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# Molecular Detection of Intron 22 Inversion of Factor VIII Gene in Egyptian Hemophilia A Patients

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## ABSTRACT

Hemophilia A (HA) is an X-linked bleeding disorder caused by a wide variety of mutations in the factor VIII (FVIII) gene, leading to absent or deficient factor VIII. We analyzed the FVIII gene of forty Egyptian patients with HA. The analysis included the investigation of intron 22 restriction fragment length polymorphism (RFLP) by PCR using Xbal restriction enzyme, screening of FVIII gene for other molecular abnormalities as deletions and point mutations by multiplex PCR and screening FVIII for a point mutation in exon 25 by using BsrDI restriction enzyme. Our research revealed the following molecular abnormalities in the studied HA patients: FVIII gene RFLP in intron 22 by using Xbal restriction enzyme: 30% Xbal (+), 67.5% Xbal (-ve), 2.5% intron 22 deletion, partial FVIII gene deletion in 10% of cases and exon 25 point mutation in 7.5% of cases. We concluded that the study of intron 22 polymorphism by Xbal restriction enzyme may be used to select the HA patients who have a severe disorder and are negative for the Xbal polymorphic marker, or who have intron 22 deletion to be investigated for FVIII gene intron 22 inversion.

***Key Words:*** *Hemophilia A – Factor VIII gene – Intron 22 inversion.*

## INTRODUCTION

Deleterious changes in the human FVIII gene reduce activity and/or circulating plasma levels of factor VIII (FVIII) protein causing (HA). Patients with HA are classified according to their plasma procoagulant levels of FVIII: severe (<0.01 IU/mL), moderate (0.01-0.05 IU/mL), or mild (0.05-0.4 IU/mL) **[1]**. The FVIII gene is located at Xq28 and is extremely large (~ 180 Kb) and structurally complex (26 exons); intron 22 of the gene contains a CpG island, which acts as a bidirectional promoter for two further genes, FVIIIA and FVIIIB. The CpG island and FVIIIA are contained within a stretch of DNA of approximately 9.5 Kb, which is repeated at least twice on the X chromosome, further towards the telomere and extragenic to the factor VIII gene, these homologues are known as int22h-1 (intragenic) and int22h-2 and int22h-3 (extragenic) **[2]**.

FVIII gene contains two types of polymorphism: single nucleotide polymorphisms (SNPs), and length polymorphisms, also known as variable number tandem repeat sequences (VNTRs), both types exist in the normal population **[3]**. One of the single nucleotide polymorphic sites is present within the int22h-1 sequence, it falls in the subcategory of restriction fragment length polymorphisms (RFLPs) and it resides within the recognition sequence for the Xba I restriction endonuclease, thus it is known as the Xba I RFLP **[4]**.

Abnormalities in FVIII gene include deletions, insertions, point mutations and inversion. FVIII gene inversions are rare defects in hemophilia, except for an inversion involving intron 22 of the FVIII gene, which causes severe HA, and which is found in 40-50% of patients with severe disease **[6]**. The intron 22 inversion arises through homologous recombination between int22h-1 and int22h-2 (proximal) or int22h-3 (distal) during meiosis. The distal inversion is more common than the proximal inversion, which may be explained by the greater genetic distance between the factor VIII gene and the distal int22h-3 homologue facilitating the formation of the loop required for alignment to take place **[4]**.

Here, we investigated intron 22 restriction fragment length polymorphism (RFLP) by polymerase chain reaction using XbaI restriction enzyme in hemophilic patients aiming to determine patients who are more likely to have intron 22 inversion, the most common genetic abnormality encountered in patients with severe HA. In addition, screening FVIII gene for other molecular abnormalities as deletions and point mutations by multiplex PCR technique followed by agarose gel electrophoresis was done and also, a point mutation in exon 25 was screened using BsrDI restriction enzyme.

## MATERIALS AND METHODS

*Patients:*

The study included 40 patients attending the out patient clinic of the National Blood Transfusion Centre with clinical and laboratory confirmation of HA with age range from 4 to 19 years and 10 age and sex matched controls after obtaining their informed consent. 14 patients were classified as severe HA, 22 patients as moderate HA and the remaining 4 were mild cases. Moreover, the patients were defined as familial (positive) or isolated (sporadic) according to their available pedigree information.

