Neonatal screening for sickle cell disease in Central Africa: a study of 1825 newborns with a new enzyme-linked immunosorbent assay test

Léon Mutesa, François Boemer, Louis Ngendahayo, Stephen Rulisa, Emmanuel K Rusingiza, Neniling Cwinya-Ay, Déographias Mazina, Pierre C Kariyo, Vincent Bours and Roland Schoos

INTRODUCTION

Sickle cell anaemia (SS) is the most common and severe haemoglobinopathy in African populations. It is an autosomal recessive genetic disorder characterized by a mutation in the gene coding for the β-globin and production of abnormal haemoglobin (Hb S) in red blood cells, which become hard and sticky and are shaped like sickles. The clinical features of SS include increased infection risk, variable degrees of haemolysis leading to pain and anaemia, and intermittent episodes of vaso-occlusion that cause tissue ischaemia, acute and chronic organs dysfunction. Heterozygous individuals have a sickle cell trait (Hb SA), a generally benign, asymptomatic carrier state.

In Central Africa, the prevalence of this pathology appears high (1.65%) and more than half of the affected children die before the age of 5 years. The survivors develop degenerative complications, increased morbidity and reduced life expectancy. However, precocious and specialized medical care reduces morbidity and mortality of these patients. Therefore, we initiated a pilot study to evaluate the feasibility of a systematic neonatal screening programme for sickle cell disease using a new ELISA test in the Great Lakes region of Central Africa.

MATERIAL AND METHODS

Population and samples

Between July 2004 and July 2006, a neonatal screening pilot study for sickle cell disease was conducted in three countries of the Great Lakes region in Central Africa. The screening programme was initially introduced in maternity units from Kigali and Butare in Rwanda, and later extended to other major maternity units of Bujumbura in Burundi and Goma in the East of the Democratic Republic of Congo. The study protocol was approved by the Institution Review Board of Medical Research.

A standard data form included the identification and geographic origin of every newborn. The parents were informed about the procedures and gave their informed consent. Dried blood samples were collected by heel prick onto filter paper (S&S, Schleicher & Schuell BioScience, Inc., 10 Optical Avenue.Keene NH, USA) during the first week of life. Feedback information was given to the paediatricians and haematologists in charge of the affected newborns, and the appropriate medical care was started for homozygous patients. The parents were also informed about the risk of recurrence in subsequent pregnancies. The parents of the heterozygous children were also contacted, when possible, for further information. At the time of the study, it was not possible to organize a systematic genetic screening in these families, but such an approach should be feasible in Rwanda in the near future.

Immunological analysis by ELISA test

Primary screening was performed using the immunological approach by ELISA with a mouse immunoglobulin G (IgG) monoclonal antibody detecting Hb S and Hb C, as previously described. The defined multiple of the median (MoM) value was used to analyse absorbance results (MoM ≥ 1.5).
Restriction fragment length polymorphism

All positive samples were analysed by restriction fragment length polymorphism (RFLP). Genomic DNA was isolated from dried blood samples with the Instagene Dry Blood Kit (BioRad, California, USA). The primers were designed as follows: 5'-TAGGGTGGCCAAATCTACTC-3' for forward, and 5'-TTAGGGTGGCCGATAACAGC-3' for reverse. The polymerase chain reaction (PCR) conditions, as previously reported, were used to amplify a 444-bp fragment of the β-globin gene covering the two mutation sites, Hb S and Hb C, at codons 6 and 26, respectively. The 444-bp fragment of the β-globin gene was digested with restriction endonuclease enzymes, Bsu36I and BsrRI (New England Biolabs, Massachusetts, USA), for discrimination of the two haemoglobin mutations. The restricted samples were then checked on a 3% agarose gel electrophoresis (BioRad, California, USA).

Statistical analysis

The stata software version 7.1 was used to create graphics and to fix the MoM cut-off level. The data were then analysed by the Epi Info software 3.3.2 version. The χ² of Pearson was used to compare the genotype incidences between countries (threshold of error = 0.05).

