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Identification and Typing Methods for the Study of Bacterial Infections: a Brief Review and Mycobacterial as Case of Study

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Abstract

Several techniques based on molecular biology and analytical chemistry has been developed to reduce some of the bacterial characterization limitations. Molecular methods represent the best alternative to identify bacterial strains isolated from diverse origins and to improve research in the context of molecular epidemiology. However, these methodologies are laborious and costly compared to phenotypic or classical techniques, and there are no reliable routine laboratories. This review shall provide basic elements for the understanding of these methodologies and raise interest in their collaborative use among analytical laboratories where bacterial identification and typing are priorities, because molecular methods are not universally implemented but are available in research and reference laboratories.

Keywords: Identification; Characterization; Bacteria; Infections

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Introduction

One of the fundamental tasks of a microbiology laboratory is to fully identify the microorganisms involved in processes associated to infection or related to humans. This allows knowing their etiopathogenic implications, their clinical evolution, as well as applying an efficient antimicrobial therapy [1].

Identification and characterization of bacteria in the past were based on diverse phenotypic and genotypic methods **(Table 1)** however, in the last decades, it has been observed that the genotypic methods can represent a better alternative to establish the identity of bacteria and to enrich epidemiological research of infectious diseases [2].

Bacterial infections cause morbidity and mortality, and are responsible for the increase in costs and hospitalization times of patients. The time needed to identify a pathogen based on its phenotypic characteristics is the first challenge, as the sample has to be seeded and incubated for at least 24 hours and, then, conventional biochemical tests must be performed in at least another 24-hour period, conditions that delay results and compromise the patient's health.

Currently, in many microbiology laboratories, the use of automated or semi-automated commercial systems for bacterial identification is common practice, as for example: API

Table 1 Methods used in clinical laboratories for bacterial identification or typing.

Phenotypic methods	Genotypic methods
Biochemical reactions	Hybridization
Serological reactions	Plasmids profile
Susceptibility to anti-microbial agents	Analysis of plasmids polymorphism
Susceptibility to phages	Restriction enzymes digestion
Susceptibility to bacteriocins	Reaction and separation by Pulsed-Field Gel Electrophoresis (PFGE)
Profile of cell proteins	Ribotyping
	Polymerase Chain Reaction (PCR) and its variants
	Ligase Chain Reaction (LCR)
	Transcription-Based Amplification System (TAS)
	Multilocus Sequence Typing (MLST)
	Spoligotyping and MIRUS-VNTR

ENTEROTUBE, VITEK, PHOENIX, MALDI-TOF MS and the GENOTYPE MYCOBACTERIUM CM system for mycobacteria. Some of the characteristics taken into account to choose the identification system are: the easiness to inoculate samples, characteristics to be determined, the required handling for the sample processing after incubation, and the availability and extent of databases [3].

Phenotypic methods are not always able to identify the microorganism to the species level, and much less to the strain level. Therefore, if a breakout, in which only one clone is responsible, is to be determined, more time and the use of genotypic (molecular) or more specific immunological techniques are required. Despite their limitation, phenotypic techniques provide an initial identification that allows taking decisions and is more available at clinical laboratories or hospitals due to their low costs and ample training of the personnel in this health area [4].

Methods for the isolation and identification of organisms from human samples, biological products, or of any other origin involve the isolation in a pure culture of the organism of interest, followed by the necessary tests to discern the microbial metabolism and/or by diverse immunological techniques that will facilitate identification. In many aspects, the culture methods and other techniques used for identification are limited in terms of sensitivity, specificity, or both. Improvements in sensitivity, specificity, and required time are based on progresses in molecular biology that have been integrated in commercial strategies for fast diagnoses. The use of molecular biology techniques for the identification and follow-up of pathogens is based on the characteristics of the genome of the particular organism to be detected or characterized. However, several aspects still complicate their application in the microbiology laboratory: the difficulty in the isolation, the slow growth, the costs of the tests, and their poor detection sensitivity for the identification of some bacterial species coming from complex samples, among others.

