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Biochemical and Molecular Genetic Characterization of G6PD Deficiency in Newborn, Benin

AKPOVI D. Casimir[1,*], FIOGBE E.M. Salomon[1], Julien G.A. SEGBO[1], Paulin SEDAH[3], Fifamè E.E. KOUGNIMON[1], Gratien G. SAGBO[2], Clément AGBANGLA[3]

[1]Research Unit of Non Communicable Diseases and Cancer, Research Laboratory in Applied Biology, "Ecole Polytechnique d'Abomey-Calavi", University of Abomey-Calavi, Benin
[2]Medical Genetics and Neonatology, "Centre National Hospitalier et Universitaire H.K. Maga", Cotonou, Benin
[3]Laboratory of Molecular Genetics and Genome Analyzes, Faculty of Sciences and Technics, University of Abomey-Calavi, Benin
*Corresponding author: casimir.akpovi@gmail.com

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Abstract Background: In Benin, the diagnosis of G6PD deficiency is based on the biochemical test performed by the quantitative spectrophotometry assay of the enzymatic activity. We used the results of the molecular gene test to verify the reliability of the biochemical diagnostic test. The distribution of genes polymorphism associated with the G6PD deficiency in neonates is also determined throughout the country. Material and Methods: This is a cross-sectional descriptive study carried on with 178 neonates aged ≤7 days. Blood samples were screened for quantitative G6PD enzymatic activity using Cypress Diagnostics kit (Cypress Diagnostics, Belgium). G6PD genotype analysis was performed using specific primers for PCR amplification of G6PD gene containing the common African mutations A376G, G202A, A542T, and T968C. We used Chi-2 test to compare the means of qualitative data and Student's t-test to ascertain difference between group's characteristics. A p-value of <0.05 was deemed significant. Results: The mean value of normal G6PD activity was 16.25 U/g Hb. Enzyme activity was significantly lower in female newborns of genotype $A^{-(376/202)}$ /$A^{-(376/202)}$ (p<0.01) and in males genotype $A^{-(376/202)}$ (p<0.001). The probability of being deficient when the test of enzymatic activity is positive among the true deficit (Se) was 0.42. The probability of being non-deficient when the test is negative among the non-deficient (Sp) was 0.89, that of being deficient when the test is positive (PPV) was 0.52 and the probability of being non-negative when the test is negative (VPN) was 0.85. Genotypes B/B, A/B, A/A, $A^{-(376/202)}$/B, $A^{-(376/202)}$/A, $A^{-(376/202)}$/$A^{-(376/202)}$ were identified in female newborns with respective proportion of 39.56%, 13.19%, 7.69%, 24.18%, 13.19% and 2.20%. In males, 64.37% were genotype B, 9.20% genotype A and 26.44% genotype $A^{-(376/202)}$. The northern departments had the highest frequencies of G6PD $A^{-(376/202)}$ (33.18% to 37.73%) while the lowest levels were seen in the south (25.58% to 28.62%). Conclusion: Enzymatic activity showed good diagnostic value in male subjects but not in heterozygous female. The highest frequency of G6PD $A^{-(376/202)}$ mutation was in the North Benin.

Keywords: *G6PD deficiency, enzymatic activity, DNA test, neonate, Benin*

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1. Introduction

Glucose-6-phosphate dehydrogenase (G6PD) is an enzyme in the pentose phosphate pathway (PPP) that plays an important role in protecting cells from oxidative damage by producing NADPH and reduced glutathione [1]. In the erythrocyte, which lacks a nucleus, mitochondria and other organelles, PPP is the only biochemical pathway for generating reducing capacity [2]. Worldwide, an estimated 400 million people are G6PD deficient with the distribution corresponding to areas in which malaria is, or has been, prevalent [3,4].

G6PD gene is X-linked, and so deficient variants are expressed more commonly in males than in females [5]. G6PD gene is highly polymorphic with almost 400 reported variants, conferring varying levels of enzyme activity [2]. Examination G6PD variants shows that the deficiency is related to the instability of the enzyme, which implies that amino acid substitutions in different locations can destabilize the enzyme molecule [1]. Different variants of deficits in G6PD have been characterized. Of these types of deficiency, some have normal activity and others have reduced activity. The WHO has classified the different variants of G6PD into three classes according to enzymatic activity and clinical manifestations: class 1 characterized by enzyme

deficiency associated with non-spherocytic chronic hemolytic anemia; class 2 characterized by a severely deficient enzymatic activity with less than 10% of normal activity and class 3 characterized by a moderate reduction of enzymatic activity that is between 20 and 60% of normal [6].

