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A Measure of Haemoglobin Instability in Individuals phenotyped as haemoglobin AC, SC and CC by Acetate Electrophoresis.

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Abstract: *One of the limitations in haemoglobin electrophoresis technique is the inability to distinguish between stable haemoglobin-C and unstable Haemoglobin-E. Haemoglobins A2, C, E and O^{Arab} migrate together during cellulose acetate electrophoresis and are often not differentiated by most diagnostic laboratories. This study investigated the instability of phenotyped haemoglobin-C individuals and the possibility of haemoglobin-E individual phenotyped as either heterozygous or homozygous haemoglobin-C. Fifty phenotyped blood samples consisting of twenty five haemoglobin-AC, fifteen haemoglobin-SC, and ten haemoglobin-CC were used for this study. Twenty two blood samples phenotyped as haemoglobin-AA served as controls. The blood samples were analyzed for haemoglobin instability using heat instability and isopropanol instability tests. The difference in turbidity of the haemoglobins measured spectrophotometrically before and after heating was taken as a measure of haemoglobin instability. The degree of instability in haemoglobin-AC individuals was insignificant ($p>0.05$) in relation the controls. A significant ($p<0.05$) level of instability was found in haemoglobins-SC and CC individuals. Two haemoglobin-AC and one haemoglobin-CC individuals showed very high concentration of haemoglobin instability. They recorded turbid reaction with isopropanol. This suggests Haemoglobin-SE, Haemoglobin-CE or Haemoglobin-EE erroneously phenotyped as haemoglobin-SC and haemoglobin-CC. This study concludes that Haemoglobin-E may be present in Nigeria.*

Keywords*: Unstable Haemoglobins, Haemoglobinopathies, phenotype, instability, isopropanol.*

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I. Introduction

Many variants of haemoglobins have no critical haematological and clinical significance because the underlining mutation causes no alteration in the function, solubility, or stability of the haemoglobin molecule [1]. Some of these mutations however affect the physical or chemical properties of the haemoglobin molecule resulting in haemoglobin solubility, instability, or oxygen-binding properties. Haemoglobin variants such as Hb-S, Hb-C, Hb-D^{Punjab}, Hb-E, and Hb-O^{Arab} have clinical or genetic significance and are readily detectable by electrophoretic and chromatographic techniques [1].

A Measure of Haemoglobin Instability in Individuals phenotyped as haemoglobin AC, SC and CC by Acetate Electrophoresis.

Unstable haemoglobins are those variants whose instability is sufficient to cause clinically recognizable haemolysis [2]. This disorder generally manifest as congenital Heinz body haemolytic anaemia. Carrell's study (as cited by Wild and Brain, 2006[1]) reported that haemoglobin variants exhibit a wide diversity of instability but the clinically unstable haemoglobins can be detected by both the heat stability test and isopropanol test. While many of these variants are separated using electrophoresis or chromatography, some are not and remain in undetected. Haemoglobin-C (Hb-C) is stable but haemoglobin E (Hb-E) is slightly unstable [2][3]. Hb-C arises from the substitution of lysine for glutamic acid in the sixth position of the β chain [2]. On the other, hand Hb-E arises from the substitution of lysine for glutamic acid in the 26th position of the β chain [2][4]. Hb-E records slightly positive reactions when subjected to heat and to isopropanol tests [3]. Though these haemoglobins have a slight degree of instability, the expression unstable haemoglobins is only used for those haemoglobins that result in Heinz inclusion bodies in patients with acute or chronic haemolytic anaemia. Hb-E is said to be found predominantly in South-East Asia, India, Burma, and Sri Lanka and amongst immigrant Indo-Chinese population in western countries [2][4][3]. Hb-E variant is regarded as the second most prevalent haemoglobinopathy throughout the world after Hb-S [3][5]. It is estimated that 15-30% of immigrants from Southeast Asia in North America have Hb-E with the highest frequency occurring in those from Cambodia and

Laos [4]. Hb-C originated in West Africa [2]. While Hb-C is found in Nigeria presence of Hb-E has not been reported. Hb-C and Hb-E migrate together during cellulose acetate electrophoresis [2][6][3] but a distinction can be made based on performing heat and isopropanol instability tests, isoelectric focusing or agar gel electrophoresis at pH 6.5[6]. Most diagnostic laboratories do not differentiate them. Hb-C is isopropanol stable while Hb-E is slightly unstable forming precipitate in isopropanol [1][3]. This study investigated the percentage of haemoglobin instability in Hb-AC, Hb-SC, and Hb-CC individuals and the possibility of Hb-E individual erroneously phenotyped as Hb-C.

