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PROTEIN AND DNA ANALYSIS FOR IDENTIFICATION OF HEMOGLOBIN VARIANTS

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ABSTRACT

Protein and DNA analyses were performed to identify the type of hemoglobin variation present among Filipino residents of Los Baños, Laguna and to determine the frequency of hemoglobin variants observed. A total of 0.58% hemoglobin (Hb) variants in a significantly large sample of 3452, which is 7% of the Los Baños population, was observed. Among the variants were 12 (0.3475%) cases of FS_1, 7 (0.2028%) cases of FS_2 and 1 (0.0290%) SS.

Three samples of FS_2 were found to be HbAE. The band pattern was A, "C" at 68.3% and 31.6%, respectively, by cellulose acetate electrophoresis; "A" by citrate agar electrophoresis; post Bc by alkaline globin electrophoresis; and E with acid globin electrophoresis.

High performance liquid chromatography (HPLC) separation of hemoglobin using an Interact and an AX-300 column showed as HbA"C". A normal and an abnormal β chain were detected using a C4 column. An HPL chromatogram of tryptic peptides showed a new peak which by amino acid compositional analysis has been identified as β T3 (27-30), indicating an amino acid substitution at B 26 (glu - -> lys).

Specimen FS_1 was found to be heterozygous for Hb D Punjab B (GH 4): glu-gln. This showed a hemoglobin D pattern by electrophoresis; an HB A, "S" and "C" composition at 40.6, 56.2 and 3.2% of the total, respectively; an abnormal beta globin chain by C4 reverse phase HPLC; an abnormal 13th peptide with a possible replacement of glutamine for glutamic acid at the 121st residue of the B chain as detected by C18 reverse phase chromatography of trypsin-digested

aminoethylated hemoglobin and compositional amino acid analysis of the peptide. A diagnostic 8.8 Kb DNA fragment for Hb D Punjab was detected by Southern blot hybridization of the ECO RI digested genomic DNA.

Considering that Hb variants often showed single occurrences in different families, no conclusions can be made regarding their pattern of inheritance in man. An allelic, autosomal codominant pattern of inheritance for FS_1 was observed in one family of 13 with 5 cases of the variant.

Electrophoresis, HPLC peptide mapping and Southern blot DNA hybridization provide powerful tools for the diagnosis of hemoglobin variation in man for genetic counseling and proper medical care.

INTRODUCTION

Polymorphic forms of hemoglobin are structural forms which vary from the normal hemoglobin Hb A. These structural variants are usually caused by point mutations which in most cases result in abnormal phenotypic effects. A hemoglobin variant Hb S results in sickling of erythrocytes and causes severe hemolytic anemia. A hematological abnormality known as a target cell formation is associated with Hb C while Hb E is frequently found in association with thalassemia (Burch et al., 1960).

At present, over 600 hemoglobin variants are known. Hemoglobin variants are heterogeneously distributed among the different populations of the world. In Africa, Hb S is more prevalent in the Western portion (Neel, 1957; Weisenfield, 1967). In the United States, the sickle cell hemoglobin is most frequently observed among blacks for whom it has been estimated that the gene frequency of the allele is of the order of 10% (Master and Holmes, 1957).

Hb D was found to be prevalent in Indian Sikhs and Punjab Hindus (Neel, 1957). HB E is common in Southeast Asia (Lie-Injo, 1969), most especially in some regions of Thailand (Supa, 1957).

In the Philippines, incidence of individuals with Hb A and Hb E was found to be highest in the Western Visayas region (Blackwell et al., 1969). However, no Hb E was found in a homozygous condition suggesting that most Hb variants are in the heterozygous condition in the trait carriers. Rodriguez (1977) reported heterozygotes for Hb E among UP Los Baños students. Normal hemoglobin (Hb A) was found to occur at a frequency of 0.99691 while Hb E occurred at a frequency of 0.00309.

