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Molecular Genetic Confirmatory Testing for the Sickle Cell Anaemia using Restriction Fragment Length Polymorphism (RFLP) in Sudan

Authors

Nadia, M . M[1]., Shamseldein. H. Ahmed[2], Galal, M. Yousif[3]

[1]College of Laboratories Studies, Karary University- Sudan

[2]College of Veterinary Medicine-Sudan University of Science and Technology - Sudan

[3]College of Laboratories Studies -The National Ribat University- Sudan

Corresponding Author

Shamseldein. H. Ahmed

College of Veterinary Medicine-Sudan University of Science and Technology - Sudan

Email: *shamshahmed@yahoo.com, shamsaldeinhassaballa@sustech.edu*

ABSTRACT

Background: *Molecular diagnosis of the disease, genetic and family studies of patients becomes an important tool for management of sickle cell disease patients.. In this study we compare the efficiency of agarose gel electrophoresis techniques with recently developed molecular biology methods. (RFLP) for the diagnosis of in Sudanese patients with SCD.*

Methods and Results: *This study was conducted in Khartoum state between 2007 to 2008.in hundred and fifty patients with homozygous SCD of age between 6 month –40 years. Questionnaires were used to collect demographic and clinical data. About 3 ml of venous ant coagulated blood were collected for DNA extraction. Each extracted DNA samples was subjected to PCR reaction. .Polymerase chain reaction was carried out for DNA amplification. , The results of RFLP In this method after the amplification of DNA with primers1 (mutant), primer 2 (normal primer), the 281bp fragment was achieved, after that we digested this fragment with DdeI restriction enzyme in two fragment (200bp&81 bp) so +/+ shows that our sample is normal and we had 200 & 81 bp both of them but we couldn't see 80 bp so we just had 200 bp! For heterozygous carrier we had 3 bands 200&281&81 that we saw just 2 bands (200 and 281), that was -/+! for homozygous disease we had just one band 281 bp that shows our restriction sits disturb and our enzyme couldn't digest it*

The study thus demonstrates the accuracy and precision of Restriction Fragment Length Polymorphism (RFLP)techniques in molecular genetic confirmatory testing for the sickle cell anaemia

Keywords: *Restriction Fragment Length Polymorphism (RFLP), sickle cell anaemia, electrophoresis.*

INTRODUCTION

SCD is believed to be the most frequent inherited blood disorder on the globe affecting an estimated 100 million people world-wide and, in particular, the black races and persons of Mediterranean origin (Ohaeri, and Shokundi, (2001). In Africa, SCD is the most prevalent genetic disease with high mortality rate at age one to five years (Cook and, Zumla,(2003): Weatherall , (2006). In the United States of America (USA), sickle cell anemia has been found to be the most frequent autosomal recessive gene disorder affecting approximately 1:375 persons of African ancestry (Doris, and Wetherland, (2000). 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 has brought to Sudan through immigrants from West African tribes, especially from Hosa, Folani and Bargo (Bereir,2007).

Cellulose acetate electrophoresis is very useful for quick screening of a small number of samples but the protein bands are relatively wide and abnormal hemoglobins overlap. Also, quantitative densitometry of abnormal hemoglobins is inaccurate at low concentrations (i.e HbA2, HbF). Since a great percentage of hemoglobin in newborns is HbF, cellulose acetate electrophoresis is not a reliable technique for the determination of genotype in neonates [Bender and, Hobbs,2003].

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 oligonucleo-tide 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 anemia 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 objectives of the study also is to provide a database for the establishment of the most appropriate genetic counseling services for SCD patients .

MATERIALS AND METHODS

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

Study sites: study conducted at Khartoum state

Study Population: The study population included 150 (HbSS) SCD Sudanese patients, fifty nine male and forty one female 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 s were asked to participate in the study. Quota sampling was used to select study participants, and data was collected during the period from December2005 to November2006. 3 ml venous blood samples 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 were discarded. The interface layer the buffy coat was then transferred to a clean tube and kept at –80C for the extraction of the DNA and PCR performance for the assessment by different molecular techniques

Ethical Considerations: 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 informed and written consent form was 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 buffy coat prepared from 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

Restriction Fragment Length Polymorphism:

The target DNA sequence was amplified by the polymerase chain reaction (PCR) with the primers beta 1 (5'-ACACAACTGTGTTCACTAGC-3') and beta 2(5.CAACTTCA TCCA CG T TCACC-3') that primed amplification of 110-base-pair (bp) segment of beta globin gene. The amplified DNA was digested with a restriction endonuclease Dde I

Agarose Gel Electrophoresis:

Confirmatory gel electrophoresis was carried out for the detection of the sickle cell, Hemoglobin S and normal hemoglobin genotypes .2 %agarose gel in TBE (Tris – boric acid – EDTA pH 8.3) with ethidium bromide (SIGMA E.1510) was prepared. TBE buffer was used as a running buffer. For loading of the samples into the gel, Bromophenol blue dye (Bromophenol blue, glycerol and xylene/cyanol) was used as a loading buffer. The electrophoresis running was in 110 volts voltage and for 45 minutes. Gel documentation system (Syngene, Chemigenius Bioimaging system) was used for visualization of the electrophoretic mobility of the gel. The DNA sizes were determined using standard markers.