This review covers the different phenotypic, also called classical, methodologies, as well as different molecular biology methods that are applied to bacterial characterization. Likewise, it is aimed at raising the interest in the collaborative use of these methodologies among laboratories where bacterial identification and typing are priorities, since, although molecular methods are not yet universally implemented, they are available at research and reference laboratories that could provide the expertise to solve with first level methodologies the health problems of a country.

Phenotypic identification

For the identification of the causal agent of an infectious process, the following must be considered: 1) sample collection, 2) determination of microscopic and colonial morphotypes, and 3) identification based on the bacterial metabolism through conventional or automated tests [2]. The phenotypic study represents the classical point of view for identification, and most identification strategies are based on it [5].

In most cases, phenotypic identification is based not only on one method but rather on the combination of more than one. The sample must come from the site where the microorganism is causing the damage or must be representative of the site or product where it is multiplying. Some samples used in clinical microbiology are: feces, urine, pharyngeal exudate, cerebrospinal fluid, tears, semen, vaginal fluid, tissues, and/or biopsies. Some methods require a pure isolation of the microorganism from the sample, whereas others do not need it. Phenotypic bacterial identification is based fundamentally on the comparison of phenotypic characteristics of unknown bacteria to those of type culture. The reliability of the identification is in direct proportion to the number of similar characteristics. In medical bacteriology, the previous expertise of the analyzer and the association among the microorganism, the site, and type of infection are instrumental for the preliminary identification. Hence, in the traditional or classical bacterial identification process, three levels of processing have been established [1].

a) Primary tests are considered in the first level. These are fast and easy tests to perform, such as uptake of dyes and stains as Gram or Ziehl-Neelsen, microscopic determination of the bacterial morphotype revealed by the stains, growth characteristics at different incubation atmospheres, different temperatures, and in diverse culture media, production of oxidase and catalase enzymes, oxidation-fermentation, glucose fermentation, productions of spores, and mobility. Through these tests, it is

generally possible to place the pathogen, provisionally, in some of the main groups of clinical relevance. Afterwards, other methods with greater discriminatory power can be used, to be able to discern among microorganisms that present a very similar aspect in the macro and microscopic analyses [6].

b) The second level of identification must specify the genus of the microorganism. In both this and the former level, the hypothesis on the probably identity of microorganisms is based on the characteristics of the culture and on the primary tests, which will allow determining the genus, group of genera, or, in some cases, the family of the isolate. Clinical data must also be taken into account. This will depend to a great extent on a stable pattern of phenotypic features and on the expertise of the microbiologist [7].

c) Finally, the third identification level is at the species level. Some biochemical tests allow identifying accurately most of the clinically significant bacteria. If this is not possible, a more ample battery of tests can be used, like those found in different commercial systems.

Numerous multi-test systems or equipments are available in the market to make bacterial identification fast and reliable. These techniques require a precise control of the inoculum, its purity, and way of inoculation, incubation, and reading of the tests, because not following these criteria may lead to errors. These systems can be manual, semi-automated, or automated. The result is compared to standardized tests or to the database of numerical profiles that the commercial methods have developed for this purpose. A limitation is the appearance of mutating strains and the acquisition of plasmids that can give origin to strains of different characteristics [5,8].

In contrast to the laboratories of clinical biochemistry or hematology that have benefitted from the technology to simplify sample processing and thus obtain results in a short time, automatization of the microbiology laboratory is more complex given the large variety of clinical samples to be analyzed and the inherent characteristics of different microorganisms. Recently, mass spectrophotometry (MS) has become part of the microbiology laboratory offering a fast and reliable alternative for the identification of microorganisms, including one of the most difficult identifiable bacterial groups, mycobacteria [9,10].

