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Original Article

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Molecular Diagnosis of Sickle Cell Anaemia using Amplification Refractory Mutation System Polymerase Chain Reaction (ARMS-PCR) In Sudan

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ABSTRACT

Background: In Africa, the Sickle Cell Disease, SCD, is the most common inherited hematological disease with a high mortality rate at age one to five years. This disease was discovered early in Sudan. The peak occurrence of SCD is among the population from Western Sudan. The objective of this study is to demonstrate the accuracy and precision of Amplification Refractory Mutation System - Polymerase Chain Reaction,(ARMS- PCR) technique for the diagnosis of SCD in Sudanese patients .

Methods and results: This study was conducted in Khartoum State between 2005 to 2008.in hundred and fifty patients with homozygous SCD from 6 months up to 40 years of age. Questionnaires were used to collect demographic and clinical data. About 3 ml of venous anti coagulated blood were collected for DNA extraction. Each extracted DNA samples was subjected to Allele-specific-PCR. Allele-specific-PCR primers were designed to amplify the HbS allele and the normal HbA allele when paired with a common reverse primer.Separate PCR reactions were set up for the HbA and HbS primer pairs, and each reaction contained control primers. Polymerase chain reaction was carried out for DNA amplification. Visible bands were seen at 207 bp regions for the AA, AS, SS genotypes yielding 207bp. Results from agarosegel electrophoresis also showed the same ARMS PCR results. Moreover,ARMS-PCRis potential method to permit rapid analysis of any known mutation in genomic DNA, it allows genotyping solely by inspection of reaction mixtures after agarose gel electrophoresis

The study thus demonstrates the accuracy and precision of Amplification Refractory Mutation System - polymerase chain reaction(ARMS- PCR) techniques in diagnosis of sickle cell anaemia.

Keywords: *Amplification Refractory Mutation System, polymerase chain reaction, sickle cell anaemia, electrophoresis.*

Introduction

Sickle cell anaemia is an inherited blood disorder due to substitution of thyamine by adenine in glutamic acid which in turn results in the substitution of valine to glutamic acid in position number six in Beta globin chain. Hemoglobin S (HbS), is the defective Hb produced as a result of this defect, is a tetramer (alpha2/beta2) that is poorly soluble and polymerizes when deoxygenated. (Bunn, 1997). In Sudan, sickle cell anaemia is the one of the major types of anaemia especially in western Sudan where the sickle cell gene is frequent (Abdelrahim, etal 2006). It is believed that the sickle cell gene was brought to Sudan through immigrants from West African tribes, especially from Hosa, Folani and Bargo (Bereir,2007).

Despite the improved sensitivity of DNA-based testing methods, quantities of obtainable foetal DNA were often insufficient. Sensitivity was greatly enhanced by the development of polymerase chain reaction (PCR), a method of in-vitro DNA amplification that employs repeated cycles of denaturation, annealing of oligonucleotide primers to the target DNA, and enzymatic primer extension to amplify DNA flanked by the primers (Embury, 2008).

The rapid, non-radioactive approach to the diagnosis of sickle cell anaemia allows the direct detection of the normal or the sickle cell β-globin allele in genomic DNA without the additional steps of probe hybridization, ligation or restriction enzyme cleavage (Dhillon,etal, 2001). Several laboratories in the developed countries have been using this technique (Bhardwaj ,etal,2009: Roberts and Kennedy,2006)

The objective of this study this study is to demonstrate the accuracy and precision of Amplification Refractory Mutation System - polymerase chain reaction. (ARMS-PCR) techniques for the diagnosis of in Sudanese patients with SCD.

Materials and methods

METHOD AND MATERIALS

Study Design: It is a community–based descriptive cross-sectional study.

Study sites: The study was conducted in Khartoum State

Study Population: The study population included 150 (HbSS) SCD Sudanese patients; fifty nine males and forty one females at different ages. The patients that attended sickle cell clinic at Khartoum Children's Emergency Hospital and other patients referred to the clinical laboratory at the Department of Biochemistry, Faculty of Medicine, University of Khartoum and wards from different teaching hospitals in Khartoum state were enrolled in this study.

