Cystic Fibrosis Case Study: Genetic Diagnosis of Disease by RFLP
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Abstract:

A three-year old male patient is suspected of having cystic fibrosis. There was no available family history. Crackling and wheezing were been observed on strong exhalation, and he had greasy/oily stools. RFLP analysis, electrophoresis, and a standard curve were used to determine if the ΔF508 mutation was present in the CFTR protein. The patient’s DNA was compared to the positive and negative controls. Cuts were made with two enzymes (Eco R1 and Bam HI). The Bam HI enzyme cuts the CFTR gene at about 1500 bp if a mutation was present because that is where the recognition site is. The Bam HI restriction enzyme searches for the ΔF508 mutation. The patient’s DNA had a cut at about 1100 bp, which is just shy of 1500bp, indicating that the patient has cystic fibrosis. Other tests, such as a sweat test can be performed as well to support the findings. Possible sources of error include determination of distance travelled in cm and incomplete digestions because of a short incubation period.

Introduction:

The object is to become more familiar with techniques that are often used in the field of cellular biology and genetic engineering to determine if a patient has cystic fibrosis. The patient is a three-year-old male that is suspected of having cystic fibrosis. His vital signs are relatively normal and he is energetic. There is no available family history. Crackling and wheezing have been observed on strong exhalation, and he has greasy/oily stools. Cystic fibrosis is an autosomal recessive disorder that mainly affects the lungs, though it also has a strong impact on the liver, intestines, and pancreas (Dalemans, 1991). It leads to thick secretions because the protein that regulates the transport of sodium and chloride across membranes is affected. Individuals with this disease have frequent lung infections and trouble breathing (Dalemans, 1991).

Genetic abnormalities that are associated with diseases such as cystic fibrosis can be identified by using restriction length polymorphisms (RFLP). Restriction enzymes are used to cut DNA and electrophoretic analysis is used to find base pair lengths. Restriction enzymes are endonucleases that are capable of cleaving the phosphate bonds in both DNA stands and require Mg$^{+2}$. At the point of cleavage, they generate a 5 prime phosphate and a 3 prime hydroxyl group. Restriction enzymes are highly specific, and only cut at special sequences called palindromes. These recognition sites are four to eight base pairs in length and both strands of DNA have the same sequence of bases when read in the 5 prime to three prime direction. When the DNA is cut by restriction enzymes, “sticky” or “blunt” ends are formed. Sticky ends occur when single-stranded regions of the ends are complementary, and blunt ends occur when cut are opposite each other. The size of DNA fragments generated depends on the distance between recognition sites. Though RFLP analysis is an excellent way of testing for cystic fibrosis, a sweat test is another diagnostic tool that can be used. Individuals with cystic fibrosis will have a much higher salt concentration in their sweat.
Genetic diseases, such as cystic fibrosis, are caused by mutations in gene encoding proteins that are essential for cellular activity. Something as small as a mutation in a single nucleotide can affect the resulting amino acid. If the sequence of amino acids is affected, the protein structure and folding will unavoidably impact the protein’s function. Mutations associate with disease can be diagnosed by RFLP analysis because point mutation associate with certain diseases affect the restriction enzyme sites. A common mutation associate with cystic fibrosis is called ΔF508 and is a deletion of three base pairs that result in the removal of the phenylalanine amino acid at position 508 of the cystic fibrosis transmembrane conductance regulator protein. There are over 600 mutations associate with cystic fibrosis; various mutations are affected to differing levels of severity. Nearly 70% of individuals with cystic fibrosis have the ΔF508 mutation.

After DNA is treated with a restriction enzyme, the fragments are separated according to size by agarose gel electrophoresis. Due to the negative charge on DNA due to the phosphate groups, it migrates towards the positive electrode. Smaller fragments can more through the gel easily and much faster because the gel acts like a sieve. Therefore, smaller fragments are closer to the positive electrode. Fragment length can be determined by the distance it migrates during gel electrophoresis due to the logarithmic relationship between molecular weight and electrophoretic mobility. A standard curve is used to determine the size of unknown fragments of DNA. If the patient’s DNA is cut with various restriction enzymes (EcoR1 and BamHI) and gel electrophoresis is performed, it will be possible to conclude that the patient has cystic fibrosis.