The study was done to identify hemoglobin variants detected in a survey on hemoglobin polymorphism among Filipinos. The target area was Los Baños, Laguna where representatives of different regions of the Philippines live because this is where a unit of the state university is located.

MATERIALS AND METHODS

Population Survey

Blood samples from 3,408 residents of Los Baños, Laguna were obtained by finger-prick using a blood lancet and placed in a heparinized capillary tube half-full. The tubes were filled with an equal amount of isotonic saline solution (0.85% NaCl). The content was blown onto a clean glass slide and mixed by an applicator stick. The mixture was then taken up by capillary action into a plain capillary tube half-full. A 1-2 mm air space was left between the mixture and an equal amount of sucrose + Tris-Triton-X lysing solution. The tubes were sealed at the sucrose end with a capillary tube sealant. These tubes were centrifuged in Hettich EBA III types 200g clinical centrifuge at setting no. 6 (3300 rpm) for 15 min to separate the erythrocytes from the plasma. The samples were then ready for electrophoretic analysis.

Samples were run by starch-gel electrophoresis (pH 8.6) using 12.5% starch in a continuous buffer system (Tris-EDTA-Boric acid).

Staining was done using 1% buffalo black stain for one minute, washed and destained in 45% acetic acid.

The bands that appeared after electrophoresis were observed. The Rf value was determined by getting the ratio of the migration of the enzyme to the migration of the tracking dye. The observed mobilities were compared to the mobilities of normal and abnormal hemoglobin presented by Burch et al. (1960).

Identification of Hemoglobin Variants

Preparation of Hemolysates

Erythrocytes from individuals with abnormal hemoglobin as detected by electrophoresis were shipped to the Comprehensive Sickle Cell Center, Children's Hospital Medical Center, Cincinnati, Ohio where hemoglobin analysis was done.

An aliquot of the erythrocyte was hemolyzed with 1.5x the volume of water and 0.4x the volume of toluene for 15 minutes with occasional stirring, centrifuged at 3,600 rpm for 15 min at room temperature and filtered using a 0.2 um cellulose acetate membrane. Hemoglobin concentration was measured spectrophotometrically at 540 nm using Drabkin's reagent.

Hemoglobin Analysis

Hemoglobin analysis was done by cellulose acetate electrophoresis at pH 8.2–8.6, citrate agar electrophoresis at pH 6 – 6.3 (Huisman and Jonxis, 1977) and high performance liquid chromatography using an interact column with Tris-NaN and NaCl solvent system. The chromatogram was developed at a linear gradient of 10-12% mixture B (0.5 NaCl in 10mM Tris, pH 8.06) in 10 ml and to 40% in 14 ml, a flow rate of 1 ml/minute. Mixture A was 500 mg NaN in 10 mM Tris, pH 8.06.

Globin chains from a sample approximately 0.150 mg Hb were separated by reverse phase high performance liquid chromatography using a Vydac large pore C4 column with a 0.1% trifluoroacetic acid (TFA) water-acetonitrile (CHCN) solvent system (Table 1). The chromatogram was developed at a linear gradient of 47-69% mixture B (0.1% TFA in 60% CHCN) in 55 ml at a flow rate of 1 ml/min. Mixture A was 0.1% TFA in 20% acetonitrile. Absorbance was monitored at 280 nm.

For peptide analysis, trypsin digestion was done on whole hemoglobin for three hours using the method as described by Schroeder et al. (1979) but with Trypsin solution added at 0 and 3 hours incubation.

Analytical chromatograms were prepared with 0.5 mg tryptic digests of hemoglobin in an Altex Ultrasphere (ODS) C18 column using different solvent systems for each hemoglobin variant. Absorbance was monitored at 214 nm. Abnormal peaks were collected, lyophilized and reconstituted with 200 ml water for amino acid analysis.

DNA Preparation and Analysis

White blood cell pellets separated from 20 ml of EDTA anticoagulated blood were frozen and transported to Cincinnati, Ohio where DNA was prepared and analyzed.

