



Review

Pulsed-field gel electrophoresis (PFGE): A review of the “gold standard” for bacteria typing and current alternatives

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ABSTRACT

Pulsed-field gel electrophoresis (PFGE) is considered the “gold standard” for bacteria typing. The method involves enzyme restriction of bacteria DNA, separation of the restricted DNA bands using a pulsed-field electrophoresis chamber, followed by clonal assignment of bacteria based on PFGE banding patterns. Various PFGE protocols have been developed for typing different bacteria, leading it to be one of the most widely used methods for phylogenetic studies, food safety surveillance, infection control and outbreak investigations. On the other hand, as PFGE is lengthy and labourious, several PCR-based typing methods can be used as alternatives for research purposes. Recently, matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) and whole genome sequencing (WGS) have also been proposed for bacteria typing. In fact, as WGS provides more information, such as antimicrobial resistance and virulence of the tested bacteria in comparison to PFGE, more and more laboratories are currently transitioning from PFGE to WGS for bacteria typing. Nevertheless, PFGE will remain an affordable and relevant technique for small laboratories and hospitals in years to come.

1. Introduction

Bacterial typing is used to discriminate between bacterial strains; it is an important tool for outbreak investigation, surveillance, and phylogenetic studies. Early typing experiments were carried out via biochemical experiments which results were based on the tested bacteria's phenotype, such as antibiotic resistance, susceptibility towards phage (phage typing) and carriage of surface antigens (serotyping) (Foxman et al., 2005). With the advent of molecular biology, DNA-based typing methods gained popularity, as results from these protocols are usually reproducible due to the stable nature of DNA, and are generally more rapid and less labourious (Adzitey et al., 2013; Olive and Bean, 1999).

Pulsed-field gel electrophoresis (PFGE) is considered the “third-generation molecular approach” for bacteria typing (Goering, 2010); the first- and second-generations being plasmid analysis and genome enzyme restriction. All first- till third-generation molecular analysis require the usage of agarose gel electrophoresis to separate enzyme-restricted plasmid or genome DNA fragments; clonal assignment of tested bacteria is subsequently based on the separated DNA banding

patterns. As an innovation to the first- and second-generation approach, movement and separation of genome fragments in PFGE, besides being dependent upon the molecular size of each restricted fragment, is also based on the pulsed-periodic reorientation of electrophoretic fields on an agarose gel (Fig. 1A) (Goering, 2010). As bacterial genomes have a size of 2–4 Mbp, pulsed electrophoresis allows clearer separation of DNA fragments of various sizes (from kb to Mb), resulting in better typing and clonal assignment of tested bacteria.

This review gives an introduction about PFGE, describes common PFGE workflows for gram-positive and gram-negative bacteria, discusses about its applications and also other molecular methods which have been developed as alternatives for bacteria typing. Recent developments in bacteria typing using whole genome sequencing (WGS) and matrix-assisted laser desorption/ionization time of flight mass spectrometry are also included in this review.

