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Short Communication

DNA sequencing analysis of several G6PD variants previously defined by PCR-restriction enzyme analysis

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Abstract

Results of a corroborative DNA sequencing analysis for five glucose-6-phosphate dehydrogenase (G6PD) mutations previously defined by PCR-restriction enzyme analysis are presented. The suitability for performing DNA sequencing analysis is discussed along with the importance of selecting the proper PCR-REA strategy in order to define the presence of a specific mutation .

Key words: G6PD, mutations, PCR-restriction enzyme analysis.

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We previously screened more than 5000 individuals for glucose-6-phosphate dehydrogenase (G6PD) deficiency in order to determine the molecular basis of G6PD deficiency in Mexico (Arámbula *et al.* 2000; Arámbula 2002; Medina *et al.* 1995, 1997; Vaca *et al.* 2002). In 67 (88%) of the G6PD deficient individuals detected, the mutation responsible being defined using PCR-restriction enzyme analysis (PCR-REA) combined with single strand conformation polymorphism (Arámbula y Vaca, 2002). Eight different G6PD deficient variants were detected , three of which were double mutants (Table 1).Five mutations (nt 202 G → A (+Nla-III), nt 376 A → G (+Fok-I), nt 542 A → T (+"BspEI"), nt 844 G → C (+Nla-III), and nt 968 T → C (+Nci-I)) were defined based on their electrophoretic banding pattern being compatible with the generation of a restriction enzyme site, and the four other mutations (nt 383 T → C (-Mnl-I), del nt 561-563 (-Mnl-I), nt 1178 G → A (-BstUI), and nt 1360 C → T (-Hha-I)) were defined by the elimination of a restriction site (Table 1).Since the methodology used could definitively define only 4 (202 G → A; 376 A → G; 542 A → T, and 844 G → C) out of 9 mutations , we decided to perform a corroborative DNA sequencing analysis for the remaining five mutations (nt 383 T → C; del nt 561-563; nt 968 T → C; nt 1178 G → A, and nt 1360 C → T).

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Exons 5, 6, 9, 10, and 11 were PCR amplified using previously described oligonucleotides primers (Beutler *et al.* 1991a) and the DNA sequenced with the Big Dye terminator kit in an ABI PRISM 310 Genetic Analyser.

The DNA sequencing analysis confirmed the genotypes previously assigned by PCR-REA , partial sequences being shown in Figures 1 and 2.

A brief description of the PCR-REA strategy previously used for the mutations corroborated by DNA sequencing analysis is given below.

The nt 383 T → C (Leu 128 Pro) (G6PD Vanua Lava) mutation destroys a Mnl-I site and shows a complex electrophoretic banding pattern (Arámbula, 2002).The restriction site can also be eliminated by mutations at nt 381-384 (Table 1). Any base change at nt 381 or 384 generates silent mutations(Ala 127 Ala or Leu 128 Leu respectively) with presumably normal G6PD activity. However nt substitutions at nt 382 or at nt 383 T → G or A generate missense mutations. To the best of our knowledge the only mutations at nt 381-384 reported to date are the G6PD Vanua Lava 383 T → C mutation and the silent polymorphisms at nt 381 C → T and nt 384 C → T (Beutler and Vulliamy 2002; Hamel *et al.* 2002; Verelli *et al.* 2002).

The del nt 561-563 (del Ser 188 or 189)(G6PD Tsukui) deletion eliminates a Mnl-I site and also shows a complex electrophoretic banding pattern (Arámbula, 2002). The observation of more rapid electrophoretic mobility of the undigested mutant PCR-product as compared

