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LETTER TO THE EDITOR

Reliable and cost-effective approach for diagnosis of heterozygous *F8/F9* large deletions by quantitative real-time PCR

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Haemophilia A (HA) (OMIM#306700) and haemophilia B (HB) (OMIM#306900) are X-linked disorders characterized by deleterious mutations in coagulation factor VIII (*F8*) and factor IX (*F9*) genes respectively. Among these mutations, *F8* large deletions cause 8–15% of severe HA (www.factorviii-db.org) [1] and *F9* large deletions 6–13% of severe HB (www.factorix.org/) [2]. Molecular diagnosis of *F8* or *F9* large deletions (*F8/F9*-LDs) is critically important because these mutations associate with a highly significant predisposition to develop inhibitors against exogenous FVIII or FIX, the most troublesome complication for haemophilia treatment [3].

F8/F9-LDs diagnosis is directly achieved in hemizygous patients with haemophilia by exon-specific PCR. However, carrier diagnosis in females is complicated by the simultaneous presence of the non-affected allele. Typical approaches to diagnose heterozygous *F8/F9*-LDs include gap-PCR (deletion-specific PCR amplification analysis) as the gold standard [4], and gene dosage methods such as multiplex ligation-dependent probe amplification (MLPA) [5,6] or quantitative real-time PCR (qPCR) [7–9]. These approaches vary in diagnostic reliability and cost-effectiveness, which is essential for developing countries.

This study presents a qPCR-based method for diagnosis of heterozygous *F8/F9*-LDs in at-risk females using healthy males and females as controls to assess evidence-based carrier probabilities.

Diagnosis of heterozygous *F8/F9*-LDs was achieved by assessing a ratio between an X-linked target (T) (on *F8* Xq28, or *F9* Xq27.1-q27.2) and an autosomic reference (R) (on cytotoxic T-lymphocyte-associated protein 4, *CTLA4* 2q33). *CTLA4* was selected as reference because it is not significantly associated with copy number variations in healthy individuals as it is shown in the Database of Genomic Variants (www.dgvbeta.tcga.ca).

Mutation screening by exon-specific PCR amplification on *F8* [1] and *F9* [2] of 364 family probands with severe HA and 58 with severe HB allowed detection of 18 families with *F8*-LDs and 6 with *F9*-LDs. In this study, 14 females at risk of being carriers from seven families (five with severe HA and two with severe HB) were selected among these families with LDs. In addition, blood samples from 30 unrelated healthy blood donors including 15 males and 15 females were used as single and double X-chromosome dose controls respectively. Genomic DNA (gDNA) was isolated from peripheral blood leucocytes using the salting-out method.

Our local Institutional Ethics Committee CEIANM approved this work and a written informed consent was obtained from all cases.

QPCR amplification protocols of four target templates (*F8*-promoter, *F8*-exon 6 and *F8*-exon 26; and *F9*-exon 8) and a reference (*CTLA4*) were applied to obtain T/R ratios from cases and controls to model the experimental distribution of T/R ratios of heterozygous carriers and non-carriers respectively.

QPCR reactions were performed in a Rotor-Gene Q instrument (Qiagen) in 25 μL containing 14 μL of *MezclaReal*™ (Biodynamics, Argentina) (with unspecified concentrations of *Taq* DNA Polymerase, dNTPs, $MgCl_2$, Tris-HCl buffer and EvaGreen® fluorescent stain), 0.6 μM of each primer and 10 ng of EcoRI-fragmented gDNA. Primer sequences and genome coordinates of qPCR amplifications on targets (*F8*-promoter: 8-P1AB, *F8*-exon 6: 8-6AB, *F8*-exon 26: 8-26-qRTUpLo, *F9*-exon 8: 9-H2AB) and reference (*CTLA4*: CTLA4-FR) are shown in Supplementary Table ST1. Thermocycling conditions were 94°C for

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2 min followed by either 45 conventional triphasic cycles (95°C 15 s, 55°C 60 s and 72°C 60 s) for *F8*-E6, *F8*-E26, *F9*-H2 and *CTLA4*-a, or 45 biphasic cycles (95°C 15 s and 60°C 60 s) for *F8*-P1AB and *CTLA4*-b. Specificity of qPCR amplifications were double checked by conventional melting curve analysis (Supplementary Fig. SF1) and agarose gel electrophoresis analysis of qPCR products (Supplementary Fig. SF2). Estimation of qPCR amplification efficiency and calibration curves (Ct vs. DNA input) were performed using five serial dilutions ½ (range 2.5–40 ng) of EcoRI-digested DNA from a control male (C85) by triplicate (Supplementary Fig. SF1).

