Gel electrophoresis in analysis of sickle cell genes

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This experiment was carried out in order to outline a scheme for the investigation of Sickle Cell Gene Analysis and to understand and carry out the agarose gel electrophoresis. Besides that, the importance of restriction enzyme and the mechanism of the enzyme in separating the specific amino acid sequence were completely understood. The fragment bands observed on the gel electrophoresis from the experiment results was interpreted and understood. The controls (tube A-C) and 3 DNA samples [tube D (Mother), E (Unborn Child), F (Father)] which was amplified and digested with the MST 11 enzyme was analysed for the sickle cell genes using gel electrophoresis. Once the electrophoresis apparatus and the gel were prepared, the controls and the samples were loaded consecutively into the respective wells. The power source was set at 125V and left to run for 30 minutes. Following that, the gel was then stained using the Ethidium Bromide staining card. The gel was left to stain for 15 to 20 minutes and was later observed under a UV Transilluminator. Tube B (control), D (Mother) and F (Father) was heterozygous for Sickle Cell Disease, Hb AS whereas tube E (Unborn child) was homozygous for Sickle Cell Disease, Hb SS. Sickle Cell gene analysis is essential for reasons such as preparation of financial source to carry out treatment for the child if the child appears to be Hb SS. Treatments such as bone marrow transplantation and blood transfusion can be done to manage and treat Sickle Cell Disease children.

Introduction

Sickle Cell Disease (SCD) is an inherited blood disorder, characterized by the presence of sickle shaped red blood cells (Figure 1) (Wun and Hassell, 2009) (Oniyangi and Omari, 2006) (Stuart and Nagel, 2004).

http://www. biologycorner. com/anatomy/blood/sickle_cell_anemia2. jpg

Normal healthy individuals carries 3 main haemoglobins; Haemoglobin A (Hb A), Haemoglobin A2 (Hb A2) and Haemoglobin F (Hb F) (Wood et al, 1976). In Hb A, the glutamic acid (GAG) is situated on the 6th position of the β- globin chain. Mutation of a single nucleotide (GAG to GTG) (Pan et al, 2007) (Stuart and Nagel, 2004) which takes place in the 6th position of the β- globin chain (Figure 2) (Wun and Hassell, 2009) (Cleon et al, 2009) of Hb A results in the formation of Haemoglobin S (Hb S) which causes SCD (Pan et al, 2007) (Cleon et al, 2009) (Stuart and Nagel, 2004). The acidic (negative charged) glutamic acid is replaced by the polar (neutral) valine (Frenette and Atweh, 2007) (Cleon et al, 2009) (Stuart and Nagel, 2004).

Sickle cell genes (autosomal recessive) are inherited from the parents (Figure 3) (Frenette and Atweh, 2007) (Wun and Hassell, 2009). One copy of gene are passed down from each parent to the fetus, therefore, a fetus will have 2 copies of genes to make the β- globin. Heterozygous SCD patients (carriers) (Hb AS) had inherited 1 copy of normal gene and 1 copy of defective gene whereas homozygous SCD patients (Hb SS) had inherited 2 copies of defective genes (Frenette and Atweh, 2007) (Makani et al, 2010) (Oniyangi and Omari, 2006). The amount of defective genes in heterozygous patients is insufficient to cause symptoms because they have a proportion of normal genes as well. (Makani et al, 2010). Therefore, most SCD carriers are unaware of the presence of Hb S in them. Homozygous SCD patients has insufficient normal genes; therefore symptoms of SCD are clearly demonstrated by them (Makani et al, 2010) (Oniyangi and Omari, 2006).

The image shows how sickle cell genes are inherited. A person inherits two copies of the hemoglobin gene-one from each parent. A normal gene will make normal hemoglobin (A). An abnormal (sickle cell) gene will make abnormal hemoglobin (S). When each parent has a normal gene and an abnormal gene, each child has: a 25 percent chance of inheriting two normal genes; a 50 percent chance of inheriting one normal gene and one abnormal gene; and a 25 percent chance of inheriting two abnormal genes.

If both parents are carriers for SCD, the child has a 50% chance of being carriers, 25% chance of being homozygous for SCD and 25% chance of having 2 copies of normal genes (National Blood, Lung and Heart Institute).

