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RESEARCH ARTICLE

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Value of DNA testing in the diagnosis of sickle-cell anemia in childhood in an environment with a high prevalence of other causes of anemia

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Abstract

Background: Sickle-cell anemia (SCA) is the most common genetic disease worldwide caused by a single mutation in the gene *HBB*. DNA testing can help to clarify the diagnosis when Hb electrophoresis is inconclusive. We evaluated the usefulness and feasibility of DNA-based diagnosis of SCA in rural Central Africa.

Methods: This is a cross-sectional study conducted from November 2016 to end October 2017 in the Hôpital Saint Luc de Kisantu, located 120 km from Kinshasa. This hospital offers the management of SCA patients, mainly identified using the Sickling test (Emmel test) combined with clinical features. We included patients aged 6 months to 18 years locally diagnosed as SCA, and we collected clinical and hematological data. All patients were offered Hb electrophoresis and DNA testing at the Center for Human Genetics of the University of Kinshasa.

Results: This study included 160 patients. Hemoglobin capillary electrophoresis suggested that 136 (85%) were homozygote SS, 13 (8.1%) were heterozygote (AS), and 11 (6.9%) were homozygote normal (AA). DNA testing confirmed these electrophoresis findings, with the exception of four patients, two AS in electrophoresis were found SS due to recent transfusion, and two SS in electrophoresis were found AS because they have compound heterozygous form S/β°-thalassemia. The diagnosis of SCA was therefore wrongly ascertained with Emmel test in 15% of patients.

Conclusion: This study reveals a high proportion of false-positive SCA diagnoses in a rural environment in Central Africa. This underlines the importance of DNA testing in conjunction with Hb electrophoresis.

KEYWORDS
Central Africa, hemoglobin electrophoresis, molecular testing, sickle-cell anemia

1 | INTRODUCTION

Sickle-cell disease (SCD) is the most common genetic disorders worldwide. SCD patients are homozygous for a recurrent mutation in the *HBB*-gene resulting in the substitution of a glutamic acid residue with a valine amino acid at position 6 of the beta globin protein (E6V).[1] The mutated protein, known as HbS, has a different electrical charge, which is exploited for the distinction of HbS from HbA by electrophoresis.[2,3] The term SCD refers to all different genotypes that cause characteristic clinical syndrome, whereas sickle-cell anemia (SCA), the most prevalent form of SCD, refers to the homozygous form of SS, and the heterozygous compound forms such as S/β-thalassemia, SC disease refer to SCD.[4]

The disorder has a very high prevalence in sub-Saharan Africa.[5,6] However, the accurate diagnosis remains challenging, due to several factors. In the DR Congo, the diagnosis of SCA is currently made by different techniques depending on their local availability. The most advanced hospitals offer hemoglobin isoelectrofocusing (IEF) or hemoglobin electrophoresis (HE). However, the Sickling test (Emmel test) remains the most commonly used method in rural areas. It relies on the observation of sickling of red blood cells in a low-oxygen tension environment. The Emmel test is known to have low accuracy. This test may be difficult to interpret and is not reliable before the age of 3 months due to high HbF levels in red blood cells and after blood transfusions.[7–9] Moreover, anemia, the key feature of the SCA,[10] is a frequent complication of infectious diseases that are prevalent in the Democratic Republic of Congo (DRC) such as malaria.[11–13] This results in a high frequency of transfusions, which in turn limits the usefulness of above-mentioned tests. These factors may cause a high rate of false results, which is concerning considering that the currently available treatment, the Hydroxy-urea, is not justified in non-affected individuals. Recently, DNA-based testing for SCA has become available at the Center for Human Genetics at the University of Kinshasa, Kinshasa, the capital of the DRC. The purpose of this study was to evaluate the value the accuracy of SCA diagnostic based on clinical presentation and with the addition of a positive Emmel test in a rural area in the DRC, as compared with Hb electrophoresis and to DNA testing for SCA.

2 | METHODS

2.1 | Framework, study design and patients

During a period of 12 months, from November 2016 to end October 2017, a cross-sectional study was conducted in the Hôpital Saint Luc de Kisantu (KSLH), a regional reference center located 120 km from Kinshasa. On average, 260 adults and children SCA patients are followed regularly.