*DNA extraction and analysis:*

DNA was extracted from peripheral blood samples using the salting out technique according to Miller et al. **[5]**.

*I- Determination of the factor VIII gene restriction fragment length polymorphism in intron 22 using XbaI restriction enzyme:*

PCR amplification was performed in a final volume of 50 ul (1 ul DNA, 25 ul Taq PCR Master Mix [Promega cat no.M7502], 1 ul primer; each sense and antisense [Qiagen Operon] and 22 ul distilled water).

The nucleotide sequence of the sense primer is 5`CACGAGCTCTCCATCTGAACATG 3`and the antisense primer is 5`GGGCTGCAGGGGGGGGGGACAACAG 3`.

The PCR reaction was carried out in the DNA thermal cycler (Perkin Elmer 9600) and the computerized thermocycler was programmed for the following conditions: 33 cycles of 1 minute at 94ºC, 1 minute at 58ºC and 1 minute at 72ºC.

17 ul of the amplified product were mixed with 1 ul XbaI restriction enzyme (Fermentas Life Sciences Cat no. number ER0683) with recognition sequence 5`T^CTAGA 3` 3` AGATC^T5` and the mixture was incubated at 37ºC for 16 hours.

The product was analyzed by gel electrophoresis using 4% agarose gel (Promega cat no. V 3121) and ultraviolet light transillumination.

*II- Factor VIII gene mutations screening by multiplex PCR technique:*

Multiplex PCR allowed simultaneous and rapid amplification of multiple exons of the FVIII gene, followed by detection of the amplified PCR product using agarose gel electrophoresis. The procedure was carried out as mentioned in the previous step.

The sequence of the oligonucleotide primers used to amplify 11 exons of the factor VIII gene is given in Table (1).

*III- Assessment of a point mutation in exon 25 using the restriction enzyme BsrDI:*

A mutant primer was used to detect nucleotide change from ATG to GTG.

PCR amplification was done using both the mutant primer and the 3`primer of exon 25 in a reaction mixture of 50 ul volume (25 u Taq PCR Master Mix, 1 ul from each of the 3 diluted sense and antisense primers [primer sequence is shown in Table (1)], 1 ul DNA, 21 ul DW).

The PCR cycles proceeded as follows: 33 cycles of 1 minute at 94ºC, 1 minute at 58ºC, and 1 minute at 72ºC and a final extension of 6 minutes at 72ºC.

PCR amplification was followed by digestion of the amplified exon 25 by the BsrDI restriction enzyme (Fermentas Life Science Cat no. ER 1261) which recognizes the sequence 5`GCAATGNN^ 3` 3` CGTTAC^NN 5`.

Then analysis of the product was done by agarose gel electrophoresis and ultraviolet light transillumination.

Table (1): Oligonucleotide sequence of the primers used to amplify factor VIII Gene.

| Exon | Sequence | PCR size (bp) |
|---|---|---|
| 2 | 5`TGAAGTGTCCACCAAAATGA 3`<br>5` TACCCAATTTCATAAATAGC 3` | 207 |
| 3 | 5` GTACTATCCCCAAGTAACCT 3`<br>5` TATTCATAGAATGACAGGAC 3` | 208 |
| 4 | 5`ACAGTGGATATAGAAAGGAC 3`<br>5` TGCTTATTTCATCTCAATCC 3` | 295 |
| 5 | 5` CTCCTAGTGACAATTTCCTA 3`<br>5` AGCAGAGGATTTCTTTCAGG 3` | 187 |
| 6 | 5` CATGAGACACCATGCTTAGC 3`<br>5` CTGGTGCTGAATTTGGAAGA 3` | 220 |
| 7 | 5` TCAGATTCTCTACTTCATAG 3`<br>5` GAAACTGTGCAAGGTCCATC 3` | 225 |
| 12 | 5` CTAGCTCCTACCTGACAACA 3`<br>5` GACATCACTTTGATTACATC 3` | 283 |
| 22 | 5` TCAGGAGGTAGCACATACAT 3`<br>5` GTCCAATATCTGAAATCTGC 3` | 288 |
| 23 | 5` CTCTGTATTCACTTTCCATG 3`<br>5` GATATTGGATGACTTGGCAC3` | 214 |
| 24 | 5` GCTCAGTATAACTGAGGCTG 3`<br>5` CTCTGAGTCAGTTAAACAGT 3` | 249 |
| 25 | 5` GAATTTCTGGGAGTAAATGG 3`<br>5` GCTTACCTTTACTTTGCCAT 3`<br>5`GGATTCCTGCAAGTGGACTTC CAGAAGGCA3` | 322 |