2. Basic components of PFGE and software for analysis

Innovations in pulse time and electrophoretic field orientation in

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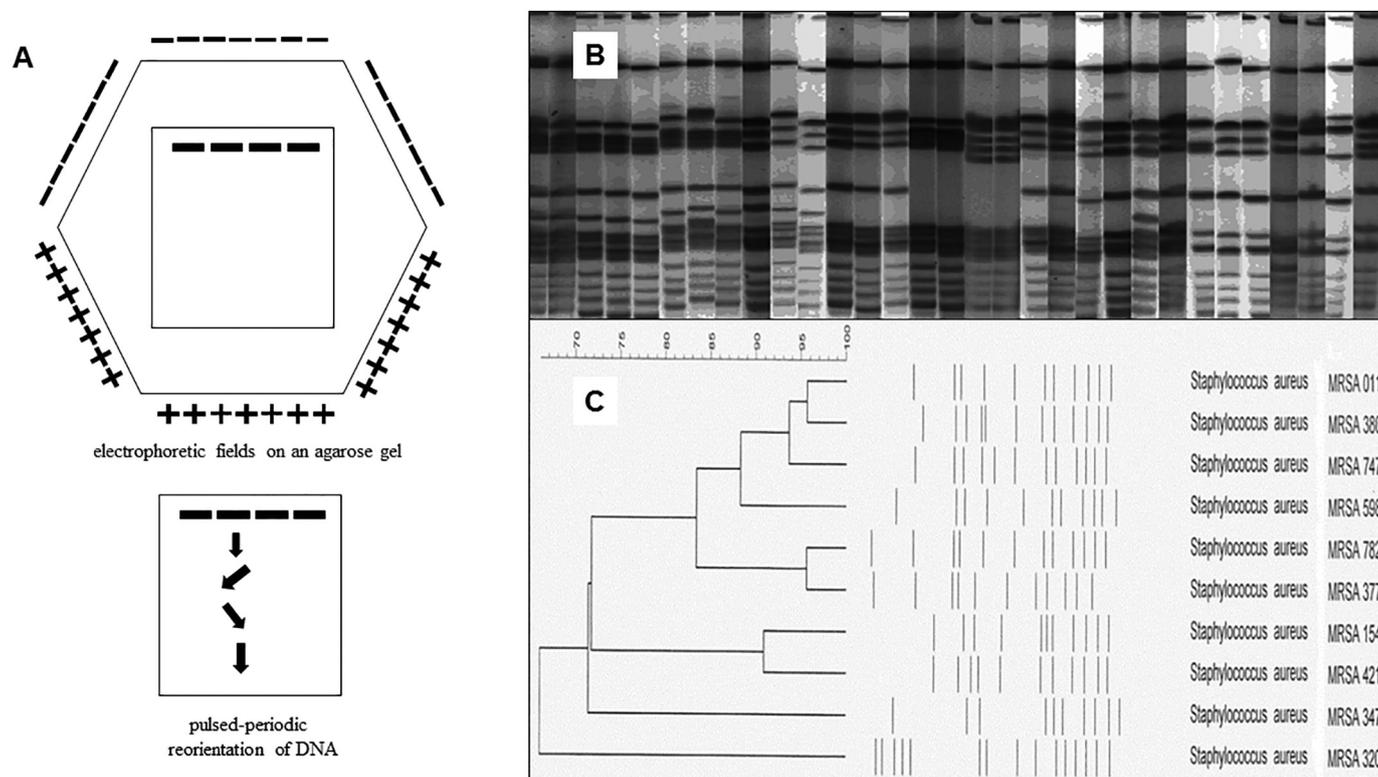


Fig. 1. Schematic diagram of DNA band movement in PFGE, PFGE banding patterns and PFGE dendrogram. A) Pulse-periodic reorientation of DNA on a PFGE gel. B) PFGE banding patterns. C) Dendrogram built based on PFGE banding patterns. MRSA 011, MRSA 380 and MRSA 747 have less than 3 band difference in banding patterns and are considered as closely-related strains. MRSA 347 and MRSA 320 have more than seven band difference between them and are considered as unrelated strains.

PFGE have given rise to variations in the technique (Basim and Basim, 2001). As DNA separation by the Contour-clamped Homogeneous Electric Field electrophoresis (CHEF) is the most discriminative amongst PFGE platforms, it is commonly used by many laboratories of the world, leading to the development of internationally-shared CHEF protocols for common bacteria (Chu et al., 1986). The following contents of this review is therefore written based on CHEF.

A typical PFGE system consists of a power supply unit with switching functions, an electrophoresis chamber and a cooling module. A gel documentation system will also be required for viewing and capture of PFGE results. Data output from the PFGE system is in the form of electrophoresis bands (Fig. 1B). Banding patterns of tested samples determines bacterial clonal assignment. According to Tenover et al.'s criteria which is widely used for interpretation of PFGE banding patterns, bacterial strains which are "closely related" will have two to three bands difference in PFGE banding patterns, strains which have four to six band difference are "possibly related", and seven or more band differences point to unrelated strains (Fig. 1C) (Tenover et al., 1995).

To ease analysis when many samples are run on one gel, or when samples from different gels are compared, algorithms have been developed to assign numerical representation of a specific PFGE pattern. For this, the band patterns being compared must be produced using the same protocol and reference standard. Gel analysis software such as GelCompar, Molecular Analyst Fingerprinting, BioImage, Phoretix and BioNumerics assigns strains according to their increment in band difference, and use band-based similarity coefficients (Dice, Jaccard or Pearson) to build dendrograms via algorithms like the Unweighted Pair-Group Method with Arithmetic Averaging (UPGMA) or the Neighbor-Joining (NJ) method. Phylogenetic trees then allow comparison of genetic relatedness and clonal assignment (Fig. 1C) (Rementeria et al., 2001; Duck et al., 2003; Felix et al., 2015; Carrico et al., 2005). A

similarity coefficient of 80% has been established for the assignment of clones (McDougal et al., 2003). The protocol listed on PulseNet Methods at the Centers for Disease Control and Prevention (CDC) website cites the Bionumerics software for its PFGE analysis (<https://www.cdc.gov/pulsenet/pathogens/pfge.html>).

