**Review**

**Insight into Phenotypic and Genotypic Discrimination of Bacterial Pathogens: From Pre-Genomic to Post-Genomic Era**

Pushpanathan Muthuirulan*

*Laboratory of Gene Regulation and Development, National Institutes of Child Health and Human Development, National Institutes of Health, Bethesda, Maryland 20892, USA

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**Abstract**

Bacterial genomes are highly compact and distinct in architecture compared to eukaryotic genomes. The ability to discriminate between pathogenic and non-pathogenic bacterial genomes has greatly facilitated the process of antibacterial therapy. The traditional methods of discriminating bacteria are based on phenotypic descriptions such as bacterial morphology and biochemical properties that imposed several limitations due to enormous biochemical diversity associated with bacterial metabolism and cell structure. In recent years, many different techniques have been developed as attractive alternatives to conventional approaches for identifying and discriminating bacteria employing microbiological methods. The advent of new technologies in ‘genomics’ based on elucidation of specific nucleotide sequences of genome have produced unprecedented levels of discrimination among different strains of pathogenic and non-pathogenic bacteria. The genome based characterization of bacteria using PCR and Next-Generation sequencing technologies have opened up a new avenue to efficiently discriminate and read the bacterial genomes in more rapid and accurate way, which had greater impact on the taxonomy and systematic of bacteria with respect to clinical diagnosis, public health and environmental monitoring of biological threat agent. These modern methods of discriminating bacteria are highly promising and provide most reliable and taxonomically relevant molecular information that serves a gold standard for rapid and sensitive detection of emerging bacterial pathogens.

**Keywords**: Bacterial Pathogen; Genome; Phenotypic Characterization; Genotypic Characterization; PCR; Next-Generation Sequencing

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**Introduction**

Infectious diseases transmitted by bacterial pathogen are one of the most important causes of human mortality worldwide [1]. Although most bacteria are harmless or often beneficial, several are opportunistic pathogens that may cause life-threatening diseases to patients with immunocompromised conditions [2]. Therefore, the ability to discriminate bacterial pathogens from other beneficial groups of bacteria is essentially important for rapid development of antibacterial agents to prevent epidemiology of bacterial infections. The genotype-phenotype distinction is the fundamentals to discriminate between pathogenic and non-pathogenic group of bacteria (Figure 1). In recent year, genome based characterization of microorganism is widely used in several disciplines of microbiological research including microbial epidemiology, microbial taxonomy, microbial evolution, phylogenetic relationships and population genetics of microorganisms [3]. Due to rapid increase in number of bacterial disease worldwide, the more accurate and efficient diagnostic methods are highly warranted to optimize clinical management of disease in an infected patient. However, the traditional diagnosis of infectious diseases has long been culturing bacteria in their growth-supporting media followed by the isolation, identification and antibiotic-susceptibility testing of the infectious agents. The different types of traditional bacterial typing systems include serotyping, biotyping, phage typing, antibiogram, multi locus enzyme electrophoresis (MLEE) and two-dimensional polyacrylamide gel electrophoresis. These methods have long been used as the gold standard for rapid and sensitive detection of emerging bacterial pathogens, but they are time-consuming and labor-intensive.

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(*Corresponding author: Pushpanathan Muthuirulan, Laboratory of Gene Regulation and Development, National Institutes of Child Health and Human Development, National Institutes of Health, Bethesda, Maryland 20892, USA, Tel: +1301-674-3108; E-mail: pushpanathan31@gmail.com

Sub Date: May 2, 2016, Acc Date: May 19, 2016, Pub Date: May 20, 2016.

Citation: Pushpanathan Muthuirulan (2016) Insight into Phenotypic and Genotypic Discrimination of Bacterial Pathogens: From Pre-Genomic to Post-Genomic Era. BAOJ Med Nursing 2: 018.

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diagnostic methods are highly expensive, time consuming, less reproducible and have lesser efficiency to discriminate bacterial pathogens, due to extreme enormous physiological factors and biochemical diversity associated with different bacterial species. Recent advancement of genomic technologies have resulted in the development of diagnostic tools that helped to improve the efficiency of discriminating bacterial infectious agents through detection of specific nucleotide sequence of DNA [4]. Expanding number of genomic sequences in the databases have also revolutionized the field of infectious diseases that enable researchers to design molecular strategies to detect genomic targets of the bacterial pathogen, which had great potential impact on the diagnosis and prevention of bacterial disease.