MS is an analytical technique that allows analyzing with great accuracy the composition of different chemical elements by permitting the measurement of ions derived from molecules and separating them in function of their mass/load (m/z) ratio [11]. The mass spectrum of each compound is named “chemical trace” and is a graphical representation of the fragments obtained, by an increasing order of mass in terms of its relative abundance. Bacterial identification based on the proteins profile obtained by MALDI-TOF mass spectrometry was proposed several decades ago. However, it has been used only recently as a fast and reliable method for bacterial identification [9]. The currently commercialized platforms use MS for the identification of microorganisms through different approaches: identification based on the specific protein profile of each microorganism (proteomic approach) or on the analysis of its nucleic acids (genomic approach). Some of the commercial systems that use MS are: MALDI-TOF for microbial identification; MicrobeLynxTM of Waters Corporation, MALDI BiotyperTM of Bruker Daltonics, and MS-ID of BioMérieux [12]. The last one allows the mycobacteria identification [13].

Genotypic (molecular) identification

In recent years and with the advent of new methodologies based on molecular methods great advances have been made in the diagnosis of clinically relevant bacteria. Among them, stand out the ribosomal RNA detection through hybridization with a DNA probe and that of nucleic acids amplification from clinical samples. These techniques improve the sensitivity and diagnostic specificity with respect to other detection techniques, including culture, and, in some cases, have allowed for the simultaneous detection of several microbial agents from the same sample [14].

The first step in the development of methodologies based on molecular biology techniques was supported by the detection of nucleic acids of microorganisms by means of a probe through hybridization. The genetic probe is a nucleic acid molecule, in a monocatenated state and marked, that can be used to detect a complementary DNA sequence. Oligonucleotide probes are obtained from natural DNA by cloning DNA fragment into appropriate plasmid vectors and then isolating the cloned DNA or through direct synthesis by means of combinatory chemistry. Probes can be labeled with substances that produce colorful reactions under adequate conditions [15].

DNA hybridization techniques are relatively easy to perform and interpret. Amplification techniques based on the detection of DNA using Polymerase Chain Reaction (PCR) and Ligase Chain Reaction (LCR) or transcription-mediated specific rRNA amplification is already available both to be performed in house or commercially obtained. These techniques provide faster results with better sensitivity and specificity than conventional techniques. Depending on the type of sample these techniques detect from 15 to 20% more infectious agents than the conventional ones and 25 to 70% more than through immunofluorescence or Enzyme Immunoanalysis (EIA) [14,16].

Construction of probes to detect virulence markers, as those directed to genes encoding toxins, allows identifying those organisms that carry these genes in the clinical samples, without having to cultivate the samples. Examples of the later are the probes for *Escherichia coli* enterotoxins, for *Vibrio cholerae* toxin, or for toxins of *Clostridium difficile*, which can be applied directly to fecal samples [17].

Different target genes are used for the detection of microorganisms, for example those causing Sexual Transmission Infections (STI), which have been used in PCR assays; among them are genes *omp*1 and *omp*2 of the Main Membrane Proteins (MOMP) to study the main etiological agents; the cryptic plasmid pCT and genes *16S rRNA* and *23S rRNA*, for assays addressed at identifying *C. trachomatis* [14,18,19]. Focusing on genes *16S rRNA* and *23S rRNA* increases sensitivity of the assay, as normally there are multiple copies in microorganisms. However, some authors suggest that the crossed reactions with other bacteria could pose a problem; whereas others have demonstrated that the use of conserved regions of gene *16S rRNA* in the amplification

reactions allows for species-specific differentiation [19, 20]. Use of genes and target regions for the detection of mycobacteria is a well studied area, particularly due to the difficulty posed for the isolation of these microorganisms from biological samples and furthermore because of the current hardships in handling these very virulent microorganisms. Several sequences, genes, and intergenic regions have been used for the identification of this bacterial genus, among them, the *rRNA 16-23S* region, genes *16S rRNA*, *gyr*B and *rpoB*, the insertion element IS6110, and the eliminate differentiation regions RD1 and RD4 [21]. The study of these genes or genic sequences by means of PCR will eventually allow comparative sequence analysis of the obtained product with the sequences of reference isolates. Several commercial probes for the diagnosis of infectious diseases have been designed, but the capacity of detecting a small number of organisms or few copies of the gene in the clinical sample is still a limiting factor of this technique. However, combination of PCR with probes hybridization can become the method of choice, particularly, for microorganisms whose culture in the laboratory is slow and difficult [15].