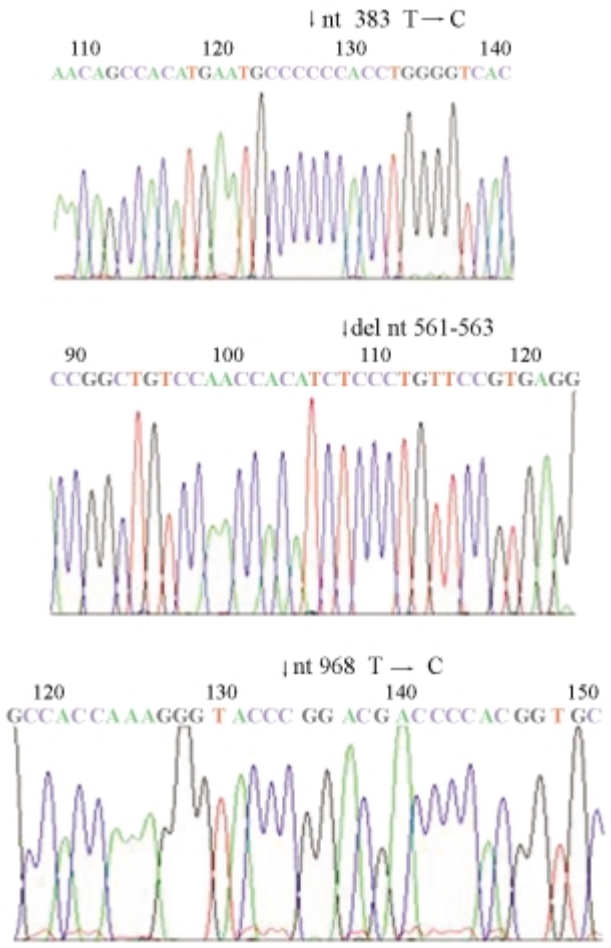

Figure 1 - Sequence analysis of a portion of exons 5, 6, and 9 showing the mutations at nt 383 T → C, del nt 561-563, and nt 968 T → C respectively.

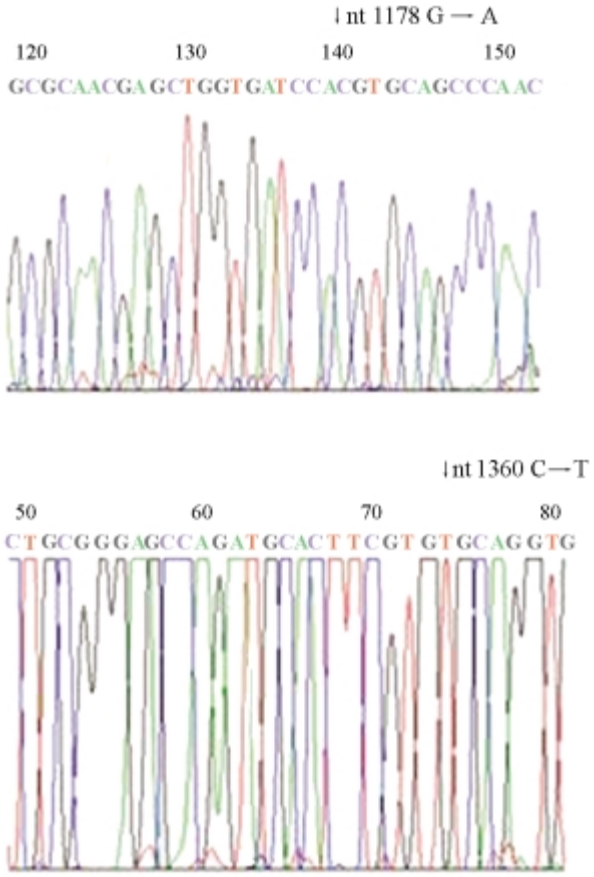

Figure 2 - Sequence analysis of a portion of exons 10, and 11 showing the mutations at nt 1178 G → A and nt 1360 C → T.

to the normal PCR-product strongly suggest the presence of this deletion. The restriction site can also be eliminated by substitutions at nt 563-566 (Table 1). Any base change at nt 563, 565, or 566 generates missense mutations (*e.g.* nt 563 C → T (G6PD Mediterranean variant , the mutation generates a Mbo-II site)), whereas nt substitutions at nt 564 generate silent polymorphisms (Ser 188 Ser).

The nt 968 T → C (Leu 323 Pro) mutation generates a Nci-I site in the double mutant G6PD $A^{-376G/968C}$ (Table 1), although it should be remembered that because the enzyme recognition sequence is 5'-CCNGG-3' where N = C or G, a base change at nt 968 T → G (Leu 323 Arg) can also generates a Nci-I site. A similar situation occurs with the mutations nt 680 G → T (Arg 227 Leu) (present in G6PD $A^{-376G/680T}$ variant) and nt 680 G → A (Arg 227 Gln) (G6PD Mexico City^{680A}), both of these mutations generating a BstNI site (5'-CCNGG-3' where N = T or A).