The PCR is the most sensitive and rapid methods of detecting bacterial pathogens in clinical specimens. PCR has extreme diagnostic value during certain circumstances where the specific pathogens are extremely difficult to culture in growth media in vitro or it's require a long time cultivation period. PCR detection of bacterial pathogen has been performed with different clinical specimens, such as blood, urine, sputum, cerebrospinal fluid (CSF). However, sensitivity of pathogen detection by PCR requires critical genomic exploration for careful design of primers to target sequences, optimization of DNA extraction procedures and PCR conditions [5]. Besides PCR based diagnosis method, quantitative DNA hybridization based techniques were used to detect bacterial pathogens, however, this method is not completely reliable and is often labor intensive and time consuming. Later, genome fingerprints-based diagnostic methods were proposed as attractive alternatives to phenotype based bacterial discrimination methods [6,7]. The genome fingerprints based diagnostic procedures involved amplification of interspersed repetitive DNA sequences present in bacterial genomes, referred to as rep-PCR [7] or amplification of random sequences by arbitrary primers, RAPD [8] or by amplification of BOX repetitive element, BOX-PCR [9] or by amplification of enterobacterial repetitive intergenic consensus (ERIC) repeated DNA sequences [10]. Other PCR-based genome fingerprint techniques include arbitrarily primed PCR (AP-PCR) and amplified fragment length polymorphism (AFLP) [11,12]. In addition, Real-Time-PCR also greatly improved the specificity and sensitivity of discriminating bacterial pathogens [13].

Development and recent advancement of Next-Generation sequencing (NGS) technologies combined with bioinformatics have revolutionized the field of genomics and afford ways for discrimination bacterial pathogens more rapidly and accurately at lower cost [4]. The advent of NGS has led to increase in the number of bacterial genomes in databases that are readily available to public for genomic exploration. There are patients with many common clinical syndromes, in which bacteria are suspected to play a role, but the extensive conventional diagnostic procedures were unable to identify those bacterial pathogens. However, NGS can serve as an effective and promising tool in diagnosing pathogens for medical and veterinary purposes [14]. In recent years, high-throughput NGS technologies coupled with metagenomic approaches have resulted in identification of enormous bacterial pathogens directly from the infected samples, bypassing the need for cultivation of infectious agent [15,16]. This paper review the relevant information on the phenotype and genotype based bacterial discrimination methods, which will provide conceptual understanding of most reliable and promising tools that can serve as gold standard techniques for discriminating emerging bacterial pathogens.

**Epidemiological Typing of Bacterial Pathogen by Phenotypic Methods**

Phenotype-based bacterial typing techniques detect mainly the specific characteristics expressed by bacteria. It includes properties such as cell size, shape, staining, biochemical and antigenic properties, which can be measured without the need of genome information (Figure 2).

**Serotyping**

Serotype or serovar are distinct variations within a species of microorganisms. Bacterial serotyping is the discrimination of bacterial species based on their cell surface structures such as lipopolysaccharides, membrane proteins, capsular polysaccharides,
flagella and fimbriae that exhibit antigenic variations. Serotyping is performed using several serologic tests such as bacterial agglutination, latex agglutination, co-agglutination, fluorescent and enzyme labelling assays that allows rapid epidemiologic classification of bacteria and often plays an essential role in determining bacteria even at the subspecies level [17]. Serogroup is a group of serovars with common antigens. For instance, Salmonella bacteria looks similar under the microscope, but can be discriminated into many serotypes based on two surface structures: (a) The outermost portion of the bacteria’s surface covering, called O antigen; and (b) a slender thread-like structure in flagella, called H antigen. The O antigens are distinguished by difference in their chemical composition. The H antigens are distinguished by the protein content of the flagella. Each O and H antigen has a unique code number. A total of 2600 serotypes have been determined from the genus, Salmonella, including Salmonella enterica serovar Typhimurium, S. enterica serovar Typhi, and S. enterica serovar Dublin [17]. Vibrio cholerae is the species of bacterial pathogen that causes cholera. This species has over 200 serotypes discriminated mainly based on its cell surface antigens. Among these serotypes, only two (0:139 and 0:1) of them have been known to produce potent enterotoxin that causes cholera. The serogroup O:139 is identified by (a) absence of agglutination in O group 1 specific antiserum; (b) by agglutination in O group 139 specific antiserum; and (c) by the presence of a capsule [18]. V. cholerae O:1 exists as two major serotypes, Inaba and Ogawa, which are associated with the O antigen of the lipopolysaccharide and are extremely capable of unequal reciprocal interconversion [19]. However, serotyping methods are used in typing of Gram-negative and Gram-positive bacteria; it has several disadvantages including low throughput, high expense, poor discriminatory power due to existence of large number of serotypes, cross reactivity of antigens, untypeable nature of some bacterial strains. It also requires considerable expertise and development of numerous antibodies by immunizing rabbits [20].