Sampling and Sample Size: Convenient samples of one hundred and fifty patients were asked to participate in the study. Quota sampling was used to select study participants, and data was collected during the period from December. 2005 to November, 2006. Samples of 3 ml venous blood were collected in EDTA (Ethylene Diamine Tetra Acetic Acid)vacutaners from all individuals included in the study and then were centrifuged at 3000 rpm for 5 minutes. The pellet was washed with normal saline (0.9 %NaCl), centrifuged again at 3000 rpm and the supernatant was discarded. The interface layer, the buffy coat, was then transferred to a clean tube and kept at –80°C for the extraction of the DNA and PCR performance for the assessment by different molecular techniques

Ethical Considerations: The collection of 2ml of blood is considered as a daily acquired risk; so the study did not pose a serious health risk to study patients. Verbal information and a written consent form were obtained from study patients prior to the commencement of the study.

DNA EXTRACTION

DNA is isolated using Ponez et al(1982)

DNA was extracted from the buffy coat prepared from the blood collected from all subjects using Ultraclean Bloodspin DNA Extraction Kit (Mo Bio Laboratories Inc., USA) following manufacturer's instructions.

DNA Quantification using Picogreen method:

DNA samples were diluted 1/100 and placed in 96 well plate, the samples were left at 4ºC for at least 12 hrs before quantification of the DNA was carried out.

PCR AMPLIFICATION

Each DNA sample was subjected to allele-specific PCR using two primer sets HbA and HbS separately and HbA and HbC primer sets owing to varying PCR conditions. Hβ14A (5'-CACCTGACTCCTGA-3') and BGP2 (5'-AATAGACCAATAGGCAGAG-3') were used as the primer set for the amplification of the normal β-globin gene (HbA primer set). Similarly, Hβ14S (5'-CACCTGACTCCTGT-3') and BGP2 (5'-AATAGACCAATAGGCAGAG-3') were used as the primer set for the amplification of the sickle cell gene (HbS primer set). Reactions were carried out for 35 cycles at optimal PCR conditions. Hemoglobin C primer set, beta 5'-a (5'-GTACGGCTGTCATCACTTAGACCTCA-3') and beta A–b (5'-TAACGGCAGACTTCTCCTC-3'), corresponding to the wild-type allele, and in the other, the primer used were beta 5'- a and beta C-b (5'-TAACGGCAGACTTCTCCTT-3'), corresponding to the hemoglobin C

allele. All the mix was run on the GeneAmp PCR System 9700 machine (Applied Biosystems, UK). Agarose gel electrophoresis was run on the PCR products with DNA bands visualized and photographed by filtered ultraviolet (UV) illumination on the Syngene gel documentation system (Syngene, UK).

Electrophoresis was carried out in 0.7% agarose slab gels in tris-borate buffer (89 mMTris, 89 mM boric acid and EDTA pH 8.0) containing about 0.5 μg/ml of ethidium bromide to enhance visualization of DNA bands under UV light. The respective PCR products were mixed with 10 μl of loading buffer, loaded and run on the electrophoresis machine at 70 V for 1 hour. The 100 bp DNA ladder was mixed with 10 μl of loading buffer and also loaded on a slot on the agarose gel. Sigma Gel Loading Solution Type I was used as the loading buffer. It contains 0.25 % (w/v) Bromophenol blue, 0.25 % (w/v) Xylene cyanole FF and 40 % (w/v) sucrose in water.

The size of DNA product (203 bp for Hb A and S; 216bp for Hb C) were read against the 100 bp DNA ladder and genotype results were obtained..

21 molecular marker,agarose gel electrophoresis shows the results of ARMS PCR(2) Lanes 1,3,5,7,9,13 SS, lane 11,12 AS ,lane 15 molecular marker.

Agarosegel electrophoresis shows the results of ARMS PCR(3) Lane 1, 3, 5,7,9,17,19 SS,13, 14,15,16 AS,

Data Analysis: Data were entered in the computer and Statistical software packages (Excel 5.0, Microsoft, Redmond, WA; and Statistical Package for the Social Sciences 20.0, SPSS, Inc., Chicago, IL) were used for data management and analysis, chi square test was used to compare percentages. P-value <0.05 was considered significant

Results

Identification of HbAA, HbAS and HbSS using ARMS-PCR:

The results of ARMS PCR revealed that ,there were two lanes M for mutation bands and N for normal bands. If were two band in M&N sample would be heterozygous but if there was one band in M that would indicate homozygous for Hb S and else if there was one in N lane would show it were normal .