2.2 | Patient recruitment

The diagnosis in these patients was made by clinical suspicion associated with a positive Emmel test, occasionally people received hemoglobin electrophoresis and/or hemoglobin isoelectrofocusing.[4,14] Inclusion criteria were children followed for SCA, aged between 6 months and 18 years, without a history of recent blood transfusion (within the last 3 months).[15] Patients were recruited during one of their regular follow-up consultations, when the study was explained and written informed consent was obtained.

At inclusion and during follow-up consultations, we obtained a detail personal history, including number and timing of hospitalizations and blood transfusions, the number of painful crises and other complications (stroke, acute chest syndrome, avascular bone necrosis, leg ulcers, pneumococcus meningitis/sepsis, priapism, osteomyelitis, and hepatobiliary complications, etc.). A complete physical examination was performed, including biometry. We measured weight and height, and then calculated Z-scores for weight for age (WAZ), height for age (HAZ), weight for height (WHZ), and body mass index for age (BMIZ) using the anthropometric software "ENA for SMART." These anthropometric indices defined wasting, stunting, thinness, and underweight, respectively as WAZ, HAZ, WHZ, and BMIZ were below two standard deviations (NCHS'CDC, 2002). Data were checked in the medical records, when available.

For each patient, we collected blood in two 4 ml EDTA tubes. We obtained a full blood cells count (red blood cells (RBC), white blood cells (WBC), platelets and reticulocytes). Biochemical analyses included lactate dehydrogenase (LDH), bilirubin, serum creatinine, aspartate aminotransferase (AST), and alanine aminotransferase (ALT) (Laboratory of Biochemistry and Hematology, Faculty of Pharmaceutical Sciences of the University of Kinshasa [UNIKIN]).

Hemoglobin electrophoresis using the automate Minicap (Sebia, Phoresis Rel 8.6.3.), DNA extracted by the salting out method, and mutation analysis for the SCA mutation (E6V) were made in the Laboratory of Human Genetics at UNIKIN.[16] Mutation analysis of the β-globin gene (NG000007.3) was made by resequencing the coding exons and by MLPA, in the Laboratory of Université Catholique de Louvain (UCL) in patients suspected for compound form of SCD Sβ-thalassemia.

2.3 | Ethics statement

The study was approved by the ethical committee of the school of public health of the University of Kinshasa (ESP/CE/079/2016), DRC. Informed consent was obtained from a parent or legal representative for all patients before their inclusion in the study.

2.4 | Statistical analysis

Data were collected using a survey form, then recorded onto an Excel file using Microsoft Excel 2013, and finally imported to SPSS software version 25.0 for statistical analysis. Qualitative data (sex, capillary electrophoresis, and molecular testing results) are presented as proportions, whereas quantitative data (age, height, and biological parameters) are presented as means ± standard deviation (SD) when the distribution was normal and as median with range when the

distribution was not normal. Columns and bars are used to provide clear data visualization. Mann–Whitney U test was used to compare means, and Chi-square and Fischer tests to compare proportions.

3 | RESULTS

3.1 | Recruitment

Hundred and sixty patients followed at KSLH for SCA were recruited. The diagnosis was made by Emmel test for all. Among them, 20% had previously also been evaluated by Hb electrophoresis and/or IEF in the frame of a research project.