II. Materials And Methods

2.1 *Type of study*

This study is a case control study. Investigations on the phenotyped Hb-AC, SC and CC were controlled using haemoglobin AA.

2.2 *Sample size*

Fifty (50) phenotyped fresh blood samples collected in disodium ethylenediaminetetraceticacid (EDTA) bottles were collected from Haematology Laboratory, Lagos State University Teaching Hospital, Idi-Araba, Full Medical Diagnostic Laboratory, Ilupeju Estate, Lagos; and Fountain of Life Medical Diagnostic Laboratory, Bariga, Lagos, for this work. The blood samples consisted of 4 milliliter (ml) of fresh 25 Hb-AC, 15 Hb-SC and 10 Hb-CC; and fresh 22 blood samples of Hb-AA serving as control. The haemoglobin phenotypes of the samples were repeated using cellulose acetate paper and Electrophoresis Machine (DY-300, Axion Medical Ltd, UK) at pH 8.6 to re-affirm their identity. Samples were tested for instability using heat and isopropanol immediately after the phenotypic determination same day.

2.3 *Heat instability test*

Heat instability and isopropanol instability tests described by Wild and Bain [1] and Chanarin [7] were used to detect the presence of haemoglobin instability.

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One ml of tests and controls blood were washed three times in normal saline using centrifuge (Ocean Med, England) at 3000 revolution per minute (rpm) for 2 minutes. The washed whole blood was lysed with distilled water and made up to 5ml. Five ml (5ml) of 0.01M phosphate buffer (pH 7.4) was added. The stroma was removed by centrifugation at 3000 rpm for 10 minutes. The optical density (OD) of the haemoglobin solution was read at 540 nm in a spectrophotometer (Camlab. co., UK). The haemoglobin solution was incubated at 50°C in a water bath (Lemfield Medical, England) for 1 hour. Haemoglobin solution was centrifuged at 3000 rpm for 10 minutes to remove the precipitate. The supernatant haemoglobin solution was read at 540 nm in the spectrophotometer. The difference in ODs before and after heating was taken as a measure of haemoglobin instability.

2.4 *Isopropanol instability test*

Isopropanol test was used to differentiate Hb-E which precipitated out of solution from Hb-C which remains in solution [1][7]. Two ml (2ml) of isopropanol buffer was added to the haemolysate of the tests and control samples separately and mixed well. The tests and controls were incubated at 37°C in water bath and observed regularly for precipitate formation over one hour.

2.5 *Statistical analysis*

The data generated were subjected to standard statistical analysis using statistical package for social science (SPSS) software version 17. Probability value was set at 0.05.

III. Results

The concentration of haemoglobin instability against haemoglobin phenotypes are represented in table 1. Two samples in Hb-AC and another one from Hb-CC accounted for the wide range of percentage haemoglobin instability in Hb-AC and Hb-CC respectively. The mean concentration of haemoglobin instability of haemoglobin phenotype AA in relation to those of haemoglobin phenotypes AC, SC and CC individuals are represented in table 2. There is no significant difference ($p>0.05$) between the level of haemoglobin instability in Hb-AC in relation to that of the controls. A high level of significance ($p<0.05$) was recorded in the level of haemoglobin instability in Hb-SC and Hb-CC individuals in relation to that of the controls.

Unusually high standard deviation of ±5.16 and ±3.54 were however recorded in Hb-AC and Hb-CC individuals respectively. All Hb-AA, Hb-AC, and Hb-CC blood samples gave clear solution in isopropanol

instability test in relation to the control except two samples of Hb-AC and one sample in Hb-CC that precipitated out of solution (Table 3). The first and second Hb-AC blood samples that precipitated in isopropanol test recorded haemoglobin instability concentration of 26.2% and 5.4% (from raw data) respectively.

