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Detection Of Mutation In DNA Sequences By RFLP Method

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Abstract: ***PCR-restriction fragment length polymorphism (RFLP)- based analysis , also known as cleaved amplified polymorphic sequence (CAPS), is a technique for genetic analysis. It has been applied for the detection of intraspecies as well as interspecies variation. There exist several techniques that are related with PCR-RFLP and also involve gel electrophoresis including techniques for DNA fingerprinting and expression profiling.***

Polymorphism is defined as inherited differences found among the individuals in more than 1% of normal population.

The technique exploits that SNPs (single nucleotide polymorphism is a variation at a single position in a DNA sequence among individuals), MNPs (Multiple nucleotide polymorphism) and microindels (small insertion and deletions of bases in the DNA) often are associated with the creation or abolishment of a restriction enzyme recognition site (Narayanan, 1991). Since the presence or absence of the restriction enzyme recognition site results in the formation of restriction fragments of different sizes, allele identification can be done by electrophoretic resolvement of the fragments.

It is the most commonly used reference standard for genotyping of Factor V Leiden and prothrombin G20210A (Emadi et al., 2009). It has also been used for a variety of other purposes including detection of the JK allele associated with a Kidd-null phenotype, determination of apolipoprotein E (APOE) alleles.

PCR-RFLP consists of several steps including design of primers, identification of an appropriate restriction enzyme (s), amplification, restriction enzyme treatment of amplified products and electrophoresis to resolve the restriction fragments. Untill recently, it was the preferred technique for identification and differentiation of microbacterial (a genus of bacteria in the family Microbacteriaceae) species.

Thalassemia is impaired production of globin chain. Children with thalassemia major are treated with regular blood transfusions to keep their Hb level around 10 g/dl. The causes of thalassemia are mutations (deletional and non deletional forms).

I. INTRODUCTION

Restriction Fragment Length Polymorphism (RFLP) is a technique in which organisms may be differentiated by analysis of patterns derived from cleavage of their DNA. Polymorphisms are inherited differences found among the individuals in more than 1% of normal population. In general, it can be defined as a discontinuous genetic variation resulting in the occurrence of several different forms or types of individuals among the members of a single species. This divides the individuals of a population into two or more sharply distinct forms.

Restriction enzymes cut double-stranded DNA at specific recognition sequences, some mutations naturally create or abolish restriction enzyme sites. Computer programs are now available that allow sequences to be screened for putative recognition sites for restriction enzymes. Genomic DNA containing the mutation the 'target' is amplified by PCR and the product is then digested by the diagnostic restriction enzyme and the resulting DNA fragments separated on gels. The presence or absence of the recognition site is determined from the pattern of the PCR digest. Restriction enzyme analysis is simple, relatively cheap and robust leading to unequivocal results: the technique is an invaluable molecular tool. However, it is limited in its application as only a proportion of the haemoglobin variants. β – thalassemia and α – thalassemia mutations naturally create or abolish restriction enzyme cutting sites. Restriction endonucleases are usually

used when there is some other evidence suggesting the causative mutation, example HPLC, Hb electrophoresis analysis and ethnic origin of the individual or it is used as a secondary confirmation test (after sequence analysis). In some instances the sequence in the vicinity of the mutation allows one to artificially create restriction sites adjacent to the mutation sequence by introducing a single base mismatch in the amplification primer. This technique is referred to as amplification created restriction site (ACRS). It is routinely used to identify the Saudi Non Deletional α thalassemia mutation in the α2 – globin gene. (AATAAA to AATAAG).

A large number of studies have reported application of microchip electrophoresis in PCR-RFLP. For example, this approach is useful in the quality control and regulatory screening in enforcement laboratories. Using PCR-RFLP in commercially available Agilent 2100 microchip electrophoretic separation system, allele frequencies in DNA pools could be reliably estimated by measurement of fluorescence intensities of the enzymatically digested DNA fragments. Thus, PCR-RFLP can be used to achieve provisional information about the allele frequencies in different groups of individuals and to guide the design of large genetic association studies.

II. PROCEDURE

Restriction Fragment Length Polymorphism is a method in molecular biology evolved for detecting the variation at the DNA sequence level of various biological samples.The principle of this method is based upon the comparison of restriction enzyme cleavage profiles following the existence of a polymorphism in a DNA sequence related to other sequence in RFLP.DNA of individuals to be compared is digested with one or more restriction enzymes and the resulting fragments are separated according to molecular size using gel electrophoresis along with a molecular weight marker. Through this approach two individuals can present different restriction profiles.

RFLP requires a restricton enzyme which cuts the DNA at a specific location. These fragments are then separated into their different sizes using an electrophoresis agarose gel. The smaller fragments travel much faster through the gel and hence appear further along the gel strip. The fragments are labeled with P32. The probe binds to only specific regions on the fragments. Photographic film. The result is like a barcode image which could be compared to the known size standards.