The nt 1178 G → A (Arg 393 His) (G6PD Nashville) mutation. The BstUI site at nt 1177-1180 can be eliminated by any base change of the enzyme recognition sequence 5'-CGCG-3' (Table 1). Nt substitutions at nt 1179 generate silent polymorphisms (Arg393Arg) whereas nt substitutions at nt 1177, 1178, or 1180 generate missense mutations such as the mutations at nt 1177 C → G (G6PD Wisconsin; Arg393 Gly), nt 1178 G → A (G6PD Nashville; Arg393His), and nt 1180 G → C (G6PD Alhambra; Val394Leu). The mutation present in the first variant also eliminates an Alw-I site whereas the mutation in the third variant generates a Pst-I site, the haplotype Alw-I/BstUI/ Pst-I +/–/ – suggesting the presence of the G6PD Nashville variant ,the presence of this haplotype being the accepted definition of the G6PD Nashville variant (Arámbula, 2002; Vaca *et al.* 2002).However an identical haplotype can also be generated by base changes such as nt 1178 G → T, or C; nt 1179 C → G, A, or T (silent mutations); and nt 1180 G → A, or T (Table 2).

The nt 1360 C → T (Arg 454 Cys) (G6PD Union) mutation. The Hha-I restriction site 5'-GCGC-3' at nt 1359-1362 can be eliminated by any base change in the enzyme recognition sequence (Table 1). Nt substitutions at nt 1359 or 1362 generate silent polymorphisms (Val 453 Val or Arg 454 Arg respectively). Base changes at nt 1360 or 1361

Table 1 - Glucose-6-phosphate dehydrogenase (G6PD) mutations detected by PCR-restriction enzyme analysis (PCR-REA). Nucleotide substitutions are in bold type and the restriction endonucleases recognition sequences are underlined.

G6PD B normal sequence	Nucleotide substitution	G6PD variant mutant sequence	Restriction site gain (+), elimination (–)
5'-CGTG-^{204} 5'-GAATG-^{379}	nt 202 G → **A** nt 376 A → **G**	5'-C**A**TG- 5'-G**G**ATG- G6PD A$^{-202A/376G}$	(+ Nla-III) (+ Fok-I)
5'-GAATG-^{379} 5'-A^{542}	nt 376 A → **G** nt 542 A → **T**	5'-G**G**ATG- 5'-**T**^{542} G6PD Santamaria$^{376G/542T}$	(+ Fok-I) ("Bsp-EI)**
5'-GAATG-^{379} 5'-CCTGG-^{970} Leu^{323}	nt 376 A → **G** nt 968 T → **C**	5'-G**G**ATG- 5'-CC**C**GG- G6PD A$^{-376G/968C}$	(+ Fok-I) (+ Nci-I)
Leu^{128} 5'-CCTC-^{384}	nt 383 T → **C**	5'-CC**C**C- G6PD VanuaLava^{383C}	(– Mnl-I)
Ser Ser^{189} 5'-CTCCTCC^{567}	del nt 561-563	5'-===CTCC- G6PD Tsukui$^{del561-563}$	(– Mnl-I)
5'-GATG-^{847}	nt 844 G → **C**	5'-**C**ATG- G6PD Seattle^{844C}	(+ Nla-III)
Arg^{393} 5'-CGCG-^{1180}	nt 1178 G → **A**	5'-C**A**CG- G6PD Nashville^{1178A}	(– BstUI)
Arg^{454} 5'-GCGC-^{1362}	nt 1360 C → **T**	5'-G**T**GC- G6PD Union^{1360T}	(– Hha-I)

**Restriction site generated by mismatching PCR when nt 542 is T.

generate missense mutations such as the mutation at nt 1361 G → A (G6PD Andalus; Arg454His) which also eliminates a BspMI site. Thus, the haplotype Hha-I/ BspMI –/+ suggest the presence of the G6PD Union variant , the presence of this haplotype being the accepted definition of the G6PD Union variant (Arámbula 2002; Vaca *et al.* 2002) but the same haplotype can also be generated by mutations at nt 1359 G → C, A, or T or by mutations at nt 1360 C → G, or A (Table 2).

