ORIGINAL ARTICLE *Women with bleeding disorders*

Heterozygous large deletions of *Factor 8* gene in females identified by multiplex PCR-LC

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Summary. Haemophilia A is the most common X-linked recessive bleeding disorder. In 5% of severely affected patients the mutations responsible for the disease are large deletions encompassing from one exon to the complete *Factor 8* (*F8*) gene. Large deletions in a male haemophilic patient are easily detected by the absence of the corresponding PCR product. However, in female carriers, identification of the various heterozygous large deletions is difficult representing a major limitation to accurate carrier diagnosis. The deletion is masked by the presence of the second allele that serves as template for the PCR reaction. Quantitative PCR can differentiate between the presence of one or two alleles. Here we report an assay based on multiplex amplification of several exons of the *F8* gene of various length and subsequent quantitative evaluation of the amplicons by liquid chromatogphy (LC). Using this approach we achieved an accurate classification of 16 female carriers and eight non-carriers for deletions in the *F8* gene in 19 investigated families. One mother and one grandmother were classified as non-carriers, underlining the high *de novo* mutation rate of large deletions in female germ cells. The large deletions in three families were confirmed by fluorescent *in situ* hybridization. In conclusion, the multiplex PCR-LC technique represents a rapid, simple and reliable method for detection of heterozygous large deletions in female carriers.

Keywords: haemophilia A carriers, large deletions, liquid chromatogphy

Introduction

Haemophilia A (HA) is the most common, severe bleeding disorder affecting one in 5000 male newborns. The X-linked disease is caused by a broad spectrum of mutations in the *Factor 8* (*F8*) gene leading to an absent or dysfunctional protein. The *F8* gene spans 186 kb on chromosome Xq28 and consists of 26 exons. Large deletions are not uncommon events in HA and occur in approximately 5% of patients with a severe form of the disease [1]. More than 100 unrelated patients with large deletions, encompassing one or more exons of the *F8* gene, have been reported in the international *F8*

mutation database [2]. Because of the X-linked inheritance, female relatives of HA patients with large deletions are at risk for being carriers. Therefore, the availability of an accurate carrier test for the various heterozygous large deletions and appropriate genetic counselling are important for comprehensive care in haemophilia.

Large deletions of *F8* in males are readily detected by the absence of a PCR product of the deleted fragment. However, to identify large deletions of complete exons on the background of a normal allele – as it is the situation in a carrier – is challenging as the presence of the non-deleted allele masks the deletion in a standard PCR. Indirect gene analysis by classical segregation investigation of polymorphisms for tracing the *F8* gene defects within a pedigree is limited because it requires examination of several family members, may be uninformative in approximately 25% of potential carriers and cannot identify *de novo* large deletion that preferably originate in female germ cells [3–5]. Historically, the method of

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choice for the detection of large deletions relied on Southern blotting. However, this approach is labour intensive, requires large amounts of DNA, has a low throughput, and is not able to detect all large deletions. Therefore, the use of Southern blotting is limited in routine molecular diagnosis. Alternative approaches for the identification of female carriers of heterozygous large deletions are quantitative PCR techniques [6–8].

In the present study, we applied a quantitative multiplex PCR and liquid chromatography (LC) method for the detection of large deletions in 24 females from 19 families in whom a large deletion was identified in the index patient.

Materials and methods

Patients

Nineteen families with severe HA (FVIII activity <1%) and an index patient exhibiting a large deletion of one or more exons were included in the study. Presence of an intron 22/intron 1 inversion mutation was excluded. The large deletion in the index patient was detected by repetitive amplification failure of a single or several contiguous exons. A total of 24 possible carriers were analysed for the presence of a large deletion. All patients and relatives gave informed consent according to the Declaration of Helsinki.

F8 gene analysis

DNA isolation High molecular genomic DNA was isolated from peripheral whole blood by a common salting out procedure [9]. DNA concentrations were standardized to 100 ng μL^{-1}.

Intron 22 and intron 1-inversion PCR The PCR for detection of the intron 22 inversion was performed as described by Liu *et al.* [10] with the following slight modifications: PCR-amplification started with 100 ng of DNA and the amplified fragments were separated on 0.8% agarose gel for 24 h. Intron 1 inversion was analysed by PCR as reported earlier with a modified annealing temperature of 59°C [11].

