Typing methods used in the molecular epidemiology of microbial pathogens: a how-to guide

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---- SUMMARY ----

Microbial typing is often employed to determine the source and routes of infections, confirm or rule out outbreaks, trace cross-transmission of healthcare-associated pathogens, recognize virulent strains and evaluate the effectiveness of control measures.

Conventional microbial typing methods have occasionally been useful in describing the epidemiology of infectious diseases. However, these methods are generally considered too variable, labour intensive and time-consuming to be of practical value in epidemiological investigations. Moreover, these approaches have proved to be insufficiently discriminatory and poorly reproducible.

DNA-based typing methods rely on the analysis of the genetic material of a microorganism. In recent years, several methods have been introduced and developed for investigation of the molecular epidemiology of microbial pathogens. Each of them has advantages and limitations that make them useful in some studies and restrictive in others. The choice of a molecular typing method therefore will depend on the skill level and resources of the laboratory and the aim and scale of the investigation.

This study reviews the most popular DNA-based molecular typing methods used in the epidemiology of bacterial pathogens together with their advantages and limitations.

KEY WORDS: Molecular epidemiology, DNA-based typing, Bacterial pathogens.

INTRODUCTION

The main role of microbial typing is to assess the relationships between microbial isolates. Understanding clonal relatedness between the microbial strains is essential to determine the source and routes of infections, confirm or rule out outbreaks, trace cross-transmission of healthcare-associated pathogens, recogni-

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ze particularly virulent strains and evaluate the effectiveness of control measures (Tenover *et al.*, 1997; Maccannell, 2013; Pérez-Losada *et al.*, 2013). Bacterial typing has also greatly contributed to increase the effectiveness of surveillance systems and has provided significant clues to public health control strategies.

Conventional epidemiological typing methods, such as antibiogram, biotyping, serotyping and phage typing have occasionally been useful in describing the epidemiology of infectious diseases. For some worldwide diffuse pathogens, serotyping, phage typing and antibiotic resistance patterns have historically provided data to be used for short-term epidemiological studies, assessment of epidemiological trends in

well-defined geographical areas and comparison between different countries (Tenover *et al.,* 1997; Maccannell, 2013). However, these methods are generally too variable, labour intensive and time-consuming to be of practical value in epidemiological investigations (Maccannell, 2013). They are able to differentiate between organisms in which there is marked variation in phenotypic expression, but most organisms causing infections are a small subpopulation of the total strains accounting for a species and may demonstrate little diversity (Tenover *et al.,* 1995). Moreover, some communicable diseases result from infection by highly fastidious bacteria and cannot routinely be confirmed by culture. Indeed, procedures to diagnose infections by such bacterial species, such as Whipple's disease, bacillary angiomatosis-cat scratch disease, ehrlichiosis, etc. and to study their epidemiology are often very demanding and, in most cases, unaffordable by routine diagnostic laboratories (Erlich *et al.,* 2013).

Consequently, DNA-based typing methods have become indispensable to study the epidemiology of most microbial pathogens. Several different epidemiological typing systems have been applied, including a wide armamentarium ranging from some non-molecular approaches to the more sophisticated molecular typing tools. The molecular typing methods most commonly used are the DNA-based methods, such as restriction endonuclease analysis of genomic and plasmid DNA, southern hybridization analysis with the use of specific DNA probes, plasmid profiling, chromosomal DNA profiling using either pulse-field gel electrophoresis or polymerase chain reaction (PCR)-based methods (Tenover *et al.,* 1997; McDade *et al.,* 1998; Maccannell, 2013). Microarray technology has more recently allowed the simultaneous assessment of large numbers of microbial genetic targets (Maccannell, 2013; Pérez-Losada *et al.,* 2013). A true revolution is announced by the adoption of the whole genome sequencing (WGS) approach (Erlich *et al.,* 2013; Pérez-Losada *et al.,* 2013).

While a broad range of methods is available, the decision whether to use one or another needs to consider several *performance* variables, such as intra- and inter-laboratory reproducibility, interlaboratory portability, unequivocal interpretation of results, high throughput and appro-

priateness, but also *convenience* variables, such as user-friendliness, cost, speed and affordability (van Belkum *et al.,* 2007). Appropriateness, i.e. the suitability of the test to answer a specific epidemiological question, should eventually tip the scale in favor of one specific method or combination of methods.

This review describes the molecular methods most commonly used for subtyping of different pathogens and addresses their main advantages and limitations.

