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Molecular Diagnosis of Blood Disorder Disease : Trial on Gabonese Sickle Cell Patients

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Abstract:- Sickle cell disease is a major health concern worldwide, and particularly In Sub-Saharan Region where Gabon lies. This disease causes considerable damage to individuals and society. Molecular diagnosis of the Sickle cell disease, genetic counseling and family studies of patients, are becoming important parameters for patient management. In this work, in addition to setting up a decision tree for the diagnosis of sickle cell disease in Gabon and determining the prevalence of this disease in the South-East zone, we are developing a molecular analysis procedure with the aim of carrying out molecular screening for sickle cell disease at CIRMF. Screening of 235 children aged 0 to 19 using the sickle cell rapid Sickle Cell Scan test showed that 17.81% of the population were sickle cell positive. The results of this study also show a high proportion of sickle-cell-affected children under 10 years of age (79.42%). There are conflicting results requiring molecular analysis. This work has also made it possible to set up a molecular analysis protocol that can serve as a basis for molecular diagnosis, with the aim of confirming the results of phenotypic analyses of people with sickle cell disease in Gabon. It also opens the way to prenatal diagnosis of sickle-cell anemia for couples in a country where 21% of the population carries the sickle-cell trait, an argument in favor of training couples at risk in this central African country.

Keywords:- *Sickle Cell Disease, DNA, Hemoglobin, PCR, Enzymatic Digestion, Diagnosis, Gabon*

I. INTRODUCTION

Sickle cell disease or Falciform Anemia is an autosomal recessive inherited disorder linked to the presence of an abnormal hemoglobin called hemoglobin S (HbS) [1]. Sickle cell disease is the most common genetic disorder in the world [2]. Sub-Saharan Africa is considered the main focus of the disease, followed by other parts of the world such as India, Saudi Arabia, the West Indies, North America (USA), South America (Brazil) and Mediterranean countries [3]. The WHO (2006) and the UN (2009) have recognized sickle cell disease as a public health problem, and have designated June 19 of each year as "World Sickle Cell Awareness Day"[4].

Sickle cell anemia is much more prevalent in southern countries [5], most of which lack appropriate screening and treatment programs. The urgent need, therefore, is to raise awareness and implement policies to combat sickle cell anemia, in order to reduce transmission of the disease and consequently lower its prevalence.

The disease is characterized by chronic anemia, which appears early in life and is associated with vaso-occlusive crisis (CVO) of varying intensity, sometimes life-threatening, and chronic complications with vital and/or functional risks [6]. In addition to CVO and chronic anemia, the major syndrome of sickle cell disease is manifested by numerous signs that can vary from one individual to another, and even from one elderly person to another [7].

Various techniques for diagnosing the disease have evolved [8]. These different types of diagnosis can be phenotypic in nature, allowing the identification of different hemoglobins including abnormal hemoglobin S (HbS) [9]. These include electro phoretic methods for migration and separation of different hemoglobins using electric fields on a gel, chromatographic methods and many others (experimental falciformation test, Itano test, isopropanol instability test). A new screening tool designed to facilitate rapid screening: the Sickle SCAN Biomedomics rapid diagnostic test (RDT). It has been developed to perform diagnosis in regions with no laboratory facilities [10].

In addition to phenotypic tests, there are other techniques for identifying the abnormality at source: this is the molecular diagnosis of sickle cell disease, which aims to identify the disease-causing mutation in the DNA sequence [11]. Phenotypic diagnosis by hemoglobin analysis is sufficient to make the diagnosis. However, the contribution of molecular genetics is essential during the prenatal period, as molecular diagnosis will enable early diagnosis using fetal DNA from chorionic villi or amniotic fluid or for confirmation of an unclear phenotypic test. In its 2006 report, the World Health Organization cites prevention through prenatal diagnosis as a means of reducing the number of children born with sickle cell disease (WHO, 2006). In view of the above, the main objective of this internship is to develop experimental procedures for the molecular diagnosis of sickle cell disease from blood samples from Gabonese sickle cell patients.

II. MATERIALS AND METHODS

A. Population and Study Area

This study was carried out on the populations of the Haut-Ogooue province received during the first scientific days on sickle cell disease in Franceville and the medical caravan, with the primary aim of determining the prevalence of sickle cell disease and the frequency of the sickle cell trait in this part of Gabon.

