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Genotyping Techniques for Determining the Diversity of Microorganisms

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1. Introduction

Typing of microbial pathogens, or identifying bacteria at the strain level, is particularly important for diagnosis, treatment, and epidemiological surveillance of bacterial infections. This is especially the case for bacteria exhibiting high levels of antibiotic resistance or virulence, and those involved in nosocomial or pandemic infections. Strain typing also has applications in studying bacterial population dynamics. The part that molecular methods have to play in elucidating bacterial diversity is increasingly important. The shortcomings of phenotypically based typing methods (generally these methods are viewed as being too time consuming and lacking in sufficient resolution amongst related strains) have led to the development of many DNA – based techniques. A suitable typing method must have high discrimination power combined with good to moderate inter- and intra-laboratory reproducibility. In addition, it should be easy to set up, to use and to interpret, and inexpensive (Olive & Bean, 1999). In this chapter, we review the current bacterial genotyping methods and classify them into six main categories: (1 and 2) DNA banding pattern-based methods, which classify bacteria according to the size of fragments generated respectively by enzymatic digestion of genomic/plasmid DNA, and PCR amplification, (3) DNA hybridization–based methods using nucleotidic probes, (4) DNA sequencing-based methods, which study the polymorphism of DNA sequences, (5) differentiation of isolates on the basis of presence or absence of particular genes and (6) high resolution melting analysis–real–time monitoring of melting process of PCR amplified polymorphic DNA fragment. We described and compared the applications of genotyping methods to the study of bacterial strain diversity. We also discussed the selection of appropriate genotyping methods and the challenges of bacterial strain typing and described the current trends of genotyping methods.

2. Description of current microbial genotyping methods

2.1 DNA banding pattern-based methods, which classify bacteria according to the size of fragments generated by enzymatic digestion of genomic or plasmid DNA

2.1.1 Pulsed-Field Gel Electrophoresis – PFGE

Pulsed–field gel electrophoresis (PFGE) was developed in 1984 and has since become the "gold standard" of molecular typing methods. PFGE is extensively used in research

laboratories that specialize in analyzing specimens sent in by hospitals or state laboratories. Clinically, it is an invaluable tool in detecting the occurrence of an outbreak and trying to determine its source (Tenover et al., 1995). The procedural steps in PFGE are the following: embed organisms in agarose plugs→enzyme digestion→restriction endonuclease digestion→electrophoresis→gel staining→interpretation. The bacterial suspension is combined with molten agarose and mixed with a protease (an enzyme that disrupts the cell membrane by attacking the membrane proteins) and with SDS (a detergent that unfolds proteins). The enzyme-detergent mixture denatures the cell membrane proteins thus forming holes in the cell through which the chromosomal DNA is released. The agarose keeps the DNA embedded in its gel matrix. Next, the plug is washed several times to remove cell debris and proteases. These diffused out of the agarose gel matrix more easily than the large DNA molecules. It is important to remove the proteases so as not to harm the restriction enzymes that cleave the DNA in the next step of the process. A small piece of the plug is cut off and added to a restriction endonuclease(s) mixture which cleaves DNA at a specific sequence resulting in 10-30 DNA fragments ranging from 0.5 to 1000 kb. The large DNA fragments are then separated by size by pulsed-field gel electrophoresis. PFGE facilitates the migration of the large DNA fragments (>600 kb) through the agarose gel by regularly changing the direction of the electrical field during electrophoresis, allowing the fragments to maneuver through the agarose. The smaller DNA fragments move faster through the agarose than the larger fragments and the result is a pattern of DNA fragments. Migration distances are compared to reference standards of known molecular weight and a profile for each strain/isolate is obtained. The PFGE pattern from one isolate can be compared to other patterns to determine whether the samples may have originated from a common source. The electrophoretic patterns are visualized following staining of the gels with a fluorescent dye such as ethidium bromide. Gel results can be photographed, and the data can be stored by using one of the commercially available digital systems. Data analysis can be accomplished by using any of a number of commercially software packages (Olive & Bean, 1999; Tenover et al., 1994, 1995, 1997).

Use of PFGE has been greatly facilitated by the incorporation of standard methods of analysis suggested by Tenover et al. (1995), which has led to its widespread adoption. In their scheme, bacterial isolates yielding the same PFGE pattern are considered the same strain. Bacterial isolates differing by a single genetic event, reflected as a difference of one to three bands, are closely related. Isolates differing by four to six bands, representing two independent genetic changes, are possibly related. Bacterial isolates containing six or more band differences, representative of three or more genetic changes, are considered unrelated. These criteria are applicable to small, local studies in which genetic variability is presumed to be limited.

PFGE is one of the most reproducible and highly discriminatory typing techniques available and is the most reliable technique for analysis of variety of foodborne pathogens: *Escherichia coli* O157:H7 (Arbeit, 1995), *Salmonella* Typhimurium (Tsen et al., 2002), *Salmonella* Enteritidis (Thong et al., 1995), *Campylobacter jejuni* (Eyles et al., 2006; Nebola & Steinhauserova, 2006), *Listeria monocytogenes* (Okwumabua et al., 2005) as well as nosocomial pathogens: methicilin-resistant *Staphylococcus aureus* (MRSA) (Saulnier et al., 1993; Tenover et al., 1994), vancomycin-resistant enterococci (Barbier et al., 1996), *Klebsiella pneumoniae* (Vimont et al., 2008), *Serratia marcescens* (Shi et al., 1997), *Acinetobacter calcoaceticus-Acinetobacter baumannii* complex (Liu et al., 1997), *Pseudomonas aeruginosa* (Grundmann et al., 1995), *Mycobacterium*

avium (Arbeit, 1994), *Neisseria gonorrhoeae* (Poh et al., 1996), *Neisseria meningitidis* Serogroup C (Shao et al., 2007), *Stenotrophomonas maltophilia* (Valdezate et al., 2004) and *Legionella pneumophilla* (Tenover et al., 1995). PFGE has also proved to be discriminatory and reproducible for typing *Clostridium difficile*, the main etiologic agent of nosocomial *diarrhoea*. Nevertheless, a high proportion of strains are non-typable by this technique due to the degradation of the DNA during the process. The introduction of several modifications in the PFGE standard procedure proposed by Alonso and coworkers (2005) increased typability from 40% (90 isolates) to 100% (220 isolates) while maintaining the high degree of discrimination and reproducibility of the technique.

The PFGE characterize with some advantages. The method can be easily applied to different species, all the strains can be typed with good reproducibility, restriction profiles are easily read and interpreted, patterns consistent within and between laboratories (strict adherence to standard conditions is necessary), PFGE generally yields a high amount of pattern diversity (Olive & Bean, 1999; Tenover et al., 1997).

However it is necessary to remember about some drawbacks of PFGE. The technique is labor-intensive, relatively slow (approximately 2 to 3 days to completion), complex patterns challenging for inter-laboratory pattern comparisons, one mutation can yield differences in several fragments (PFGE can not determine phylogenic relationships), the agarose gel used for PFGE has a lower resolving power compared to those of polyacrylamide-urea gels (this may be a disadvantage for reliable comparison of patterns and computer analysis) and high initial cost of the equipment can also be an important limitation for many laboratories and investigators (Olive & Bean,1999; Tenover et al., 1997).

2.1.2 Restriction Enzyme Analysis of Plasmid

The development of novel genotypic methods, characterized by high discriminatory power, universality and reproducibility, caused that the REAP method (Restriction Enzyme Analysis of Plasmid) is currently not so popular. However it is one of the first method of molecular biology which was applied for differentiation of pathogenic bacteria such as *Yersinia enterocolitica* (Kapperud et al., 1990) or methicilin resistant *S. aureus* (Harstein et al., 1989). From the technical point of view, the REAP method is easy and includes five relatively simple steps: isolation and analysis of presence of plasmid/s DNA in the cells →digestion of the plasmid/s with a selected endonuclease→ electrophoresis→gel staining→interpretation.

In the REAP method, strains are distinguished due to presence, number and size of plasmids, and size of fragments generated in the result of plasmid digestion with a restriction enzyme. The most important limitation of this method is that it can be used only for strains carrying plasmids. The differentiation power of the method can be modified by selection of restriction enzymes, or by using a combination of several of them. Although the method is not very popular, it still should be considered an important tool of microorganisms' differentiation. The REAP method can be very useful for investigation of spreading of plasmid - dependent antibiotic resistance or virulence of pathogenic microorganisms, or analysis of transmission of strains harboring plasmids. Plasmids belong to the mobile genetic elements and very often contain genes encoding virulence factors and genes, expression of which results in antibiotic resistance. Some bacteria eg. staphylococci and *Enterobacteriaceae* are very prone to the plasmid DNA transfer. It is possible that two

strains of bacteria with identical chromosomal DNA, differ in the virulence potential or sensitivity to some antibiotics, only because of the presence of a plasmid DNA. The plasmid DNA transfer often leads to the creation of endemic strains in hospital environment. For example the REAP analysis performed by Trilla and coworkers revealed that 95% of MRSA strains isolated after an outbreak of MRSA nosocomial infections in Hospital Clínic in Provincial of Barcelona presented an unique homologous pattern of DNA fragments (Trilla et al., 1993). High virulence potential and decreased susceptibility towards antibiotics and chemoterapeutics are very often characteristic for such endemic strains.

