

SHORT COMMUNICATION

RAPID IDENTIFICATION OF THE ASSOCIATION OF HEMOGLOBIN D PUNJAB AND HEMOGLOBIN S (HbD Punjab/HbS) BY THE POLYMERASE CHAIN REACTION

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ABSTRACT

Hemoglobin D-Punjab has been observed in several ethnic groups, either in heterozygosis or in association with Hb S or β -thalassemia. In this report, we describe the case of a 10 year-old Black Brazilian girl who presented the classical clinical and hematological features of sickle cell disease, but whose hemoglobin electrophoretic profile suggested the association Hb S/Hb D. The Hb D mutation was confirmed by EcoRI digestion of the PCR amplified β globin gene and by sequencing of the resulting fragment. The mutation involves a simple base change at codon 121 which eliminates a normal EcoRI site. The abnormal gene can be detected by agarose gel electrophoresis. This finding represents the second proven case of Hb S/Hb D association in Brazil. We suggest that Hb D should be investigated by PCR techniques in sickle cell disease patients presenting an anomalous hemoglobin electrophoretic profile.

INTRODUCTION

Hemoglobin D-Punjab or D-Los Angeles ($\alpha_2 \beta_2 121(\text{GH4}) \text{Glu-Gln}$) is a hemoglobin variant commonly encountered in Pakistan and Northwest India, particularly

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in the Punjab region (Lehmann, 1986; Brittenham, 1987). In the Sikh population of the Punjab, Hb D- Punjab has a prevalence of 2-3% but it has also been observed in several other ethnic groups (Lehmann, 1986). Hb D-Punjab is not common in Brazil but has been described in both the simple heterozygous state and in association with hemoglobin S (Hb S) and β -thalassemia (Zago and Costa, 1985, 1988).

The association of Hb D and Hb S (Hb D/Hb S) is clinically and hematologically indistinguishable from Hb S homozygosis. Hb D comigrates with Hb S under standard electrophoretic conditions, but it can be suspected on the basis of its agar gel electrophoretic mobility and its high solubility in phosphate buffer (Lehmann, 1986). However, the identification of this variant can only be accomplished by protein structural or by DNA analysis (Zeng *et al.*, 1989). We describe the rapid identification by DNA analysis of Hb D in a patient with association of Hb S and Hb D.

CASE STUDY

The patient was a 10 year old black girl who presented anemia and jaundice since the age of two years. She presented several episodes of vaso-occlusive crises and received occasional blood transfusions. The clinical examination showed moderate anemia and jaundice without splenomegaly. The parents were asymptomatic.

MATERIAL AND METHODS

Laboratory data of the patient and of her immediate relatives are shown in Table I. The hemoglobin electrophoretic profile and solubility of hemoglobin in phosphate buffer suggested an Hb S/Hb D association. Red blood cells, hemoglobin concentration, and MCV were determined electronically (Coulter Counter S.Sr). Hemoglobin electrophoresis was performed on cellulose acetate with Tris-EDTA-boric acid buffer at pH 8.9 and on agar gel with sodium citrate buffer at pH 6.1 (Weatherall and Clegg, 1972). HbA₂ was measured spectrophotometrically after elution from cellulose acetate strips following electrophoresis (Marengo-Rowe, 1965). Hb F was determined by alkali denaturation (Pembrey *et al.*, 1972) and the solubility of hemoglobin S was measured in 2.87M phosphate buffer (Zago *et al.*, 1982).

In order to identify the hemoglobin variant we carried out an amplification of the β globin gene by the polymerase chain reaction (PCR), followed by digestion with the restriction enzyme EcoRI. For the globin gene amplification we used the following reaction primers: P3 = 5' - CTAGACAGAGAAGACTCTTG - 3' (located at position IVS1, nt 52) and P7 = 5' - GACCTCCCACATTCCCTTTT - 3' (located at position +1662, with respect to the Cap site). These primers flank exons 2 and 3 and yield an amplified fragment of 1469 base pairs (bp). The reaction mix contained 100 pmol of each primer,

Table I - Laboratory data of the family with Hb S/Hb D association.

