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Genomic Analysis Methods of Microorganisms

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ABSTRACT

Molecular approaches used to identify bacterial species use 16S rRNA and MLST to determine the genetic linkage of bacteria; MLST characterizes clonal linkages by examining differences in various gene loci. MLVA determines the genetic relationships of bacterial strains and biovar-level differences and assesses the copy number of repeated DNA sequences. Sequencing provides genetic data by identifying DNA sequences; Sanger sequencing is the basis for next-generation approaches. CRISPR modifies the genetic code and can correct mutations or control genes using Cas9. These methods are important for identifying bacterial species and annotating genomic information. The methods used for this purpose are brought together in this study. The explanation and detailed description of the methods examined will contribute to their use in the field of microbiology.

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Introduction

Molecular techniques, especially based on nucleic acid amplification protocols, provide sensitive, rapid and quantitative analytical methods to study pathogens, including new or existing strains. Microscopic, cultural and serological techniques for viral, bacterial and protozoan agents constitute the traditional diagnostic microbiological assays to detect pathogen-specific markers in the media. However, the low specificity of these techniques, slow growth, the need for very sensitive media for growth or contamination with apathogenic and saprophytic microorganisms limit their use. Antimicrobial treatments or non-specific cross-reactions are also major problems of conventional microbiological analysis. In contrast, molecular techniques offer high specificity and sensitivity, along with rapid results, underscoring the necessity for their integration into diagnostic procedures (Gerace et al., 2022).

The methods used are basically based on the detection and analysis of specific parts of the pathogen's genome (DNA or RNA). By amplifying certain regions of DNA or RNA, it enables the differentiation of strains on the basis of genus, species and subspecies according to the target sequence. The detection and quantification of numerous pathogens using molecular methods is a standard procedure in many laboratories within the clinical field (Zhang et al., 2021) Molecular techniques have disadvantages as well as advantages. Although these techniques are methods that facilitate diagnosis, they are quite costly methods compared to traditional methods, the use of most of them in low-budget laboratories is very limited, and the need for qualified technical personnel is also required for most techniques (Liu et al., 2023). This study was prepared in order to emphasize the microbiological importance of these techniques and to gather the necessary information for the use of the methods.

Multilocus Sequence Typing

The field of bacterial genetics benefited from a significant advancement when Carl Woese and colleagues identified sequences based on the 16S rRNA gene. . This technique is widely used for the identification of species based on bacterial genetics and for the investigation of taxonomic relationships. 16S rRNA sequences are used for typing genetically closely related species. The Multilocus Sequence Typing (MLST) method was proposed in 1998, arising from the need for more precise typing of isolates to detect clonal relationships between bacteria (M. C. J. Maiden et al., 2013). The genetic variations between bacteria can be identified through the analysis of nucleotide sequences, which led to the development of Multilocus Sequence Typing (MLST), a method that examines the differences in multiple gene loci. MLST is based on the analysis of housekeeping genes. For each gene, a region of approximately 450 bases is amplified by PCR and the data obtained from MLST are visualised with a dendogram (Blanchard et al., 2018) (Figure 1).