3. Common PFGE protocols for Gram-positive and Gram-negative bacteria

Due to its superior discriminatory power in bacterial typing, various PFGE protocols have been developed and harmonized by different laboratories for many clinical pathogens. In general, the protocols mainly differ in methods for bacterial cell lysis, selection of restriction enzyme, and, to a smaller effect, the parameters of switch and running time during electrophoresis. Most protocols use a cell lysis buffer of 50 mM Tris: 50 mM EDTA (pH 8.0) with 1% Sarcosyl and 20 mg/ml of proteinase K. Lysis of gram positive pathogens require the addition of enzymes or detergents such as lysostaphin (for *Staphylococcus aureus*), lysozyme or Brij 58 (for *Clostridium botulinum*).

Restriction enzymes also play an important role in the resulting PFGE banding patterns (pulsotypes) of tested bacteria. Established protocols published in the PulseNet International and CDC websites use designated restriction enzymes for specific bacteria; this enables pulsed-type comparison between different laboratories and identification of bacterial strains associated with foodborne diseases. With the increasing availability of whole genome sequences, researchers are now able to explore new restriction enzymes for PFGE typing of bacterial isolates using in silico platforms hosted on websites such as <http://insilico.ehu.es/digest/> (Bikandi et al., 2004; Chang et al., 2013). This approach will be useful for typing mutated pathogens which cannot be digested using established protocols (Marti et al., 2013).

CDC has published a unified PFGE protocol for typing gram-positive

Table 1
Major differences in PFGE protocols for various pathogens.

Pathogen	Cell lysis	Restriction enzyme	Thiourea requirement	Running conditions for CHEF-DR III		
				Initial switch time (s)	Final switch time (s)	Run time (hours)
<i>Campylobacter jejuni</i>	50 mM Tris:50 mM EDTA, pH 8.0 + 1% Sarcosyl, proteinase K	SmaI, KpnI or XbaI	no	6.8	35.4	17–20
<i>Clostridium botulinum</i>	12 mM Tris, 2M NaCl, 200 mM EDTA, 1% Brij 58, 0.4% Deoxycholate, 5% Sarcosyl, lysozyme, mutanolysin, proteinase K	XbaI or XhoI, SmaI	yes	0.5	40	18–19
<i>Cronobacter sp.</i>	50 mM Tris:50 mM EDTA, pH 8.0 + 1% Sarcosyl, proteinase K 20 mg/ml	XbaI (primary enzyme), SpeI (secondary enzyme)	no	1.8	25	18–19
<i>E. coli O157</i>	50 mM Tris:50 mM EDTA, pH 8.0 + 1% Sarcosyl, proteinase K 20 mg/ml	Xba I-primary enzyme, BlnI/AvrII-secondary enzyme, SpeI-tertiary enzyme	no	2.2	54.2	17–20
<i>Non-O157 E. coli</i>	50 mM Tris:50 mM EDTA, pH 8.0 + 1% Sarcosyl, proteinase K 20 mg/ml	Xba I-primary enzyme, BlnI/AvrII-secondary enzyme, SpeI-tertiary enzyme	no	6.76	35.38	17–20
<i>Salmonella sp.</i>	50 mM Tris:50 mM EDTA, pH 8.0 + 1% Sarcosyl, proteinase K 20 mg/ml	Xba I-primary enzyme, BlnI/AvrII-secondary enzyme, SpeI-tertiary enzyme	no	2.2	63.8	17–20
<i>Shigella sonnei</i>	50 mM Tris:50 mM EDTA, pH 8.0 + 1% Sarcosyl, proteinase K 20 mg/ml	Xba I-primary enzyme, BlnI/AvrII-secondary enzyme, SpeI-tertiary enzyme	no	2.2	54.2	17–20
<i>Shigella flexneri</i>	50 mM Tris:50 mM EDTA, pH 8.0 + 1% Sarcosyl, proteinase K 20 mg/ml	NotI-primary enzyme, XbaI-secondary enzyme, SpeI-tertiary enzyme	no	5	35	17–20
<i>Listeria monocytogenes</i>	lysozyme, proteinase K, 50 mM Tris:50 mM EDTA, pH 8.0 + 1% Sarcosyl	AscI (primary enzyme), ApaI (secondary enzyme)	no	4	40	17-20
<i>Vibrio cholerae</i>	proteinase K, 50 mM Tris:50 mM EDTA, pH 8.0 + 1% Sarcosyl	SfiI (primary enzyme), NotI (secondary enzyme)	no	2 (block 1); 20 (block 2)	10 (block 1); 25 (block 2)	13 (block 1); 6 (block 2)
<i>Vibrio parahaemolyticus</i>	proteinase K, 50 mM Tris:50 mM EDTA, pH 8.0 + 1% Sarcosyl	SfiI (primary enzyme), NotI (secondary enzyme)	no	10	35	17-20
<i>Staphylococcus aureus</i>	lysozyme (S. aureus - lysostaphin), Proteinase K, (50 mM Tris: 50 mM EDTA: 1% sodium sarcosyl)	SmaI	no	5	40	19-20
<i>Enterococcus s.</i>	lysozyme (S. aureus - lysostaphin), Proteinase K, (50 mM Tris: 50 mM EDTA: 1% sodium sarcosyl)	SmaI	no	3.5	23.5	19-20
<i>Campylobacter jejuni</i>	50 mM Tris:50 mM EDTA, pH 8.0 + 1% Sarcosyl, proteinase K	SmaI, KpnI or XbaI	no	6.8	35.4	17-20