Biotyping

Bacterial biotyping methods make use of pattern of metabolic activities expressed by bacteria, colonial morphology and environmental tolerances. Biotyping methods are highly useful in epidemiological studies and appropriate for screening of bacterial strains. A few examples of biotyping methods include sugar fermentation, amino acid decarboxylation/deamination, standard enzymatic tests (such as IMViC, Citrate, urease), pH tolerance, chemicals and dyes, haemagglutination and hemolysis. Acinetobacter baumannii is the most commonly reported species from clinical sources. Biotyping of these species based on protein profile led to identification of nine biotypes of A. baumannii strains [21]. Yersinia enterocolitica is a diverse species subdivided into two subspecies, Y. enterocolitica ssp. enterocolitica and Y. enterocolitica ssp. Palaearctica. Further subtyping of these species by biotyping method had led to identification of six biotypes of Y. enterocolitica including 1A, 1B, 2-5. Biotype 1B has been associated with higher pathogenicity and biotypes 2-5 with low pathogenicity. However, strain 1A is usually non-pathogenic, but in rare cases it serves as an opportunistic pathogen [22]. Fallon (1973) [23] have classified 209 strains of Klebsiella isolated from sputum and studied their relationship with pathogenicity. Biotyping methods are highly reproducible and ease in performance. However, these methods have lesser discriminating power mainly associated with variations in gene expression of isolates that impose strains to be differing in one or more biochemical reactions.

Bacteriophage Typing

Bacteriophage typing is widely used in epidemiological studies to detect single strains of bacteria. It is also used to identify different strains of bacteria within a single species [24]. In phage typing, the strains are classified based on their pattern of resistance or susceptibility to a standard set of bacteriophages. This strategy mainly relies on the presence or absence of specific bacterial cell surface receptor that are used by viruses to bind and infect bacterial species. This method has been successfully employed to type isolates such as Staphylococcus aureus, V. cholera, Listeria monocytogenes, Campylobacter spp., Mycobacterium tuberculosis and Salmonella spp. [24,25,26,27,28,29]. The bacterial strains typed using phages are often referred as ‘phage types’. Phage typing method has greater reproducibility, high discriminating power and ease of interpretation. The major limitations associated with this method are maintenance of biologically active phages and the demand for technical expertise to handle phages [30].

Antibiogram

Antibiogram involves sensitivity profiling of a bacterial strain to different antibiotics. The bacterial strains differing in their susceptibilities to antibiotics are considered as different strains. The quantitative antibiogram is a valuable epidemiological tool for typing of bacterial pathogens. It is a simple, rapid and readily available method suitable for the prospective surveillance of bacterial pathogens [31]. This method is highly reproducible and ease for performance. Almost all species of bacterial strains can be typed using this method. The major limitation associated with this method is the resistance development by bacterial strains to one or more class of antibiotics that may lead to false discrimination between bacterial species. In other cases, as consequences of different genetic mechanisms, the bacterial strains may develop similar resistance pattern that may reduce its discriminating efficacy.

Multilocus Enzyme Electrophoresis (MLEE)

Multilocus enzyme electrophoresis (MLEE) is the method of characterizing organisms based on the difference in electrophoretic mobility of large sets of intracellular metabolic enzymes. These differences in mobility are directly related to mutation at gene locus that induces amino acid substitution in the enzyme coded by the gene. The mobility variants generated by mutations are called as electromorphs. The unique profile produced for each electromorphs is designated as electromorphs type (ET) [32]. MLEE has been successfully used to type bacterial pathogens such as L. monocytogenes, S. aureus, C. jejuni, Streptococcus mutans [33, 34, 35, 36, 37]. MLEE is highly reproducible and can be used to type almost strains of bacteria. However, this method provides only an estimate of overall genetic relatedness and diversity. It has
moderate discriminatory power for epidemiological classification of bacterial pathogens [38].

