ARTICLE ORIGINAL

Neonatal screening for sickle cell anemia : Evaluation of the IEF protocol

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Abstract

Background : The sickle cell anemia is a genetic disease with a recessive autosomal transmission, dominated by severe anemia, painful crises and big susceptibility to bacterial infections.
The premature care of the homozygous (SS) is the best way to minimize the clinical severity and this passes by the neonatal screening. Several approaches had yet been developed.

Aim : In our lab, eight years after the implementation of the neonatal screening of the sickle cell anemia, we try here to evaluate the technique used to detect this disease, the isoelectrofocusing analysis (IEF), and reveal the optimal conditions which allow detecting 100 % of the cases.

Methods : This work was conducted on 210 newborns born at the maternity center, La Rabta. Two types of blood sampling were collected for every subject: a cord blood used for HPLC analysis and PCR / RFLP analysis and dried blood samples used in IEF followed by a confirmation by HPLC.

Results : The HPLC revealed 10 samples among 210 tested ones, confirmed by the molecular method: 8 heterozygous (AS) and 2 heterozygous (AO), while the IEF analysis revealed only 3 cases of sickle cell trait, a homozygous S/S and a heterozygous (AO).

Conclusion : The difference between the obtained results shows that homozygous (SS) detection cannot be missed by IEF analysis. Optimization has to be brought to this technique to ensure fully detection of the whole cases of βS carriers by concentrating samples.

Keywords: sickle cell anemia – NBS – IEF – HPLC – PCR/RFLP

INTRODUCTION

Sickle cell disease (SCD) is an autosomal recessive disorder that affects the β-globin gene resulting in abnormal hemoglobin (Hb): Hb S (sickle) which affects haemoglobin (Hb) structure [1].

SCD occurs when an individual is homozygous for β^s allele or because of compound heterozygosity of β^s with other mutant β-globin allele such as hemoglobin βC or β-thalassemia. The sickle cell trait due the presence of β^s in simple heterozygosity is benign.

SCD is characterized by chronic hemolytic anemia and recurrent vascular occlusion that leads to ischemia and distal tissue infarction in multiple organs [2].

SCD is the most common genetic disease worldwide[3], with over 300,000 new cases born each year, two-third of them has an African origin [4].

In Tunisia, the βS carriers rate is around 1.9 %, but it is not uniformly distributed across the country; in some regions, this rate can reach a level as high as 7.7%. Each year, around 73 SCD cases born [9,10].

Newborn screening (NBS) identifies newborns sickle-cell disease carriers before complications occur. Without NBS, many earlier studies have shown that the mortality approached the 20% by 3 years age. Most deaths were attributable to pneumococcal sepsis. [5] NBS is then conducted to identify infants with sickle cell disease and other hemoglobinopathies as early as possible to prevent complications6 allowing the reduction of morbidity and mortality of this disease [7,8].

As part of a research project , neonatal screening for sickle cell disease was initiated in February 2005 in Tunisia in the Laboratory of Biochemistry and Molecular Biology at Children's Hospital “Bachir Hamza “of Tunis in collaboration with two maternity Centers: the Neonatology and maternity center of la Rabta and the Neonatology and maternity Hospital Aziza Othmana in Tunis.

Eight years after the implementation of the neonatal screening of the sickle cell anemia using the Isoelectric focusing (IEF) technique set in our Lab [10], we try here to evaluate the used technique by comparing IEF analysis to HPLC and β^s allele detection with PCR/RFLP technique, in order to determine the optimal procedure that allow 100 % cases detection..

SUBJECTS AND METHODS

Subjects

The study concerned 210 newborns collected in the Maternity Center La Rabta without preselection.

Two types of sample were collected from each newborn:

• Cord blood at birth (50 ml on EDTA)

•Dried blood collected on GHUTRIE" paper (Whattman grade BFC 180 cat no. 9535-9362) (3 spots) at the third day of life.

All mothers have responded to a questionnary concerning parents' geographic origin, consanguinity between them and by the way, they have given their written consent for this study.

After analysis and for every identified carrier of β^s, convocation is sent to parents for a family study. For parents having reached the Lab, 5 ml of total blood were collected to HPLC analysis.

METHODS

Dried blood spot was analyzed by IEF as previously described 10, abnormal cases were confirmed by HPLC analysis for each sample presenting a defect: The system uses the Hb VARIANT II (Hemoglobin testing system, BIO RAD Augusta, GA, USA) using VARIANT™ II HbA2 / HbA1C dual kit (Hercules, CA). This check was performed on dilution of a spot of dried blood in 50 µl of wash solution (Wash Bio Rad Variant ™ II HbA2 / HbA1C Program).

Cord blood samples were directly analyzed by HPLC. This check was performed on total blood with a dilution of 1/200 in 1 ml of wash solution (Wash Bio Rad Variant™ II HbA2 / HbA1C Program) .