Sickle Cell gene analysis is an important screening test for a married couple. The fetus should be screened for SCD if one parent is a carrier for SCD (Tshilolo et al, 2008) (Streetly et al, 2009). It is important to ensure whether the child is heterozygous or homozygous for SCD for reasons such as financial preparation for treatment of the child (if child is homozygous for SCD) (Streetly et al, 2009) (Tshilolo et al, 2008).

Sickle Cell gene analysis can be done using gel electrophoresis. The Deoxyribonucleic Acid (DNA) sample can be obtained from white blood cells (in adults) and from the amniocentesis fluid (in fetus). The samples can then be amplified with Polymerase Chain Reaction (PCR) (Rahimi et al, 2008) (Bruzzone et al, 2008). PCR produces copies of DNA which had been obtained from a small sample, to ensure the amount of sample is sufficient for the SCD gene analysis (Bruzzone et al, 2008) (VanGuilder et al, 2008).

The multiplied DNA sample will then be digested with a restriction enzyme (Sasnauskas et al, 2007) (Rahimi et al, 2008), for instance MST 11. This enzyme recognizes the CCT-GAG-G sequence of the amino acid on the β-globin and will bind to cleave (cut) that DNA strand which it recognizes (Figure 4). Due to the substitution of amino acid in SCD (CCT-GTG-G), the MST 11 enzyme cannot recognize that mutated sequence so therefore, will not cut that mutated DNA strand (Sasnauskas et al, 2007). As a result, the normal genes will be cut by the enzyme and will become short fragments whereas the sickle cell genes will be an uncleaved long fragment. The DNA from a normal individual (Hb AA), SCD carrier (Hb AS), and SCD homozygous individuals (Hb SS) produces fragments of different sizes and lengths (Rahimi et al, 2008) after digested with the restriction enzyme.

Figure 4: The picture on the (left) shows the sites recognized and would be cleaved by the restriction enzyme in a normal β- globin allele, whereas the enzyme would not cut the altered β- globin allele (SCD). The picture on the (right) shows the different fragments bands of different sizes and lengths demonstrated in a gel electrophoresis (www. bio. miami. edu)

These fragments are then separated by gel electrophoresis (Figure 4). Gel electrophoresis is used to separate molecules such as DNA by using an electric field applied to a gel matrix. Smaller DNA fragments are able to squeeze through the pores of the gel, compared to the larger fragments (Rahimi et al, 2008). The sickle cell genes (long fragments) will be slowest to move compared to the normal genes which had been cut (short fragments). The electrophoresis gel will finally be stained and observed under an Ultra Voilet Transilluminator to view the bands produced.

This experiment was carried out in order to outline a scheme for the investigation of Sickle Cell Gene Analysis and to understand and carry out the agarose gel electrophoresis. Besides that, the importance of restriction enzyme and the mechanism of the enzyme in separating the specific amino acid sequence were completely understood. The fragment bands observed on the gel electrophoresis from the experiment results was interpreted and understood.

Method

Gel Bed Preparation

The open ends of a clean and dry gel bed (casting tray) was closed by using a tape. The ¾ inches wide tape was extended over the sides, and the bottom edges of the bed. The extended edges of the tape were then folded back onto the sides and bottom. The contact points were pressed firmly to form a good seal. Next, the well former template (comb) was placed in the first set of notches at the end of the bed. The comb was placed firmly and evenly across the bed.

Casting Agarose Gels

Agarose gel (0. 8g), concentrated buffer (2ml of 50X), and distilled water (98ml) were added into a 250 ml flask to prepare the gel solution. The volume in the flask which gave the total volume of 100 ml was marked with a marker pen. Next, the mixture was swirled to disperse the agarose powder clumps. The level of the solution on the outside of the flask was indicated with a marker pen. The mixture was then heated to dissolve the agarose powder. The flask was covered with a plastic wrap to minimize the

evaporation rate. The mixture was then heated in high temperature for a minute. After that, the mixture was swirled every 25 seconds while it was heated in high temperature, until all the agarose was completely dissolved. The final solution produced was clear (like water), without any undissolved particles in it. The agarose solution was then cooled to 550 C by swirling it carefully, so even dissipation of heat was promoted. Distilled water was added to bring the solution up to the original volume as marked on the flask when evaporation was detected. The interface of the gel bed sealed with the tape, was checked once the gel has cooled slightly to avoid leakage of the agarose solution. A transfer pipette was used to deposit a small amount of cooled agarose solution to both ends of the bed and left for a minute for the agarose to solidify. Finally, the bed was placed on an even surface and the cooled agarose solution was poured onto the bed. The gel was allowed to completely solidify. The gel appeared firm and cool after approximately 20 minutes.