Abbreviations: PFGE, pulsed-field gel electrophoresis; EDTA, Ethylenediaminetetraacetic acid

bacteria such as *Staphylococcus* sp., *Enterococcus* sp., and *Clostridium* sp. (https://www.cdc.gov/hai/pdfs/labsettings/unified_pfge_protocol.pdf). PulseNet International, the International Molecular Subtyping Network for Foodborne Disease Surveillance publishes PFGE protocols for important foodborne pathogens on its website (<http://www.pulsenetinternational.org/protocols/>), with the updated versions (2017) on the CDC PulseNet website (<https://www.cdc.gov/pulsenet/pathogens/pfge.html>). Table 1 summarizes major differences in PFGE protocol parameters for typing of clinically important pathogens.

In addition, several research laboratories have harmonized and inter-laboratory-verified their PFGE protocols. Using *S. aureus* as an example, the HARMONY typing group developed a standardized protocol for typing of *S. aureus* isolated from laboratories in Europe, while 15 laboratories in Canada collaborated together to establish a standardized protocol for the bacteria (Mulvey et al., 2001; Murchan et al., 2003). Using the CDC PFGE standardized protocol for *S. aureus*, a PFGE database of oxacillin-resistant *S. aureus* (ORSA) was built, where major lineages of ORSA isolated from the United States were identified using the protocol, and designated as pulsotypes USA100 through USA800 (McDougal et al., 2003).

4. Applications of PFGE

PFGE has various applications. As it is a tool to produce DNA fingerprints for bacterial isolates, the technique has been used to establish bacteria databases based on each strain's PFGE banding patterns. Information from this database has been used to identify pathogenic bacteria in food safety monitoring and also for phylogenetic studies of bacteria. Due to its ability to separate large DNA molecules (up to 10 Mbp), it is also used in the analysis of large chromosomes in yeasts and some mammals (Levene, 1992; Wagner and Lai, 1994; Gardiner, 1991).

4.1. Phylogenetic, evolutionary and dissemination studies

Bacteria diverge from their ancestors via mutations, transfer of mobile genetics elements and genetic exchange via homologous recombination. As per Tenover's criteria, using PFGE, closely related strains will have less chromosomal differences, resulting in similar PFGE banding patterns (Tenover et al., 1995).

PFGE provided information about the evolution of *Vibrio cholerae* strains circulating in Mexico from 1983 to 1997, where the researchers found that CL biotype strains in their study existed prior to and evolved independently from Asian strains (Alam et al., 2012). The technique was also used in a following study by the authors on *V. cholera* isolated in the country from 1998 to 2008, where they discovered divergence of a subset of strains which were negative for the cholera toxin (Alam et al., 2014).

Similarly, in our study conducted in 2009, PFGE revealed possible divergence in methicillin-resistant *S. aureus* (MRSA) strains isolated from a teaching hospital, where two dominant MRSA clones with only a single PFGE band difference between them were observed (Tan et al., 2013). Temporal distributions of MRSA isolated from patients of teaching and non-teaching hospitals in Cologne, Germany, from 1984 to 1998 have also been identified using PFGE (Wisplinghoff et al., 2005).

Comparison of PFGE profiles will also provide information on international dissemination of certain bacteria clones. Denmark researchers identified the prevalence of the community-acquired MRSA (CA-MRSA) USA300, a dominant CA-MRSA in Canada and USA, in healthy carriers in the country (Larsen et al., 1992). Interestingly, the same clone has also been identified using PFGE typing in Japan (Murai et al., 2019) and Singapore (Grant et al., 2013).

4.2. Infection control, transmission checking, outbreak investigation

PFGE typing is used for infection control to track transmission of pathogens in the hospital. Comparison of PFGE banding patterns from bacteria isolated from patients, healthcare workers and the ward environments helps infection control team identify source of infection and transmission routes (Yan et al., 2019; Pirs et al., 2019). This will enable eradication of the source, halt transmission, and prevent morbidity and mortality (Kevat et al., 2018; De la Rosa-Zamboni et al., 2018). Routine swabs from healthcare professionals and ward environments, together with PFGE typing, are useful to monitor dominant clones "residing" in hospital environments (De la Rosa-Zamboni et al., 2018; Kong et al., 2018).

