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Detection of β-Hemoglobin Gene and Sickle Cell Disorder from Umbilical Cord Blood

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Abstract

Sickle cell disease (SCD) is one of the most common hemoglobinopathies, which is caused by the replacement of glutamic acid with valine at the sixth position of the beta-globin amino acid chain which sickling of the entire red blood cells in the homozygous (Hb S/S) condition. There are many analyses and screening procedures were developed to detect sickle cell anemia in the early age of birth, especially from heel prick blood, but in case of developing countries, it would be more acceptable to detect sickle cell disorder using umbilical cord blood just after birth rather than using heel prick blood. In this study, umbilical cord blood (UCB) was used to detect β-hemoglobin gene and sickle cell disorder. Polymerase chain reaction (PCR) based analysis was done using two primers (wild-type and mutant type) to detect this disorder. A total number of 22 samples were enrolled in this experiment for PCR amplification among which nineteen samples were identified by amplification of both 267 bp and 517 bp fragments revealing heterozygous sickle cell trait (Hb A/S), whereas three samples were found to amplify of 517 bp only revealing healthy individuals. The result from PCR analysis was then collaborated with the information of the mothers of each sample to analyze the result more conveniently and found that the mothers of all individuals except the three samples had anemia or mild form of anemia, thus it was expected that the newborn might have anemia trait (Hb A/S) the exception was found in case of sample No. 9 and sample No. 15. Both samples showed the bands on 267 bp and 517 bp thus expressed the sickle cell disease trait although the mothers of these samples were not anemic. However, no samples were recorded having sickle

cell anemia (9 Hb S/S). The inherent simplicity and low cost of this PCR based analysis with umbilical cord blood will be considered as an effective tool in future newborn screening in Bangladesh.

Keywords

β-Hemoglobin Gene, Sickle Cell Disorder, Umbilical Cord Blood, New Born Screening

1. Introduction

Hemoglobinopathies are a variant group of inherited blood disorders that result from an alteration in the structure or synthesis of hemoglobin. There are more than 700 defects in globin genes which are found to be responsible for hemoglobinopathies [1]. More than a quarter of a million people are found to be born with one of the disorders in the world per year. Various public health problems are appeared because of these disorders, particularly in the Mediterranean area, in the Middle East and in parts of India, Africa and Southeast Asia [2]. Among several types of hemoglobinopathies, sickle cell disease is one of the most common of them. Sickle cell disease is a group of genetic disorders caused by the predominance of HbS [3] [4]. A point mutation is responsible for this disease. This results in the replacement of hydrophilic glutamic acid with the hydrophobic amino acid valine at the sixth position (*β*6Glu → Val) in the beta-globin amino acid chain [2]. Clumping of HbS molecules into rigid fibers causes "sickling" of the entire red blood cells in the homozygous (Hb S/S) condition [5]. In Bangladesh, anemia is found to have greater significance in public health problem.

It has been estimated in recent years that anemia is affecting the lives of 27 million children and women, especially the rates are higher for girls than for boys and approximately half of the pregnant women have anemia which is commonly due to iron deficiencies [6] [7]. But the severe conditions found in children who carry sickle cell anemia, showing much difficulty in different stages of the lifespan. In an extrapolated statistical analysis, it has been shown that Bangladesh is in an alarming condition in case of sickle cell anemia within southern Asia and also estimated that there is a prevalence of sickle cell anemia may happen approximately 1 in 1000 [8]. For survival and development of present and future generations, it has become very important to give more emphasis on the prevention and control to anemia in Bangladesh. Alkaline and acid hemoglobin electrophoresis methods are the most traditional and commonly used method to identify and investigate normal and mutant HbS [9] [10]. Moreover, hemoglobin fraction analysis of different hemoglobin variants by cation-exchange HPLC is another option to study hemoglobinopathies and other related disorders [10] [11]. Though these sophisticated techniques are being used in the developed countries, however, it is difficult to apply these advanced