## RESULTS

Analysis of the clinical data showed that 77.5% (31) of the patients had one or more relatives affected on the maternal side of the family so their mothers could be obligate carriers; this is of importance for genetic counseling for the future risk of giving birth to a hemophiliac son as obligate carriers may transmit the hemophiliac gene to 50% of their children, males or females. On the other hand, 22.5% (9) of the patients had negative family history for the disease, these are considered as isolated cases which could be explained by the fact that isolated cases of hemophilia may result from transmission of the hemophilia gene through asymptomatic females in whom the gene has remained undetected; from a new mutation in the mother, resulting in her being a carrier; or a new mutation in the hemophiliac (true de novo mutation). The existence of somatic mosaicism has also to be taken in consideration.

Study of the intron 22 RFLP by the XbaI restriction enzyme showed that 27 out of 40 patients (67.5%) were negative for the XbaI polymorphic marker; (11 patients had marked severity, 14 cases had moderate severity and 2 cases had mild severity), while 12 patients (30%) were positive for this marker; (3 cases had marked severity, 7 cases had moderate severity and 2 cases had mild severity) and one patient (2.5%) had absent DNA polymorphic fragment indicating deleted intron 22. No significant relation was found between the XbaI polymorphic status and none of the clinical or laboratory data ($p$ value >0.05) Fig. (1).

FVIII gene screening revealed that 10% (4/40) of hemophilia A cases had partial gene deletions. The deletion represented 28.5% (4/14) of severe hemophilia A patients and were found in exon 24, exons 23-24, exons 12-22 and a fourth deletion in one or more of the exons 2, 3, 6 or 7, however the identity of this deletion could not be determined precisely as the 4 amplified PCR fragments had the same electrophoretic migration site on the agarose gel (4%) used to analyze the PCR products. A significant relation was found between the presence of FVIII gene deletion and the severity of the disease where all patients had a severe disorder ($p$ value ≤0.01) Figs. (2,3).

Assessment of the point mutation affecting exon 25, at codon 2238 where the amino acid methionine is replaced by valine using PCR technique where the BsrD I restriction enzyme and a mutated primer creating an artificial site for the enzyme were used, fragments of 140 bp and 29 bp were obtained for the normal gene, when A→G mutation was present, the sequence was not recognized by the enzyme and only a fragment of 169 bp was obtained (Fig. 4). Our research revealed that 3 patients (with a diagnosis of moderate hemophilia) out of 40 patients (7.5%) were found to have this point mutation, however, no significant association was found between this point mutation and the clinical or laboratory data of these patients ($p$ value >0.05), nor with FVIII gene deletion ($p$ value =0.86) or XbaI polymorphic status ($p$ value =0.69).

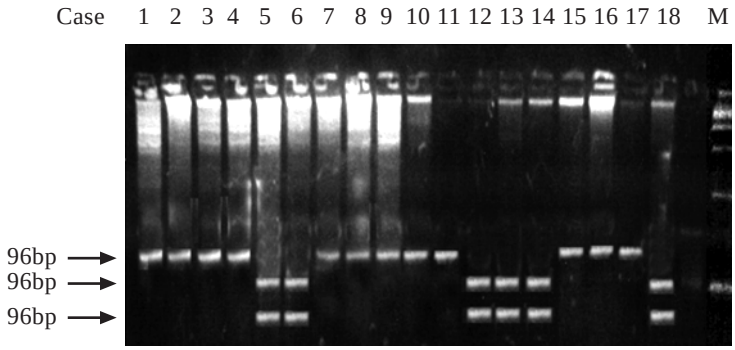


Fig. (1): Characterization of the polymorphism in intron 22 of the factor VIII gene using xbai restriction enzyme.

