Hemoglobin F level in different hemoglobin variants

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Background
Fetal hemoglobin (HbF) levels in different hemoglobin variants in Osogbo, Nigeria, were estimated using two principal methods of estimation using existing information for HbF concentration and distribution of various hemoglobin variants in the area, as well as diagnosed cases of thalassemia. Two hundred and sixty samples collected from HbSS, HbSC, HbAA, HbAS, and HbAC subjects were analyzed. HbF level and hemoglobin type were determined in this study.

Methods
The hemoglobin type was determined using cellulose acetate electrophoresis at an alkaline pH, and HbF was determined by the acid elution and alkaline denaturation methods.

Results
The mean±SD of HbF in the respective hemoglobin variants was as follows: HbSS, 2.09±1.94%; HbSC, 0.85±0.54%; HbAA, 0.69±0.46%; HbAS, 0.52±0.31%; and HbAC, 0.57±0.26%. The mean HbF level across the hemoglobin variants was statistically significant ($P<0.05$). Investigating the sex distribution of the HbF level in the studied population revealed a slightly higher mean HbF level in females than in their male counterparts.

Conclusion
Within the study population, the HbF level was found to be highest in HbSS and lowest in HbAS. The two methods of estimating HbF are equally reliable, since there was no significant difference between the results obtained from the two methods.

Key Words   Hemoglobin F, Alkaline denaturation, Acid elution, Hemoglobin variants, Cellulose acetate electrophoresis

INTRODUCTION

Inherited hemoglobin disorders are the most common single gene disorders, estimated by the World Health Organization (WHO) to be carried by approximately 7% of the world's population. Some of these diseases, particularly sickle-cell anemia and the more severe forms of thalassemia, cause life-threatening medical emergencies, chronic disability to families, and a major drain on health resources [1]. However, patients with severe inherited disorders of β-globin chain structure or synthesis, in particular sickle cell anemia and β-thalassemia (β-thal), may have a milder illness if they produce unusually high levels of fetal hemoglobin (HbF) [2]. This β-globin chain structure disorder comprises a heterogeneous group of conditions, in which HbF production persists through adult life in the absence of hematological abnormalities called hereditary persistence of fetal hemoglobin (HPFH) [3]. Persistent production of variable levels of HbF into childhood and adult life is a characteristic finding in sickle cell anemia and more severe forms of β-thal. HbF levels are also useful for predicting the clinical severity of sickle cell disease (SCD) [4].

Hemoglobin is a tetrameric protein compound made up of a complex of four polypeptide chains and four heme groups. It is the oxygen-carrying molecule of RBC, and it makes up approximately 95% of the RBC proteins [5]. Of this protein population, normal adult blood is about 96-98% HbAA, with the molecular structure $\alpha_2\beta_2$, up to 3.5% HbA$_2$, with the molecular structure $\alpha_2\delta_2$, and less than 1% HbF [6]. HbF, with a molecular structure of $\alpha_2\gamma_2$, is heterogeneous, with two types of γ-chains that differ in their amino acid composi-

tion, having either glycine (γ^G chains) or alanine (γ^A chains) at position 136. The γ^G and γ^A chains are the products of separate loci. HbF comprises less than 1% of hemoglobin in normal adults and has a high affinity for oxygen due to its inability to interact with 2,3-diphosphoglycerate [1]. High oxygen affinity of HbF allows it to bind to oxygen more avidly than normal hemoglobin, leading to tissue hypoxia and, in turn, increased erythropoietin and an elevated mass of RBCs. The resistance to acid elution and alkaline denaturation seen in fetal cells is attributed to the presence of HbF [7].

Over 300 structural hemoglobin variants have been described, most of which result from single amino acid substitutions [8]. Many of these variants, which are not associated with disease conditions, were discovered during a survey of the electrophoretic patterns of human hemoglobins. These abnormal hemoglobins can be classified based on the type of underlying mutation or according to their clinical consequences. The heterozygous state is called the trait and the homozygous conditions are called the disease; for example, patients with heterozygous HbS (genotype AS) are said to have the sickle cell trait, while the homozygous HbS state (genotype SS) is the SCD. Examples of structural hemoglobin variants include HbS, HbD, Punjab, HbE, HbH, HbM, HbBart, HbG Philadelphia, and HbHasheron [9-12].

Using existing information about the HbF concentration and distribution in various hemoglobin variants in Osogbo, Nigeria, the aims of this study were to determine the HbF levels of normal (AA), sickle cell trait (AS), SCD (SS), compound heterozygous state (SC), and AC subjects and to evaluate the results of the two principal methods of estimating HbF level and the sex distribution in the study area. We also provide information on the underdiagnosis of thalassemia cases in this area, owing to limited facilities and resources.

