Biotechnological Applications of Recombinant DNA

Dr. Selma Hamimed
Department of Molecular and Cellular Biology
Faculty of Natural Sciences and Life
University of Jijel
Email: selma.hamimed@univ-jijel.dz[1]

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I Combating infectious diseases

1. Objectives

- Provide a detailed understanding of how recombinant DNA techniques are utilized to identify, modify, and produce therapeutic proteins, vaccines, and diagnostic tools for infectious diseases.
- Evaluate the effectiveness of recombinant DNA-based interventions.

2. Introduction to infectious diseases

Infectious diseases, which arise from various pathogens, including bacteria, viruses, parasites, and fungi, represent a significant global health challenge and are among the leading causes of morbidity and mortality worldwide. Recent statistics indicate that approximately 60 million deaths occur annually, with at least 25% attributable to infectious diseases. The emergence and re-emergence of these diseases pose considerable obstacles to public health systems and can destabilize socioeconomic structures.

Despite advancements in public health, including the development and administration of vaccines and antimicrobial and antiviral agents, the efficacy of these interventions is often compromised by the emergence of novel infectious agents and the rise of drug-resistant pathogens. Furthermore, the development of vaccines and therapeutic agents typically involves extensive clinical trials, which can prolong the timeline for effective intervention and hinder the rapid response necessary for managing infectious disease outbreaks.

In scenarios where specific vaccines and therapeutic agents are not readily available, the prompt selection of appropriate detection methodologies becomes paramount. Rapid and accurate identification of pathogens is essential for enhancing treatment efficiency, minimizing disease transmission, and facilitating swift responses to critical public health events [74]*.

Every infectious disease exhibits particular vulnerabilities that can be exploited for control. The fundamental strategy in managing infectious diseases involves identifying these vulnerabilities and disrupting the weakest links in their transmission chains. The application of molecular tools in epidemiology was first articulated by Edwin Kilbourne in 1973 in the context of influenza research. This approach emphasizes understanding the causative factors, distribution patterns, and impacts of diseases. Molecular epidemiology focuses on elucidating genetic and environmental risk factors at the molecular level, enabling the identification of the genetic underpinnings of diseases. This includes investigating host and pathogen variants that influence infection dynamics, transmission routes, and prevention strategies. Such insights are critical for developing targeted interventions and improving public health outcomes.

(cf. Combating Infectious Diseases on a Global Level)

3. Techniques used to combat infectious diseases

Advances in science and technology have significantly bolstered our ability to combat infectious diseases, particularly through the use of modern techniques such as molecular biology, genetic engineering, and biotechnology.

Among these, recombinant DNA technology has emerged as a cornerstone in the fight against infectious diseases ,by allowing for the manipulation of genetic material, this technology has revolutionized vaccine development, enabling the production of safer and more effective vaccines. Additionally, molecular diagnostics have greatly improved the speed and accuracy of pathogen detection, facilitating early interventions that limit disease transmission and improve patient outcomes.

Biomarkers, genetic markers, specific genes, virulence genes, … using gel electrophoresis or 3D SDS-PAGE, Western blotting, Southern Blotting.

3.1. Plasmid profiling

Plasmid profiling, introduced in the 1970s, was the first DNA-based bacterial subtyping method. This technique distinguishes bacterial strains by analyzing the number and size of plasmids they harbor. Plasmid DNA is separated from bacterial isolates using agarose gel electrophoresis (AGE), often visualized with fluorescent dyes. Plasmids play a key role in the dissemination of antibiotic resistance genes (ARGs), which is of growing concern due to the increasing prevalence of antibiotic-resistant bacteria (ARB). The emergence of multidrug-resistant (MDR) bacteria poses a significant threat to public health, limiting the effectiveness of available antibiotics[75]*.

