Genotyping the Hemophilia Inversion Hotspot by Use of Inverse PCR

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Background: Factor VIII intron 22 inversions (Inv22) cause 40%–45% of severe cases of hemophilia A in all human populations. Currently, Inv22 can be analyzed either by Southern blotting or by rapid long-distance-PCR–based approaches. We describe an alternative method using inverse-PCR (I-PCR).

Methods: I-PCR involved 3 steps: (a) BclI restriction; (b) self-ligation of restriction fragments, providing BclI rings; and (c) standard multiplex-PCR analysis. PCR was achieved by use of a set of 3 primers that yielded a 487-bp amplicon for the nonrearranged intragenic allele and a 559-bp amplicon for the Inv22 allele. Specific primer sites were targeted by masking relevant regions for human repeats and low-complexity DNA. Inv22 I-PCR was applied to samples from 16 individuals (8 women and 8 men) representing 24 X chromosomes previously genotyped by Southern blotting. Additionally, we evaluated the sensitivity and the ability to assess eventual Inv22 carrier mosaicisms by experiments using artificial DNA mixtures (Inv22 + no-Inv22 male samples).

Results: Results for previously genotyped samples agreed with results of Southern blot analyses. As expected, cell composition of the artificial mosaic was linearly reflected by the relative intensities of Inv22 signals. I-PCR was estimated to detect Inv22-positive cells at concentrations as low as ~5%.

Conclusion: The proposed technique provides a rapid tool for Inv22 genotyping.

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Hemophilia A (HA) is an X-chromosome–inherited disorder associated with deleterious mutations in the coagulation factor VIII gene (F8). Carrier detection and prenatal diagnosis are now possible through characterization of F8 causative mutations by the use of simplified PCR-based tests developed with Human Genome sequencing data (1).

The most important recurrent HA mutation is the intron 22 inversion (Inv22) (2, 3), which accounts for ~42% of severe cases worldwide (4, 5). Inv22 originates almost exclusively in male meiosis (6) and is mediated by an event of intrachromosomal homologous recombination between a region of 9.5 kb within F8 intron 22 (int22h-1) and 1 of 2 inversely oriented copies of this sequence (int22h-2, proximal or int22h-3, distal) (7). Currently, the Inv22 can be detected either by Southern blot analysis, considered the reference method (2), or by single-tube (multiplex overlapping) long-distance PCR (LD-PCR) (8). Although Southern blot analysis is robust and can be used to evaluate carrier mosaicisms (9) and to identify all types of inversions, this technique is labor-intensive and uses hazardous radiochemicals. LD-PCR is rapid but quite sensitive to minor reductions in DNA quality and presents some difficulties in the interpretation of results associated with analysis of a system of 3 signals with different relative intensities. Given the shortcomings of these detection methods, a new system for Inv22 analysis that shares the merits of previous techniques may be particularly valuable.

This report presents an alternative method using inverse-PCR (I-PCR) for Inv22 genotyping.
Materials and Methods

Studied Population and DNA Samples
Samples from 2 groups of individuals were studied. One group was previously Inv22 genotyped by Southern blot analysis and included 16 cases, 8 women (3 Inv22 carriers, 2 with the proximal pattern and 1 distal, and 5 non-Inv22) and 8 men (5 Inv22 (3 distal and 2 proximal) and 3 non-Inv22). The other group had not been studied previously and included 12 individuals, 9 men who were severe HA probands and 2 mothers and 1 female relative of probands. Genomic DNA was obtained from peripheral blood leukocytes by either phenol–chloroform or salting-out extraction (11) and ethanol precipitation. DNA purity, quality, and concentration were assessed by ultraviolet spectrophotometry (260 and 280 nm) and agarose gel electrophoresis.

Southern Blot Analysis of Inv22
We investigated Inv22 by Southern blot analysis as described by Lakich et al. (2). BclI-digested DNA was probed with a random-primed 32P-radiolabeled EcoRI and SalI 0.9-kb fragment of p462.6 (ATCC no. 57203). This classic approach yields specific signals of 21.5, 16.0, and 14.0 kb for no-Inv22 alleles; 20.0, 17.5, and 14.0 kb for Inv22 distal alleles; and 20.0, 16.0, and 15.5 kb for Inv22 proximal alleles.