PCR for amplification of the F8 gene in haemophilia patients All 26 exons and flanking regions of the *F8* gene were amplified by touchdown PCR. The reaction was performed using 100 ng of genomic DNA, 20 pmol of primer, 50 μM of each dNTP and 2.5 U of Ampli*Taq*-Gold DNA polymerase (PE Applied Biosystems, Foster City, CA, USA) in a total volume

of 50 μL. The primers were designed to anneal at 59°C for all exons. The PCR programme was set to initial denaturation at 94°C for 5 min, followed by 38 cycles of amplification with 30 s of denaturation at 94°C, 30 s of annealing with temperature ramping from 65 to 59°C by 3°C every two cycles, 30 s elongation at 72°C and 5 min final extension at 72°C. The amplicons were checked on an ethidium bromide stained 1.5% agarose gel.

Semi-quantitative multiplex PCR A semi-quantitative multiplex PCR was performed for detection of heterozygous *F8* large deletions in female relatives. The touchdown PCR was performed using the same conditions as described above, differing only by number of cycles, which were reduced to 25 cycles to ensure that the amplification reaction remained within the exponential phase. Less than 22 cycles did not yield enough PCR product for good resolution on LC and more than 25 cycles were too close to the plateau phase. In designing the multiplex reaction three important criteria were taken into consideration: (i) One or more exons in which a deletion was expected were selected for amplification. (ii) A human growth hormone (*HGH*) gene fragment with length of 440 bp (corresponding to *HGH* exons 3 and 4) was coamplified as an internal PCR control. (iii) Undeleted exon(s) were amplified as a second internal control. (iv) The length of the fragments differs in at least 30 bp to allow a clear separation by LC. Due to its size, exon 14 was divided into eight fragments (Table 1).

LC analysis Aliquots of 10 μL of the multiplex PCR product were injected into a semi-automated high throughput LC system (WAVE; Transgenomic Ltd, Omaha, USA). Separation was performed on DNAsep cartridge (Ion pair reverse phase C18 column – Transgenomic Ltd) at constant oven temperature of 50°C ensuring non-denaturing conditions and UV detection at 260 nm. The mobile phase was 0.1 M triethylammonium acetate (TEAA), pH 7 (buffer A) and 0.1 M TEAA and 25% acetonitril (buffer B). The PCR product was eluted at a flow rate of 0.9 mL min^{-1} with a gradient of 40–72% for buffer B. NAVIGATOR 1.5.3. Software (Transgenomic) was used for data analysis. The chromatograms of investigated patient samples were normalized according to the internal HGH peak and superimposed on those of a normal control. The peak height of each exon of the normal control and the investigated sample were compared and a ratio for each peak was calculated (peak height of test sample/peak height of control). The yield of each amplicon in

Table 1. Amplification primer sequences for *Factor 8* and *HGH* genes.