III. MATERIALS REQUIRED

There are six basic components of PCR procedure (including RFLP):

- ✓ DNA template (clean DNA, no inhibitors).
- ✓ Primers. (A pair of synthetic oligonucleotides to primer DNA synthesis).
- ✓ DNA polymerase. (Taq polymerase – a thermostable DNA polymerase to catalyse the template)
- ✓ Magnesium. (MgCl2)

(NOTE: All thermostable DNA polymerases require free divalent cations- usually Mg2+ for activity. Some polymerases will work albeit less efficiently with buffers containing Mg2+. Ca ions are quite ineffective. The dNTPs and oligonucleotides bind Mg2+, the molar concentration of the cations must exceed the molar concentration of the phosphate groups contributed by dNTPs and primers.)

- ✓ dNTPs.
- ✓ Buffer - To maintain pH (Tris –Cl)
- ✓ Monovalent Cations.

IV. METHOD

The Steps Involved Are:

- ✓ DNA is extracted from specimen.
- ✓ DNA is digested with restriction enzyme to produce fragments.
- ✓ Double stranded DNA fragments separated by electrophoresis through slab gel.
- ✓ Denaturation of single stranded DNA fragments.
- ✓ Covering of the DNA fragments in a membrane and incubation.
- ✓ Washing to remove excess probe.
- ✓ Probe bound to complimentary DNA fragments but positions on membrane invisible.
- ✓ Visualization of banding pattern by exposure in X-ray film.
- ✓ The film is developed and DNA typing pattern is observed.

V. OBSERVATION

The primer sequences used for diagnosing Hb variants are listed:

The β globin gene cluster haplotype normally consiste of 5 RFLP's located in the 5' cluster (Hind II/eplison-gene; Hind III/G^Y-gene; Hind III/A^Y-gene; Hind II/3'psi beta-gene; Hind II/5'psi beta-gene) and two RFLPs in the 3' cluster (Ava II/β-gene; BamH I/β gene).

The restriction sites used in the procedure are:

- ✓ Xmn I
- ✓ Hind II(eplison)
- ✓ Hind III(GY)
- ✓ Hind III(AY)
- ✓ Hind II(5' psi β)
- ✓ Hind II(3'psi β)
- ✓ Rsa I (5' β).

Family

Female	CVS	Male
(+----+-)		(+-----+)
+	(+----+-)	+
(+-----+)	(------+)	(-----+-)

Table 1

In order, the sequences found were:

- ✓ (+-----+) (Most Common)

- ✓ (+----++)
- ✓ (+++--+-)
- ✓ (-----+-)
- ✓ (+----++)

VI. DISCUSSION

About 200 mutations account for beta thalassemia world wide but only 30 of these reach a frequency of 1% or more in the at risk groups (Flint et al. 1998). With the exception of the 619 bp deletion, which accounts for 20% of the beta thalassemia in Asian Indians, the common mutations are all point mutations because of single base substitutions, insertion or deletion of a few bases. The types of thalassemia and their description are stated below:

Name	Description	Allele
Thalassemia Minor	Only one of β- globin alleles bear a mutation. Individuals will suffer from Microcytic Anaemia. Detection usually involves lower than normal MCV value(<80 ft).	β^+/β or β^0/β
Thalassemia Intermedia	Affected individuals can often manage a normal life but need occasional Transfusions, at times of illness/pregnancy.	β^+/β^+ or β^0/β
Thalassemia Major	Occurs when both alleles have Thalassemia mutations. This is a severe Microcytic Hypocronic Anaemia. Untreated, it causes Anaemia, Splenomegaly and severe bone related anomalies. It progresses to death before 20 years of age. Treatment consists of periodic blood transfusions, Splenectomy for Splenomegaly and chelation pf transfusion caused iron overload.	B^0/β^0

Table 2

A small number of the beta- thalassemia mutations create or abolish a restriction endonuclease site in the globin gene sequence. Provided that the enzyme is commercially available and that there is not another site too close to the mutation , the loss or creation of a site can be used to diagnose the presence or the absence of the mutation . This is useful for diagnosis of a few of the common beta- thalassemia mutations. It exploits variations in homologous DNA sequences; It refers to a difference between the samples of homologous DNA molecules from differing locations of restriction enzyme sites. As because the Restricton Fragment Length Polymorphism is based on sequence homology, it is used in phylogenetic analysis between related species. These are locus specific markers and can be at the species and / or population levels (single-locus probes), or individual level (multi locus probes).

VII. CONCLUSION

The discussed process is one of the first DNA profiling techniques. RFLPs find their use in tracing inheritance patterns, identify specific mutations, and for other molecular genetic techniques. The compactness of the globin genes means that haemoglobinopathy detection is largely a PCR – based approach that can utilize direct sequencing analysis. Almost all haemoglobinopathies can be detected with the current PCR- based assays with the exception of a few rare deletions. However the molecular diagnostic service is still under development to try and meet the demands of the population it serves.

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