DNA-BASED TYPING METHODS

Analysis of plasmid profiles
Plasmid DNA analysis has been applied for typing of diverse species of Gram-negative and Gram-positive bacteria (Wachsmuth *et al.,* 1991; Liu *et al.,* 1996). Although plasmids can be used as a marker for comparing strains in field and healthcare settings, their most important use in epidemiology is for evaluating the potential spread of a resistance gene (Farshad *et al.,* 2011; Mnif *et al.,* 2013; Wang *et al.,* 2013). This is especially useful for organisms like *Staphylococcus* spp. and enterobacteria. Plasmid profiling was one of the earliest genotyping methods used for epidemiological studies. However, it has a number of disadvantages, mainly intrinsically related to the properties of the plasmids. These mobile elements are transferable by conjugation, can be gained or lost spontaneously and under selective pressure they may spread rapidly from one strain to the next (Tenover *et al.,* 1997). The gain or loss of plasmids can generate confusion in the attribution of genetic relatedness to the isolates, which limits the applicability of the method to short-term epidemiological studies. Moreover, plasmids may show discrepancy as time passes and either carry or lose genetic sequences such as transposons (Bopp *et al.,* 1999). However, small plasmids which appear on agarose gels as bright bands mostly below chromosomal DNA, are suitable to be used for typing purposes because of their stability unlike larger plasmids which tend to be lost during cell storage and subculturing or extraction procedures (Farshad *et al.,* 2006). Moreover, more recently, the old classification scheme based on plasmid incompatibility, which allowed plas-

mids to be recognized and classified in homogeneous groups (Datta *et al.,* 1971), has been re-proposed in a molecular version. Indeed, since 2005, a PCR-based replicon typing scheme has become available, targeting the replicons of the major plasmid families (Carattoli *et al.,* 2005). This user-friendly version has revived interest in the epidemiology of plasmids, leading to identification of largely prevalent plasmid families as well as of plasmids prevalently associated with specific resistance genes and "epidemic" plasmids closely associated with selective pressure exerted by antimicrobial use (Villa *et al.,* 2010).

Analysis of chromosomal DNA
The ability of molecular typing systems to differentiate between epidemiologically unrelated organisms is a reflection of the genetic variation in the chromosomal DNA of the bacterial species. Several techniques are available for pathogen typing based on analysis of chromosomal DNA, including restriction endonuclease analyses (REA) of chromosomal DNA using frequent cutting enzymes and traditional gel electrophoresis, restriction fragment length polymorphism (RFLPs) analysis using DNA probes, PCR and other related nucleic acid amplification-based typing methods, pulse field gel electrophoresis (PFGE) and DNA sequencing.

Analysis of RFLPs
The most common molecular epidemiology methods rely on the study of RFLPs after enzymatic restriction of the chromosomal DNA. The number of fragments obtained reflects the frequency and distribution of the cutting site specifically recognized by the enzyme in the nucleotide sequence of the bacterial chromosome. The fragments obtained are then separated by gel electrophoresis which provides a banding profile or "fingerprint" of the DNA that can be easily visualized. When "frequent cutting" enzymes are used, hundreds of fragments can be generated. Since two clonally related strains contain almost identical DNA sequences, restriction sites should be conserved generating identical electrophoretic profiles. On the contrary, in distinct strains it is highly probable that variations in the nucleotide sequence of the chromosome alter the distribution of restriction sites, generating different

restriction profiles. Any bacterial species can be typed by this method whose main limitation lies in the difficulty of interpreting complex electrophoretic profiles, since patterns with very large numbers of DNA fragments are difficult to read, both visually and instrumentally, in standard gel electrophoresis. Many methods have been developed to simplify the patterns and their comparison, reducing the number of bands. Two main approaches have been followed: the selection of a limited number of fragments by southern blot and hybridization, as in ribotyping and insertional sequence (IS)-RFLP typing, and the reduction of the number of fragments produced through "rare cutting" endonucleases and special electrophoretic conditions for appropriate resolution, as in pulsed-field gel electrophoresis (PFGE).

RFLP analysis using DNA probe (southern analysis)
In this method, DNA from a bacterial strain is digested with restriction enzymes. The fragments are separated by electrophoresis and transferred to a membrane filter. The filter is then incubated with a probe that hybridizes to a specific gene. Depending on the restriction enzyme chosen, the pattern of hybridizing bands can be species- or strain-specific. Two or more different restriction enzymes in separate digestions can be used to generate different patterns from the same isolates and obtain greater confidence when strains are being tracked during epidemiological investigations.
Ribotyping is one of the typing techniques based on RFLP analysis of ribosomal DNA. It simplies the restriction fragments pattern generated by enzymatic digestion of the chromosomal DNA selecting only those containing the gene sequences of the ribosomal RNA (rRNA). Among repetitive elements, the operons coding for ribosomal RNA are those most frequently used, since most of the bacterial species contain multiple copies dispersed throughout the entire chromosome. Sequence differences in the regions flanking the rRNA gene lead to variability in the size of the fragments which produce distinct patterns useful to discriminate between related strains. Consequently, for a mutation or other genetic change to be recognized, it must alter the size of fragments containing a portion