Name	Primer sequences (5′ to 3′)	Length of PCR products (bp)
F8-Exon 1F	ccctcctgggagctaaagat	293
F8-Exon 1R	caccttcctccaagcagact	
F8-Exon 2F	ttcaaatttgcctccttgct	338
F8-Exon 2R	tttggcagctgcactttta	
F8-Exon 3F	gcttctccactgtgaccttga	250
F8-Exon 3R	tgacaggacaataggagggtattt	
F8-Exon 4F	acatgtttctttgagtgtacagtgg	373
F8-Exon 4R	caggtgaaggaacacaaatgc	
F8-Exon 5F	tcctcctagtgacaatttcctaca	193
F8-Exon 5R	gtagcagaggatttctttcagga	
F8-Exon 6F	taaggtgtgagcacactggg	314
F8-Exon 6R	tatcctctgagatgccgagc	
F8-Exon 7F	tgtcctagcaagtgttttccatt	400
F8-Exon 7R	aatgtccccttcagcaacac	
F8-Exon 8F	cccatatagcctgcagaaacat	473
F8-Exon 8R	tggcttcaggatttgttggt	
F8-Exon 9F	agagttggatttgagcctacctag	296
F8-Exon 9R	cagacttttcttcttacctgacctt	
F8-Exon 10F	ctagcctcaaattactataatg	347
F8-Exon 10R	actttagactggagcttgag	
F8-Exon 11F	gagacccttgcaacaacaac	364
F8-Exon 11R	tttcttcaggttataaggggaca	
F8-Exon 12F	tgctagctcctacctgacaaca	298
F8-Exon 12R	cattcattatctggacatcactttg	
F8-Exon 13F	atcatgacaatcacaatccaaaa	366
F8-Exon 13R	catgtgagctagtgggcaaa	
F8-Exon 14_1F	ctgggaatggggagagaacct	436
F8-Exon 14_1R	tttggcttcttggagatcaga	
F8-Exon 14_2F	tggtttgcacacagaacacc	500
F8-Exon 14_2R	aagctcagaggtccaccaga	
F8-Exon 14_3F	cagacaatttggcagcaggt	600
F8-Exon 14_3R	tttgtgcatctggtggaatg	
F8-Exon 14_4F	tttgaggctaaatcatatgtcaaa	399
F8-Exon 14_4R	cttgattgtgtgtattattttcatgt	
F8-Exon 14_5F	aaagagatggtttttccaagca	560
F8-Exon 14_5R	ctcggggtcaaatgtttcat	
F8-Exon 14_6F	tcacgcaacgtagtaagagagc	396
F8-Exon 14_6R	tgactgaatttgtggcacttg	
F8-Exon 14_7F	aaccttggagatgactggtga	399
F8-Exon 14_7R	ttccactcttcttttggtatctga	
F8-Exon 14_8F	ctgcaaagactccctccaag	544
F8-Exon 14_8R	tgtcacaagagcagagcaaa	
F8-Exon 15F	ggatgtgaggcatttctaccc	299
F8-Exon 15R	tgggaatacattatagtcagcaaga	
F8-Exon 16F	aaagacaggtatttctttagggatg	399
F8-Exon 16R	tgcacgtaggataaatatcaaaattc	
F8-Exon 17F	caggttggactggcataaaaa	399
F8-Exon 17R	ccctggatcaagtctcatttg	
F8-Exon 18F	tgtgggagtggaatcctcat	367
F8-Exon 18R	gtgttcccagtgcctagacc	
F8-Exon 19F	tctgatatttcgcataaaccaatg	280
F8-Exon 19R	gcaaccattccagaaaggaa	
F8-Exon 20F	ttgacgttctcccatttttca	243
F8-Exon 20R	ttcattatctgagattctccacca	
F8-Exon 21F	ccacagcttagattaacctttctca	263
F8-Exon 21R	tttgagcttgcaagaggaataa	
F8-Exon 22F	ttggtgactgcttcacttgc	371
F8-Exon 22R	ccaatatctgaaatctgccaaa	
F8-Exon 23F	aaggaagatatgattgacagaaattg	292

Table 1. Continued

Name	Primer sequences (5′ to 3′)	Length of PCR products (bp)
F8-Exon 23R	aactagaacagttagtcaccctaccc	
F8-Exon 24F	gccctagaatatcagtggaagc	397
F8-Exon 24R	caaatgcctgtgtggttgtc	
F8-Exon 25F	agggatttgggaatttctgg	354
F8-Exon 25R	ttttgctctgaaaatttggtca	
F8-Exon 26F	aagcgtctgtgctttgcagt	276
F8-Exon 26R	gctgaggagggagaggtgac	
HGH-Exon2-3F	tgccttcccaaccattccctt	434
HGH-Exon2-3R	ccactcacggatttctgttgtgtttc	

HGH, human growth hormone.

Table 2. Sequences and positions of primers applied for the amplification of the probes used for hybridization in fluorescent *in situ* hybridization.

Name	Sequence	Position according to NG-005114	Size of the product
Exon1 F	TTCCAAATTTTTCCTTCTCAAAGC	5′UTR	∼ 5000
Exon1 R	ATGGCAAAAACCCATCTCTAAAAA	3091–3114	
Exon2 F	TCCAGTAGGATACACCAACAGCAT	25716–25739	5035
Exon2 R	CAGAGACTGGAGGAGGTGAGAGTT	20704–20727	
Exon4 F	CCATTTGAATTGGAATGAAGTTGA	32275–32298	5024
Exon4 R	CCTATTCTACCAACAACCAAAGGG	27274–27297	
Exon5 F	CATGGATGTGGAAGTCTGATTCTC	38635–38658	5083
Exon5 R	CACAGGGTCTTCCTACTTAGCCAT	33575–33598	
Exon6 F	TTTGTTTAGGGCCATGGCTTTGTG	33925–33948	5157
Exon6 R	GTCAGAGCCCTGCCTGTCCCAAGT	39059–39082	
Exon7 F	GCACAGCAAAGGAAACAATCAA	50217–50238	5025
Exon7 R	TTGATTATGAGGAACAGGGAAGGT	55219–55242	
Exon13 F	AAACATCGGCATACCCTATTGACT	72433–72456	5052
Exon13 R	AAACTGATTCCACAAGGCCAGTAT	77462–77485	
Exon14 F	TAAGCATTCACAGGGCTTTTGGGA	90151–90174	5046
Exon14 R	CTGTGGAGGTTCATTGCACAGCAT	95174–95197	
Exon15 F	CCACGTACATGAATTGGAAAACA	114144–114166	3450
Exon15 R	TCAAAGTTTTTAATACCCTGGAAGAA	117569–117594	
Exon25 F	AACCAGCATAGTCCTGGGCAAACC	159765–169788	5166
Exon25 R	TGTGAGTGCTCAACGAAGGGTGAC	164908–164931	
Exon26 F	AAGAAACAGAAGGGGTAAGAGCCT	183531–183554	∼ 5000
Exon26 R	AAAGGTGAGGTGAAGTGGAGAAAG	3′UTR	