Gel Preparation for Electrophoresis

The tape was carefully and slowly removed from the gel bed once the gel was completely solidified. The comb was slowly pulled straight up to be removed. This was done carefully and evenly to prevent the sample wells from tearing. The gel (on the bed) was placed into the electrophoresis chamber, orientated properly and centred on a level and even platform. The 50X buffer was then diluted in distilled water to prepare a 500 ml of 1X buffer. Once the buffer was prepared, it was poured into the electrophoresis apparatus chamber. The gel was completely covered with the buffer. The

samples were then loaded to conduct the electrophoresis, as discussed below.

Loading of Samples

The sample volumes were checked to ensure small amounts of samples were not clanged onto the walls of the tubes. The entire volume of the samples was ensured to be at the bottom of the tubes, before the gel was loaded with the samples. 25µl of DNA samples from tubes A to F was loaded into the wells in a consecutive order (Table 1).

Running the Gel

The cover was snapped down carefully onto the electrode terminals after the DNA samples were loaded. The negative and positive colour coded indicators on the cover and the apparatus chamber was properly orientated. The plug of the black wire was inserted into the black input of the power source (negative input) and the plug of the red wire was inserted into the red input of the power source (positive input). The power source was set at 125V and the electrophoresis was conducted for 30 minutes. Bubbles observed on the two platinum electrodes indicated that the current was flowing properly. Once the electrophoresis was completed, the power was turned off and the plug was unplugged from the power source. The leads were disconnected and the cover was removed. The gel was removed from the bed to be stained with Ethidium Bromide.

Staining the Gel

After electrophoresis, the gel was placed on a flat surface and was moistened with several drops of the electrophoresis buffer. The Ethidium Bromide staining card adhesive was removed and placed onto the surface of the gel (where the wells were). Fingers were firmly run over the entire surface of the card several times. Once that has been done, an empty beaker or the casting tray was kept on top of the gel with the staining card to maintain good contact between the gel surface and the card. The gel was left to stain for about 15 to 20 minutes. The card was then removed from the gel surface and the surface of the gel was rinsed with the buffer. The gel was then examined on a Visible Light Gel Visualization System or an Ultra Violet Transilluminator. The fragment bands formed on the surface of gel was observed, and interpreted.

Discussion

Based on the results obtained, Sample A (Control for Normal Gene) had only 2 bands on the gel when observed under the UV Transilluminator. This is because, the restriction enzyme, MST 11 only cuts the amino acid sequence which it recognises. The MST 11 had cut the CCT- GAG- G sequence from the DNA strand of this normal Hb A gene. Therefore, two bands are formed instead of 1 band. The long fragment had now been cut to two short fragments. Since the MST 11 only recognises the CCT- GAG- G sequence which is on the beginning of the strand, this band appears shorter than the remaining band after it has been cut. Short fragments are able to squeeze through the gel pores easily compared to the longer fragments. Since glutamic acid (GAG) is negatively charged, and is therefore shorter than the

remaining fragment, this fragment travels faster and further from the well towards the anode (positive terminal) compared to the remaining fragment.

Sample D (Mother’s DNA) had the same results as Sample F (Father’s DNA) and Sample B (Control for Sickle Cell Trait Gene). There were 3 bands observed on the gel under the UV Transilluminator. Since these samples are heterozygous for SCD (Hb AS), they have inherited 1 copy of normal gene and 1 copy of defective gene. The restriction enzyme did not recognize the sickle cell gene (CCT- GTG- G). Therefore, the MST 11 enzyme did not cut that sequence. As a result, that fragment appeared to be 1 long fragment and could not easily squeeze through the pores of the agarose gel. As a result, this fragment was not able to move fast and further from the well. However, the MST 11 restriction enzyme recognised and had cut the normal gene which was present. Therefore, when the DNA strand was cut, two shorter fragments were formed which easily travelled through the pores and were able to travel away from the wells. The A gene with the shorter fragment travelled further compared to the remaining fragment.