PCR is an *in vitro* method of the DNA synthesis with which a particular segment of DNA is amplified by being delimited with a pair of flanking primers. Copying is achieved exponentially through repeated cycles of different incubation periods and temperatures in the presence of a thermostable DNA polymerase enzyme. In this way, millions of copies of the desired DNA sequence can be obtained in a couple of hours. This is a highly specific, fast, sensitive, and versatile molecular biology technique to detect the smallest amounts of a specific DNA, fostering its easy identification and avoiding the use of radioisotopes [22]. Despite the benefits that the PCR technique offers in comparison to culture for the detection of some microorganisms, the commercially available techniques are scarce and are limited to research laboratories or to reference laboratories specialized in molecular diagnoses, among other causes, due to their high cost. An alternative to make the use of molecular diagnoses feasible as routine techniques could be the acquisition of reagents in a separate manner and standardization of nucleic acids extraction and amplification protocols designed in each diagnostic laboratory; this would lead to a significant reduction in technological dependence and to an increase in the sensitivity and specificity of the used diagnostic techniques [23].

The multiple amplification for the concomitant detections of some microorganisms enhances, in some cases, the sensitivity and specificity of those addressed at a single microorganism. This PCR variant is called multiple PCR (mPCR), in which more than one target sequence can be amplified simultaneously by the inclusion of more than a pair of primers in the reaction [24]. This technique has been applied successfully in many diagnostic areas, like the study of infectious diseases, species genotyping, diagnosis of hereditary diseases, identification of mutations, paleontology, anthropology, and forensic sciences, among others; here, the technique has shown the potential to save considerable time, without compromising the usefulness and efficiency of the test [24]. On the other hand, plataforms for pathogen identification are becoming available like pyroseguencing and spectroscopy [25].

In the amplification-pyrosequencing platforms, bacterial identification is achieved by PCR of three variable regions of the *16S rRNA* (V1-V3, or V1, V2 and V6). Lower amplicons of 500 bp are obtained, their nucleotides composition can be determined by means of the emission of light by the release of pyrophosphates (extension byproducts by polymerization of the DNA chain). This platform has been increasingly innovated based on the type of clinical sample and on the determination of different genic fragments corresponding to the different virulence factors and the resistance to antimicrobials, which has improved the versatility of this platform [2,25]

Another innovating platform for bacterial identification is the conjunction of amplification (by PCR) and mass spectrophotometry (PCR/ESI-TOF). The latter allows for the universal detection of one or more pathogens encountered in a wide variety of samples (environmental, clinical, foodborne, o in cultures). It consists in the extraction of nucleic acids and PCR amplification with primer pairs of ample range; one or several PCR products are obtained that correspond to genomic identification regions of the different microbial domains in relation to the complexity of the problem sample. The products are desalted and then ionized and aerosolized towards the mass spectrometer, generating signals that are processed to determine their mass and composition. Results are interpreted with the TIGER (Triangulation Identification for the Genetic Evaluation of Risks) strategy, and accessing the information into a genomic database that assigns the species. The advantages of this platform are that it does not require culture, is efficient in polymicrobial samples, and, in the case of non-characterized new pathogens, it allows assigning them to bacterial genera or families. In addition, it also permits to detect some virulence and resistance genes, and typing of the identified microorganism [2,12].