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B. Study Framework and Recruitment

Analyses were carried out at the International Center of Medical Research of Franceville (CIRMF) within the sickle cell disease diagnostic platform of the Parasitology Department and the Natural Host Resistance Team (RNH).

To carry out this study and select the patient sample, our initial inclusion criteria were patients of both sexes aged between 0 and 19 years, who had not undergone a blood transfusion within the last 3 months, and whose parents had signed an informed consent form.

Data are collected following the signing of an informed consent form, and patients or parents provide a certain amount of personal information, as well as information relating to sickle cell disease. Once the confidentiality of responses and the objectives of this study have been established, this information is recorded on a patient form and entered into a database.

The patient form consists of several questions grouped into six parts: The first part concerns questions linked to the patient's identity; the second part provides information on the patient's parents; the third part is concerned with clinical parameters (clinical signs developed by patients, vaccinations, biological signs) and the fourth and fifth parts are linked to sickle cell screening, respectively rapid diagnosis (RDT) and capillary electrophoresis;

Finally, the last section looks at patients' hospitalizations, their frequency and duration, and their transfusion history.

C. Experimental Process

After providing a certain amount of information, patients underwent blood sampling. This was done by venipuncture, and the blood collected in EDTA (Ethylene-Diamine-Tetra-Acetic) tubes, which is also a nuclease inhibitor, allowing the DNA to remain intact and undegraded.

Phenotypic analysis was performed using the Biomedomics rapid test for sickle cell disease (first-line) according to the protocol described by Delicat-Loembet et al. in 2022 [12]. The different phenotypes possible with the Sickle SCAN rapid test are the following hemoglobin combinations: AA, AC AS, SS, SC and CC (Fig.1).

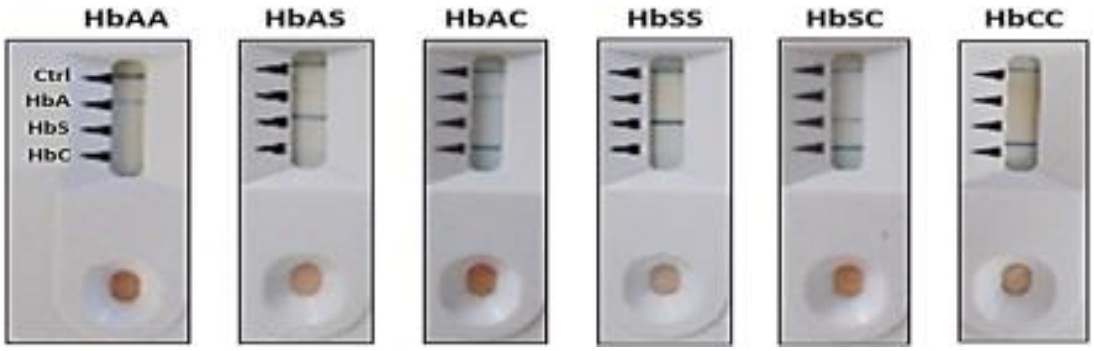

Fig. 1: Examples of different hemoglobin phenotypes diagnosed by Sickle SCAN

Molecular diagnosis of sickle cell disease involves several steps:

- ***DNA extraction*:** is a technique used to isolate DNA from cells or tissues (blood). The extracted DNA can then be used for molecular biology research techniques such as sequencing, PCR, cloning and genetic disease diagnostics. DNA extraction from the blood pellet was performed using the Qiagen commercial kit according to the manufacturer's recommendations [13].
- ***DNA sample quantification and purity*:** For our analyses, quantification was performed using a NanoVue plus spectrophotometer. According to the Qiagen manual, a pure DNA sample has a 260/280 ratio of between 1.7 and 2.0. Therefore, any ratio below 1.7 indicates protein contamination, and any ratio above 2.0 suggests RNA contamination. Samples presenting this type of results will not be retained for the rest of our experiment.
- ***Polymerase chain reaction (PCR):*** This step consisted in searching for sickle cell mutations in the β-globin gene. The previously extracted DNA is subjected to PCR amplification. In the case of our work, a primer pair with a size of 21 bp (HbA_2F: 5'-*ACCTCAAACAGACACCATGGT* and HbA_2R: 5'-*GCAGAGAGAGTCAGTGCCTAT*) was used to hybridize from region 62171 in order to induce polymerization of a 212 bp fragment up to region 62382, which corresponds to the sequence of interest, the one likely to harbor the mutation.

