed by direct sequence of the PCR products.

Results and Discussion:

PCR was use to amplify the target fragment which was specified by using specific primers.The fragment size amplified was 308 bp in the Exon 4 region , 229 bp in the Exon 5 region and 218bp in the exon 7 region of f9 (FIX) gene,as shown in Figure(1),(2) and (3).

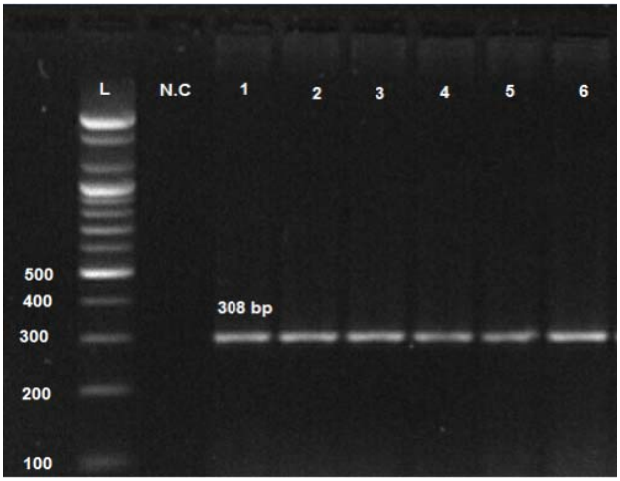

Figure (1): PCR product (308bp) exon 4,visualized under UV light after staining with ethidium bromide.The electrophoresis was on 2% agarose gel at 1 volt/cm^2 for 75 min;L= DNA ladder =100bp;N.C=negative control.

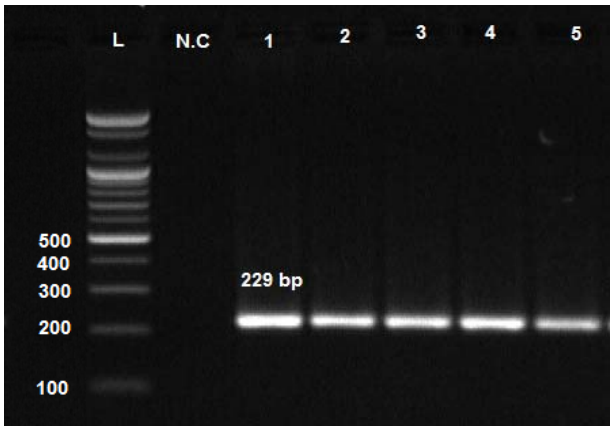

Figure (2): PCR product (229bp) exon 5 visualized under UV light after staining with ethidium bromide.The electrophoresis was on 2% agarose gel at 1 volt/cm^2 for 75 mints;L=DNA ladder = 100bp ;N.C =negative control.

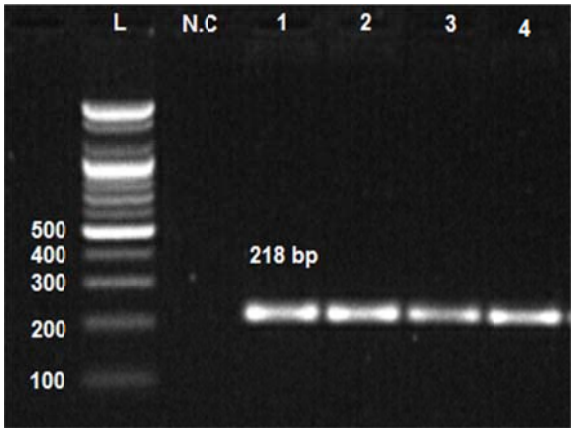

Figure (3): PCR product (218bp) exon 7 visualized under UV light after staining with ethidium bromide.The electrophoresis was on 2% agarose gel at 1 volt/cm^2 for 1hour 15 mints;L= DNA ladder = 100bp ;N.C=negative control.

Dna Sequencing:

PCR products were sequenced at macrogen (south korea). In Figure (4), Figure (5), and Figure (6), show the deletion and substitution mutation ocured in exon 4 ,exon 5 and exon 7 of F9 gene for 14 patients .