	Hb g/dl	MCV fl	MCH pg	Hb F %	Hb A2 %	Hb electrophoresis
Patient	8.5	102	34	9.0	2.0	A2 + (S + D)
Father	15.4	93	32	0.4	2.0	A2 + S + A
Mother	13.7	89	29	0.7	2.2	A2 + D + A
Brother	14.6	85	30	0.4	2.4	A2 + D + A
Sister	13.2	88	29	0.5	2.1	A2 + S + A

200 μ M of deoxynucleotides, four units of Taq polymerase (BRL), 50 mM Tris-HCl pH 8.3, 0.01% gelatin, 1.5 mM HCl, 1.5 mM MgCl₂ and 0.5 μ g of DNA in a total volume of 100 μ l. This mixture was overlaid with light mineral oil and the PCR reaction were performed in a DNA thermal cycler (Perkin Elmer Cetus). The reaction conditions consisted of initial denaturing at 94°C for 6 min followed by 40 cycles of 94°C for 90s, 55°C for 90s and 72°C for 120s, with a final polymerization step at 72°C for 7 min.

For direct sequencing of the previously amplified fragment, we sequenced the double-stranded amplified DNA with an internal sense primer (5'-AATCCAGCTACCATTCTGC - 3', located at +1450 with respect to the Cap site), and the antisense primer P7, Taq DNA polymerase and the double strand sequencing kit (GIBCO-BRL) following the manufacturer's recommendations.

RESULTS AND DISCUSSION

The mutation that causes Hb D-Punjab is a single base substitution in codon 121 of the gene (GAA-CAA). This alteration removes the EcoRI recognition site (5'-GAATTC - 3') normally located in this DNA region (Zeng *et al.*, 1989). Thus, normal DNA amplified with the primer pair P3 and P7 can be cleaved by EcoRI giving two fragments, one each of 1201 and 268 bp. However, amplified DNA from Hb D-Punjab carriers must originate three fragments after digestion: the two normals (1201 and 268 bp) and the intact 1469 bp fragment.

In the patient studied here, we observed the typical pattern of Hb D heterozygosis, thus confirming that the patient is a compound heterozygote for Hb S and Hb D (Figure 1). Two other very rare hemoglobin variants, Hb Bcograd (β 121 Glu-Val) and Hb D-Arab (β 121 Glu-Lys), also can be detected by EcoRI digestion as a result of base changes in the same codon as in Hb D-Punjab. We therefore confirmed the Hb

D-Punjab variant by direct sequencing of the DNA (Figure 2). As the Hb Beograd and Hb D-Arab variants are extremely rare compared to the frequency of Hb D-Punjab, we suggest that it is not necessary to perform such DNA sequencing or dot-blot hybridization to confirm each case of Hb D-Punjab.