Identification of HbAA, HbAS and HbSS using ARMS-PCRFig (1)shows amplification of HbA and HbS genotypes using ARMSPCR, typing of the AA, AS, SS genotypes yielded 207bp .Agarose gel electrophoresis shows the results of ARMS PCR(1)Lanes 1,2 normal AA, lanes 3,5,7,9,11,13,15,17,19 SS, 8lane

12 AA and the same reading for other results(4)A.B.C.

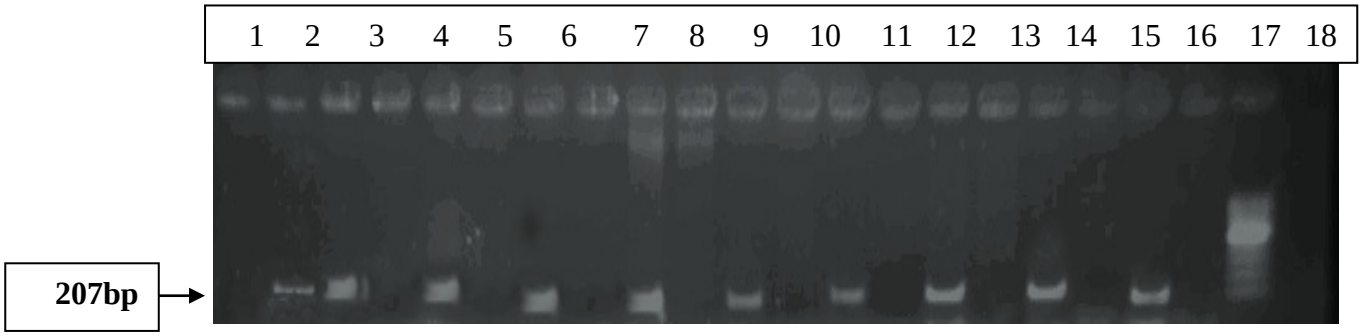
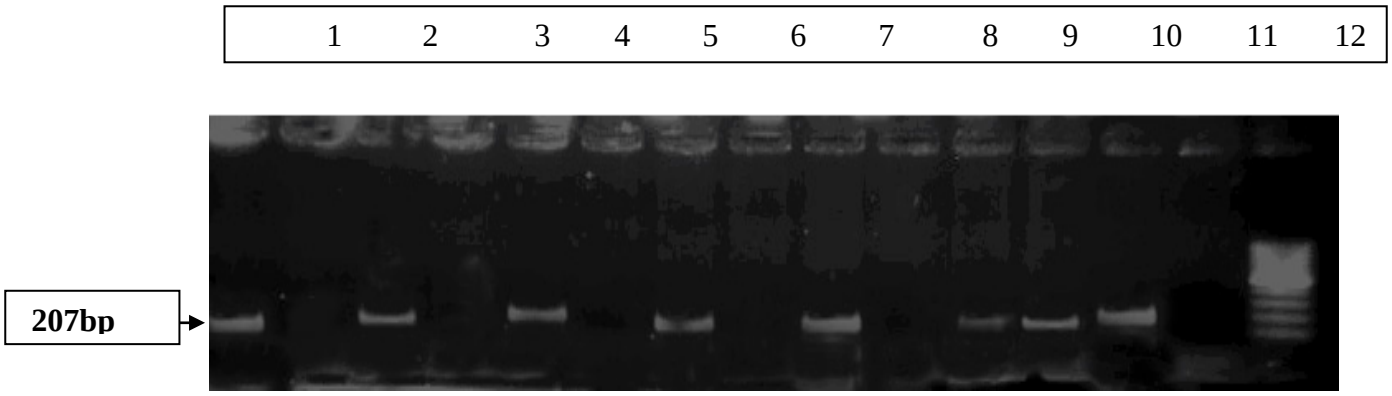


Fig No (1); Amplification of HbA and HbS genotypes using ARMSPCR, typing of the AA, AS, SS genotypes yielded 207bp.Agarose gel electrophoresis 2% shows the results of ARMS PCR(1)Lanes 1,2 normal AA, lanes 3,5,7,9,11,13,15,17,19 SS, 8lane 21 molecular marker



Fig(2)Agarose gel electrophoresis2% shows the results of ARMS PCR Lanes 1,3,5,7,9,13 SS, lane 11,12 AS,lane 15 molecular marker.