various samples and controls was evaluated and the presence of a deletion was predicted by a minimum of 1.5-fold decrease of peak height, corresponding to a ratio of 0.6. Consequently, a ratio <0.6 was taken to represent a large deletion of the corresponding fragment.

FISH

Cell cultures A 10 mL-sample of heparin anticoagulated fresh venous blood was used for the cultivation of lymphocytes. Cell division was stopped after 72 h by the addition of colcemid and chromosome slices were prepared by standard procedures.

PCR amplification for probe generation Probe amplifications were performed using Expand™ Long Template PCR System (Boehringer Mannheim,

Mannheim, Germany). The reactions were carried out in 25 μL volume containing 10 ng BAC DNA, 20 pmol of each primer, 22 μL Long Template buffer1 and 5 mM betaine. Primers used for PCR assays are shown in Table 2. An initial denaturation of 5 min at 96°C was followed by 35 cycles comprising 30 s at 96°C, 30 s at 62°C, 4 min at 72°C and a final extension step of 5 min at 72°C.

DNA labelling via nick translation and preparation of the probes Two micrograms PCR amplified probe-DNA was incubated in a finale volume of 100 μL wih 10 μL 10x dNTPs (0.5 mM each of dATP, dCTP, dGTP), 10 μL 10x NT buffer (0.5 M Tris/HCl, 50 mM MgCl$_2$, 0.5 mg mL^{-1} BSA), 1 μL 1 M mercaptoethanol, 5 μL DNase I (1:1000), 2 μL DNA polymerase I and 5 μL Biotin-16-dUTP 1 mM at

15°C for 1.5 h. After stopping the reaction with 1 μL 10% SDS and 1 μL 0.5 M EDTA nucleotides which were not incorporated were removed via a Sephadex G 50 colum. After saturating repetitive sequences with human Cot-DNA and salmon testis DNA the labelled DNA was precipitated and resuspended in 40 μL of Hybridization-Mix (50% formamide, 2x SSC, 10% dextran sulphate).

Hybridization and detection of the signals Ten microlitres of the labelled probe were mixed with 1 μL of a 2-kb *Bam*HI fragment of the alpha-satellite DNA from X_c which served as an internal control and hybridized to metaphase chromosomes of the probands for 48 h at 37°C. The labelled chromosomes were detected by binding of FITC labelled avidin to bound biotin-16-dUTP.

Results

Detection of large deletions in the index patients

Absence of intron 22 and intron 1 inversion mutations were confirmed for all index patients. Thirty-three fragments representing all 26 exons and exon/intron boundaries were amplified from each patient for further mutation screening analysis (Table 1). Repetitive failures in PCR amplification of one or more neighboured exons indicated the presence of a large deletion of the corresponding exons. In 14 patients, single exon deletions were identified including exons 1, 6, 13, 14, 21, 22 and 26. Multi-exon deletions were detected for each of five patients that comprised exons 1–6, exons 15–18, exons 15–22, exons 23–24 or exons 1–25.

Detection of large deletions in female relatives

Based on the localization and extension of the large deletion in the index patient, a set of primers that allowed amplifying the deleted and flanking exons were used (Table 1).

For determination of the deletion in exon 1 (families 1–4), exons 1–5 and 8 were amplified in a multiplex reaction. In family 3, the mother of the index patient showed a peak height ratio of 0.3 and was identified as a carrier, while the sister showed a ratio of 1.0, indicating no deletion. In families 1, 2 and 4, three female relatives were identified as carriers with ratios for exon 1 in the range of 0.4–0.5 (Table 3, Fig. 1a).

The deletion of exon 6 was examined in three females belonging to families 5 and 6 by amplification of exons 3, 5, 6 and 9. The two mothers were

Table 3. Localization of deletions in index patients and the results in their female relatives analysed by multiplex PCR-liquid chromatogphy and fluorescent *in situ* hybridization.