of the rRNA gene. The limited number of bands in the restriction patterns facilitates their analysis, but it is also a potential drawback when using ribotyping for epidemiological purposes. On the contrary, ribosomal RNA genes have become the best targets for studying long-term epidemiology and phylogenetic relationships. Ribotyping is time-consuming and demands skilled personnel, but the method has been automated in the RiboPrinter Microbial Characterization System (Qualicon, Inc., Wilmington, DE, USA). Automated ribotyping can be a very useful tool as the first-step method in epidemiological surveys based on its ability to analyse large numbers of bacterial isolates in a very short time and with minimal human effort, with the major disadvantage being the comparatively high cost per bacterial isolate (Grimont *et al.*, 1986; Mammina *et al.*, 2009; Schumann *et al.*, 2013; Ranjbar *et al.*, 2008).

Pulse field gel electrophoresis (PFGE)
In PFGE, the restriction pattern of the whole bacterial genome is analyzed without the use of probes. The bacterial chromosome is digested by rare cutting enzymes which recognize specific DNA sequences of 6-8 bases, yielding a low to moderate number of fragments. The technique takes care to protect the chromosomal DNA from mechanical damage by immobilizing it into agarose blocks during lysis. After enzymatic digestion, blocks are inserted into the wells of the migration gel and submitted to an alternating voltage gradient in which the orientation of the electrical field switches direction under the control of a programmable device. This approach can resolve DNA fragments up to 800 kb in size. Point mutations, deletions, insertions and loss or acquisition of plasmids might account for minor differences in profiles within a subtype or among epidemiologically related strains. These changes usually result in two to three fragment differences in PFGE banding patterns. Therefore, one generally accepted interpretation rule is that one isolate is closely related to another when the difference is around two to three fragments, possibly related when it is four to six, and unrelated when the difference is seven or more fragments (Tenover, 1995).
PFGE has been successfully used in short-term

epidemiological investigations for many bacterial pathogens, proving a very accurate and reproducible method (Ranjbar *et al.*, 2007). A visual comparison of profiles with usually only 10 to 20 bands is relatively easy and computer-based analysis with the possibility of creating database libraries is available. A drawback of PFGE is that it is labour intensive, requiring multiple days to perform the procedure and skilled personnel to interpret the results and for computer-assisted analysis of banding patterns (Goering, 2010). Additionally, only base substitutions involving the restriction sites of the selected enzyme or genetic variation affecting restriction fragments' length (insertion or deletion) can identify separate pulsotypes. Therefore, strains that appear indistinguishable following analysis with one enzyme may not be truly identical and have to be confirmed by a second enzyme (Goering, 2010). Poor portability is an additional disadvantage. Technical problems with PFGE vary in their complexity and ease of diagnosis. They can include the weak intensity of banding patterns due to low cell concentration, artifactual bands due to incomplete digestion of DNA, skewed lanes due to faulty electrodes or uneven gel thickness and/or buffer height due to uneven surfaces used for gel casting or electrophoresis. Furthermore, isolates belonging to some bacterial species, mainly with an environmental habitat, such as *Pseudomonas aeruginosa* and *Vibrio cholera*, are often untypeable due to DNA degradation by their DNAse production (Romling *et al.*, 1994; Kam *et al.*, 2013).
PFGE used with various restriction enzymes is considered the golden standard for subtyping of bacteria and is the currently used method in the PulseNet USA network (http://www.cdc.gov/pulsenet), the molecular surveillance network for foodborne infections in the United States (Swaminathan *et al.*, 2001, Gerner-Smidt *et al.*, 2006).

AMPLIFICATION-BASED TYPING METHODS

Amplification-based typing methods are also identified as PCR-based methods. The PCR method, which is widely known as the most fre-

quently applied nucleic acid amplification and detection method, has had a substantial impact on the diagnosis and epidemiological investigation of infectious disease. The ability of this method to amplify minute amounts of specific microbial DNA sequences has made it a powerful molecular tool.

PCR gave rise to a variety of methods with many diagnostic and epidemiologic applications such as PCR-RFLP, PCR-ribotyping, randomly amplification of polymorphic DNA (RAPD), repetitive extragenic palindromic (Rep)-PCR, enterobacterial repetitive intergenic consensus (ERIC)-PCR, PCR-sequencing and other techniques. (Towner *et al.,* 1995; Vaneechoutte *et al.,* 1997; Bala *et al.,* 2001; Singh *et al.,* 2006, Ranjbar *et al.,* 2010).

PCR-RFLP

PCR-RFLP is a variation of RFLP in which the restriction analysis is performed on PCR amplicons obtained using primers for specific sequences of interest. A DNA banding profile is generated cutting the PCR amplicons with restriction enzymes. To generate adequate discrimination after cutting the amplicon, the amplified gene needs to have variable sequences flanked by highly conserved regions to be targeted by universal primers. This method can identify a genetic correlation among pathogenic bacteria and is used in molecular epidemiology study of different genera and species (El-Adawi *et al.,* 2013; Pereyre *et al.,* 2013).