**Two-Dimensional Polyacrylamide Gel Electrophoresis (2-DE)**

Two-dimensional polyacrylamide gel electrophoresis (2-DE) is a gel-based technique used for large scale analysis of proteome in an organism. It is capable of resolving highly complex mixture of proteins into individual protein spots through combination of two orthogonal biophysical separation techniques such as isoelectric focusing (first dimension) and polyacrylamide gel electrophoresis (second dimension). 2-DE is highly suited for analyzing the entire proteome of a bacterial cell. The relative simplicity and good reproducibility of 2-DE have made it a widely used tool for proteomics exploration within a wide range of medically-relevant bacteria [39]. 2-DE was extensively used to discriminate bacterial isolates, which is critical in the study of the molecular taxonomy and epidemiology of bacterial pathogens [40]. The bacterial isolates typed using 2-DE includes *S. porcinus*, *Bacillus thuringiensis*, *Helicobacter pylori*, *Haemophilus influenza*, *Pseudomonas aeruginosa* and *S. pneumoniae* [41, 42, 43, 44]. The major drawback associated with 2-DE is that, it mostly detects proteins expressed at higher concentrations. Nevertheless, several regulatory proteins are present in the cell at very low concentrations [45]. This drawback can be overcome by development of sensitive high-throughput methods for accurate protein detection and quantification.

**Epidemiological Typing of Bacterial Pathogen by Genotypic Methods**

Genotype–based bacterial typing techniques are mainly based on the composition, homology and presence or absence of specific nucleotide sequences of bacterial genome (Figure 2). The genotypic methods can also be performed without any specific knowledge of the genome sequences of target organisms.

**16S-23S Intergenic Spacer-PCR (ITS-PCR)**

The rRNA genes (16S, 23S, and 5S) are ideal candidates for bacterial identification, because they are highly conserved within the species. The major disadvantage of rRNA gene sequences is that these “variable” regions are not sensitive enough to allow clear discrimination of closely related bacterial species. The internal transcribed spacer (ITS) is also known as ISR present between 16S and 23S rDNA region of ribosomal genes. The arrangement of complete unit of ribosomal genes such as 16S-ITS-23S-ITS-5S are scattered in the genome of bacteria that varies from 1 - 15 copy numbers. The 16S-23S rRNA gene ITS sequences are not subject to the same selective pressure as the rRNA genes and consequently have a 10-times greater evolution rate that can overcome the apparent limitation of rRNA genes. Sequence and length polymorphisms found in the ITS are increasingly being used as tools for bacterial species/subspecies identification and evolutionary studies [46]. The PCR primers targeting ITS regions of a wide range of bacteria have been designed in such a way that the 5’ primer corresponds to a conserved sequence motif from the 3’ end of 16S rRNA and the 3’ primer corresponds 5’ end of the 23S rRNA. The differences among the length of the PCR amplified product itself can be used for the recognition of bacterial genera and species [47]. 16S-23S rDNA ITS PCR has been used in typing of bacterial isolates such as *Staphylococcus* sp., *Streptococcus* sp., *P. putreri*, *Abiotrophia*, *Enterococcus*, *Gramicidicella*, *Frankia* sp. *Pasteurella pneumotropica* [48,49,50,51,52,53]. The potential limitations associated with this method is due its specificity highly depends on the primer design. Non-specific amplification of contaminant bacterial DNA could result in a misdiagnosis of bacterial pathogen that may lead to misinterpretation of results [54].

**Plasmid analysis**

Plasmids are circular or extrachromosomal self-replicating structure present within bacterial cells. Plasmids can confer antibiotic resistance or virulence mechanism to bacterial cells, whose study is highly essential in the field of medical microbiology. ‘Plasmids profiling’ or ‘plasmid fingerprinting’ methods uses plasmid molecules as marker to type various bacterial strains. Plasmid profiling can be performed by two procedures. In first procedure, the partially purified plasmid DNA species are separated on agarose gel based on their molecular size. In a second procedure, plasmid DNA are subjected to cleavage by restriction endonucleases followed by electrophoretic separation and fingerprint analysis based on pattern of restricted fragments to study the identity of bacterial isolates. However, most species of bacteria contain plasmids; plasmid profiling method has been used to investigate epidemiologic surveillance of disease outbreaks and in tracing the transmission of antibiotic resistance [55]. Plasmid profiling has been used in epidemiological survey or strain differentiation of bacteria isolates such as *Shigella*, *Clostridium perfringens*, *Vibrio* spp, *S. enterica*, *P. aeruginosa* [56,57,58,59,60,61]. This method has less reproducibility due to existence of plasmid in different molecular forms (such as supercoiled, nicked or linear) and difference in their migration on electrophoresis. In addition, bacterial cells can lose their plasmid DNA spontaneously or can acquired readily from other species suggesting that related strains can exhibit different plasmid profiles that would affect the discriminatory power of this typing method.

