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Comparison between PCR– based Single Tube Genotyping of Sickle Cell Disease and Alkaline Haemoglobin Electrophoresis

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Authors' contributions

This work was carried out in collaboration between all authors. Author EWA designed the study, wrote the protocol, solve the technical problems and review the final manuscript. Author MFA collect blood samples, perform the technical work, and wrote the first draft of the manuscript. Author EAA managed the literature searches and collect and analyze patients' data. All authors read and approved the final manuscript.

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ABSTRACT

Background: Sickling test and haemoglobin solubility test are screening techniques used to detect the presence of haemoglobin S, but can't identify exact phenotype or genotype. Haemoglobin electrophoresis can determine the phenotype of sickle cell disease but it cannot be performed on infants aged less than 6 months because of the presence of hemoglobin F as the predominant hemoglobin before this age and thus it is not suitable for prenatal and neonatal screening.
This study aimed to compare PCR-based single tube genotyping of sickle cell disease with routine alkaline haemoglobin electrophoresis.

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Materials and Methods: This is a cross sectional-analytic study conducted at Khartoum state, Sudan in the period from November 2014 to March 2015.
The total number of study subjects was 70, 35 of them with sickle cell anaemia (Hb S/S), 15 sickle cell trait (Hb A/S), and 20 healthy volunteers (Hb A/A).
Blood samples were collected in EDTA blood tubes and genomic DNA was extracted from leucocytes by salting out method. Allele specific polymerase chain reaction (PCR) was used for genotyping of all samples. Alkaline haemoglobin electrophoresis was also performed on all subjects to confirm the phenotypes and the findings were compared with genotyping results.
Results: Using alkaline Hb electrophoresis, 33(94.3%) out of 35 patients with provisional diagnosis of sickle cell anaemia were found to be S/S and 2(5.7%) were A/S; using PCR all the 35 patients were found to be S/S; the two patients who their phenotype was A/S while by PCR their genotype was S/S were found to have a history of recent allogenic blood transfusion.
The results of Hb electrophoresis and PCR were in full agreement for both sickle cell trait individuals (A/S), and the healthy controls (A/A).
Conclusion: Diagnostic accuracy of PCR based single tube genotyping of SCD is superior to routine alkaline haemoglobin electrophoresis particularly in situations of recent allogeneic blood transfusion and prenatal and neonatal screening.

Keywords: Sickle cell disease; alkaline Hb Electrophoresis; polymerase chain reaction; Genotyping.

1. INTRODUCTION

Sickle cell anemia is one of the most common heritable hematologic diseases affecting humans. Detection of the single base pair mutations at codon 6 of the beta-globin gene is important for the prenatal diagnosis of sickle cell disease [1]. Homozygous inheritance of the sickle mutation is termed sickle cell anaemia. In heterozygous state (sickle cell trait- Hb A/S), there is only one sickle gene and one normal Hb. A gene and in this situation, usually, the individual is clinically and haematologically normal. In doubly heterozygous state (e.g Hb SC) the individual inherit sickle gene and another globin gene defect, this situation characterized by variable severity [2].

Sickle cell anemia has provided the most well documented example of chronic haemolysis and clinical vasculopathy. The biological importance is emphasized by the redundant and over lapping mechanisms [3]. Sickle cell disease (SCD) affects the structure of erythrocytes by altering the normal discoid shape to a crescent or sickle shape. During this process the haemoglobin S (Hb S) mutation leads to polymerization and precipitation of haemoglobin during deoxygenation resulting in sickling, abnormal adhesion of leukocytes and platelets, increased inflammatory tone, hypercoagulability, chronic haemolysis, chronic anaemia with resultant hypoxia, microvascular occlusion and progressive organ damages [4].

Each year, approximately 300,000 individuals with sickle SCD are born, and >75% of those are in Africa [5], Sickle Cell Anaemia is by far the most prevalent and severe form of SCD [6]. Only limited early detection and treatment initiatives have been implemented in Africa and as consequence death rates are high before the age of 5 years in this region [7].

Sickling test and haemoglobin solubility test are screening techniques used to detect the presence of haemoglobin S, but can't identify exact phenotype/genotype, and thus not enough to make proper result. Haemoglobin electrophoresis can determine the phenotype of sickle cell disease but it should not be performed on infants until they are at least 6 months old because of the presence of hemoglobin F as the predominant haemoglobin at birth, or subjects who are recipients of allogeneic blood transfusion in the preceding three months [8].