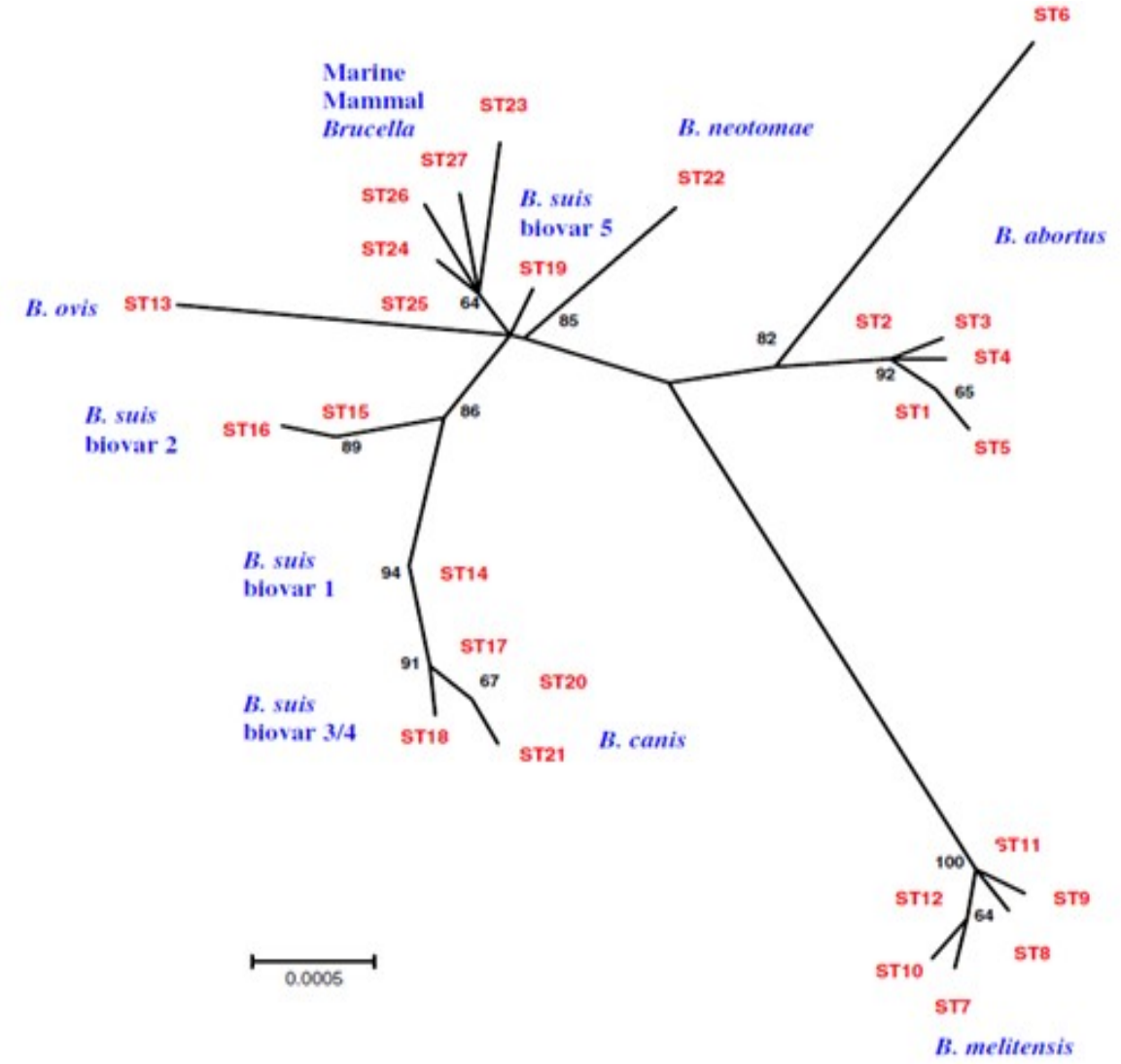

Figure 1. Phylogenetic tree of Brucella strains typed by MLST (Whatmore et al., 2007).

The MLST scheme was first developed for *Neisseria meningitidis* and subsequently applied to many bacteria and eukaryotic organisms. Since its initial discovery, MLST has been employed for a number of purposes, including epidemiological analysis of pathogens, investigation of their evolutionary history through genealogical analysis, and the study of the population structure of non-pathogenic bacteria (Feijao et al., 2018). MLST plays a critical role in the field of microbiology to systematically identify genetic variations. This method determines the genetic relationships between bacteria through the gene sequences of alleles. By comparing allele sequences, bacterial strains with similar genetic profiles can be grouped and their evolutionary relationships analysed. MLST is widely used in scientific research by making allele sequences available online through reference datasets. This method makes a significant contribution to the identification of bacterial species and a more detailed understanding of their genetic affinities (Gonzalez-Escalona et al., 2017).

One of the advantages of MLST is that it analyses fewer loci and has higher discrimination power. With this method, it is determined whether the causative agent of pandemics, epidemics and hypervirulent strains are from the same lineage by looking at the allele sequences. The identification process is fast and the newly identified allele is uploaded to the database, where different variations of the conserved gene are also detected and uploaded to the database (Pelerito et al., 2021). The sequence data used for MLST is used to analyse the degree of differences between alleles, MLST is considered the gold standard for typing bacterial pathogens. It avoids the need to analyse information at all loci and categorise which variations are due to point mutations and which are due to recombination. MLST data can also be used in sequence-based analyses as allelic variant sequences are stored with MLST data. Accommodates a variety of variations as it covers both close and distant pedigrees (Liu & Chen, 2021).