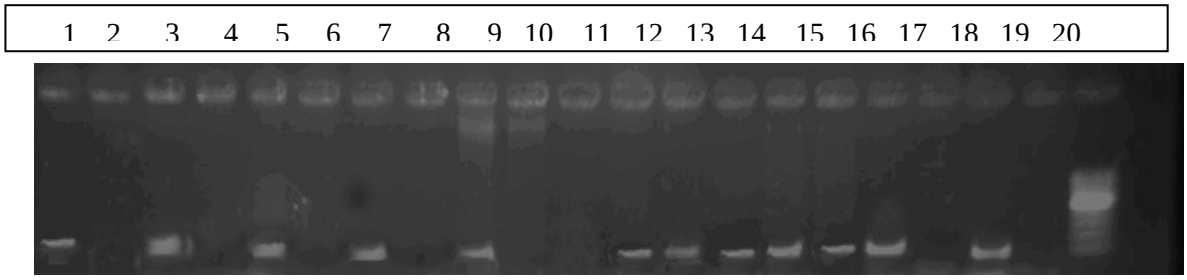
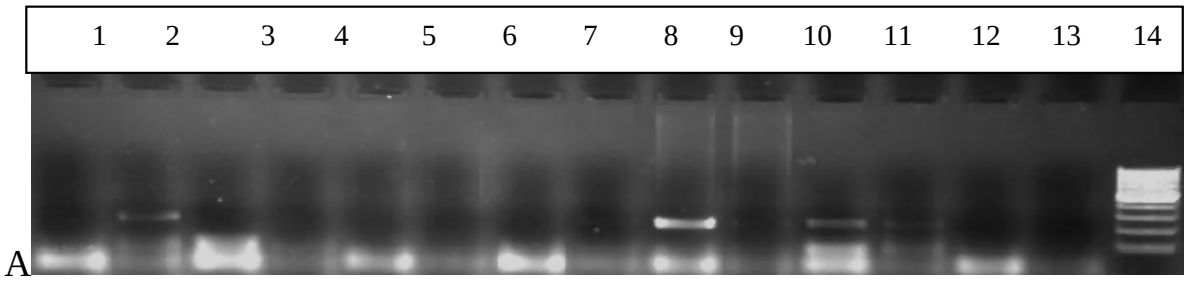
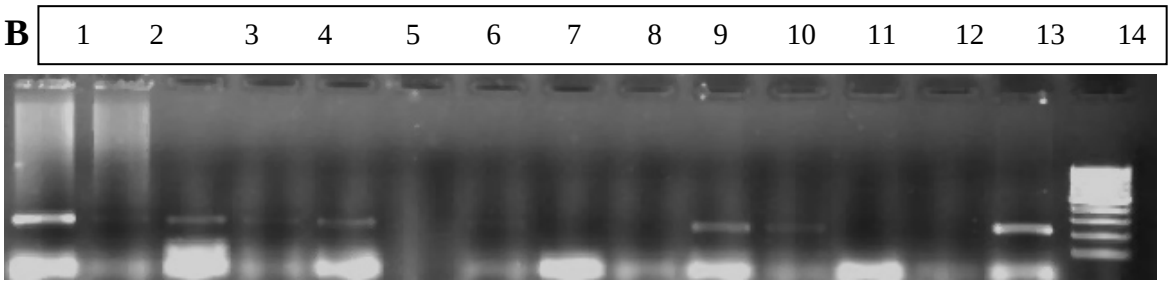


Fig (3) Agarose gel electrophoresis 2% shows the results of ARMS PCR.Lane 1,3, 5,7,9,17,19 SS,13, 14,15,16 AS, 12 AA