2.2 DNA banding pattern-based methods, which classify bacteria according to the size of fragments generated by a PCR amplification

2.2.1 PCR ribotyping and PCR – RFLP

Rybotyping is based on the analysis of sequences of a rRNA operone, a very important element of genomes of all bacteria (Fig. 1). The method was developed by Kostman et al. (1992) and Gurtler (1993) in the 1990s as a response in part to the need in the clinical microbiology laboratory setting for expeditious epidemiological discrimination among pathogenic microorganisms without the use of probes, thus making the analysis more widely applicable. The genes coding for 16S rRNA, 23S rRNA and 5S rRNA are absolutely essential for existence of each bacterial cell due to its involvement in protein synthesis, and their sequences are generally highly conserved among bacteria, however some variable regions are present within these sequences. The sequence of 16S rRNA gene is conserved even at the strain level. As a result sequencing of 16S rRNA gene is at present one of the most important method used for bacterial species identification. However also less advanced techniques such as analysis of restriction fragments length polymorphism of PCR amplified fragments of 16S rRNA gene (PCR – RFLP) was successfully applied for identification of bacteria species. Okhavir et al. (2000) performed PCR – RFLP analysis on 53 strains of 14 bacterial species (eight Gram-positive and five Gram-negative) collected from both *keratitis* and *endophthalmitis* patients. Two pairs of oligonucleotide primers based on the 16S rDNA gene were used to PCR-amplify 1.2- and 1.0-kb fragments of bacterial genomic DNA. All bacteria tested could be identified and speciated using RFLP analysis except for *E. coli* and *S. marcescens,* which could not be interdifferentiated using PCR – RFLP.

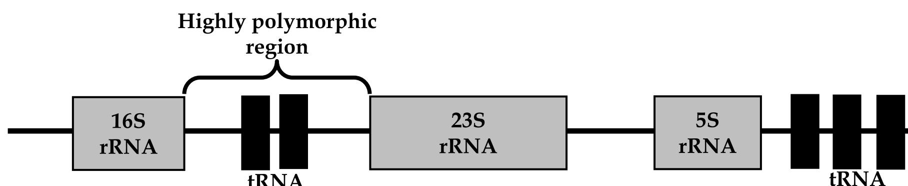

Fig. 1. Organization of bacterial rRNA operon

From the point of view of analysis of genetic differentiation of microorganism especially interesting is the fragment of the operon located between 16S and 23S, called internal transcribed spacer (ITS) or intergenic space region (ISR). In the case of some kinds of bacteria ITS characterize with surprisingly high polymorphism of both length and sequence even at the species level. Additionally some of the species of bacteria contain in the genome more than one copy of the operon. *In silico* analysis of ISR length variability in 27

genomically sequenced bacterial species revealed that while in some species ISR length is variable within and between isolates, in others ISR lengths are limited to one or two sizes, usually dependent on the number of tRNAs present (Christensen et al., 1999). For example, while the multiple copies of the *S. enterica* serovar Typhimurium, serovar Infantis, and serovar Derby ITS fragments are polymorphic, they are conserved in other *Salmonella* species, as well in *Listeria*, *Streptococcus*, and certain species of *Staphylococcus* (Giannino et al., 2003; Gurtler & Stanisich, 1996; Lagatolla et al., 1996; Marsou et al., 1999). As consequence depending of the construction of rRNA operon amplification of its noncoding fragments can be a very useful technique for identifying bacteria to the species level (if the sequence is conserved) or for their differentiation if the target fragment of gene characterize with high polymorphism. As it was mentioned above the sequences of 16S and 23S rRNA are generally conserved among bacteria thus one universal pair of PCR primers can be used for amplification of a target polymorphic region of rRNA operon of different species of bacteria. If the examined strains contain several polymorphic copies of rRNA operon several products of ITS – PCR amplification are generated, and strain specific patterns of DNA bands is obtained after electrophoresis. The discriminatory power of the ITS – PCR method can be improved by digestion of PCR products, and analysis of restriction fragment length polymorphism (PCR – RFLP) after agarose or polyacrylamide electrophoresis. The differences in sequences of the amplified fragments of intergenic spacer regions of the same length can be alternatively analyzed by using the single sequence conformation polymorphism (SSCP) (Daffonchio et al., 1998).

The PCR ribotyping method has proven to be suitable for the routine investigation of foodborne outbreaks of *Shigella* isolates (De Paula et al., 2010). Cho & Tiedje (2000) successfully used a PCR ribotyping approach to define the biogeography of fluorescent pseudomonad isolates, and found it as discriminating as the BOX rep-PCR. The other species with which this methodology has found some success is *C. difficile*, having a total of 11 ribosomal operons, with differing tRNAs and ISR lengths found among ribosomal operons of the same organism (Sebaihia et al., 2006) Almost 43% of all published PCR ribotyping studies have been performed with *C. difficile* (Bidet et al., 2000; Kikkawa et al., 2007; Rupnik et al., 1998; Stubs et al., 1999). An analysis of 45 isolates of *C. difficile* from which the ITS had been sequenced did not reveal striking sequence length diversity; however, it appears that the selection of PCR ribotyping as a typing technique relies more on the comparative ease of the technique and repeatability rather than on the discriminatory ability (Bidet et al., 2000), due to the occurrence of DNA degradation in *C. difficile* interfering with PFGE analysis.

Beside ITS fragment of rRNA operon, there are also some other targets which can be used for PCR – RFLP genotyping of different species of bacteria. The popular criterion often used for examining genetic variability of staphylococci is RFLP analysis of polymorphism of size an sequence of coagulase gene (Goh et al., 1992). Nebola and Steinhauserowa (1996) used PFGE technique and PCR – RFLP analysis of the flagellin gene (*fla*) for differentiation of 92 poultry and 110 human strains of *C. jejuni*, and obtained results were comparable.

And lately the restriction analysis of PCR amplified gene coding for major outer membrane protein was successfully applied for genotyping *Chlamydia trachomatis* isolated from cervical specimens in New Delhi (India) (Gita et al., 2011).

PCR ribotyping and RFLP technique is easy in comparison to other molecular methods and consists of: PCR amplification→restriction endonuclease digestion→agarose gel

electrophoresis→gel staining→interpretation. The technique is not time-consuming and inexpensive, but the most important drawback is fact that only limited fragment of genome is analyzed as a result its discriminatory power is usually poorer than for example PFGE, RAPD, ERIC or BOX rep-PCR (Bouchet et al., 2008; Kikkawa et al., 2007, Van den Berg et al., 2007).

2.2.2 Random Amplified Polymorphic DNA – RAPD

The random amplified polymorphic DNA (RAPD) assay, also referred to as arbitrary primed (AP) PCR and DNA amplification fingerprinting (DAF), is a powerful tool for genetic studies. It was first described by Williams et al. (1990) and Welsh & McClelland (1990). RAPD analysis is useful as a screening genotyping method (Speijer et al., 1999). RAPD is fast, simple and less labor than the usual fingerprinting method with non radioactive isotopes used (Leal et al., 2004). It detects differences along the entire bacterial genome, not only in particular sequences. Thus, this system is helpful in characterizing bacteria isolates over long periodes (Ortiz-Herrera et al., 2004). RAPD can generate various fingerprint profiles with unlimited number of primers (Leal et al., 2004). A simple short primers are used without the need of prior knowledge of the template DNA. The selection of an appropriate primer and optimization of PCR conditions are the important factor in RAPD analysis (Blixt et al., 2003). The primers that work for some bacteria may fail for others and because of that, the screening process needs to determine the appropriate primers (Shangkuan & Lin, 1998). RAPD uses oligonucleotide (9 to 10 bases in length) primers with arbitrary sequence, 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. If two RAPD primers anneal within a few kilobases of each other in the proper orientation, the result is a PCR product with a molecular length corresponding to the distance between the two primers results. The number and location of these random primer sites vary for different strains of a bacterial species. Thus, following separation of the amplification products by agarose gel electrophoresis, a pattern of bands which, in theory, is characteristic of the particular bacterial strain results (Caetano et al, 1991; Meunier & Grimont, 1993; Welsh & McClelland 1990; Williams et al., 1990). The relationship between strains may be determined by comparing their unique fingerprint information (Leal et al., 2004).

Several investigators found poor reproducibility with RAPD. However, the technique is reliable if the PCR conditions are optimized (Benter et al., 1995; Leelayuwet et al., 2000; Ortiz-Herrera et al., 2004;). Leelayuwet et al. (2000) have optimized the RAPD conditions using eighteen deca-oligo nucleotide primers with 70% GC content, eight 60%GC RAPD primers, and four random deca oligomers to produce reproducible but complex patterns showing a high degree of variation between strains of *Burkholderia pseudomallei* isolated from five patients with localized and four with septicemic *melioidosis*. They found that reproducible RAPD patterns are dependent upon the optimal concentrations of DNA in accordance with Taq polymerase and magnesium as well as PCR cycling conditions. Thus, DNA samples should be quantified, and the same lot of Taq enzyme should be used for the entire study. If a new batch of Taq is introduced, re-optimization is required. Furthermore, a ramping time of 7 min is essential in obtaining reproducible RAPD patterns. The RAPD patterns were analyzed by high resolution polyacrylamide gel electrophoresis using a laser based automated fragment analyzer, GS2000.