Many MLST databases are available (PubMLST, MLST homepage, Institut Pasteur MLST databases). These databases allow comparison of data from different laboratories (Jolley et al., 2018). MLST can detect subtle changes in DNA compared to other genotyping methods and enables serotyping with sequence data. It is easily reproducible and does not require special reagents. Since MLST is a PCR-based method, direct access to live bacterial isolate or high quality genomic DNA is not required. The data obtained are easily accessible on the internet (Ibarz Pavón & Maiden, 2009).

MLST is very difficult to develop dendograms except for closely related bacteria, due to the differences in "housekeeping genes" in different bacteria. Even within genera there should be multiple MLST schemes targeting different loci. MLST cannot distinguish between asexual pathogens such as *Bacillus anthracis* and *Yersinia pestis*, which have single-stranded DNA, or pathogens believed to be of the same lineage. Furthermore, this system is costly and complex to implement, particularly in low-budget laboratories (M. C. Maiden et al., 2013).

Multi Lokus Variable Number Tandem Repeat Analysis

Multi Locus Variable Number Tandem Repeat Analysis (MLVA) is a powerful method that detects strains at the biovar level and is used to evaluate genetic relationships between strains of the same species. The first study on the use of MLVA, an effective molecular typing method, was conducted in 1977 by Van Belkum et al. (Belkum et al., 1997). It is one of the preferred methods in local epidemiological studies and in the detection of the causative agent of epidemics. MLVA, which is used to categorise bacterial strains according to their subtypes, detects the number of copies of repeated DNA sequences distributed throughout the bacterial genome. While the difference in the number of consecutive repeats is usually observed in epidemiologically unrelated strains, this is preserved in epidemic-associated strains of the same lineage. The number of consecutive sequence repeats at a given locus varies from strain to strain, with variable number sequence repeats (VNTR) for a given bacterium being present in more than one locus or region. Therefore, loci of isolates from epidemically different sources differ in size and number of sequential repeats (Pontieri, 2016).

The initial step in applying this technique is to amplify Variable Number Tandem Repeat (VNTR) loci. This is followed by the targeted amplification of repeat regions using PCR. The resulting PCR products are then loaded into either agarose or automatic capillary electrophoresis and separated into DNA fragments. The number of consecutive sequence repeats is then determined based on the size of the separated DNA fragments, and an MLVA profile is created with the number of consecutive sequence repeats (Figure 2). MLST provides a great advantage for determining genotypic differences between closely related isolates when evaluated by capillary electrophoresis. It is also a very advantageous method for rapidly analysing changes in VNTR regions and determining the short-term evolution of bacteria due to storage conditions (Mirkalantari et al., 2021). There are also some disadvantages such as the high cost of the MLVA process, the need for sufficient technical personnel to interpret the results of the analyses and the difficulty of comparing the results between laboratories (Stoikov et al., 2020).

The databases and analysis platforms where MLVA results are stored and published include (www.minisatellites.u-psud.fr/MLVAnet), (www.pasteur.fr/mlva), and (www.pulsenetinternational.org/protocols). These databases and analysis platforms, which are part of the PulseNet international network, are currently in use. They allow for the comparison of MLVA profiles of strains worldwide and the determination of the geographical and temporal distribution of bacterial pathogens (Simar et al., 2021).

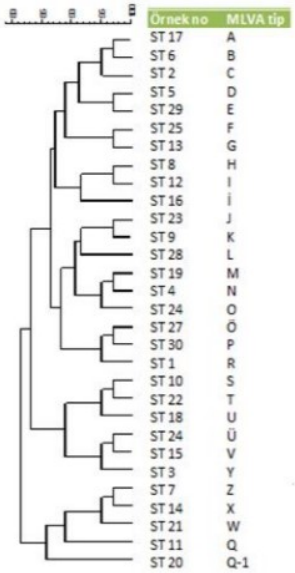

Figure 2. Dendogram of MLVA results (Savaş, 2020).