Although there are more than 400 G6PD variants worldwide in sub-Saharan Africa, three variants were reported with polymorphic frequencies >0.1%: G6PD(B), G6PD(A) and G6PD(A-). G6PD(B) is the wild type form. G6PD(A) has a single adenine to guanine substitution at nucleotide number 376 (A376G), causing changes in the amino acid asparagine by aspartic acid in position 126 on the peptide chain. G6PD(A) showed phenotype with about 90% of the G6PD(B) enzyme activity [7]. However, a second mutation found on this same allele due to the substitution of adenine by guanine at position 202 (G202A) corresponds to the variant G6PD(A-). The G6PD(A-) variant represents 30% of the African black population. The instability of the corresponding protein is characterized by a reduction of 62 to 13 days of the protein half-life (Doupa et *al*., 2008) with about 8-20% of the wild type residual enzymatic activity [8].

De Araujo *et al.*, [9], found two other common deficiency alleles in the Sereer population in Senegal G6PD Santamaria (542T/376G, 1% frequency) and G6PD Betica-Selma (968C/376G, 10%). Others study reported those variants in west Africa population [3,10,11].

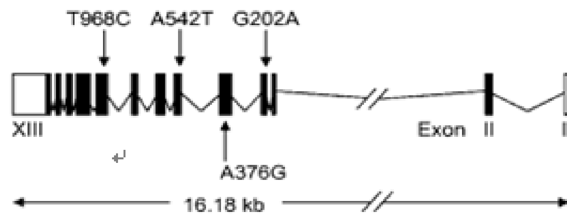

Figure 1. G6PD exons and relative positions of coding variation studied

The frequency of G6PD varies from one population to another, which can be related to the frequency of genetic factors or behaviors in each population. This great variability is related to G6PD deficiency detection techniques.

Although different laboratory techniques or methods are employed for the biochemical screening, a strict relationship between biochemists, clinicians, and molecular biologists is necessary for a definitive diagnosis [12]. Furthermore, due to lyonization phenomenon [13], molecular tests are particularly important for the deficit diagnosis in heterozygous female [12]. G6PD heterozygous female should be early warned and, when necessary, treated as if they are G6PD deficient. Early identification of female carriers of a defective G6PD variant represent a very important diagnostic challenge. Minucci and al. reported that, in steady state conditions, the screening test is low sensitive, because less than 46% of the deficient females results as abnormal at fluorescent semi-quantitative biochemical test [12]. It was reported that up to 60% of heterozygotes were not detected using the spectrophotometric G6PD assay [14]. Genetic testing is also needed to identify at-risk populations and which ethnic groups are preferred for routine screening at birth if it is not possible to do for the general population.

The main objective of this study is to determine genetic polymorphisms of G6PD deficiency in newborns in Benin in order to identify population at risk.

2. Methods

2.1. Subjects and Sample Collections

Blood samples were collected from 178 neonates recruited in different maternity and neonatology services in Benin. This descriptive cross-sectional study was carried out from February to October 2019.

The research work was approved by the National Research Ethics Review Boards of Benin and parents provided informed consent before been enrolled into the study. A written informed consent was obtained from each participant authorizing the collection of a 2ml whole blood sample in ethylenediaminetetraacetic acid (EDTA) as anticoagulant.

2.2. Biochemical Analysis of G6PD Deficiency

Blood samples were screened for red cell G6PD enzymatic activity by a quantitative enzymatic kinetic assay method using a commercial kit from Cypress Diagnostics (Cypress Diagnostics, Langdorp, Belgium) following manufacturer's instructions. Results of G6PD activity were expressed in U/g Hb (units per gram haemoglobin). Hemoglobin (Hb) was measured by spectrophotometry on the same samples. The overall mean value for G6PD normal activity was 16.11 U/g Hb. According to WHO classification, three levels of G6PD activity were considered (normal > 9.27 U/g Hb, moderate deficiency: 3.22-9.27 U/g Hb and severe deficiency < 3.22 U/g Hb).