MDR bacteria are defined by their resistance to multiple antibiotics, typically from at least two of the three major groups: β-lactams, aminoglycosides, and quinolones. The misuse and overuse of antibiotics, along with selective pressure from their widespread use, have accelerated the spread of antibiotic resistance. This rise is further driven by genetic mutations and horizontal gene transfer mechanisms, including the exchange of resistance-carrying plasmids between bacteria. The global effort to combat antibiotic resistance now focuses on slowing the spread of ARGs and improving antibiotic stewardship[76]*.

Plasmid profile analysis is a genotyping method that isolates and analyzes the plasmid DNA from bacterial cells. The technique generates unique plasmid patterns based on the size and number of plasmids present, which can be further refined by digesting the plasmid DNA with restriction enzymes for additional differentiation. This method is particularly useful for epidemiological studies, as it has been applied to typing methicillin-susceptible and methicillin-resistant *Staphylococcus aureus* (MSSA and MRSA). Despite its utility, plasmid profiling is limited to strains containing plasmids, and the ability of bacteria to acquire or lose plasmids must be considered when interpreting results[75]*.

Overall, plasmid profiling remains a sensitive, specific, and straightforward method for tracking bacterial subtypes and monitoring the spread of antibiotic resistance.

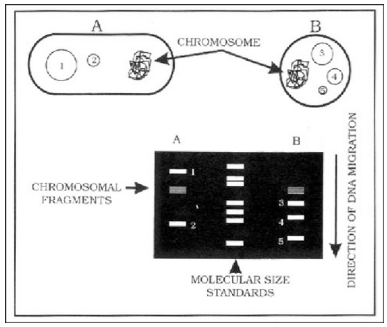


Image 1 Plasmid profiling

3.2. RFLP (Restriction Fragment Length Polymorphism)

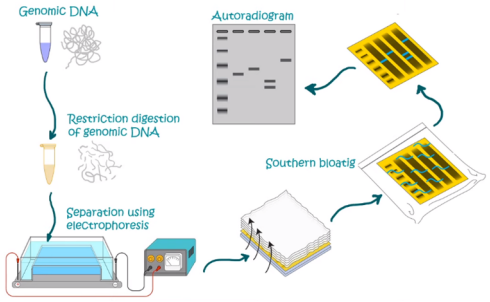
Restriction Fragment Length Polymorphism (RFLP) is a molecular technique used to differentiate organisms by analyzing patterns generated from the cleavage of their DNA by specific restriction enzymes. If two organisms differ in the distance between cleavage sites for a particular restriction endonuclease, the resulting DNA fragments will vary in length when digested with the enzyme. The comparison of these fragment patterns enables the differentiation of species and even strains within species[77]*.

Polymorphisms are inherited differences that occur in more than 1% of the population, making them useful for genetic studies. RFLP has a wide range of applications, including:

1. **Forensic Applications**: RFLP can be employed in paternity testing and criminal investigations to determine the origin of a DNA sample, as the distinct patterns generated provide a genetic fingerprint.
2. **Disease Detection**: RFLP can be used to identify specific mutations associated with diseases, particularly known genetic mutations, making it a valuable tool for diagnosing genetic disorders.
3. **Genetic Mapping**: RFLP is also used to measure recombination rates, helping to construct genetic maps that show the distances between loci in centiMorgans, a unit of genetic linkage.

The method relies on restriction endonucleases, enzymes that cut DNA at specific nucleotide sequences, typically between 4 and 6 base pairs in length. Shorter recognition sequences produce more fragments, and differences in nucleotide sequences between organisms result in fragments of varying sizes. These fragments are separated using gel electrophoresis, providing a visual representation of the DNA pattern[77]*.

Restriction enzymes are derived from various bacterial species and are thought to play a role in the bacterial defense mechanism against viral infection. They are named by combining the first letter of the genus, the first two letters of the species, and the order of discovery. The ability of RFLP to produce distinct DNA patterns makes it a powerful tool for species identification, genetic analysis, and forensic investigation.