MATERIALS AND METHODS

1. Study area and collection of samples

The study was conducted at the Hematology Department Laboratory of the Ladoke Akintola University of Technology Teaching Hospital (LTH), Osogbo, Osun State, Nigeria. The 260 study subjects consisted of 80 HbAA, 40 HbAS, 20 HbAC, 80 HbSS, and 40 HbSC. Subjects in crises, as well as those on HbF reactivators, were excluded from the study using questionnaires. Informed consent was obtained from the participating subjects with ethical approval from the appropriate committee of our institution. The samples were collected between July and October 2010. Approximately 2.5 mL of blood was collected into a bottle containing 0.04 mL of ethylenediamine tetra acetic acid (EDTA) as an anticoagulant. The blood was well mixed with the anticoagulant and analyzed immediately or stored at 4°C for subsequent analysis.

2. Cellulose acetate paper electrophoresis

Five microliters of lysed sample was transferred into well plates using a unit applicator; the sample was applied into the cellulose acetate membrane, along with suitable controls, and immediately placed in the electrophoresis chamber. The chamber was connected to a power supply and electrophoresed for 15 min at 350 V. This was used to separate and identify the different hemoglobins by their migration within an electric field. The amino acid composition of hemoglobin variants results in differences in their surface electrical charges, corresponding to different separation rates. Good separation of HbA, HbF, HbS, and HbC was obtained using this technique [13].

3. Acid elution cytochemical method

Fresh blood films were prepared. These were air dried, fixed in 80% ethanol for 5 min, rinsed rapidly, and then air dried on blotting paper for 10 min. They were then placed in elution solution for 20 s, rinsed thoroughly in water, counterstained for 2 min, rinsed in water, and air dried. Slides were observed microscopically using a ×100 objective to count the number of fetal cells per 1,000 ghost cells [7].

4. Alkali denaturation

A solution of hemoglobincyanide (HiCN) was prepared by adding 0.25 mL of hemolysate to 4.75 mL cyanide solution; 2.8 mL of HiCN solution was transferred to glass test tubes and 0.2 mL of 1.2 mol/L NaOH was added and mixed for 3 s. After 2 min, 2 mL of saturated ammonium sulfate solution was added to each tube and mixed for 10 min. The solution was filtered twice through the same Whatman no. 42 filter paper, and the filtrate (the alkali-resistant hemoglobin) was obtained. HiCN solution (0.4 mL) was diluted with 13.9 mL of water to obtain total hemoglobin, and its absorbance, as well as the absorbance of the alkali-resistant hemoglobin, was measured spectrophotometrically at 420 nm against water blank. The percentage alkali-resistant hemoglobin was calculated as follows:

$$\% \text{ Alkali-resistant hemoglobin } [14] = \frac{A_{420 \text{ alkali-resistant Hb}} \times 100}{A_{420 \text{ total Hb}} \times 20}$$

5. Statistical analysis

A paired t-test was used for the data analysis with pie and box plot representations constructed using SPSS version 16. $P < 0.05$ denotes a significant difference between groups.

RESULTS

The HbF levels in the different hemoglobin variants involved in this study are shown in Table 1 and Figs. 1-4. Table 1 represents the mean±SD of HbF in the different hemoglobin variants (HbSS, HbSC, HbAA, HbAS, and HbAC), along with their respective subjects' population. The

Table 1. The mean+S.D HbF level in different hemoglobin variants in the studied population.

Hemoglobin types		HbF (%) (Acid elution method)	HbF (%) (Alkaline denaturation method)	P
SS	N	80	79	
		1.98±1.96	2.09±1.94	>0.05
SC	N	38	40	
		0.75+0.61	0.85+0.53	>0.05
AA	N	77	80	
		0.48±0.43	0.69±0.46	>0.05
AS	N	37	40	
		0.35±0.23	0.52±0.31	>0.05
AC	N	20	20	
		0.37±0.19	0.57±0.26	>0.05

Data are given as Mean+SD. Comparison of the two methods was done using paired 't' test method. "P<0.05" denotes significant difference between the two methods.
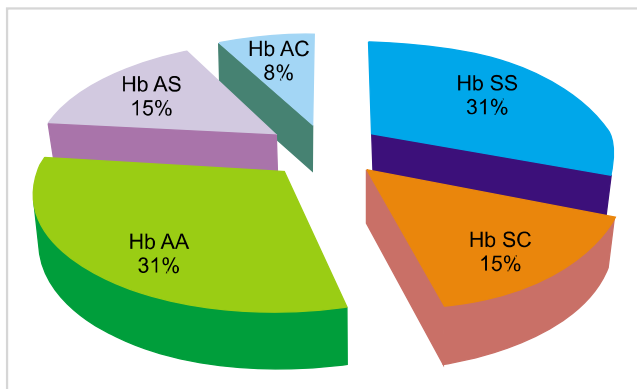

Fig. 2. Hemoglobin F concentrations using cytochemical acid elution method in different hemoglobin variants.