DNA was prepared by Proteinase K digestion of the white blood cell pellets, repeated phenol extraction and isopropanol precipitation (Mears et al., 1981).

Ten μg of DNA was digested with the restriction endonuclease enzyme Eco RI for 4 hours, then electrophoresed overnight on 0.8% agarose gels. The DNA was transferred to nitrocellulose filters by Southern blotting and hybridized with the 32P labelled β cDNA-containing plasmid probe JW102. The filter was under stringent conditions and autoradiographed.

RESULTS AND DISCUSSION

A total of 0.58% Hb variants in a significantly large sample of 3454, which is 7% of the Los Baños population, was observed. Among the variants were 12 (0.3475%) cases of FS_1, 7 (0.2028%) cases of FS_2 and 1 case (0.0290) of SS (Fig. 1 and Table 2).

Three samples of FS_2 were found to be HbAE. The band pattern was A, "C" at 68.3% and 31.6%, respectively, by cellulose acetate electrophoresis (Fig. 2); "A" by citrate agar electrophoresis; post Bc by alkaline globin electrophoresis; and E with acid globin electrophoresis.

High performance liquid chromatography (HPLC) separation of hemoglobin using an Interact and an AX-300 Column showed as HB A "C". A normal and an abnormal β chain were detected using a C4 column. An HPL chromatograph of tryptic peptides showed a new peak which by amino acid compositional analysis

has been identified as βT3 (27-30), indicating an amino acid substitution at β26 (glu-lys) (Fig. 3 and Table 3).

Specimen FS_1 was found to be Hb D Punjab B (GH 4): glu-gln. This showed a hemoglobin D pattern by electrophoresis; an Hb A, "S" and "C" composition at 40.6, 56.2 and 3.2% of the total (Fig. 2); and an abnormal β globin chain by C4 reverse phase HPLC. It also showed an abnormal 13th peptide with a possible replacement of glutamine for glutamic acid at the 121st residue of the B chain as detected by C18 reverse phase chromatography of trypsin-digested aminoethylated hemoglobin and compositional amino acid analysis of the peptide (Fig. 4 and Table 3). A diagnostic 8.8 Kb DNA fragment for Hb D Punjab was detected by Southern blot hybridization of the ECO RI digested genomic DNA (Fig. 5).

Considering that Hb variants often showed single occurrence in different families, no conclusions can be made regarding their pattern of inheritance in man. An allelic, autosomal pattern of inheritance for FS_1 was observed in one family of 13 with 5 cases of the variant (Fig. 6).

SUMMARY AND CONCLUSION

Protein and DNA analyses were performed to identify the type of hemoglobin variation present among Filipino residents of Los Baños, Laguna and to determine the frequency of hemoglobin variants observed. A total of 0.58% hemoglobin (Hb) variants in a significantly large sample of 3452, which is 7% of the Los Baños population, was observed. Among the variants were 12 (0.3475%) cases of FS_1, 7(0.2028%) cases of FS_2 and 1(0.0290%) case of SS. Considering that Hb variants often showed single occurrences in different families, no conclusions can be made regarding their pattern of inheritance in man. An allelic, autosomal codominant pattern of inheritance for FS_1 was observed in one family of 13 with 5 cases of the variant.

Electrophoresis, HPLC peptide mapping and Southern blot DNA hybridization served as powerful tools for the identification of FS_1 as Hb AD [Punjab B (GH 4)]: glu-gln FS_2 as HbAE.