Typing of microorganisms

After bacterial identification, microorganisms have to be typed for epidemiological studies; hence, molecular typing systems constitute one of the molecular techniques contributions to microbiology widely used in the last years. These systems involve a large variety of techniques aimed at comparing the structure of genomes of highly inter-related organisms.

Typing methods (phenotypic and genotypic) allow differentiating one bacterial strain from another. Before using a typing technique it is important to ensure that the method can differentiate among non-related isolations, that it is able to detect the same strain in different samples, and that it reflects the gene relations among isolations with epidemiological relation [26].

From a practical point of view, a typing system should be reproducible, have a high discrimination capacity, and be easy to use and to interpret the results [26]. Notwithstanding, election of the molecular method depends also on other factors, such as the microorganism to be studied, the clinical sample, the target to be studied (a single gene or the whole genome), the area of application, the infrastructure available at the clinical laboratory, and the speed needed to reach a result. Once the microorganism has been identified, it is important to know whether it is responsible for a breakout; therefore, the corresponding

epidemiological research has to be performed. To accomplish this process diverse molecular techniques have been used; these tools are aimed at determining the clonal relation that exists among several isolates of a given species. This information is useful in sporadic infections and even more during disease breakouts and epidemics because it allows determining the number of circulating clones, detecting the pathogens' transmission route, identifying the source of infection, recognizing particularly the virulent strains, and, thereby, leading to the most appropriate treatment [27,28].

The typing technologies based on the whole genome of the microorganism yield better results in establishing the clonal relation. However, for this analysis, digestion of the genome with restriction enzymes is needed to obtain DNA fragments of diverse sizes that provide patterns or profiles, once they have been separated by electrophoresis. On the other side, there is the inconvenient that the diverse fragments obtained from the restriction procedure are large-sized fragments that have to be analyzed by Pulsed-Fields Gel Electrophoresis (PFGE) [26]. PFGE is a technique widely used for typing clinically relevant bacteria. Its importance relies on that this type of electrophoresis is capable of separating fragments of a length higher than 50 kb up to 10 Mb, which is not possible with conventional electrophoresis, which can separate only fragments of 100 bp to 50 kb. This capacity of PFGE is due to its multidirectional feature, changing continuously the direction of the electrical field, thus, permitting the re-orientation of the direction of the DNA molecules, so that these can migrate through the agarose gel, in addition to this event, the applied electrical pulses are of different duration, fostering the reorientation of the molecules and the separation of the fragments of different sizes [29]. Along time, different types of PFGE equipment have been developed **(Table 2)**, mainly to improve the resolution of gels and to diminish costs of reagents and electricity. The most used apparatus is the Contour Clamped Homogeneous Electric Fields (CHEF, BioRad), because it can separate molecules of 7000 kb, this characteristic is provided by its 24 electrodes that are hexagonally distributed and generate homogeneous electrical fields allowing for the samples to be run uniformly [29,30]. Some advantages of PFGE are: it possesses a high discrimination power, excellent reproducibility, easiness to measure the genome and it allows working with a large number of samples. Disadvantages include that most of the protocols require more than 4 days to get and analyze the pulse types, in comparison to other methods that can be less costly, but not appropriate to study clonally related strains **(Tables 2 and 3)** [27,29]. Application of PFGE to the study of mycobacterial infection has been limited by the need to count upon high mycobacterial DNA concentrations that are difficult to obtain, as effective breakage of the cell wall and disaggregation of the lipids covering the mycobacterium are complicated procedures; additionally, mycobacterial growth tends to aggregate in clusters, which also hinders the assay [31].