2.3. Molecular Analysis of G6PD Variants

G6PD genotype analysis was performed at Laboratory of Molecular Genetics and Genome Analyzes, Faculty of Sciences and Technics, University of Abomey-Calavi. The G6PD genotyping protocol was based on published methods. DNA was extracted using Formol-Chloroform method from 200 µl of whole blood. Specific forward primers and reverse primers were used for PCR amplification of a section of G6PD gene containing the common African mutation (A376G) and the other deficient variants (G202A, A542T, and T968C). Amplicons were subsequently analysed by restriction fragment length polymorphism (RFLP) and genotypes scored after agarose gel electrophoresis. All restriction endonucleases, Taq polymerase and dNTP were sourced from New England Biolabs (NEB Inc, Ipswich, England). Restriction endonucleases were used at optimal temperature and digestion period.

Genomic DNA was first amplified using forward and reverse primers for A376G based on protocol using by Bouanga *et al.* [15]. The amplification reaction mixture (50 µl) contained 50 ng DNA, 10x buffer for Taq DNA Polymerase 250 U, 10 pM of each primer, 2 mM dNTP and 0.25 U of Taq DNA Polymerase. Thermocycling was performed at 94°C for 5 min (initial denaturation), then 30 amplification cycles of denaturation at 94°C for 10 sec, annealing at 56°C for 20 sec, and elongation at 72°C for 12 sec, with a final extension at 72°C for 7 min. Amplification products were recovered and PCR fragments incubated at 37°C for 16 h with FokI for

identification of the A376G mutation (G6PD A). All samples positive for A376G were then subjected to another PCR amplification to look for G202A, G542T and T968C mutations using specific primers. Amplification of the sequences containing G202A and G542T mutations was independently done from 50 ng of genomic DNA in a reaction mix containing 1x buffer, 1.5 mM of magnesium ions, 0.05 mM of each dNTP, 0.16 µM of each specific primer and 1 unity of Taq polymerase. Amplification condition was 96°C for 5 min, followed by 30 cycles of 96°C for 1 min, 60°C for 1 min, 72°C for 1 min, and a final extension step of 7 min at 72°C [11]. Amplification products were recovered and PCR fragments incubated at 37°C for 16 h with NlaIII for identification of the G202A mutation [16] and 37°C for 16 h with BspEI for identification of the G542T mutation [17]. Sequence containing T968C mutation was amplified using 0.75 U/µL Taq Polymerase, 1.5 mM MgCl2, 200 µM dNTP (mix 10 mM), 2.5 µM of each primer, 10% PCR buffer, and 2.0 µL of DNA. Thermocycling was performed at 94°C for 10 min, then 30 cycles at 94°C for 45 sec, 65°C (annealing temperature) for 45 sec, and 72°C for 45 sec, with a final extension at 72°C for 7 min. Amplicons were then incubated at 37°C for 16h with NciI for the T968C mutation (Table 1).

2.4. Statistical Analysis

Data were analyzed by SigmaPlot statistical analysis software 2014 (Systat Software, Inc. San Jose, CA, USA). Means and standard errors of the mean (SEM) of blood parameters were calculated. If Normality Test (Shapiro-Wilk) is correct, Student's t-test was used to ascertain difference between group's characteristics, if not, Mann- Whitney Rank Sum Test was used. The Chi 2 test was used to compare the means of the qualitative variables. A p-value of <0.05 was deemed significant.

Table 1. PCR/RFLP conditions used to identify the *G6PD* genetic variants of G6PD deficiency unrelated individuals in the present study

Mutations	Forward and reverse primers (5'-3')	Restriction enzyme	Amplicon size (pb)
376 A → G	CAG-TAC-GAT-GAT-GCA-GC CAG-GTA-GAA-GAG-GCG-GT	FokI	90
202 G → A	CTG-CCC-GCA-CTG-GTT-ACA AGG-AGA-GGA-GGA-GAG-CAT-CC	NlaIII	259
542 G → T	GTC-TGA-ATG-ATG-CAG-CTG-TGA CCA-GGT-GAG-GCT-CCT-GAG-TA	BspEI	296
968 T → C	TCC-CTG-CAC-CCC-AAC-TCA-AC CCA-GTT-CTG-CCT-TGC-TGC-GC	NciI	295