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 RFLP:

The results of RFLP In this method first we amplified our DNA with IVSI-I primer that we used for IVSI-I mutation and in that method we have two primers1 is mutant, primer 2 is normal primer!

And we had 281bp fragment, after that we digested this fragment with DdeI restriction enzyme in two fragment (200bp&81 bp) so +/+ shows that our sample is normal and we had 200 & 81 bp both of them but we couldn't see 81bp so we just had 200 bp !For heterozygous carrier we had 3 bands 200&281&81 but we saw just 2 bands (200 and 281), that was !+/- For homozygous disease we had just one band 281 bp that shows our restriction sits disturb and our enzyme couldn't digest it !

Fig (1) Identification of HbAA, HbAS and HbSS using RFLP

Shows amplification of HbA and HbS genotypes using RFLP-PCR, typing of the AA, AS, SS genotypes yielded 281,200 bp .Agarose gel 2% electrophoresis shows the results of RFLP lanes 2,3,4,6,7,9,10,12,13SS, lane 8 AS, lane 13,16 molecular marker ,lane 14,15 normal control.

The other two figures, (figure 2) agarose gel 2% electrophoresis shows the results of RFLP lane 1 AA, lane 2,3,4,5,6,7,9,10,11,12,SS, lane 8 AS and figure (3) lanes 1,2,3,4,5,6,7,8,9,10 SS, lane 11,12 AA, lane 13 marker.

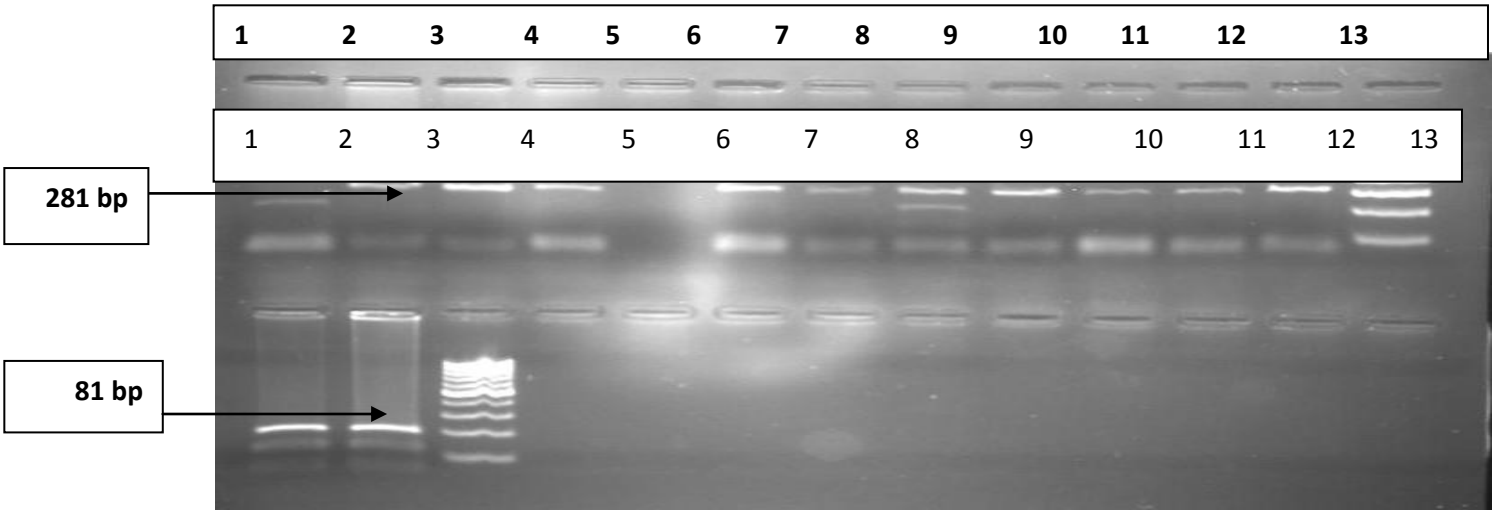

Fig (1) Identification of HbAA, HbAS and HbSS using RFLP

Agarose gel electrophoresis shows the results of RFLP:typing of the AA, AS, SS genotypes yielded 281,200 bp.lanes2,3,4,6,7,9,10,12,13SS, lane 8 AS, lane 13,16 molecular marker,lane 14,15 normal control.