**Pulse Field Gel Electrophoresis**

Pulse Field Gel Electrophoresis (PFGE) is a gel based technique used to generate a DNA fingerprint for a bacterial isolate. PFGE make use of molecular scissors, called restriction enzymes, to generate a small number of DNA pieces from bacterial genome that can be separated based on size. These DNA pieces or restriction fragments are large enough to generate a DNA fingerprint. PFGE subtyping has been successfully applied to subtyping of wide variety of pathogenic bacteria and has high concordance with epidemiologic relatedness. This method has been shown to have greater discriminating power than other methods such as ribotyping or multi-locus sequence typing. The DNA fingerprints generated by PFGE are highly stable and reproducible [63]. Most species of bacteria can be typed using PFGE and the only concern with this method is the choice of restriction enzyme and optimization of electrophoresis condition.
for all species. The bacterial species successfully typed using PFGE includes *C. jejuni*, *Listeria* spp., *Salmonella enterica*, *C. difficile*, *S. aureus* and *Cronobacter* sp. [63,64,65,66,67,68]. PFGE methods are highly time consuming, requires skilled technician, does not discriminate between unrelated isolates and also certain strains cannot be typed by PFGE [69].

**Random Amplified Polymorphic DNA (RAPD) and Arbitrarily Primed (AP)- PCR**

RAPD markers are DNA fragments from PCR amplification of random segment of genomic DNA with single primer of arbitrary nucleotide sequence. Unlike traditional PCR, RAPD does not require any specific knowledge of the genome or DNA sequences of target organisms. In RAPD, the identical 10-mer arbitrary primers may or may not amplify a segment of DNA, depending on nucleotide position that is complementary to the primer’s sequence. For instance, no PCR amplified fragment is produced if primers annealed too far apart or 3’ ends of the primers are not facing each other. Therefore, if a variation has occurred in the template DNA at the site that was previously complementary to the primer, a PCR product will not be produced, resulting in a different pattern of amplified DNA segments on the gel. RAPD is an inexpensive yet powerful and widely recognized method for typing most bacterial species [70]. Developing locus-specific marker from polymorphic RAPD maker band isolated from gel is called as sequenced Characterized Amplified Region (SCAR) marker. RAPD technique has been successfully used to type bacterial isolates such as *Listeria* spp., *Lactobacillus* spp., *Enterococcus* spp. *S. thermophilus*, *Serratia Marcescens*, *Klebsiella pneumoniae*, *S. aureus* [71, 72, 73, 74, 75]. The factor that greatly influence the outcome of RAPD includes, the quality and concentration of template DNA, concentrations of PCR components, and the PCR cycling conditions. RAPD technique is highly laboratory dependent and needs careful and critical development of laboratory protocols to achieve greater reproducibility [76].

AP-PCR is a technique for producing species-specific DNA fingerprints. AP-PCR amplifies fragments of DNA from genome, using single non-specific arbitrary primer. Arbitrary primers are degenerate primers that hybridize randomly with genomic DNA.
and generate DNA fingerprints that are specific to particular species. The AP-PCR procedure involves two cycles of stringency amplification followed by PCR at high stringency conditions. If the DNA fingerprints produced by AP-PCR for two different genomes are not similar, the two genomes are likely to have come from two different species. The universal primers (M13R, T7 and T3) most often used for DNA sequencing can be also used as arbitrary primers for AP-PCR. Use of individual or combination of primers gives different pattern of AP-PCR products, each with the potentials of detecting polymorphisms between different strains. AP-PCR with use of single primers at three different annealing temperatures simultaneously in a triplicate reaction would allow identification of bands that are sensitive to small changes. This variant of AP-PCR is designated as triplicate AP-PCR (TAP-PCR). DNA amplification fingerprint (DAF) in another variant of AP-PCR that uses arbitrary primers of length 7-8 nucleotides long and it requires primer/template ratio greater than 5, whereas RAPD requires ratio less than or equal to 2 and AP-PCR primer/template ratio fall in between the range for DAF and RAPD. The advantages and limitations of AP-PCR and DAF are identical to RAPD [77].