Fig. 1. Percentage distribution of hemoglobin variants in the studied population using cellulose acetate paper electrophoresis.

Fig. 3. Age distribution of Hb SS variants with Hb F concentration obtained from using cytochemical acid elution method.

table also highlights and compares HbF concentration in the test hemoglobin types, showing results of the cytochemical acid elution and alkaline denaturation methods. There was no significant difference between results obtained using the two methods for estimating HbF levels, cytochemical acid elution and alkaline denaturation. Fig. 1 displays the distribution of respective hemoglobin variants for the 260 subjects studied.

Fig. 2 shows variations in the HbF concentrations in different hemoglobin variants, with HbSS showing the highest variability of greater than 1 SD. A normal distribution of HbF concentration was observed in other hemoglobin variants, HbSC, HbAA, HbAS, and HbAC, since they have a lower SD. A high level of HbF was observed in the HbSS variant (Fig. 2) for the 1-10 and 11-20 years age groups. The 20-30 years age group had a normal distribution of HbF (Fig. 3).

Fig. 4A shows the effect of sex on the distribution of HbF in HbSS subjects. The figure shows that females had higher HbF levels than the male counterparts. The combina-
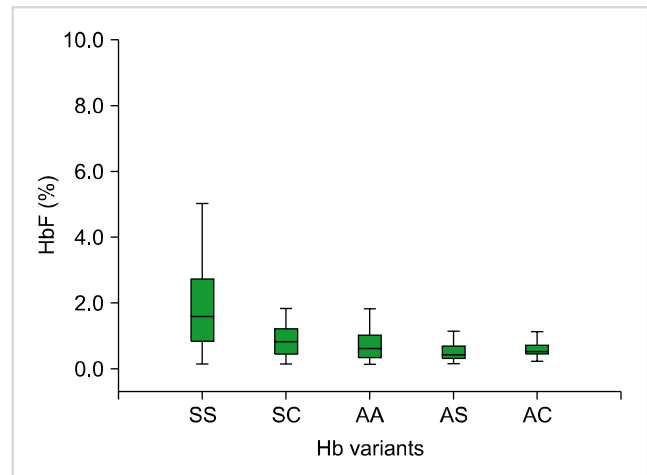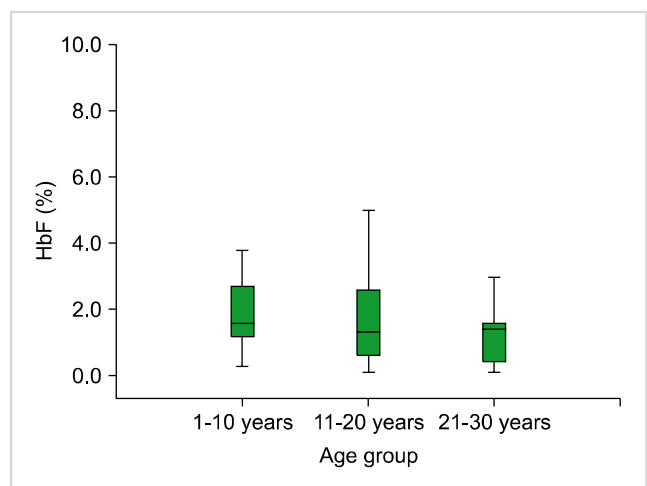
tion of gender and age distribution of the mean HbF level in the studied HbSS variant population is shown in Fig. 4B. In the 1-10 years age group, males had a higher HbF distribution than females; this trend reversed in the 11-20 years age group, in which females had higher HbF levels than males.

