A modified IS-PCR protocol for easy detection and interpretation of inversion 22 in severe haemophilia A

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TOPIC: Laboratory Issues

INTRODUCTION

Haemophilia A is the most common inherited bleeding disorder caused by mutations within the factor VIII gene (*F8*). A high rate of mutation has been reported. The molecular diagnosis is important for the management of subjects with this condition and useful for genetic counselling of at-risk family members.

Inversion of intron 22 (Inv22) is a frequent mutation encountered in up to 45 % of families with severe haemophilia A. Identification of Inv22 is challenging due to particular molecular characteristics of the *F8* gene such as size of intron 22 (32.8 kb) and presence of GC rich sequence of 9.5 kb. Several methods have been developed to identify Inv22, such as Southern-blot, long distance-PCR (LD-PCR) and more recently inverse-shifting PCR (IS-PCR)[1]. IS-PCR is the most recent technique described for Inv22 genotyping, which is used to detect Inv22 type 1 (Inv22-1), type 2 (Inv22-2), int22h-mediated deletions (Del22) or duplications (Dup22) in subjects as well as in carriers[2].

We described herein a modification of the IS-PCR (mIS-PCR) to improve the precision and reliability of Inv22 detection.

MATERIAL AND METHODS

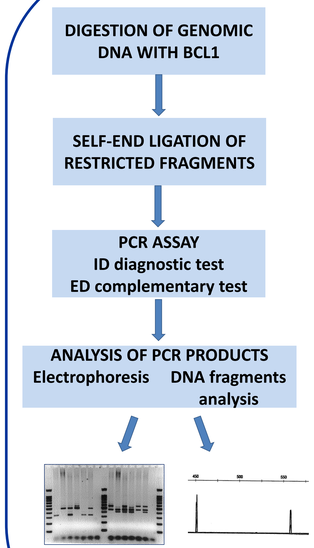

Figure 1. Steps of Inv22 diagnostic test by mIS-PCR

Inv22 detection was performed by IS-PCR modifed from *Rossetti et al.*[2] (mIS-PCR).

Subjects and DNA samples extraction: mIS-PCR was performed on genomic DNA isolated from whole blood samples (40 cases), from cultured amniocytes (5 cases) and from chorionic villi samples (2 cases) according to standard procedures.

mIS-PCR protocol: mIS-PCR consists of 4 steps (Figure 1).

1. Digestion of genomic DNA (2µg) by 30 units of Bcl1 at 50°C for 4 h in a final volume of 50µL.

2. Circularization of restricted fragments with T4DNA ligase (3 units) at 15°C overnight.

3. PCR assay: 2 separate PCR (ID diagnostic and ED complementary test) using 3 intragenic primers (IU) and 1 intragenic downstream primer (ID) or 1 extragenic downstream primer (ED). ID and ED primers are labeled on 5' end with the fluorescent dye Hexafluorescein (HEX) (Table 1). PCR were performed with 4 µL of circularized DNA, 0.6 nM of each primer, 1U of Taq DNA polymerase, 0.8 mM dNTP, 1.5 mM $MgCl_2$. The initial denaturation step of 10 min at 95°C was followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at 56°C for 1 min and an extension at 72°C for 1 min 30 with a final extension step at 72°C for 7 min.

4. Analysis of PCR products :
- Electrophoresis was performed on a 1.5% agarose gel with ethidium bromide. Amplicons were visualized under UV light.
- 1 µL of PCR products were mixed with HD formamide and GENESCAN 1200LIZ size standard. A denaturation step was performed at 94°C for 3 min, kept on ice for 5 min before loading on a ABI3730 DNA analyzer.

Table 1. Sequences of PCR primers for Inv22 detection

Primer	Sequence
ID-**HEX**	5'-**(HEX)**-ACA TAC GGT TTA GTC ACAAGT-3'
ED-**HEX**	5'-**(HEX)**-TCC AGT CAC TTA GGC TCA G-3'
1U	5'-CCT TTC AAC TCC ATC TCC AT-3'
2U	5'-ACG TGT CTT TTG GAG AAG TC-3'
3U	5'-CTC ACA TTG TGT TCT TGT AGT C-3'

RESULTS

- 47 subjects including 7 prenatal diagnosis were analyzed. Results from all positive LD-PCR Inv22 (n=25/47) were concordant with the mIS-PCR results.
- DNA fragment analysis gave precise distinction and identification of the different amplicons of the ID PCR and ED PCR.

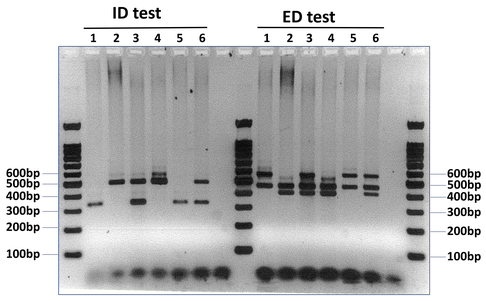

Figure 2. Analysis of PCR products by agarose gel electrophoresis. Samples 1 and 5: Inv22-1 male (ID:333, ED:457, 559bp); Sample 2: Normal male (ID:487, ED:457, 405bp); Samples 3 and 6: Inv22-1 carrier female (ID:333, 487, ED: 405bp, 457bp and 559bp); Sample 4: Normal female (ID:487, ED:457, 405bp).

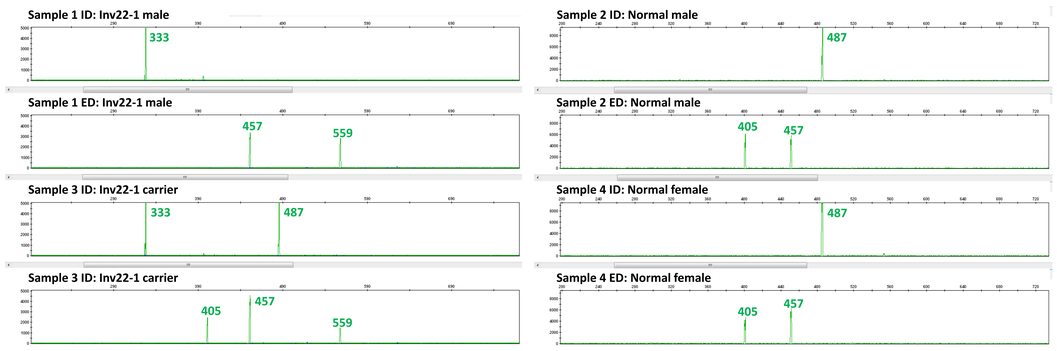

Figure 3. DNA fragment analysis of ID PCR and ED PCR products from samples 1-4. Numbers indicate fragment length.

CONCLUSIONS

Gel electrophoresis results based on the apparent size of PCR products are occasionally difficult to interpret. The advantages of this more discriminant technique are: 1) the DNA fragment analysis gives precise numerical size of PCR products rendering the detection and interpretation of results very simple, reliable, and providing a double check; 2) the use of labeled oligonucleotides does not increase the workload and is safer than the radioactive material used for Southern-Blot, and 3) mIS-PCR is easily reproducible with classical molecular diagnostic laboratory equipment.

REFERENCES

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2. Rossetti LC, Radic CP, Larripa IB, De Brasi CD (2008). Developing a new generation of tests for genotyping hemophilia-causative rearrangements involving int22h and inth1hotspots in the factor VIII gene. J Thromb Heamostasis 6, 830-6.

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