**Repetitive Sequence-Based PCR (Rep-PCR)**

Various classes of repeated DNA sequences have been characterized in diverse prokaryotic genomes. Repetitive element sequence-based PCR (rep-PCR) is a typing method that differentiates bacteria employing primers complementary to interspersed repetitive consensus sequences that enable amplification of diverse-sized DNA fragments consisting of sequences between the repetitive elements [78]. REP elements are repetitive elements with 38-bp sequences consisting of six degenerate positions and a 5-bp variable loop between each side of a conserved palindromic stem. ERIC sequences are repetitive elements with 126-bp elements consisting a highly conserved central inverted repeat and are located in extragenic regions of the bacterial genome. These sequences were obtained from the genome of *Escherichia coli* and *S. typhimurium* [79]. BOX element is the highly conserved repeated DNA element identified in the chromosomes of *S. pneumoniae* (pneumococcus). Approximately 25 of BOX elements are found in non-coding regions dispersed throughout the entire pneumococcal genome. The BOX repeat is found to consist of three discriminate regions: boxA, boxB, and boxC, which are 59, 45, and 50 base pairs in length, respectively. Various combinations of these three elements are found to be present in different BOX loci and limited sequence heterogeneity is encountered among different elements from the same strain or elements sequenced from different strains [80]. Numerous studies have shown that the application of rep-PCR using oligonucleotide primers based on the REP elements (REP-PCR) (or) BOX element (BOX-PCR) (or) ERIC sequences (ERIC-PCR) has been successful in typing a variety of bacterial species such as *L. monocytogenes*, *S. pneumoniae*, *A. baumannii*, *Bartonella*, *Citrobacter diversus* [81,82,83]. It has been well recognized that combination of all three methods i.e., REP, ERIC and BOX PCR would increase the discriminatory power of typing bacterial species.

**Amplified Restriction Fragment Polymorphism (AFLP)**

AFLP is a PCR-based DNA fingerprinting technique, which makes use of restriction enzymes to digest genomic DNA, followed by the ligation of adaptors to the ends of the restriction fragments. Further, a subset of the restriction fragments is selected to be amplified using primers containing adapter defined sequences with one to three arbitrary nucleotide followed by analysis of amplified fragments by gel electrophoresis [84]. Unlike RAPD, AFLP technique is robust and reliable because of highly stringent PCR conditions used for primer annealing. The combination of different restriction enzymes and the choice of selective nucleotides in the primers for PCR make AFLP a useful system for molecular typing of bacterial pathogen. AFLP technique has been widely employed in microbiological research to determine ‘inter-’ and ‘intra’ species relatedness. AFLP techniques have been used to investigate bacterial isolates such as *E. cloacae*, *C. perfringens*, *L. monocytogenes*, *Legionella* sp., *V. cholera*, *P. multocida*, *Bacillus anthracis*, *M. avium* [85,86,87,88,89,90,91]. AFLP techniques are highly reproducible, need lesser concentration of DNA template, no prior knowledge of DNA sequence is required prior to analysis, the number of bands generated by AFLP is too high that gives highly informative fingerprints. The drawback associated with this technique is inability to differentiate different fragments with similar size (homology), has scoring bias and the organism to be typed must be isolated, since DNA from other sources disturbs the AFLP pattern.

**Real-Time PCR**

Real-time PCR is a process in which the target DNA is amplified and quantified simultaneously during PCR reaction. Real-time PCR employs specific primer set, one or two probes and/or fluorescent dyes (Eg. SYBR Green, Syto9 and LC Green) to improve detection signals. This is a valuable quantitative PCR-based technique widely used for diagnoses and quantification of bacterial pathogens in clinical microbiology laboratory [92]. Real-time PCR have been used in detection of various bacterial isolates such as *C. difficile*, *S. aureus*, *Streptococcus* sp., *P. aeruginosa*, *Francisella tularensis*, *K. pneumoniae* [93,94,95,96,97,98]. Advantages of Real-time PCR includes rapid detection and absolute or relative quantification of bacteria in various samples compared to conventional standard PCR, it requires no post-PCR processing of products and has greater sensitivity, reproducibility and high discriminatory power. The major disadvantage of Real-time PCR is associated with its high cost for equipment and reagents used for experiments. Further, this technique uses either DNA or RNA as the initial template. However, detection of pathogen using RNA templates requires minimum skill for handling RNA due to its greater instability compared to DNA [99].