RFLP technique(Restriction Fragment Length Polymorphism)

⚙Méthode : How to design the probe in RFLP?

The probe and restriction enzyme are chosen to detect a region of the genome that includes a variable number tandem repeat [VNTR] segment.

It has some limitations:

- Slower and cumbersome.
- Involves southern blot and autoradiography.
- Requires a large amount of the genome.

3.3. AFLP (Amplified Fragment Length Polymorphism)

A molecular marker, such as an AFLP (Amplified Fragment Length Polymorphism) marker, is a DNA-based tool used in genetic analysis. AFLP markers are highly polymorphic, meaning they detect a wide range of genetic variation within populations, making them particularly useful in studies related to population genetics, evolution, and biodiversity. The AFLP technique, first introduced by Vos et al.[78]*, is a PCR-based fingerprinting method that generates large numbers of marker fragments without prior knowledge of the organism's genome, enabling its application across diverse species.

The AFLP method consists of five main steps[78]*:

1. **Restriction and Ligation**: Genomic DNA is digested using restriction enzymes, and adaptors are ligated to the fragmented DNA.
2. **Preselective PCR Amplification**: A subset of these restricted DNA fragments is amplified using specific PCR primers.
3. **Selective PCR Amplification**: A more refined selection of DNA fragments is further amplified to reduce the number of fragments.
4. **Electrophoresis**: The amplified DNA fragments are separated by electrophoresis based on their size.
5. **Data Scoring and Interpretation**: The resulting fragment patterns are scored and interpreted to assess genetic variation.

One of the strengths of AFLP is its ability to generate large quantities of marker data quickly, requiring only small amounts of starting DNA. AFLP also exhibits high reproducibility compared to other fingerprinting techniques such as RAPD (Random Amplified Polymorphic DNA) and ISSR (Inter-Simple Sequence Repeats). While AFLP is labor-intensive, it can be multiplexed, allowing the simultaneous analysis of numerous samples. Optimization of primer combinations is key to the success of an AFLP assay, as it maximizes the detection of polymorphic fragments[79]*.

AFLP analysis is widely used to target genome-wide loci. However, several modifications have expanded its utility. One such variant is **cDNA-AFLP**, a method that focuses on expressed genes, enabling the analysis of gene expression between different phenotypes. This technique uses cDNA, synthesized from mRNA, to analyze transcriptomic variation, providing insights into functional gene differences that drive phenotypic diversity. cDNA-AFLP is valued for its speed, reproducibility, and minimal requirements for prior sequence data.

Another notable AFLP-derived protocol is **Methylation-Sensitive Amplified Polymorphism (MSAP)**, which targets DNA methylation variations. DNA methylation, particularly of cytosines, plays a crucial role in regulating gene expression and development, and MSAP helps to identify methylation patterns that may differ between individuals or cell types. This method provides valuable insights into epigenetic regulation, including gene silencing and the control of mobile genetic elements[79]*.

In summary, AFLP is a versatile and robust molecular marker technique with broad applications in genetic analysis, population studies, and molecular ecology. Its variants, such as cDNA-AFLP and MSAP, extend its reach into transcriptomics and epigenetic research, making AFLP a key tool in modern genomics.

3.4. RAPD (Random Amplified Polymorphic DNA)

Random Amplified Polymorphic DNA (RAPD) is a versatile and cost-effective PCR-based method commonly used for genotyping bacterial species. RAPD markers are produced by amplifying random segments of genomic DNA using a single universal primer with an arbitrary nucleotide sequence. This technique generates DNA fragments that reveal polymorphisms between bacterial isolates, providing insights into their genetic diversity.

In RAPD analysis, two isolates are considered genetically indistinguishable if they produce the same number of DNA bands, and these bands exhibit the same apparent size on a gel electrophoresis profile. Variations in band patterns, such as a difference of three bands, can be used to differentiate closely related strains or clones. For instance, if the genetic similarity between *Staphylococcus aureus* isolates is 65% or greater, the isolates are likely to share a common ancestor, indicating potential genetic relatedness$80^{80}{}_{*}$.