Using single tube PCR-based genotyping enable the early diagnosis of sickle cell disease using cord blood, or neonatal sample after delivery or even can be used for the prenatal diagnosis of fetus of sickle cell trait carrier parents [9].

This study aimed to compare findings from PCR-based single tube genotyping of sickle cell disease with routine alkaline haemoglobin electrophoresis phenotyping.

2. METHODS

This study is cross-sectional-analytic study conducted at Khartoum state, Sudan in the period from November 2014 to March 2015.

2.1 Study Subjects

A total of 70 Sudanese subjects attended Ahmed Gasim hospital, Khartoum, Sudan, during the study period were enrolled in this study; 35 of them with sickle cell anaemia (S/S), 15 were sickle cell trait (A/S), and 20 were healthy adult volunteers (A/A).

2.2 Sample Collection and Hb Electrophoresis

Study subjects were selected using non-probability accidental sampling method. Venous blood samples were collected from each subject in ethylene diamine tetra acetic acid (EDTA) blood tubes. From a part of the sample, haemolysate was prepared and cellulose acetate haemoglobin electrophoresis was run on all samples at a pH 8.6 with appropriate controls. The phenotypes were read alongside controls and recorded.

2.3 Molecular Analysis

DNA was extracted by salting out method and stored at -30ºC until PCR is performed.

PCR was used for the determination of the genotype of sickle cell disease patients and healthy individuals using the following primer sequences:-

2.3.1 Wild type

Forward: (5′-ATG GTG CAC CTG ACT CCT GA-3′)

Reverse: (5′-CCC CTT CCT ATG ACA TGA ACT-3′)

This is designed for amplification of a 517 bp fragment from the normal β globin gene.

2.3.2 Mutant type

Forward: (5′-CAG TAA CGG CAG ACT TCT CCA-3′)

Reverse: (5′-GGG TTT GAA GTC CAA CTC CTA-3′)

This is designed for amplification of a 267 bp fragment from homozygous mutant DNA(Hb S/S) [10].

The thermocycling conditions included: initial denaturation at 95ºC for 2 minutes, followed by 30 cycles of [denaturation at 95ºC for 30 second, primer annealing at 60ºC for 30 second and extension at 72ºC for 35 second], and then final extension at 72º for 10 minutes [11].

The amplified fragments are separated on 2% agarose gel stained with ethedium bromide and demonstrated using gel documentation system; 100 bp DNA ladder was run with each batch of samples for determination of fragments size.

2.4 Ethical Considerations

This study was approved by the ethical committee of the ministry of health, Khartoum state, and after detailed explanation of the intended study, informed consent was obtained from each adult participant or participants' parents in case of children before sample collection.

3. RESULTS

A total of 70 subjects were enrolled in this study; 35 patients (23 female and 12 male) with homozygous sickle cell anaemia (Hb S/S); 15 sickle cell trait (Hb A/S), and 20 healthy individuals (Hb A/A). Five (33.3%) of those with heterozygous Hb A/S were males, and 10 (66.7%) were females and all of them were parents of sickle cell anaemia patients. Of the 20 healthy individuals eight (40%) were males, and 12 (60%) were females.

Based on the results of PCR patients with Hb S/S revealed only 267 bp band, and healthy individuals revealed 517 bp band, while heterozygous ones revealed both 267 and 517 bp bands (Fig. 1).

According to the results of Hb electrophoresis, out of 35 patients with provisional diagnosis of sickle cell anaemia (Hb S/S), 33 (94.3%) were found to be S/S phenotype, and two (5.7%) were found to have A/S phenotype. With PCR based single tube genotyping all the 35 patients were S/S.

The results of Hb electrophoresis and PCR were in full agreement for both sickle cell trait individuals and the healthy controls (Table 1).

4. DISCUSSION

Several high through put methods such as cation-exchange high performance chromato-graphy (CE-HPLC) or more recently capillary

electrophoresis (CE) are now available for diagnosis of haemoglobinopathies. Nevertheless, electrophoretic studies are still performed in many laboratories [12,13,14,15], particularly in developing countries where these advanced techniques are not available.

In this study we compared PCR-based genotyping of sickle cell disease with alkaline Hb electrophoresis to verify the reliability of PCR-based genotyping as screening tool for SCD carrier and also for screening of the disease in cord blood samples to early identify children with SCD who their parents are sickle cell trait (AS).