RAPD-PCR

RAPD-PCR is a typing method based on the use of short random primers which hybridize with sufficient affinity to chromosomal DNA sequences at low annealing temperatures such that they can be used to initiate amplification of regions of the bacterial genome. A couple of other techniques, such as arbitrarily primed PCR (AP-PCR) and DNA amplification fingerprinting (DAF), can be considered variants of RAPD-PCR, differing mainly for the length of the primers and times and temperatures of the amplification protocol. The amplification products are separated by agarose gel electrophoresis to generate a bacterial fingerprint and the banding patterns are used to compare the relatedness of bacterial strains The method does not require prior special knowledge of specific DNA target sequences (Ranjbar *et al.,* 2011; Ranjbar *et al.,* 2013). This makes it a flexible tool with general applicability (Lin *et al.,* 1996; Pourshafie *et al.,* 2007). RAPD-PCR has the additional advantage of a short turnaround time and requiring a limited amount of bacterial DNA to carry out the analysis. The discriminatory power is variable according to number and sequence of arbitrary primers and amplification conditions. RAPD typing suffers from problems in low inter-run and inter-laboratory reproducibility and from a lack of consensus rules for interpretation of pattern differences (Lin *et al.,* 1996).

Rep-PCR

This method consists of PCR amplification of spacer fragments lying between repeat motifs of the genome using two outwardly-directed primers at high stringency (Farber *et al.,* 1996). The repetitive extragenic palindromic (Rep) elements are 38-bp sequences consisting of six degenerate positions and a 5-bp variable loop between each side of a conserved palindromic stem in the bacterial genome.

The amplicons are then separated by electrophoresis to generate migration patterns that are compared to one another to determine genetic relatedness. Banding patterns differ as a result of the number of repetitive elements and their relative position within the bacterial genome. Rep-PCR shares with many other PCR typing methods the advantage of a relatively short time to results and requires a minimum amount of DNA for typing.

Discriminatory power depends on the method used and the number of repetitive sequences present in the strain (Woo *et al.,* 2006). Rep-PCR reproducibility can be affected by variability in PCR reagents, thermal cycling and gel electrophoresis conditions. In addition, if a strain lacks a number of repetitive sequences in close enough proximity to one another, the amplicons generated may be insufficient for epidemiological or phylogenetic inferences. To overcome the reproducibility problems, a semi-automated rep-PCR commercial system has been developed using microfluidic chip-based DNA fragment separation, rather than the traditional gel electrophoresis (DiversiLab® Sys-

tem, bioMérieux, Marcy l'Étoile, France). This semi-automated rep-PCR system also provides data analysis software to compare the banding patterns and generate dendrograms and an on line database for strain identification and typing.

ERIC-PCR

Another strategy to exploit specific bacterial genomic sequences is ERIC-PCR fingerprinting. The targets are highly conserved central inverted repeats of 126-bp located in extragenic regions of bacterial genomes. The position of these elements in enterobacterial genomes varies between different species and strains and has been used as a genetic marker (Versalovic *et al.*, 1991). Consensus primers have been successfully used for subtyping Gram-negative enteric bacteria by amplification of DNA sequences located between successive repetitive elements (Hulton *et al.*, 1991, Ranjbar *et al.*, 2009; Ranjbar *et al.*, 2011). ERIC-PCR has been especially useful as a typing method for multiresistant *Enterobacteriaceae* strains and a rapid identification of the pandemic clone ST131 of *E. coli* (Eckert *et al.*, 2004). An interesting application has been developed by Wei *et al.* (2004), who used ERIC-PCR to fingerprint the enteric microbial community from a sample of healthy and diseased human subjects and piglets. ERIC-PCR profiles obtained from total DNA were transfer-blotted onto nylon film to form an array-like organization made of amplified genomic DNA fragments distributed to reflect community structural differences. All ERIC-PCR amplicons from one healthy individual were DIG-labeled to hybridize with the DNA arrays. Consequently, DNA bands in the fingerprints sharing sequence homology with the probes developed signals, while bands with no sequence homology in the probes were "erased" from the fingerprints. This allowed for a straightforward identification of genome fragments useful as genome-specific markers for dynamic monitoring of bacterial populations in complex communities, such as human gut microflora.

PCR Melting Profile Technique (PCR-MP)

The PCR-MP technique is based on using low denaturation temperatures during ligation-mediated PCR (LM PCR). By using this method, genomic DNA varying in thermal stability can be gradually amplified starting from the less stable DNA fragments amplified at lower denaturation temperature to more stable ones which are amplified at higher temperatures. The high discriminative power of PCR-MP fingerprinting has been demonstrated on clinical strains of *E. coli* and, more recently, of *Candida albicans* (Krawczyk *et al.*, 2009). The PCR-MP fingerprinting technique has several advantages: it does not require prior knowledge of sequences under analysis, results can be easily analyzed on polyacrylamide gels and the same enzyme/adaptor/primer toolkit can be applied to DNA from diverse bacterial species.