The reaction medium is Econo Taq Green PCR Master mix (2x) (a 2X concentrated solution containing Taq polymerase, dNTPs, PCR buffer and Mgcl2). Using a master mix saves time and reduces contamination, as fewer pipetting steps are required for the PCR set-up. The mixtures obtained are subjected to automated temperature changes using the EPPENDORF MASTERCYCLER PRO S thermocycler (1 cycle 95°C for 2min, then 30 cycles of 95°C for 30 sec and 55°C for 30 sec 72°C for 30sec and 1 cycle 72°C for 10 min).

- ***Enzymatic Digestion*:** After PCR, the DNA is mixed with the restriction enzyme. The following sequence: CTNAG where N represents any nucleotide. This sequence is cut by the HpyF3I enzyme, which is an isoschizomer of the DdeI enzyme because it does not contain the sickle cell mutation [14], thus obtaining two fragments of 33 and 179 bp.

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As a result, for the dominant homozygous form (HbAA), we have two fragments (33 and 179 bp); for the heterozygous form (HbAS), digestion is carried out on the normal gene, followed by three fragments (212, 33 and 179 bp); finally, for the homozygous form (HbSS), there is no restriction site, so the only type of fragment observed is that resulting from amplification (212 bp). The enzymatic reaction conditions are carried out according to the proportions defined in the laboratory.

- ***Migration*:** Agarose gel electrophoresis (1,5%) is the technique we used, as it allows previously amplified DNA fragments to be separated by restriction enzymes according to their size.
- ***Anomaly identification*:** Figure 2 shows an example of the position and number of bands revealing the patient's genotype. Indeed, due to the presence of the HpyF3I restriction site on the A allele, an AA homozygote is identified by the presence of two bands (33 and 179 bp), while the restriction site is absent on the S allele, so there is no cut on it. Consequently, an SS homozygote has a single band (212 bp), while AS heterozygotes have three bands.

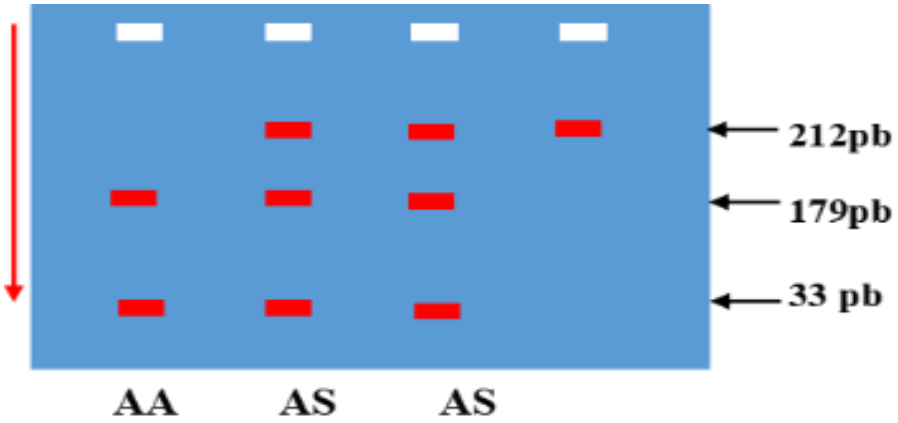

Fig. 2: Example of DNA migration on agarose gel after enzymatic digestion

For statistical analysis, our data, classified in a database using Microsoft Excel, were processed and analyzed using Epi Info version 7 and R version 3.2.2. The chi-square (X^2) test was used to compare the various subject parameters. The significance level for statistical tests was set at $\alpha = 5\%$.

III. RESULTS AND DISCUSSION

A. *Phenotypic analysis*

The sickle cell disease scientific days and medical caravan in Franceville were very well attended by children and young people under the age of 20. Indeed, 300 individuals were seen for a sickle cell diagnosis. Of these, 235 were unaware of their status, i.e. 78.33% were present for screening. Apart from those present to determine their hemoglobin status, a second group of 65 individuals (21.67%) came specifically to confirm a previously established diagnosis. The proportions associated with these two groups show a highly significant difference ($p < 2.2\ 10\text{-}16$).

