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Identification of patients with sickle cell anaemia using restriction enzyme discrimination

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ABSTRACT

Tracking down a mutation for Single-cell anaemia (SCA) is a full-day, inquiry-based biology experiment that has given scientist a lot to do in the field of medicine. Gene therapy as so far proved to be the own escape route through the use of Hematopoietic stem-cell transplantation (HSCT) although it is limited to the donor availability. Clinical trials have been conducted concerning testing manufacturing, optimization and characterization of transduction hematopoietic stem cell FCP (final cell product) and its degree of vivo toxicology to ascertain the method. This study aims at establishing a prenatal diagnostic facility through determination of PCR performance and sensitivity and gel electrophoresis that will help in decision making regarding the fetus affected with sickle cell anaemia. Several samples were taken from affected patients using the chorionic villus biopsy, DNA extracted and subjected to amplification refractory mutation system (ARMS-PCR), to identify a mutation in the GAG-GTG gene for sickle cell anaemia. Gel electrophoresis was done from which it was found out that out of the 41 cases analyzed, 26 [70.73%] had AS Hb genotype, 4 [9.76%] had AA Hb genotype and 3 [7.32%] had SS Hb genotype and the reminded of 5 [12.20] had either AA or AS genotype. It was concluded that the ARMS-PCR has a high performance and 75% sensitivity hence can be used to study and detect patients with SCA mutation as a prenatal diagnostic facility. Gel electrophoresis was done from which it was found out that out of the 41 cases analyzed, 26 [70.73%] had AS Hb genotype, 4 [9.76%] had AA Hb genotype and 3 [7.32%] had SS Hb genotype and the reminded of 5 [12.20] had either AA or AS genotype. Detection of sickle anaemia requires a lot of information about the restriction enzymes to be restudied. It is easy to detect an irregularity in the human genome whenever any alteration is done more especially due to mutation. Sickle cell anaemia is one of the most studied genetic diseases that can be treated if noticed in the early stage through gene therapy.

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INTRODUCTION

The study of sickle cell anaemia condition using genetic test has been made effective, efficiency and reliable due to the application of the principle behind polymerase chain reaction (PCR), restriction digestion and gel electrophoresis which has significantly helped in the improvement of the study (Taylor D, 2013). Several scientific steps are involved in the study of HbS and HbA genes. These steps lie by discrimination by the restriction enzyme that is used to identify patients with sickle cell anaemia (Keiden, 1998). Anaemia is an SNP disorder which can be identified by subjecting gene to

restriction digestion. The principle behind polymerase chain reaction (PCR), restriction digestion and gel electrophoresis have significantly helped in the improvement of the genetic test for sickle cell anaemia. A DNA polymerase is extracted from heat stable bacteria called Thermus aquaticus (Taq); it is then treated with the target sequence where it will make copies of a complementary sequence from the primers and the nucleotide bases (Taylor D, 2013).

DNA materials extracted from the patients are so small to be used in the study hence require amplification before the genetic test begins (Gravitz, 2014). In the genetic test, the amplified DNA is treated with a restriction enzyme (RE) which cut the DNA strands at a specific sequence the RE recognizes the. The last step involves the identification of the cleaved DNA fragments depending on their size on the gel electrophoresis (Keiden, 1998).

MATERIALS AND METHODS

Part A: Amplification of the gene involved in the Sickle Cell Anaemia.

This forms the first step in the simulation of a genetic test of the sickle cell anaemia. In this step, the extracted DNA from the patients is copied into several identical copies that can be used in the restriction enzyme treatment (Gravitz. 2014). Materials required in this step include the Red-mix, ten µl pipette, 100 µl pipette, PCR tubes, DNA sample form 41 patients, DNA templates, distilled water, reverse primers and forward primers (Keiden, 1998).

When all the materials are assembled, the cocktail is prepared procedurally with a keen observation of the correct ratio and dilution of the buffer. Generally, the required materials are used in the preparation of a master mix before the coping of the DNA by mixing distilled water, template DNA, reverse and forward primer as well as the red mix in the correct ratio as shown in Table 1.