Amplified Fragment Length Polymorphism (AFLP)

AFLP was originally applied to the characterization of plant genomes, but over time has become of more common use in the field of microbial typing (Koeleman *et al.*, 1998; Gürtler *et al.*, 2001).

There are two variants of AFLP, one with two different restriction enzymes and two primers for PCR amplification, and the second with a single primer and restriction enzyme. In the most common configuration, whole DNA is digested with one or more enzymes, and then specific DNA fragments, termed adapters, are ligated to DNA and used as targets for PCR primers. Classically, in AFLP two restriction enzymes, a "frequent cutter" and a "rare cutter", are combined (Vos *et al.*, 1995). To reduce the number of amplicons generated from the large number of restriction fragments, one to three nucleotides are added on the 3'-end of the PCR primers that recognize sequences in the adapter, so generating a selective amplification of a fraction of the restriction fragments. The amplified fragments are subjected to high-resolution gel electrophoresis and characteristic separation profiles are generated and compared (Vos *et al.*, 1995).

Fluorescent-labeled PCR primers can allow detection of the fragments using an automated DNA sequencer. In most instances, the technique has showed high reproducibility (Torpdahl *et al.*, 2004, Ross *et al.*, 2005, Giammanco *et al.*, 2007). Discriminatory power is excellent

since even a single base mutation can be detected and the use of different combinations of restriction enzymes and primers can generate large numbers of different AFLP fingerprints. Furthermore, it combines high resolution and high throughput. Among the main limitations of AFLP is the complexity of the procedure involving a great number of steps, the requirement for an automated DNA sequencer in analysis of a large number of isolates and the consequent costs, and the quality of the target DNA, which is a crucial factor to warrant reproducibility.

Variable number of tandem repeat (VNTR) analysis
Many bacterial genomes contain regions with repetitive sequence DNA motifs ranging from a few bases to more than 100 base pairs in length. The repeats are tandem, i.e. a number of copies of each of the repeat motifs are clustered together and oriented in the same direction. The number of repeats in a tandem can be highly variable, even among strains of the same species. VNTR is a PCR-based technique that relies on the amplification of DNA that encompasses short tandem repeats of a DNA sequence. PCR primers are designed to anneal non-repetitive sequences just outside the repeat region, and amplified products are separated and sized to determine the number of repeats present in the amplicon.

The tandem repeats are prone to higher-than-background mutation rates due to DNA strand slippage during replication (van Belkum *et al.*, 1998) so the amplified fragments will vary in length depending on the number of repeats at a given locus. In VNTR analysis, differences in the number of repeated copies at specific loci are used to distinguish isolates. Because of the relatively high mutation rate, strains can accumulate distinctive patterns within a relatively short period of time (Call *et al.*, 2008). Multiple regions of repeated motif can be examined at the same time to gain increased discrimination for studying genetic diversity. The most common approach using multiple VNTR loci for typing is referred to as multiple locus VNTR analysis (MLVA) or MLV Fingerprinting (MLVF) (Sabat *et al.*, 2012). Detection of the fragment sizes using capillary electrophoresis

or microfluidic technology facilitates standardization and allows the fragment analysis to be more comparable between laboratories. Whole genome analysis of *Mycobacterium tuberculosis* has also revealed short sequence repeats, termed mycobacterial interspersed repeat units (MIRUs) and variable number tandem repeat units (VNTRs), which are tandemly repeated sequences of 40-100 bp (Frothingam *et al.*, 1998). The location and number of repeats varies in different *M. tuberculosis* strains and can be measured by PCR-based methods.

The data generated are portable between laboratories. MIRU-VNTR typing is being increasingly adopted, often in combination with spoligotyping, as the new reference method for molecular epidemiology and phylogenetic studies of *M. tuberculosis*. Approaches based on 15 to 24 loci have been shown to have comparable or better predictive value than IS6110 RFLP and proposed as a new golden standard for the study of TB transmission (Wirth *et al.*, 2008; Zamani *et al.*, 2013).

Sequencing-based methods
Microbial genomes are subject to variability due to mutation or recombination. The sequence variability within particular genes can be used in molecular typing schemes to determine the relatedness of bacteria.

Nowadays, it is simple using the PCR methodology to accumulate a large amount of material that can be sequenced directly after purification.

An increasing number of truly complete bacterial genomes are being placed in the International Nucleotide Sequence Database Collaboration, a public database which can be searched on the web (http://www.ncbi.nlm.nih.gov/sites/entrez?db=genome). This method is also acquiring practical implications for the identification and typing of microorganisms (Laure *et al.*, 2005; Roetzer *et al.*, 2013).