Confirmation follows an initial diagnosis by a laboratory, because when the initial diagnosis reveals the presence of the homozygous "S" allele (HbSS) or a "C" allele, a confirmatory test is recommended to the patient by the Medical Biologist.

B. *Molecular confirmation of phenotypic diagnosis*

Of the 219 patients we received, 40 were sickle-cell patients, including 39 (17.81%) SS homozygotes and 1 (0.46%) SC double heterozygote. The frequency of healthy carriers is very close to that of sick individuals, estimated at 38 (17.35%). Normal individuals are the most numerous, accounting for over half of the population screened, i.e. 141 (64.38%) individuals (Fig. 3).

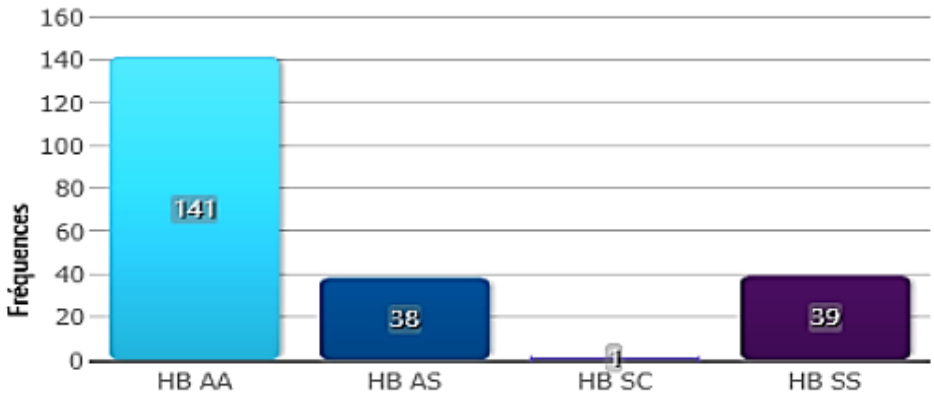

Fig. 3: Proportion of sickle cell disease phenotypes

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The presence of a single carrier of the C allele is linked to its geographical distribution, as it is much more common in the West African region [15]. The proportion of healthy carriers in Haut-Ogooué (17.35%) remains close to the national average, estimated at 21.1% [16], but also close to that of newborns (15.1%) screened between May and December 2007 at CIRMF [17]. It is essential to screen heterozygotes and recommend couple screening, as sickle cell children are often born to carrier parents [18]. Carriers do not suffer from any clinical signs of the disease, unlike homozygous sickle cell (SS) patients [19]. In this study, the most revealing figure is that of sickle cell children (39 cases or 17.81%). This result calls into question the absence of systematic neonatal screening, so diagnosis is often made in the presence of suspicious signs of the disease [20]. In several studies, notably those carried out in Gabon [17], Congo Brazzaville [21], or in Arab countries [22], the proportion of sickle-cell subjects does not exceed 2%. This rate of sickle-cell-affected children is far higher than the country's prevalence, estimated at between 2% and 3% [23], so it is necessary to put in place means that will ultimately reduce the transmission rate (prevention), but also harmonize the management of children with MDS for better follow-up [24]. It should be added that the fact that this screening day only concerned sickle cell patients' reason why sickle cell children were susceptible to be the most encountered population.

All the individuals tested were sickle-cell patients (homozygous SS or SC), but the results confirmed 61 sickle-cell patients and 3 healthy carriers. It is possible that the carriers had undergone blood transfusions in the three months prior to the test, which is the source of the hemoglobin A contribution (Fig. 4).

Hb Status	Fréquence	Pour cent	Cum. Pour cent
HB AS	3	4,69 %	4,69 %
HB SC	1	1,56 %	6,25 %
HB SS	60	93,75 %	100,00 %
TOTAL	**64**	**100,00 %**	**100,00 %**

Fig. 4: Confirmation Test Results

In the molecular analysis process, In the results obtained, more than 75% of samples are considered uncontaminated due to their 260/280 ratios meeting standards. Also; 7.75% of samples are contaminated by proteins and 16.67% by RNA. Uncontaminated DNA is required in the sense that protein or nucleic contaminants inhibit PCR.

- **PCR verification gel:** In order to adopt a standard PCR protocol, a few DNA samples sorted according to genotype and high concentration were subjected to PCR and then observed on a 1.5% agarose gel (Fig. 5).