RAPD is considered as a simple (PCR amplification with single or two primers→agarose gel electrophoresis→gel staining→interpretation), rapid, highly discriminating, less costly and simple technique for molecular typing of various microorganisms (Van Belkum et al., 1995). It has been successfully applied in the genetic differentiation of *Salmonella* (Tikoo, 2001), *E. coli* (Renqua-Mangia et al., 2004), *A. baumannii* (Reboli et al., 1994), *S. marcescens* (Debast et al., 1995), *Proteus mirabilis* (Bingen et al., 1993), *Enterobacter cloacae* (Hou et al., 1997), *Haemophilus somnus* (Myers et al., 1993), *Leptospira* species (Ralph et al., 1993), *L. pneumophilia* (Tram et al., 1990, Van Belkum et al., 1993), *B. pseudomallei* (Haase et al., 1995, Leelayuwet et al., 2000), *Aeromonas salmoniciola* (Miyata et al., 1995), *Aeromonas hydrophila* (Miyata et al., 1995), *Vibrio cholerae* (Leal et al., 2004), *S. aureus* (Saulnier et al., 1993), *Lactobacillus plantarum* (Elegado et al., 2004; Lawrence et al., 1993), *Bacillus cereus* (Svensson et al., 2004), *Listeria monocytogenes* (Hiroshi et al., 2007), *Candida albicans* (Lehmann et al., 1992, Robert et al., 1995), *Histoplasma capsulatum* (Kersulyte et al., 1992) and *Cryptococcus neoformans* (Yamamoto et al., 1995). RAPD is highly discriminatory and therefore especially useful in the investigation of short term or local outbreaks of disease. Several authors have used RAPD fingerprinting of material obtained by gastric biopsy (Akopyanz et al., 1992) or gastric aspirates (Konno et al., 2005) to investigate transmission of *Helicobacter pylori*, supporting the hypothesis of intra-familial transmission. RAPD assay can be also useful in identification of *Proteus penneri* strains (Kwil et al., 2002). This typing presented a high discriminatory power between strains of *Malassezia furfur* and can be applied in epidemiological investigation of skin disease caused by these bacteria (Gandra et al., 2006). *Lactobacillus*-probiotic strains from 5 *Lactobacillus* species (*L. brevis*, *L. reuteri*, *L. gallinarium*, *L. salivarius* and *L. panis*) were specifically, rapidly, immediately and conveniently differentiated after optimization of the RAPD parameters such as $MgCl_2$, Taq polymerase, primer concentration and type of primer (Manan et al., 2009). It is a potentially useful assay for the rapid characterization of neonatal infections associated with group B streptococci (Zhang et al., 2002). Random amplified polymorphic DNA (RAPD) and enterobacterial repetitive intergenic consensus (ERIC) can be applied successfully in study of genetic distribution and epidemiology of *Vibrio parahaemolyticus* (Zulkifli et al., 2009). This technique utilizing a universal typing primer was successfully used for genotyping the isolates *Arcobacter butzleri*, *A. cryaerophilus* and *A. skirrowii* (Atabay et al., 2008).

RAPD-PCR can be practically applied in most laboratories since it requires no special and/or complex equipment and takes less time and is less labourous as compared with some other genotyping methods such as PFGE and AFLP (Atabay et al., 2008). Vila et al. (1996) and Speijer et al. (1999) found that the RAPD assay was more discriminating than RFLP analysis of either the 16S rRNA genes or the 16S-23S rRNA spacer region but less discriminating than PFGE, AFLP and Rep-PCR analysis in the case of studing of *P. aeruginosa* and *A. calcoaceticus-A. baumannii* complex.

2.2.3 Rep–PCR

Versalovic et al. (1991) described a method for fingerprinting bacterial genomes by examining strain or subtype-specific patterns obtained from PCR amplification of repetitive DNA elements present within bacterial genomes. There are three main sets of repetitive DNA elements used for typing purposes. The 35-40 bp repetitive extragenic palindromic (REP) elements are palindromic units, which contain a variable loop in the proposed stem-loop structure (Stern et al., 1984). The 124-127 bp enterobacterial repetitive intergenic consensus (ERIC) sequences also known as intergenic repeat units (IRUs) are characterized

by central, conserved palindromic structures. They are present in many copies in the genomes of many enterobacteria (Hulton et al., 1991). The position of ERIC elements in enterobacterial genomes varies between different species and has been used as a genetic marker to characterize isolates within a bacterial species (Son et al., 2002; Versalovic et al., 1991). The 154 bp BOX elements consist of differentially conserved subunits, namely boxA, boxB, and boxC (Martin et al., 1992). Only the boxA-like subunit sequences appear highly conserved among diverse bacteria (Versalovic et al., 1994). BOX elements were the first repetitive sequences identified in a Gram-positive organism (*Streptococcus pneumoniae*) (Martin et al., 1992). REP- and ERIC-sequences were originally identified in Gram-negative bacteria (*E. coli* and *Salmonella* Typhimurium) and then found to be conserved in all related Gram-negative enteric bacteria and in many diverse, unrelated bacteria from multiple phyla (Olive & Bean, 1999; Versalovic et al., 1994). The repetitive elements may be present in both orientations, and oligonucleotide primers have been designed to prime DNA synthesis outward from the inverted repeats in REP and ERIC, and from the boxA subunit of BOX, in the polymerase chain reaction (PCR) (Versalovic et al., 1994). The use of these primer(s) and PCR leads to the selective amplification of distinct genomic regions located between REP, ERIC or BOX elements (Scheme 5). The corresponding protocols are referred to as REP-PCR, ERIC-PCR and BOX-PCR genomic fingerprinting respectively, and rep-PCR genomic fingerprinting collectively (Versalovic et al., 1991, 1994). The amplified fragments can be resolved in a gel matrix, yielding a profile referred to as a rep-PCR genomic fingerprint (Versalovic et al., 1994). These fingerprints resemble "bar code" patterns analogous to UPC codes used in grocery stores (Lupski et al., 1993). The rep-PCR genomic fingerprints generated from bacterial isolates permit differentiation to the species, subspecies and strain level.

The procedural steps in rep-PCR are: PCR amplification with REP or ERIC or BOX primers→agarose gel electrophoresis→gel staining→interpretation. Rep-PCR can be performed with DNA extracted from bacterial colonies or by a modified method using unprocessed whole cells (Woods et al., 1993). Studies carried out by many laboratories on a variety of different bacterial genera and species have revealed that, at fine taxonomic resolution, phylogenetic trees derived from BOX-, ERIC- and REP-PCR genomic fingerprinting are not always identical. This is to be expected, since different numbers of bands may be generated with each primer set, the annealing conditions vary between primers or sets, and the prevalence/distribution of the target repetitive elements in question may vary. To compare multiple different gels with each other, the best results are obtained when all experimental parameters are standardized as much as possible. This is especially important, when large databases are to be generated or, data generated by different laboratories need to be compared. The standardized conditions used should include sample preparation and processing, use of similar growth conditions, the same DNA isolation methods and use of the same rep-PCR conditions. Moreover the use of standardized electrophoresis conditions and size markers is essential (Versalovic et al., 1994). REP or ERIC amplification can be performed with a single primer, a single set of primers, or multiple sets of primers. ERIC patterns are generally less complex than REP patterns, but both give good discrimination at the strain level. Application of both REP and ERIC-PCR to samples to be typed increases the discriminatory power over that of either technique used alone. BOX-PCR is the most robust of the three rep-PCR methods. BOX-PCR patterns are not affected by the culture age of the strain to be analyzed (Kang & Dunne, 2003) and fingerprinting output can be easily analyzed by computer assisted methods (Ni Tuang et al., 1999). These features

make BOX-PCR a frequently used tool in biogeography studies in environmental microbiology (Cherif et al., 2003; Dombek 2000; Oda et al., 2003; Singh et al., 2001). The highest data point scatter was observed with ERIC-PCR fingerprint similarity values, which are more sensitive to disturbances.

Rep-PCR genomic fingerprinting protocols have been developed in collaboration with the group led by Dr. J.R. Lupski at Baylor College of Medicine (Houston, Texas) and have been applied successfully in many medical, agricultural, industrial and environmental studies of microbial diversity (Versalovic et al., 1994). In addition to studying diversity, rep-PCR genomic fingerprinting has become a valuable tool for the identification and classification of bacteria, and for molecular epidemiological studies of human and plant pathogens (Louws et al., 1996; Versalovic et al., 1997). It has been applied in the classification and differentiation of strains of many Gram-positive and -negative bacteria including *Bartonella* (Rodriguez-Barrados et al., 1995), *Bacillus subtilis* (Pinna et al., 2001), *B. sporothermodurans* (Herman & Heyndrickx, 2000), *E. coli* (Leung et al., 2004; Panutdaporn et al., 2004; Silveira et al., 2003), *Citrobacter diversus* (Woods et al., 1992), *Enterobacter aerogenes* (Georghiou et al., 1995), *Salmonella* (Chmielewski et al., 2002; Kerouanton et al., 1996; Millemann et al., 1996; Rasschaert et al., 2005), *Vibrio cholerae* (Colombo et al., 1997; Rivera et al., 1995), *Pseudomonas corrugata* (Achouak et al., 2000), *Vibrio parahaemolyticus* (Khan et al., 2002; Son et al., 1998), *Pseudomonas syringae-Pseudomonas viridiflava* group (Marques et al., 2008), *Aeromonas* spp. (Taco et al., 2005), *Xanthomonas* (Rademaker et al., 2000), *Rhizobium meliloti* (De Bruijn, 1992; Niemann et al., 1997), *Pandoraea apista* (Atkinson et al., 2006), methicillin-resistant *S. aureus* (Van Belkum et al., 1992), *S. pneumoniae* (Versalovic et al., 1993), *A. baumanii* (Dijkshoorn et al., 1996), *Burkholderia cepacia* (Hamill et al., 1995), *B. pseudomallei* (Currie et al., 2007), *L. pneumophilia* (Georghiou et al., 1994), *Helicobacter pylori* (Kwon et al., 1998), *N. gonorrhoeae* (Poh et al., 1996), *N. meningitidis* (Woods et al., 1996), *Enterococcus* spp. (Svec et al., 2005), *Paenibacillus larvae* subsp. *larvae* (Genersch & Otten, 2003) and *Lactobacillus* spp. (Gevers et al., 2001). Rep-PCR is emerging as a potential tool for identification of the source of environmental *E. coli* populations owing to its success in classifying the correct host source, reproducibility, cost-effectiveness and easy operational procedures (Baldy-Chudzik et al., 2003; Carson et al., 2003; Dombek et al., 2000; Johnson et al. 2004; Mohapatra et al., 2007). Dombek et al. (2000) compared the ability of REP-PCR and BOX-PCR to discriminate 154 *E. coli* isolates of 7 source groups (human, duck, geese, chicken, pig, sheep and cow) and concluded from the ARCC results that the discriminatory efficacy of BOX-PCR (ARCC Ľ 93.3%) was superior to REP-PCR (ARCC Ľ 65.8%). Leung et al. (2004) documented that ERIC-PCR was not an effective tool in distinguishing *E. coli* isolates between animal and human sources. In contrast to above study, the ERIC sequence was used successfully for differentiation of *E. coli* isolates obtained from patients showing clinical signs of urinary tract infection (UTI) (Dalal et al., 2009). Baldy-Chudzik et al. (2003) have evaluated REP-PCR and ERIC-PCR and found greater discriminatory power for REP-PCR than ERIC-PCR for genotyping of 93 aquatic *E. coli* isolates. Results of Wieser & Busse (2000) demonstrated that ERIC- and BOX-PCR are excellent tools for rapid identification of *Staphylococcus epidermidis* strains at the species level. For species differentiation ERIC-PCR appeared to be more suitable than BOX-PCR, however, the combination of these two PCR methods provide more reliable results in classifying and identifying staphylococcal isolates. ERIC-PCR and BOX-PCR were rapid, highly discriminatory and reproducible assays that proved to be powerful surveillance screening tools for the typing of clinical *P. aeruginosa* isolates (Dawson et al.