A

B



C

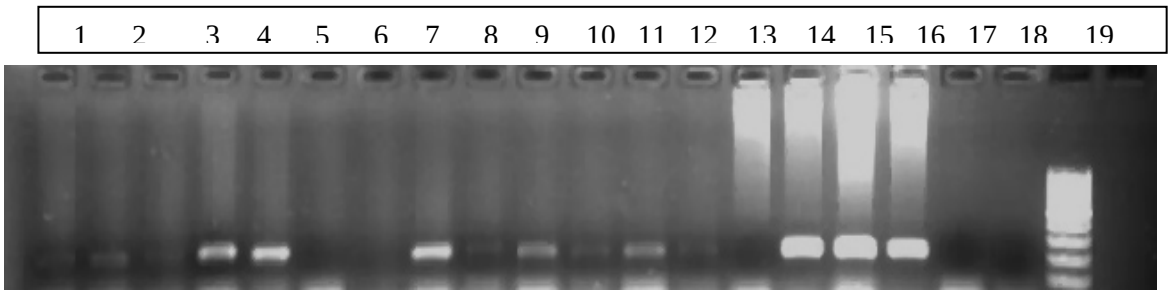


Fig (4) **A**garose gel electrophoresis 2% shows the results of ARMS PCR **A**, 2,9,11 SS, **B** .lanes 1, 2 AS, 3, 4 AS, lane 5 SS, lane 10 SS, lane 11 AA, Lane 14SS lane 15 molecular marker .**C** lane 2 SS, lane 4 SS, lane 5 AA, lanes 8 SS, lane 10 SS, lane 12 SS, lane 14 AA, lanes 16, 17 AS. Lane 18,19 negative controls, lane 20 molecular marker.

Discussion

SCD is the third leading cause of hospitalization, after malaria and diarrhea,and the third leading cause of death in children, ≤ 12 years of age,in Africa (Athale et al., 1994; Diallo et al., 2002;and Rahimy et al., 2003). Early detection and comprehensive care can reduce morbidity and mortality in patients with SCD(Almeida et al., 2001). Molecular diagnostic testing has shifted dramatically in the past decade from the research arena to the clinical arena. The success of the Human Genome Project, forensic applications, genetic identification of various disease-causing microbes, expanded public health epidemiology and surveillance activities have all contributed to the incorporation of molecular diagnostics into the routine practices of medical and public health laboratories at a rapid speed. Personnel in clinical laboratories around the world are being asked to provide rapid identification of emerging and re-emerging disease-causing agents associated with “common” disorder. There are many challenges to implement new diagnostic tests designed to provide more sensitive and specific tests for detecting and monitoring the disease.

All are recommended for the genotyping and discrimination of sickle cell disease mutation. Restriction Fragments Length Polymorphism is a powerful technique for the characterization of DNA at the molecular level,RFLP analysis is particularly useful for diagnosis of disease because it assays directly for a genotype (DNA sequence)and does not depend on expression of a gene or even phenotypic expression of the disease itself. Thus, a disease can be identified in a variety of techniques based on the amplification of DNA by the polymerase chain reaction (PCR)have been developed to identify the globin gene mutations. We applied different techniques for the analysis of the Beta S mutation in different Sudanese sickle cell patients. The Amplification Refractory Mutation System (ARMS).ARMS-PCR is potential method to permit rapid analysis of any known mutation in genomic DNA. It allows genotyping solely by inspection of reaction mixtures after agarose gel electrophoresis. The system is simple, reliable and non-isotopic. It will clearly distinguish heterozygotes at a locus from homozygotes for either allele. The system requires neither restriction enzyme digestion, allele-specific oligonucleotides, as conventionally applied, northe sequence analysis of PCR products.

Each of these methods has its own advantages and disadvantages and individual before symptoms of the disease are apparent. Additionally, a fetus can be monitored for diseases before birth.

Conclusion:

ARMS PCR molecular diagnostic technique is considered rapid and reliable in diagnosing and determination of mutation and inherited sickle cell gene.

RECOMMENDATION

As amethod of choice we can do prenatalandpostnatal diagnosisto be standard method in our research and diagnostic centers coupled with counseling units inside the laboratories.

Implementation The implementation, of neonatal screening combined with comprehensive care of patients with SCD, by health institutions programs is worthy of consideration.

REFERENCES

AbdelrahimO.Mohammed, Bekhieta Attalla, FathyaM.K.Bashir, Fatima E.Ahmed, Ahmed M.Elhassan,GafarIbnauf,. Relation of Sickle Cell Gene to the Ethnic and Geographic Groups Populating the Sudan.Public Health Genomics 2006 9:113-120.

Almeida AM. Henthorn JS, Davies SC(2001). Neonatal screening for hemoglobinopathies :the results of a 10-year programme in English Health Region .British Journal of Haematology, 112:32-5.