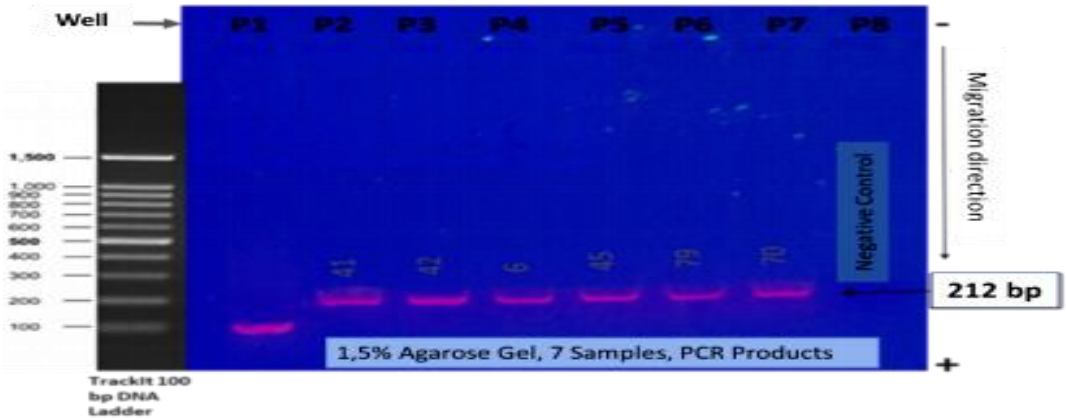

Fig. 5: Gel Migration Number 7

After several inconclusive tests, we arrived at the result shown in the figure above: an agarose gel showing bands of PCR-derived DNA. Analysis of this gel (Fig. 5) revealed the presence of bands of the expected size, i.e. 212 base pairs corresponding to the DNA sequence amplified by the chosen primer pair. In view of this result, this PCR protocol was retained as the standard protocol for all subsequent experiments, as the PCR is considered to have been successful. These amplified samples underwent the enzymatic digestion step to enable validation of the enzymatic digestion protocol, the aim of which is to bring out the different genotypes.

- **Enzymatic digestion verification gel:** The following gel (Fig. 6) shows the DNA bands obtained after digestion of the amplicons with the HpyF3I enzyme for 16 hours at 37°C. Well 1 contains the size marker, well 2 contains DNA from a healthy subject (Homozygote AA), wells 3, 5 and 7 contain DNA from homozygous sickle cell patients (SS), and wells 4 and 6 contain amplicons from a carrier subject (AS) and a composite heterozygote (SC) respectively.

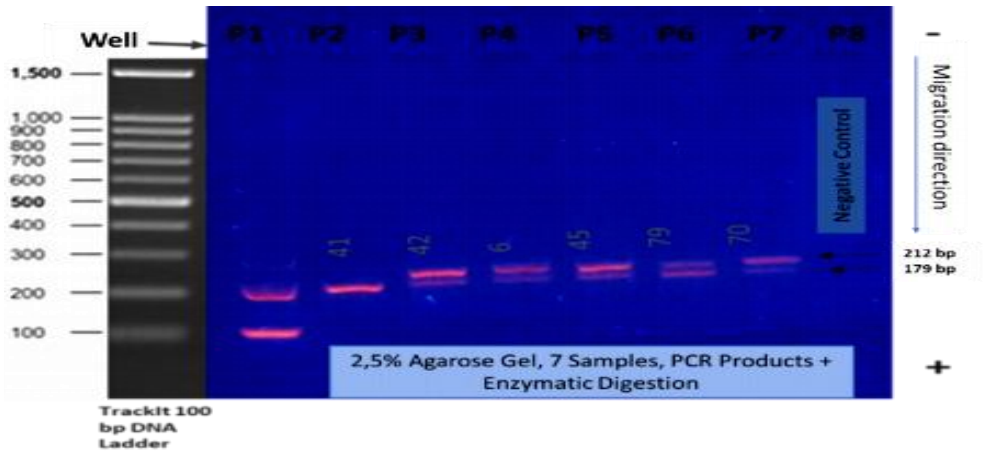

Fig. 6: Agarose gel (2.5%) with digested DNA fragments.