RESULTS

From July 2004 to July 2006, 1825 (914 females and 911 males) newborn dried blood samples were collected from major maternity units in Rwanda, Burundi, and the Democratic Republic of Congo. Among these samples, 97 (5.32%) newborns screened positive for haemoglobin S or C using an ELISA test with MoM values between 1.5 and 3.4 (Table 1).

Table 1 Sample characteristics and incidence of ELISA-positive tests (MoM>1.5)

<table>
<thead>
<tr>
<th>Sex</th>
<th>n</th>
<th>%</th>
<th>n</th>
<th>%</th>
<th>P value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
<td>914</td>
<td>50.1</td>
<td>38</td>
<td>4.16</td>
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</tr>
<tr>
<td>Male</td>
<td>911</td>
<td>49.9</td>
<td>59</td>
<td>6.48</td>
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<tr>
<td>Total</td>
<td>1825</td>
<td>100</td>
<td>97</td>
<td>5.32</td>
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</table>

<table>
<thead>
<tr>
<th>Ethnic origin</th>
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<th>%</th>
<th>n</th>
<th>%</th>
<th>P value*</th>
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<tbody>
<tr>
<td>Burundi</td>
<td>637</td>
<td>34.9</td>
<td>38</td>
<td>5.97</td>
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<tr>
<td>DRC</td>
<td>84</td>
<td>4.6</td>
<td>11</td>
<td>13.10</td>
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</tr>
<tr>
<td>Rwanda</td>
<td>1104</td>
<td>60.5</td>
<td>48</td>
<td>4.35</td>
<td>0.002</td>
</tr>
<tr>
<td>Total</td>
<td>1825</td>
<td>100</td>
<td>97</td>
<td>5.32</td>
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The P value refers to a comparison of the incidence between the three countries.

<table>
<thead>
<tr>
<th>AS-AC SS AA</th>
<th>n</th>
<th>%</th>
<th>n</th>
<th>%</th>
<th>P value*</th>
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<tr>
<td>AS-AC</td>
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<tr>
<td>AA</td>
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*DRC = Democratic Republic of Congo

Figure 1 Results of ELISA test with MoM values ≥1.5. The distribution of MoM values for AS-AC and SS samples, as confirmed by the molecular test, are shown, as well as the numbers of false-positive samples when a cut-off of 1.5 is chosen.

All ELISA-positive samples (MoM above 1.5) were checked by a PCR-restriction molecular test, and the final result was considered as positive if confirmed by the molecular analysis. Among the 48 positive samples from Rwanda, 28 were Hb S heterozygous carriers (Hb SA, 2.54%); two were Hb C carriers (Hb CA, 0.18%) and one had SS anaemia (Hb SS, 0.09%) (Table 2). From Burundi, out of the 38 ELISA-positive patients, 28 were Hb S heterozygous carriers (Hb SA, 4.40%), while one had sickle cell disease (Hb SS, 0.16%).

We received only 84 samples from the Democratic Republic of Congo, a small number that does not allow any appropriate allele frequency evaluation. However, the Democratic Republic of Congo Hb S incidence was higher (8.33% of positive ELISA tests and 7.14% of confirmed positive samples), and the difference was statistically significant between countries (P = 0.002 based on the ELISA tests, Table 1, and P = 0.036 on the basis of molecular confirmations, Table 2). Indeed, among the 84 samples from the Democratic Republic of Congo, 11 were ELISA positive and the PCR test revealed that four of these were Hb S heterozygotes (Hb SA, 4.76%), while two were Hb C heterozygotes (Hb CA, 2.38%).

For the whole cohort, our results indicated that the overall incidence of SS in the area was 0.11%. The prevalence of sickle cell trait (Hb SA) was 3.23%, whereas the haemoglobin C carriers were less frequent (0.22%).