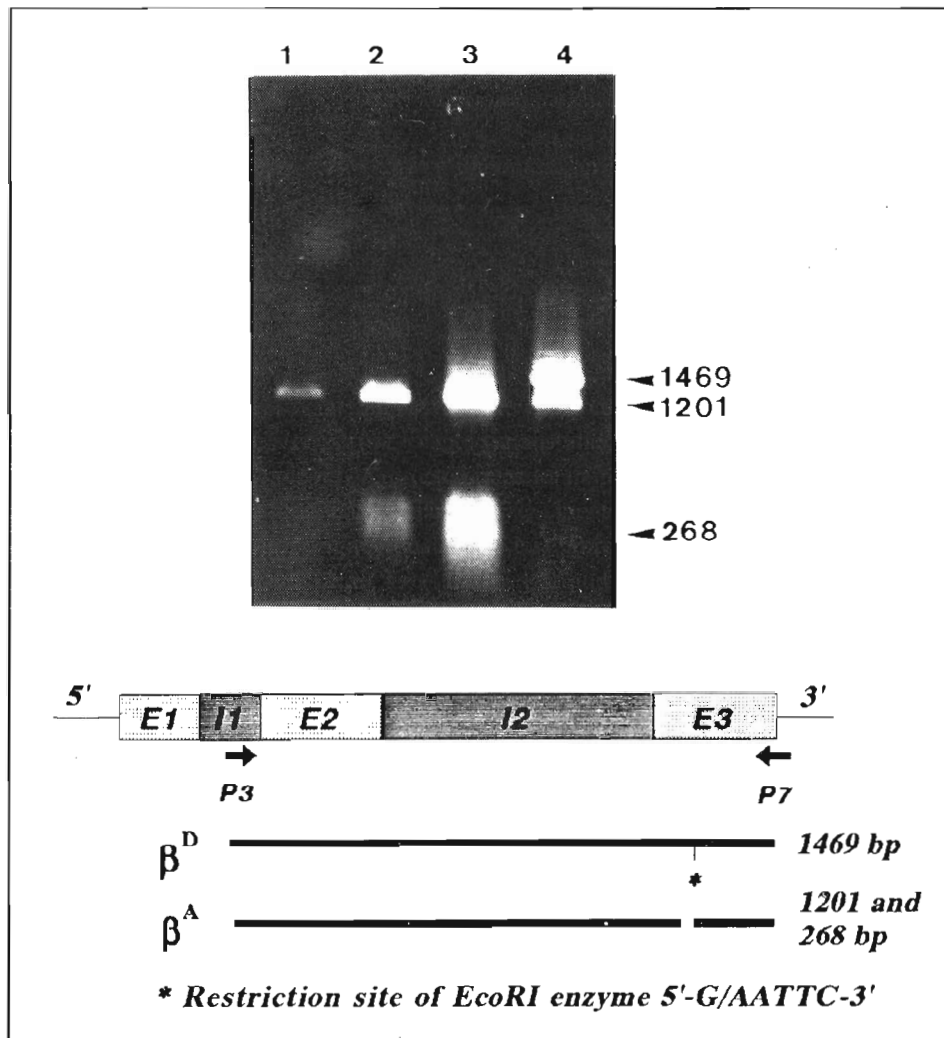


Figure 1 - Above: PCR amplified genomic DNA digested with *EcoRI* and electrophoresed in 0.8% agarose. Lanes 1 and 3 - normal controls, Lane 2 - patient homozygote for hemoglobinopathy S and Lane 4 - propositus (Hb S/Hb D). Below: Representation of β -globin gene showing the primers positions and the length of DNA fragments after *EcoRI* digestion in the normal (β^A) and mutant gene (β^D).

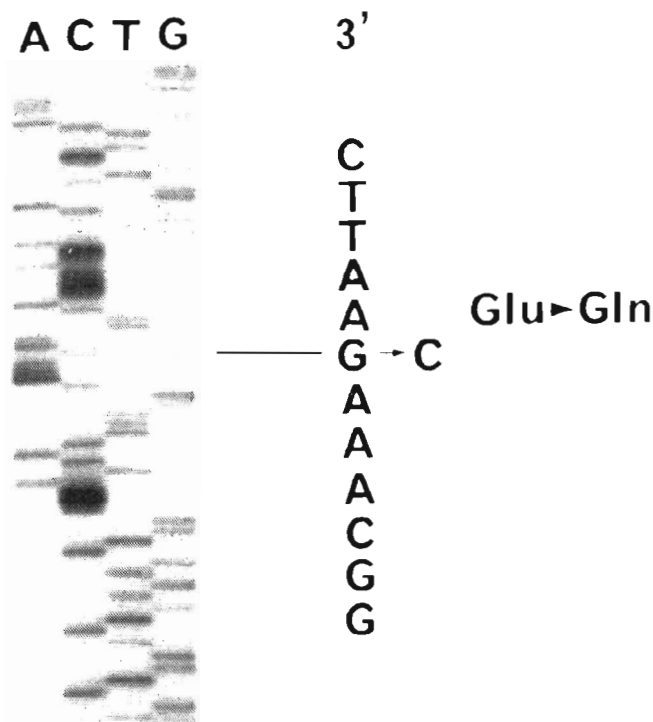


Figure 2 - Nucleotide sequence of PCR amplified β -globin gene of the patient studied. The G - C substitution at codon 121, i.e. Hb D Punjab, is indicated by the arrow.