methods in low-income countries like Bangladesh for detection of hemoglobin disorders. In many developed countries, there are many analyses and screening procedure being developed to detect sickle cell anemia in the early age of birth and treat them [12] [13]. Most of those screening involve in collecting heel prick blood from the newborn. But in aspects of many other developing countries including Bangladesh, the general people are usually denied providing heel prick blood from their children for early detection and are not aware of going through the prenatal or postnatal diagnosis. In that case, cord blood can be the most importance that the parents may not deny giving for their children's safety as its blood from 50 - 70 cm long and about 2 cm in diameter umbilical cord attaches the developing fetus or embryo to the placenta [14] which contains all the whole blood elements and hematopoietic stem cells [15] [16]. Therefore, in this study, simple PCR based agarose gel electrophoresis of codon 6 of the β-hemoglobin gene was observed for screening of sickle cell disease during birth using cord blood. PCR is one of the fundamental and cost effective methods with high accuracy approach for diagnosis of sickle cell disease [17] [18], where a small fragment of 517 bp can detect normal β-hemoglobin gene and 267 bp can detect mutant allele.

2. Methods

2.1. Sample Collection and Preservation

The research work was conducted in the Animal Biotechnology Laboratory (ABL), USDA laboratory and TWAS Laboratory of the Department of Genetic Engineering and Biotechnology (GEB) at Shahjalal University of Science and Technology, Sylhet (SUST), Bangladesh. The collection of cord blood was performed in the Department of Obstetrics and Gynaecology at Jalalabad Ragib-Rabeya Medical College and Hospital in Sylhet. UCB samples were collected directly from the labor room and OT (Operation Theater) just after delivery after consulting with the parents of the newborns. 5 ml of UCB from each newborn was drawn into a sterile vial containing 50 µl (10 µl/ml blood) of 10% solution of Potassium EDTA (K_2EDTA). The vials were preserved at −20˚C freezer until DNA extraction.

2.2. Subject Information

Some related information about the samples was collected from the patient's medical history just after the delivery (**Table 1**). All information was arranged against the sample number so that the data could be analyzed according to the information. Hemoglobin level in the data helped to determine whether the mother was anemic or not and the intensity of anemia. This information could be used to analyze about the possibility of the newborn to have anemia and to collaborate with the results so that the analysis would be more accurate.

2.3. DNA Extraction

Genomic DNA was extracted from the UCB samples through a modified phe-

Table 1. Information of the subjects collected from the patients' medical history.

Sample No.	Mother's Medical History				Samples' Information	
	Age (Years)	Blood Group	Anemia	Hb Level (g/dl)	Sex	Status of Birth
01	20	B +ve	Absent	12.3	F	Mature, 2.5 kg
02	20	B +ve	Present	6.8	F	Mature and Healthy
03	30	A +ve	Present	6.2	M	Mature, 2.3 kg
04	21	B +ve	Present	8.4	M	Mature and Healthy
05	21	O +ve	Mild	9.4	M	Mature and Healthy, 3 kg
06	25	A +ve	Absent	11.6	M	Mature and Healthy, 2.9 kg
07	26	O +ve	Present	8.2	F	39 Weeks and Mature
08	25	O +ve	Absent	11.9	F	2.7 kg
09	20	O +ve	Present	7.2	M	Mature, 2.8 kg
10	28	B +ve	Mild	9.6	M	Healthy, 3.2 kg
11	30	AB +ve	Present	6.5	F	2.6 kg
12	32	O −ve	Present	7.0	M	Triplet baby, 2 died and 1 premature, 1.5 kg
13	25	B +ve	Present	7.2	F	Mature, 3.4 kg
14	22	A +ve	Present	8.5	M	Mature and Healthy, 3.3 kg
15	30	B +ve	Absent	11.3	F	Mature, 3 kg
16	25	B +ve	Present	9.4	M	Mature, 2.8 kg
17	25	A +ve	Present	10.6	F	Mature, 3 kg
18	22	O +ve	Absent	12.6	M	Mature and Healthy, 2.9 kg
19	22	AB +ve	Present	9.7	M	Mature, 3.3 kg
20 (F)	22	B +ve	Present	6.2	F	Mature, 2.5 kg
20 (M)	22	B +ve	Present	6.2	M	Mature, 2.8 kg
21	30	A +ve	Present	10.4	F	Mature, 3 kg