2002; Syrmis et al., 2004; Wolska & Szweda, 2008; Yang et al., 2005). Rep-PCR was also used to study the epidemiology of *Vibrio parahaemolyticus* isolated from cockles in Padang, Indonesia. For the ERIC primer, it produced bands ranged from 3-15 with sizes from 0.1 - 5.0 kb and twenty seven different ERIC patterns (Zulkifli et al., 2009).

Rep-PCR has been shown to have similar or even better strain differentiation power, as well as to be easier, quicker and cheaper to perform, than ribosomal intergenic spacer analysis (RISA), restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP), random amplified polymorphic DNA (RAPD) and other techniques (Chmielewski et al., 2002; Niemann et al., 1997; Olive & Bean, 1999). Several studies have shown Rep-PCR to have good correlation with PFGE results but, in general, with slightly less discriminatory power and less reproducible (Kidd et al., 2011).

The rep-PCR technique, in which the amplified products are separated by agarose gel electrophoresis, suffers from several limitations like poor band resolution and run standardization for comparison of the different profiles in different gels. To overcome these limitations separation of fluorescent labelled products in automated DNA sequencer can be used (Del Vecchio et al., 1995; Versalovic et al., 1995). This method allows consistent pattern formation and storage of the data in a database as a digitized image. Unknown strains can be compared against the stored database for identification purposes.

The recently introduced DiversiLab (DL) system (bio-Mérieux) is based on the repetitive-sequence-based PCR. The DL system is a semiautomated rep-PCR with a high level of standardization, in particular for the electrophoresis step by using a Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA). This reduces reproducibility problems due to variation in assay conditions. The analysis software allows the comparison of individual amplification product patterns (peak patterns), which enables easier interpretation of the patterns, but a virtual gel image is also generated. The patterns can be stored in a database and used for comparison. An important advantage of DL is that a result can be obtained in 1 day starting from a pure culture. DL is a useful tool to help identify hospital outbreaks of *Acinetobacter* spp., *S. maltophilia*, the *Enterobacter cloacae* complex, *Klebsiella* spp., and, to a somewhat lesser extent, *E. coli*. DL was inadequate for *P. aeruginosa*, *Enterococcus faecium*, and MRSA (Fluit et al., 2010).

2.2.4 Ligation mediated–PCR

This is a group of highly sensitive methods widely used for detection of DNA polymorphism of both prokaryotic and eukaryotic organisms. Several techniques classified to this group have been described to date. The procedure of all of them is based on five steps: digestion of the total cellular DNA with restriction enzymes (one or more, depending of the type of LM-PCR) → ligation of restriction half-site specific adaptors to all restriction fragments generated during digestion of the cellular DNA → selective amplification of some of the DNA fragments generated in the ligation step → visualization of the amplified fragments of DNA →interpretation.

2.2.4.1 Amplified Fragment Length Polymorphism – AFLP

The AFLP-PCR (Amplified Fragment Length Polymorphism) is the first described (Vos et al., 1995) and the most popular method belonging to the LM-PCR (Ligation mediated) group. In the first step the total cellular DNA is digested with a combination of two

restriction endonucleases, a frequent cutting enzyme with a 4 – base recognition and a rare-cutting enzyme of 6 to 8 - base recognition. Three types of restriction fragments are generated following digestion:

a. with a cohesive ends left by a rare cutter at both ends,
b. with a cohesive ends left by a frequent cutter at both ends,
c. with two distinct ends left by each of used enzymes.

More than 90% of fragments are expected to have frequent – cutter sites at both ends (A) (Blears et al., 1998). The short double - stranded fragments of synthesized DNA (10-30 base pairs long), called adapters, complementary to the corresponding restriction site, are ligated to the generated fragments of DNA of analyzed organisms. The adapters serve as a primer binding sites for PCR amplification carried out in the next step. The ligation of adapters and restriction products is performed in the presence of both restriction enzymes used previously for the digestion of the template DNA. It prevents ligation of restriction fragments obtained in the first step. The adaptor is designed to ensure that ligation of a fragment to an adaptor does not reconstitute the restriction site and the obtained products are not digested with the endonucleases. The products of ligation are amplified with primers consisting of an adapter sequences at the 5′ ends and extended with a variable number of 3′ nucleotides, usually 1 to 3. The addition of nucleotides at the 3′ end of the primers reduces a number of amplified fragments of DNA. The number of amplified fragments is reduced approximately four fold with each additional selective base, assuming a random base distribution (Vos et al., 1995). The limitation of a number of amplified fragments is necessary to make the analysis of DNA fragments pattern easier or rather possible. Even after using the extended primers, the number of amplicons is still very high, in a range of 50 to 100. As a result, special methods have to be used for analysis of the results of amplification, such as high resolution electrophoresis with silver staining or autoradiography. The fluorescent AFLP method (FAFLP), which uses fluorescently labeled primers for PCR amplification and an automated DNA sequencer for fragment detection can be used as a solution to such problems. In some cases, less complex patterns can be obtained by AFLP protocols employing just one restriction enzyme, which can be analyzed by simple agarose gels, as it has been demonstrated for *Chlamydia psittaci*, *H. pylori* and *L. pneumophila* (Boumedine & Rodolakis, 1998; Gibson et al., 1998; Jonas et al., 2000; Valsangiacomo et al., 1995). Each time, the AFLP – PCR must be carried out under stringent conditions, permitting only a selective amplification of those genomic fragments that are perfectly complementary to the 3′ ends of the primer sequences. The stringent PCR conditions used in AFLP – PCR lead to highly reproducible results that are readily comparable among different samples.

This method has been successfully applied for genotypic analysis of different species of bacteria, yeast, fungi and plants. A model system for AFLP is genus of *Xanthomonas* (Rademaker et al., 2000). Lomonaco and coworkers have shown that AFLP-PCR can be a very useful tool for the subtype of *L. monocytogenes* isolated from environmental and food samples (Lomonaco et al., 2011). Also coagulase-positive and coagulase-negative staphylococci from different origins: veterinary (Cuteri et al., 2004; Piessens, 2010) and clinical samples (Sloos et al., 1998) were differentiated using the AFLP technique. Analyzing the group of over 50 clinical isolates of *S. aureus,* Melles and coworkers (2007) obtained similar results for three genotypic methods: AFLP, PFGE and MLTS, and AFLP was shown

to be more reproducible than PFGE. Lan and Reeves (2007) successfully applied radioactively and fluorescent dye-labeled AFLP method for analysis of a genetic polymorphism of one of most dangerous species of foodborn pathogens – *Salmonella*. An interesting modification of the AFLP method has been recently proposed and applied for typing of 70 clinical isolates of *E. coli* by Brillowska-Dabrowska and coworkers (2008). The authors digested the template DNA with TspRI, the enzyme which recognizes and digests 9 base degenerated sequences, generating the DNA fragments with degenerated cohesive ends. The long adaptor containing the primer binding site was cloned to DNA fragments containing only one selected sequence of a cohesive end. The remaining cohesive ends were covered with short - lacking primer site adaptors with degenerated cohesive ends. In the result, only the selected TspRI digested DNA fragments were amplified. The analysis of results was performed using a classical polyacrylamide gel electrophoresis and the grouping obtained was identical with that resulting from REA–PFGE. Identification and epidemiological examination of *Candida* species isolated from clinical samples using the AFLP method were carried out by several authors eg. Ball and coworkers (2004). Satisfactory results were also obtained in the case of analysis of genetic similarity between 55 isolates of *Aspergillus fumigatus* obtained from 15 different patients suffering from the proven invasive aspergillosis (De Valk et al., 2007). The AFLP can be also applied for analysis of more complicated higher organisms: plants, animals and human. For example the method has been used to study human DNA samples in forensic investigations and in paternity tests (Brinkman et al, 1991), however the analysis of diversity of higher organisms is not a subject of this chapter and will not be discussed in details.

In addition to their widespread use in DNA fingerprinting, the AFLP-based approaches have also been used to produce gene expression fingerprints. AFLP gene expression fingerprints are generated using cDNA (rather than genomic DNA) as the PCR template. With this approach, researchers can study gene expression from multiple *loci* as a means of comparison between two different individuals or populations (Rice, 2010).

The most important advantage of AFLP – PCR is the high discriminatory power of this method, that is comparable with that of PFGE – the method currently considered to be "the gold standard" for epidemiological studies and can be easily determined by combination of a pair of restriction enzymes and by design of primers. The selection of restriction enzymes is usually based on the in *silico* analysis (San Millan et al., 2005), however the combination of MseI and EcoRI enzymes is the most popular in the literature. If fact, the AFLP analysis can be performed without any prior knowledge about DNA sequence of a tested organism. However, on the other hand pre - isolation of the pure culture is necessary. The analyzed DNA cannot be contaminated with any plasmid or chromosomal DNA of another organism. Therefore the method is not suitable for analysis of DNA samples isolated directly from an infected tissue, food or environmental sample, which in some applications is an obvious limitation.