PFGE has been used during investigation of multi-state outbreaks. The technique was used to determine the source and dissemination of an *Escherichia coli* O157:H7 outbreak in the USA in 2006, where a specific brand of fresh spinach was determined to be the source of the outbreak (Sharapov et al., 2016). PFGE was also used in a recent outbreak caused by consumption of Romaine Lettuce contaminated by the same bacteria in the USA (<https://www.cdc.gov/ecoli/2018/o157h7-04-18/index.html>). In outbreak investigations using PFGE, attention to collection dates of tested isolates is of importance, as banding patterns for bacteria such as vancomycin-resistant *Enterococcus faecium* might change in as short as 150 days, and obscure clonal assignment (McLaughlin et al., 2013).

4.3. Food safety

PFGE typing is used in the food industry to monitor potential bacteria contaminants of food produce. PFGE banding patterns derived using published, established protocols of important food pathogens, such as *Listeria monocytogenes* isolated from food, feed and livestock have been published and curated for reference in food safety surveillance (Roussel et al., 2014). The College of Agriculture and Life Sciences of Cornell University maintains a database of 6000 and 1700 PFGE banding patterns of *Salmonella* sp. and *L. monocytogenes* for food safety monitoring (<https://foodsafety.foodscience.cornell.edu/laboratory-molecular-typing-lmt/pulsed-field-gel-electrophoresis-pfge-typing/>).

4.4. Analysis of large chromosomes

PFGE has been used in the analysis of large chromosomes in yeasts and some mammals (Levene, 1992; Crowe et al., 2017; Gardiner, 1991). It has been used to construct Yeast Artificial Chromosome (YAC) libraries and even transgenic mice (Gardiner, 1991). The technique is also useful for mapping of human chromosomes (Larsen et al., 1992).

5. PFGE: comparison with other PCR-based typing techniques (rep-PCR, ERIC PCR, MLVA)

Results from PFGE typing are in the form of enzyme-restricted whole genome DNA bands separated on an agarose gel; it is the most widely used gel-based typing method for clonal comparison of bacterial isolates. Nevertheless, the PFGE method for bacterial typing is cumbersome and requires harmonization of various parameters (Table 1) for inter-laboratory comparison of results.

As an alternative to PFGE, PCR-based typing methods have been introduced, following the discovery of sequences consisting repetitive DNA fragments (tandem repeats) in bacterial genomes which can be used for bacterial typing. These PCR-based typing methods, which are more robust and easier to perform, include repetitive extragenic palindromic (REP) elements (rep-PCR), enterobacterial repetitive intergenic consensus (ERIC) sequences PCR (ERIC-PCR) and multi-locus variable number of tandem repeats analysis (MLVA).

Even so, till today, PCR-based methods such as the MLVA is only

used for research purposes or as a complementary technique to PFGE in reference centres like the CDC. Protocol standardization and the availability of large databases for PFGE banding patterns of pathogens retain the method's superiority in bacteria typing.

5.1. Extragenic palindromic (REP) elements (rep-PCR)

REP elements are 21–65 bp repetitive and palindromic sequences located in the extragenic space of bacterial genomes (Tobes and Ramos, 2005). Species-specific REPs have been found in human and plant pathogens as well as soil bacteria; the sequences were first discovered in *E. coli* and *Salmonella* sp. (Tobes and Ramos, 2005; Hielt and Seal, 2009). Similar to PFGE band patterns, bands from PCR amplification and gel electrophoresis separation of REP elements are specific for bacterial clones and can be used for typing and clonal identification of bacteria.

rep-PCR can be performed by any small laboratory which has a PCR machine with gel electrophoresis and documentation setup. The technique has also been automated commercially using the DiversiLab system (biomerieux, France) with readily-available rep-PCR typing primers for nosocomial pathogens such as *E. coli*, *Enterococcus faecium*, *S. aureus* (Shutt et al., 2005), or *Neisseria meningitidis* (Healy et al., 2005; Shutt et al., 2005). Clonal concordance between PFGE and rep-PCR might not be 100% for some bacteria, with either technique being more discriminatory than the other (Table 2). The best method to use depends very much on the tested bacteria and also research question of each investigation, i.e., for phylogenetic analysis across strains isolated from different countries, or identification of highly epidemic and virulent strains in a single centre (Bae et al., 2014; Ahmed et al., 2017).