3. Results

3.1. Genetic Variants

The present study included 178 infants aged 7 days and under recruited from maternity and neonatology services of Benin throughout the country. Females accounted for 51.12% of the study population with a sex ratio (F/M) of 1.05. Genetic variants A (mutation at position 376), $A^{-(376/202)}$ (mutation at position 202 coupled with variant A), Santamaria (mutation at position 542 coupled with variant A) and Betica-Selma (mutation at position 968 coupled with variant A) were analyzed. We did not detect cases of Santamaria and Betica-Selma in our study population. Only variants A and $A^{-(376/202)}$ were observed. 60.44% of female newborns had a normal genotype compared to 73.56% of male neonates. The incidence of female heterozygosity and hemizygosity in male was 37.36% and 26.44% respectively. Only two (02) cases of female homozygous were found in the study representing 2.20% (Table 2).

Table 2. G6PD variant by gender

	Female		Male	
	Number	**Percentage (%)**	**Number**	**Percentage (%)**
Normal	55	60.44	64	73.56
Heterozygote	34	37.36	--	--
Hemizygote	--	--	23	26.44
Deficient Homozygote	2	2.20	--	--

Genotypes B/B, A/B, A/A, $A^{-(376/202)}$/B, $A^{-(376/202)}$/A, $A^{-(376/202)}$/$A^{-(376/202)}$ were identified in female newborns with respective frequencies of 39.56%, 13.19%, 7.69%, 24.18%, 13.19% and 2.20%. In males, 64.37% were genotype B, 9.20% genotype A and 26.44% genotype $A^{-(376/202)}$ (Table 3).

Table 3. Frequency of genotypes by sex

	Female		**Male**	
	Number	**Percentage (%)**	**Number**	**Percentage (%)**
B/B or B	36	39.56	56.00	64.37
A/A or A	7	7.69	8.00	9.20
A/B	12	13.19	--	--
$A^{-(376/202)}$ /B	22	24.18	--	--
$A^{-(376/202)}$ /A	12	13.19	--	--
$A^{-(376/202)}$ / $A^{-(376/202)}$ or $A^{-(376/202)}$	2	2.20	23.00	26.44

Table 4. G6PD deficiency status

	Normal		Moderate		Severe	
	Number	**Percentage (%)**	**Number**	**Percentage (%)**	**Number**	**Percentage (%)**
Female	67	49.26	21	67.74	3	27.27
Male	69	50.74	10	32.26	8	72.73
Total	136	100.00	31	100.00	11	100.00

Table 5. G6PD deficiency by enzymatic activity and genetic tests

	Female			Male		
	Genetic test	Enz. activity	p-Value	Genetic test	Enz. activity	p-Value
Normale	60.44%	73.63%	0.08	73.56%	79.31%	0.47
Heterozygote	37.36%	-	-	-	-	-
Deficiency	2.20%	26.37%	<0.001	26.44%	20.69%	0.47

Table 6. G6PD enzymatic activity performance

Test		Enz. activity		Total	Performance criteria	
		Normal	Deficients		Se	0.42
Genomic analysis	Normal	101	12	113	Sp	0.89
	Deficients	18	13	31	VPP	0.52
Total		119	25	25	VPP	0.52

Sensitivity (Se), Specificity (Sp), Positive Predictive Value (PPV) and Negative Predictive Value (NPV).