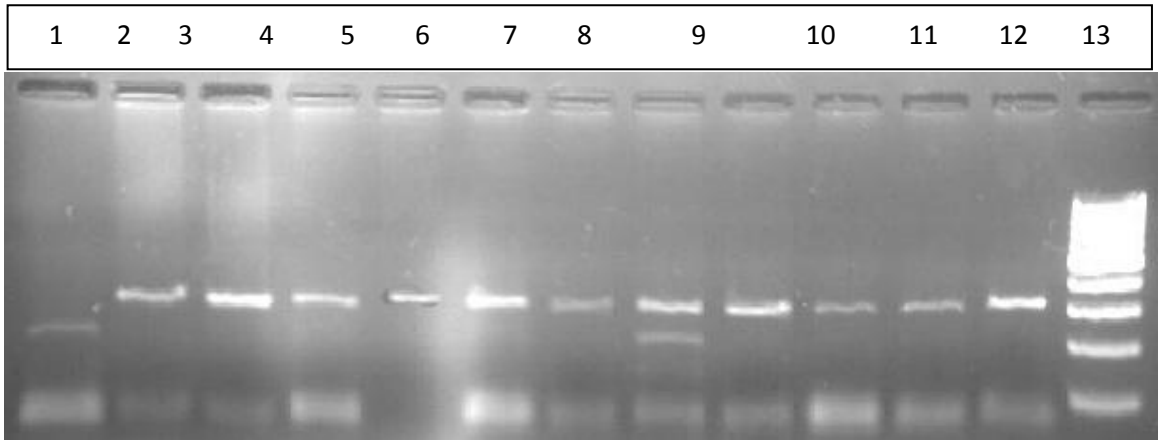

Fig (2) Agarose gel electrophoresis shows the results of RFLP lane 1 AA, lane 2,3,4,5,6,7,9,10,11,12,SS, lane 8

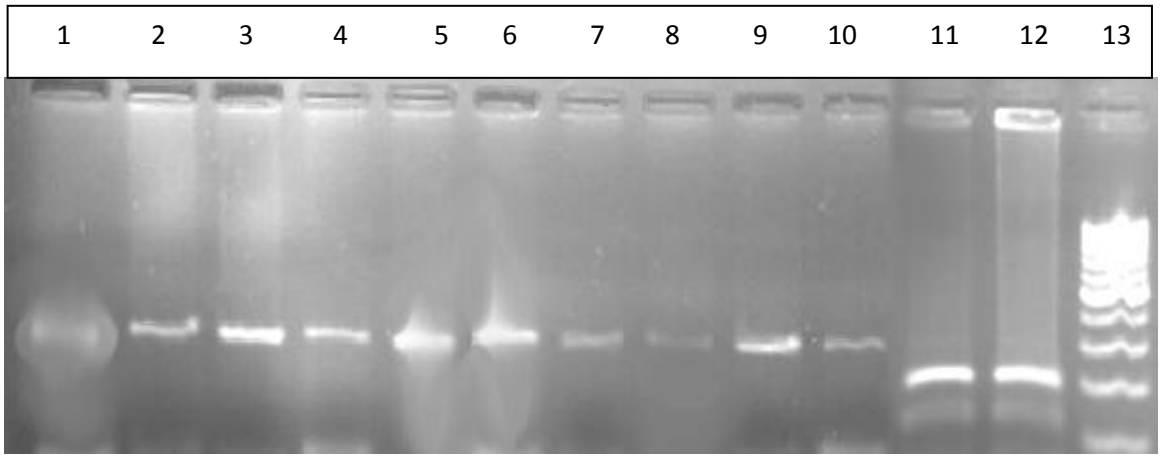

Fig (3) Agarose gel electrophoresis shows the results of RFLP lanes 1,2,3,4,5,6,7,8,9,10 SS, lane 11,12 AA, lane 13 marker.

DISCUSSION

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 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. Additionally, RFLPs provide a beginning point for the isolation of the gene responsible for a disease. If the mutation that causes an RFLP in fact lies within the gene responsible, an RFLP at this gene must occur in all cases of the disease .Therefore, it is difficult to prove that a defect in a particular gene is in fact responsible for causing a disease. However, mapping and RFLP analysis can exclude certain genes as candidates. They may also provide a point from which researchers may proceed along the DNA to identify the causal gene itself .it can be used as a molecular procedure to detect the disease, either in a prenatal screen or after birth.

There is considerable scope for more effective use of DNA-based methods for diagnosis of genetic diseases. These technologies offer rapid results with potentially high sensitivity and specificity, at relatively low cost. Recognition of these advantages has led to rapid adoption of available DNA-based tests.

Implementation of standardized practices that produce reliable, useful and comparable data will require a significant investment in research, training and infrastructure development. Effective implementation will also be assisted by enhanced communication between health sectors, researchers and practitioners in the region and scientists with expertise in molecular diagnostic technologies.

DNA-based methods have been used in diagnosis and for detection of many economically diseases . Many tests have been developed for SCD and other genetic diseases and now used routinely in a number of laboratories around the world .

DNA- based techniques will have an important role to play in efforts to develop sustainable life. The further development and use of DNA-based diagnostic techniques will also assist international efforts for the management of genetically inherited diseases .Reliable and rapid techniques are needed by national and regional diagnostic laboratories for screening of neonates and adults.

CONCLUSION

RFLPs provide a beginning point for the isolation of the gene responsible for a disease. If the mutation that causes an RFLP in fact lies within the gene responsible, an RFLP at this gene must occur in all cases of the disease..

RECOMMENDATION

The further development and use of DNA-based diagnostic techniques will also assist international efforts for the management of genetically inherited diseases .Reliable and rapid techniques are needed by national and regional diagnostic laboratories for screening of neonates and adults.

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