Samples from well 2 (HbAA) show a single band at 179 bp and an absence of the fragment at 33bp, which could be explained by the width of the gel pores allowing this fragment to escape. Well 7 (HbSS) were well digested by the enzyme and are clearly different. It is clear that the DNA in line 7 HbSS lacks the restriction enzyme site required to cut the DNA fragment, as the β-S mutation destroys the enzyme's cutting site [25]. The HbC mutation is also found in codon 6, but it is important to know whether it also destroys the HpyF3I restriction enzyme site in codon 6. Indeed, the HbC mutation is due to the substitution of guanine for adenine on the sixth codon (GAG to AAG) leading to the production of a lysine instead of glutamic acid. HpyF3I recognizes CTNAG where N represents one of the nucleotides A, T, C or G. Consequently, the HbC mutation does not affect the action of the restriction enzyme. Thus, DNA electrophoresis results for HbAS and HbSC individuals after digestion with Hpyf3I are similar. Hence the similarity in the appearance of the bands produced in HbAS and HbSC individuals.

IV. CONCLUSION

This study has enabled us to develop a technique for the molecular diagnosis of sickle cell disease, which will subsequently help to clarify the diagnosis of this pathology. Through the implementation of molecular strategies, we have successfully established a standard molecular diagnostic procedure within our laboratory. This technique has empowered us to routinely identify a range of mutations in sickle cell patients. Molecular results complement phenotypic exploration (RDT) and will be used in the event of a request for genetic counselling or in the event of an ambiguous diagnosis of sickle cell disease in our context. This tool is part of the drive to improve access to screening for at-risk couples, ideally starting very early in their children's first years of life. This work opens up new prospects in the field of Gabonese public health and even sub-regional research, and we propose in future studies to increase the number of samples from our population and carry out a genetic study to assess the sensitivity of the test previously developed, to extend molecular diagnosis to other allele forms (C, D, E..) and to search for thalassemic mutations. It also opens the way to prenatal diagnosis of sickle-cell anemia for couples in a country where 21% of the population carries the sickle-cell trait, an argument in favor of training couples Who are susceptible of giving birth to children with sickle cell disease Gabon.

- **Conflicts of Interest Statement:** The authors have no conflicts of interest to declare.
- **Acknowledgment:** The author is pleased to acknowledge Isaac Okello for editing this paper.

REFERENCES

[1]. S. Huynh-Moynot et al., “Dŕpanocytose: Des aspects moĺculaires à la pratique clinique. À propos d’un cas et revue de la littŕature,” Ann. Biol. Clin. (Paris)., vol. 69, no. 6, pp. 679–684, 2011, doi: 10.1684/abc.2011.0633.

[2]. D. Labie and J. Elion, “The problem of sickle cell disease in Africa,” Med. Trop. Rev. du Corps sante Colon., vol. 70, no. 5–6, pp. 449–453, 2010, [Online]. Available: http://www.ncbi.nlm.nih.gov/pubmed/21520645

[3]. B. Modell and M. Darlison, “Global epidemiology of haemoglobin disorders and derived service indicators,” Bulletin of the World Health Organization. 2008. doi: 10.2471/BLT.06.036673.

[4]. M. Coppieters, L. Godin, Y. Coppieters, and B. Gulbis, “Qualitative analysis of the offer for prevention and caring of sickle cell anaemia in Brussels,” Rev. Med. Brux., vol. 32, no. 3, 2011.

[5]. A. Lainé and G. Tchernia, “‘L’émergence’ d’une maladie multimillénaire.” Université Jean-Moulin-Lyon 3, 2010.

[6]. V. Thuilliez, V. Ditsambou, J. R. Mba, S. Mba Meyo, and J. Kitengue, “Aspects actuels de la drepanocytose chez l’enfant au Gabon,” Arch. Pediatr., 1996, doi: 10.1016/0929-693X(96)87087-4.

[7]. D. Bonnet, “Rupture d’alliance contre rupture de filiation. Le cas de la drépanocytose,” Crit. la santé publique une Approch. Anthropol., no. June, pp. 257–280, 2001.

[8]. H. Wajcman, “diagnostic et dépistage de la drépanocytose,” Rev Prat, vol. 54, pp. 1543–1547, 2004.

