STUDY OF CLONING VECTORS, RESTRICTION ENDONUCLEASES AND DNA LIGASE. RECOMBINANT DNA TECHNOLOGY. APPLICATION OF GENETIC ENGINEERING IN MEDICINE, BRIEF INTRODUCTION TO PCR

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STUDY OF CLONING VECTORS

- **Gene Cloning**-The production of exact copies (clones) of a particular gene or DNA sequence using genetic engineering techniques.
- The DNA containing the target gene(s) is split into fragments using **restriction enzymes**. These fragments are then inserted into **cloning vectors** which transfer the recombinant DNA to suitable host cells.
- Inside the host cell the recombinant DNA undergoes replication; thus, a bacterial host will give rise to a colony of cells containing the cloned target gene.

CLONING VECTOR

- It is the central component of a gene cloning process.
- A small piece of DNA into which a foreign DNA fragment can be inserted.
- The insertion of the fragment is carried out by treating the vector and the foreign DNA with a restriction enzyme that creates the same overhang, then ligating the fragments together.

CHARACTERISTICS OF CLONING VECTORS:-

- Ori (Origin of replication) is a specific sequence of nucleotide from where replication starts
- It should have restriction sites: a synthetic multiple cloning site (MCS) can be inserted into the vector
- Replicate inside the host cell to form multiple copies of the recombinant DNA molecule.
- Less than 10kb in size.
- **Origin of Replication**: Allow the vector as well as the foreign DNA to amplify in the host cell
- **Multiple Cloning Sites**: Allow insertion of foreign DNA

- **TYPES OF CLONING VECTORS**:-They allow the exogenous DNA to be inserted, stored, and manipulated mainly at DNA level.
- **Types-**

1. Plasmid vectors
2. Bacteriophage vectors
3. Cosmids
4. Phagemids (type of plasmid)
5. Fosmids
6. Bacterial Artificial Chromosomes & Yeast Artificial Chromosomes

- **PLASMID VECTOR**:- Plasmid vectors are double-stranded, extra-chromosomal DNA molecules, circular, self-replicating.
- Contains an origin of replication, allowing for replication independent of host's genome
- **Advantages**: – Small, easy to handle
  – Easy purification
  – Straight forward selection strategies
  – Useful for cloning small DNA fragments (<10kb)
- **Disadvantages**: Less useful for cloning large DNA fragments (>10kb)

- **BACTERIOPHAGE**:-These are the viruses that specifically infect bacteria and during infection inject the phage DNA into the host cell where it undergoes replication.
- The phages are simple in structure and consist of DNA molecule having several genes for phage replication which is surrounded by a capsid made up of proteins.
- **Advantages**: – Useful for cloning large DNA fragments (10 - 23 kb)

  – Inherent size selection for large inserts
- **Disadvantages**: Less easy to handle

RESTRICTION ENDONUCLEASES

- Restriction enzymes are molecular scissors
- Molecular scissors that cut double stranded DNA molecules at specific points.
- Found naturally in a wide variety of prokaryotes.
- First restriction enzyme Hind II was isolated in 1970 by Hamilton O. Smith, Thomas Kelly and Kent Wilcox, from the bacterium *Haemophilus influenzae*.
- He also done the subsequent discovery and characterization of numerous restriction endonucleases.
- From then Over 3000 restriction enzymes have been studied in detail, and more than 600 of these are available commercially and are routinely used for DNA modification and manipulation in laboratories.