Cord blood was also used for molecular analysis. The DNA extraction was performed using standard phenol-chloroform method.

The sickle cell mutation was tested using PCR/RFLP procedure. DNA fragment containing the exon I of the β-globin chain was amplified using 0.1 µM of each of this 2 primers: PCO4 (5'CAACTTCATCCA CGTTCACC 3') and CHINA I (5'GTACGGCTGTCA TCACTTAGACCTCA 3'), 1 mM of $MgCl_2$, 0.1 mM of dNTPs, 100 ng of ADN, 1 unit of ampli Taq polymerase and 1X of its buffer in a total volume of 25 µL. PCR

revealed a band of 256 bp. The PCR program is composed by three stages: the initial denaturation performed at 94° C during 5 min, 30 cycles of denaturation at 94° C during 30 seconds, hybridation at 57 ° C during 30 seconds and elongation 72 ° C during 30 seconds and a final elongation at 72 ° C during 5 min.

Restriction analysis was done using 8 µl of amplified product, 0.1 mg/ml of BSA, 5 units of the enzyme Bsu 36 I and 1 X of its buffer and total volume of 25 µL. This mix was incubated in a water bath during one hour and a half at 37° C.

RESULTS

Studied Population

Among the 210 newborns included, 53.81% were boys against 46.19% girls, a sex ratio of 1.16 in favor of boys. The origins of newborns are spread across all regions of Tunisia. (Table 1)

Analysis of dried blood samples

The first drop has served to IEF analysis.

Among the 210 tested subjects, 5 subjects carried a hemoglobin defect (Table 1):

- 3 sickle cell heterozygotes (AS)
- 1 homozygous sickle cell (SS)
- 1 carrier of Hb C or Hb O_{Arab} (by IEF analysis, it is difficult to distinguish Hb C from Hb O_{Arab}).

Samples, on which defects were detected in the IEF, were tested by HPLC on the second drop of dried blood which confirmed the IEF results and the Hb O_{Arab} diagnosis for the fifth sample.

Cord blood analysis by HPLC

For each dried blood sample, cord blood was also collected.

HPLC analysis of cord blood revealed 10 anomalies:

- 8 sickle cell heterozygous (AS)
- 2 carriers Hb O_{Arab} subjects (AO)

Table 1: Distribution of samples and their analysis results according to their regions

Region	Studied number	Cases reveale by IEF analysis	Cases revealed by HPLC analysis	Cases revealed by PCR/RFLP analysis	prevalence
Tunis	51 (24.29%)	1	1	1	1.96%
North East	17 (8.09%)	0	0	0	0
North West	100 (47.62%)	3	5	5	5%
Central East	7 (3.33%)	0	0	0	0
Central West	21 (10%)	0	1	1	4.76%
South East	8 (3.81%)	0	0	0	0
South West	6 (2.86%)	0	1	1	16.66%
Total	210 (100%)	4	8	8	3.8%

Analysis by PCR / RFLP of cord blood

PCR amplification of a DNA fragment spanning the region of the mutation, after a check on 1.5% agarose gel revealed a band of 256 bp. Separation of restriction fragments is controlled by electrophoresis on 2% agarose gel and revealed a heterozygous sample (AS) three bands: 256pb and 200bp and 56pb, a homozygous sample (SS) a band 256pb and a normal sample, two bands at 200 bp and 56pb.

Among the 210 analyzed samples by PCR / RFLP, 8 β^s carriers were revealed. (Table 1)

Family Study

In total, 10 families were called to the family study, but only 4 families have responded positively to this meeting.

DISCUSSION

IEF/HPLC+ RFLP comparison

By IEF, the total number of sickle cell trait carriers corresponds to a prevalence of 1.9% and this is in accordance with previous study (1.92%) [10]. That is is explained by the studied population almost the same (Tunis Maternity center) and the same analysis technique.

On the other hand, HPLC and molecular analysis revealed a prevalence 4.76% of abnormal Hb variants carriers. The β^s is the predominant defect in our series and its prevalence 3.8% of AS is significantly higher than previous studies (1.92%) (10) and (2.32%) [9]. This high prevalence of Hb S carriers at birth case can be explained in part by the use of different technique: IEF and cellulose acetate for prior studies and HPLC for the present one), and this shows that HPLC is more efficient than the other two approaches in Hb S detection at birth. HPLC is a rapid technique that uses a small amount of blood for accurate detection of the different fractions present in a sample. However HPLC is relatively expensive. Indeed, a sample costing 5.4 Dinars Tunisian (DT) against 0.855 DT for the analysis by IEF. In this work, the IEF, a simple and high resolving power that can process multiple samples simultaneously for a relatively modest cost, has revealed a significant loss of information. Indeed, we lose a half of information on the carriers of hemoglobinopathies.

In order to explain the difference in results by IEF and HPLC, we have compared the means of HbS rate between cases of AS detected by IEF and those not detected by IEF, the average of HbS rate is statically lower in the second group. The low concentration of HbS could be the cause of non-detection of HbS fraction agarose gel ($p = 0.012$).