Table 1. Solvent systems for HPLC separation of variant hemoglobin

Hemoglobin Variant	*Developer* A	*Developer* B	*Development*
FS_1	10mM NH_4OAc, pH 6.07	100% CH_3CN	0.40% B in 1 DO min at 1ml/min
FS_2	0.1% TFA in H_20	0.1% TFA in CH_3CN	0.55% B in 120 min at 1ml/min

Table 2. Percentage frequency of Hb electrophoretic banding patterns in human population, Los Baños, Laguna

AREA	*ELECTROPHORETIC BANDING PATTERN*									
	FF		*SS*		*FS_1*		*FS_2*		*TOTAL*	
	No.	*%*	*No.*	*%*	*No.*	*%*	*No*	*%*	*No.*	*%*
Anos	161	4.6612	0		1	0.0290	0		162	4.6902
Bambang	418	12.1019	0		1	0.0290	1	0.0290	420	12.1598
Batong Malake	907	26.2594	0		3	0.0868	2	0.0579	912	26.4041
Bayog	413	11.9572	0		0		2	0.0579	415	12.0151
Lalakay	320	9.2646	0		5	0.1448	0		325	9.4094
Maahas	24	0.6948	1	0.0290	0		0		25	0.7238
Mayondon	127	3.6769	0		2	0.0579	0		129	3.7348
San Antonio	33	0.9554	0		0		1	0.0290	34	0.9844
Tadlak	145	4.1980	0		0		0		145	4.1980
Timugan	227	6.5720	0		0		0		227	6.5721
Tuntungin	659	19.0793	0		0		1	0.0290	660	19.1083
TOTAL	3434	99.4237	1	0.0290	12	0.3475	7	0.2028	3454	100.0000

Table 3. Amino acid composition of the abnormal peptides

AMINO ACID	*Hb E BT (26) glu-lys* *Residues 27-30*	*Hb D BT (121) glu-gln* *V*		*A*
asx				
glx		2.7	(3)	3
ser				
gly	1.03 (1)			
his				
arg	1.12 (1)			
thr		1.25	(1)	1
ala	1.06 (1)	2.38	(2)	2
pro		2.02	(2)	2
tyr		0.65	(1)	1
val		0.91	(1)	1
leu	0.78 (1)			
phe		0.79	(1)	1
lys		1.28	(1)	1

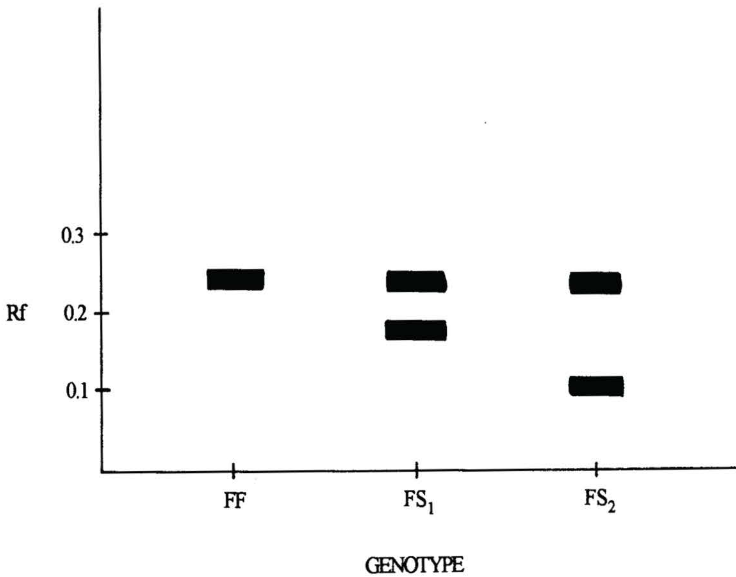


Figure 1. Electrophoretogram of hemoglobin among residents of Los Baños, Laguna, Philippines