Hemoglobin capillary electrophoresis revealed that 136 (85%) were homozygote SS, 13 (8.1%) were heterozygote (AS), and 11 (6.9%) were homozygote normal (AA) (Table 1). DNA testing confirmed these electrophoresis findings, with the exception of two seemingly heterozygote patients. These patients were AS on Hb electrophoresis but presented homozygous SS after DNA analysis. While trying to explain this discrepancy, we discovered that these patients had a recent transfusion, respectively, 21 and 8 days before. That information was not reported at the recruitment. The two individuals had besides presence of HbS, an elevated level of HbA with a HbF of, respectively, 1.9% and 1.4% and a Hb 9.4 and 9.1 g/dl. In addition, in two patients with SS on Hb electrophoresis, DNA analysis revealed heterozygote AS. These two patients had a HbF of, respectively, 8.5% and 16.6%, with a HbA_2 of 5.9 and 4.9% and a Hb 7 and 11.1 g/dl. They were therefore suspected for being compound heterozygous for an S allele and a β-thalassemia null mutation. Sanger sequencing established that one patient was heterozygous for the c.315+1G>A variant in intron 2 of the HBB gene (VSV000619686.5), the second patient carried the c.92+2 T>C variant in intron 1 (VSV000619860.5). Both variants are known splicing variants, classified as pathogenic for Beta thalassemia in Clinvar (two stars).[17,18] Although segregation analysis was not performed, we retained the compound heterozygosity for HbS and β-thalassemia.

We thus identified three clinical groups of patients: 136 (85%) with SCA or homozygous SS, two (1.25%) with a distinct type of sickle-cell disease (compound heterozygous HbS/Beta° thalassemia) and 22 (13.75%) without SCA or SCD (Table 1).

The diagnosis of SCA was evaluated by a combination of DNA analysis and Hb electrophoresis. This revealed eventually 11 cases without SCA, 11 heterozygous patients (AS), 2 with HbS/Beta° thalassemia ([b]), and 136 with SCA (SS), of whom two showed AS on Hb electrophoresis, resulting from a recent blood transfusion ([a]) (Table 1).

3.2 | Clinical and biological data

Based on corrected diagnostics, clinical and biological data were compared between SCA patients and non-SCA patients (AA or AS) (Table 2). The mean age was 7.5±4.5 y in *SS group* compared to 9.6±4.5 y in *Non-SS group* ($p = 0.03$) and the mean weight was 22.7±10.6 Kg in *SS group* and 24.1±10.2 Kg in *Non-SS group* ($p = 0.04$). During the study period, the occurrence of clinical events was significantly lower in the *Non-SS group* than in the *SS group*. In fact, the patients within the *SS group* were more hospitalized, received more transfusions, and presented more painful crisis than in the *Non-SS group*. Laboratory variables showed significantly more anemia, leukocytosis, and reticulocytosis in *SS patients* compared to the *No SS patients*. After logistic regression, SS status was statistically associated with painful crises (OR 48.119; $p = 0.017$) and HbF levels (OR 4.048; $p = 0.046$) (Table 3).

4 | DISCUSSION

To evaluate the current practice of diagnosing SCA in a rural area in the DRC, we verified the supposed SCA diagnosis in 160 children followed in a single reference center by means of Hb electrophoresis in combination with a DNA-based test for SCA. Of interest, 15% of patients were not homozygous SS, but were either heterozygous or even homozygous normal. This has major implications. For the child, the real underlying cause of the anemia may not be dealt with adequately. He may enter an unnecessary follow-up scheme for children with SCA, which comes at a burden and financial cost for the family. Moreover, as a diagnosis of SCA in the DR Congo is associated with a poor outcome for most children, this may threaten his open future. For the family, they may receive the wrong message of an inherited disorder, with a recurrence risk of 25% for each other child.

These false-positive diagnoses could be due to a combination of factors, which we did not study in detail for each patient individually. There is a high incidence of other causes of anemia, the key feature of SCA, for example, iron deficiency and infectious diseases such as malaria. However, in an environment where SCA is frequent, with an estimated incidence of 0.96%–1.4% in childhood,[19,20] this may result in false attribution of the anemia to SCA. Misinterpretation of the Emmel test is likely to be another important factor. In AS heterozygotes, the low-oxygen environment under the coverslip during the Emmel test, provokes the sickle shape also for AS-red blood cells, resulting in a false-positive test. The Emmel test cannot discriminate AS from SS, with a positive result in the both cases due to low-oxygen environment created under the coverslip during Emmel test.[21–23]

TABLE 1 Diagnostic evaluation in 160 patients followed for sickle-cell anemia

Test	Results				
Hb electrophoresis	AA ($n = 11$)	AS ($n = 13$)		SS ($n = 136$)	
DNA	AA ($n = 11$)	AS ($n = 11$)	SS ($n = 2$)[a]	SS ($n = 134$)	AS ($n = 2$)[b]

[a]Two patients showed AS on Hb electrophoresis and SS in DNA testing due to transfusion.
[b]Two patients with HbS/Beta° thalassemia.