2.2.4.2 Amplification of DNA Surrounding Rare Restriction Sites ADSRRS

At the beginning of the first decade of the 21st - century Masny and Plucienniczak described two new methods of LM–PCR, that are called respectively ADSRRS (Amplification of DNA Surrounding Rare Restriction Sites) and PCR MP (Melting Profiles) (Masny and Plucienniczak, 2001, 2003). In comparison to the AFLP technique, the main advantage of both proposed methods is reduction of number of amplified fragments of DNA, that leads

to much easier analysis of obtained results. In this procedure, similary like in the AFLP technique, the products of digestion of the template DNA with a pair of restriction enzymes (one frequent and one rare cutting), are ligated with adapters. Adapter design is the crucial element of the ADSRRS method. The adapter complementary to the cohesive ends of rare cutter is short and not really rich in GC pairs, in contrast to the adapter complementary to the ends generated by the other enzyme, which is long and contains a lot of G and C nucleotides. It is important to mention that adapters are not phosphorylated and in fact only one (shorter) strand is ligated to the fragments of digested template DNA. The other strand of adapter, called helper, is only necessary for binding of the adapter with complementary cohesive end before ligation. The helper is than thermally dissociated and the ends of DNA fragments ligated with one strand of adapter are filled with thermostable DNA polymerase. In the effect, three sets of DNA fragments are generated:

a. containing sequences of a long adapter at both ends. These are the products of digestion of the template DNA with only frequent cutter. As it was mentioned they constitute about 90% of the obtained population of DNA fragments,
b. containing sequences of short adapter at both ends,
c. with ends containing sequences of two different adaptors at their ends.

In consequence of ligation of the same adaptors to the both ends, the fragments belonging to the groups A and B contain in fact the terminal complementary sequences within each of a single DNA strand. It leads to the creation of secondary DNA structures, called sometimes the tennis racket structures, during the PCR procedure and inhibition of amplification of these fragments of DNA. The PCR amplification is carried out with primers complementary to the sequences of ligated adaptors. Especially effective is suppression of amplification of fragments belonging to the group A, which contain the long and GC rich complementary sequences at both ends of each DNA strand. Amplification of fragments of group B is not inhibited so effectively. The terminal complementary sequences are shorter and do not contain so much GC so that they are unstable in the conditions of PCR reaction. The fragments of group C are amplified exponentially. Finally, in the electrophoretic analysis one can find only the products of amplification of fragments belonging to the groups B and C, which constitute less than 10% of fragments generated after digestion of template DNA. Usually the number of amplified fragments range between 20 and 30, so the interpretation of results is much easier than in the case of AFLP, which usually generates 50 to 200 products of amplification. In spite of reduction of the number of amplicons, the ADSRRS method has high discriminatory power. The method still is not very popular, however it has been successfully applied for genotyping of several important species of bacteria: *Enterococcus faecium* (Krawczyk et al, 2003 a), *S. marcescens* (Krawczyk et al., 2003 b), *K. pneumonia* (Krawczyk et al., 2005), *S. aureus* (Krawczyk et al., 2007a), *Corynobacterium pseudotuberculosis* (Stefanska et al., 2008) and *C. jejuni* and *C. coli* (Krutkiewicz & Klimuszko, 2010). The above authors have revealed that the discriminatory power of the ADRRS method is comparable with that of a gold standard RAE-PFGE.

2.2.4.3 PCR Melting Profiles – PCR MP

In the case of PCR MP, the template DNA is digested with only one enzyme and generated DNA fragments are ligated with only one adaptor. The mechanism of ligation is identical as

in the ADSRRS method. The adaptor is not phosphorylated and only one strand is ligated with fragments of the digested template DNA. After thermal dissociation of helper sequences, the ends of DNA fragments are filled with a thermostable polymerase. Finally, the terminal complementary sequences within a single DNA strand are present in the whole population of the obtained DNA fragments. The reduction of number of amplified fragments in this method is achieved by using low denaturation temperature of template DNA during PCR reaction. Typically during PCR, denaturation temperatures around 94 – 95ºC are applied, whilst in PCR MP the temperature of this step is reduced to the level of 80 – 86ºC, depending of analyzed species of microorganism. As a result most of the fragments of DNA, especially long and containing a high number of GC pairs, are not melted and are not amplified because only single – stranded DNA may serve as a template in PCR. In other words, the primers cannot effectively bind to the complementary sequences of template DNA fragments. Additionally, as in the case of AFLP, the limitation of the number of amplified fragments is possible by using extended primers with a variable number of 3' nucleotides (Masny & Plucienniczak, 2003). The PCR MP has been widely investigated in the group of Krawczyk and coworkers, who revealed a comparable discriminatory power of this method with RAE PFGE in differentiation of staphylococci (Krawczyk et al., 2007 a), vancomycin – resistant *E. faecium* (Krawczyk et al., 2007 b), and *C. albicans* (Krawczyk et al., 2009), and higher discriminatory power than ITS – PCR in genotypic analysis of *Klebsiella oxytoca* (Stojowska et al., 2009).

2.2.5 Cleavase Fragment Length Polymorphism – CFLP

Cleavase fragment length polymorphism (CFLP) is a subtyping system based on the single-stranded DNA patterns resulting from digestion with the enzyme cleavase, a structure-specific, thermostable nuclease. This enzyme recognizes and cleaves secondary structures that consist of double-stranded hairpin regions interspersed with single-stranded regions of DNA and that are formed after denaturation and cooling to an intermediate temperature, in a pattern unique to the nucleotide sequence. CFLP analysis is a method with the capacity for direct assignment of alleles based on the nucleotide sequences of genes. CFLP can be applied to the rapid screening of a large number of strains during investigations of outbreaks and/or surveillance systems (Tondella et al., 1999).

The use of a thermostable endonuclease, Cleavase I (engineered endonuclease which consists of the 5'-nuclease domain of *Thermus aquaticus* DNA polymerase), allows the cleavage reactions to be performed at elevated temperature, which is fundamental to realizing the full benefit of this assay. For example, if a particularly stable secondary structure is assumed by the DNA, a single nucleotide change is unlikely to significantly alter that structure or the cleavage pattern it produces. Elevated temperatures can be used to bring structures to the brink of instability, so that the effects of small changes in sequence are maximized and revealed as alterations in the cleavage pattern. Furthermore, the use of high temperature reduces long-range interactions along the DNA strands, thereby increasing the likelihood that observed cleavage differences will reflect the locations of the base changes. The potential for multiple localized pattern changes in response to a single sequence change presents an additional advantage of this method of mutation detection over other methods in use. Direct sequencing of this region would provide a single variant peak on which to base a conclusion. In contrast, the multiplicity of effects seen here provides redundant confirmation of the base change, allowing multiple checkpoints within the

pattern to be compared. This is especially useful in the cases of heterozygosity, or the presence of drug-sensitive and drug-resistant bacteria in a single sample. While the analyses shown above have been done with nonisotopic labels, the method is fully compatible with radiolabeled DNAs and standard autoradiography. When staining or uniform labeling are used, the patterns produced are more complex, as all fragments are visible, and the localization feature is lost because bands cannot be measured from a discrete labeled end, yet variations in the cleavage patterns are readily detected (Brow et al., 1996).

CFLP can be performed on differently labeled wild-type and mutant alleles in the same reaction, with no discernible effect of the patterns of each. When fragment size analyses are performed on a fluorescence based DNA sequencer, the wild-type pattern can serve as an internal control for enzyme digestion and as an internal reference that allows accurate discrimination of pattern differences in the mutant samples. When calibrated against a sequence ladder or other known markers, the wild-type pattern also allows precise sizing of the mutant bands. With the CFLP patterns normalized in this way, experimental samples can be compared day-to-day and lab-to-lab, with a high degree of confidence. The ability to rapidly perform an independent analysis of both DNA strands with the use of differently labeled PCR primers facilitates complementary confirmatory information analogous to sequencing both DNA strands. Analysis of DNAs through the probing of their structures with the Cleavase I enzyme promises to be a powerful tool in the comparison of sequences (Tondella et al., 1999). The procedural steps in CFLP are the following: PCR amplification→exonuclease digestion→spin column purification→cleavase digestion→eectrophoresis→overnight transfer to nylon membrane→probe hybridization→interpretation.

CFLP can rapidly screen for the major epidemic clones of serogroups B and C *N. meningitides*, e.g., clonal complexes ET-5 and ET-37 and also, more importantly, can provide guidance regarding the choice of genes for the DNA sequence-based approach to the molecular subtyping of *N. meningitides (Tondella et al., 1999)*.

The low cost, rapidity, and reliability of the analysis make this method very suitable for the rapid screening of DNAs, not only for the examination of disease-associated mutations but also in applications such as tissue typing, genetic identity, bacterial and viral typing, and mutant screening in genetic crosses. The method is able to detect single base changes in DNA molecules over 1 kb long. It has been able to apply CFLP analysis to detection of mutations responsible for rifampin resistance in *M. tuberculosis* (Brow et al., 1996) and for determining the genotype of HCVs and have successfully analyzed DNAs as long as 2.7 kb (Marschall et al., 1997; Sreevtatsan et al., 1998). The potential of the CFLP method was examined for differentiating many bacteria. In order to provide the greatest utility, a bacterium-typing method should be able to differentiate both species and strains. The genotype of the 16S rRNA genes, while useful for general classification, would not be expected to necessarily allow identification to the species and strain levels. For these purposes, a 550-bp amplicon derived from the intergenic region of several isolates of *Shigella* and *Salmonella* spp. was examined. The structural fingerprints generated by Cleavase I treatment clearly differentiated all species of *Salmonella* and *Shigella*. Again, a familial resemblance is clear in all of these evolutionarily related bacteria, yet differences in the structural fingerprints of each genus and species are clearly visible. The power of CFLP analysis is even more striking when the analysis of *Shigella* spp. was examined. *Shigella*

sonnei was clearly differentiated from the three *Shigella dysenteriae* isolates examined. Furthermore, although serotypes 1, 2, and 8 of *S. dysenteriae* yielded related structural fingerprints, the antisense patterns for the individual serotypes could, however, be distinguished (Tondella et al., 1999).