Materials & Methods:

Samples of a negative control and the patients DNA were obtained. Four microcentrifuge tubes were labeled and reaction buffer was added to each one. The patient’s DNA was transferred to two of the microcentrifuge tubes and the negative control was transferred to the other two microcentrifuge tubes. Enzyme 1 (EcoR1) was added to the to a tube containing the patients DNA and a negative control; Enzyme 2 (BamHI) was added to the two remaining microcentrifuge tubes that contained the patient’s DNA and a negative control. The tubes were incubated. While the restriction digests were incubating, a .8% agarose gel was prepared. This was done by mixing agarose and TAE buffer and then heating it. Ethidium bromide was added and the liquid was placed in a gel holder and left to cool. After it was cooled, the comb was removed and set aside and the gel was transferred to an electrophoresis chamber and covered with TAE buffer. The four tubes with the patients DNA and negative control were removed from the water bath and loading dye was added to each tube. A tube of marker DNA and positive control DNA cut with Enzyme 1 and 2 were obtained. Each of the samples was loaded into a separate well. The apparatus was placed on top of the gel and an electric current ran through the gel at 120V. After the DNA fragments travelled far down the gel, the apparatus was turned off and the gel was taken to a UV transilluminator to be viewed under UV light.

Results:
DNA that was positive and negative for the particular cystic fibrosis mutation as well as the patient’s DNA were cut by two different restriction enzymes (Eco R1 and Bam HI) for a total of six digests. After the digests, the samples were ran on an agarose gel and fragment sizes were calculated by making standard curve graph from the known molecular weight ladder values. The values for the molecular weight ladder (in bp) were: 23100, 15000, 12000, 8000, 4700, 3500, 3000, 1800, and 1500. When the positive and negative control DNA were cut by the Eco R1 restriction enzyme, the base pair lengths of the two observed fragments were 6800 and 2600 base pairs in length. When the DNA was cut by the Bam HI restriction enzyme, the positive control DNA had two fragments that were 7600 and 1100 base pairs in length. The negative control DNA that was cut by the Bam HI enzyme had three fragments that were 6800, 1200, and 1000 base pairs in length. When the DNA was cut by the Bam HI restriction enzyme, the positive control DNA had two fragments that were 7600 and 1100 base pairs in length. The negative control DNA that was cut by the Bam HI enzyme had three fragments that were 6800, 1200, and 1000 base pairs in length. When the patient’s DNA was cut by the Bam HI restriction enzymes there were two resulting fragments that were 7600 and 1100 base pairs in length. The patient’s DNA matched both of the positive controls. It is also important to note that two phantom bands appeared above the 6800 bp fragment of the patient’s DNA that was cut by Eco R1.

**Figure 1: Gel with DNA cut by Restriction Enzymes**

<table>
<thead>
<tr>
<th>Ladder</th>
<th>Eco R1</th>
<th>Bam HI</th>
<th>Patient Eco R1</th>
<th>Patient Bam HI</th>
</tr>
</thead>
<tbody>
<tr>
<td>(+) control</td>
<td>(-) control</td>
<td>(+) control</td>
<td>(-) control</td>
<td>(+) control</td>
</tr>
</tbody>
</table>

Pictured above is an image of the gel that depicts a molecular weight ladder and the distance travelled by each DNA fragment after being cut by either the Eco R1 enzyme of Bam HI enzyme.
Figure 2 is the standard curve of the known molecular weight ladder. The Y axis is logarithmic and the trendline is exponential. The best fit line is \( 53973e^{-1.09x} \).

### Table 1: Size (bp) of Each Band

<table>
<thead>
<tr>
<th>Length of DNA fragment (bp)</th>
<th>Ladder</th>
<th>(+) Control Eco R1</th>
<th>(-) Control Eco R1</th>
<th>(+) Control Bam HI</th>
<th>(-) Control Bam HI</th>
<th>Patient Eco RI</th>
<th>Patient Bam HI</th>
</tr>
</thead>
<tbody>
<tr>
<td>23,100</td>
<td>15,000</td>
<td>12,000</td>
<td>8,000</td>
<td>4,700</td>
<td></td>
<td>6,800</td>
<td>7,600</td>
</tr>
<tr>
<td>3,500</td>
<td>3,000</td>
<td>1,800</td>
<td>1,500</td>
<td></td>
<td></td>
<td>2,600</td>
<td>1,100</td>
</tr>
<tr>
<td>2,600</td>
<td>2,600</td>
<td>1,100</td>
<td></td>
<td></td>
<td></td>
<td>2,600</td>
<td>1,100</td>
</tr>
</tbody>
</table>

Table 1 represents the known base pair length of the molecular weight ladder and the calculated values of every DNA fragment in the sample that were cut by restriction enzymes Eco R1 and Bam HI.