Athale UH, Chintu C(1994). Clinical analysis of mortality in hospitalized Zambian children with sickle cell anemiaanaemia .African Medical journal-71:388 ;91

Bereir RE, Hassan HY, Salih NA, Underhill PA, Cavalli-Sforza LL,. Co-introgression of Y-chromosome haplogroups and the sickle cell gene across Africa's Sahel. Eur J Hum Genet. 2007;15:1183–1185.

Bhardwaj U, Zhang YH, Lory F, McCabe LL, McCabe ERB. Molecular genetic confirmatory testing from newborn screening samples for the common African-American, Asian Indian, Southeast Asian and Chinese Thalassemia mutations. American Journal of Hematology, 2005, 78(4):249-255.

Bunn HF. Pathogenesis and treatment of sickle cell disease. New Engl J Med 1997.

Dhillon Ye S, Ke S, Collins X, Day AR.. IN. An efficient procedure for genotyping single nucleotide polymorphisms. Nucleic Acids Res, 2001, 29 (17): 88-8.

Diallo D. Tchernia G. Sickle Cell Disease in Africa. Current Opin.Hematolo (2002), 9:1116

Embury SH. Molecular diagnosis of inherited hemoglobin disorders. Am J Haematol, 2008, 98:280-283.

Ernest Beutler, MarashallA.Lichtman, Barry S.Coller , ThomoasJ,Kipps and Uriseligsohn“editor” Williams .Hematology .6th edOKDOKeY; P 469.

Ponez M, Solowiejczky D, Harpel B.(1982). Construction of human gene librariesfrom small amounts of peripheral blood .Hemoglobin;6:27–36.

Almeida AM. Henthorn JS, Davies SC(2001). Neonatal screening for hemoglobinopathies :the results of a 10-year programme in English Health Region .British Journal of Haematology, 112:32-5.

Athale UH, Chintu C(1994). Clinical analysis of mortality in hospitalized Zambian children with sickle cell anemia .African Medical journal-71:388 ;91

Diallo D. Tchernia G. Sickle Cell Disease in Africa. Current Opin.Hematolo (2002), 9:1116

Ernest Beutler, MarashallA.Lichtman, Barry S.Coller , ThomoasJ,Kipps and Uriseligsohn“editor” Williams .Hematology .6th edOKDOKeY; P 469.

AbdelrahimO.Mohammed, Bekhieta Attalla, FathyaM.K.Bashir, Fatima E.Ahmed, Ahmed M.Elhassan,GafarIbnauf,. Relation of Sickle Cell Gene to the Ethnic and Geographic Groups Populating the Sudan.Public Health Genomics 2006 9:113-120.

Bunn HF. Pathogenesis and treatment of sickle cell disease. New Engl J Med 1997.

Bereir RE, Hassan HY, Salih NA, Underhill PA, Cavalli-Sforza LL,. Co-introgression of Y-chromosome haplogroups and the sickle cell gene across Africa's Sahel. Eur J Hum Genet. 2007;15:1183–1185.

Embury SH. Molecular diagnosis of inherited hemoglobin disorders. Am J Haematol, 2008, 98:280-283.

Dhillon Ye S, Ke S, Collins X, Day AR.. IN. An efficient procedure for genotyping single nucleotide polymorphisms. Nucleic Acids Res, 2001, 29 (17): 88-8.

Bhardwaj U, Zhang YH, Lory F, McCabe LL, McCabe ERB. Molecular genetic confirmatory testing from newborn screening samples for the common African-American, Asian Indian, Southeast Asian and Chinese Thalassemia mutations. American Journal of Hematology, 2005, 78(4):249-255.

Roberts RL, Kennedy MA. Rapid detection of common cytochrome p450 2d6 alleles in Caucasians. ClinicaChimicaActa, 2006, 366(1–2): 348-51.

Ponez M, Solowiejczky D, Harpel B.(1982). Construction of human gene librariesfrom small amounts of peripheral blood .Hemoglobin;6:27–36.

Rahimy MC, Gangbo A, Ahouignan G, Adjou R, Deguenon C, Goussanou S, Alihonou E. (2003). Effect of comprehensive clinical care program on disease course in severely ill children with sickle cell anemiaanaemia in a sub-Saharan African setting .Blood;102:834-8.

Roberts RL, Kennedy MA. Rapid detection of common cytochrome p450 2d6 alleles in Caucasians. ClinicaChimicaActa, 2006, 366(1–2): 348-51.