Sequencing

Sequencing is the determination of nucleic acid sequences in the polynucleotide chains that make up DNA. Sequencing allows the discovery of information about the hereditary and biochemical properties of life. Sequencing is of great importance in biological research (Heather & Chain, 2016) The history of sequencing began with the discovery of DNA. Sequence analysis was first applied to a yeast cell in 1965 (Matthews & Vosshall, 2020). Based on this work, new methods were developed. The Sanger sequencing method, discovered by Sanger et al. in 1977, was used extensively until a new generation of sequence analysis methods were discovered, and then automated systems were introduced (Fridman et al., 2021). With the discovery and subsequent widespread use of PCR technology, genome mapping studies have also gained momentum. In DNA sequencing studies with automated systems initiated by The Institute for Genomic Research (TIGR), 337 human genes, *Haemophilus influenzae, Mycoplasma genitalium, Escherichia coli* and *Bacillus subtilis, Saccharomyces cerevisiae*, Drosophila melanogaster (fruit fly) and many other organisms have been sequenced (Matthews & Vosshall, 2020).

While all these studies are continuing, studies have started for technological methods that are alternatives to the Sanger sequencing method which is used intensively. Because the Sanger sequencing method works with fewer samples and at the same time it works more expensive by making shorter readings with lower sensitivity (Barba et al., 2014).

The sequencing technique can be broadly categorised into two main groups: traditional and next generation sequencing methods.

Traditional Sequencing Techniques

Sanger Sequencing Method

This technique is called chain termination reaction. In order to apply the technique, single-stranded mould DNA, dNTPs, ddNTPs, DNA polymerase and primer containing a free OH group are required.

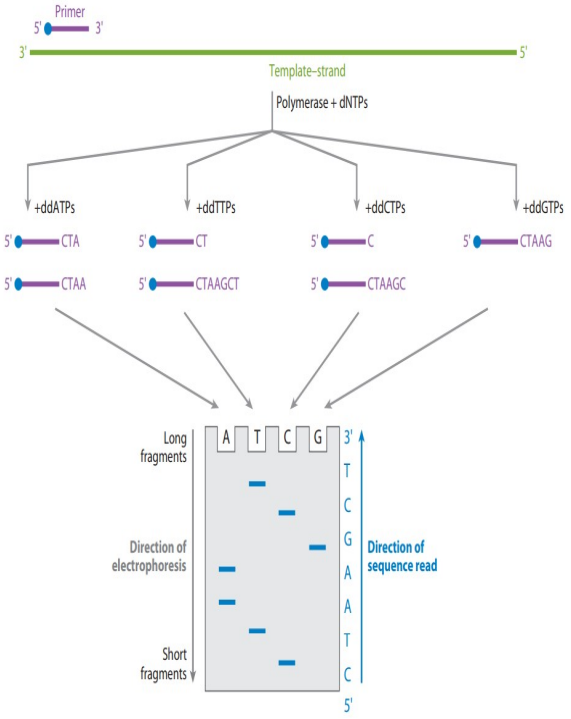

Figure 3. Visual explanation of Sanger sequencing method (Mardis, 2013).

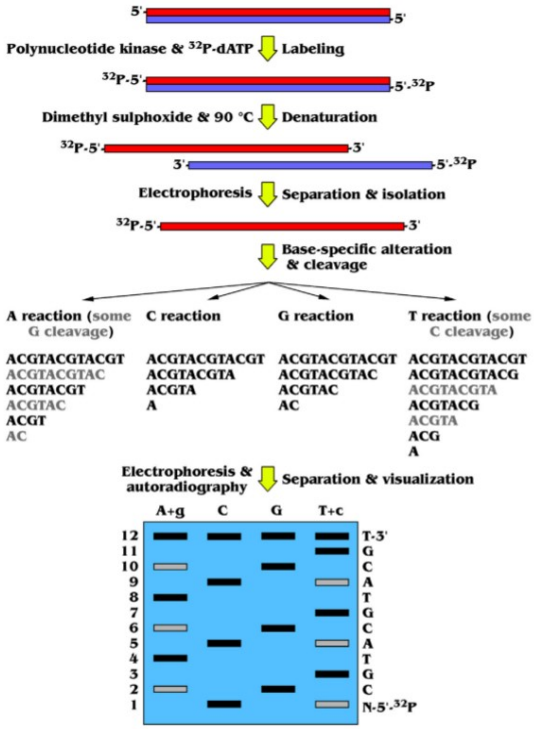

Figure 4. Visual explanation of Maxam-Gilbert method (Dorado, Gálvez, Budak, Unver, & Molina, 2019).