Family number	Deletion in index patient	Female relative	Multiplex PCR-LC	Ratio for deleted exon(s)	FISH
1	Ex 1	Sister	Carrier	0.4	n.d.
2	Ex 1	Mother	Carrier	0.5	n.d.
3	Ex 1	Mother	Carrier	0.3	n.d.
		Sister	Normal	1.0	n.d.
4	Ex 1	Sister	Carrier	0.4	n.d.
5	Ex 6	Mother	Carrier	0.5	Carrier
		Sister	Normal	0.9	n.d.
6	Ex 6	Mother	Carrier	0.6	n.d.
7	Ex13	Mother	Normal	1.0	n.d.
8	Ex 14.5–14.8	Mother	Carrier	0.4–0.5	n.d.
		Sister	Carrier	0.4.0.6	n.d.
9	Ex 14	Mother	Carrier	0.4–0.6	n.d.
10	Ex 14	Mother	Carrier	0.4–0.6	Carrier
11	Ex 21	Sister	Normal	0.9	n.d.
12	Ex 22	Mother	Carrier	0.4	n.d.
13	Ex 26	Mother	Carrier	0.5	n.d.
14	Ex 26	Mother	Carrier	0.6	n.d.
15	Ex 1–6	Mother	Carrier	0.3–0.5	n.d.
16	Ex 15–18	Mother	Carrier	0.3–0.4	n.d.
17	Ex 15–22	Mother	Carrier	0.3–0.5	n.d.
18	Ex 23–24	Aunt	Normal	0.9–1.0	n.d.
19	Ex 1–25	Grand mother	Normal	0.8–1.0	n.d.
		Mother	Carrier	0.3–0.6	Carrier
		Sister	Normal	0.8–1.0	n.d.

n.d., not determined.

found to be carriers for the deletion (ratios of 0.5–0.6). The sister of the proband of family 5 was identified as a non-carrier (ratio of 0.9) (Fig. 1b).

The deletion of exon 13 in family 7 was not present in the mother of the index patient. Repetitive analysis confirmed a normal peak height ratio of 0.95–1.00.

Three index patients exhibited a deletion of exon 14 (families 8–10). In two of the families, the deletion spans all of exon 14 (family 9 and 10), while in family 8, only the second half of exon 14 was deleted (lack of PCR products from 14-5 to 14-8). Both the mother and the sister in family 8 exhibited the deletion of fragments 14-5 (ratio 0.4) and were identified as carriers. In families 9 and 10, both mothers carried a deletion corresponding to fragments 14-1 and 14-8 indicating a deletion of the entire exon 14. In the amplified flanking exons 13 and 15, the peak height ratios were 1.0.

Deletions of exons 21, 22 and 26 were identified in families 11, 12, 13 and 14. The sister of the index patient in family 11 did not carry the deletion of exon 21 (ratio 0.9). A deletion was assigned to the
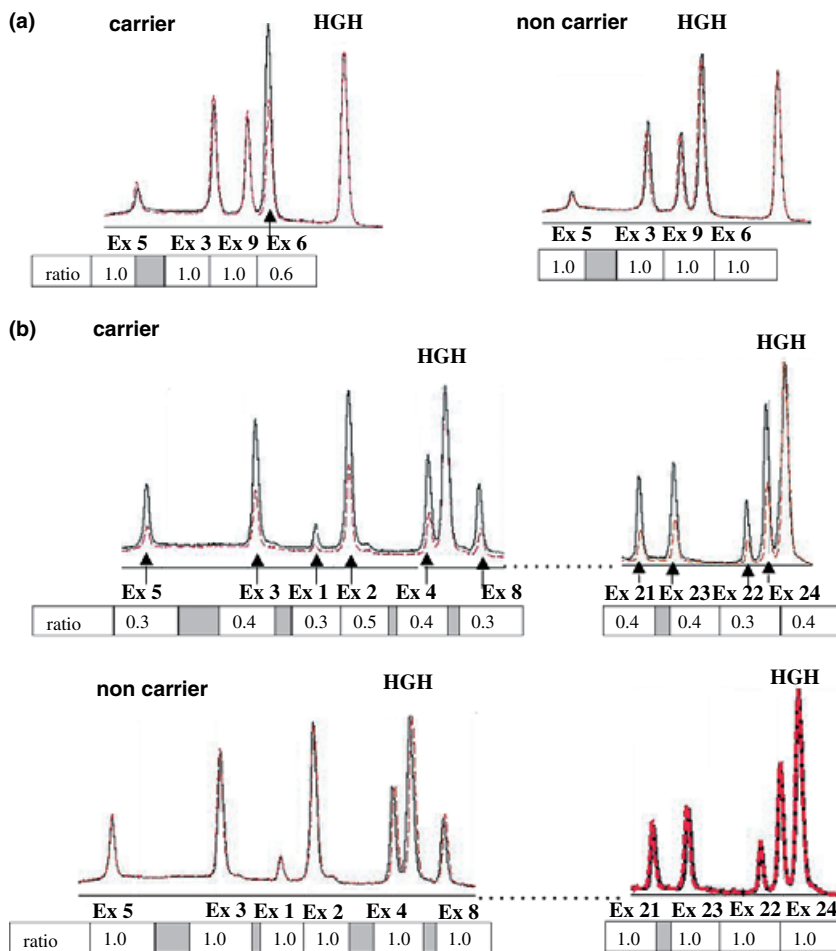
Fig. 1. Chromatograms of representative families with various deletions. The black line marks the control and the red line indicates the test sample. The deleted exon(s) are pointed out by arrows. The ratio is given under each fragment. (a) Family 3: heterozygous deletion of exon 1 in a carrier (left side); non-deleted exon 1 in a non-carrier (right side). (b) Family 5: heterozygous deletion of exon 6 in a carrier (left side); non-deleted exon 6 in a non-carrier (right side). (c) Family 17: hetero-zygous deletion of the whole *F8* gene in a carrier (upper part) and absence of the deletion in a non-carrier (lower part).