Multilocus sequence typing (MLST)
In MLST genetic relatedness among strains is determined analyzing the sequences of multiple genes which are compared for nucleotide substitution. Since genes possess varying degrees of genetic drift, housekeeping genes are most often sequenced because they are present

in all isolates within a species. Since they are not under strong selective pressures, their rate of genetic variability is relatively low and may not provide adequate discrimination among unrelated isolates. For MLST to be effective as an epidemiological tool, the selection of genes and their number needs to be adequate to distinguish among isolates with more recent genetic divergence. For this purpose, genes under greater selective pressure, such as virulence genes, may provide a better result. In this case the method is generally referred to as multi-virulence-loci sequence typing (MVLST) (Chen *et al.*, 2007).

In MLST, for each of the genes sequenced the allelic group including the isolate can be established as a sequence type (ST). Relatedness among STs can be revealed using different methods of clustering:

a) the unweighted pair group method with arithmetic mean, using distance matrices containing the pairwise differences of allelic profiles;

b) the minimum spanning tree approach, constructing a tree that connects all entries in such a way that the summed distance of all links of the tree is the shortest i.e. minimum;

c) the based upon related sequences types (eBURST or the more recent global optimized version, goeBURST) algorithm, inferring patterns of evolutionary descent among isolates by a model of clonal expansion and diversification and assigning isolates to clonal complexes (Francisco *et al.*, 2012).

Internet-based MLST databases have also been set up to facilitate the sharing of MLST results among laboratories (http://www.mlst.net). MLST has been successfully used for global epidemiology and population genetic studies of many Gram positive and Gram negative bacteria.

It is less suitable for routine typing in outbreak investigations or local surveillance studies because of its relatively low discriminative power, high cost and workload.

Single nucleotide polymorphism (SNP) analysis
SNP analysis differentiates among strains looking for nucleotide substitutions at specific hypervariable loci in the bacterial genome. An affordable picture of the relatedness of strains

can be obtained by examining multiple SNPs. The loci to be included in SNP genotyping protocols are generally selected among those known to have a high rate of polymorphism or based on data provided from genome sequencing. Sequencing of the gene targets can be obtained either using traditional methods or by pyrosequencing, where short fragments of immobilized single strand DNA are read one base at a time by synthesizing the complementary strand along them and detecting which base was actually added at each step, allowing sequencing to be performed in a short amount of time (Roos *et al.*, 2006).

Besides sequencing, many alternative methods can be used to detect polymorphisms at defined SNP locations, such as restriction enzyme digestion and real-time PCR assays (Octavia *et al.*, 2010). High-resolution melting analysis was also applied as a closed-tube method to detect polymorphisms at defined locations (Sangal *et al.*, 2013). Eventually, microarray analysis can be utilized for high throughput screening of samples on arrays that contain probes corresponding to each of the nucleotides of potential gene SNP targets.

Microarrays
The principle of the microarray is based on generating labelled cDNA or cRNA molecules that are subsequently hybridized to an arrayed series of thousands of microscopic spots with specific complementary oligonucleotides (probes). DNA microarrays have been used to measure changes in expression levels and to detect SNPs, but also for genotyping. Microarray technologies have the power to perform simultaneous analysis of large numbers of DNA sequences in a sample and also the potential for automation of the analytical chain. Probes can be generated identifying genomic markers representing small nucleotide polymorphisms. Microarray technology offers a wide range of analysis for simultaneous detection of multiple gene products, such as antibiotic resistance determinants and virulence factors whose identification can be useful for epidemiological investigations (El-Adawy *et al.*, 2013; Voets *et al.*, 2012).

Probe panels have also been intended for phage typing or serovar identification. Initially, SNP

TABLE 1 - *Epidemiological questions molecular typing methods are expected to answer.*

Setting	Purposes
Outbreak	Track common source and identify specific vehicles and risk factors
	Refine epidemiological investigation design
	Identify risk factors even when sample size is small or inadequate for a conventional epidemiological investigation
Endemicity	Study dynamics of disease transmission on a local, regional, national or more geographically widespread area
	Detect unrecognized outbreaks or epidemics
	Assess attributable risk fractions in sporadically or endemically occurring disease cases
	Identify and assess variables contributing to the persistence and spread of specific strains/clones
Surveillance	Obtain baseline information
	Identify and analyze trends and changing patterns
	Detect the introduction and spread of specific strains/clones into healthcare settings and the community
	Identify changes in the prevalence of drug resistant infections and generate hypotheses about their causes and transmission routes
	Support implementation of corrective actions and assess their effectiveness
	Source attribution studies in some common diseases, such as zoonoses and food- and water-borne infections