**Sample Calculation for Unknown DNA Fragment Length (bp)**

Ex:

Line of Best fit from graph: \( y = 53973e^{-1.09x} \)
Discussion:

After the patient’s DNA was cut with the Eco R1 and Bam HI restriction enzymes, it was concluded that the three year old male patient has cystic fibrosis. DNA that was positive and negative the ΔF508 mutation was cut by two different enzymes (Eco R1 and Bam HI). The patient’s DNA was also cut using the same enzymes. After the six digests, the samples were ran on an agarose gel and fragment sizes were calculated by making standard curve graph from the known molecular weight ladder values. When the positive and negative control DNA were cut by the Eco R1 restriction enzyme, the base pair lengths of the two observed fragments were 6800 and 2600 base pairs in length. When the DNA was cut by the Bam HI restriction enzyme, the positive control DNA had two fragments that were 7600 and 1100 base pairs in length. The negative control DNA that was cut by the Bam HI enzyme had three fragments that were 6800, 1200, and 1000 base pairs in length. The patient’s DNA was cut by the Eco R1 enzyme and the fragment sizes were 6800 and 2600 base pairs in length. When the patient’s DNA was cut by the Bam HI restriction enzymes there were two resulting fragments that were 7600 and 1100 base pairs in length. The patient’s DNA was identical to both of the positive controls, indicating that the enzyme cut at the exact same place. Because the patient’s DNA was cut at the same points as the positive controls, it can be concluded that the patient has cystic fibrosis. Two phantom bands appeared in the gel image above the 6800 bp fragment of the patient’s DNA that was cut by Eco R1. These phantom bands can be attributive to an incomplete digest, and would not have appeared if the incubation time was extended (Dalemans, 1991).

Restriction enzymes search for recognition sites that are palindromes in order to determine where to make a cut. The Eco R1 enzyme made two identical cuts for the negative and positive controls, which indicates that the recognition site was not for the cystic fibrosis mutation (ΔF508 ) in the CFTR gene (Dalemans, 1991). The first enzyme (Eco R1) was merely used to ensure that DNA was present and that there was human DNA in the sample. The Bam HI enzyme cuts the CFTR gene at about 1500 bp if a mutation is present because that is where the recognition site is. The Bam HI restriction enzyme searches for the ΔF508 mutation. The patient’s DNA has a cut at about 1100 bp, which is just shy of 1500bp (Luca, 2013).

The positive and negative controls are necessary in a diagnostic experiment because the patient’s DNA must be compared to the positive and negative control in order to determine if the mutation is present in the patient’s DNA (Dalemans, 1991). In a positive control, and variable is changed to reflect an expected outcome. In this case, the positive control is the presence of the ΔF508 mutation in the CFTR gene (Luca, 2013). The negative control is compared to the positive control because it does not have any variables changed (it doesn’t reflect expected result) and can thus be contrasted to the result and positive control. The patient’s DNA had the same cuts made by both enzymes, and was identical to the positive control DNA. The loss of a single amino acid in a protein can be utterly devastating because there is a direct correlation between protein structure and function (Luca, 2013). The absence of phenylalanine due to the ΔF508 mutation affects the overall structure and folding of the resulting CFTR protein, which inhibits its ability to function and regulate chloride and sodium transport across membranes (Luca, 2013).
There are many possible sources of error. The main source of error could be due to the standard curve. The distance travelled in cm can be off slightly, which would have a huge impact on the calculated bp length of the fragments. Another source of error could be the that the digests were not left to incubate for a long enough time, which led to the appearance of the phantom bands. The incomplete digests present there may indicate that there are other incomplete digests and that not all fragments travelled to where they were meant. Another source of error may be failing to change tips frequently, that would lead to contamination that would ruin the integrity of the samples. Overall, it can be concluded that the patient has cystic fibrosis because of the mutation present that led to the deletion of the phenylalanine amino acid. The ΔF508 mutation is present in 70% of individuals with cystic fibrosis. In order to have a more conclusive diagnostic, at least one more of the 600 possible cystic fibrosis mutations should be tested to see if they are present. A sweat test could be performed as well to fully conclude that the patient has cystic fibrosis.

References:
