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Review Article

Identification and characterization of microorganisms: DNA-fingerprinting methods

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Abstract

Identification and classification of the microorganisms are of utmost importance in the field of environmental, industrial, medical and agricultural microbiology, and microbial ecology. Traditional phenotype-based methods encounter many challenges and shortcomings which limit their usability. Molecular methods offer better solutions in identifying and characterizing microorganisms. Several DNA fingerprinting methods have been developed and are in use already. In principle, most of these methods are based on PCR and restriction site analysis. Some of these methods are still not economic in use and require huge set-up cost. Continuous research is going on around the world to improve the methodology and applicability of these methods as well as to make them economic in use.

Keywords: fingerprinting, RFLP, SSCP, RAPD, MLST, PFGE

1. Introduction

Before the advent of molecular biology techniques, microorganisms were generally characterized based on their morphological, physiological and cultural characteristics, Biotyping, serotyping, bacteriocin typing, phage typing, antimicrobial susceptibility patterns, and other protein-based methods are all examples of commonly employed phenotypic methods. These phenotypic methods are associated with one or more problems related to their reproducibility, discriminatory power or untypeability. Moreover, as these phenotypic methods characterize organisms based on the products of gene expression, these are highly sensitive to variations in the environmental conditions like growth temperature, growth phase and spontaneous mutation. Such shortcomings of phenotypically based methods have therefore led to the development of genotypic methods based on DNA. With the exception of DNA sequencing, all of these methods rely on visualization of DNA bands whether they are from restriction digestion; hybridization or PCR amplification. These banding patterns or the 'DNA fingerprints' are used to compare one isolate with another. Most of the DNA fingerprinting techniques are based on the presence/absence of the restriction sites (polymorphic restriction sites) while others are based on the homologies to short oligonucleotide primers. In this review, we briefly describe the commonly used DNA fingerprinting methods.

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2. DNA Fingerprinting Techniques

2.1 Restriction endonuclease analysis of chromosome (REAC)

REAC involves isolation of chromosomal DNA, digestion with one or more restriction enzymes followed by their resolution into discernible banding patterns or 'fingerprint' after electrophoresis on agarose or polyacrylamide gel. Hundreds of fragments ranging from 0.5-5 kb in length are produced. The bands obtained are stained in situ or denatured within the gel, blotted onto a suitable membrane (nitrocellulose/nylon) and then stained. The resulting pattern of bands, reflecting the cutting sites of the particular enzymes in the chromosome, is highly characteristic of the given strain and is referred to as the strain's "fingerprint". Strains are differentiated based on their fingerprints. Isolates showing one band difference in the fingerprint are considered subtypes of each other. Patterns having 2 or more band difference are generally characterized as different strains. The variations in the fingerprints obtained by these methods account for even the minor changes in the genetic content of a microbe like point mutation, insertions, deletions, site-specific recombination and transformation. Thus, REAC is a highly sensitive technique and even a single event causing a change in DNA can be traced, thus it is an important tool for epidemiological investigation for strain typing. REAC helped in tracking the outbreak of *Pseudomonas aeruginosa* mastitis among Irish Dairy herds (Daly *et al.*, 1999).

One major advantage of this method is that it involves the whole chromosome, so the strains are compared on a broad basis and no prior knowledge of sequence data is required. Using REAC all strains can be typed. However, sometimes fingerprints are very complex and difficult to interpret. Presence of plasmid DNA in the reaction-mixture can sometimes interfere with the results. Recently several computer-based analysis methods have been developed that make comparison of REAC patterns easy and help to build up databases for identity searches and epidemiological typing.

2.2 Restriction endonuclease analysis of plasmid DNA (REAP)

This method can be used for those bacteria which harbor plasmids. Plasmids can be identified readily by alkaline lysis procedure followed by the agarose gel electrophoresis of the lysate (Tenover, 1985). The numbers and the sizes of the plasmids present are used as the basis of strain identification. This strain typing technique has been used successfully for analysis of outbreaks of nosocomial infections and community-acquired infections caused by various species of gram-negative bacteria (Schaberg *et al.*, 1981; Fornansini *et al.*, 1992). Some strains of bacteria carry only a single large plasmid, in the range of 100-150kb. Because of the difficulty to differentiate plasmids in this range, a restriction endonuclease digestion step is added to increase the discriminatory power of agarose gel electrophoresis. At present, this method is primarily used for staphylococcal isolates, which frequently carry multiple plasmids and for selected species of *Enterobacteriacea*, which often have large distinctive plasmids (Pfaller *et al.*, l99l).

2.3 Restriction fragment length polymorphism (RFLP)

This technique is based upon the polymorphic nature of restriction enzyme digestion sites within a defined genetic region. RFLP involves restriction digestion of the chromosomal DNA followed by southern blotting for detection of specific genetic loci. Whole chromosomal DNA is restriction digested, fragments are then separated on agarose-ge1 and transferred on to the nitrocellular / nylon membrane (Sambrook *et al.*, 1989). The DNA fragments immobilized on the membrane are then hybridized with one or more labeled probes. Only the genomic DNA fragments, which will have specificity for the probe, will be detected, simplifying the restriction endonuclease analysis greatly. This differentiates the strains as the position and length of target sequences will vary in the fragments of different isolates. RFLP has been used to subtype *Brucella* sp. (Grimont *et al.*, 1992), *Legionella pneumophila* (Tram *et al.*, l990), *Pseudomonas* (Loutit *et al.*, l99l), *Mycobacterium tuberculosis* (van Embden *et al.*, 1993). Ribotyping is also a form of RFLP typing in which the probe's target is in a multicopy operon i.e. the *rrn* operon.

Ribotyping is a particular form of RFLP- based typing in which probe's target sequence is the multicopy *rrn* operon. *rrn* operons are highly conserved and most bacteria have multiple copies of these, permitting both inter- and intra-species discrimination. Also intergenic spacer regions (the region present between 16S and 23S regions) are variable in length among different copies of the *rrn* operon and thus this property can also be exploited for typing. Use of multiple restriction enzymes provides additional discriminatory capacity to this technique. Strains differentiated by ribotyping are referred as 'ribotypes'.

Jorden and Leaves (1997) observed that the traditional ribotyping was most discriminatory to detect variations between strains of *H. influenzae*. Ribotyping is used for typing and subtyping of several microbes like *L. pneumophila* (van Belkum *et al.*, 1996), *Yersinia ruckeri* (Garcia *et al.*, 1998), *V. parahaemolyticus* (Marshall *et al.*, 1999) for epidemiological studies.

Ribotyping offers a good typing method and is highly useful for taxonomical and long term epidemiological studies. Ribotyping and other RFLP techniques generate interpretable banding patterns that are highly reproducible. However, this technique is time-consuming and requires considerable technical expertise. Recently an automated typing system, the Riboprinter (Qualicon. Welmington, DE, USA) has been designed for characterizing the bacteria to the strain level in 8 hours. However, 'riboprinter' has low discriminatory ability

and low cost-effectivity as compared to the traditional ribotyping hindering its usage in most laboratories

2.4 PCR-RFLP

PCR is an important tool in molecular biology for amplification of short sequences of DNA. PCR-RFLP does not require the cumbersome blotting technique used in RFLP. Moreover, in RFLP, the DNA used is in the methylated form due to *in vivo* modification by cellular methylases, which methylate adenine or cytosine, protecting it from the cells' own restriction enzymes. Thus, the RFLP analysis requires a careful choice of restriction endonuclease to avoid inhibitory effects of methylations in the DNA. Both these problems are overcome by PCR-RFLP. PCR-RFLP involves the use of specific primers to amplify specific genetic loci. Amplicons are then subjected to restriction endonuclease analysis with different enzymes and fingerprints are then compared. PCR-RFLP of polar flagellar genes (*fla* genes) is a valuable tool for typing bacteria like *Campylobacter* (Owen *et al.*, 1994) and *Vibrio parahaemolyticus* (Marshall *et al.,* 1999). Also, locus specific RFLP of *kat*G gene of *Mycobacterium tuberculosis* is used to index point mutations corresponding to various levels of resistance to the anti-tubercular drug isoniazid (Cockerilli *et al.*, 1995).

Another version of PCR-RFLP is PCR-ribotyping, where the specific locus used for amplification is intergenic spacer region of ribosomal DNA. PCR-ribotyping has been used for the characterization of different serotypes of *Salmonella* (Lagatolla *et al.*, l996), *H. influenzae* (Jorden and Leaves, 1997), *Pseudomonas cepacia* (Kostman *et al.*, 1992) and *Yersinia enterocolitica* (Lobato *et al.*, 1998). PCR targeted to intergenic spacer region of 16S-23S rDNA of *Clostridium difficile* was used for construction of reference library (Stubbs *et al.*, 1999). A modification of PCR-ribotyping is ARDRA (amplified ribosomal DNA restriction analysis), in which 16S rRNA gene of *rrn* operon is amplified and digested with restriction endonuclease. The molecular identification and cluster analysis of thermophilic Lactobacilli has been carried out by comparison of their ARDRA patterns (Andrighetto *et al.*, 1998). Combining several polymorphic genes and analyzing their restriction patterns simultaneously can enhance the discriminatory power of PCR-RFLP. Multiplex PCR-RFLP analysis has a discriminatory power comparable to Pulse field gel electrophoresis (PFGE), which is described in the sections to follow. Such a multiplex PCR was developed for *Campylobacter jejuni* using polymorphic genes *gyr*A, *pfl*A and *fla* (Ragimberbeau *et al.*, 1998).

2.5 Random amplified polymorphic DNA (RAPD)

This technique is based on arbitrary amplification of polymorphic DNA sequences. Amplification is carried out using single or multiple, non-specific primers whose sequences are random and not designed to be complementary to any particular site in the chromosome. These primers bind at various 'best-fit' sequences on the denatured DNA under low stringency conditions and extend efficiently to give short amplicons. In subsequent cycling, conditions are made more stringent so that primers continue to bind to best-fit sequences and generate products of fixed lengths. Their (products) electrophoresis and staining produces the fingerprint. RAPD has the main advantage that no prior sequence information is required. Moreover, the entire genomic sequence is explored for comparison. Since the primers are not directed against any particular genetic locus, several priming events can result from variations in experimental conditions, making rigorous standardization of the method essential. The major disadvantage of this method is lack of inter-laboratory reproducibility. A little change in protocol, primers, polymerase or DNA extraction may give different results.

RAPD has been used for typing of a number of bacteria using 10-mer primers (oligonucleotides consisting of 10 nucleotides). It was carried out for *Campylobacter coli* and *C. jejuni* (Madden *et al.,* 1996), *Listeria monocytogenes* (Czajka *et al.,* 1993), *Staphylococcus haemolyticus* (Young *et al.*, 1994), *Vibrio vulnificus* (Warner and Oliver, 1999). In *V. vulnificus* typing, discriminatory power of RAPD was highlighted; a difference in band patterns was obtained between encapsulated and non-encapsulated isogenic morphotypes.

Another version of RAPD is AP-PCR i.e. arbitrarily primed PCR in which PCR is carried out with arbitrary primers. Here, PCR is carried out using >20-mer primers instead of 10-mer (RAPD). Other details of the methodology remain similar (Welsh and McClelland, 1990; Williams *et al.,* 1990, Welsh and McClelland, 1993). Recently, to promote reliability and reproducibility of arbitrarily primed PCR, various procedures have been recommended by Tyler *et al.* (1997).

Direct amplification fingerprinting (DAF) is another method based upon amplification of DNA using arbitrary primers. Here the arbitrary primers used are very short (5-8 bases in length). This is not frequently used because this method gives complicated fingerprints consisting of a large number of bands, which could only be distinguished by silver staining technique (not by ethidium bromide/fluorescence). Moreover, separation of these small fragments requires the use of polyacrylamide (instead of agarose) gel.

2.6 Repetitive sequence-based PCR (rep-PCR)

rep-PCR genomic fingerprinting is a DNA amplification-based technique involving repetitive DNA elements present within bacterial genome (Versalovic *et al.*, 1991). Repetitive DNA sequences are universally present in eubacteria and have been applied to fingerprinting of bacterial genomes. Three main types of repetitive sequence used for typing purposes are repetitive extragenic palindromic sequences (REP elements), enterobacterial repetitive intergenic consensus (ERIC) sequence and BOX elements. All

these motifs are genetically stable and differ only in their number and chromosomal locations between species, permitting differentiation of bacterial isolates to species, subspecies and strain levels. REP elements are 33-40 bp sequences consisting of a conserved palindromic stem, 5-bp variable loop and six degenerate positions (Stern *et al.*, 1984). ERIC sequences are 124-127 bp elements containing a highly conserved central inverted repeat, located in extragenic regions of various enterobacteria in approximately 30-150 copies (Hulton *et al.*, 1991; Sharples *et al.*, 1990). They were first identified in *Salmonella typhi* and *E. coli* and constitute approximately 1% of the bacterial genome. Although REP and ERIC sequences are most widely used targets in gram negative bacteria, however gram-positive equivalents of these elements are BOX sequences. BOX elements are intergenic sequences of 154 bp each present in approximately 25 copies. These sequences appear to be located in distinct intergenic positions around the genome. The repetitive elements may be present in both the orientations and oligonucleotide primers have been designed to prime DNA synthesis outward from the inverted repeats in REP and ERIC and from the Box A subunit of BOX.

Rep-PCR genomic fingerprinting is becoming a method of choice for bacterial typing. Rep-PCR has been applied to differentiate strains of *L. monocytogenes* (Jersek *et al.,* 1999), *V. parahaemolyticus* (Marshall *et al.*, 1999), *Acinetobacter baumanii* (Reboli *et al.*, 1994), *Burkholderia cepacia* (Hamill *et al.*, 1995), *Citrobacter diversus* (Woods *et al.*, 1992), and *Rhizobium meliloti* (De Bruijn, 1992). ERIC-PCR is used for typing *Haemophilus somnus* (Appuhamy *et al.,* 1997), viridans Streptococci (Alam *et al.,* 1999) and *Vibrio parahaemolyticus* (Marshall *et al.,* 1999). Rep- PCR was used to study the role of genetic rearrangement in adaptive evolution. One distinct advantage of rep-PCR is its broader species applicability. Moreover this technique has a good discriminatory power which can be further increased by the use of multiple sets of primers.

2.7 Pulsed field gel electrophoresis (PFGE)

Schwartz and Cantor (1984) first described PFGE for examining yeast chromosomal DNA. Subsequently, it has been applied to a number of bacterial species and because of its reproducibility and discriminatory power, PFGE is considered as the 'Gold standard' of molecular typing techniques. PFGE involves extraction of the genomic DNA from bacterial cells immobilized into agarose plugs. Within these plugs the bacterial cells are lysed to extract DNA and subjected to digestion with rare-cutting restriction endonucleases like *Xba*I, *Not*I. *Spe*I whose recognition sites are infrequent. The digested bacterial plugs are then subjected to agarose gel electrophoresis in a highly specialized apparatus, in which both the orientation and the duration of the electric field are periodically changed during electrophoresis. 'Pulse time' or 'Switch time'- the length of time for which the current is applied in each direction, determines the range of sizes of molecules that can be separated. Short pulses separate small molecules while long pulses separate larger DNA molecules. The Gel is then stained with ethidium bromide to detect the electrophoretic pattern. The pulse-field allows large DNA fragments to orient and reorient themselves resulting in better resolution. PFGE allows the separation of DNA fragments ranging from 10 to 800 Kb. PFGE fingerprints are highly reproducible and their interpretation is relatively straight-forward. However, when several isolates are used interpretation becomes time-consuming necessitating the use of computerized gel analysis softwares.

Tenover *et al.* (1995) gave criteria to interpret the PFGE pattern: if strains give identical pattern of fingerprint, they are considered as identical strains. If patterns differ by 1 to 3 bands, the strains are considered closely related and differ by a single genetic event. If the difference is of 4-6 bands, the strains are possibly related and their genomes differing by 2 independent genetic events. Strains are unrelated if their patterns differ by >6 bands.