The time taken for the cloudy appearance was 25 and 30 minutes respectively. The third blood sample of Hb-CC that precipitated in isopropanol test recorded haemoglobin instability concentration of 15% (from raw data). Precipitation was observed after 25 minutes of incubation. Light turbidity was observed in Hb-SC after a mean time of 38 minutes.

A Measure of Haemoglobin Instability in Individuals phenotyped as haemoglobin AC, SC and CC by Acetate Electrophoresis.

IV. Tables

Table 1: The concentration of haemoglobin instability against haemoglobin phenotypes.

Haemoglobin phenotypes	Range of unstable haemoglobin concentration (%)	Mean unstable haemoglobin concentration (%)
Hb-AA (Control, n = 22)	0.00 – 1.13	0.36
Hb-AC (n = 25)	0.00 – 26.20	1.88
Hb-SC (n = 15)	0.00 – 4.76	1.53
Hb-CC (n = 10)	0.00 – 15.00	5.02

A Measure of Haemoglobin Instability in Individuals phenotyped as haemoglobin AC, SC and CC by Acetate Electrophoresis.

Table 2: The mean concentration of haemoglobin instability of phenotype AA in relation to haemoglobin phenotypes AC, SC and CC individuals.

Genotype	Mean unstable haemoglobin		
	Mean	t	p-value
Hb-AA n = 22	0.36 ± 0.07		
Hb-AC n = 25	1.88 ± 5.16	-1.47	0.154
Hb-SC n =15	1.53 ± 0.17	-25.34	< 0.001
Hb-CC n = 10	5.02 ± 3.54	-4.16	0.002

A Measure of Haemoglobin Instability in Individuals phenotyped as haemoglobin AC, SC and CC by Acetate Electrophoresis.

Table 3: Isopropanol instability against haemoglobin phenotypes

Hb Phenotypes	Isopropanol insolubility		Result
	Precipitate	Time	
Hb-AA (n = 22)	No precipitate	1 hour	Stable haemoglobin
Hb-AC (n = 23)	No precipitate	1 hour	Stable haemoglobin
Hb-AC (n = 2)	Precipitate recorded	25, 30 Minutes	Unstable Haemoglobin
Hb-SC (n = 15)	Precipitate recorded	38 minutes (mean)	Unstable haemoglobin
Hb-CC (n = 9)	No precipitate	1 hour	Stable haemoglobin
Hb-CC (n = 1)	Precipitate recorded	25 minutes	Unstable haemoglobin

A Measure of Haemoglobin Instability in Individuals phenotyped as haemoglobin AC, SC and CC by Acetate Electrophoresis.

V. Discussion

Haemoglobin instability was significantly present in Hb-SC and Hb-CC individuals in relation to Hb-AA. There is paucity of information on measurement of haemoglobin instability in Hb-C, and Hb-E individuals. Bachir and Galacteros [3] however observed that Hb-E demonstrates slight level of instability when subjected to heat and isopropanol treatment. Hb-C on the other hand is known to be stable [1]. Significant level of haemoglobin instability is not expected when Hb-C is subjected to heat and isopropanol treatment. It then follows that some of the phenotyped Hb-C in homozygous or heterozygous states were an error as Hb-C and Hb-E may not be distinguished during cellulose acetate paper electrophoresis[2][6].

Significant haemoglobin instability associated with Hb-SC and Hb-CC in this study may require that individuals phenotyped as such without further discrimination to rule out Hb-E may need to be further investigated to rule out presence of Hb-E.

Isopropanol instability test was compared with the result of heat instability test for each set of tests to discriminate between Hb-C and Hb-E. Considering the haemoglobin phenotype using alkaline cellulose acetate paper electrophoresis in which Hb-C and Hb-E migrate together, the heat instability test and isopropanol

instability test, the three samples that gave precipitate with isopropanol are not likely to be Hb-C. Twenty three (23) Hb-AC out of twenty five (25) recorded stability to both heat and isopropanol treatment. These may be actual Hb-C individuals. The two blood samples with Haemoglobins-AC and one Haemoglobin- CC behaved differently by consistently demonstrating instability under heat and isopropanol treatment.