A similar variation to CFLP analysis may occur with restriction-enzyme-dependent fingerprinting methods like RFLP, ARDRA and AFLP, depending on the restriction enzyme that is used (Tondella et al., 1999).

2.3 Hybridization – Based method

Southern blotting is a technique named after its inventor and developer, the British biologist Edwin M. Southern in 1975 (Southern et al., 1975). Southern blotting is a technique which allows the detection of a specific DNA sequence (gene or other) from a variety of prokaryotic and eukaryotic organisms. This classic method has been adapted to the differentiation of bacterial strains on the basis of the observation that the locations of various restriction enzyme recognition sites within a particular genetic locus of interest can be polymorphic from strain to strain, resulting in gel bands that differ in size between unlike strains. Thus, the name restriction fragment length polymorphism (RFLP) refers to the polymorphic nature of the locations of restriction enzyme sites within defined genetic regions. Only the genomic DNA fragments that hybridize to the probes are visible in RFLP analysis, which simplifies the analysis greatly.

The procedural steps in RFLP-Southern blotting are: **restriction endonuclease digestion**→agarose gel electrophoresis→DNA fragments transfer to nitrocellulose or nylon membrane→probe hybridization→interpretation. DNA (genomic or other source) is digested with one or more restriction enzymes and the resulting fragments are separated according to size by electrophoresis through an agarose gel. The DNA is then denatured into single strands by incubation with NaOH and transferred to a solid support (usually nitrocellulose filter or nylon membrane). The relative positions of the DNA fragments are preserved during their transfer to the filter. The membrane-bound nucleic acid is then hybridized to one or more labeled probes homologous to the gene to be examined. The probe is labeled before hybridization either radioactively or enzymatically (e.g. alkaline phosphatase or horseradish peroxidase). Finally, the location of the probe is detected by directly exposing the membrane to X-ray film or chemiluminescent methods. The optimum size range of DNA fragments detected this way is 2 – 25 kb. Although RFLP analysis does not require knowledge of the genome sequence, RFLP studies can be extremely time consuming and challenging in the absence of such data. When sequence data is not available, the experimenter must physically clone a region of the genome under study that is large enough to be cut up by enzymes, and this process requires a great deal of time and resources (Campbell, 2001; Olive & Bean, 1999).

Genetic specific probes have been used to subtype *Brucella* species (Grimont et al., 1992), *L. pneumophilla* (Tram et al., 1990) and *P. aeruginosa* (Loutit & Tompkins, 1991). RFLP was commonly found in the *tcdB* gene of *C. difficile* (Rupnik et al., 1998). It was earlier the gold standard for *M. tuberculosis* complex species genotyping - *IS6110*. One of the factors that have limited the use of RFLP was it being laborious and time consuming and the resulting complex banding patterns made interlaboratory comparisons difficult (Van Embden et al., 1993). Ribotyping, a variation of RFLP-Southern blotting in which the probes are derived

from the 16S and 23S rRNA genes, has been applied successfully in many studies for differentiating strains of *Salmonella* (Landers et al., 1998), *P. aeruginosa* (Dawson et al., 2002) and *Pseudomonas fluorescens* (Dawson et al., 2002). Ribotyping results in only a small number of bands (one to four varing between about 6 and 9 kb), which simplifies the interpretation. However, this also limits the ability of the technique to distinquish between closely related strains (Olive & Bean, 1999). Analysis of the 16S rRNA nucleotide sequence, the 16S to 23S rRNA intergenic spacer region (ISR) sequence, and the 5′-terminal region of 23S rRNA allowed the strains of *Bacillus thuringensis* to be subdivided into three groups based on the pattern of nucleotide substitutions (Boulygina et al., 2009). Furthemore, a phylogenetic approach based on 16S rRNA genes (rDNA) has been applied to investigate the diversity of cultivable and non-cultivable species in the human oral cavity without cultivation (Sakamoto et al., 2003).

Advantages of RFLP-Southern blotting: all strains carrying loci homologous to probe are typeable; they are reproducible, have good ease of interpretation. Disadvantages of RFLP-Southern blotting: the discriminatory power depends on the choice of probes; the process requires costly reagents and equipment besides being labour intensive (Campbell, 2001).

Both RFLP-Southern blotting probe sets yielded information about the evolutionary relationships of the strains examined, yet neither technique was as efficient at discriminating strain differences as PFGE (Olive & Bean, 1999).

2.4 Sequencing methods

The results of "classical" genotypic methods are presented as specific patterns of DNA fragments after electrophoresis. However, the profiles of DNA fragments generated in PFGE or AFLP techniques are very complicated and consist of at least 50 bands. It is very difficult to use these results for long – term or interlaboratory investigations. This problem can be solved by application of the newest group of genotypic methods applying nucleotide sequencing approach. The results of sequencing are presented as a sequence of combination of four letters: A, T, G, C, corresponding to the sequence of nucleotides, respectively: adenine, thymine, guanine and cytosine. The phylogenetic classification of microorganisms is based on differences of sequences of a particular fragment of the genome. Various types of differences among DNA sequences may be identified: insertion, deletion, duplication and a situation when a single nucleotide A, T, G, or C differs between members of a given species. This form of results is much easier to analyze and can be used for studying long-term or global pathogen epidemiology, such as the worldwide distribution and frequency of bacterial lineages, virulence properties associated with certain lineages, etc. The data, i.e. sequences can be easily stored in the online accessible databases and compared among laboratories. Some of the most important databases containing sequences widely used for global investigation of genetic variability of microorganisms are presented below. Although the cost of sequencing decreased substantially during the least 20 years it is still very high, what is the biggest drawback of sequencing – based methods. At present there are two main strategies for DNA sequencing: the traditional Sanger method and the newly developed pyrosequencing method. The key principle of the Sanger method is using of dideoxy nucleoside triphosphates (ddNTPs) as DNA chain terminators. Dideoxynucleotides are essentially the same as nucleotides except they contain a hydrogen (H) atom on the 3′ carbon instead of a hydroxyl group (OH). These modified nucleotides, when integrated into

a sequence, prevent the addition of further nucleotides. Each of the four ddNTPs is also labeled with specific fluorescent dye for detection in automated sequencing machines. After the PCR amplification, a set of DNA fragments is generated. Each of them is labeled with a specific dye depending on the last nucleotide. The newly synthesized and labeled polynucleotides are separated by capillary electrophoresis according to their size and particular ddNTPs are recognized by a fluorescence detector. The results of sequencing are presented as a fluorescent peak tracing.

Another technique of DNA sequencing called pyrosequencing was developed by Pal Nyren and Mostafa Ronaghi at the Royal Institute of Technology in Stockholm in 1996. In this method, a DNA fragment of interest (sequencing primer hybridized to a single-stranded DNA template immobilized on solid matrice) is incubated with DNA polymerase, ATP sulfurylase, firefly luciferase, and a nucleotide-degrading enzyme. Repeated cycles of deoxynucleotide addition are performed. A deoxynucleotide will only be incorporated into the growing DNA strand if it is complementary to the base in the template strand. The synthesis of DNA is accompanied by release of PP_i in molar concentration equal to that of the incorporated deoxynucleotide. Thereby, real-time signals are obtained by the enzymatic inorganic pyrophosphate detection assay. In this assay, the released PP_i is converted to ATP by ATP sulfurylase and the concentration of ATP is then sensed by the luciferase. The amount of light produced in the luciferase-catalyzed reaction can readily be estimated by a suitable light-sensitive device such as a luminometer or a CCD (charge-coupled device) camera. The sequence of solutions of deoxynucleotide which produce chemiluminescent signals allows the determination of the sequence of the template (Ronaghi et al., 1998).

Recently developed sequencing – based methods of differentiation of microorganisms represent a real progress because of their accuracy, reproducibility, versality and exchangeability of the results. Their discriminatory power depends of the size of the fragment of the genome that is sequenced. Theoretically, the analysis of the bigger fragment should guarantee the higher discriminatory power. However there are also some examples of single-locus-sequence-based (SLST) typing methods which give unexpectedly good results. Probably the most widely used method of the SLST group, called the *spa* typing, is sequencing of the polymorphic X region of the protein A gene (*spa*) of *S. aureus*, which contains a variable number of 24-bp-repeat regions flanked by well-conserved fragments (Harmsen et al, 2003). Several authors have demonstrated fairly good correlation between the clonal groupings classification of MRSA obtained by *spa* typing and those obtained by other typing techniques including PFGE (Hallin et al, 2007, Strommenger et al, 2008). At the moment it is possible to compare the sequence of fragment X of the *spa* gene of any analyzed strain with 9073 *spa* sequence types, from 17,9659 strains isolated in 81 countries (August 2011), which are stored on the Ridom Spa Server (http://spa.ridom.de). The SpaServer was developed to collate and harmonize data from various geographic regions. This WWW site is freely accessible for internet users and the *spa*-repeat sequences and the -types can be downloaded. Chromatograms of new *spa*-repeats and/or -types can be submitted online for inclusion into the reference database. Another example of a SLST method that can be used for typing staphylococci is analysis of the polymorphism of sequence of the *coa* gene encoding *S. aureus* coagulase. Short review of other genes which can be used for bacterial strain typing was recently presented by Li and coathors (Li et al, 2009). Sequence analysis of the *ompt* gene, which encodes a major antigenic outer membrane protein of *Rickettsia* species, was demonstrated to be an efficient tool for identification and subtyping of these

bacteria (Fournier et al, 1998). Karjalainen and coworkers revealed that serotyping of *C. difficile* can be replaced by sequencing of a variable fragment of *slpA* gene, that encodes a surface layer protein of this bacteria (Karjalainen et al, 2002). Great potential for genotyping of bacteria represents also the *rpoB* gene encoding β – subunit of RNA polymerase. The gene exist in only one copy in most bacteria and its sequence polymorphism offers higher discriminatory power than the 16S rRNA gene. The analysis of its polymorphism enabled genotyping of *Bacillus licheniformis* strains from different sources (De & De, 2004; Li et al, 2009).