5.2. Enterobacterial repetitive intergenic consensus (ERIC) sequences (ERIC-PCR)

ERIC sequences are found in the genomes of Enterobacteriaceae and Vibrionaceae (Wilson and Sharp, 2006). The sequences are palindromic, ~120bp and are first described in *E. coli*, *Salmonella*

typhimurium, *Yersinia pseudotuberculosis*, *Klebsiella pneumoniae* and *V. cholera* (Hulton et al., 1991). Found only in intergenic, transcribed regions of the genome and specific for bacterial species and strains, the sequences can be PCR-amplified and used for bacteria finger printing (Versalovic et al., 1991). The method can also be used to type *Mycobacterium* spp. (Sampaio et al., 2006). Similar to the comparison between PFGE and rep-PCR, neither PFGE nor ERIC-PCR is superior to one another to determine genetic relatedness in bacterial strains. Table 2 shows the concordance and discriminatory power between PFGE and ERIC-PCR for several bacteria.

5.3. Multi-locus variable number of tandem repeats analysis (MLVA)

MLVA typing is based on the number of VNTRs (variable number of tandem repeats) in specific loci of a bacteria (Frothingham and Meeker-O'Connell, 1998). VNTRs are adjacent regions of coding and non-coding nucleotide repeats; these regions vary in number between different microorganisms. A multiplex PCR will be used to amplify various, selected VNTR loci followed by gel electrophoresis, generating band patterns with sizes concordant to the number of VNTRs in the loci, and enable clonal differentiation between tested bacteria. Strains which carry similar numbers and patterns of VNTR band amplifications will be grouped as the same clone, and vice versa (van Belkum et al., 1998; Frothingham and Meeker-O'Connell, 1998).

Gel-based MLVA workflow is similar to rep- and ERIC-PCR, involving only multiplex PCR amplification of targeted regions followed by gel electrophoresis and clonal assignment based on band patterns. However, gel-based typing is not ideal for comparison of results between different laboratories. To this end, for MLVA, usage of fluorescent primers and capillary electrophoresis with a DNA sequencer has enabled assignment of integers to the numbers of VNTR repeats in targeted loci. Digitizing of these results and protocol harmonisation allow inter-laboratory comparison of typing results using the MLVA website (www.mlva.net/) hosted by The Netherlands's National Institute for Public Health and the Environment (RIVM) (Nadon et al.,

Table 2
Concordance and discriminatory power of PFGE against rep-PCR, ERIC-PCR and MLVA for typing of bacterial pathogens

Bacteria	Discriminatory power compared to PFGE (concordance)			Reproducibility	Reference
	REP PCR	ERIC PCR	MLVA		
<i>Enterococcus faecium</i>	Higher (high)	n.a.	Lower (moderate)	n.a.	(Bourdon et al., 2011; Top et al., 2008)
<i>Clostridium difficile</i>	Lower (low) / lower (high)	n.a.	n.a.	n.a.	(Pasanen et al., 2011; Spigaglia and Mastrantonio, 2003)
<i>Staphylococcus aureus</i>	Lower (73–100%)	n.a.	Similar (high)	Yes (PFGE and rep PCR)	(Ross et al., 2005; Malachowa et al., 2005; Roussel et al., 2015)
<i>E. coli</i> O157 and non-O157	Lower (n.a.)	n.a.	n.a.	n.a.	(Anderson et al., 2015)
<i>Pseudomonas aeruginosa</i>	Lower (low)	Lower (high)	Similar (low)	n.a.	(Brossier et al., 2015; Kidd et al., 2011; Maatallah et al., 2013)
<i>Klebsiella pneumoniae</i>	Lower (low)	n.a.	Lower (low)	n.a.	(Koroglu et al., 2015; Derakhshan et al., 2017)
<i>Campylobacter jejuni</i>	n.a.	Similar (high)	n.a.	Yes (PFGE)	(Shi et al., 1996)
<i>Laribacter hongkongensis</i>	n.a.	Higher (high)	n.a.	Yes (PFGE and ERIC PCR)	(Feng et al., 2013)
<i>Stenotrophomonas maltophilia</i>	Similar (high)	Lower (low)	n.a.	n.a.	(Lin et al., 2008)
<i>Salmonella typhimurium</i>	Similar (n.a.)	Lower (n.a.)	Higher (n.a.)	Yes (PFGE and MLVA)	(Woo and Lee, 2006; Ferrari et al., 2017)
<i>Salmonella enterica</i>	n.a.	n.a.	Higher	Yes (MLVA)	(Ranjbar et al., 2016; Kjeldsen et al., 2015; Boxrud et al., 2007)
<i>Mycobacterium chelonae</i>	n.a.	Similar (n.a.)	n.a.	n.a.	(Sampaio et al., 2006)
<i>Mycobacterium abscessus</i>	n.a.	Similar (n.a.)	n.a.	n.a.	(Sampaio et al., 2006)
<i>Vibrio parahaemolyticus</i>	n.a.	Similar (moderate)	n.a.	Yes (ERIC PCR)	(Marshall et al., 1999)
<i>Vibrio parahaemolyticus</i> O1:KUT	n.a.	n.a.	Higher (high)	n.a.	(Preeprem et al., 2019)
<i>Vibrio cholerae</i>	n.a.	n.a.	Similar (low)	n.a.	(Teh et al., 2010)
<i>Yersinia enterocolitica</i>	n.a.	n.a.	Higher (n.a.)	yes (MLVA)	(Raymond et al., 2018)
<i>Listeria monocytogenes</i>	n.a.	n.a.	Similar (low)	yes (MLVA)	(De Cesare et al., 2017; Saleh-Lakha et al., 2013; Lindstedt et al., 2008)
<i>Acinetobacter baumannii</i>	n.a.	n.a.	Lower (low)	n.a.	(Johnson et al., 2016)
<i>Streptococcus pneumoniae</i>	n.a.	n.a.	Higher (high)	yes (MLVA)	(Costa et al., 2016)