Notes: sample 20 (F) and 20 (M) are twins. Here, F symbolizes female while M symbolize male.

nol-chloroform method according to the protocol established previously [19]. DNA quality was checked by electrophoresis on 0.8% agarose gel where the gel was run at 80 V for 25 minutes. The gel was viewed on an Ultraviolet Transilluminator (UVP, High-Performance Transilluminator, USA) and a photograph was taken by gel documentation system with canon camera (Nikon Coolpix P100, 26X Zoom, 10.3 Megapixel camera). DNA quality was compared with 1kb plus DNA ladder (Promega) and good quality bands were observed for each of the samples.

2.4. PCR Amplification

In this experiment, PCR amplification was done using two sets of primers for conferring the healthy individuals and the patients with sickle cell disease. Wild-type primers, forward (5'-ATG GTG CAC CTG ACT CCT GA-3') and re-

verse (5'-CCC CTT CCT ATG ACA TGA ACT-3') were designed for amplification of a 517 bp fragment from the normal β globin gene and mutant type primer sets, forward (5'-CAG TAA CGG CAG ACT TCT CCA-3') and reverse (5'-GGG TTT GAA GTC CAA CTC CTA-3') were designed for amplification of a 267 bp fragment from homozygous mutant DNA [20].

PCR reactions were performed with 25 µl reaction mixtures for each sample with 12.5 µl of master mix (Promega Hot Start), 2 µl of each primer set, 2 µl of template DNA and 6.5 µl nuclease-free water for both primer sets. PCR reaction was conducted for pre-heating at 95˚C for 2 minutes, denaturation at 95˚C for 30 seconds, annealing at 60˚C for 30 seconds and 35 seconds for elongation or extension at 72˚C. A final step of 10 minutes at 72˚C was added to allow complete extension of the amplified fragments. The PCR was run for 30 cycles. PCR products were checked by electrophoresis on 1.2% agarose gel with 7 µl DNA loaded with 6X loading buffer where 1kb plus ladder was used to compare migration of DNA. The gel was run in 1X TBE buffer at 75 V for 35 minutes. This gel was then placed in gel documentation system and a photograph was taken by digital a camera (Nikon Coolpix P100, 26X Zoom, 10.3 Megapixel camera).

3. Results

In this experiment, extraction of DNA from umbilical cord blood and detection of the β-hemoglobin gene and sickle cell disorder were done using different types of techniques. The results of this experiment were described in the following segments in a systematic way.

3.1. PCR Product Analysis for Detection of β-Hemoglobin Gene and Sickle Cell Disorder

After checking the DNA quality, twenty-two samples were analyzed for detection of beta hemoglobin and sickle cell disorder. Two specific primers were used, one to amplify 517 bp fragment from the normal β-globin gene and another mutant primer to amplify a 267 bp fragment from mutant DNA for sickle cell disorder. In the outer lane, a 1 kb plus ladder range started from 250 kb length was used for this experiment. Nice bands were shown in agarose gel for all samples at the level of 517 bp. In the level of 267 bp, all samples except sample No. 1, 6 and 18 showed bands (**Figure 1**).