To date, for most species of bacteria, yeast and fungi it is still impossible to select one appropriate gene which could be used for differentiation of isolates with enough high discriminatory power. More detailed analysis of genetic diversity of these microorganisms can be performed by the MLST method (Multilocus sequence typing), which was developed for *N. meningitidis* as the model species (Maiden et al, 1998). MLST is an unambiguous procedure for characterizing isolates of bacterial species using the sequences of internal fragments of several, usually seven house-keeping genes coding for proteins that are required for maintenance of basic cellular function. Approximately 450 - 500 bp internal fragments of each gene are used, as these can be accurately sequenced on both strands using an automated DNA sequencer. Depending on the species of microorganism, different sets of housekeeping genes are selected as targets for MLTS. For example the *S. aureus* MLST scheme uses internal fragments of the following seven house-keeping genes: *arc* (carbamate kinase), *aro* (shikimate dehydrogenase), *glp* (glycerol kinase) , *gmk* (guanylate kinase), *pta* (phosphate acetyltransferase), *tpi* (triosephosphate isomerase) and *yqi* (acetyl coenzyme A acetyltransferase) (Enright et al, 2000a), whilst *S. pneumoniae* scheme uses: *aroE* (shikimate dehydrogenase), *gdh* (glucose-6-phosphate dehydrogenase), *gki* (glucose kinase), *recP* (transketolase), *spi* (signal peptidase I), *xpt* (xanthine phosphoribosyltransferase) and *ddl* (D-alanine-D-alanine ligase) (Enright et al., 2000b). For each house-keeping gene, the different sequences present within a bacterial species are assigned as distinct alleles and each strain is assigned a seven – number allelic profile designated as a sequence type (ST) (Maiden et al, 1998). MLST is an especially useful tool for long - term investigation of bacterial population structures and is rather rarely used for analyzing local, short - term epidemics. The results of MLST of 27 microorganisms species (August 2011) are available on the MLST homepage (http://www.mlst.net), and the DNA sequences of all housekeeping genes of all analyzed strains and all necessary software are available online, that facilitates the global epidemiology survey of infections caused by these groups of pathogens (Li et al, 2009).

Besides sequences coding for particular proteins, different forms of RNA or sequences important for regulation of expression of some genes, the genomes of all living organisms contain surprisingly large amounts of non-coding DNA sequences, i.e. intergenic spacers. With high probability it can be assumed that intergenic spacers are a subject of much weaker selection pressure than genes and their variability is higher than that of genes. Therefore the intergenic spacers have a great potential as targets for genotyping. A particularal example of noncoding sequence of prokaryotic organisms useful for their genotyping is a genomic region separating 16S and 23S rRNA genes called the internal transcribed spacer (ITS). Possibilities of application of analysis of polymorphism of size and sequences of this target by classical genotypic techniques was presented in the earlier parts of this chapter. The presence of more than one copy of the rRNA operone in most bacterial species cause that it is not really good target for sequencing analysis. In order to achieve a good discriminatory power usually several intergenic spacers are analyzed (MTS –

Multispacer typing). The crucial point of the MTS method is selection of non-coding sequences characterized by the high level of variability. Amplification and sequencing of selected spacers provide unique sequence types (ST) for each of them. Finally combination of STs from each studied spacer provides a multispace genotype (MTS genotype) (Li et al., 2009). First successful analysis of polymorphism of sequences of several selected highly variable intergenic spacers was presented by Drancourt and coworkers (2004). The authors performed subtyping of *Y. pestis* from dental pulp collected from remains of eight persons who likely died during two historical plagues caused probably by this species of bacteria. The MST system was also applied with success to strains of other human pathogens, including *Bartonella henselae* (Li et al., 2007), *M. tuberculosis* (Djelouadji et al., 2008), *Rickettsia conorii* (Wenjun et al., 2009) and *M. avium* (Cayrou et al., 2010).

As a result of the presented advantages with great certainty it can be assumed that sequencing – based methods will gain popularity in the future. It should also be stressed that all sequencing – based methods do not require isolation of a pure culture of analyzed microorganism. The biggest drawback of this group of methods is the fact that they are time demanding and still too expensive to be used in routine genotyping in most laboratories. However a huge progress in the sequencing techniques has been observed during the last years and it is quite realistic that in not too remote future the analysis of polymorphism of the whole genomes will become a routine genotypic method, at least in some specialized institutions.

2.5 Detection of presence or absence of particular genes

Another PCR – based genotypic method relies on detection of presence or absence of sets of genes, usually coding for virulence factors or genes involved in antibiotic resistance. However the main goal of this kind of search is identification of genes being important factors in particular types of infection. They can be also used for characterisation of genetic differentiation of isolated pathogens. More or less detailed analysis of genetic features has been carried out and described for probably all known groups of microorganism, and it is impossible to present all of them in this short review. *S. aureus,* common and important human and animal pathogen may serve as a model for description of this genotyping method. One of the most spectacular, classical analyse of genetic features of staphylococci was presented by the group of Ote , who determined the genetic profiles of 229 strains of *S. aureus* collected from bovine cases of mastitis. The presence of about forty virulence-associated genes was investigated by specific polymerase chain reaction (PCR) amplification, and a high number of genetic subtypes were observed (Ote at al., 2011). This method does not require using of expensive and sophisticated equipment, but on the other hand, this is an extremely time consuming and laborious approach for analysis of genetic diversity of microorganisms. The mentioned authors had to carry out about ten thousand of PCR reactions, assuming that they were not repeated. The time required for analysis of presence of selected genes can be dramatically reduced by using macro or microarrays. These technologies can provide detailed, clinically relevant information on the isolate by detecting the presence or absence of a large number of virulence-associated genes simultaneously in a single assay. Trad and coworkers designed and prepared the DNA macroarray containig 465 intergenic amplicons of genes characteristic for *S. aureus*. The genes selected included those encoding *S. aureus*-specific proteins, staphylococcal and enterococcal proteins mediating antibiotic resistance and factors involved in their

expression, putative virulence proteins and factors controlling their expression, and finally proteins produced by mobile elements. The macroarray was hybridized with the labeled cellular DNAs of 80 *S. aureus* clinical isolates. In a gene content dendrogram, the isolates were distributed into 52 clusters, and outbreak-related isolates were linked in the same or a closely related cluster(s) (Trad et al., 2004). The most spectacular example of application of macroarrays, which in fact does not concern staphylococci but in our opinion should be presented because of its importance and popularity is spoligotyping of mycobacteria (Groenen et al., 1993). Spoligotyping (spacer oligonucleotide typing) is a method that can be simultaneously used for detection as well as typing of the *M. tuberculosis* complex. This is a PCR-based method, which depends on the amplification of a highly polymorphic Direct Repeat (DR) locus in *M. tuberculosis* genome. The DR region contains direct repeat sequences of 36 bp, that are interspersed by the non-repetitive DNA spacers of 35-41 bp in length. One DR and its neighboring non repetitive spacer is termed as Direct Variant Repeat (DVR). Spoligotyping can be used for detecting the presence or absence 43 spacers of known sequences in DR region by hybridizing the PCR amplified spacer DNA to the set of immobilized oligonucleotides, representing each of the unique spacer sequences. In the effect, a distinct spoligotyping pattern is generated.

Use of microarrays – miniaturized version of macroarrays enables performance even more detailed analysis. Fitzgerald and coworkers demonstrated that 2,198 (78%) of the 2,817 of chromosomal open reading frames (ORFs) of reference COL *S. aureus* strain represented on a DNA microarray were shared by the 36 analyzed *S. aureus* isolates from various sources and geographical regions. The investigation also revealed that 10 out of 18 large regions of difference carry genes that encode putative virulence factors and proteins that mediate antibiotic resistance (Fitzgerald et al., 2001). The microarrays which are used for detection of presence or absence of particular genes are called cDNA microarrays. The microarray technology seems to be also a very promising tool for analysis of SNP, however a modified construction of array, called oligonucleotide microarray, is required. The most important difference of both kinds of arrays is the size of the DNA probe that is immobilized on surface of the chip. The probes of the oligonucleotide microarray are definitely shorter (20 – 70 nuclotides), in comparison to cDNA arrays. In consequence of using shorter DNA probes, the hybridization on the oligonucleotide microarray is more stringent than that on the cDNA microarray. The detection of SNP within the sequences of analyzed genes can be achieved by preparing and immobilization on the chip surface of short nucleotide sequences complementary to the fragments of genes of reference strain (without mutations) and also a number of modifications of each of these nucleotide sequences containing all possibilities at the potential polymorphic sites including short deletions, insertions and replacement of particular nucleotides. The hybridization of labeled DNA of analyzed strain of microorganism and the immobilized DNA probes is the most efficient in the case when their sequences are identical. The special equipment and software enable quantitative analysis of hybridization of analyzed DNA to the oligonucleotides spotted on the surface of the chip. At present it is possible to prepare a microarrays containing up to 1 000 000 oligonuclotides per chip, so that the actual sequences of a large number of genes can be deduced simultaneously in one experiment. The advantages of macro or microarrays are undeniable, however with some exceptions like the presented above spoligotyping of mycobacteria, they are still rarely used for routine genotyping of viruses, bacteria, yeast or fungal pathogens. It is mainly caused by the high cost of these methods – especially microarrays and some technical

problems related with their preparation and analysis of obtained results. In addition it can be difficult to achieve satisfactory quality control in DNA microarray analysis because many factors affect nucleic acid hybridization reaction (Li et al., 2009). The macroarrays can be self-prepared by the methodology presented for example by Trad and colleagues (2004), however it requires specific equipment and advanced bioinformatic and laboratory skills. The microarrays are specially treated microscope slides (chips) that carry an ordered mosaic of sequences of selected genes of analyzed species of microorganism. As it was mentioned, the analysis of presence of two - three thousands of genes is not a problem, so that in the case of the most pathogenic bacteria it is possible to analyze the presence of all possible open reading frames. The preparing of microarrays can be definitely classified as a hi - tech technology and can be performed only in specialized institutions. The particular microarray can be prepared and supplied by these institutions, however performance of experiment and then analysis of results is definitely more complicated than in the case of macroarrays, it requires using specific equipment and software and therefore can be done in specialized laboratories only. In spite of all presented actual limitations, there are no doubts that macro and especially micro array technologies will gain in popularity and in near future they will become routine techniques of genotyping of microorganisms.