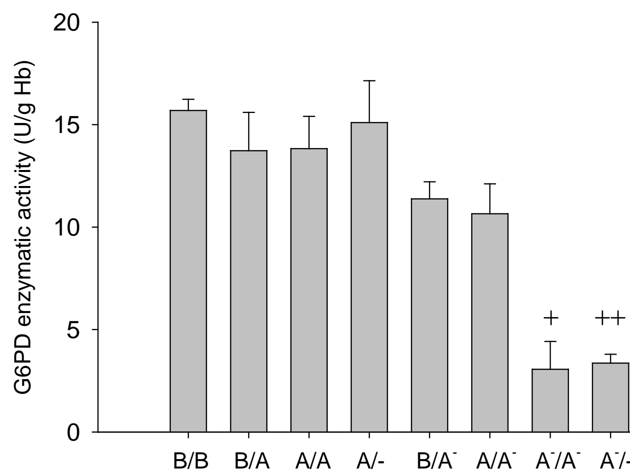

Figure 2. G6PD enzymatic activity level by genotypes

3.2. Diagnostic Value of G6PD Enzymatic Activity

Based on G6PD enzymatic activity, 20% and 60% thresholds of normal activity were established for the diagnosis of G6PD deficiency (WHO Working Group, 1989). The mean value of normal G6PD activity was 16.25 U/g Hb. The upper and lower thresholds for a moderate deficit were respectively 3.22 U/g Hb (20% of the normal average) and 9.27 U/g Hb (60% of the normal average). The upper limit of severe deficit was 3.22 U/g Hb (20% of normal mean G6PD activity in neonates). Male newborns accounted for 50.74% of normal newborns and 72.73% of severe cases. Female newborns accounted for 67.74% of all moderate deficit (Table 4). Genetic test showed 60.44% of non-deficient female newborns compared to 73.63% for the biochemical test (p =0.08) with 37.36% of heterozygotes and 2.20% of deficient female newborns compared to 26.37% for the biochemical test (p<0.001) (Table 5). A contingency table between the results of the genomic analysis and the enzymatic activity of G6PD was performed. This contingency table allowed us to calculate the enzymatic test performance criteria of sensitivity (Se), specificity (Sp), positive predictive value (PPV) and negative predictive value (NPV). The probability of being deficient when the test of enzymatic activity is positive among the true deficit, Se, was 0.42. The probability of being non-deficient when the test is negative among the non-deficient, Sp, was 0.89. The probability of being deficient when the test is positive, PPV, was 0.52 and the probability of being non-negative when the test is negative, VPN, was 0.85 (Table 6).

The variation of the G6PD enzymatic activity was analyzed according to the genotype statut. Enzyme activity was significantly lower in female newborns of genotype $A^{-(376/202)}$ /$A^{-(376/202)}$ (p<0.01) and in newborn male genotype $A^{-(376/202)}$ (p<0.001) (Figure 2).

3.3. G6PD Enzymatic Activity Level Heterozygous

Female neonates with a heterozygous genotype ($A^{-(376/202)}$/B, $A^{-(376/202)}$/A) accounted for 37.36% of female group and 19.10% of the study population in general. The G6PD enzymatic activity level ranged from 2.41 U/g Hb to 18.68 U/g Hb. The cases of severe deficit and moderate deficit were respectively 8.57% and 25.71%. Normal women accounted for 65.72% of heterozygous females. The mean enzyme activity was 2.58±0.09 in the severe deficit (p<0.01), 7.57±0.37 in the moderate deficit (p<0.01) and 13.61±0.58 in the normal (p<0.01) (Table 7).

Table 7. Enzyme activity level in heterozygous female

Parameters	G6PD Enzyme Activity		
	Severe	**Moderate**	**Normal**
Activity level (U/g Hb)	2.58±0.09	7.57±0.37$^{\alpha}$	13.61±0.58$^{\beta}$
% Proportion (Number)	8.57 (03)	25.71 (09)	65.72 (23)

p(α): Severe vs. Normal; p(β): Moderate vs. Normal;
p(μ): Severe vs. Moderate, p(α), p(β): <0.001.

3.4. G6PD Variant $A^{-(376/202)}$ Distribution

Based on the results of the genetic test, we determined the prevalence of mutation carriers by department. The northern departments had the highest frequencies with 35.76% in Alibori, 37.73% in Donga, 34.93% in Atacora and 33.18% in Borgou. Zou department had the lowest frequency of 25.58% G6PD $A^{-(376/202)}$ (Table 8).

Table 8. Prevalence of G6PD $A^{-(376/202)}$ variant by department

Department	Percentage (%)
Plateau	26.91
Oueme	26.72
Atlantique	27.97
Littoral	28.62
Mono	27.76
Couffo	27.37
Zou	25.58
Collines	28.24
Donga	37.73
Atacora	34.93
Borgou	33.18
Alibori	35.76