All T and R qPCRs showed amplification efficiencies within the range 0.96–1.24 and regression R^2 within 0.990–0.998 (Supplementary Fig. SF1). *T* and *R* values of patients and controls were obtained by averages of duplicates. No overlapping was observed between the ranges of *T*/*R* ratios from male and female control groups demonstrating the reliability of the approach to detect single and double relative doses of X-linked targets. The ranges of minimum–maximum *T*/*R* values of the 15 males and females normal controls were as follows: *F8*-promoter 0.71–1.12 and 1.36–2.42, *F8*-exon 6 0.78–1.43 and 1.69–2.37, *F8*-exon 26 0.70–1.23 and 1.57–2.41 and *F9*-exon 8 0.40–0.73 and 0.95–1.54. *T*/*R* data of healthy males and females were represented by Probability curves using their mean and standard deviations (sd) (Table 1) in the Gaussian formula $f(T/R) = \frac{1}{\mathrm{sd}\times\sqrt{2\pi}} \times e^{-0.5\times((T/R-\mathrm{mean})/\mathrm{sd}^2}$, after checking for Normality (Shapiro–Wilk test, $P < 0.05$). *T*/*R* ratios from females at risk were interpolated into male (Px) and female (Pxx) Probability curves to calculate a deletion carrier probability (Pc) as Px/(Px + Pxx) (Fig. 1).

The carrier diagnosis of 14 at-risk females from seven families with probands affected with *F8*/*F9*-LDs is shown in Table 1: nine of them were diagnosed as carriers (Pc = 100%) and five as non-carriers (Pc = 0%). These nine females diagnosed as carriers of *F8*/*F9*-LDs were studied on *F8* targets outside each family-specific *F8*/*F9*-LDs and Pc values of non-carriers were obtained in all cases (Pc = 0%) further confirming the consistency of the method for diagnosis of heterozygous LDs.

The validation of the presented qPCR-based approach was achieved by comparing results of the carrier status obtained by other techniques, as follows.

In Family 1, the proband's mother at risk of *F8*-exon 4_10 deletion was determined to be a carrier by qPCR (T/R: 0.89, Pc: 100%) confirming results of gap-PCR by long-range PCR amplification of the deletion junctions [4].

In Family 2, affected by a deletion involving *F8*-exon 3_26, the proband show a deletion-specific PCR product of about 650 bp, whereas the three at-risk females (the proband's mother and two sisters) showed the deletion-specific PCR product along with the normal-allele signal. All these three gap-PCR-based carrier diagnosis were equal to those obtained by qPCR (Table 1).

In Family 5, an obligate carrier of a *F9*-exon 7_8 deletion, and three non-carriers females diagnosed by MLPA were equal to qPCR-based diagnosis (Table 1).

Table 1. Diagnosis of heterozygous deletions in *F8* and *F9* in at-risk females from affected families.