The most important part of this method is dideoxynucleoside triphosphates, because they do not carry an OH group and prevent the formation of phosphodiester bonds, thus the necessary environment for the binding of nucleotides is not created. Therefore, the elongation of the DNA chain is terminated and the reaction cannot continue. The mould DNA is stained with labelled dNTP to make its readings more effective. As a result, the sequence is terminated, and since the sites of these terminations are of various lengths, these fragments are assembled and sequenced (Taishan et al., 2021) (Figure 3).

Maxam-Gilbert method

This method, known as chemical sequencing, was discovered by Allan Maxam and Walter in 1977. The method first starts with radioactive labelling of the 50-P ends of double-stranded DNA (dsDNA) with 32P-dATP using polynucleotide kinase. The DNA is then denatured with dimethyl sulfoxide (DMSO) at 90°C, the resulting single-stranded DNA (ssDNA) molecules are separated by polyacrylamide gel electrophoresis into ssDNA fragments and visualised by autoradiography (Figure 4). The Maxam-Gilbert sequencing method has become the methodology of choice as it allows direct sequencing of purified DNA without the previous steps of in vivo cloning and ssDNA preparation. While this method was extensively used at the time of its discovery, its use has declined due to the use of hazardous chemicals and technical difficulty in its application (Dorado, Gálvez, Budak, Unver, & Molina, 2019).

Next Generation Sequencing Methods

Next generation sequencing (NGS) is a sequencing method developed against the disadvantages of traditional methods. Next-generation sequencing is a high-read, high-throughput and faster DNA sequencing technology that can sequence multiple DNA sequences in a single reaction. NGS usually consists of three steps: the first step is the fragmentation of DNA; the second step is the preparation of a library followed by ligation with specific ligands; the library is amplified by PCR to increase its volume; Finally, fluorescently labelled nucleotides and DNA polymerase are added for sequencing (Analara et al., 2023). NGS sequencing with fluorescent reflections of labelled fluorescently labelled nucleotides gives readings similar in shape to real time PCR.

NGS (Next Generation Sequencing) is a groundbreaking DNA sequencing method. It allows large amounts of parallel sequencing, sequencing billions of DNA fragments simultaneously. An NGS platform has the ability to process and isolate thousands or millions of different DNA samples in a single response. This approach allows for the rapid and efficient examination of vast quantities of genomic material (Robert Thomas, 2021).

The high throughput of NGS enables a variety of genetic analyses. For example, an NGS system can sequence thousands of genes simultaneously. Furthermore, a large number of patient samples, each labelled with a distinct tag, can be combined into a single response and then digitally separated using computer software. . It is particularly important for understanding the genetic characteristics of inherited disorders and complex diseases (Dubbink et al., 2014).

The versatility and high performance of NGS provide researchers with important contributions in genetic analysis studies. It offers a wide range of uses, from genomic studies to personalised medical treatments, and enables faster and more comprehensive collection of genetic data (Amanda & Robert, 2019).

There are several NGS methods and operating principles (Table 1.)

Table 1. NGS techniques used and working principle (Dorado, Gálvez, Budak, Unver, & Hernández Molina, 2019)