4. Discussion

In Benin, as in the West African region, the diagnosis of G6PD deficiency is made by a quantitative spectrophotometry assay of the enzymatic activity. We used molecular genetic testing to verify the reliability of the biochemical diagnostic test. Since G6PD deficiency is an X-linked disorder [5], the level of the enzymatic activity in male subject must be better reflected by the genome. Despite this, fewer male-deficient newborns were detected by the biochemical test (20.69%) compared to the DNA test (26.44%). The difference is represented by the individuals identified in the biochemical test as having a subnormal activity level (moderate deficit) that proportion was 5.61%. This result showed that in male neonates, only values of G6PD activity <3.22 U/g Hb [18] can be considered G6PD deficient. So, male neonate with G6PD enzyme activity higher than 3.22 U/g Hb is non deficient. Interpretation of the effect of genetic variants on the rate of G6PD activity is less simple in females. Because of the phenomenon of lyonisation (random inactivation of one of the two X chromosomes in each cell), heterozygous women present a genetic mosaic. The level of expression of the enzyme in each cell depends on the inactivated X chromosome, which can be reduced to the same level as a male subject with a deficit in case of inactivation of healthy X [19]. Heterozygous women thus have a double erythrocyte population, and the overall enzymatic activity resulting from it is usually subnormal. The clinical expression of the deficit in these female subjects is variable, most often absent or moderate. In our study, all homozygous deficient female neonates had G6PD activity <3.22 U/g Hb. It is the same for normal homozygotes in which the enzymatic activity was normal (> 9.27 U/g Hb) [18]. In some heterozygous women, asymmetric X inactivation has been observed, with the X chromosome carrying the deficit being preferentially expressed, producing severe clinical manifestations comparable to those of men with a deficit [20]. This phenomenon changes with age, with 70 to 90% of women over 70 years of age showing a significant asymmetry of X inactivation. It is therefore possible that a heterozygous woman with normal activity may progress to intermediate or low activity and become at risk of haemolysis [21]. This suggests that in heterozygote newborns, there is a preponderance of normal X chromosome expression relative to the carrier X. This explains why gene test showed 60.44% of non-deficient female newborns compared to 73.63% for the biochemical test with 37.36% of heterozygotes. We showed here that, in female subjects, the biochemical test has a good diagnostic value in homozygotes non-deficient and deficit. In heterozygotes however, only the gene test is valid. Our finding is in agreement with the report that a high percentage of partially deficient female neonates are missed during routine screening [14].

One of the main objectives of our study was to determine the most affected areas by the G6PD deficit in Benin. The aim here was to identify the most at-risk ethnic groups in which systematic screening may be preferred as general screening of the population could not considered. For this reason, we determined the proportion of newborn carrying the deficient gene of G6PD by department throughout the Benin country. On a representative sample of 178 newborns enlisted throughout the national territory, we determined 59 (33.15%) newborns carrying the deficient allele of G6PD including 23 (12.92%) male (hemizygous); 2.24% female homozygous deficient and 17.97% female heterozygotes. Overall, the southern and central departments had the lowest rates of carriers of the G6PD deficient gene ranging from 25.58% to 28.62%. In contrast, the 4 northern departments of Benin have higher rates ranging from 33.18% to 37.73%. The prevalence of G6PD deficiency varies within ethnic group [22]. Marriage of close family members is a tradition in many ethnic groups in Benin and around the world [23,24]. The higher incidence of G6PD deficiency carriers in northern Benin may be related to the predominance of first cousin's marriage in these populations.

5. Conclusion

The diagnostic value of the enzymatic activity of G6PD is determined in neonates in comparison with the genetic test. Enzymatic activity showed good diagnostic value in male subjects. In female however, the effect of lyonisation phenomenon rendered biochemical test results

unpredictable. The carrying frequency of the G6PD A^{-} $_{(376/202)}$ mutation was determined with the highest proportion in North Benin.

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Statement of Competing Interests

This study is included in UAC student graduate research program and is not funded by an organization. No competing interest to report.

Author Contribution

Conceptualization: Casimir D. AKPOVI.
Formal analysis: Casimir D. AKPOVI, Salomon E. M. FIOGBE, Paulin SEDAH.
Investigation: Casimir D. AKPOVI, Julien G.A. SEGBO.
Methodology: Casimir D. AKPOVI, Salomon E. M. FIOGBE.
Validation: Casimir D. AKPOVI, Julien G.A. SEGBO, Clément AGBANGLA.
Writing - original draft: Salomon E. M. FIOGBE, Casimir D. AKPOVI.
Writing - review and editing: Casimir D. AKPOVI, Fifamè E.E. KOUGNIMON, Julien G.A. SEGBO.

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