Gaussian distribution curve, mean (SD)										
46; XY	46; XX	Case no. (family no.)	Family relation	At-risk deletion	T	R	T/R	Pc	Diagnostic conclusion	Validation
F8-exon 6										
1.07 (0.16)	2.05 (0.17)	137 (1)	M	exon 4_10	7.08	7.93	0.89	100%	Carrier	Gap-PCR*
F8-exon 26										
0.97 (0.13)	1.98 (0.28)	98 (2)	M+S	exon 3_26	33.27	35.81	0.93	100%	Carrier	Gap-PCR†
		563 (2)	S+SM	exon 3_26	4.57	5.14	0.89	100%	Carrier	Gap-PCR†
		676 (2)	S+SD	exon 3_26	1.25	1.67	0.75	100%	Carrier	Gap-PCR†
		108 (3)	S	exon 26	15.76	6.54	2.41	0%	Non-carrier	nd
F8-promoter P1AB										
0.91 (0.11)	1.84 (0.38)	14 (6)	M	exon 1	7.05	9.478	0.81	100%	Carrier	nd
		408 (6)	S	exon 1	5.31	6.95	0.77	100%	Carrier	Gap-PCR†
		242 (7)	S	exon 1	16.64	22.29	1.55	0%	Non-carrier	Gap-PCR†
		81 (7)	M	exon 1	15.47	9.96	0.64	100%	Carrier	Gap-PCR†
F9-exon 8										
0.57 (0.13)	1.25 (0.19)	382 (4)	S	exon 1_8	15.66	33.05	0.47	100%	Carrier	nd
		662 (5)	M+S	exon 7_8	3.46	7.89	0.44	100%	Carrier	MLPA‡
		663 (5)	S+SD	exon 7_8	5.15	4.74	1.09	0%	Non-carrier	MLPA‡
		664 (5)	SD+SDD	exon 7_8	3.96	3.57	1.11	0%	Non-carrier	MLPA‡
		665 (5)	SD+SDD	exon 7_8	2.61	1.89	1.39	0%	Non-carrier	MLPA‡

Mean corresponds to the average and (SD) corresponds to the standard deviation calculated from male and female control groups ($n = 15$), used to set the Gaussian distribution curves. P_c indicates the probability of carrier. Not done, nd. Family relation code: M indicates patient's mother; S, sister; SM, maternal aunt; SD, maternal niece; SDD, daughter of maternal niece. Plus sign (+) joins the familiar relationships with each one of two different affected individuals in the family.

*Case no. 137 was diagnosed as *F8* exon 4_10 deletion carrier by direct gap PCR (Rossetti *et al.*, 2004).

†Cases no. 98, 563, 676, 408, 242 and 81 were diagnosed by gap-PCR.

‡Case no. 662, 663, 664, 665 were diagnosed by MLPA at the *Sant Pau* Hospital, Barcelona, Spain (data kindly provided by Eduardo Tizzano).