RAPD relies on short primers, typically 8-12 nucleotides in length, to amplify random genomic regions. This technique is advantageous for its simplicity and affordability, as it does not require prior knowledge of the organism's genome. RAPD is widely used in epidemiological studies, bacterial typing, and population genetics due to its ability to distinguish between bacterial strains and assess genetic relatedness within species.

RAPD (Random Amplified Polymorphic DNA)

3.5. PCR

As we explored in the chapter before, PCR has become the most widely used nucleic acid amplification method for pathogen detection. With the development of PCR technology, molecular diagnostic techniques such as quantitative PCR (qPCR), digital PCR (dPCR) and high-resolution melting (HRM) based on the principle of conventional PCR (cPCR) have been widely used for the rapid and straightforward identification and drug resistance detection of known infectious disease pathogens. PCR performs an important role in the early diagnosis of infectious diseases.

a) qPCR

qPCR uses fluorescently labeled probes or double-stranded DNA-specific fluorescent dye to qualitatively and quantitatively analyze the fluorescence signal of amplification products in real time without the need to detect PCR products through complex electrophoresis steps. This method is more automated and has a lower risk of contamination compared with cPCR. qPCR has been widely used for the early diagnosis and drug resistance detection of common clinical pathogens and has the advantages of higher sensitivity, specificity, simplicity and rapidity compared with traditional diagnostic methods.

i) dPCR

dPCR performs absolute quantification of target genes in samples by dividing the amplification reaction into thousands of independent sections using microplates, capillaries, oil emulsions or microarrays, amplifying each target gene in separate compartments, distinguishing the generated droplets as negative or positive based on the setting of the fluorescence threshold, and calculating the target gene content through the ratio of negative and positive droplets. This partitioned amplification reduces template competition, increases the sensitivity of the reaction and allows dPCR to detect low levels of pathogens, minor mutations and rare allele targets.

1 HRM (High Resolution Melting Analysis)

HRM is a novel molecular diagnostic technique based on the principle that different double-stranded DNA molecules have different melting temperatures. The technique uses fluorescent dyes or probes to monitor changes in the shape of the melting curve to rapidly and accurately detect and identify various pathogens. With the advantages of rapidity, and high sensitivity and specificity, HRM is often used for species identification, genotyping and drug resistance gene detection of known pathogens*81*[81]*.

3.6. Pulsed field gel electrophoresis

In PFGE, the organisms are embedded in agarose, lysed in situ to extract the DNA and followed by digesting the DNA by using a restriction enzyme that cleaves infrequently. Slices of agarose are then inserted into wells of an agarose gel and electricity applied at three different angles to resolve the DNA into discrete bands. Multiple strategies have been developed to produce homogenous electrical field by different companies. For rapid separation of fragments 100bp to 250 kb in size, electrical field is fixed at 180^{o} angle and is inverted in the forward and reverse directions. In another approach different voltages are applied to forward and reverse directions*82*[82]*.