A total of 22 samples were enrolled in this experiment for PCR amplification and among which nineteen samples were identified by amplification of both 267 bp and 517 bp fragments. As bands were identified at both 267 bp and 517 bp, the samples revealed heterozygous sickle cell trait (Hb A/S). Three samples such as sample No. 1, 6 and 18 were found to amplify of 517 bp product only where it was considered that these three individuals were totally healthy and do not carry the mutant form of hemoglobin of Hb S/S or Hb A/S. No sample was identified by amplification of 267 bp only, and thus, there was no homozygous condition (Hb S/S) to develop sickle cell anemia in any sample. These were seen from the

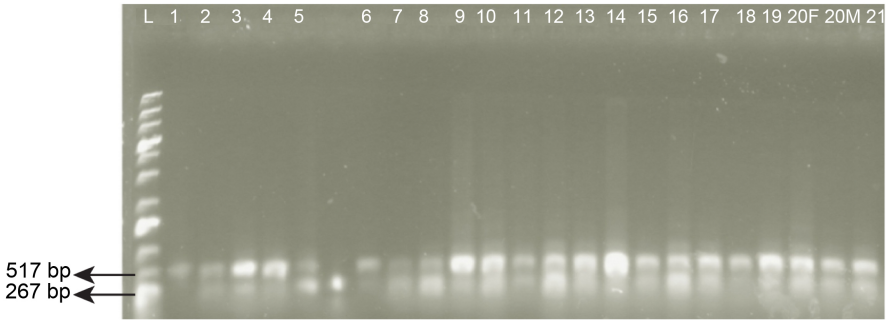

Figure 1. Gel image data of PCR amplification. In the outermost lane, denoted as L is a 1kb plus ladder DNA. Lanes from 1 to 21 are the samples number. Good bands are shown in agarose gel for all samples at the level of 517 bp. In the level of 267 bp, all samples except sample No. 1, 6 and 18 showed bands which revealed heterozygous sickle cell trait (Hb A/S). Sample No. 1, 6 and 18 are totally healthy and do not carry the mutant form of hemoglobin of Hb S/S or Hb A/S as those only show bands in the level of 517 bp.

data that the mothers of all individuals except those three samples had anemia or mild form of anemia, so, it was expected that the newborn might have anemia trait (Hb A/S). The exception was found in case of sample No. 8 and sample no. 15. Both samples showed bands on both 267 bp and 517 bp, therefore, expressed the sickle cell disease trait although the mothers of these subjects were not the patient of anemia that could be considered that the father of the child might be a patient of SCD, so the subjects inherit the trait. The results of PCR analysis might agree for both sickle cell trait individuals and the healthy controls.

3.2. Correlation between the Subject Information and PCR Data Analysis

After associating the subject information with the PCR data analysis, it was found that the samples such as sample No. 01, 06 and 18 showed normal Hb A/A condition in PCR whose mothers were recorded as free from anemia in medical history (Table 2).

On the contrary, some samples were detected to have Hb A/S condition in PCR result analysis, whose mothers were recorded as anemic in medical history (Table 3). Moreover, an exception was found in case of sample No. 08 and 15 that the samples show to be the carrier of anemia whereas their mothers were not anemic. It could be predicted that the fathers of the samples might have anemia or carrier of it (Table 4). This collaboration helped to intensify the accuracy of the PCR result analysis.

4. Discussion

In this experiment, extraction of DNA from umbilical cord blood using phenol-chloroform method and detection of β hemoglobin gene and sickle cell disorder using PCR analysis by gel electrophoresis were done. Several experiments were in practice to detect hemoglobinopathies using venous blood samples [17] [21] [22] and in case of newborn screening, the blood is mainly collected from

Table 2. Correlation between the newborn and their normal mother.

Sample No.	Disease Condition of Newborn	Mother's Medical History			
		Age (Years)	Blood Group	Anemia	Hb Level (g/dl)
01	Normal (Hb A/A)	20	B +ve	Absent	12.3
06	Normal (Hb A/A)	25	A +ve	Absent	11.6
08	Normal (Hb A/A)	22	O +ve	Absent	12.6

Table 3. Correlation between the newborn and their mother with anemia.

Sample No.	Disease Condition of Newborn	Mother's Medical History			
		Age (Years)	Blood Group	Anemia	Hb Level (g/dl)
08	Hb A/S	25	O +ve	Absent	11.9
15	Hb A/S	30	B +ve	Absent	11.3

Table 4. Correlation between the newborn with sickle cell trait and their normal mother.