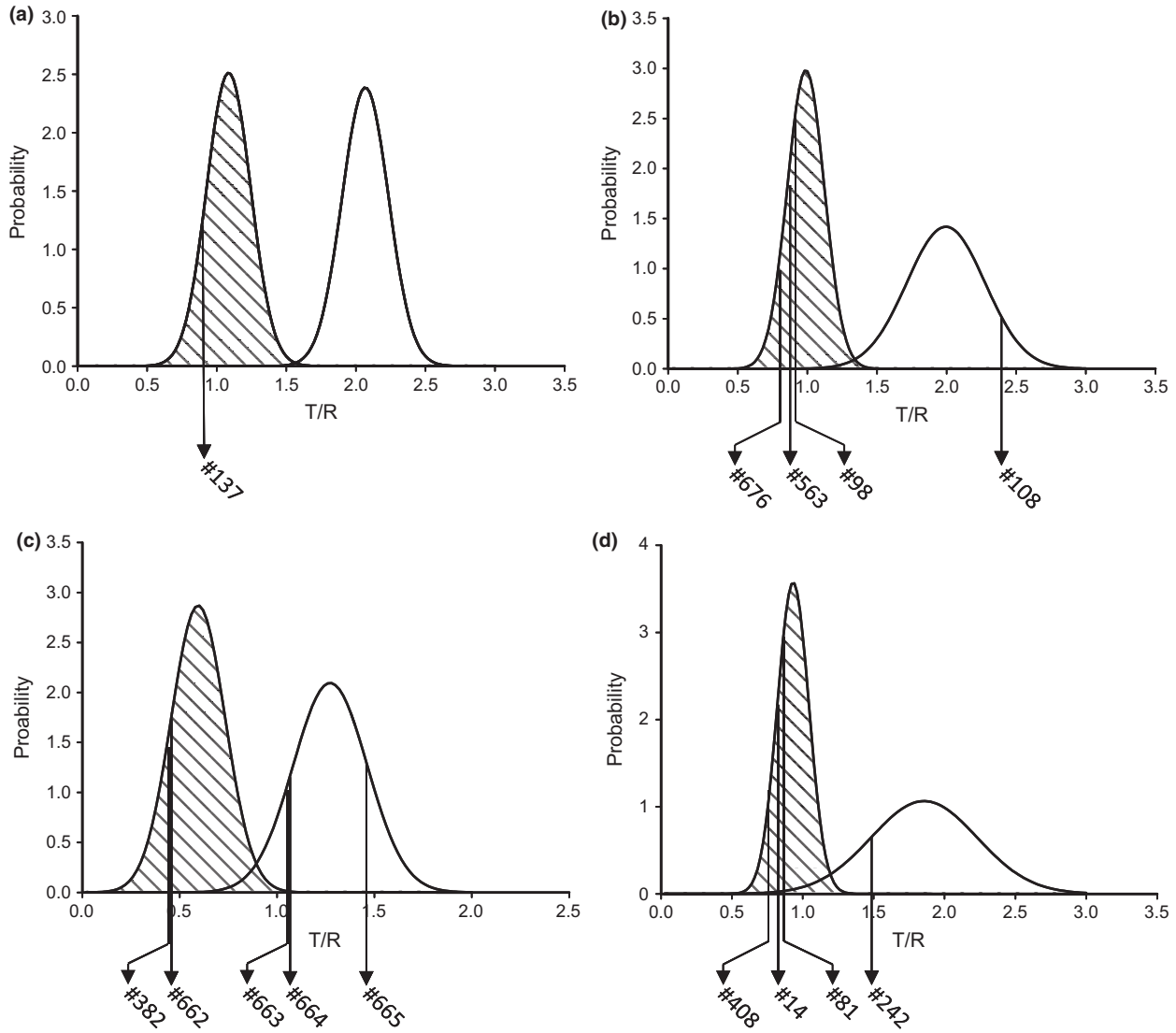

Fig. 1. Single and double X-chromosome dose probability curves of target-reference (*T/R*) ratios and interpolation of *T/R* ratios of female relatives at risk of large deletions in *F8* and *F9*. Hatched and open Normal (Gaussian) curves characterize *T/R* ratios from the general population of human males (46,XY) ($n = 15$) and females (46,XX) ($n = 15$) respectively. Panel (**a**): shows the carrier analysis of deletion on *F8* exon 6 (one family, case no.137); (**b**): analysis on *F8* exon 26 (two families, cases no. 98, 108, 563 and 676); (**c**): analysis on *F9* exon 8 (two families, cases no. 382, 662, 663, 664, 665); and (**d**): analysis on *F8* promoter (two families, cases no. 14, 81, 242 and 408).

In family 6, the proband, affected by a deletion involving *F8* promoter and exon 1, showed a gap-specific signal of about 4.5 kb, which is also present in his sister, case #408 along with a wild-type-specific signal on *F8*-intron 1 indicating her carrier status. QPCR-based diagnosis of case #408 was identical (Table 1).

In Family 7, affected by a deletion involving *F8* promoter and exon 1, the proband showed a gap-specific signal of about 400 bp, his mother showed the deletion-specific PCR product and the wild-type PCR product indicating the carrier status, and his sister showed only the wild-type allele signal indicating her non-carrier status. Both diagnostic conclusions were equal to those obtained by qPCR (Table 1).

The perfect matching of these results obtained by established techniques of these 11 female cases (seven carriers and four non-carriers) validates our qPCR-based approach on the four *F8*/*F9* targets.

We have recently estimated the *F8*/*F9* genotype associated inhibitor risks in Argentine patients with HA and HB, and found inhibitor odds ratios, OR (CI95), of 3.66(1.07–12.55) for *F8*-multi-exon deletions, and 32.7(2.26–471) for deletions on the entire *F9* respectively [2,10]. Inhibitor development impacts the quality of life of patients with haemophilia and the National health care system because of the higher costs involved for their specific treatment. Consequently, the provision of reliable information for genetic counselling in families affected by *F8*/*F9*-LDs

is critically important and all gene testing laboratories for haemophilia must include a reliable technique for carrier diagnosis of these HA/HB-causative mutations. We presented a reliable and cost-effective approach to detect heterozygous *F8*/*F9*-LDs. The qPCR-based method described herein employs control populations of healthy males and females to adjust and validate the approach, and to estimate experiment-based carrier probabilities as a diagnostic conclusion. The only drawback of qPCR-based approaches is the difficulty to screen all potential LDs in an obligate carrier, as MLPA or microarray technologies can do testing multiple targets in a single multiplex experiment. However, in haemophilia gene testing laboratories, investigation of the extent of the *F8*/*F9*-LDs in family probands provides information to localize the involved targets in most cases.