As described in a previous study on a large cohort of Caucasians, samples containing Hb S or C had MoM values in excess of 2.0, and the reference cut-off was fixed at that level for screening in that population. In this African population, we tested different hypotheses at various levels of MoM ranging between 1.5 and 3.4 (Figure 1). Ninety-seven patients had MoM values over a cut-off of 1.5: of these 97 patients, 66 (68%) were confirmed by PCR to carry...
Hb S or C mutations and we expect to have detected over 90% of positive patients, heterozygotes and homozygotes (Table 2, Figures 1 and 2). At this cut-off, 31/97 (32%) false-positive results were generated. However, when the cut-off was fixed at 1.8, the rate of false-positives decreased markedly (9.4%), but we lost 13.64% (9 heterozygous patients) of positive samples. Beyond an MoM cut-off of 2, the number of false-positive results was slightly lower (4 instead of 6 at a cut-off of 1.8), but the detection of Hb S- and C-positive patients decreased significantly as we identified only 43 out of 66 positive samples (a loss of 34.8% of positive samples) (Figures 2 and 3).

DISCUSSION

The main objective of neonatal screening is the early detection of newborns with sickle cell disease in order to start appropriate medical care, including penicillin prophylaxis around 2–3 months of age,6 preferably before the onset of symptoms. In the African population, although the symptoms of sickle cell disease occur during the first year of life in 80% of the children, diagnosis is rarely achieved before the age of 2–3 years. As a consequence, it is likely that many children die undiagnosed in early infancy from meningitis, pneumonia or acute anaemia, as did one of...
the two homozygous patients detected during the present study. In addition, neonatal screening can alert the parents of an affected baby about the risk for the next pregnancy and the need for antenatal diagnosis.

However, in Central Africa, the prevalence of inherited sickle cell disorders has been underestimated. Neonatal screening is necessary to obtain correct numbers. In this pilot study, a new immunological technique, using an ELISA test recognizing the Hb S and Hb C, was performed as the primary screening method. As shown in Figure 2, an acceptable detection level of Hb S and Hb C was obtained at the MoM cut-off of 1.8 in this population. These values are slightly different from our reported data in a mostly Caucasian population, which showed that an MoM cut-off reduced at 2.0 markedly increased the number of false-positives (12.9–44.2%) and did not allow any additional detection of Hb S or C. Moreover, in the present study, two children were found to be homozygous for Hb S, with MoM values of 2.3 and 2.4, respectively, which were different from those reported in a Caucasian population. These differences could be explained by population disparities. Indeed, it has been shown that there are differences between normal values obtained from subjects with European or African ancestry. Therefore, different reference ranges need to be considered for these two groups, and they may have implications for how different ethnic groups could respond to this genetic testing.

The ELISA test was validated as a simple and rapid technique for neonatal screening with limited costs compared with commonly used high-performance liquid chromatography (HPLC) and isoelectric focusing (IEF) techniques, and it would be applicable by laboratories with limited facilities. The incidence of sickle cell disease in the studied population is high, and thus a neonatal screening programme appears fully justifiable, particularly as prophylactic measures lead to a reduction of mortality and morbidity in childhood. It is important to mention that a correct sampling is most important as insufficient amounts of blood on the filter paper can generate false-negative results.

Recently, in a series of 987 Rwandan neonates screened for red blood cell disorders using IEF method, the incidence of sickle cell trait was 2.7%, but no other Hb variant was detected. In West Africa, 4.7% children were found to be heterozygous for Hb S, whereas 0.3% were heterozygous for Hb C. In our population, sickle cell trait was found in 3.28% and Hb C trait in 0.22% of screened newborns, and these incidences were thus close to the previously reported ones.

Our data provide information about the incidence of sickle cell disease in the region of Great Lakes in Central Africa, and they suggest that a neonatal screening programme based on an ELISA test is suitable and should be extended at the national level in all countries of Central Africa, especially in Rwanda and Burundi, where the sickle cell disease has been previously considered to be rare.

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REFERENCES