2.6 High Resolution Melting analysis – HRM

High Resolution Melting (HRM) analysis is a next powerful technique for the detection of epigenetic differences in double-stranded DNA samples. It was discovered and developed in 2003 by the group of Carl T Wittwer from University of Utah in collaboration with Idaho Technology (Gundry et al., 2003; Wittwer et al., 2003). The method is based on the real – time monitoring of the melting process of PCR amplified fragment of a DNA region in which the potential mutation of interest lies. The measurement is carried out in the range of temperatures from about 50 to 95°C. This can be achieved by using fluorescent dyes, that specifically bind to double - stranded DNA. The complexes of the double - stranded DNA and the dye characterize with high value of fluorescence. At the beginning of the HRM analysis there is a high level of fluorescence in the sample because of the billions of copies of the amplicon present. Heating cause progressive melting of the double - stranded DNA and in consequence a reduction of fluorescence of the sample is observed. Using a Real-time PCR machinery enables execution of the whole analysis consisting of PCR amplification and melting monitoring in one tube in the time period shorter than 1 hour. The kinetics of DNA melting and reduction of fluorescence level is presented as a fluorescence *vs.* temperature curve. The melting profile of a given DNA fragment and a shape of the obtained curve depend on length and sequence, especially the GC content of the analyzed PCR product. The method is very sensitive and even a single nucleotide difference can be detected. Thus the method is very popular for detection of SNP. Several authors successfully applied the HRM method for genotyping important human pathogens. The HRM analysis of 35 - fold repetitive *B1* gene was successfully used for genetic differentiation of the protozoan parasite *Toxoplasma gondi* (Costa et al., 2011). HRM analysis of 44 diverse MRSA isolates carried out by Stephens and coworkers generated 20 profiles from 22 *spa* sequence types. The two unresolved HRM *spa* types differed by only 1 bp (Stephens et al., 2008). Surprisingly good results of genotyping of *C. jejuni* were obtained by HRM scanning of polymorphism of several targets: a regularly interspaced short-palindromic-repeat (CRISPR) locus (Price et al., 2007), highly variable fragment of flagellin-encoding *flaA* gene (Merchant – Patel et al.,

2010). Levesquote and coworkers revealed that HRM can complement full MLST characterization of *C. jejuni* by identifying the most common alleles more rapidly and at the lower cost (Levesquote et al., 2011). The group of Levesquote performed the HRM analysis of polymorphism of seven house - keeping genes which are used in MLTS analysis of *C. jejuni*: *aspA* (aspatase), *glnA* (glutamine synthetase), *gltA* (citrate synthase), *glyA* (serine hydroxymethyl transferase), *pgm* (phosphoglucomutase), *tkt* (transketolase), *uncA* (ATP synthase alpha subunit).The HRM method also provides a rapid, robust, and inexpensive way to detect the dominant mutations known to confer MDR in *M. tuberculosis* strains and offers evident advantages over current molecular and culture-based techniques. First of all, the time of assay is very short. This method was used to screen 252 *M. tuberculosis* clinical isolates, including 154 rifampicin - resistant strains and 174 isonizaid - resistant strains based on the agar proportion method of drug susceptibility testing (DST). The rifampicin resistance determinant region (RRDR) of *rpoB* and specific regions of *katG* and the *inhA* promoter were HRM scanned for the detection of mutations conferring rifampicin (RIF) and isoniazid (INH) resistance, respectively. Of the 154 RIF-resistant strains, 148 were also resistant to INH and therefore classified as multidrug resistant (MDR). The assay demonstrated sensitivity and specificity of 91% and 98%, respectively, for the detection of RIF resistance and 87% and 100% for the detection of INH resistance (Ramirez et al., 2010). The HRM scanning of PCR amplified fragments of *rpoB, katG and inhA genes* was also successfully applied for rifampicin and isoniazid resistance among 217 clinical isolates of *M. tuberculosis* from South Korea (Choi et al., 2010) and lately Wang and coworkers revealed usefulness of the HRM method for detection of mutations of *rplS* gene resulting in streptomycin resistance of *M. tuberculosis* (Wang et al., 2011). Because of some important adavantages: rapidity, relatively low costs, possibility of differentiation pathogens without isolation of a pure culture and simplicity of interpretation of results it can be expected that HRM method will be more and more popular. However it is necessary to remember that expensive PCR – real time machinery is required and first of all it can be difficult to use the results in interlaboratory investigations.

3. Conclusion

DNA-based strain typing techniques are a remarkably useful set of tools for complementing the epidemiological analysis of nosocomial outbreaks. This chapter highlights the strengths of molecular typing methods. The ideal technique to assess genetic diversity should meet the following criteria. First of all, should be typed, that is, give the possibility to assign tested isolates for genotypic group of microorganisms. It should also have appropriate high discriminatory power, which will show the differences between unrelated microorganisms and the similarity between organisms isolated from the same source. The other important criterion is the reproducibility, the possibility of obtaining the same results for the same tested microorganisms, with each test and using the same procedure, reagents and apparatus. For epidemiological studies it is necessary that the method was quick and easy to interpret, not require specialized equipment or expensive reagents, and which is extremely important, gave the opportunity to be tested at the same time many trials and did not require specialized and expensive training of medical staff (could be used by routine microbiology laboratory personnel). So far there has not been found a universal and ideal typing method, each has its advantages and disadvantages. Moreover, each method has its pluses and minuses with regard to ease of application, reproducibility, requirement for equipment and level of resolution (Akkermans et al., 1995).

Table 1 shows the characteristics of the methods described above, in the context of their suitability for genetic differentiation of microorganisms. Currently, the gold standard for microbial typing methods is the PFGE technique and methods based on DNA-DNA hybridization. However, they are labour intensive and limited by high cost and extended turnaround times. The methods based on PCR appear to be more common and available. A simple and fast technique with high discriminatory power, and low cost is rep-PCR. But its disadvantage is a relatively small reproducibility in scale inter-laboratory. AFLP analysis has also a highly reproducible discriminatory power however the large number of band differences obtained by use primers without a selective base may be confusing and will usually not give additional information about whether strains are epidemiologically related. The other method, CFLP has the average discriminatory power and its need for DNA purification, careful optimization, and complex interpretation and its questionable reproducibility can be difficult to implement in a clinical laboratory. RAPD analysis is characterized by the lowest discriminatory power and poor reproducibility, however it gives the fastest typing results with the least hands-on time. It everything stimulates the search for new solutions, without the mentioned drawbacks. Most modifications are aimed towards the improvement of verified and reliable methods of analysis or applications of the combined techniques, which aims to increase the power of differentiation, and thus the universality of the method.

No.	Methodology	Discrimination power	Intralaboratory reproducibility	Interlaboratory reproducibility	Ease of use	Ease of interpretation	Time to result (days)	Setup cost	Cost per test
1	PFGE	High	Good	Good	Moderate	Moderate	3	High	High
2	REAP	Moderate	Good	Good	Easy	Easy	1	Low	Low
3	PCR rybotyping	Moderate	Good	Good	Easy	Easy	1	Moderate	Low
4	RAPD	High	Moderate	Poor	Easy	Moderate	1	Moderate	Low
5	Rep-PCR	High	Good	Moderate	Easy	Easy	1	Moderate	Low
6	AFLP	High	Good	Good	Moderate	Moderate	2	High	Moderate
7	ADSRRS	High	Good	Good	Moderate	Easy	2	Moderate	-*
8	PCR-MP	High	Good	-*	Moderate	Easy	2	Moderate	-*
9	CFLP	Moderate	Good	Poor	Moderate	Moderate	2	Moderate	High
10	IRS-PCR	High	Good	Good	Moderate	Easy	2	Moderate	-*
11	Sequencing	High	Good	Good	Difficult	Moderate	2	High	High
12	HRM	High	Good	Moderate	Moderate	Moderate	1	High	Moderate

*(-) – not shown

Table 1. Summary of the characteristics of the various molecular typing methods

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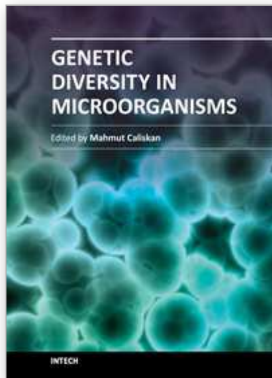

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Genetic Diversity in Microorganisms presents chapters revealing the magnitude of genetic diversity of microorganisms living in different environmental conditions. The complexity and diversity of microbial populations is by far the highest among all living organisms. The diversity of microbial communities and their ecologic roles are being explored in soil, water, on plants and in animals, and in extreme environments such as the arctic deep-sea vents or high saline lakes. The increasing availability of PCR-based molecular markers allows the detailed analyses and evaluation of genetic diversity in microorganisms. The purpose of the book is to provide a glimpse into the dynamic process of genetic diversity of microorganisms by presenting the thoughts of scientists who are engaged in the generation of new ideas and techniques employed for the assessment of genetic diversity, often from very different perspectives. The book should prove useful to students, researchers, and experts in the area of microbial phylogeny, genetic diversity, and molecular biology.

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