It is important to carefully select the PCR-REA strategy to be used to define the presence of a specific point mutation. The use of programs (*e.g.* NEB cutter: www.neb.com) to analize the restriction enzymes that could be used to excise the PCR-product is also important because simply assuming the presence of a "specific" mutation based on electrophoretic banding patterns compatible with the elimination of a restriction site is inadequate , strategies based on the generation of a restriction site by a specific mutation being better. Retrospectively we have realized that the G6PD Nashville and G6PD Union variants can be defined by PCR-REA using the restriction enzymes Mae-II and BsgI site respectively, a Mae-II site (5'-^{1178}**A**CGT-3') occurring in the G6PD Nashville variant and a BsgI site (5'-^{1359}G**T**GCAG-3') in the G6PD Union variant. Similarly, BsmFI PCR-REA would permit discriminate between the mutations at nt 968 T → C or 968 T → G (both are Nci-I +) since the second mutation also generates a BsmFI site (5'-^{968}**G**GGAC-3'). Obviously, when possible, direct DNA sequencing is a good option, particularly in those cases in which mutations can not be categorically defined by PCR-REA.

To date a total of 18 different G6PD variants have been observed in Mexico (Arámbula *et al.* 2000; Arámbula 2002; Beutler *et al.* 1991b; Beutler *et al.* 1992; Lisker *et al.* 1981; Medina *et al.* 1995; Medina *et al.* 1997; Vaca *et al.* 2002; Vaca *et al.* 2003) and 3 of them (both G6PD A^{-} variants along with G6PD Santamaria variant) account for more than 80% of the overall prevalence of G6PD deficiency in Mexico (Arámbula 2002; Lisker 1981; Vaca *et al.* 2002).This pattern of common G6PD variants is similar to that seen in other parts of the Americas such as Costa Rica (Beutler *et al.* 1991c), Cuba (Rovira *et al.* 1994), and Brazil (Hamel 2002; Weimer 1998).

In conclusion, it should be stressed that it is important to select the proper PCR-REA strategy because even a PCR-REA strategy based on the presence of a restriction site generated by a mutation could, in some cases, be inadequate to categorically define the presence of a particular mutation. Even so, PCR-REA can be used not only to detect the presence or absence of certain alleles (and their frequency) in specific populations but also for the definition or exclusion of a molecular diagnosis for a specific monogenic disease as well as for other estimates, definitions or exclusions. This methodology is important because thera-

Table 2 - PCR-REA genotyping of the glucose-6-phosphate dehydrogenase (G6PD) G6PDNashville and G6PD Union variants. Nucleotide substitutions are in bold type and the restriction endonucleases recognition sequences are underlined.

G6PD B normal sequence	Nt subst.	G6PD variant mutant sequence	Haplotype		
			Alw-I	BstUI	Pst-I
GATCC[1177] Alw-I 5'GATCCGCGTGCAG[1185] CGCG[1180] BstUI			+	+	–
	Nt 1177 C → **N1** C → **G** G6PD Wisconsin	GATC**G**[1177] [1177]**G**GCG	–	–	–
	Nt 1178 G → **N2** G → **A** G6PD Nashville	GATCC[1177] [1177]C**A**CG	+	–	–
	Nt 1179 C → **N3** Undescribed	GATCC[1177] CC**N**G[1180]	+	–	–
	Nt 1180 G → **N4** G → C G6PD Alhambra	GATCC[1177] CGC**C**[1180] [1180]**C**TGCAG Pst-I	+ +	– –	+ or – +
			Hha-I	BspMI	
GCGC[1362] Hha-I 5'-GCGCAGGT-[1366] GCAGGT[1366] BspMI			+	+	
	Nt 1359 G → **N1** Undescribed	**N**CGC[1362] GCAGGT[1366]	–	+	
	Nt 1360 C → **N2** C → **T** G6PD Union	G**T**GC[1362] GCAGGT[1366]	–	+	
	Nt 1361 G → **N3** G → **A** G6PD Andalus	GC**A**C[1362] [1361]**A**CAGGT	–	–	
	Nt 1362 C → **N4** Undescribed	GCG**N**[1362] [1361]G**N**AGGT	–	–	

For sequence nt 1173-1185: N1 = G, A, T; N2 = C, A, T; N3 = G, A, T; N4 = C, A, T. Base changes N1-N4 in the BstUI recognition sequence.
For sequence 1359-1366: N1 = C, A, T; N2 = G, A, T; N3 = C, A, T; N4 = G, A, T. Base changes N1-N4 in the Hha-I recognition sequence.

peutic decisions and genetic counseling could be based on the assigned genotype.

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