Abbreviations: n.a., data not available; PFGE, pulsed-field gel electrophoresis; rep-PCR, repetitive extragenic palindromic PCR; ERIC-PCR, enterobacterial repetitive intergenic consensus sequences PCR; MLVA, multi-locus variable number of tandem repeats analysis

2013; Peters et al., 2017). In this website, MLVA database for *Bordetella pertussis* (Schouls et al., 2004), *Haemophilus influenza* (Schouls et al., 2005), *N. meningitidis* (Schouls et al., 2006), *S. aureus* (Schouls et al., 2009) and *Streptococcus pneumoniae* (Elberse et al., 2011) can be accessed and utilized for MLVA profile assignment. The technique was first described for *Bacillus anthracis* typing by Keim et al. (Keim et al., 2000); subsequently, protocols were developed for *Yersinia pestis*, (Klevytska et al., 2001), *Francisella tularensis* (Farlow et al., 2001) and *Borrelia* sp. (Farlow et al., 2002) by researchers in the same laboratory from Northern Arizona University. MLVA protocols were then developed for various types of bacteria: *Pseudomonas aeruginosa* (Onteniente et al., 2003); *E. coli* O157 (Noller et al., 2003); *Salmonella* spp. (Lindstedt et al., 2004); *Enterococcus* spp. (Top et al., 2004) and *H. influenza*- (Schouls et al., 2005). Table 2 shows the concordance and discriminatory power between PFGE and MLVA for several bacteria.

6. PFGE: comparison with “next-generation” typing methods – MALDI-TOF MS typing and whole genome sequencing

The first- to third-generation of bacteria typing are gel-based techniques. Following this, fourth generation of bacterial typing have mostly diverged from the usage of gels, and some techniques, such as proteomics typing, is based on protein sequences instead of DNA.

6.1. Matrix assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) typing

The usage of MALDI-TOF MS has revolutionized the process for bacterial species identification, where the specific whole cell mass spectra of a bacteria (constructed based on the mass to charge ratio of its protein peaks) can be used for its identification (Seng et al., 2009; Holland et al., 1996). Briefly, a colony of tested bacteria is treated with formic acid and dried in a matrix. Laser irradiation of the colony-containing matrix in a TOF chamber vaporizes and releases ions, which are then accelerated towards a detector, generating a mass spectrum (Patel, 2015). The observation of spectral similarity between bacteria of the same clone has enabled the usage of MALDI-TOF MS for bacterial typing and clonal assignment, and, in the beginning phase it has been successfully used to type bacteria such as *Streptococcus pyogenes* (Moura et al., 2008), *S. aureus* (Boggs et al., 2012; Wolters et al., 2011), *Clostridium difficile* (Reil et al., 2011), *E. coli* (Karger et al., 2011), *Salmonella enterica* (Dieckmann and Malorny, 2011; Kuhns et al., 2012) and *Yersinia enterocolitica* (Stephan et al., 2011). As only simple sample preparation is needed and the results can be obtained in a short time, MALDI-TOF MS can be considered a probable alternative to gel-based bacterial typing methods (Sauget et al., 2017).

