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Real-time inverse PCR, a substitute and rapid detection method for intron 22 inversion mutation in patients with severe Hemophilia A

Sayed Hamid Mosavi[1]

1- Scientific board member of medicine faculty, Kateb University, Kabul, Afghanistan
dr.mousavi@kateb.edu.af

Abstract

Hemophilia A (HA) is one of the most deleterious X-linked bleeding disorders in male patients. Early and rapid detection could provide management strategies for this disease. This study aims to improve the classic method for detection of important intron 22 inversion mutations (INV22). Whole blood samples were taken from 21 male children with a history of severe HA, referred to the Iranian comprehensive hemophilia care center. Two groups were involved for detection of INV22 and two methods were examined in a double-blind manner. The first method used a classical method, inverse PCR, and the second method was Real-time inverse PCR. Results showed that both methods could separately detect the INV22 in 11 out of 21 patients with severe HA (52%) in the same accuracy, but with this difference that only one day was needed for detecting of INV22 by Real-time inverse PCR and on other hand 3 days were needed for the classical methods. Because of the medical importance of early detection or prenatal/postnatal diagnosis of this disease, this study recommends the Real-time inverse PCR technique for the rapid detection of this mutation in well-equipped genetic laboratories everywhere.

Keywords: *Hemophilia A, Factor VIII, Intron 22 Inversion, Real-time inverse PCR*

Introduction

Hemophilia A (HA) is the most common bleeding disorders in developing countries caused by a deficiency/defect in coagulation factor VIII (FVIII) (1). The frequency of this disease is about 1 in 5,000–10,000 newborns. Spontaneous bleeding episodes after minor trauma was shown in patients with severe HA. (2). The patients have to undergo the substitution coagulation factor for their lifelong, through injections of FVIII into the bloodstream (3, 4). For diagnosing the disease, measurement of the activated prothrombin thromboplastin time (aPTT) comes first, which could be prolonged and also prothrombin time (PT), which is generally normal. Then, the estimation of FVIII activity is important, which will reduce this disease (5). The severity of the disease is determined, in terms of the level of normal FVIII activity in plasma, severe having < 1% of normal levels, moderate 1–5%, and mild 6–30% (6). The FVIII gene located at Xq28 position at the telomeric end of the X chromosome comprising 26 exons and 25 introns encoding 2332 amino acids. There are high GC regions within the 9.1 kb coding region of FVIII make it hyper mutable representing 140 different potential base-pair changes (7). The known and causative mutations of the FVIII gene in severe HA are very heterogeneous (8), but the most prevalent HA mutation is the intron 22 inversion (Inv22), which accounts for about 42-50 % of severe cases worldwide (9) and 39.5% in Iran (10). Intron 22 inversion originates in male germ cells (11) and from homologous recombination between the int22h-1 region within the FVIII locus and either int22h-2 (Inv22 type 2) or int22h-3 (Inv22 type 1), which lie approximately 400 kb distal to FVIII (12). The classical method for detecting of INV22 mutation in patients with hemophilia is the inverse PCR (I-PCR), but , some researchers have tried to modify this method using other techniques such as long-distance PCR (LD-PCR) (13) or LD Real-time PCR (14), which are relatively rapid, but their inappropriate sensitivities may cause difficulty in the interpretation of results. This report presents a reliable method for detecting Inv22, using Real-time inverse-PCR in individuals affected with severe HA.

Materials and methods

Whole blood samples of 21 unrelated male babies have randomly taken over one year (started in 2015) with their parents' consent. They were affected by severe HA, and referred to the Iranian Comprehensive Haemophilia Care Center (ICHCC).

Some of the samples were analyzed immediately, while others stored at minus 80°C. Genomic DNA extracted from the samples using the High Pure PCR Template-Preparation Kit (Roche Applied Science, Germany) according to the manufacturer's instructions. The quality and quantity of extracted DNA assessed by agarose gel electrophoresis and spectrophotometrically, and stored in aliquots at -20◦ C until required.