Using Hb electrophoresis, 33(94.3%) patients out of 35 patients with provisional diagnosis of sickle cell anaemia were found to be S/S and 2(5.7%) were A/S. Using PCR all the 35 patients were found to be S/S. By reviewing patients' medical files we found that the two patients who revealed as A/S by Hb electrophoresis were transfused with blood before a short time period. This gives the PCR the advantage that the blood transfusion doesn't affect the result as in haemoglobin electrophoresis. A limitation of PCR that can't detect the doubly heterozygous state such as Hb-S/C or Hb-S/beta thalassemia which sometimes may have the same severity as Hb-SS but fortunately haemoglobinopathies other than SCD are very rare in Sudan.

The results of Hb electrophoresis and PCR were identical for both sickle cell trait individuals (A/S), and the healthy controls (A/A). This indicates that, allele specific PCR can be used for screening of carriers. Despite the availability of SCD screening methods such as the solubility, sickling and peripheral blood film methods and their reliability in the demonstration of patients with SS, they showed variability in their ability to detect the carrier state of haemoglobin (AS) [16].

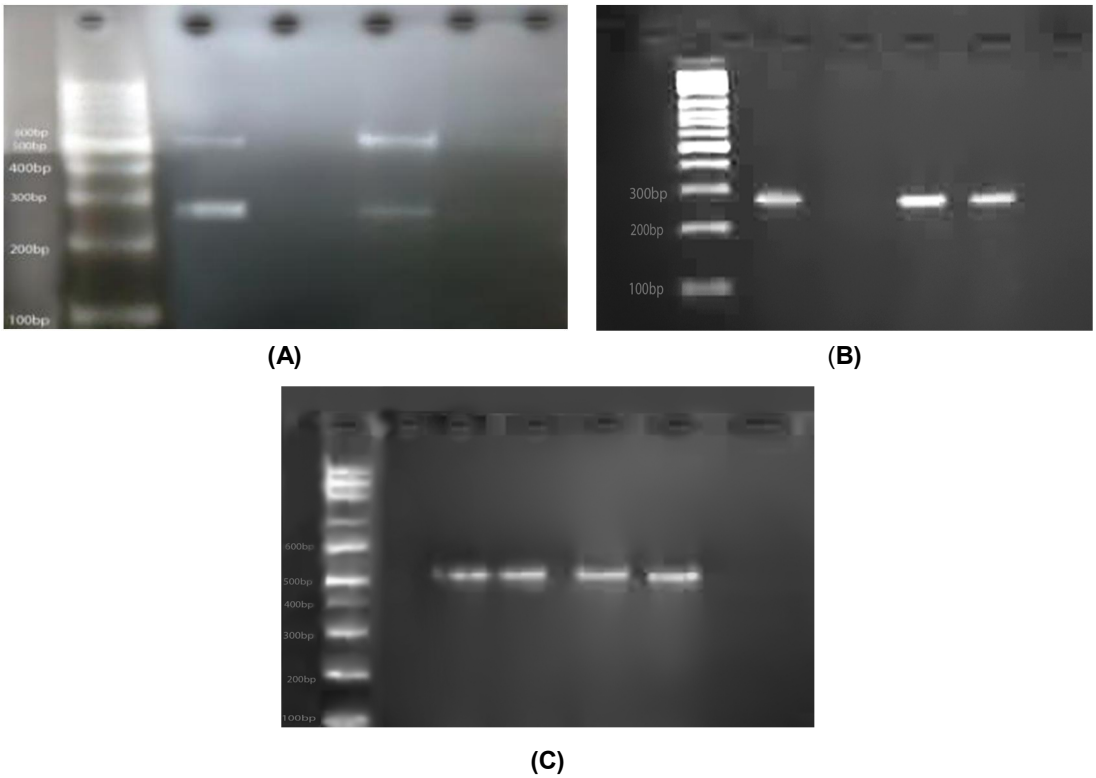

Fig. 1. [A] Homozygous SS (267 bp band); [B] Heterozygous sickle cell trait (267bp & 517bp bands); [C] Wild-type (517 bp band)

Table (1). Comparison of PCR and Hb electrophoresis results

Group	N	Alkaline Hb electrophoresis	Polymerase chain reaction
Hb S/S	35	33(94.3%)	35(100%)
Hb A/S	15	15(100%)	15(100%)
Hb A/A	20	20(100%)	20(100%)

While Hb electrophoresis method can't be used for prenatal screening or cord blood because the predominant Hb is Hb F [7], the PCR could be used in both situations. The increased sensitivity provided by PCR analysis has both current and potential applications for prenatal diagnosis, confirmation of genotype in neonatal screening and antenatal diagnosis of SCD [17].