It has been observed that some unstable haemoglobins show clearly observable precipitation even at 5 minutes while milder haemoglobin variants show precipitation at 20 minutes[1]. Precipitates were recorded in the three "unusual Hb-C" samples after 25 and 30 minutes. Hb-SC recorded precipitation at a mean time of 38 minutes after incubation. The Hb electrophoresis results, the heat instability test results, isopropanol instability test results in this study suggest that some of the phenotyped Hb-C individual might be Hb-E.

The unusually high standard deviation recorded in Hb-AC and Hb CC (Table 2) may not be pointing to any procedural error. Rather it is most likely due to the three unusual blood samples with marked deviation from the haemoglobin instability concentration recoded for other haemoglobins in their group. If they are not Hb-C they cannot behave like Hb-C but bound to behave differently. The deviation might be caused by Hb-E erroneously phenotyped as Hb-C.

People do haemoglobin phenotype for the purpose of taken decision on the choice of marital partners to avoid inherited haemoglobinopathies. Haemoglobin phenotype test is conducted in children often for investigation of haemoglobinopathies. While Hb-E trait (heterozygous state) is asymptomatic with normal level of haemoglobin, Hb-E disease (homozygous state) may be characterized by compensated hemolysis with a mild level of microcytic anaemia, jaundice, and marked hypochromia [2]. Exodus of people from one country to the other and subsequent cross-ethnic intermarriages might be the reason for the discovery of haemoglobin phenotypes alien to any ethnic group or country. In view of the implication of wrong haemoglobin phenotype results it might be necessary to further discriminate Hb-C phenotype results from cellulose acetate paper electrophoresis in Nigeria from Hb-E before release of such results.

A Measure of Haemoglobin Instability in Individuals phenotyped as haemoglobin AC, SC and CC by Acetate Electrophoresis.

VI. Conclusions

This study showed that significant haemoglobin instability is found in Hb-SC and Hb-CC individuals. Some individuals whose haemoglobins were phenotyped as Hb-AC, Hb-SC and Hb-CC using alkaline cellulose acetate paper electrophoresis alone might actually be Hb-AE, Hb-SE or even Hb-EE.

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A Measure of Haemoglobin Instability in Individuals phenotyped as haemoglobin AC, SC and CC by Acetate Electrophoresis.

References

[1] B. Wild and B. Bain, Investigation of abnormal haemoglobins and thalasaemia, in S. M. Lewis., B. J. Bain., I. Bates, (Eds), Dacie and Lewis Practical Haematology, 10th Ed, (Churchill Livingstone: Elsevier, 2006), 271-310.

[2] R. Saxena., H. P. Pati., and M. Mahapatra, *de Gruchy's clinical haematology in medical practice* (Daryaganj, New Delhi: Wiley India pvt Ltd, 2013).

[3] D. Bachir, and F. Galacteros, Hemoglobin E disease.Orphanet Encyclopedia. November 2004), http://www.orpha.net/data/patho/GB/uk-HbE.pdf

[4] R. J. Laudicina, Hemoglobinopathies: Qualitative Defects. in S. B. Mckenzie and J. L. Williams, (Eds), Clinical Laboratory Haematology, 2nd Ed, (New Jersey: Pearson Education, 2010), 210-230.

[5] J. Flint., R. M. Harding., A. J. Boyce and J. B. Clegg, The population genetics of the haemoglobinopathies, *Baillieres Clin Haematol*, *11*, 1998, 1-59.

[6] A. Lal., and E. P. Vichlnsky, Sickle cell disease. in V. Hoffbrand., D. Catovsky., E. G. D. Tuddenham., A. R. Green (Eds), Postgraduate Haematology, 6th ed, (Wiley-Blackwell: Chichester, 2005), 109-125.

[7] Chanarin, I. *Laboratory haematology: an account of laboratory techniques* (Churchill Livingstone: Edinburgh, 1989).

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