TABLE 2 Comparison of clinical and hematological features between SS and Non-SS

Variable	SS patients ($n = 136$)	Non-SS patients ($n = 22$)	p-Value
Clinical features			
Age	9.6 ± 4.5	7.4 ± 4.5	**0.042**[a]
Gender			
Male	65 (48.5)	17 (70.8)	**0.035**[a]
Female	69 (51.5)	7 (29.2)	
WAZ	−1.68 ± 1.11	−0.98 ± 1.31	**0.010**[b]
HAZ	−1.52 ± 1.58	−0.98 ± 0.78	0.106[b]
BMIZ	−1.47 ± 1.72	−0.67 ± 1.82	**0.046**[b]
Nutritional status			
Normal	47 (34.6)	12 (50.0)	0.113[b]
Wasting	60 (44.1)	4 (16.4)	**0.008**[b]
Stunting	47 (34.6)	4 (16.4)	0.063[b]
Underweight	52 (38.2)	5 (20.8)	0.076[b]
Hospitalization in the previous year	3.0 (1.0–4.0)	2.0 (1.0–2.0)	**0.019**[a]
Transfusion in the previous year	1.0 (0.0–3.0)	1.5 (1.0–2.0)	0.724[a]
Painful crisis in the previous year	2.0 (0.0–4.0)	0.0	**<0.001**[a]
Hematological features			
HbF(%)	9.7 ± 6.5	0.9 ± 1.3	**<0.001**[b]
Hb (g/dl)	7.4 ± 1.86	10.5 ± 2.1	**<0.001**[b]
Ht (%)	22.5 ± 5.9	32.7 ± 6.7	**<0.001**[b]
MCV (fl)	83.6 ± 10.9	76.1 ± 12.5	**0.007**[b]
CCMH (pg)	33.1 ± 2.4	31.1 ± 2.1	**0.001**[b]
Reticulocytes per mm^3	180418.6 ± 118144.9	70810.7 ± 4303.8	**<0.001**[b]
Platelets per mm^3	392656.8 ± 157831.9	321600.0 ± 144593.2	0.065[b]
WBC per mm^3	13958.3 ± 6727.1	7141.0 ± 3282.1	**<0.001**[b]
LDH (UI/L)	1872.3 ± 847.8	1590.6 ± 816.5	0.064[b]
Bili conjugate (mg/dl)	0.47 ± 1.52	0.40 ± 0.50	0.856[b]
Bili non conjugate (mg/dl)	2.31 ± 1.42	2.0 ± 1.36	0.419[b]
GOT (AST) (UI/L)	57.85 ± 27.11	41.90 ± 29.38	0.086[b]
GPT (ALT) (UI/L)	22.10 ± 14.86	19.20 ± 12.64	0.556[b]
Crea (mg/dl)	0.763 ± 0.424	1.0 ± 0.0	0.083[b]

Note: "Bold values" means that the difference is statistically significant, p-value is under 0.05.

Abbreviations: BMIZ, Body mass index Zscore; HAZ, Height for age Zscore; LDH, Lactate dehydrogenase; MCV, Mean corpuscular volume; WAZ, weight for age Zscore; WBC, white blood cell.

[a]p-value for comparison of proportions with Chi-square and Fischer tests.

[b]p-value for comparison of means with Mann–Whitney U test.

With an incidence of AS in the adult population of 23.3%,[20] one can expect that blood donors may sometimes be AS.[21,24] This may result in an elevated level of AS-RBC, equally leading to false-positive Emmel test. This may result in a false normal or carrier result in SCA screening test. These results clearly indicate that a DNA-based diagnostic test is more valuable in this setting. Thus, a high degree of suspicion for SCA is justified in this population, but confirming the diagnosis requires correct use of diagnostic tests, preferably by DNA analysis with or without Hb electrophoresis.