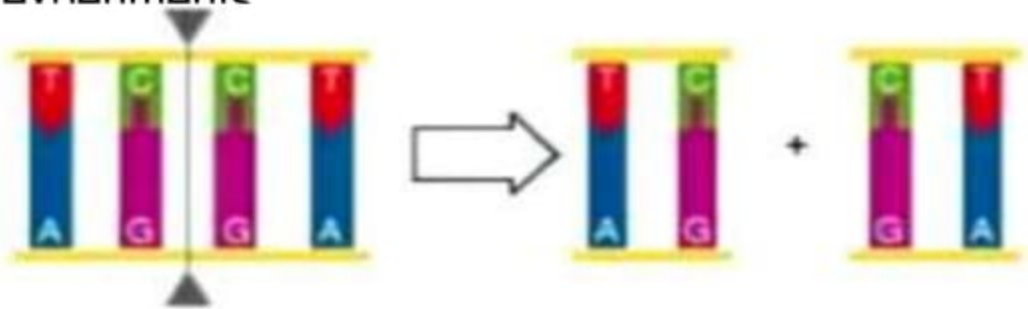
- **Mechanism of Action**:- Restriction Endonuclease scan the length of the DNA , binds to the DNA molecule when it recognizes a specific sequence and makes one cut in each of the sugar phosphate backbones of the double helix – by hydrolyzing the phoshphodiester bond.

PALINDROME SEQUENCE:-

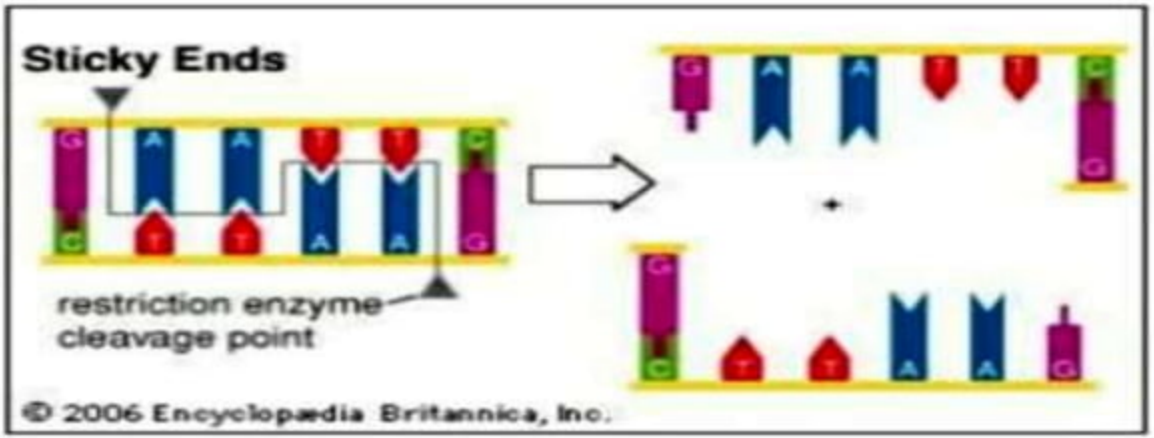
- The **mirror like palindrome** in which the same forward and backwards are on a single strand of DNA strand, as in GTAATG
- The **Inverted repeat palindromes** is also a sequence that reads the same forward and backwards, but the forward and backward sequences are found in complementary DNA strands (GTATAC being complementary to CATATG)
- Inverted repeat palindromes are more common and have greater biological importance than mirror- like

- **Fragments**:- (1)Blunt ends
  (2)Sticky ends

- **Blunt ends** - Some restriction enzymes cut DNA at opposite base
- They leave blunt ended DNA fragments
- These blunt ended fragments can be joined to any other DNA fragment with blunt ends.
- Enzymes useful for certain types of DNA cloning experiments



- **Sticky ends -** Most restriction enzymes make staggered cuts
- Staggered cuts produce single stranded "sticky-ends"
- "Sticky Ends" Are Useful DNA fragments with complimentary sticky ends can be combined to create new molecules which allows the creation and manipulation of DNA sequences from different sources.