Polymerase Chain Reaction-RFLP

Consists of a PCR for the amplification of a gene or parts of it, combined with the subsequent digestion of the PCR products using one or several restriction enzymes. The electrophoretic analysis of the restriction products reveals the polymorphisms of the gene or of the gene fragments (RFLP) and evidences the genetic changes among isolates. This technology is capable of revealing sequence polymorphisms rapidly; it is technologically simple and highly reproducible. Besides, it compares well with other techniques like: Denaturing Gradient Gel Electrophoresis (DGGE), Temperature Gradient Gel Electrophoresis (TGGE), or single strand conformation polymorphism (SSCP), and Cut Fragments Length Polymorphism (CFLP), which also reveal sequence polymorphisms among strains without having to determine the whole sequence [32]. For mycobacteria, PCR-RLFP has been widely used, particularly for the study of the insertion element IS6110, where the Pvull enzyme is used to generate restriction fragments from genomic DNA [33]. Other sequences or genes used to identify and genotype *Mycobacterium tuberculosis*, as well as other non-tuberculous mycobacteria are 16S rDNA, and genes *rpoB, recA,* and *hsp65*, which have yielded variable results [34]. Of these sequences, the most consistent has been the gene that encodes the 65-kDa heat shock protein (hsp65), analyzed through a PCR-based assay and its posterior restriction with enzymes BstEII and HaeIII, an assay known as PRA (Polymerase Chain Reaction Restriction Enzyme Analysis—PRA) [35]. Of the non-commercial molecular methods, the PRA method is one of the most used for the identification of non-tuberculous mycobacteria of fast growth, due to its speediness, low cost, and above all, because the data base: http://app.chuv.ch/prasite/index.htm, is available and contains the restriction profiles of at least 113 species [36].

Table 2 Most relevant features of some typing methods based on PCR-amplification of nucleic acids in comparison to Pulsed-Field Gel Electrophoresis (PFGE).

Method	Ease of the technique	Results interpretation	Duration of the test (days)	Reproducibility among laboratories	Intra-assay reproducibility	Cost per test
PFGE	Moderate	Easy	3	Good	Good	Moderate
PCR-RFLP	Easy	Easy	1	Good	Good	Low
rep-PCR	Easy	Easy	1	Good	Moderate	Low
AP-PCR	Easy	Easy	1	Moderate	Low	Low
AFLP	Moderate	Moderate	2	Good	Good	Moderate
MLST	Difficult	Moderate	2	Good	Good	High

PCR= Polymerase Chain Reaction; **PFGE**= Pulsed-Field Gel Electrophoresis; **PCR-RFLP**= PCR-Restriction-Fragment Length Polymorphism; **rep-PCR**= PCR-Repetitive Extragenic Palindromic Elements; **AP-PCR**= PCR-Arbitrary Primers; **AFLP**= Amplified Fragment Length Polymorphism; **MLST**= Multilocus Sequence Typing. Modified from [27].

Table 3 Comparative analysis of some molecular biology techniques and bacteria of hospital relevance where they are applied.

Typing method	Description	No. Of markers	Time scale	Source of variation	Discrimination power	Reproducibility	Application	Database
MLST	PCR amplification of *housekeeping* genes to create allele profiles	7	GE LE	DtNA sequence	Moderate to high	High	*Acinetobacter baumannii* *Clostidium difficile* Coagulase negative staphylococci *Enterococci* *Pseudomonas aeruginosa* *Staphylococcus aureus*	pubmlst.org www.mlst.net
rep-PCR	PCR amplification of repeated sequences in the genome	NA	LE	Banding patterns	Moderate to high	Medium	*Staphylococcus aureus* *Mycobacterium tuberculosis* *Acinetobacter baumannii*	NA
PFGE	Comparison of macro-restriction fragments	NA	LE	Banding patterns	Moderate to high	High	*Acinetobacter baumannii* *Staphylococcus aureus* Coagulase negative staphylococci	NA
AFLP	Enzyme restriction digestion of genomic DNA, binding of restriction fragments and selective amplification	NA	LE	Banding patterns	Moderate to high	Low	*Acinetobacter baumannii* *Klebsiella pneumoniae* *Staphylococcus aureus* *Mycobacterium tuberculosis*	NA
MLVA	PCR amplification of loci VTR, visualizing the polymorphism to create an allele profile	Oct-80	GE	DNA sequence	Moderate to high	High	*Clostidium difficile* *Enterococci* *Mycobacterium tuberculosis* *Staphylococcus aureus*	minisatellites.upsud.fr www.mlva.net www.pasteur.fr/mlst
RFLP	Genomic DNA digestion or of an amplicon with restriction enzymes producing short restriction fragments	NA	LE	Banding pattern	Low	High	*Staphylococcus aureus* *Pseudomonas aeruginosa* *Mycobacterium tuberculosis*	http://app.chuv.ch/prasite/index.htm