Figure (4): Exon 4 , DNA Sequencing(Deletion)

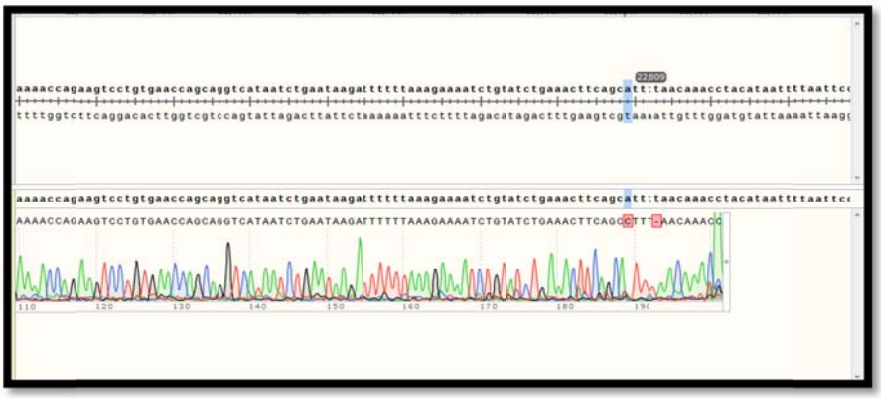

Figure (5): Exon 5 , DNA Sequencing(substitution)

Figure (6): Exon 7 , DNA Sequencing (Deletion).

Table (2): Mutations in *F9* gene .

Mutations	Locatin	Type	Effect	Originality
g.15257 C>G	Intronic	transversion	-	Novel
g.15260 A>T	Intronic	transversion	-	Novel
g.15262 C>T	Intronic	transition	-	Novel
g.15264 C>G	Intronic	transversion	-	Novel
g.15265 *del* A	Intronic	deletion	Frame shift	Novel
g. 15265 A>G	Intronic	transition	-	Novel
g. 15339 *del* A	Intronic	deletion	Frame shift	Novel
g. 15340 *del* G	Aspartic acid > Methionine	deletion	Missense	Novel
g. 15314 C>T	Intronic	transition	-	Novel
g. 15515 T>G	Intronic	transversion	-	Novel
g. 15517 ins G	Intronic	insertion	Frame shift	Novel
g. 22626 *del* A	Intronic	deletion	Frame shift	Novel
g. 22632 *del* A	Valine > Valine	deletion	Silent	Novel
g. 22635 *del* A	Threonine > Threonine	deletion	Silent	Novel
g.22639 *del* A	Asparagine > Threonine	deletion	Missense	Novel
g. 22809 *del* A	Intronic	deletion	Frame shift	Novel
g.22809 T>C	Intronic	transition	-	Novel
g.22810 T>G	Intronic	transversion	-	Novel
g.22813 *del* T	Intronic	deletion	Frame shift	Novel
g. 34997 *del* T	Intronic	deletion	Frame shift	Novel
g. 35001 *del* C	Intronic	deletion	Frame shift	Novel
g. 35003 *del* C	Intronic	deletion	Frame shift	Novel
g. 35015 *del* A	Asparagine > Methionine	deletion	Frame shift	Novel
g. 35170 *del* A	Intronic	deletion	Frame shift	Novel

As point mutations account about 90% of cases, direct sequencing can be the first-line diagnostic modality for molecular diagnosis in HB (18). Screening for mutation within the F9 gene in 20 Iraqi hemophilia B patients by PCR and sequencing of the entire coding sequence of F9 gene led to

characterization of all responsible mutations,Table 2.According to our information in Iraq no studies were done about hemophilia B.The genotyping for HB in various countries performed over the past decades is documented in HB databases(19,20).The molecular analysis revealed 24 point mutations in these cohorts. These mutations included 5 transition mutations, 4 transition mutations, and 15 deletion (table 2). CpG dinucleotides are considered to be hot spots for mutations and are responsible for the recurrent mutations in unrelated families due to the spontaneous deamination of 5-methylcytosine to thymidine (21). Of the 24 point mutations in our study, two missense variant (15340 *del* G , p.Aspartic acid convert to Methionine acid) and (.22639 *del* A, p. Asparagine convert to Threonine) . For missense mutations, the risk is almost zero (22). In other hand, two mutations were silent (g. 22632 del A, g. 22635 del A), In particular, these rearrangements can be the genetic backgrounds of female (Carrier)HB with or without family history. while the rest of mutations were intronic mutations. These mutations could cripple the normal cleavage process of FIX during its secretion or activation ,related with haemophilia B disease.

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