- **NOMENCLATURE OF RESTRICTION ENZYME** :- Each enzyme is named after the bacterium from which it was isolated using a naming system based on bacterial genus, species and strain.
- For e.g. **EcoRI**

ABREVIATION	MEANING	DESCRIPTION
E	*Escherichia*	Genus
co	*coli*	Species
R	RY13	Strain
I	First identified	Order of identification in the bacterium

DNA ligase

- In biochemistry, ligase from the Latin verb *ligare* — "to bind" or "to glue together") is an enzyme that can catalyze the joining of two large molecules by forming a new chemical bond.
- DNA ligase is a specific type of enzyme, a ligase, that facilitates the joining of DNA strands together by catalyzing the formation of a phosphodiester bond DNA ligase has applications in both DNA repair and DNA replication.
- In addition, DNA ligase has extensive use in molecular biology laboratories for recombinant DNA experiments.
- Purified DNA ligase is used in gene cloning to join DNA molecules together to form recombinant DNA.

TECHNOLOGY

- **TOOLS OF RECOMBINANT DNA TECHNOLOGY**:- Restriction enzymes, Cloning vector, Competent Host cell
- **Competent Host cell-** since DNA is hydophilic molecule, it does not pass through the cell membranes easily (as its made up of lipids and proteins).

  - In order to force the bacteria to take up the plasmid, bacterial cells must be made 'competent' to take up the DNA.

  - It is done by treating them with specific concentration of a divalent cation, such as calcium, which increases the efficiency with which DNA enters the bacterium through pores in its cell walls.

  - Recombinant DNA can then be forced into such cells by incubating the cells with recombinant DNA on ice, followed by placing them briefly at 42ºC (heat shock), and putting them back on ice

- This is not the only way to introduce alien DNA into the host cell. In a method known as **micro-injection**, recombinant DNA is directly injected into the nucleus of an animal cell.
- In another method, suitable for plants, cells are bombarded with high velocity micro-particles of gold or tungsten coated with DNA in a method known as **Biolistics** or **gene gun**.

PROCESS OF RECOMBINANT DNA TECHNOLOGY

- **Isolation of DNA**
- **Fragmentation of DNA by restriction endonucleases**
- **Isolation of a desired DNA fragment**
- **Ligation of the DNA fragment into a vector**
- **Transferring the recombinant DNA into the host**
- **Culturing the host cells in a medium at large scale**
- **Extraction of the desired product**

1. Isolation of DNA

- In order to cut the DNA with restriction enzymes, it needs to be in pure form, free from other macro-molecules (such as RNA, proteins, lipids, polysaccharides).
- Since the DNA is enclosed within the membranes, we have to break the cell open to release the DNA
- This is achieved by treating the bacterial cells/plant/animal tissue with enzymes such as lysozyme (makes hole in the outermost layer of the cell), chitinase, cellulase (helps in separation)
- RNA can be removed by treatment with ribonuclease and proteins can be removed by using protease.
- Other molecules can be removed by appropriate treatment and purified DNA ultimately precipitates out after the addition of chilled ethanol