DISCUSSION

The HbSS variant was observed to have the highest mean± SD HbF level (2.09±1.94%), followed by HbSC (0.85±0.54%), HbAA (0.69±0.46%), HbAS (0.52±0.31%), and HbAC (0.57± 0.26%), with statistically significant differences among the hemoglobin types ($P<0.05$) (Table 1). When the HbF levels were estimated in 130 sickle cell anemia patients in Ibadan, Nigeria, using the alkaline denaturation technique, the mean HbF level was 5.9±3.8% [15]. A similar study performed
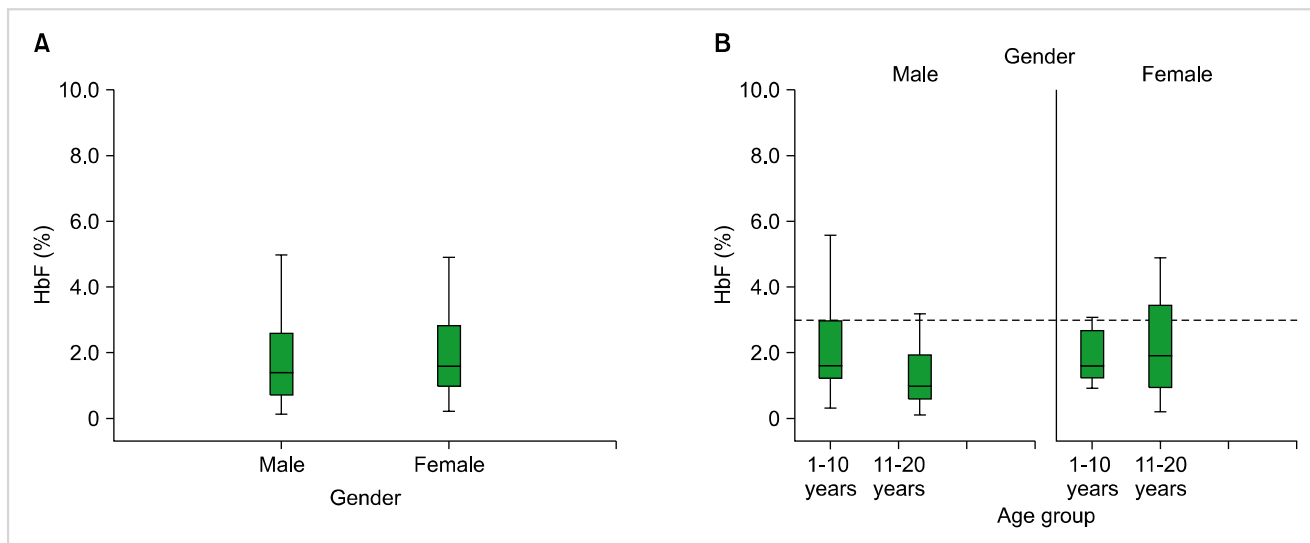
Fig. 4. **(A)** Gender effect on the mean Hb F level as obtained using cytochemical acid elution method in Hb SS variant. **(B)** Gender and age distributions of the mean Hb F level obtained using cytochemical acid elution method among the studied population of Hb SS variant.

in Calabar, Nigeria, reported that the mean HbF value in HbSS subjects was higher (3.05±1.61%) than in HbA and HbAS subjects, with means±SD of 0.20±0.25% and 1.07± 0.98%, respectively, and with a statistically significant mean HbF ($P<0.02$) [16]. HbF levels in healthy Nigerian adults, as assessed by the alkaline denaturation method, were 2.70±3.40% (mean±SD) in individuals with HbA and 2.40± 2.20% (mean±SD) in HbAS subjects; this high level of HbF in healthy adults was reported to be genetic [4]. Additionally, in a similar study conducted among adult Nigerians with SCD, [4] the mean±SD HbF value was 7.40±3.60%; however, the distribution was not significant. Previous findings from other groups corroborate the results from this study. The variations in the HbF levels in HbSS patients and others from different localities could be due to common single-nucleotide polymorphisms (SNPs) at the *BCL11A* and *HBS1L-MYB* loci, which have been implicated previously in HbF level variation in non-anemic European populations [17]. An association between a *BCL11A* SNP and HbF levels in a SCD cohort study in the USA has also recently been demonstrated. A report on human HbF expression also supports this claim, suggesting that the *BCL 11A* gene is a potential regulator of HbF expression [18].

In our study, the mean HbF level was higher in females than in males, with female HbSS and HbSC subjects having the highest mean HbF level (Fig. 2). This is in agreement with a study showing that, after the age of 10, HbF levels were consistently higher in females than in males, and this was statistically significant (Fig. 4B) [19]. The difference between males and females was suspected to be due to the hormonal effects of puberty. In a study estimating HbF levels in SCD, male sickle cell patients were found to have significantly lower levels of HbF than their female counterparts (Fig. 4A) [15]. In a similar study, males were found to have higher HbF levels (7.6±3.9%) than their female counterparts (6.7±3.6%), but the difference was not statistically significant

[4]. When age is considered, the 1-10-year age group had the highest mean HbF level among all hemoglobin genotypes, with the exception of HbAC. The 21-30-year age range had the lowest HbF levels among all hemoglobin genotypes except for HbAC (Fig. 2 and 3), and the relationship was statistically significant ($P<0.05$). In a study comparing the hematological indices in homozygous sickle cell patients, it was discovered that beyond age 10, there is no consistent age-related trend in HbF levels [20].

When the two methods to estimate HbF level (the acid elution method and the alkaline denaturation method) were compared, there was no difference between the results obtained from the two methods. These methods were well correlated, and this implies that either method can be reliably used to estimate HbF levels (Table 1).

In conclusion, the HbF level is higher in SS subjects compared with subjects with other hemoglobin variants. This increased HbF level is a compensatory mechanism for sickling in SS subjects [2]. Further investigations into the diagnosis of co-existent thalassemia among subjects are recommended.

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