The amplification of the DNA material involves the following step; Samples are taken from 41 patients in the correct process and kept in the cuvette away from contamination and labelled well to avoid confusion (Bright JA, 2015). For sickle cell anaemia test, any patient is legible for the test. When the master mix is ready for use, the second step of amplification will proceed by introducing the patients' uncontaminated sample of the DNA in the PCR tubes with each tube having DNA from a different patient (Gravitz, 2014). The thermocycler is used to amplified DNA sequence. The system was fed with 0.2 µl of each sample into the sample individual. Each tube should be labelled appropriately. Once the tubes are filled, the thermocycler in switched on then allowed amplify the DNA without interruptions (Keiden, 1998). The setting of the thermocycler should as shown below in Table 2.

At the end of the thermocycler, the amplified DNA should be cooled before proceeding to the next step. The next step is to carry out the restriction di-

Table 1: The correct ration in which the master mix is prepared

Reagents	Starting concentration	Final concentrations	PCR cocktail	Master mix.
Red mix (RM)	2x	1x µM	12.5	62.5
Forward primary.	10 µM	0.2 µM	0.5	2.5
Reverse primers.	10 µM	0.2 µM	0.5	52. 5
Template DNA	-----	……..	10 µl	----
Distilled water.	----	……..	25 µl	120 µl

Table 2: Setting of the Thermocycler

Step number	Step type	Temperature	Time
1	Activation	94°C	1 min
2	Denaturation	94°C	15 s
3	Annealing	60°C	15 s
4	Extension	72°C	10 s
5	Go to step 2 for 29 times		
6	Final extension	72°C	1 min

Table 3: Analysis of Results

Gene type	Sample treatment	Number of patients	Percentage
AS Hb genotype	Sample without enzyme	0	0%
	Sample with enzyme	26	70.73%
AA Hb genotype	Sample without enzyme	0	0%
	Sample with enzyme	4	9.76%
AA or AS genotype	Sample without enzyme	0	0%
	Sample with enzyme	3	7.32%

gestion of the PCR products by the use of the specific polymerase (Gravitz, 2014). The ten µl of the patient's samples from the four tubes in the thermocycler is treated with one µl of the enzyme *Bsu*36I. It was spun up to make sure that all the content mixed using a vortex. Also, a negative tube was used. (The negative does not contain any DNA sample). The results were incubated for one hour in temperature close to the body level (37°C) (Bright JA, 2015).

Part B: gel electrophoresis

The last principle is the separation of the DNA on the gel electrophoresis. In this case, gel agarose is prepared by mixing 500 ml of 1 µl x TBE buffer. Eight wells and two combs were put into the gel. Hence two pairs of work can be done. Then the sample is loaded into the gel and run with a voltage of 90 for 40 minutes. After which the gel was viewed under the UV light.

RESULTS

Gel electrophoresis was done from which it was found out that out of the 41 cases analyzed, 26 [70.73%] had AS Hb genotype, 4 [9.76%] had AA Hb genotype and 3 [7.32%] had SS Hb genotype and the reminded of 5 [12.20] had either AA or AS genotype.

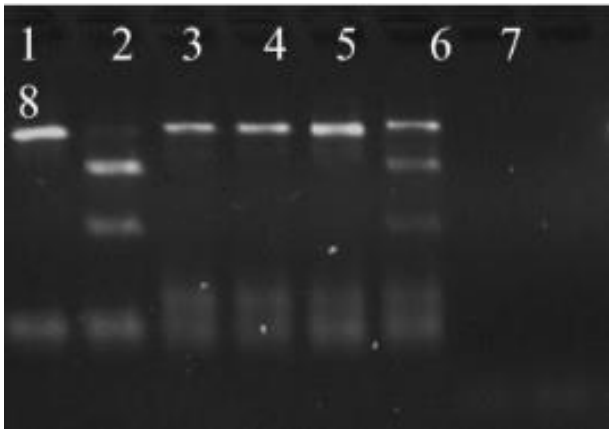

Figure 1: Analysis of Gel electrophoresis

The results of each patient were displayed in the gel electrophoresis were bands were formed on a different point on the gel while some formed smearing. No patient showed bands on the wells treated without enzyme but had at least three bands on the well that had the restriction enzyme. 26 patients had two bands on both the wells with and without the restriction enzyme. Three patients formed two bands on the well without RE and four bands on the well with the RE. Well, 7 and eight which had no patient sample but the enzyme only did not record any band, probably due to some error or faults. Although some smearing was observed in wells 3, 4 and 5, maybe due to the high concentration of the buffer, the result was recommendable visible and clearer. As shown in the table below.