Sample E (Unborn Child’s DNA) had the same results as Sample A (Control for Sickle Cell Anaemia Gene). Only 1 band was observed on the gel under the UV Transilluminator. Since the sample is homozygous for sickle cell (Hb SS), the baby had inherited 2 copies of defective gene from the parents. The restriction enzyme did not recognize the Sickle cell gene (CCT- GTG- G) and did not cut the DNA strand. As a result, the fragment remained as 1 long fragment and was not able to squeeze though the pores of agarose gel easily. This fragment moved extremely slow and could not move very far

from the wells. The inheritance pattern which had caused the child to be Homozygous for SCD had been discussed above (Figure 3).

SCD can lead to complications such as anaemia, jaundice and stroke if left untreated (Pan et al, 2007) (Nagababu et al, 2008). This is because, when substitution of amino acid occurs due to the mutation, a new longitudinal polymer which has valine in the 6th position of the beta chain instead of glutamic acid is polymerized . Haemoglobin S is formed due to this. During hypoxia (less oxygen supply to tissues), aggregation of haemoglobin takes place due to the presence of the hydrophobic valine instead of the polar glutamic acid in the 6th position of the β -globin chain (Cleon et al, 2009) (Stuart and Nagel, 2004). As a result, red blood cells with sickle shape which are fragile and not flexible are formed (Nagababu et al, 2008) (Pan et al, 2007) (Manchikanti et al, 2007).

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Figure 5: The complications of Untreated Sickle Cell Disease (ICAGEN Ion Channel Advances, 2010)

Children with SCD will normally be closely observed by a paediatrician in order to ensure they are healthy. These children will be subscribed with folic acid to be taken daily to stay healthy. Besides that, penicillin is also required in order to prevent them from getting illnesses due to their weak and immature immune system (Ndefo et al, 2009).

Complications due to Sickle Cell Disease

Treatment

Streptococcus Pneumoniae sepsis

Newborn – 5 years (Penicillin VK)

2years and above (23- valent Streptococcus Pneumoniae polysaccharide vaccine (PPV23)

Bone marrow Aplasia and Megaloblastic erythropoiesis

Folic acid

Stroke

Blood transfusions

Pain Episodes

Hydroxyurea

(complete blood count monitored every 2 weeks, Hb F monitored every 6 -8 weeks, and serum chemistries monitored every 2- 4 weeks)

Other than that, hydroxyurea are also given to children with SCD. Hydroxyurea works by activating the fetal haemoglobin production to replace the production of Haemoglobin S which results in SCD (Ndefo et al, 2008). Children with SCD who has a high risk for developing stroke, will be given blood transfusion frequently (Vichinsky et al, 2007) (Kirkham, 2007). A blood transfusion replaces the sickle cells with normal red blood cells which leads to a good supply of oxygen to the brain, which will reduce the chances of stroke in children (Kirkham, 2007) (Nagababu et al, 2008). Unfortunately, blood transfusions causes iron overload in the body because the body lacks

mechanism to excrete iron (Vichinsky et al, 2007). An overload of iron results in iron deposition in various organs which can be fatal if untreated (Vichinsky et al, 2007). Therefore, children with regular blood transfusions will be given chelating agents (Vichinsky et al, 2007) such as desferrioxamine to excrete iron from the body through the urine and faeces.

Bone marrow transplantation is the only cure for SCD (Ndefo et al, 2008). The affected bone marrow will be replaced with a closely matched Human Lymphocyte Antigen (HLA) bone marrow from a healthy individual (Ndefo et al, 2008). Bone marrow transplants enables , new and normal healthy red blood cells to be produced. The disadvantages of bone marrow transplantations are the occurrence of Graft – Versus- Host Disease (GVHD). Based on the study by Ndefo et al (2008), it was suggested that there will be an 85% chance of survival free from SCD, 7% chance of developing bone marrow transplant complications and a 9% chance of GVHD failure rate with a bone marrow transplant procedure.

Conclusion

As a conclusion, gel electrophoresis is found to be an excellent way to aid in the analysis of sickle cell genes. Using gel electrophoresis, many samples can be analysed at the same time, and its results are specific and accurate. The disadvantages of this method are that analytical errors might occur due to the large amount of sample handling and it is costly. Although it is costly, this method can increase the awareness of the carriers of SCD about the presence of Hb S in them, which can aid in their future family planning. Children with homozygous SCD, can be prevented from suffering from SCD complicatio