**Next-Generation Sequencing (NGS)**

Bacterial typing using Next-Generation Sequencing (NGS) technology is an emerging alternative to all other bacterial typing methods. It offers simpler workflow, greater resolution and universally applicable bacterial subtyping methods. Recent advancement and growing number of applications has demonstrated the utility and need for NGS technology in clinical microbiology. The advancement in NGS technology makes bacterial whole genome sequencing (WGS) more feasible even at
small research laboratories. The advantage of NGS over traditional Sanger sequencing is its ability to generate millions of reads (app. 35–700 bp in length) in single runs at comparatively cheaper costs. The multiple short reads obtained after sequencing were assembled based on overlapping regions (de novo assembly) or based on reference genomes (Reference assembly) to construct the complete nucleotide sequence of bacterial genomes. However, the reads generated by the NGS are relatively short, which makes the de novo genome assembly more complex and challenging [77]. Single-molecule sequencing technologies (third-generation sequencing) offers relatively longer reads with average lengths of 2–3 kb, which could overcome the drawback associated with the de novo genome assembly. Nanopore sequencing technologies can generate reads up to 100 Kb. The potential drawback associated with the third-generation sequencing is relatively its high cost and low accuracy in generating sequence reads [100]. Salipante et al. [101] have recently studied the outbreak involving three important pathogens (vancomycin-resistant E. faecium, methicillin-resistant S. aureus, and A. baumannii) associated with nosocomial infections by WGS. Their results suggested that sequencing whole bacterial genomes at the level of single-nucleotide resolution demonstrates that PFGE is prone to false-positive and false-negative results; rather there is need for a new gold standard approach for molecular epidemiological typing of bacterial pathogen. WGS has wide application in public health surveillance, outbreak detection, bacterial identification and characterization, source attribution and containment. The present way of storing and comparing NGS sequence data was greatly facilitated by Bacterial Isolate Genome Sequence Database (BIGSdb) comparator and the web accessible PubMLST database (http://pubmlst.org/software/database/bigsdb/). WGS has allows the researcher to compare different genomes at a single-nucleotide resolution, which provides accurate characterization of disease transmission events and epidemic outbreaks. In addition, NGS can be effective in defining characteristics of bacterial pathogen such as the virulence or antibiotic resistance genes (resistome) or toxin genes (toxome) [102].

In recent years, food industries are growing rapidly to meet customer demands for wide range of food types. It is critically important to ensure the sensitivity and specificity of available techniques for detection of bacterial strains and its associated toxins to promote human health and hygiene. Development and recent advancement of new technologies have substantially improved the way of detecting bacterial pathogens and its associated toxins. Conventional ELISA-based tests were available to detect the bacterial toxins, but they do not measure the functional activity of toxins. However, use of highly sensitive and specific DNA probes and PCR for toxin gene can detect only potentially toxigenic bacterial strain, but it does not indicate that toxin gene was actually expressed or not. Real-time PCR might be useful in studying toxin gene expression in bacterial pathogen. However, this technique has limitation to study expression of toxin gene which is not known. All these challenges can be overcome by use of NGS technologies, which would provide detail insight into the existence, diversity and functional activity of toxin genes in bacterial pathogens.

The science of metagenomics is currently in its pioneering stages of development and many tools and technologies in this field are undergoing rapid evolution. The combination of 16S rRNA profiling strategies with metagenomics approach can be used to identify and characterize bacterial community prevailing at particular environmental sources. Recently, metagenomic pyrosequencing/ion sequencing of 16S rRNA amplicon libraries has been used to characterize an incredible array of bacterial communities from diverse sites (such as mouth, airway, bacterial infection sites, clinical specimens, intestinal tract, etc), which provided deeper insight into the bacterial populations important for research into human health [103]. Bacterial shotgun metagenomics provides insights into existing bacterial community and its associated metabolic processes that can be used to study the existence and role of bacterial pathogens in diseases [104]. Epidemiological typing of bacterial pathogen using NGS is shown in Fig. 3. The application of NGS is continuously expanding and in the near future, is likely to replace currently existing bacterial typing methodologies due to ultimate resolution, high-throughput efficiency and greater discriminatory power. The epidemiological bacterial typing methods from pre-genomic to post-genomic era have been listed in Table. 1.

**Conclusion**

Development and recent advancement of new technologies have substantially improved the way of defining bacterial pathogens. Genome-based bacterial discrimination methods offer attractive alternatives to phenotype-based methods due to its greater specificity and sensitivity in discriminating bacterial pathogens. However, there is no single ideal bacterial typing method is available and each method has its own advantages and potential drawbacks. In recent years, the PFGE technique has been proven to be prone to false-positive and false-negative results, suggesting the need for a new gold standard approach for molecular epidemiological bacterial typing. Event though, PCR based methods have high discriminatory power, but still has potential limitations including scoring bias and lack of statistical interpretation. Compared to all other genotypic methods, NGS has greater resolution, high-throughput, less expensive, much faster, has adequate bioinformatics tool for comparative genomics and statistical analysis, provides highly reliable taxonomically relevant information, flexibility to integrate with other approaches (such as Metagenomics). NGS can also be effective in defining genotypic characteristic of bacterial pathogen such as virulence and antibiotic resistance. To conclude, NGS can be considered as new gold standard techniques for getting insight into aspects of clinical diagnosis, molecular taxonomy and epidemiological surveillance of bacterial pathogens towards clinical management of widespread bacterial diseases.
### Table 1: Epidemiological bacterial typing from pre-genomic to post-genomic era