The study is carried out on two independent groups to detect the known inversion mutation in intron 22 (INV22) in a double-blind manner using two method. The first method was detecting classic method, I-PCR, and the second method was detecting Real-time inverse PCR. For the first group I-PCR method was used that is described by Rossetti et al with some modification (15). Without purification step of digestion of DNA by a restriction enzyme, the *Bcl I* -digested DNA ligated with T4 DNA ligase and B-rings amplified using special primers under standard PCR condition. The sequences of the primers used in this method are as follows: IU (intragenic upstream; 5 -CCTTTCAACTCCATCTCCAT-3; Accession No. BX842559, nucleotides 35744 –35763); ID (intragenic downstream; 5 -ACATACGGTTTAGTCACAAGT-3; Accession No. BX842559; nucleotides 14622–14602), and ED (extragenic downstream; 5 -TCCAGTCACTTAGGCTCAG-3 , proximal Accession No. BX682237, nucleotides 15364 –15347; distal accession No. BX276110, nucleotides 14604 –14621). As mentioned in the procedure, it is expected that the products with 487 bp band specified for the normal and 559-bp for the mutant alleles visualized in 1.5% agarose gel electrophoresis. Other group used the Real-time PCR after getting B-ring for detecting INV22 in affected samples with severe HA (named Real-time inverse PCR method). They had to have more purified DNA samples. Therefore, 2µg of extracted DNA digested overnight in a 50µl reaction volume containing *Bcl* I enzyme (10 U/µl; Fermentas) under conditions recommended by the manufacturer. Then, fragmented DNA was undergone to the ligation reaction by T4 DNA ligase (Fermentas), and incubated overnight at 4 °C. Ligated DNA extracted in phenol-chloroform. Real time-inverse PCR performed on the 3-6 µl of eluted DNA (nearly 200 ng of B-rings) in a 20 µl of reaction volume, using specific primers as previously described and using Light Cycler ® Fast Start DNA Master plus SYBR Green. The initial denaturation step was 10 min at 95 °C followed by 40 cycles of 95 °C for 20 s, 65 °C for 20s, and 72 °C for 20s; After that, melting curve obtained for the validity of the amplification results. Finally, 5µl of Real-time inverse PCR products were visualized on the 1.5% agarose gel electrophoresis.

Results

The results of this study considered in two concepts: First, the rate of prevalence of the INV22 mutation in patients with severe HA. Second, evaluation of using the recommended method for rapid detection of INV22 mutation in the same patients. The results show that both classical and recommended method accurately detected the INV22 mutation in patients with severe HA. In this regard, only 11 out of 21 (52%) patients had the INV22 mutation in both experimental groups. Consequently, using Real-time inverse PCR on all of the samples, we obtained the same results in the less-time consuming manner (for only one day instead of 3 days) in comparison with I-

PCR method. Figures 1a and 1b show the bands for samples with (559 bp) and without INV22 mutation (487 bp) on the gel agarose electrophoresis, respectively. Figure 2 shows the melting curves of most of the samples applied in Real-time inverse PCR with (Tm= 83°C) and without (Tm= 85 °C) INV22 mutation.

Discussion

We have described a reliable method (Real-time inverse PCR) for genotyping of INV22 mutation in patients with severe HA. Results of the first step of this study showed that 11 out of 21 DNA samples (52%) had the Inv22 mutation in subjects affected with severe HA which were confirmed by I-PCR method and Real-time inverse PCR. Regarding the frequency of INV22 mutation, results indicate that the prevalence of INV22 mutation is variable, ranging from 36% in Spain patients to 55% in the Netherlands (16). Our results in this study are similar to those of Xue F. et al in China (2010), which was 51% (17). The advantage of described method is the rapid and much more sensitive diagnosis of Inv22 mutation in comparison with I-PCR. Meanwhile, the time required for I-PCR exceeds 3 days including overnight incubation to achieve *BclI* digestion, the same for fragments ligation, two-step purification, doing PCR and finally post PCR phases, while Real-time inverse PCR amplification usually takes only one day. This is the first report showing that the I-PCR method can be improved by using Real-time inverse PCR

Conclusion

This study recommends the Real-time inverse PCR technique for the rapid detection of mutation in well-equipped genetic laboratories.

Conflict of Interest

The authors declared no conflict of interest.

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Figure 1. Gel agarose electrophoresis for patient samples with INV22 (a) and without INV22 (b) after doing Real-time inverse PCR. M= DNA 50 bp ladder marker.

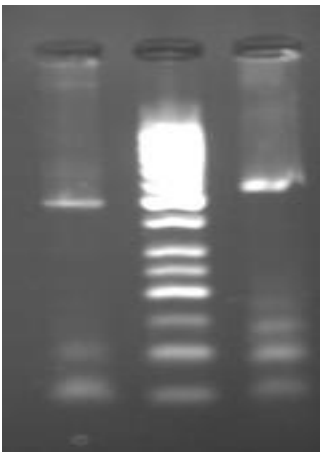

Figure 2. Real-time inverse PCR melting curves analysis of most of samples: 7 cases with INV22 mutations (Tm= 83°C) and 4 cases without ones(Tm= 85 °C).