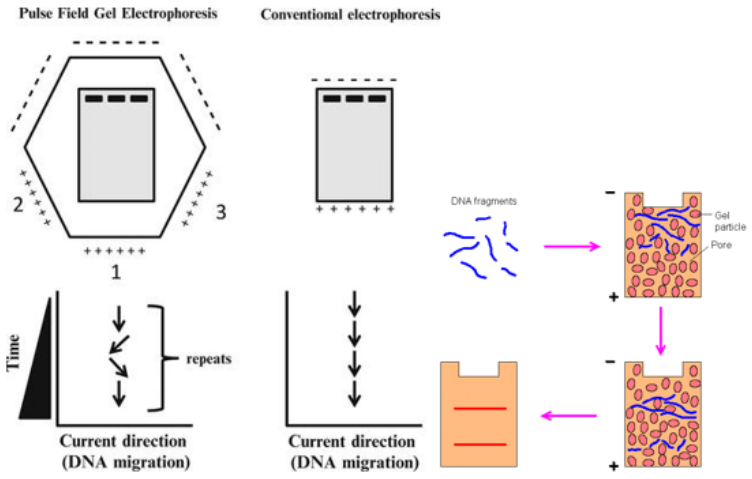


Image 2 Pulsed Field Gel Electrophoresis

3.7. Ribotyping

Ribotyping is a highly specific fragment-based molecular technique used for bacterial typing and strain differentiation. This method leverages the use of restriction enzymes to target and cleave conserved regions within the ribosomal RNA (rRNA) genes, specifically 5S, 16S, and 23S, as well as the intergenic spacer regions, including Glu-tRNA. By cutting these regions, ribotyping generates a unique DNA fingerprint that can be used to differentiate organisms down to the strain level.

The restriction enzymes cleave the genomic DNA at specific recognition sites within the rRNA gene regions, producing DNA fragments of varying lengths that reflect the genetic composition of the organism. These fragments are then separated by gel electrophoresis, yielding a distinctive banding pattern or "fingerprint" that is characteristic of the organism. This pattern can be compared across different isolates to assess genetic relatedness and identify specific strains.

Ribotyping is particularly valuable in epidemiological studies, microbial classification, and tracking the spread of bacterial pathogens in clinical and environmental settings. Its high resolution at the strain level makes it an important tool for monitoring outbreaks, identifying sources of contamination, and studying bacterial evolution and diversity. Furthermore, as rRNA genes are highly conserved across bacterial species, ribotyping provides a robust platform for distinguishing closely related strains, making it an essential technique in molecular microbiology*83*[83]*.

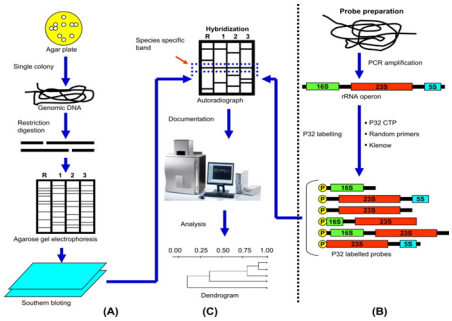


Image 3 Ribotyping

4. Comparison of molecular typing techniques for the best infectious disease combating

Technique	Principle	Advantages	Limitations
PFGE (Pulsed-Field Gel Electrophoresis)	Separates large DNA fragments by changing electric field direction	High discriminatory power, suitable for large genomes	Time-consuming, requires specialized equipment
Plasmid Profiling	Separates plasmids based on size and number	Quick and simple, identifies plasmid content	Limited resolution for plasmids of similar sizes
RFLP (Restriction Fragment Length Polymorphism)	Analyzes variations in DNA fragment lengths generated by restriction enzymes	Robust and widely applicable, established method	Limited discrimination for highly similar strains
RAPD (Random Amplified Polymorphic DNA)	Amplifies random genomic DNA fragments using short primers	Simple and rapid, requires minimal DNA preparation	Low reproducibility, less discriminatory power
AFLP (Amplified Fragment Length Polymorphism)	Combines restriction digestion and PCR amplification to generate fingerprints	suitable for diverse genomes	high cost

Tableau 1 Difference between various molecular techniques

5. Exercice : MDR report

Write a report on the transition from plasmid profiling to PCR-ribotyping for the detection of multidrug-resistant strains, in 800 words.

Glossaire

Polymerase chain reaction (PCR)

A method widely used to make millions to billions of copies of a specific DNA sample rapidly, allowing scientists to amplify a very small sample of DNA (or a part of it) sufficiently to enable detailed study.

Abréviations

DNA : Deoxyribonucleic acid ; is the molecule that carries genetic information for the development and functioning of an organism.

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