patients' mothers in family 12 for exon 22 (ratio 0.4) and in families 13 and 14 for exon 26 (ratio 0.5–0.6) (Table 3).

Families 15–19 exhibited multi-exon deletions ranging from 4 to 25 exons. A deletion of exons 1–6 was found in the index patient of family 15. The mother was proven to be a carrier with peak height ratios in the range of 0.3–0.5. Exon 6 was excluded from analysis because of its similar size to exon 1. Exon 7 was analysed as an unaffected flanking control exon and exhibited a ratio of 1.0.

In families 16 and 17, both mothers were found to carry a deletion, that in family 16 extending from exon 15 to 18 (ratio 0.3–0.4) and in family 17 from exon 15 to 22. Flanking exons 14-8 and 21 showed no deletion (Table 3).

In family 18, deletion of exons 23 and 24 was not found in the proband's aunt (ratio 0.9–1.0). In family 19, we studied three females. The deletion in the index patient covered nearly the whole *F8* gene (from exon 1 to 25). Subsets of the first exons (1–5 and 8) and of the last exons (21–24) were examined and the deletion was found to be present

in the mother but not in either grandmother or sister (Fig. 1c).

FISH

A fluorescent *in situ* hybridization (FISH) analysis was performed to confirm the large deletions in females in three families (families 5, 10 and 19). Probes were designed to be located within or flanking the deletions and were hybridized to the X-chromosomes of the female relatives of the index patients. Female carriers of a deletion exhibit centromeric signals on both X-chromosomes, while the *F8* signal is only found on the normal X-chromosome. In contrast, non-carrier females exhibit two *F8* signals and two centromeric signals (Fig. 2). Hybridization with a probe for exon 6 of metaphase chromosomes of the family 5 patient's mother resulted in a signal only on one X-chromosome (Fig. 2a1), while hybrid-ization with a probe for exon 5 resulted in signals on both X-chromosomes (Fig. 2a2) indicating that she is a carrier of the deletion of exon 6. The proband's mother from family 10 exhibited a signal on only one