PFGE is highly sensitive technique and is used for differentiating a wide range of bacteria. These include *Yersinia enrerocolitica* (Najdenski *et al.,* 1994), methicillin-resistant *S. aureus* (MRSA) (Schmitz *et al.*, 1998), *Shigella dysenteriae* type 1 (Talukder *et al.*, 1999), *Burkholderia cepacia* (formerly known as *Pseudomonas cepacia*) (Kumar *et al.,* 1997), *Vibrio parahaemolyticus* (Marshall *et al.,* 1999), *Serratia marcescens* (Shi *et al.,* 1997), and *Neisseria meningitidis* (Yakubu *et al.*,1995). Contour-clamped homogeneous electric field electrophoresis (CHEF), is one form of PFGE in which hexagonal chamber with electrodes is used. This version of PFGE has been used for typing *Neisseria gonorrhoea* (Pou and Lau, 1993).

Although very sensitive, PFGE is a time consuming (2-3 days) technique. It is also susceptible to endogenous nucleases that can degrade target DNA. Isolation of DNA requires expertise as shearing forces can cause disruption and give artefactual fragments. The equipment for electrophoresis is very costly and out of the reach of most laboratories. However, in the future it will be practised in many laboratories, as cheaper versions of the equipment become available.

2.8 Amplified fragment length polymorphism (AFLP)

Vos *et al.* (1995) described a method of fingerprinting bacterial genomes based on the PCR amplification of a selective subset of genomic restriction fragments. For AFLP analysis, a small amount of purified genomic DNA is digested with two different restriction enzymes, one is a frequent cutter such as *Eco*R1, *Hind*III and other a rare cutter (like *Mse*I or *Taq*I). The restriction fragments are then ligated with linkers or adapters, possessing a sequence corresponding to the recognition site of the restriction enzyme and a point mutation such that the initial restriction site is not restored after ligation. Ligated fragments are then subjected to PCR amplification under high stringency conditions with two primers.

Each primer is complementary with one or other adapter molecules. Also the 3' end of each of the primer extends to one to three nucleotides beyond the restriction site, resulting in selective amplification of only those restriction fragments that contain nucleotides complementary to selective 3' nucleotide of the primer. One of the PCR primers contain either a radioactive (Janssen *et al.*, 1996; 1997) or fluorescent (Koeleman *et al.*, 1998) label giving rise to a detectable fingerprint, or alternatively, the gels may be stained with ethidium bromide and examined under UV 1ight (Gibson *et al.*, 1998). Typically fingerprints contain 50-100 amplified restriction fragments.

Although, AFLP technique is a random fingerprinting technique, its reproducibility, robustness and universal applicability make it a promising technique in future. The differentiation power of AFLP appears to be greater than other random fingerprinting techniques (Janssen *et al.*, 1997; Koeleman *et al.*, 1998). Recently employed fluorescent labeled AFLP called FAFLP appears to have great potentials for high-resolution epidemiological typing and phylogenetic analysis (Arnold *et al.*, 1999, Goulding *et al.*, 2000, Ahmed *et al.*, 2002), but the cost of equipments makes its use prohibitive for most laboratories.

Further detailed principle, applications, advantages and disadvantages of AFLP are summarized by Savelkoul *et al.* (1999).

2.9 Single-strand conformational polymorphism (PCR-SSCP)

Single-strand conformation polymorphism refers to differences in the conformation between (related) strands of nucleic acids due to the differences in their intra-strand base pairing. Such differences may arise due to mutation in even a single base. SSCP may be detected as difference in the mobility when conformationally diverse strands are subjected to gel electrophoresis. It is used to examine homologous sequences of nucleotides from different strains of an organism. 100-300 bp sequence from each strain is amplified by PCR, denatured to single stranded state and electrophoresed in a non-denaturing gel which promotes/preserves intra-strand base-pairing. It was used to detect strains of *M. tuberculosis* having mutant *rpo*B gene, which confers resistance to rifampicin (Kim *et al.,* 1997). PCR-SSCP is also used for subtyping of *Neisseria meningitidis* using a variable sequence (VR1) in the *por*A gene (Newcombe *et al.*, 1997).