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<td></td>
<td>2-DE</td>
<td>Typing method based on analysis of entire proteome of bacterial cells</td>
<td>High-throughput, highly reproducible, requires high sensitive protein detection method</td>
<td>Taxonomic discrimination</td>
<td><em>S. porcinus, B. thuringiensis, H. pylori, H. influenza, P. aeruginosa and S. pneumoniae</em></td>
<td>[39, 45]</td>
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<tr>
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<td>16S-23S ITS PCR</td>
<td>Typing method based on sequence and length polymorphisms in internal transcribed spacer region of bacterial rRNA genes</td>
<td>High specificity, non-specific PCR amplification due to primer bias</td>
<td>Taxonomic discrimination, environmental monitoring</td>
<td><em>Staphylococcus sp., Streptococcus sp., P. stutzeri, Abiotrophia, Enterococcus, Granulicatella, Frankia sp., P. pneumotropica</em></td>
<td>[46, 54]</td>
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<td>Plasmid analysis</td>
<td>Typing method based on profiling of plasmid molecules present within bacterial cells</td>
<td>Easy in performance, Lesser reproducibility due to the existence of plasmid in different molecular forms, low discriminatory power due to the ability of bacterial cells to lose or acquired plasmid DNA from other sources</td>
<td>Environmental monitoring</td>
<td><em>Shigella, C. perfringens, Vibrio spp., S. enterica, P. aeruginosa</em></td>
<td>[55]</td>
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<td>PFGE</td>
<td>Typing method based on restriction endonucleases generated DNA fingerprints of bacterial isolates</td>
<td>Highly reproducible, time consuming, requires skilled technician, does not discriminate between unrelated isolates</td>
<td>Clinical diagnosis, taxonomic discrimination and environmental monitoring</td>
<td><em>C. jejuni, Listeria spp., S. enterica, C. difficile, S. aureus and Cronobacter sp.</em></td>
<td>[63, 69]</td>
</tr>
</tbody>
</table>
Genotype-based methods (Post-genomic) | Typing method based on PCR amplification of random segment of genomic DNA of bacterial isolates using single primer of arbitrary nucleotide sequence | Highly laboratory dependent, low reproducibility, PCR bias, needs high stringent PCR conditions | taxonomic discrimination | Listeria spp., Lactobacillus spp., Enterococcus spp. S. thermophilus, S. Marcescens, K. pneumoniae, S. aureus [70, 76]


Rep-PCR | Typing method based on PCR amplification of interspersed repetitive consensus sequences from genomic DNA of bacterial isolates | Combination of different rep-PCR methods such as REP, ERIC, BOX would achieve greater discriminatory power | taxonomic discrimination | E. coli, S. typhimurium, S. pneumoniae, L. monocytogenes, A. baumannii, Bartonella, C. diversus [78, 83]

AFLP | PCR-based DNA fingerprinting technique, which make use of restriction enzymes to digest genomic DNA, followed by the ligation of adaptors to the ends of the restriction fragments and PCR amplification using adapter specific primers | High reproducibility, requires less amount of DNA, no knowledge of DNA sequence is required prior to analysis, produce highly informative fingerprints, inability to differentiate different fragments with similar size (homology), scoring bias | taxonomic discrimination | E. cloacae, C. perfringens, L. monocytogenes, Legionella spp., V. cholera, P. multocida, B. anthracis, M. avium [84, 88, 89]

Real time-PCR | Typing method based on PCR amplification and quantification of target DNA from bacterial isolated using specific set of primers with fluorescent probes | High sensitivity, reproducible, greater discriminatory power, high cost, requires much skilled expertise | Clinical diagnosis, taxonomic discrimination and environmental monitoring | C. difficile, S. aureus, Streptococcus sp., H. pylori, P. aeruginosa, F. tularensis, K. pneumoniae [92, 99]

Next-Generation Sequencing | Typing method based on bacterial community profiling, whole genome sequencing and metagenome shotgun sequencing of bacterial isolates | single-nucleotide resolution, high-throughput, high reproducibility, high sensitivity, cheaper cost, faster performance, interactive bioinformatics tool, statistical analysis, high flexibility, highly informative | Clinical diagnosis, taxonomic discrimination and environmental monitoring | vancomycin-resistant E. faecium, methicillin-resistant S. aureus, and A. baumannii [77, 103, 104]

References