MLST: Multilocus Sequence Typing; MLEE: Multilocus Enzyme Electrophoresis; PFGE: Pulsed Field Gel Electrophoresis; AFLP: Amplified Fragment Length Polymorphism; MLVA: Multilocus Tandem Repeat Sequence Analysis; RFLP: Polymorphism of DNA Restriction Fragments; rep-PCR= PCR-Extragenic Palindromic Repetitive Elements; GE: Global Epidemiology (macro-epidemiology); LE: Local Epidemiology; NA: Not Applied. Modified from [43]

rep-Polymerase Chain Reaction

Versalovic et al. [37] described a method to study the bacterial genome by examining the specific patterns of a given strain obtained through PCR amplification of repetitive DNA elements present in bacterial genomes. They used two main sets of repetitive elements for typing purposes, the REP with 38-bp sequences that consist of six degenerated positions and a variable loop of 5 bp between each side of a conserved palindromic portion. REP sequences have been described both for enteric bacteria and for Gram-positive [28,38] and, more recently, for mycobacteria including nontuberculous mycobacteria, in the latter with good results [39]. ERIC sequences are a second set of DNA sequences that have been used successfully for the typing of strains; they are 126-pb elements that contain a highly conserved central inverted repetition and are located in extragenic regions of the bacterial genome. ERIC analysis has also been used for genotyping of mycobacteria [40]. REP or ERIC amplification can be performed with only one primer or with one set or multiple sets of primers. ERIC patterns are generally less complex than REP patterns, but both provide a good discrimination at the level of strains. The combined use of both methods (REP-PCR and ERIC-PCR) in the strains to be typed, increases their discrimination power [28]. Although REP and ERIC sequences are the most commonly used targets for DNA typing, BOX sequences are also used, the latter have been used to differentiate strains of *Streptococcus*

pneumoniae. BOX elements are located in intergenic regions and they can also form stem-loop structures due to their even symmetry. They are a mosaic of repetitive elements composed of several combinations of three sequences known as *box*A, *box*B and *box*C. The three-subunit sequences have molecular lengths of 59, 45 and 50 nucleotides, respectively. BOX elements have no sequence relation with REP and ERIC sequences [28].

Amplified Fragment Length Polymorphism

Amplified Fragment Length Polymorphism (AFLP) is a genomic fingerprinting technique based on the selective amplification of a subset of DNA fragments generated through restriction enzymes digestion. Originally, applied to the characterization of plant genomes, currently AFPL has been used for bacterial typing. Two variations of AFLP have been described: the first, with two different restriction enzymes and two primers for the amplification, and the second, with only one primer and one restriction enzyme. Bacterial DNA is extracted, purified, and then subjected to digestion with two different enzymes, such as EcoRI and MseI. Afterwards, the restriction fragments are bound to adaptors that contain each restriction site and a sequence that is homologous to a binding site of the PCR primer. The PCR primers used for the amplification contain DNA sequences that are homologous to the adaptor and contain one or two selective bases in their 3' ends [30,41]. The AFLP method has been adapted for the study of *M. tuberculosis*, however, it has been scarcely used due to its poor genotyping resolution power in *M. tuberculosis* [42].