NGS techniques	Principle of working
Roche 454 life sciences emulsion-pcr sequencing	Random dsDNA fragments are nebulized, immobilized on streptavidin-coated beads, and subsequently denatured to isolate ssDNA, which is then amplified via emulsion PCR with biotinylated primers and read out using a glow-generating reaction, followed by genome assembly using bioinformatics tools.
Illumina reversible terminator sequencing	Illumina is defined as reversible terminator sequencing. It generates much greater genome coverage, albeit with shorter reads of 35-150 b. The technique is based on the generation of random dsDNA fragments by nebulisation, the addition of 'Y' adapters to the ends of the generated DNA fragments and hybridisation with a primer attached to a solid support.
Life technologies solid sequencing	DNA is fragmented by nebulization and adapters are ligated to the ends, followed by capture of single dsDNA molecules on primer-coated beads within a water-in-oil emulsion, leading to amplification, denaturation, and immobilization of ssDNA on a glass surface with blocked OH ends; subsequent sequencing via SOLiD involves ligation and fluorescent detection using labeled primers.
Life technologies ıon torrent chip sequencing	dsDNA is fragmented via nebulization, with adapters ligated to the ends, followed by capture of biotin-labeled dsDNA on streptavidin-coated beads using biotincontaining adapters; subsequent isolation of non-biotinylated ssDNA through denaturation, amplification via EmPZR with biotinylated primers, and genome assembly through read alignment using bioinformatics tools.
Helicos biosciences true single-molecule sequencing	DNA is randomly fragmented via nebulization, followed by denaturation and labeling of OH ends with fluorescent tags, enabling detection of labeled ssDNA on a solid support and subsequent light emission during polymerization with fluorescent dNTPs in billions of parallel reactions on flow cells, facilitating assembly of reads into chromosomes and genomes using bioinformatics tools.
Pacific biosciences single-molecule real-time sequencing	Rolling Circle Amplification (RCA) and Multiple Displacement Amplification (MDA) utilize loop adapters to convert dsDNA fragments into ssDNA, followed by incorporation of fluorescent nucleotides during polymerization to enable real-time sequencing in nanophotonic chambers for high-throughput readouts.
Complete genomics combinatorial probe-anchor ligation(cpal) sequencing	A library of randomly fragmented DNA with semi-adapters is first generated through autoligation and off-target cutting methods. Subsequently, ssDNA NanoBubbles (DNB) are created via clonal ssDNA RCA amplification, where fluorescence signals from bound probes are split and detected, enabling parallel reactions on millions of color-coded probes for genome assembly into chromosomes using bioinformatics tools.
Oxford nanopore technologies sequencing	Oxford Nanopore Technologies detects nitrogenous base residues of free nucleotides or nucleic acid polymers on a silicon chip via nanopores, analysing ssDNA from nebulized, digested dsDNA through ion current variations; this high throughput process enables sequencing reads to be assembled into chromosomes and genomes using bioinformatics tools.

Clustered Regularly İnterspaced Short Palindromic Repeats

Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) is an important technology that can be used to edit genes within sequencing techniques, basically with a gene editing approach. CRISPR is based on the logic of finding a specific DNA fragment in the cell, modifying and rearranging it. However, CRISPR also has functions such as turning genes on or off without changing sequences (Horodecka & Düchler, 2021). CRISPR allows cells to be fingerprinted and recorded by decoding the genetic code. It thus clarifies the evolutionary process (K. S. Makarova et al., 2011). The key to CRISPR is the many variants of "Cas" proteins found in bacteria and known to help defend against viruses (Jansen et al., 2002). The Cas9 protein is the most widely used protein by scientists. This protein can be easily programmed to find and bind to the desired target sequence. The most important function of the CRISPR-Cas system is the elimination of mutations. The mechanism that accomplishes this function consists of several steps. In the first step, when the Cas protein is introduced into the cell along with the guide RNA, it travels along the DNA strands until it combines with the RNA and discovers and binds to a DNA sequence 20 nucleotides long. CRISPR and the bound RNA then identify the target sequence, in the next step the faulty genetic material is cut by CRISPR-linked nucleases. Finally, the cut is repaired, removing the mutations that render the gene inoperable (Hale et al., 2012; Kira S. Makarova et al., 2011). This process is referred to as genome editing or gene editing and represents the most prevalent application of CRISPR.

Conclusion

Next-generation techniques offer significant advantages in microbiology, replacing many traditional methods. In particular, Next Generation Sequencing (NGS) has revolutionized microbiological diagnostics and epidemiological studies by genomic characterization of

pathogens. Studying pathogens with NGS allows their genetic structure to be determined in detail. This allows for greater precision and accuracy in the identification and classification of pathogens. It also plays a critical role in identifying antimicrobial resistance mechanisms and helps determine appropriate treatment options. In epidemiologic studies, next-generation methods are an extremely powerful tool for identifying the source and spread of infections. For example, they can be effectively used to detect and monitor outbreaks of nosocomial infections such as MRSA. While it can be very difficult to understand interspecies relationships with traditional methods, next-generation techniques can clearly reveal the isotypes and relationships of pathogens. Next-generation techniques are of great importance in the diagnosis of bacteria that take a long time to culture or are difficult to culture. As a result, next-generation methods offer significant advantages over traditional methods in critical areas such as microbiological diagnostics, drug resistance monitoring and infection control. The development of these technologies is considered a revolutionary advance in microbiological diagnostics and epidemiological monitoring.

Declarations

Conflict of Interest

The authors declare that they have no conflict of interest.

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