2. Fragmentation of DNA by restriction endonucleases

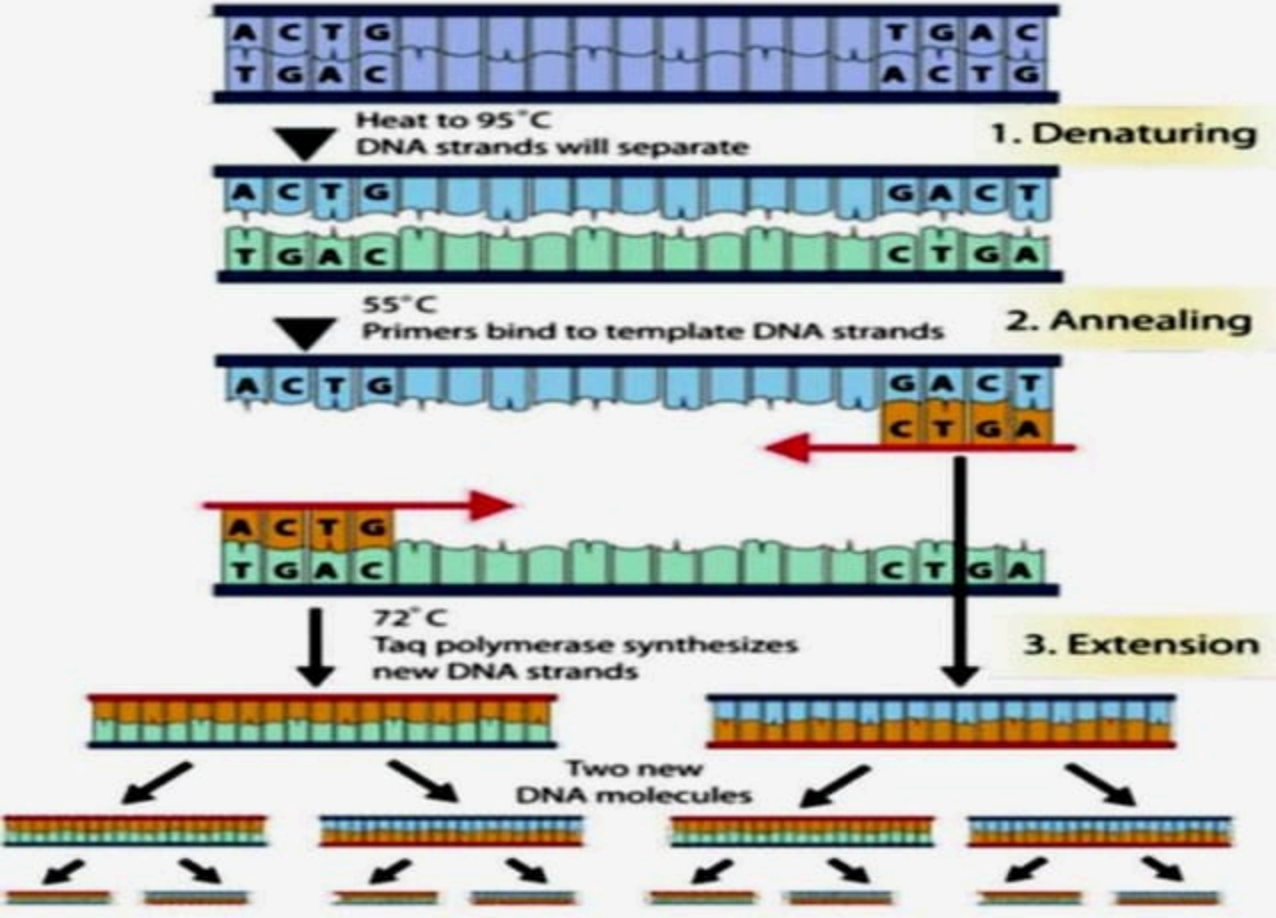
- This is done by incubating purified DNA molecules with the restriction enzyme, at the optimal conditions for that specific enzyme.
- Agarose gel electrophoresis is employed to check the progression of a restriction enzyme digestion.
- DNA is a negatively charged molecule, hence it moves towards the positive electrode (anode)

Ligation of the DNA fragment into a vector

- Same process is repeated with the vector DNA also.
- The joining of DNA involves several processes
- DNA as well as vector DNA with a specific restriction enzyme, the cut out 'gene of interest' from the source DNA and the cut vector with space are mixed and ligase is added.
- This results in the formation of recombinant DNA.

using PCR

- PCR-**Polymerase Chain Reaction**
- In this reaction, multiple copies of gene (or DNA) of interest is synthesized in vitro using 2 sets of primers and the enzyme **DNA polymerase**.
- The enzyme extends the primers using the nucleotides provided in the reaction and the genomic DNA as template.
- If the process of replication of DNA is repeated many times, the segment of DNA can be amplified to approximately billion times i.e. 1 billion copies can be made.
- Such repeated amplication is achieved by using a thermostable DNA polymerase (Taq, isolated from a bacteria *Thermus aquaticus*), that remains active during the high temperature induced denaturation of double