TABLE 2 - *Categorization of the main features of molecular typing systems.*

Feature	Definition
Performance-related	
Typeability	The ability of the method under study to generate a result for each isolate tested
Repeatability	The ability to generate identical results when an isolate is tested repeatedly in the same laboratory
Reproducibility	The ability to generate identical results when an isolate is tested repeatedly in different laboratories
Discriminatory power	The ability to generate distinct units of information from epidemiologically unrelated isolates, at least at the sub-serotype level. The suitability of a method for epidemiological or surveillance purposes does not necessarily correlate with a high discriminatory power
Stability	The ability to recognize a clonal relationships between isolates in spite of genetic differences accumulating during their spread in the time/space dimension
Ease of interpretation of data generated	Intended as unequivocal interpretation
Convenience-related	
Ease of use	The ability of the method to be handled in non specialized and non research laboratories, such as in field setting and by minimally-trained workers
High throughput	The ability to process a large number of strains in a reasonable interval of time. It depends upon simplicity of procedures, but also on susceptibility to automation
Cost and affordability	They depend upon cost and accessibility of reagents and equipment and the specific skill required
Setting-related	
Typing system concordance	The ability to discriminate epidemiologically related strains from those that are not when the typing method is tested against evidence obtained with a previously validated method
Epidemiological concordance	The ability to discriminate epidemiologically related strains from those that are not when the typing method is tested against epidemiological evidence, e.g. using an outbreak strains as a reference
Phylogenetic information	The ability to provide information about evolutionary relationship between isolates
Appropriateness	The ability of the method or the combined methods to address the specific epidemiological question to be answered

analysis was very expensive to develop and apply for species typing, but it has progressively become more cost-effective for a variety of species. SNP analyses have been used in bacterial population genetics with highly informative results (Filliol *et al.*, 2006).

Whole genome sequencing (WGS)
Advances in WGS are rapidly resulting in a reduction of the fully economic costs and time of sequencing (the cost and time to sequence a typical bacterial genome is now less than $100 in one day's time). In brief, WGS is increasingly becoming competitive with any diagnostic technology, including traditional methods of culturing bacteria. Consequently, the hypothesis to eliminate all the intermediate approaches to typing that necessitate whole genome data, such as MLST and SNP analysis, and to use the WGS data as such, is rapidly become concrete. According to some researchers, in a few years WGS could become the sole diagnostic and molecular epidemiological tool, including identification, genetic characterization and drug susceptibility testing (Schürch *et al.*, 2012). However, an integrated approach combining "old" methods with

WGS appears to be opportune, especially while sequencing costs are still high and the capabilities to reliably and easily interpret the results are still under development (Pearson *et al.*, 2009). It is realistic to think that WGS will only gradually replace the current diagnostic tests starting with fastidious, hazardous and slow-growing bacteria, whose paradigm is *M. tuberculosis* (Köser *et al.,* 2012). The innovative impact of WGS on microbial diagnostic and public health microbiology has been described in some recent comprehensive reviews (Köser *et al.,* 2012; Erlich *et al.,* 2013). However, it is likely that the WGS "revolution" will not be extensively effective until several organizational and bioinformatics challenges have been solved. This implies primarily that the results have to be translated into a format that can be understood by health professionals without bioinformatics skills. Moreover, apart from the foreseeable enormous boost toward a higher resolving power of epidemiological investigations and, consequently, to a more timely and effective management of infection control measures, WGS will not replace, but only complement, more traditional forms of epidemiological inquiry.

TABLE 3 - *Characteristics of the*

Feature	Plasmid analysis	(IS) RFLPs	Ribotyping	PFGE	PCR-RFLP	RAPD-PCR
Typeability	Many	All	All	All	All	All
Repeability[4]	Moderate	Excellent	Excellent	Excellent	Excellent	Moderate
Reproducibility[4]	Moderate	Good	Excellent	Excellent	Excellent	Moderate
Discriminatory power[4]	Poor	Moderate to excellent[2]	Moderate to excellent[2]	Excellent	Poor to moderate[2]	Good
Stability[4]	Moderate	Good	Good	Good	Good	Moderate
Ease of interpretation of data generated[4]	Moderate	Moderate	Moderate to good	Moderate	Good	Moderate
Ease of use[4]	Moderate	Poor	Poor to moderate[4]	Poor	Good	Good
High throughput	No	No	No	No	No	Yes
Cost[4]	Low	Moderate	High	Moderate	Low to moderate	Low
Time required (days)[3]	1	3-5	1 to 3-5	3	1-2	1

[1]If automated. [2]The discriminatory power may perform differently based upon clonality of organisms (for example, some serotypes of *Salmonella* or some clones of MRSA). [3]The approximate number of days to get typing results is estimated by excluding the interval of time to

CONSIDERATIONS ON THE APPLICATIONS OF MOLECULAR TYPING METHODS

The most frequent questions which molecular typing is called to answer are summarized in *Table 1*. Epidemiological and surveillance applications using molecular typing methods have eventually to assess issues related to interpretation and attribution of a molecular subtype and criteria to be adopted for similarity. The strain similarity is a measure of the degree of "equality" among strains as detected or determined by a given method, but it can be argued whether similarity actually represents the real relatedness of the strains. All of the strain similarity comparisons ultimately deal with the probability that a molecular subtype cluster is a proxy of a true epidemiological relationship or, when in the field of taxonomy or phylogeny, of an evolutionary relationship. The desirable attributes of a molecular typing system are illustrated in *Table 2*.