Our findings were further supported by many studies conducted to evaluate molecular methods applied for screening and diagnosis of SCD. Martinez et al. conducted study in Venezuela dealing with the application of the PCR for the diagnosis of sickle cell anemia and reported that PCR is one of the fundamental technical bases for establishing a newborn screening program [18]. Yue et al. evaluated PCR- high resolution melting (HRM) analysis as a rapid tool for screening of SCD; he concluded that HRM is a simple, high efficiency approach for screening of SCD and particularly suitable application in the African area [5]. A study by Ayatollah et al. conducted study for molecular analysis of Iranian families with SCD; he reported that PCR- restriction fragment length polymorphism (RFLP) is a simple, sensitive, and rapid, and also has application that is important for the prenatal diagnosis of SCD [19].

5. CONCLUSION

Diagnostic accuracy of PCR based single tube genotyping of SCD is superior to routine alkaline haemoglobin electrophoresis in situations of recent allogeneic blood transfusion, prenatal and newborn diagnosis of SCD. As such, ample facilities should be made available for genotyping studies in this locality. Where indicated, PCR-based single tube genotyping for SCD will provide physicians with accurate diagnostic conclusions, which translates to better clinical management of SCD.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Luzzatto L. Hemoglobinopathies including thalassemia. Part 3. Sickle cell anemia in tropical Africa. Clin Haematol. 1981;10: 757-84.
2. Glassberg J. Evidence-based management of sickle cell disease in the emergency department. Emergency Medicine Practice. 2011;13(8):1-20.
3. Schaer DJ, Alayash AI, Buehler PW. Gating the radical hemoglobin to macrophages: The anti-inflammatory role of CD163, a scavenger receptor. Antioxid. Redox Signal. 2007;9:991-999.
4. Bartolucci P, Galactéros F. Clinical management of adult sickle-cell disease. Current Opinion in Hematology. 2012;19: 149-55.
5. Yue L, Lin M, Chen J.T. et al. Rapid screening for sickle cell disease by polymerase chain reaction-high resolution melting analysis. Mol Med Rep. 2014; 9(6):2479-84.
6. 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.
7. Grosse SD, Odame I, Atrash HK, et al. Sickle cell disease in Africa: A neglected cause of early childhood mortality. American Journal of Preventive Medicine. 2011;41:S398-405.
8. Steinberg MH. Sickle cell disease and other haemoglobinopathies. (Accessed August 2013).
   Available: http;//www.md consult.com
9. Newton CR, Graham A, Heptinstall LE, et al. Analysis of any point mutation in DNA. The amplification refractory mutation system (ARMS). Nucleic Acids Res. 1989; 17:2503-2516.
10. Cobb BD, Clarkson JM. A simple procedure for optimizing the polymerase chain reaction (PCR) using modified Taguchi methods. Nucleic Acids Res. 1994;22:3801-3805.
11. Henegariu O, Heerema NA, Dlouhy SR, et al. Multiplex PCR: Critical parameters and step-by-step protocol. Biotechniques. 1997;23:504-511.
12. Riou J, Godart C, Hurtrel D, et al. Cation-exchange HPLC evaluated for presumptive identification of haemoglobin variants. Clin Chem. 1997;43:34-9.
13. Joutovsky A, Hadzi-Nesic J, Nardi MA. HPLC retention time as a diagnostic tool for haemoglobin variants and haemoglobinopathies: A study of 60000 samples in a clinical diagnostic laboratory. Clin Chem. 2004;50:1736-47.

14. Louahabi A, Philippe M, Lali S, et al. Evaluation of a new Sebia kit for analysis of haemoglobin fractions and variants on the capillary system. Clin Chem Lab Med. 2006;44:340-5.
15. Wajcman H. Electrophoretic Methods for Study of Haemoglobins. In: Nagel RL, editor. Methods in molecular medicine. Totowa, NJ: Humana Press Inc; Haemoglobin disorders: Molecular methods and protocols. 2003;82:93–100.
16. Chasen S, Loeb ZS, Landsberger E. Haemoglobinopathy screening in pregnancy: Comparison of two protocols. Am J Perinat. 1999;16:175-180.
17. Steinberg M.H. DNA diagnosis for the detection of sickle hemoglobinopathies. Am J of Hematology. 1993;43:110-115.
18. Martínez J, Blanco Z, Hakshaw P, Moreno N. Application of the polymerase chain reaction to the diagnosis of sickle cell anemia in Venezuela. Sangre (Barc). 1998;43(1):63-6.
19. Ayatollahi M, Zakerinia M, Haghshenas M. Molecular analysis of Iranian families with sickle cell disease. J Trop Pediatr. 2005;51(3):136-40.

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