A C T G
T G A C
T G A C
A C T G
Heat to 95°C
DNA strands will separate
1. Denaturing
A C T G
T G A C
G A C T
C T G A
55°C
Primers bind to template DNA strands
2. Annealing
A C T G
G A C T
C T G A
A C T G
T G A C
C T G A
72°C
Taq polymerase synthesizes
new DNA strands
3. Extension
Two new
DNA molecules

3 steps of PCR-

- **Denaturation**-Two strands of DNA are separated by heating
- **Annealing**-Two sets of primer are attached/annealed to the separated DNA strands
- **Extension**-Taq polymerase catalyses the extension of primers using genomic DNA as template and nucleotides provided in the reaction

5. Transferring the recombinant DNA into the host

- There are several methods of introducing the ligated DNA into the recipient cells.
- Recipient cells after making them 'competent' to receive, take up DNA present in its surrounding.

6. Culturing the host cells in a medium at large scale

- When you insert a piece of alien DNA into a cloning vector and transfer it into a bacteria, plant or animal cell, the alien DNA gets multiplied.
- The main aim of recombinant technology is to produce a desirable protein.
- After cloning the gene of interest and having optimised the conditions to induce the expression of the target protein, one has to produce it on large quantity.
- The cells can be multiplied in a continuous culture system wherein the used medium is drained out from one side while fresh medium is added from the other to maintain the cells in their physiologically most active log/exponential phase.

- This type of culturing method produces a larger biomass leading to higher yields of desired protein
- To produce large quantities, fermenters can be used, where large volumes (100-1000 litres) of culture can be processed.
- A fermenter provides optimum conditions for achieving the desired product by providing optimum growth conditions (temperature, pH, substrate, salts, vitamins, oxygen)
- After completion of this stage, the product has been subjected through a series of processes before it is ready for marketing as a finished product.
- The processes include separation and purification.

APPLICATION OF GENETIC ENGINEERING IN MEDICINE

- **Genetic disorder**-A genetic disorder is a disease that is caused by an abnormality in an individual's DNA.
- Abnormalities can be as a small as a single-base mutation in just one gene, or they can involve the addition or subtraction of entire chromosome
- Most common genetic disorders are Cystic fibrosis, Down syndrome, Duchenne muscular dystrophy

- **Monoclonal antibody product-** These are made by cell culture that involves fusing myeloma cells with mouse spleen cells immunized with the desired antigen.
- Rabbit/mouse B-cells can be used to form a hybridoma. Unfused spleen cells cannot grow indefinitely because of their limited life span.
- Mab can be used to treat cancer.
- Mab is used in pregnancy test kits to identify small level of a hormone called as Human Chorionic Gonadotrophin, which is present in the urine of pregnant women.
- Mab can be used to locate blood clots as they bind to the clots.

- **Gene therapy**-It is a method that allows correction of a defective gene that has been diagnosed in a child.
- Here genes are inserted into a person's cells and tissues to treat a disease.
- Correction of a genetic defect involves delivery of aa normal gene into the individual to take over the function of and compensate for the non-functional gene.
- A person with a hereditary disease can be given a gene therapy

- **DNA Fingerprinting**-It is a technique used for the identification (as for forensic purposes) by extracting and identifying the base-pair pattern of an individual's DNA- called as DNA typing, genetic fingerprinting.
- In DNA fingerprinting, scientists collect samples of DNA from different sources- for example, from a hair left behind at a crime scene and from the blood of victims and suspects.

- **Vaccines**- It is a product that produces immunity from a disease and can be administered through needle injections or by mouth.
- A vaccination is the injection of a killed or weakened organism that produces immunity in the body against that organism.
- An antigen is a chemical substance that will trigger an immune response in the human body and this will cause the body to produce antibodies. Usually virus proteins or a weakened virus are used as vaccine antigens.