Data obtained by molecular typing, which may include electrophoretic banding patterns, nucleic acid sequences or hybridization matrices, are habitually arranged by similarity or difference indices. Several computer-assisted analyses are generally used to support all categories of analytical methods aimed at inferring the relatedness of microorganisms, i.e. comparison and analysis of data generated by a molecular typing pattern, phylogenetic and numerical taxonomy methods, and generation of dendrograms.

To date, typing methods producing a DNA fingerprint have been those most widely used due to their higher discriminatory power and application within laboratory-based surveillance networks, such as PulseNet. When fingerprints are compared, similarities and differences must be evaluated.

Two isolates sharing the same DNA fingerprints are described as "indistinguishable", whereas, conversely, two isolates having largely different fingerprints are categorized as "not related". More generally, the relative relatedness may be expressed in terms of the number of genetic events or changes to the chromosome occurring from one generation to the next. Consequently, two strains differing by one genetic event may be defined as "probably related", strains differing by two genetic events as "possibly related"

main molecular typing systems.

Rep-PCR	ERIC-PCR	AFLP	VNTR	MLST	SNP analysis	Microarrays
All	All	All	All	All	All	All
Good to excellent	Excellent	Excellent	Excellent	Excellent	Excellent	Excellent
Good to excellent	Good	Good to excellent	Excellent	Excellent	Excellent	Excellent
Moderate to excellent[2]	Good	Excellent	Excellent	Good to excellent[2]	Good to excellent[2]	Good to excellent[2]
Good	Good	Good	Moderate	Good	Good	Good
Moderate to good	Good	Moderate to good	Good	Good	Good	Good
Good	Good	Poor to moderate4	Good	Moderate	Moderately easy	Moderate
Yes	Yes	Yes	Yes	Yes	Yes	Yes
Low to moderate	Low to high	Moderate to high	Low to moderate	Moderate	Moderate to high	Eigh
1	1	2	≥3	≥3	1-≥35	1-3

obtain a pure culture suitable to be handled by the method. [4]Parameter categorized in a three-level relative scale, the top level including those methods fully accomplishing the feature. [5]Depending upon the detection method.

and those differing by three or more genetic events are most likely "unrelated". Tenover *et al.* (1995) made a detailed discussion on molecular typing methods and the way in which mutations affect banding patterns. The guidelines for PFGE are probably those most widely applied. However, interpretive guidelines can give misleading information if applied on large time-space contexts without epidemiological supporting data.

In order to address the typing method's properties that best fit the objective and scale of a study, a classification of the techniques presented in this review according to the possible selection criteria is provided in *Table 3*.

The ability to characterize isolates,as belonging to a defined clone, generates information on transmission chains and sources of exposure that would be extremely difficult or impossible to detect by the traditional phenotype-based methods.

From a practical point of view, some steps are not to be missed when applying molecular typing techniques for epidemiological or surveillance purposes: first, to define objectives, time and population scale; second, to select a method or methods according to the objective and scale of the study; third, to choose interpretative criteria according to these last variables; fourth, to relate results to epidemiological data.

CONCLUSIONS

It has been shown that in order to study the epidemiology of a disease, genotyping methods show a better performance than phenotypic characterization. Genetic variation in the chromosomal DNA of a bacterial species influences the ability of a molecular typing system to distinguish among epidemiologically unrelated isolates. Usually, this variability is sufficiently high, and differentiation of unrelated strains can be accomplished using any of a variety of techniques. Many techniques are available, but each of them has its advantages and limitations that make it useful in some studies and restrictive in others. Although a particular typing method may have high discriminatory power and good reproducibility, the complexity of the method and interpretation of results

as well as the costs involved in setting up and using the method may be beyond the capabilities of the laboratory. The choice of a molecular typing method, therefore, will depend upon the skill level, and resources of the laboratory and the aim and scope of the study. With the new DNA-based technologies, with special reference to WGS, a new era seems to be opening in the field of diagnostic and molecular epidemiology, when a single machine will likely replace multiple different approaches, providing in the meantime a wealth of information. In an age in which the spread of drug resistance and the emergence of new pathogens are everyday challenges, they promise to equip the epidemiological, diagnostic and clinical field with tools able to detect, monitor, and control these threats in real time.

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