The practical protocol to set up a new X-linked amplification target to be measured by qPCR may take into account three basic hints: (i) choose target and reference regions associated with matching characteristics for efficient PCR amplifications (average human GC content on a repeat free region); (ii) adjust qPCR amplification protocols to get appropriate calibration curves (efficient range 0.75–1.25, Ct values lower than 30 cycles and qPCR products with a single melting peak (>80°C) and a specific molecular size); (ii) T/R values from male and female groups ($N = 15$) should not overlap and each control data group should pass the normality test before estimating a Pc of an analytical female sample. This procedure allows the presented approach to be applied with minimal efforts to diagnose heterozygous LDs on other targets in *F8* and *F9* as well as other X-linked genes such as *DMD* (dystrophin), *BTK* (Burton agammaglobulinemia tyrosine kinase) or *XIST* (X-inactive specific transcript) among others.

QPCR-based diagnosis of heterozygous LDs involves lower costs than MLPA and can be easily performed in laboratories of low/medium complexity worldwide. Gene dosage-based approaches such as MLPA and qPCR represent an advantage over long-range-based gap-PCR because this latter requires more DNA input quality and quantity being significantly more time consuming.

The use of a probability associated with the diagnostic conclusion is beneficial to estimate the accurateness and the extent of the diagnosis, which is particularly valuable for genetic counsellors, but also to repeat the protocol if the result poses any doubt (e.g., $5\% < Pc < 95\%$).

In conclusion, our data supports the inclusion of the presented approach into the molecular analysis algorithm for haemophilia after detection of a *F8*/*F9*-LDs in a hemizygous proband. This inclusion will help in reducing costs, without compromising diagnostic reliability.

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Disclosures

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

Author contribution

MMA designed and performed the research, analysed the data and wrote the manuscript; TT and CPR designed the research, analysed the data and approved the final version of this manuscript; VM performed the research and approved the final version of this manuscript; IBL, AFF and LCR designed the research, discussed the results and approved the final version of this manuscript; and CDB designed and performed the research, analysed the data and wrote the manuscript.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table ST1. Oligonucleotide primers for qPCR, long-range PCR and standard-PCR.

Fig. SF1. Crossing threshold (Ct) vs. concentration calibration curves and Melting curves. Calibration curves made with serial dilutions of a genomic DNA sample of a male from the general healthy population (C85) by triplicate (blue dots) on the left panel and their associated melting curves on the right panel. Calibration curve parameters are shown: slope (M), amplification efficiency and R^2. Row **A** shows amplification of *F8* exon 6; **B** with *F8* exon 26; **C** with *F9* exon 8; **D** with *F8* promoter; **E** with *CTLA4*a (a reference for *F8* exon 6, *F8* exon 26 and *F9* exon 8 targets); and **F** with *CTLA4*b (a reference for *F8* promoter).

Fig. SF2. Specificity analysis of qPCR products by agarose gel electrophoresis. Lane 1: 100 bp marker. Lanes 2–15: qPCR products are organized as Normal Control (lanes 2, 5, 8, 11 and 14, control C85) used to performed the calibration curves, a female at risk of being carrier (lane 3, case #408; lane 6, case #137; lane 9, case #563; lane 12, case # 382) and negative controls (no DNA) (lanes 4, 7, 10, 13 and 15). qPCR products: *F8* promoter (lanes 2–4); *F8* exon 6 (lanes 5–7); *F8* exon 26 (lanes 8–10); *F9* exon 8 (lanes 11–13); and *CTLA4 (*lanes 14–15).