- **Pharma products**- A pharmaceutical drug is a drug used to diagnose, cure, treat or prevent disease.
- Drug therapy is an important part of the medical field and relies on the science of pharmacology for continual advancement and for appropriate management.
- Examples include insulin, interferons, human growth hormone

APPLICATION OF RDNA TECHNOLOGY AND GENETIC ENGINEERING IN THE PRODUCTION OF- Interferon, Vaccine- Hepatitis B, Hormone -insulin

INSULIN-

- Human insulin produced by recombinant DNA technology was first approved for general medical use in 1982.
- It was the first product of recombinant DNA technology to be approved for therapeutic use in humans

PRODUCTION OF INSULIN USING RDNA TECHNOLOGY

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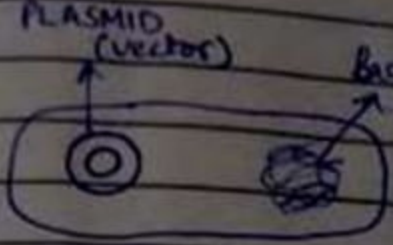
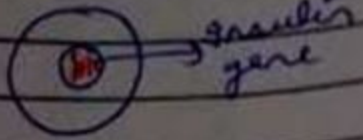




E. coli

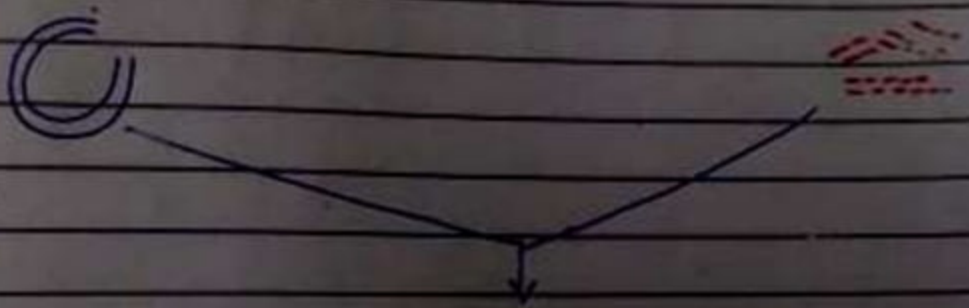
PLASMID

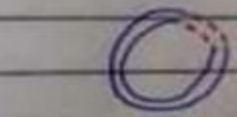
RESTRICTION ENDONUCLEASE

Human cell

INSULIN GENE

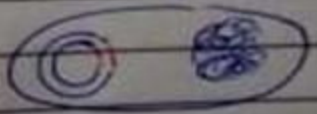
RESTRICTION ENDONUCLEASE

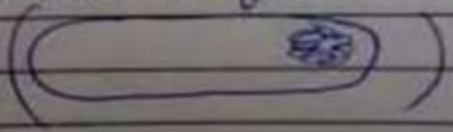




RECOMBINANT DNA

↓ Inject into Plasmid free E. coli





RECOMBINANT DNA
E. coli CELL

↓

Culturing of this bacteria and extraction of insulin

↓

- Insulin injected into patients

INTERFERONS

- Interferons (IFNs) were the first family of cytokines to be discovered.
- In 1957 researchers observed that if susceptible animal cells were exposed to a colonizing virus, these cells immediately become resistant to the attack by other viruses.
- This resistance was induced by a substance secreted by virally-infected cells, which was named 'interferon' (IFN).
- Humans produce at least three distinct classes, IFN-α, IFN-β and IFN-γ.

- Biological effects,

  -Induction of cellular resistance to viral attack.

  -Regulation of most aspects of immune function.

  -Regulation of growth and differentiation of many cell types.

- A DNA sequence coding for the product was synthesized and inserted into E. coli. The recombinant product accumulates intracellularly as inclusion bodies
- Large-scale manufacture entails an initial fermentation step. After harvest, the E. coli cells are homogenized and the inclusion bodies recovered via centrifugation.
- After solubilization and refolding, the interferon is purified to homogeneity by a combination of chromatographic steps.
- The final product is formulated in the presence of a phosphate buffer and sodium chloride.
- It is resented as a 30 mg/ml solution in glass vials and displays a shelf- life of 24 months when stored at 2–8°C`.