Therefore, we should improve the IEF technique by concentrating tested samples so that the low Hb S rates are detectable and do not read those with low intensity and reanalyze them.

Certainly, the detection of homozygotes (SS) cannot be missed by IEF analysis given the high concentration of the Hb S fraction (more than 5%) in the sample as previously demonstrated in this study.

The heterozygous form (AO) has a relatively high prevalence of 0.95% compared to the rate indicated by Fattoum (0.08%) (9) and revealed by Siala (0.14%) (10). Two carriers of the trait O were found in a relatively small number and it is difficult to refer to this result.

Concerning the sample N° 215 detected as SS case on dried blood by IEF while on cord blood analysis by HPLC and PCR-HPLC presented an AS profile : it could be explained by the low sample concentration of HbA (3%) making this undetectable by IEF technique in contrary at HPLC which is a technique used for a precise detection of different fractions. We take note that the rate of Hb A for tested samples presents an average of 15.61 % ± 5.85.

Hb S trait prevalence

Concerning the prevalence of Hb S trait in Tunis, considered as a representative mix of the hole Tunisian population, our results show a very high prevalence (3.8%) compared to earlier published data conducted on adult subjects (1.89%) [9].

This high prevalence could attribute to the significant presence (47.62%) of newborns from the North-West (high risk region for SCD).

If obtained results are projected on representative Tunisian population, the Hb S prevalence became 2.79%. This rate corresponds probably to a real Hb S prevalence in Tunisia. It remains significantly higher than established data by adult study (1.89%) (9) and could be explained by a childhood death.

Family Study

The families of hemoglobinopathies carriers were convened for a family study. But only 40% of families have come. This response rate is fully consistent with the usual rate of the laboratory.

Parents study has not revealed any couple at risk.

Geographical origins of Hb S carriers

SCD is not uniformly distributed in our country. Three main centers: the southwest (2.30% in Kebelli), northwest (6.66% in Beja) and northeast (7.27% in Sejnen) [9].

In addition, here is Jendouba governorate (northwest) presenting the highest prevalence of AS (17.39%: 4 carriers of 23 subjects screened). While Beja (previously described as the fireplace for this disease) is shown with a prevalence of carriers of hemoglobinopathies (5.4%: 2 carriers of 37 subjects screened) what is close to the rate previously found in Beja (5.68%) (9).

CONCLUSION

Through this study, we wanted to ensure that the IEF technique, first line of screening, is effective enough to achieve the different objectives of newborn screening. Certainly, the detection of homozygous (SS) cannot be missed by IEF analysis but this technique doesn't be effective enough cause a disparity of information for heterozygous was revealed and therefore couple at risk and consequently increases the number of homozygous that could be prevent. Optimization of this technique will be conducted in order to ensure fully detection of the whole cases of hemoglobinopathies carriers.

NBS for SCD would be establishing on the governmental level and especially in Jendouba. Also, this measure will be benefic to correct the SCD rate in Tunisia.

REFERENCES BIBLIOGRAPHIQUES

1. Serjeant GR. Sickle cell disease. Lancet 1997; 350(9079): 725–730.

2. Ahmed S, Shahid RK, Russo LA. Unusual causes of abdominal pain: sickle cell anemia. Best Best Pract Res Clin Gastroenterol 2005; 19(2): 297–310

3. Piel FB, Patil AP, Howes RE et al. Global epidemiology of sickle haemoglobin in neonates: a contemporary geostatistical model-based map and population estimates. Lancet 2013; 381: 142–51

4. Howard J, Oteng-Ntim E. The obstetric management of sickle cell disease. Best Pract Res Clin Obstet Gynaecol 2012; 26:25.

5. Reed W, Lane PA, Lorey F et al. Sickle-cell disease not identified by newborn screening because of prior transfusion. J Pediatr 2000; 136 (2) : 248–50

6. Panigrahi S, Patra PK, Khodiar PK. Neonatal Screening of Sickle Cell Anemia: A Preliminary Report. Indian J Pediatr 2012; 79(6):747–50

7. Berthet S, monpoux F, Soummer AM, Bérard E, Sarles J, Badens C. Dépistage néonatal de la drépanocytose au CHU de Nice : bilan des 8 dernières années. Arch Pediatr 2010;17:1652-56

8. Bardakjian J, Benkerrou M, Bernaudin F et al. Le dépistage néonatal de la drépanocytose en France métropolitaine. Arch Pediatr 2000; 7: 1261-63

9. Fattoum S. Les hémoglobinopathies en Tunisie. Revue actualisée des données épidémiologiques et moléculaires. Tunis Med 2006; 84 : 687-96

10. Siala H, Tabboubi N, Hadj Fradj S et al. A lower-cost protocol for sickle cell disease neonatal screening in Tunisia. Ann Saudi Med 2012; 32 (1): 49-52