Compared to the more established genome-based techniques for bacterial typing, less studies investigated the discriminatory power of PFGE compared to MALDI-TOF MS typing. Steensels et al. found a concordance of 93% between the two techniques in a study to type 15 MRSA isolates from a neonatal intensive care unit outbreak. Nevertheless, the MALDI-TOF MS typing results were found to have poor reproducibility (Steensels et al., 2017). Clustering via MALDI-TOF MS was found to be different from PFGE in a study to type *E. coli* isolates of B2 (ST131) and D (ST69, ST405, ST393) phylogenetic groups (Novais et al., 2014). With more studies attempting to use MALDI-TOF MS for bacterial typing, a few drawbacks have been observed. It was reported to have poorer discriminatory power for *V. cholera* (Schirmeister et al., 2014), *S. aureus* and *E. faecium* typing (Lasch et al., 2014). In addition, the technique utilizes protein which is very changeable according to bacterial culture, storage and test conditions. This variation in results arising from protein changes will obscure clonal assignment for tested strains. Furthermore, result reproducibility, statistical analysis, and difficulty in portability of MALDI-TOF MS results require the technique to be carefully assessed and protocol-harmonised across laboratories before it can be used as a robust typing

tool (Sauget et al., 2017; Spinali et al., 2015).

6.2. Whole genome sequencing (WGS)

With the advent of next- and third-generation sequencing technologies, whole genomes of bacteria can be sequenced in less than 24 hours, and these techniques provide higher discrimination power of bacterial clones compared to PFGE (Salipante et al., 2015; Quainoo et al., 2017). Results from this approach are considered comprehensive and accurate, as not only selected sequences (as in MLVA, ERIC-PCR and rep-PCR) or enzyme restriction patterns of the whole genome (as in PFGE) are used for clonal assignment; rather, information from the whole/core genome can be used for strain comparison and identification (Schurch et al., 2018; Mellmann et al., 2017). Strain typing and clonal relatedness determination using WGS is usually performed based on single nucleotide polymorphism (SNP) analysis compared to a control strain's reference genome; or typed via thousands of gene-by-gene comparison against a pre-defined, curated set of core genes, designated as the core genome MLST (cgMLST) (Schurch et al., 2018; Kohl et al., 2014; de Been et al., 2015; Wendel et al., 2018). Due to the stable nature of DNA compared to proteins, results from WGS will be more reproducible than those from MALDI-TOF MS typing. Inter-laboratory comparison of results will also be achievable (Deurenberg et al., 2017; Mellmann et al., 2017). WGS protocol can be identical for most pathogens and provides not just information on clonal assignment, but also information such as resistance and virulence factors about tested pathogens in a single test (Salipante et al., 2015; Leopold et al., 2014; Deurenberg et al., 2017; Klemm et al., 2018).

Japanese researchers characterized and compared *C. botulinum* isolated from 10 cases in 2006-2011 using PFGE, MLVA and whole-genome SNP analysis (Kenri et al., 2014), and found WGS to be more discriminatory than PFGE for these 10 isolates. Indeed, seven strains previously regarded to be similar strains via PFGE were revealed to be of two separate distinct lineages. Even though this finding did not translate to differences in clinical outcome of the botulism cases, the study demonstrated the high discriminatory power of WGS for bacterial typing. Prospective WGS surveillance of *L. monocytogenes* isolated from food, human and environmental samples carried out by the Australian Microbiological Diagnostic Unit Public Health Laboratory also showed that WGS typing provided greater level of discrimination compared to PFGE or MLST (Kwong et al., 2016). Similarly, WGS was found to be more discriminatory than PFGE for typing of other pathogens such as *Salmonella* sp. (Leekitcharoenphon et al., 2014; Morganti et al., 2018), *E. coli* O157:H7 (Rumore et al., 2018), *Campylobacter jejuni* (Pendleton et al., 2013) and also MRSA (Harris et al., 2010; Bosch et al., 2013). Decreasing costs for WGS and its culture-independent protocol will encourage its usage over PFGE for bacterial typing. Nevertheless, high costs for instrument purchase and the need for bioinformatics analysis will delay its replacement of PFGE (SenGupta et al., 2014; Roy et al., 2016; Fricke and Rasko, 2014). At the time of writing of this review, PulseNet International and CDC are in the transitioning from using PFGE to WGS for bacteria typing (Nadon et al., 2017; Crowe et al., 2017; Whaley et al., 2018).

6.3. Concluding remarks

PFGE is the “gold-standard” for bacterial typing; it has demonstrated superiority and staying power compared to other methods which were developed in the same time. Protocol harmonisation across laboratories has led to its wide usage for phylogenetic studies, infection control, outbreak investigation and also for food safety surveillance. With the advent of WGS, many laboratories, including PulseNet International and CDC have started incorporating WGS in their typing protocols. Nevertheless, PFGE will remain an affordable and relevant technique for small laboratories and hospitals in years to come.

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Declaration of Competing Interest

None.

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