The individual reported here represents the second proven case of Hb S/Hb D association in Brazil. In our patient, as in the family previously described (Zago and Costa, 1988), an African origin for the Hb D-Punjab gene may be presumed since both parents were Black and a prevalence of 0.1 - 0.4% has been reported for this variant among American Blacks (Lehmann, 1986). On the basis of these results, we suggest that the occurrence of this hemoglobin association should be investigated in all individuals with suspected homozygosity for Hb S who present an anomalous hemoglobin electrophoretic pattern or a high solubility in the phosphate buffer test. The present paper describes a single and rapid procedure to identify the Hb D-Punjab mutations by PCR, which may be useful to detect the cases of Hb D carriers in Brazil.

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RESUMO

A hemoglobina D-Punjab foi descrita em vários grupos étnicos, tanto em heterozigose simples quanto em associação com Hb S ou β -talassemia. Neste trabalho descrevemos uma paciente brasileira, negra de 10 anos de idade que apresentava características clínicas e hematológicas clássicas de doença falciforme. O padrão eletroforético sugeriu a associação Hb S/Hb D. A mutação da Hb D foi confirmada através da digestão do fragmento amplificado do gene da globina β com a enzima EcoRI e sequenciamento direto do fragmento amplificado. A mutação muda uma base no codon 121 e abole o sítio de reconhecimento normal da enzima, possibilitando o reconhecimento do gene anormal em eletroforese de gel de agarose. Estes dados representam a segunda descrição comprovada da associação Hb S/Hb D no Brasil e indicam que a presença da Hb D deve ser investigada, pelo método aqui descrito, em casos de doença falciforme com padrão eletroforético anômalo.

REFERENCES

- Brittenham, G.M. (1987). Globin gene variants and polymorphisms in India. In: *Hemoglobin Variants in Human Populations* (Winter, W.P., ed.). Vol. 2. CRC, Boca Raton, pp. 79-110.
- Lehmann, H. (1986). Human hemoglobin variants. In: *Hemoglobin: Molecular, Genetic and Clinical Aspects* (Bunn, H.F. and Forget, B.G., ed.). W.B. Saunders Company, Philadelphia, pp. 381-421.
- Marengo-Rowe, A.J. (1965). Rapid electrophoresis and quantitation of haemoglobin on cellulose acetate. *J. Clin. Pathol.* 18: 790-792.
- Pembrey, M.E., MacWade, P. and Weatherall, D.J. (1972). Reliable routine estimation of small amounts of foetal haemoglobin by alkali denaturation. *J. Clin. Pathol.* 25: 738-740.
- Weatherall, D.J. and Clegg, J.B. (1972). *The Thalassemia Syndromes*. 2nd edn. Blackwell Scientific Publications, Oxford.
- Zago, M.A. and Costa, F.F. (1985). Hereditary haemoglobin disorders in Brazil. *Trans. Royal Soc. Trop. Med. Hyg.* 79: 385-388.
- Zago, M.A. and Costa, F.F. (1988). Hb D-Los Angeles in Brazil: simple heterozygotes and associations with β -thalassemia and with Hb S. *Hemoglobin* 12: 399-403.
- Zago, M.A., Costa, F.F. and Bottura, C. (1982). Teste de solubilidade quantitativo modificado em hemolisados normais e em variantes de hemoglobina. *Rev. Paulista Med.* 100: 15-17.
- Zeng, Y., Huang, S., Ren, Z. and Zi, H. (1989). Identification of Hb D-Punjab gene: Application of DNA amplification in the study of abnormal hemoglobin. *Am. J. Hum. Gen.* 44: 886-889.

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