Cases 5, 6, 12, 13, 14 and 18 are XbaI (+) where 2 DNA fragments of 68 and 28bp were obtained, while the XbaI (-) ve cases showed single DNA fragment of 96bp.
M: PCR marker (50-100-150-200-250-300-350-400-450-500-550-600-650-700-750-800 bp).

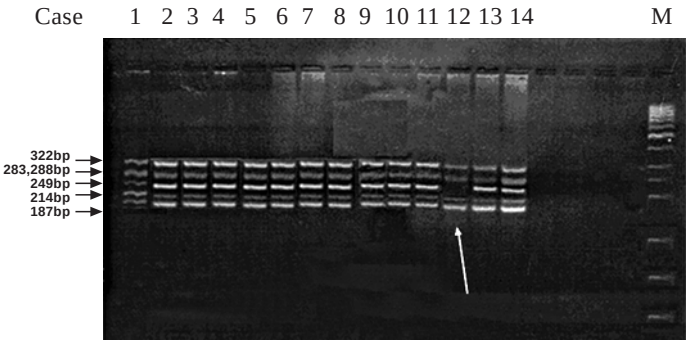


Fig. (2): Agarose gel electrophoresis 4% analysis of multiplex PCR products resulting from amplification of the part of factor VIII gene using primers for exons 5, 12, 22, 23, 24 and 25.

The fragments corresponding to exons 5, 12, 22, 23, 24 and 25 are identified on the left side.
Case 12 shows absence of DNA fragment corresponding to exon 24.
M: PCR marker (50-100-150-200-250-300-350-400-450-500-550-600-650-700-750-800 bp).

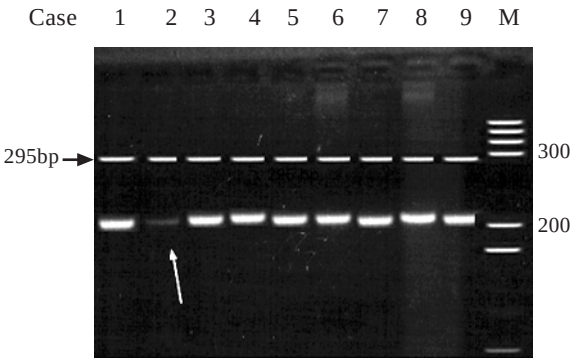


Fig. (3): Agarose Gel Electrophoresis 4% analysis of multiplex PCR products resulting from amplification of the part of factor VIII gene using primers for exons 2, 3, 4, 6 and 7.

The fragment corresponding to exon 4 is present in all cases while the other fragment represents exons 2, 3, 6 and 7 where the electrophoretic migration site was the same (207, 208, 220, 225 bp respectively).
Case 2 shows a faint DNA fragment, denoting the deletion of one or more of either exons 2, 3, 6, or 7.
M: PCR marker (50-100-150-200-250-300-350-400-450-500-550-600-650-700-750-800 bp).

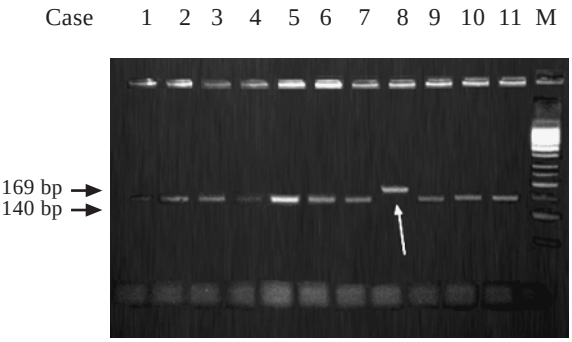


Fig. (4): Characterization of the mutation at exon 25, codon 2238.

After digestion with BsrDI, fragments of 140 and 29 bp were obtained for the normal nucleotide sequence. When the nucleotide substitution A_G is present, digestion with BsrDI results in a fragment of 169 bp as seen in case 8.

M: PCR marker (50-100-150-200-250-300-350-400-450-500-550-600-650-700-750-800bp).