In our study, 1.3% (2 of 160) of patients had compound heterozygous HbS/β°- thalassemia. This form of SCD is more prevalent in the eastern Mediterranean region and India. It results in a severe SCD comparable with SCA due to SS.[2,4,25] The c.315+1G>A variant (VSV000619686.5) was described before as a β°-allele, and was associated with elevated levels of fetal hemoglobin in most but not all cases.[18,26,27] In contrast, the patient in this study, a female patient of 16 years, presented a mild phenotype (no hospitalizations, no transfusions and no painful crises), with HbF 16.6%, Hb 11.1 g/dl, MCV 67 fl, WBC 8100/mm^3, reticulocytes 15,616/mm^3. The c.92+2 T>C variant (VSV000619860.5) was described before in a 21-year-old African American female patient with β°- thalassemia.[28] The present case was a female patient of 15 years, with HbF 8.5%, Hb 7 g/dl, MCV 76 fl,

TABLE 3 Factors associated to SS status

Variable	*p*-Value	OR	CI 95%
Hospitalization in the previous year	0.977	1.017	0.977–1.220
Transfusion in the previous year	0.436	0.631	0.198–2.009
Painful crisis in the previous year	**0.017**	48.119	2.02–1145.5
HbF (%)	**0.046**	4.048	1.024–16.002
Hb (g/dl)	0.991	1.012	0.142–7.0.197
MCV (fl)	0.056	1.400	0.991–1.978
Reticulocyte per mm^3	0.127	1.000	1.000–1.000
Platelets per mm^3	0.205	1.000	1.000–1.000
WBC per mm^3	0.916	1.000	1.000–1.000
Bili Ind. (mg/dl)	0.394	0.154	0.002–11.389
LDH (IU/L)	0.525	1.001	0.998–1.004

Note: "Bold values" means that the difference is statistically significant, *p*-value is under 0.05.
Abbreviations: LDH, Lactate dehydrogenase; MCV, Mean corpuscular volume; WBC, white blood cell.

WBC 11130/mm^3 and reticulocytes 175,320/mm^3, with a history of painful crises, four hospitalizations and two transfusions over 1 year.

Despite an apparent clinical confusion that exists between "SS patients" and "non SS patients", we observed significant clinical and biological differences between these two groups, allowing to distinguish these two groups even after transfusion. Obviously, painful crises are limited to SCA patients. "Non SS patients" have on average, higher levels of Hb, no leukocytosis and normal reticulocyte levels and absent levels of HbF in the majority of them. However, as the currently available techniques to quantify HbF are costly, the HemoTypeSC test, based on monoclonal antibodies detecting HbS, HbA and HbC, may provide a cheap alternative.[29]

In conclusion, this study reveals a high proportion of wrongly diagnosed SCA patients in a rural environment in Central Africa, and underlines the importance of a DNA test in addition to Hb electrophoresis in helping to clarify the diagnosis of SCA. Improving the skills of healthcare professionals in the clinical recognition of SCA in children remains a crucial step in the management of SCA, especially in rural area.

AUTHOR CONTRIBUTIONS

Data collection: Gloire Mbayabo. Drafting: Gloire Mbayabo, Paul Lumbala Kabuyi, Koenraad Devriendt, Prosper Lukusa Tshilobo. Laboratory diagnosis of sickle-cell disease: Gloire Mbayabo, Mamy Ngole Zita. Diane Maisin, Damien Gruson. Conception and design of the study, review of the manuscript: Gloire Mbayabo, Paul Lumbala Kabuyi, Mamy Ngole Zita, Aimé Lumaka, Valerie Race, Gert Matthijs, Tite Mikobi Minga, Koenraad Devriendt, Prosper Lukusa Tshilobo, Chris Van Geet. All authors revised and approved the final version of the manuscript.

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CONFLICT OF INTEREST

The authors declare that they have no competing interests.

DATA AVAILABILITY STATEMENT

Data available on request from the authors. The data that support the findings of this study are available from the corresponding author upon reasonable request.

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