I-PCR Analysis of Inv22
Typically, 1–2 μg of genomic DNA was digested for 4 h (or overnight) in a 50-μL reaction containing BclI enzyme (10 U/μg of DNA; Promega) under conditions recommended by the manufacturer. Fragmented DNA was extracted once in phenol–chloroform and once in chloroform–isoamyllic alcohol (24:1 by volume), precipitated in 0.3 mol/L NaCl with 2 volumes of ethanol, and resuspended in 50 μL of distilled water. Self-ligation was performed overnight at 15 °C in a total volume of 600 μL under conditions recommended by the manufacturer. Self-ligated BclI fragments (B-rings) were recovered in 30 μL of distilled water by chromatography using GFX spin columns (Amersham). The PCR products were prepared for DNA sequencing by purification using GFX spin columns (Amersham) or alternatively by phenol–chloroform extraction and ethanol precipitation.

B-rings were amplified under standard PCR conditions using primers IU (intragenic upstream; 5′-CTCTTCTAACAATCCTCATCTCAT-3′; GenBank entry BX842559, nucleotides 35744–35763), ID (intragenic downstream; 5′-ATCAGTCGATTAGTCAAGT-3′; GenBank entry BX842559, nucleotides 14622–14602), and ED (extragenic downstream; 5′-TCCAGTCATTAGGCTCAG-3′, proximal GenBank entry BX682237, nucleotides 15364–15347; distal entry BX276110, nucleotides 14604–14621). The underlined nucleotide indicates a mismatched base according to the GenBank file. Briefly, PCR was performed with 6 μL of eluted DNA (B-rings; 200–400 ng of input DNA) in a volume of 25 μL, containing 0.5 U of Taq DNA polymerase (Promega), 0.6 μM each primer, 200 μM deoxynucleotide triphosphates, 1.5 mM MgCl2, and standard Taq polymerase buffer [50 mM Tris-HCl (pH 9.0), 50 mM NaCl, 0.1 μg/μL bovine serum albumin]. The initial denaturation step of 2 min at 94 °C was followed by 30 thermocycles of 94 °C for 30 s, 55 °C for 1 min, and 72 °C for 90 s; final extension was at 72 °C for 5 min.

Restriction Analysis and Electrophoresis of PCR Products
PCR products (5–10 μL) were analyzed by use of 5 U of restriction enzyme in a total volume of 15 μL under conditions recommended by the manufacturers (Promega, Gibco BRL, and New England Biolabs). PCR products and their restriction digests were analyzed by rapid (45–50 min) gel electrophoresis on ethidium bromide-stained 1.5% agarose gels with a 50-bp DNA ladder (Invitrogen) as molecular size marker. Gel images were documented by use of a digital camera (HP Photosmart 735) equipped with ultraviolet filters, and the intensities of electrophoretic signals were estimated by GelPro software (Syrex).

Bioinformatics
In silico restriction mapping, determination of int22h boundaries, and primer annotations were performed with EditSeq and MapDraw software (LaserGene; DNA Star). The National Center for Biotechnology Information web page (http://www.ncbi.nie.edu/blast) was used to gain access to the BLAST (Basic Local Alignment Search Tool) algorithm (12) and to get the relevant genomic sequences to design the inverse PCR strategy. The repeat-masker web server was used to map human repeats in the target sequences to limit the primer sites to regions of single-copy DNA.

DNA Sequencing
PCR products were prepared for DNA sequencing by purification using GFX spin columns (Amersham). The sequencing primers were those used for PCR amplifications (IU, ID, and ED). Manual DNA sequencing was performed on the fmol DNA Cycle Sequencing System (Promega) with 5′-end 32P-radiolabeled primers, under conditions recommended by the manufacturer. DNA sequences were assessed in both orientations.