2.10 Multilocus sequence typing (MLST)

MLST is based on the direct sequencing of ~500 nucleotides of a number of housekeeping genes. The sequence of each of these gene fragments is considered as a unique allele, and dendrograms are constructed from the pairwise difference in the multilocus allelic profiles by cluster analysis. As this method indexes the variations that accumulate very slowly, MLST is a suitable method to study long term and global epidemiology or evolution of microbes. Also, MLST is well suited for the construction of global databanks assessable to different laboratories for result comparison and classification (Maiden *et al.,* 1998). Recently MLST has become a gold standard technique for classification of *Neisseria meningitidis* (Maiden *et al.*, 1998). MLST has also been established for *Streptococcus pneumoniae* (Enright and Spratt, 1998; Enright *et al.,* 1999) and is in progress for *Streptococcus pyogenes*, *Haemophilus influenzae* and *Campylobacter jejuni*. Recently Cocolin *et al.* (2000) have used a similar technique for identifying 39 strains of *Lactobacillus* species isolated from naturally fermented Italian sausages. A small fragment from 16S rRNA was amplified via PCR. Tannock *et al.* (1999) identified *Lactobacillus* isolates from gastrointestinal tract, silage and yoghurt by amplifying and sequencing spacer region between 16S and 23S rRNA genes (The sequences obtained were compared with the reference strains in databases such as Genbank and a similarity of 97.5% or more was considered to provide identification). The 16S-23S intergenic spacer region sequencing has been used for identification of *Clostridium difficile* and *Staphylococcus aureus* (Gutler and Stanisich, 1996). Although expensive and labor-intensive, none of the DNA fingerprinting technique described above is as reliable or as reproducible as MLST. Once the cost and the difficulty of the large scale sequencing have been reduced by technological developments, MLST will become the method of choice in many laboratories around the globe.

3. Whole Genome Sequencing

Whole genome sequencing would be the most sensitive method for distinguishing microbes unequivocally. The nucleotide sequence is determined with an automated sequencing instrument. DNA sequencing instruments are based on the modification of dideoxynucleotide chain terminator chemistry in which the sequencing primer is labeled at 5' end with one of the four fluorescent dyes. Each of the fluorescent dye represents one of the four nucleotides and thus four different annealing and extension reactions are performed. Finally, the four sets of reactions are combined, concentrated and loaded in a single well on a polyacrylamide gel. During electrophoresis the fluorescently labeled products are excited and the corresponding signal is automatically detected. Subsequently, the resulting data is processed into a final sequence with the aid of computer software. Although regarded as the ultimate technique for identification, DNA sequencing is highly expensive and requires a high degree of technical competency. Thus, in future sequencing methods need to be simplified and automated further for their extensive applicability. Currently the genomes of approximately 200 microbes have been sequenced completely.

4. Computer Assisted Analysis

Most of the DNA fingerprints obtained by the above-described methods (except sequencing technique) can be compared primarily by visual inspection. However, recent efforts to compare a large number of patterns generated in the same laboratory or different laboratories have led to the development of several computer-assisted analysis methods. Some popular computer-based fingerprint analysis programs commercially available for this purpose are Gel Compar TI (Applied Maths, Kortrjk, Belgium), Bionumeric, Molecular Analyst Fingerprinting plus (Biorad Laboratories, Hercu1es, CA), Whole Band Analysis (BioImage, Ann Arbor, MI) and Dendron (Solltech, Inc., Oakdale, IA). These systems have some basic functions in common to all of them. Each system allows gels, photographs and autoradiographic images to be scanned and stored as digitized files. Bands in each lane can be detected automatically. However, there is a flexibility to add, delete or move bands, after visual inspection. Within gels and between gel band matching can be performed. Also, with these programs it is possible to build databases of the fingerprint patterns and carry out identity searches. In addition, these software packages can perform sophisticated similarity calculations such as Jaccards and Dice coefficients and can construct dendograms from cluster analysis like UPGMA (unpaired weight grouping method using arithmetic averages) of the fingerprints stored in their databases.

5. Conclusion

From the foregoing account, it is clear that in the last decade or so, several DNA-based fingerprinting techniques have been developed to assist in the identification and characterization of the microbes. Although the conventional phenotypic methods like serotyping would continue to be used for several years to come, molecular techniques will be increasingly used in the future. Further research on the methodologies of DNA fingerprinting methods would reveal the pitfalls to which these are prone and would certainly be refined, making them more robust and applicable in most of the laboratories of the world. Several of these methods will then enable creation of large reference libraries or databases of the typed organisms for comparison, quick identification, characterization and classification of new isolates across the world.

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