Multilocus Sequence Typing

Multilocus Sequence Typing (MLST) is a genetic method with a high resolution power; it is based on sequencing fragments of 7 genes of 450 to 500 bp (with a high degree of variability). The analysis detects variations in the different loci and permits the identification of identical microorganisms (clones) or of highly related ones (clonal lines or genotypes). Therefore, they are markers that have remained stable along evolution and are used for the comparison of strains in large time scales or from different geographical regions [43]. Sequencing allows detecting variants of just one change in the database of the analyzed gene. Hence, it has been calculated that if 30 different alleles are found per locus, and seven genes are studied, then up to 307 different genotypes could be distinguished [44]. Each allele is numbered considering its previous presentation in the database and each type of Sequence (ST) is defined by a bar code of seven digits that are unique to the seven loci [45].

Spoligotyping and MIRU-VNTR

Three methods are the most commonly used for mycobacterial genotyping: spoligotyping, MIRU-VNTR analysis, and analysis of restriction fragments obtained with the PvuII enzyme to detect the insertion element *IS6110* by hybridization. Spoligotyping was the second method widely used for fast genotyping of mycobacteria, originally described for the typing of *M. bovis* isolates [46]. This technology combines a PCR amplification of the Direct Repeat (DR) region of each isolate and the hybridization of the spacer regions, the latter are unique sequences that separate DRs, there are 47 DRs for *M. tuberculosis* and 41 DRs for *M. bovis;* these spacers depict a large polymorphism, which enables their use as variability indicators. The method was named "spoligotyping" derived from "spacer oligotyping" [47]. The hybridization pattern shown by each isolate is interpreted on a matrix that can be worked with the binary system or by means of the "octal code" to ease handling of the patterns [48]. Analysis of genomic loci of *M. tuberculosis* containing Variable Number Tandem Repeat Sequences (VNTR) is a fast genotyping method, similar to spoligotyping, and is based on the analysis of repeated sequences found in the mycobacterial genome (MIRU-Mycobacterial Interspersed Repetitive Units) that are different in the DR regions. The minimal set of MIRUS-VNTR to achieve a differentiation is of 12 loci, which yields a code that establishes the pertinence to the different genotypes through an informatics analysis [49]. The number of MIRUS loci has been increased to 24, granting a higher resolution to this protocol and has widened its application to evolutionary-type studies [50].

Conclusions

Isolation, identification, and analysis of isolates of a single sample are some of the main functions and objectives of a microbiology laboratory. However, it is fundamental to recall that the best bacteriological result is obtained when the sample received by the laboratory has been procured under the best conditions. In addition, it is a major task of the laboratory to differentiate a pathogenic microorganism per se or the potential pathogenicity of colonization and a contaminant, and to describe, if applicable, the possible resistance mechanisms to be able to propose the most efficacious treatment.

The medical bacteriology diagnostic laboratory currently counts upon diverse methodologies that constitute a fundamental cornerstone in the diagnostic process of bacterial-origin infections. Within its daily routine, the clinical laboratory applies phenotypic techniques to reach its goals. However, these techniques do present some limitations in terms of sensitivity, specificity, and time. These limitations are more evident for some types of bacteria of difficult or slow growth, of the so-called non-cultivable bacteria, or for the processing of samples from multi-treated patients. In the last decade, diverse techniques have been developed in the field of molecular biology and analytical chemistry with a great potential to diminish some of these limitations, and which have allowed for the search and identification of the causal agent, as well as the evaluation of clonality, for epidemiological research and objectives. Because these techniques are still laborious and of a higher cost than some phenotypic ones, they are usually not available at laboratories of public hospitals. Although their implementation is not universal they are available at research and reference laboratories.

To be clinically useful, the identification of a microorganism must be as fast as possible. Economic aspects and praxis propose the use of a minimal number of diagnostic tests. By necessity, identification in the clinical laboratory will always represent a compromise of accuracy and precision, on one side, and the speed and economy on the other side; therefore, the collaborative efforts among public clinical laboratories and research and reference laboratories is undoubtly the correct course of action to better diagnoses.

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