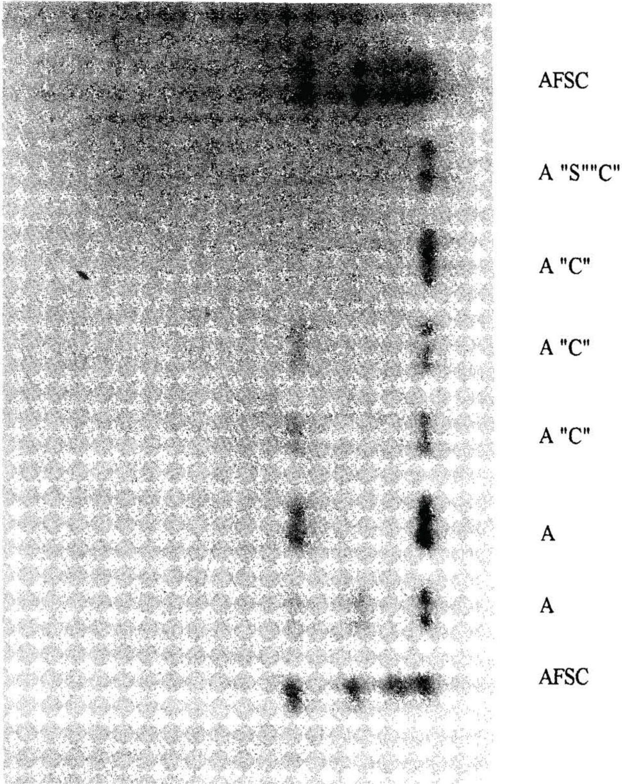


Figure 2. Cellulose acetate electrophoresis of hemolysates with Hb AE showing A "C" pattern and Hb AD showing A "S" "C" pattern

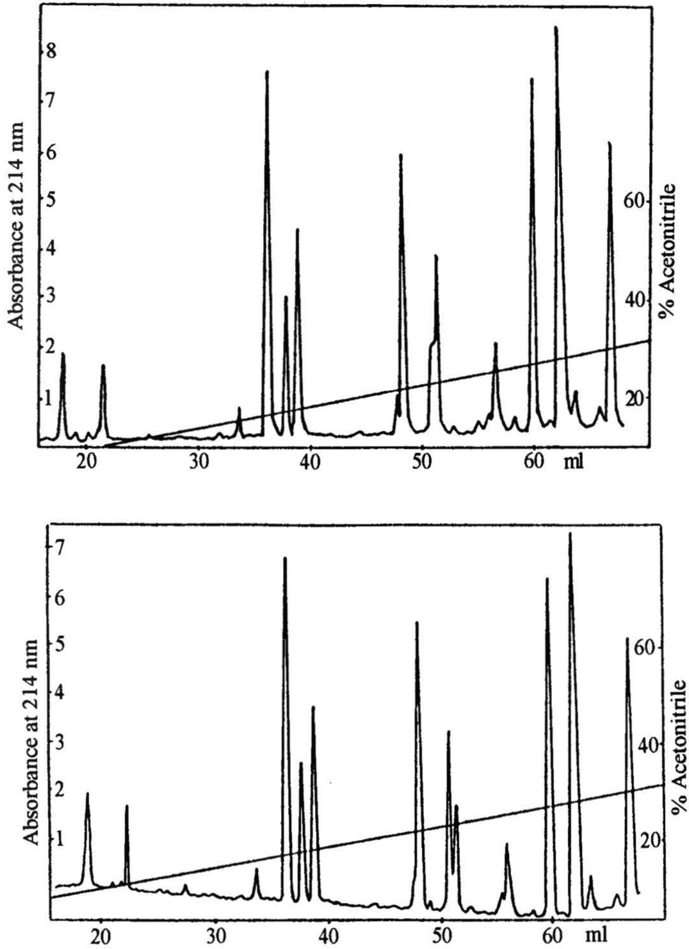


Figure 3. **Separation of tryptic peptides of: A) Hemoglobin A; and B) Hemoglobin E on a C_{18} column. Developer A was 0.1% TFA in 2d H_20 and developer B was 0.1% TFA in CH_3CN. The chromatogram was developed at a linear gradient of 0.55% B in 120 min at 1ml/min. Sample was 0.5 mg.**