Fig. 2. Fluorescent *in situ* hybridization results of families 5, 10 and 19. Left-hand side: hybridization with a probe that binds to the deleted exon/region (a1 – exon 6; b1 – exon 14; c1 – exon 25) results in a signal on only one of the two X-chromosomes. Right-hand side: hybridization with a probe binding to the deletion's flanking exons/regions (a2 – exon 5; b2 – exon 13; c2 – exon 26) resulted in signals on both X-chromosomes.
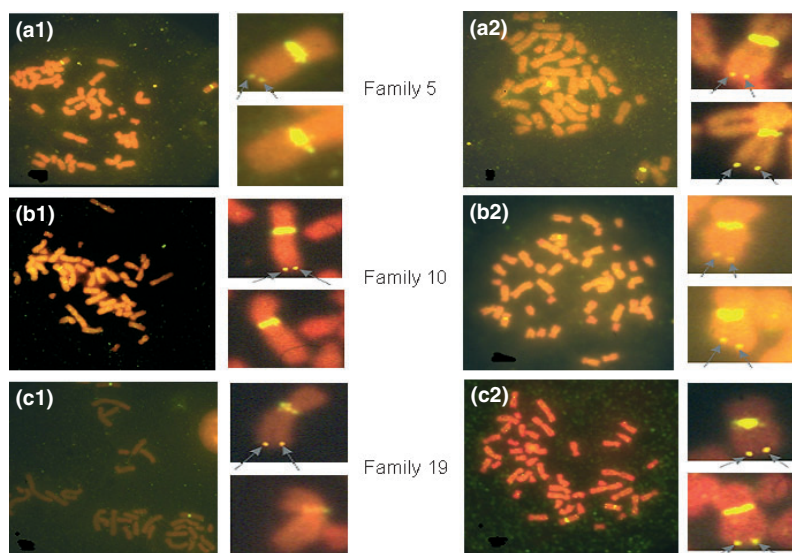
X-chromosome when probed for exon 14 hybridization (Fig. 2b1). Hybridization with the flanking exon 13 resulted in signals on both X-chromosomes (Fig. 2b2). In family 19, the deletion was confirmed in the patient's mother as verified by only one signal upon hybridization to a probe of exon 25 and by two signals when the flanking exon 26 was probed.

Discussion

In the present study we describe the successful diagnosis of 16 heterozygous large deletions of the *F8* gene in 24 female relatives of 19 severe HA patients by a multiplex PCR-LC method. As the *F8* gene encompasses 26 exons and large deletions are not clustered in any specific region of the gene, carrier detection in females represents a challenge. This situation is even more complicated when the index patient is not available.

We employed a rapid multiplex PCR-LC technique that had been previously established in our lab for the detection of heterozygous large deletions of the antithrombin gene [12]. By quantification of potentially deleted exons, accurate classifications of carriers and non-carriers of heterozygous large deletions in the *F8* gene is achieved. This technique also provides reliable results when no index patient is available. Unlike other available techniques including capillary gel electrophoresis, multiplex amplifiable probe hybridization and multiplex ligation-dependent probe amplification, the multiplex PCR-LC method enables carrier detection without the use of radioisotopes, fluorescence-labelled or gel-based reagents [13,14]. Compared with classical Southern blotting,

a significant reduction in handling time and improved detection of all deletions, including the determination of their size, is achieved.

All patients' mothers were carriers of the heterozygous large deletions, except for family 7 where the absence of a large deletion in the patient's mother indicated a *de novo* mutation either in the germ cells or a somatic mosaicism. Large deletions within the *F8* gene are known to originate predominantly from female germ cells because homologous recombination of both X-chromosomes during meiosis can enhance appearance of large deletions [15–18]. Therefore, it is of particular importance to determine the carrier status of the mothers of HA patients presenting large deletion genotypes. Among the investigated sisters of patients in the present study, three were identified as carriers and four as non-carriers. The aunt in family 18 and the grandmother in family 19 were also found to be non-carriers (Table 3). In three families, the deletion was additionally confirmed by FISH-technique. Although FISH allows direct visual analysis, it is rather laborious, especially when a deletion is not confirmed *a priori* and when several regions of the *F8* gene must be analysed. However, FISH does provide a suitable technique for the reliable identification of heterozygous large deletions in females [19].

Accurate carrier detection of females with large deletions is important for purposes of genetic counselling as large deletions in male descendents always lead to the severe clinical phenotype of haemophilia, often complicated by inhibitor development. Additionally, the risk of inhibitor formation for such patients correlates with the size of the deletion. For multi-domain deletions, the patients exhibit an

elevated risk (88%) for inhibitor development, while this risk is decreased (41%) for single domain deletions [20–22].

In conclusion, the multiplex PCR-LC technique described here can reliably identify HA female carriers of heterozygous large deletions, an attractive alternative to previously reported methods. The method is rapid, simple and robust and is, therefore, well suited for routine diagnostics. It can also be successfully applied to screening and identification of asymptomatic carrier females even when the index patient has not been genetically analysed and, thus, represents a new, powerful tool for genetic counselling for HA families.

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Disclosures

The authors stated that they had no interests which might be perceived as posing a conflict or bias.

References

1 Becker J, Schwaab R, Möller-Taube A *et al.* Characterization of the factor VIII defect in 147 patients with sporadic hemophilia A: family studies indicate a mutation typedependent sex ratio of mutation frequencies. *Am J Hum Genet* 1996; **58**: 657–70.