Sample No.	Disease Condition of Newborn	Mother's Medical History			
		Age (Years)	Blood Group	Anemia	Hb Level (g/dl)
02	Hb A/S	20	B +ve	Present	6.8
03	Hb A/S	30	A +ve	Present	6.2
04	Hb A/S	21	B +ve	Present	8.4
05	Hb A/S	21	O +ve	Present	9.4
07	Hb A/S	26	O +ve	Present	8.2
09	Hb A/S	20	O +ve	Present	7.2
10	Hb A/S	28	B +ve	Present	9.6
11	Hb A/S	30	AB +ve	Present	6.5
12	Hb A/S	32	O −ve	Present	7.0
13	Hb A/S	25	B +ve	Present	7.2
14	Hb A/S	22	A +ve	Present	8.5
16	Hb A/S	25	B +ve	Present	9.4
17	Hb A/S	25	A +ve	Present	10.6
19	Hb A/S	22	AB +ve	Present	9.7
20 (F)	Hb A/S	22	B +ve	Present	6.2
20 (M)	Hb A/S	22	B +ve	Present	6.2
21	Hb A/S	30	A +ve	Present	10.4

Notes: sample 20 (F) and 20 (M) are twins. Here, F symbolizes female while M symbolize male.

heel prick of the newborn [23]. In case of a developing country like Bangladesh, most of the people are not concern about newborn screening after birth but newborn screening has also been established in recent years in this developing country [24]. Though newborn screening has been practiced nowadays, the

parents are unwilling to give blood from heel prick or vein of the newborn. Considering these circumstances, this experiment was done using umbilical cord blood which is considered as wasted after delivery.

In recent time, DNA extraction is usually done using different sophisticated DNA extraction kit [25] [26] [27], but in this experiment, all the buffers and solutions were prepared in the lab and DNA extraction was done manually following phenol-chloroform method. This method might be time-consuming but more cost-effective and DNA can be extracted more accurately than extracting the DNA using the DNA extraction kit.

Various methods of high quality such as capillary electrophoresis, cation-exchange high-performance chromatography (CE-HPLC) are in recent practice for diagnosis of hemoglobinopathies. In developing countries where these advanced techniques are not available or limited, electrophoretic and PCR studies are still performed in many laboratories for initial newborn screening [9] [10] [11] [28]. Despite PCR based methods, several other similar approaches likerestriction enzyme digestion [29], denaturing gel electrophoresis [30], allele-specific amplification [20], and the ligase chain reaction method [31] are also available for the characterization of mutations in the β-globin gene. But, though in most patients with sickle cell anemia and other related genetic disorders, the breakpoints are clustered in a very small section of the chromosome and the PCR technique deals with the small amount as well as a small section of DNA, thus it can be used for the detection in most of the cases with high accuracy [32]. Therefore, a simple method was established for this experiment using PCR technique and gel electrophoresis analysis so that newborn screening might not consider impossible or expensive.

Besides these, the methods can detect the mutation at the gene level which facilitates prenatal diagnosis and diagnosis at age less than 3 months when HbS is not yet in peripheral blood. Moreover, the method is easily maintained and cost-effective as well as put no effect to the newborn. This makes the technique more suitable for neonatal screening which ensures a better diagnosis and prognosis in presence of suitable medical care before the clinical onset of the disease. The knowledge of the molecular pathology of hemoglobinopathies has been applied to molecular diagnosis, carrier identification, and neonatal diseases diagnosis and has resulted in a decrease in the incidence of the homozygous state in several at-risk populations [33].