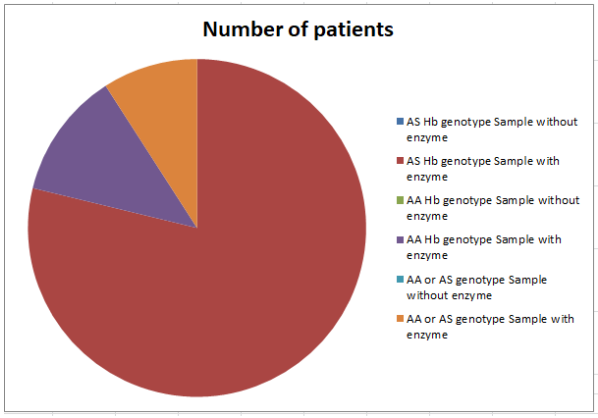

Figure 2: Analysis of Gel electrophoresis

Table 4: Result Patient Wise

Electrophoresis well number	Type of sample treatment
1	Patient 1 sample without enzyme.
2	Patient 1 sample with the enzyme.
3	Patient 2 sample without enzyme.
4	Patient 2 sample with the enzyme.
5	Patient 3 sample without enzyme.
6	Patient 3 sample with the enzyme.
7	A negative control without enzyme.
8	Negative control with enzyme

DISCUSSION

In this discussion section, the principle behind the separation of the DNA is demonstrated (Scott, 1977). Now that all DNA molecules are negatively charged, and the amount of charge depends on the size of the strand (Fritsch, 2009). Long stranded DNA sequence has more charge as compared to the short chain DNA. In the gel electrophoresis, electric current is used to separate the DNA materials based on the fact that when a DNA molecule is subjected to an electric current, it will migrate to the positive terminal where the short fragments will move faster as compared to the long chain. (Bright JA, 2015) The long chain, due to their weight, they will lag in the gel as the short chains are carried faster by the buffer used (Scott, 1977). When they are treated with the same restriction enzyme (*Bsu*36I), the normal DNA fragment will be cut at a specific sequence (CCTˆNAGG) resulting to a 331 bp and 200 bp from the 531 bp fragment (Jeffreys,

1772). Hence the HbA will be cut once by the RE, while the mutated HbS will not be cut. The control PCRs were important because they give a point of reference to the counting of the number of fragments present. (Taylor D, 2013)

From our results, those patients that showed two bands are heterozygous sickle (AS) because of one more strand in the well with RE. Patients that showed two bands on both wells mean that he/she is homozygous SS (Jeffreys, 1772). The other patients on the other side will be AA or AS due to the presence of one more bands due to lack of cutting by the RE.

Therefore, the sample that shows two bands represents the patients who are homozygous AA as the DNA fragment was only cut to form 331bp and 200bp. The one that shows three bands means the patients are a heterozygous carrier for the anaemic gene (AS) due to many incomplete RE recognition sequence that made many fragments (Scott, 1977). The last patients' category will show a single band which means that he/she is homozygous sickle cell anaemia as it had no recognizable sequences for cleaving by the RE (Jeffreys, 1772). It can be concluded that the ARMS-PCR has a high performance and 75% sensitivity hence can be used to study and detect patients with SCA mutation as a prenatal diagnostic facility.

CONCLUSION

Detection of sickle anaemia requires a lot of information about the restriction enzymes to be restudied. It is easy to detect an irregularity in the human genome whenever any alteration is done more especially due to mutation. Sickle cell anaemia is one of the most studied genetic diseases that can be treated if noticed in the early stage through gene therapy. Although gene therapy is an escape technology from genetic disorders like sickle anaemia, I recommend the legislative banning of all human trials of somatic cell therapy before the technique is fully understood. Genetic modifications of a somatic cell are very lethal hence there is a need to reduce the risks that patients undergo during the trials. Also in the present status of the gene therapy, germ-line gene therapy should be outlawed, and the government should fully control the application of the biotech since privatization may lead to misapplication.

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