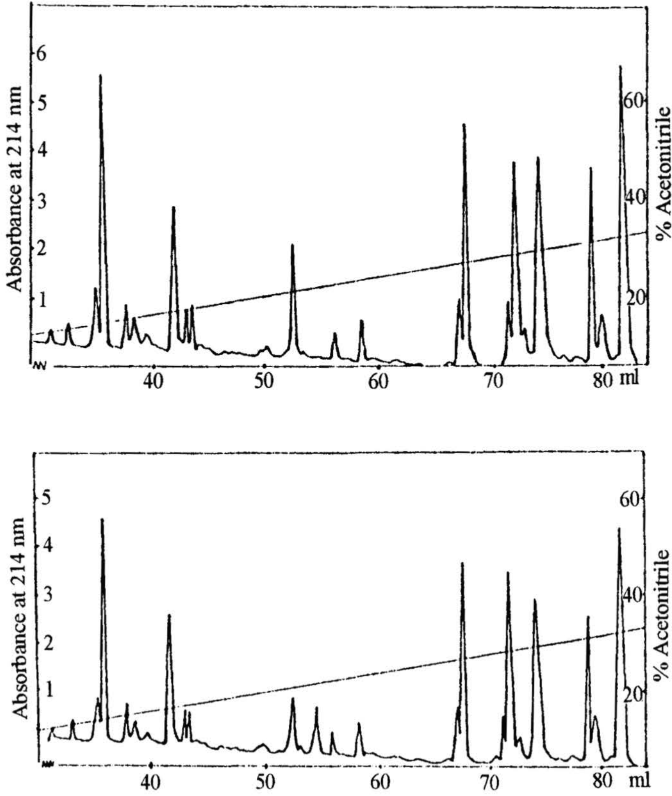
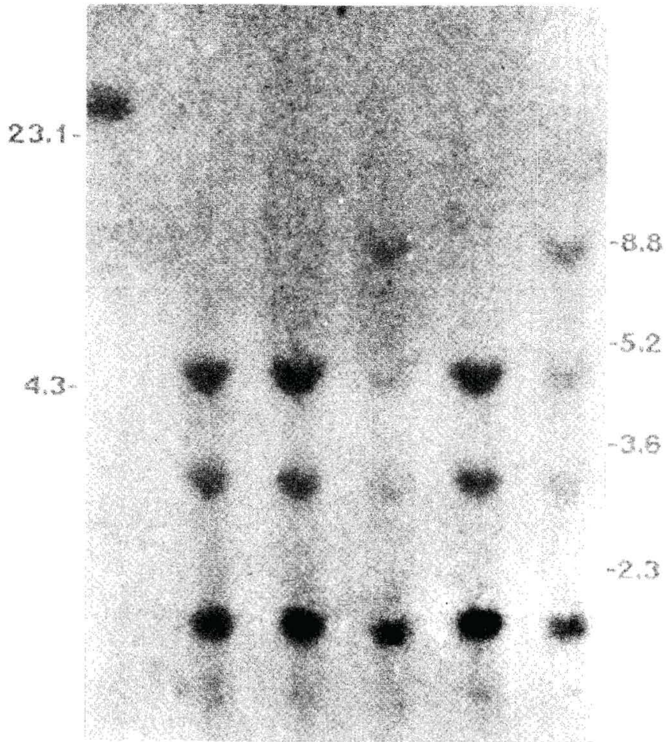


Figure 4. **Separation of tryptic peptides of: A) Hemoglobin A; and B) Hemoglobin D on a C_{18} column. Developer A was 10 mM NH_4DAC, ph 6.07 and developer B was 100% CH_3CN. The chromatrogram was developed at a linear gradient of 0.40% B in 100 min at 1ml/min. Sample was 0.5 mg.**



Target: Eco RI digest of genomic DNA
Probe : pJW 102/Eco RI Hyb: 65 - 68 C
Exp : 4 days

Figure 5. Autoradiogram of EcoRI digested DNA of Hb AD showing a diagnostic 8.8 Kb DNA fragment

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