The final target of this study was to detect β-hemoglobin gene and the mutant form of hemoglobin for sickle cell disorder which can be used as screening tool for SCD carrier and for screening of the disease in cord blood samples to early identification of children with SCD whose parents are sickle cell trait or having sickle cell disorder. In this study, wild-type primer that cut at 517 bp fragment from the normal β globin gene and the mutant type primer that amplify a 267 bp fragment from mutant DNA and showed band like some other experiments that were done in detecting β-hemoglobinopathy specifically sickle cell disorder [20]. The results of PCR were shown that in most of the individuals have bands iden-

tified at both 267 bp and 517 bp, thus the samples revealed heterozygous sickle cell trait (Hb A/S). Three samples, sample No. 1, 6 and 18 which showed fully normal individuals by amplifying at 517 bp product only. By reviewing patient's medical files, it was found that most of the mother of the newborn either has anemia or mild anemia. According to the data of the mothers of the sample No. 1, 6 and 18 were fully normal and healthy and were not the patient of anemia. The exception was found in case of sample No. 8 and sample No. 15. Both samples showed bands on both 267 bp and 517 bp thus expressed the sickle cell disease trait although the mothers of these subjects were not the patient of anemia. That could be considered that the father of the child might be a patient of SCD, so the subjects inherit the trait. This could not be identified whether most of the mother showing anemia or mild anemia had sickle cell disorder or they had anemia for iron deficiency or some other reasons.

The findings of this research were further supported by many experiments conducted to establish molecular methods used for screening of sickle cell disorder. The study was conducted in Venezuela using PCR analysis for the diagnosis of sickle cell anemia and reported that PCR is one of the fundamental technical basis for establishing a newborn screening program [34]. A study has also experimented on molecular analysis of Iranian families with SCD where PCR-RFLP (restriction fragment length polymorphism) is considered as a simple and rapid method and important for the prenatal diagnosis of SCD [35]. The PCR-high-resolution melting (HRM) analysis was studied which was revealed as a rapid tool for screening of SCD and it was concluded that HRM is a particularly suitable application in the screening of SCD in the African area [36].

The overall result of this experiment showed that PCR analysis of umbilical cord blood samples might establish as an effective method to detect the normal condition of the newborn as well as the newborn having sickle cell disorder or trait inherited from their parents and might be suitable for the screening of sickle cell disorder.

5. Conclusions

In this study, detection of β-hemoglobin gene and sickle cell disorder from umbilical cord blood was observed by using PCR analysis. It could be helpful for studying hemoglobin disorders and newborn screening in aspects of Bangladesh as it can be used as an easy and cost-effective tool. Individuals contain healthy condition with normal β hemoglobin gene and mostly with sickle cell trait were found in this experiment. As only twenty-two individuals were investigated by PCR analysis, therefore, it is not sure that the people of Bangladesh will show the same kind of higher sickle cell disorder tendency. It is, therefore, necessary to conduct the experiment with a large number of samples and more experiments.

Due to the limitations of lab facilities, various problems occurred during the research work. Besides this, if the analysis could be done with the parent's blood samples against the UCB samples of the newborn, the experiment would be more authenticated. Furthermore, other molecular approaches like RFLP,

PCR-high-resolution melting (HRM) and Hemoglobin gel electrophoresis etc. should also be studied. However, it is a matter of great advent that this research can also be used as a baseline study for future investigation on disorders related to hemoglobin in Bangladesh.

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Availability of Data and Materials

Data and materials related to this work are available upon request.

Authors' Contribution

All authors contributed equally to this research study and during the preparation of the manuscript. All authors read and approved the final manuscript.

Competing Interests

The authors declare that they have no competing interests.

Consent for Publication

All authors approve the manuscript for publication.

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List of Abbreviations

UCB: Umbilical Cord Blood; SCD: Sickle cell disease; Hb: Hemoglobin; DNA: Deoxyribonucleic acid; Taq: *Thermusaquaticus*; EDTA: Ethylenediaminetetraacetic acid; TBE: Tris borate EDTA; bp: base pair; kb: kilobase; TE: Tris EDTA; SSC: Saline Sodium Citrate; PCR: Polymerase Chain Reaction; %: Percent; ˚C: Degree Celsius; cm: centimeter; gm: Gram; mg: Milligram; ml: Milliliter; µl: Micro liter; V: Volt.