Strategy Design and Set-up of I-PCR for Inv22
We designed the multiplex I-PCR strategy for Inv22 genotyping, using human genomic DNA sequence files that span either a relevant part of or the entire BclI restriction fragment obtained by classic Southern blot analysis for Inv22 (21.5 and 20.0 kb). The complete nucleotide sequence of the 21.5-kb segment spanning int22h-1 was found in GenBank entry BX842559 (complementary) and bears the relevant BclI sites at bases 36204 (IU) and 14595 (ID; Fig. 1A). On int22h-mediated crossing over, the Inv22-associated or 20.0-kb BclI fragment represents a chimera between an IU arm, a rearranged copy of int22h
(int22h-1 and int22h-2 or -3), and an ED arm (either distal or proximal; Fig. 1A). These proximal and distal ED regions showed virtually identical sequences because the DNA sequence homology of these extragenic loci extends through the downstream limits of int22h. This segment of extended homology spans at least 2.2 kb of int22h and includes the ED BclI site. Genomic sequences from Xq28 clones served to precisely locate the ED BclI recognition site of both the distal copy (int22h-3; nucleotide 14703; GenBank entry BX276110), and the proximal copy (int22h-2; nucleotide 15265; GenBank entry BX682237 complementary). To design a reliable I-PCR–based approach with a highly specific primer set, we masked relevant regions from the human genomic DNA file sequences mentioned above for human repeats (long and short interspersed nuclear elements, and long terminal repeats) and simpler repeats identifying low-complexity regions. Short interspersed nuclear elements (Alu and MIR), a long terminal repeat (MaLR), and long interspersed nuclear elements (L1 and CR1) were found within 1 kb of the BclI-relevant sites in BX842559, BX682237, and BX276110, and thus would restrict potential primer target sequences (data not shown). Additionally, an applicable set of primers should be designed to define distinguishable amplicons from the Inv22 allele (either proximal or distal type) and the no-Inv22 allele in BclI self-ligated fragments (B-rings). Taking into account these restrictions, we designed a set of 3 primers (IU, ID, and ED) that yielded amplimers of 559 and 487 bp from the Inv22 and no-Inv22 B-rings, respectively (Fig. 1, A and B). In our first attempt, standard conditions gave a neat, specific, intense signal system on the multiplex PCR analysis (Fig. 1B). When the Inv22 signal was very intense, it was accompanied by 1 or 2 satellite bands in the zone of segments longer than 600 bp (Fig. 1, B and C). These satellite signals did not interfere with the interpretation of Inv22 genotypes because they were not associated with negative PCR amplifications (Table 1 in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol51/issue7/) or with no-Inv22 signals (Fig. 1, B and C); we therefore made no additional effort to eliminate them.
The sequence identities of both specific amplifiers (487 and 559 bp) were determined by both restriction mapping (using BclI and HaeIII) and DNA sequencing (data not shown). These experiments confirmed restriction fragments determined experimentally and DNA sequences predicted in silico.

**Results**

**Validation and Performance**

Samples from 16 individuals (8 women and 8 men) representing 24 X chromosomes that had been analyzed by Southern blot previously and showed several different Inv22 genotypes were blindly studied by I-PCR. There was perfect concordance of Inv22 results obtained from both methods. An important result was that, as expected, Inv22 Southern blot patterns (distal and proximal) yielded the same Inv22-specific amplimer (559 bp) without significant differences in relative signal intensities.

In addition, we applied the I-PCR method to new clinical samples from patients with severe HA and relatives of these patients. Of this group of 12 individuals (9 with severe HA), samples from 3 gave the Inv22-specific signal (Fig. 1C) and 6 did not. Cases successfully genotyped for Inv22 by I-PCR included 1 severe HA patient’s mother, who was found to be an Inv22 carrier; 1 female cousin, who was genotyped as a noncarrier of hemophilia because she showed a single signal of 487 bp (Fig. 1C); and 1 mother who was found to be an Inv22 carrier (Fig. 1C). Some of these clinical cases were refractory to long-range PCR amplification, presumably because of the quality of their genomic DNA samples.

**Specificity, Limit of Detection, and Sensitivity**

To further evaluate the specificity and reliability of the analytical system, we performed a PCR experiment with a random combination of primers and samples (Table 1 in the online Data Supplement). As was expected based on the assay design, when both IU and ED primers were used, only treated (BclI-digested and ligated DNA) samples from Inv22-positive hemophiliacs and carriers gave the Inv22-specific amplimer (559 bp); similarly, only treated samples from a no-Inv22 control and an Inv22-positive carrier female gave the wild-type amplimer (487 bp). When other primer combinations were used, neither the untreated (nondigested and nonligated DNA) genomic DNA from controls nor the spectrum of treated samples yielded any PCR product (Table 1 in the online Data Supplement).