[9]. N. Mario and N. Sala, “Diagnostic biologique des hémoglobinopathies,” Rev. Francoph. des Lab., vol. 2016, no. 481, 2016, doi: 10.1016/S1773-035X(16)30127-7.
[10]. T. N. Williams, “An accurate and affordable test for the rapid diagnosis of sickle cell disease could revolutionize the outlook for affected children born in resource-limited settings,” BMC Medicine, vol. 13, no. 1. 2015. doi: 10.1186/s12916-015-0483-4.
[11]. M. H. Steinberg, “DNA diagnosis for the detection of sickle hemoglobinopathies,” American Journal of Hematology, vol. 43, no. 2. 1993. doi: 10.1002/ajh.2830430208.
[12]. L. M. Delicat-loembet, A. D. N. Mabiala, and U. Bisvigou, “Diagnosis of Sickle Cell Disease in Gabon Using Sickle SCAN ® : A Point-of-Care Blood Test,” vol. 6, no. 2, pp. 1–9, 2022, doi: 10.21106/ijtmrph.315.
[13]. E. Elguero et al., “Malaria continues to select for sickle cell trait in Central Africa,” Proc. Natl. Acad. Sci., p. 201505665, 2015, doi: 10.1073/pnas.1505665112.
[14]. R. K. Saiki et al., “Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia.,” Science, vol. 230, no. 4732, pp. 1350–1354, Dec. 1985, doi: 10.1126/science.2999980.
[15]. J. P. De Caluwé, M. Alexander, and H. Bondue, “[Hemoglobin C (alpha 2 beta 2 6Glu-->lys). Study of 19 heterozygote AC carriers and of 5 cases of double hemoglobinopathy SC],” Acta Clin. Belg., vol. 48, no. 5, pp. 297–306, 1993, doi: 10.1080/17843286.1993.11718322.
[16]. L. M. Délicat-Loembet et al., “Prevalence of the sickle cell trait in Gabon: a nationwide study.,” Infect. Genet. Evol. J. Mol. Epidemiol. Evol. Genet. Infect. Dis., vol. 25, pp. 52–56, Jul. 2014, doi: 10.1016/j.meegid.2014.04.003.
[17]. Y. VIERIN NZAME, I. BOUSSOUGOU BU BADINGA, J. KOKO, P. BLOT, and A. MOUSSAVOU, “Dépistage néonatal de la drépanocytose au Gabon,” Med. Afr. Noire, vol. 59, no. 2, pp. 95–99, 2012.
[18]. A. Niakaté, A. Stanislas, C. Rémus, and R. Girot, “Les porteurs du trait drépanocytaire AS,” Santé Et Déveppement, p. 2019, 2019.
[19]. F. B. Piel, “[Sickle-cell disease: geographical distribution and population estimates].,” Medecine sciences: M/S, vol. 29, no. 11. France, pp. 965–967, Nov. 2013. doi: 10.1051/medsci/20132911012.
[20]. M. Y. P. Shongo et al., “Profil h??matologique et nutritionnel du dr??panocytaire homozygote SS ??g?? de 6 ?? 59 mois ?? Lubumbashi, r??publique D??mocratique du Congo,” Pan Afr. Med. J., vol. 21, pp. 142–145, 2015, doi: 10.11604/pamj.2015.21.276.6363.
[21]. A. Mpemba Loufoua, P. Makoumbou, J. R. Mabiala Babela, C. Nakahonda, F. Mayanda, and S. Nzingoula, “Dépistage Néonatal de la Drépanocytose au Congo Brazzaville,” Ann. l’Université Mar. NGOUABI, vol. 11, no. 5, 2010.
[22]. H. A. Hamamy and N. A. S. Al-Allawi, “Epidemiological profile of common haemoglobinopathies in Arab countries,” Journal of Community Genetics, vol. 4, no. 2. 2013. doi: 10.1007/s12687-012-0127-8.
[23]. J. Koko, “Prof. Jean KOKO Département de Pédiatrie Faculté de Médecine Université des Sciences de la Santé (USS) Libreville (GABON) E.mail: jeankoko06@yahoo.fr,” no. April 2013, 2015, doi: 10.13140/2.1.1350.4806.
[24]. S. Minto’o Rogombé et al., “Article Original Suivi Médical et Scolarité de l’Enfant Drépanocytaire au Gabon Relation between medical monitoring and schooling of sickle cell children in Gabon,” J. Med. Heal. Sci., vol. 19, no. January, pp. 74–78, 2018.
[25]. J. M. Old, “Screening and genetic diagnosis of haemoglobin disorders,” Blood Rev., 2003, doi: 10.1016/S0268-960X(02)00061-9.