## DISCUSSION

Hemophilia A is an X-linked recessively inherited bleeding disorder characterized by deficiency of procoagulant factor VIII with an incidence of 1/5 000-10.000 male births. Except for the common inversion mutation in intron 22 of FVIII gene, most other changes are point mutations and deletions **[6]**.

The inversion of intron 22 was found in 40-50% of patients with severe HA **[7]** with a prevalence of about 47.5 to 53% in India population **[8]** and 40-50% in European **[9]**.

Direct mutation detection is essential for carrier detection and prenatal diagnosis in the family members of Egyptian hemophilic patients and this if achieved will decrease the incidence of the disease. In addition, it will avoid the hazards complicating therapy, including disease transmission and the development of inhibitors to FVIII substitutes.

In order to find mutations in a gene as large as FVIII gene, a simple method has to be applied. The use of PCR to search for RFLP of intron 22 is known to be useful to select cases likely to have intron 22 inversions. This was followed by screening for small mutations and deletions in FVIII gene using multiplex PCR followed by agarose gel electrophoresis and assessment of a point mutation in exon 25 using the restriction enzyme BsrDI.

The intron 22 inversion arises through homologous recombination between int22h-1 and int22h-2 (proximal) or int22h-3 (distal) during meiosis **[7,10]**. Thus, in haemophilia A families in which the intron 22 inversion is the causative gene defect, the defect can often be shown to have originated in an unaffected male relative, and in sporadic cases, this is often the patient's grandfather on his mother's side of the family **[11]**. This explains the presence of 22.5% of the cases included in our study with no family history for hemophilia A.

The work of several authors agreed with our findings regarding the study of the intron 22 RFLP by the XbaI restriction enzyme; El Maari et al. **[12]** studied intron 22 by RFLP, they showed that 52% of cases were negative for XbaI polymorphic marker FVIII gene haplotype study, they studied intron 22 inversion by Southern blotting technique and the study revealed that 29% of cases had intron 22 inversion. At the same time the haplotype of factor VIII gene revealed that the prevalence of one haplotype in which XbaI is negative was higher in the inversion patients. Also, when intron 22 polymorphism was studied **[13]**, int22h-1 gene deletion was reported and was associated with factor VIIII gene inversion. The author concluded that deletions involving intron 22 of the factor VIIII gene are associated with increased incidence of gene inversion and are associated with severe hemophilia A. These results had led us to speculate that the study of intron 22 polymorphism by XbaI restriction enzyme may be used not only to track the abnormal gene during family study, but also to select hemophilia A patients who have a severe disorder and negative for XbaI polymorphic marker, or who have intron 22 deletion to be investigated for intron 22 inversion.

On the other hand, a similar study was performed **[14]** and the results disagree with this hypothesis as they found no association between the presence of the intron 22 inversion and RFLP haplotype.

Multiplex PCR screening for FVIII gene revealed that 10% of the studied cases had partial gene deletion and this was associated with severe disease as FVIII gene deletions have a high probability of destroying the genetic function, removing part of FVIII protein, or introducing a frameshift, all of which are associated with a severe disease **[6]**. Exon 24 deletion was also reported **[15]** and was associated with a severe disease. The worldwide database for hemophilia A in 1995 also reported deletions affecting exon 24; 11 to 22, 23-24 all were associated with severe disease. This was also consistent with the results of a previous study where three partial deletions of factor VIII gene were characterized: a deletion of at least 7 Kb eliminates exons 24 and 25 and a deletion of 16 Kb deletes exons 23-25 and a 5.5 Kb deletion eliminates exon 22. In all these deletions non sense codons were generated resulting in a severe disease **[16]**.

The point mutation detected in exon 25, codon 2238 in three of our patients was also reported in a former study **[17]** that stated that this point mutation is responsible for the disease.

The results of FVIII gene mutation screening by multiplex PCR and the screening for exon 25 point mutation suggest that the spectrum of gene defects in Egyptian hemophiliacs is as heterogeneous as reported in other populations. In addition multiplex PCR followed by agarose gel electrophoresis offers a simple and sensitive way for mutation screening which is essential for carrier detection and prenatal diagnosis of this fatal disorder.

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