We assessed the ability of the I-PCR to assess Inv22 carrier mosaicisms by a DNA/DNA dilution experiment (Fig. 2). Serial mosaic carrier samples were simulated in vitro by mixing decreasing quantities of Inv22 DNA with complementary amounts of control DNA (Fig. 2). By visual inspection of ethidium bromide-stained analysis results, we estimated the limit of detection as ~2.5% Inv22 DNA, which corresponded to 5% of cells being heterozygous for Inv22 in a female carrier mosaic. Relative signal densities of the 559-bp amplimer were calculated (Inv22/no-Inv22) and depicted (Fig. 2B). The parameters of the linear regression (slope and R²) indicate that the relative intensity of the Inv22 signal reflects the sample’s mosaic-cell composition (Fig. 2B).

![Fig. 2. Estimation of sensitivity and detection limit of I-PCR.](image-url)

(A), agarose gel electrophoresis of ethidium bromide-stained products from artificially constructed Inv22 mutation carrier mosaicisms. Inv22/Normal indicates a typical pattern and relative signal intensities of an Inv22 carrier. Mosaic composition: 50% Inv22 simulates a nonmosaic Inv22 mutation carrier, which corresponds to 100% heterozygous cells; 40% Inv22, 60% heterozygous cells; 30% Inv22, 70% heterozygous cells; 20% Inv22, 80% heterozygous cells; 10% Inv22, 90% heterozygous cells; 5.0% Inv22, 95% heterozygous cells; 2.5% Inv22, 99% heterozygous cells; 1.2% Inv22, 99.9% heterozygous cells; 0% Inv22, no heterozygous cells. PCR cycles and conditions as described in the Materials and Methods. Marker indicates 50-bp ladder. The arrow indicates the sample with the minimal easily recognizable amount of Inv22-positive DNA. (B), linear regression of the relative signal densities obtained in A. The relative density of the Inv22 allele is calculated as: (Inv22 signal/no-Inv22 signal). Equation for the regression line: y = 0.956x. The parameters R² (0.9692) and slope of the line (0.956) indicate both good linear adjustment and correlation between the Inv22-positive cell composition of eventual carrier mosaicisms and the relative I-PCR signal densities.
Discussion

The design of the I-PCR system reported here was inspired by the typical 21.5- to 20-kb signal shift on Southern blot autoradiograms that indicates the presence of distal or proximal Inv22. I-PCR analysis provides an alternative method for molecular diagnosis of Inv22 in severe HA families and evaluation of Inv22 carrier mosaics. Currently, most molecular genetics laboratories that test for hemophilia use LD-PCR–based approaches (13, 14) for molecular diagnosis and Southern blot analysis for evaluation of carrier mosaics (2, 9), whereas other groups use Southern blot analysis for all applications.

The lengths of DNA templates would seem to indicate that Inv22 LD-PCR–based approaches would require input DNA of lesser quality than Inv22-specific BclI restriction fragment-based techniques (Southern blot analysis and I-PCR). In practice, however, at least for Inv22, Southern blot analysis results are clear and reliable even with degraded DNA substrate, and I-PCR genotyping is accurate for DNA of a wide quality range. A possible explanation for this observation is that evenly nicked double-strand DNA templates can retain physical integrity until the formation of B-rings (the key step for I-PCR analysis) but cannot act as templates for long-range PCR amplifications.

The time required for LD-PCR amplifications is usually 2 days because of DNA polymerization and the slow electrophoresis necessary to resolve signals of 10, 11, and 12 kb (~8–9 h). Similarly, I-PCR for Inv22 genotyping requires 1 overnight incubation to achieve BclI fragment ligation, and the other 2 steps, BclI treatment and standard PCR analysis, can be completed in a few hours. Southern blot analysis is more time-consuming, requiring ~1 week, and requires the use of hazardous radiochemicals that should be handled by trained personnel.

In addition to specific use in cases of Inv22-uninformative families with severe HA, I-PCR for Inv22 provides a remnant source of self-ligated BclI fragments, which may be used as substrate to obtain mutation-specific signals to investigate heterozygous female samples and to isolate DNA for sequencing of large deletions with only 1 breakpoint mapped to a relatively small region.

In our hands, this I-PCR method for Inv22 genotyping has been notably robust, working over a wide range of conditions and qualities of input DNA. In addition, the proposed multiplex approach is amenable to quantification and may be easily adapted to more accurate analytical systems, such as capillary electrophoresis using 1 fluorescent primer (for example, the common primer IU). These features make I-PCR–based Inv22 genotyping a valuable first-line tool in cases of severe HA. This method provides rapid, reliable information for genetic counseling.

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