2 Kemball-Cook G, Tuddenham EGD, Wacey AI. The factor VIII structure and mutation resource sit: HAMSTeRS version 4. *Nucleic Acids Res* 1998; **26**: 216–9. http://europium.csc.mrc.ac.uk.

3 Peake I. Registry of DNA polymorphisms within or close to the human factor VIII and factor IX genes. *Thromb Haemost* 1992; **67**: 277.

4 Lalloz M, McVey J, Pattinson J, Tuddenham E. Haemophilia A diagnosis by analysis of a hypervariable dinucleotide repeat within the factor VIII gene. *Lancet* 1991; **338**: 207.

5 Peake IR, Lillicrap DP, Boulyjenkov V *et al.* Report of a joint WHO/WFH meeting on the control of haemophilia: carrier detection and prenatal diagnosis. *Blood Coagul Fibrinolysis* 1993; **4**: 313–44.

6 Armour JAL, Barton DE, Cockburn DJ, Taylor GR. The detection of large deletions or duplications in genomic DNA. *Hum Mutat* 2002; **20**: 325–37.

7 Costa C, Jouannic JM, Stieltjes N, Costa JM, Girodon E, Goossens M. Quantitative real-time PCR assay for rapid identification of deletion carriers in hemophilia. *Clin Chem* 2004; **50**: 1269–7.

8 Tizzano EF, Barcelo MJ, Baena M *et al.* Rapid identification of female haemophilia A carriers with deletions in the factor VIII gene by quantitative real-time PCR analysis. *Thromb Haemost* 2005; **94**: 661–4.

9 Miller SA, Dykes DD, Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Res* 1989; **16**: 1215, 1218.

10 Liu Q, Nozari G, Sommer SS. Single-tube polymerase chain reaction for rapid diagnosis of the inversion hotspot of mutation in hemophilia A. *Blood* 1998; **92**: 1458–9.

11 Bagnall RD, Waseem N, Green PM, Giannelli F. Recurrent inversion breaking intron 1 of the factor VIII gene is a frequent cause of severe hemophilia A. *Blood* 2002; **99**: 168–74.

12 Pavlova A, El-Maarri O, Luxembourg B *et al.* Detection of heterozygous large deletions in the antithrombin gene using multiplex polymerase chain reaction and denatured high performance liquid chromatography. *Haematologica* 2006; **91**: 1264–7.

13 Purroy J, Bisceglia L, Jaeken J, Gasparini P, Palacin M, Nunes V. Detection of two novel large deletions in SLC3A1 by semi-quantitative fluorescent multiplex PCR. *Hum Mutat* 2000; **15**: 373–9.

14 Sellner LN, Taylor GR. MLPA and MAPH: new techniques for detection of gene deletions. *Hum Mutat* 2004; **23**: 413–9.

15 Leuer M, Oldenburg J, Lavergne JM *et al.* Somatic mosaicism in haemophilia A: a fairly common event. *Am J Hum Genet* 2001; **69**: 75–87.

16 Schwaab R, Oldenburg J, Lalloz MRF *et al.* Factor VIII gene mutations found by a comparative study of SSCP, DGGE and CMC and their analysis on a molecular model of factor VIII protein. *Hum Genet* 1997; **101**: 323–32.

17 Casey GJ, Rodgers SE, Hall JR, Rudzki Z, Lloyd JV. Grandpaternal mosaicism in a family with isolated haemophilia A. *Br J Haematol* 1999; **107**: 560–2.

18 Oldenburg J, Rost S, El-Maarri O *et al.* De novo factor VIII intron 22 inversion in a female carrier presents as a somatic mosaicism. *Blood* 2000; **96**: 2905–6.

19 Ligon AH, Kashork CD, Richards CS, Shaffer LG. Identification of female carriers for Duchenne and Becker muscular dystrophies using a FISH-based approach. *Eur J Hum Genet* 2000; **8**: 293–8.

20 Oldenburg J, Schroder J, Hermann Brackmann H, Muller-Reible C, Schwaab R, Tuddenham E. Environmental and genetic factors influencing inhibitor development. *Semin Hematol* 2004; **1**(Suppl. 1): 82–8.

21 Salviato R, Belvini D, Are A, Radossi P, Tagariello G. Large FVIII gene deletion confers very high risk of inhibitor development in three related severe haemophiliacs. *Haemophilia* 2002; **8**: 17–21.

22 Goodeve AC, Peake IR. The molecular basis of hemophilia A: genotype-phenotype relationships and inhibitor development. *Semin Thromb Hemost* 2003; **29**: 23–30 (Review).