Human fibroblast

↓

Human interferon beta gene

+

E. coli

↓

E. coli plasmid

Restriction enzyme

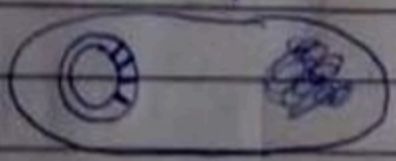
Restriction enzyme

Recombinant DNA molecule (Plasmid containing Human interferon)

↓

Insert into plasmid free E. coli

↓



Culture this bacteria for production of Interferon

Purification and Recovery

End Product

- **Interferon toxicity-**

  - Like most drugs, administration of IFNs can elicit a number of unwanted side effects.

- **Minor side effects –**

  - Range of flu-like symptoms, e.g. fever, headache, chills.

- **Serious potential side effects –**

  - Anorexia
  - Strong fatigue
  - Insomnia
  - Cardiovascular complication
  - Autoimmune reactions
  - Hepatic disorder

- **CONCLUSION OF INTERFERONS**

- Interferons represent an important family of biopharmaceutical products.
- They have a proven track record in the treatment of selected medical conditions, and their range of clinical applications continue to grow.
- It is also likely that many may be used to greater efficacy in the future by their application in combination with additional cytokines.

VACCINE- HEPATITIS B

- Hepatitis B virus (HBV) is one of the most common infectious diseases known to man.
- The World Health Organization (WHO) estimates that there are as many as 285 million chronic carriers of this virus worldwide.
- Hepatitis B is 50 to 100 times more infectious than AIDS.
- Hepatitis B is irritation and swelling (inflammation) of the liver due to infection with the hepatitis B virus (HBV).
- Other types of viral hepatitis include: Hepatitis A; Hepatitis C; Hepatitis D.
- It produces several chronic liver disorders such as Fulminant chronic hepatitis, cirrhosis and primary liver cancer.

- **Hepatitis B Recombinant Vaccine-** It's a novel and significant developed vaccine which is produced from the antigenic proteins of Hepatitis B virus by recombinant process that duplicates the chemical messages and secreted factors (Interleukin-2) for the communication and activity of immune cells.
- **A recent approach for Recombinant Hepatitis B Vaccine production-** A special type of tropical monocot banana under the genus Musa in the Musaceae family, can be ideally engineered by genetic mechanism process for the production of hepatitis B vaccine--- it was suggested. With this technology, the cost of vaccination could be reduced.

- General features of nucleic acid of Hepatitis B Virus:-

  -HB virus has been identified as a 42-nm particle containing a double stranded circular DNA molecule of about 3Kb size.

  -DNA genome has a relative molecular mass of approximately 2 X 10^6

- Production of these genes is needed in order to get production of vaccines on a large scale.
- **A general procedure for the production of recombinant Hepatitis B vaccines are described here-**

1. HBs antigen producing gene is isolated from the HB virus by normal isolation process (cell lysis, protein denaturation, precipitation, centrifugation and drying).
2. A plasmid DNA is extracted from a bacterium- E.coli and is cut with restriction enzyme- Eco RI forming the plasmid vector
3. The isolated HBs antigen producing gene is located and inserted into the bacterial plasmid vector on forming the recombinant DNA.

4. This recombinant DNA, containing the target gene, is introduced into a yeast cell forming the recombinant yeast cell.
5. The recombinant yeast cell multiplies in the fermentation tank and produces the HBs antigens.
6. After 48 hours, yeast cells are ruptured to free HBs Ag. The mixture is processed for extraction.
7. The HBs antigens are purified.
8. HBs Ag are combined with preserving agent and other ingredients and bottled. Now it is ready for vaccination in humans.

THANKS