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Pulsed-Field Gel Electrophoresis (PFGE): Principles and Applications in Molecular Epidemiology: A Review

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Abstract

The study of epidemiology related to bacterial outbreaks has come a long way since Mary Mallon (Typhoid Mary) was sent to a tuberculosis sanatorium at North Brother Island (East River of New York City) because city officials did not know what to do with her. However, it was just these types of unfortunate situations which spurred epidemiologists and microbiologists to develop techniques to answer the basic question posed to a molecular epidemiologist which is whether "strain A is related to strain B." The main hypothesis of molecular epidemiology is the following: within the "epidemiologic window," the isolates being considered will have either indistinguishable characteristics (i.e. the same genetic fingerprint) and thus be considered clonally related (same strain), or the isolates will have distinguishable characteristics and be considered different strains. Currently, multiple molecular techniques have been developed to assess this question. Although it is believed that DNA sequencing of highly variable genes will become the method of choice for molecular epidemiologists in the near future, currently the "gold standard" technique used in this discipline is pulsed-field gel electrophoresis (PFGE). In this review, the author described the principle, types and application of PFGE in molecular epidemiological studies of bacteria. Also the issues commonly influencing the quality of PFGE data and its analysis are discussed.

Keywords: PFGE, Molecular Epidemiology, Bacteria.

1.Introduction

The usefulness of agarose-gel electrophoresis to visualize the intracellular nucleic acid content of bacterial cells (Goering**,** 2010) was a revolutionary milestone in molecular biology that rapidly found clinical application including molecular epidemiology. The use of agarose-gel electrophoresis to comparatively analyze patterns of bacterial chromosomal restriction fragments was an important step toward genome-based epidemiological analysis. However, this approach encountered several difficulties. DNA sequences recognized by commonly employed restriction enzymes (e.g., EcoRI, HindIII) are abundantly dispersed around a typical 2–4 MB bacterial chromosome (e.g., on average >600 copies) and are thus difficult to accurately discriminate by electrophoresis. The average frequency with which restriction enzymes are expected to cut genomic DNA can be mathematically estimated and restriction enzymes with infrequent recognition sites in bacterial genomes have been known since the late 1970s (Roberts et al., 2010 and Stephenson, 2004). However, the megabase-size restriction fragments they produce cannot be accurately separated by conventional agarose-gel electrophoresis which is unable to

resolve DNA molecules larger than 40–50 kb due to their size-independent co-migration (termed "reptation") (Schwartz and Koval, 1989, Schwartz et al., 1983).

In 1982, Schwartz *et al.* introduced the concept that DNA molecules larger than 50KB can be separated by using two alternating electric fields (i.e. PFGE). Since that time, a number of instruments based on this principle have been developed, and the value of using pulsed fields has been demonstrated for separating DNAs from a few KB to over 10 megabase pairs (Mb). The development of PFGE has increased by two orders of magnitude the size of DNA molecules that can be routinely fractionated and analyzed. This increase is of major importance in molecular biology because it simplifies many previously laborious investigations and makes possible many new ones. Its range of application spans all organisms from bacteria and viruses to mammals (Smith *et al.,* 1986). PFGE has shown excellent ability to separate small, natural linear chromosomal DNAs ranging in size from 50-kb parasite microchromosomes to multimillion-bp yeast chromosomes. However, intact human chromosomes range in size from 50 million to 250 million bp (Mb), too large for direct PFGE separations (Smith *et al.*, 1986).

PFGE provides the means for the routine separation of fragments exceeding 6,000KB. Therefore, PFGE separates DNAs from a few kilo base (kb) to over 10 megabase pairs (Mb) (Kaufmann and Pitt, 1994). The technique of PFGE takes advantage of the elongated and oriented configuration of large DNA molecules in agarose gels at finite field strengths. An important bonus of this technique is the ease with which the genome size can be measured, a parameter that was previously subject to considerable error when measured by other techniques. One important outcome of the use of PFGE and restriction endonuclease digestion is the construction of a physical map. General applications of PFGE can be in the separation of whole chromosomes, the large - scale restriction mapping of chromosome regions and in using DNA fragment purification as an aid in cloning. PFGE will greatly facilitate the precise selection of very large fragments for cloning, and it provides rapid analysis of a large chromosomal region. PFGE has proven extremely powerful in the analysis of large DNA molecules from a variety of sources, including specifically fragmented genomes of bacteria (Smith *et al.*, 1987), mammals (Smith *et al.*, 1986), parasite protozoa (VanderPloeg *et al.*, 1984) and intact chromosomal DNAs from fungi (Schwartz and Cantor, 1984). The introduction of PFGE techniques for separating large DNA molecules has had an invigorating effect on the study of chromosomal DNA molecules, genome structure and electrophoretical theory. In this review, the author described the principle, types and application of PFGE in molecular epidemiological studies of some clinically important bacteria.

2. Types of PFGE Systems

PFGE resolves DNA molecules of almost a millimeter in length through the use of pulsed-field electric fields, which selectively modulate mobilities in a size-dependent fashion. The pulsed electrophoresis effect has been utilized by a variety of instruments (FIGE, TAFE,CHEF, OFAGE, PACE and rotating electrode gel) to increase the size resolution of both large and small DNA molecules (Basim, 2001). It is important when choosing a PFGE system to evaluate cost and performance in the light of projected use. There are different types of PFGE systems are described below.

2.1 Field-Inversion Gel Electrophoresis (FIGE)

In 1986, Carle, Frank and Olson developed a simpler system, FIGE, in which the two fields were 180° apart (Carle *et al.*, 1986). Electrode polarity was reversed at intervals, with a longer forward than reverse pulse time to generate a net forward sample migration. Net forward migration is achieved by increasing the ratio of forward to reverse pulse times to 3:1. To improve the resolution of the bands by FIGE, the duration of pulse times is increased progressively during a run. This is called switch time ramping. By changing pulse durations continually during the course of an experiment, FIGE has the advantages of straight lanes and simple equipment. All that is needed are standard gel boxes and a pulse controller. Today, FIGE is very popular for smaller fragment separations. FIGE provides

acceptable resolution up to 800 kilobases (600-750 kb).

2.2 Transverse-Alternating Field Gel Electrophoresis (TAFE):

This form of PFGE allows separation of large DNA fragments in a simple, convenient format without the drawbacks of earlier pulsed-field techniques. In TAFE, the gel is oriented vertically and a simple four-electrode array is placed not in the plane of the gel, but in front and at the back of it. Sample molecules are forced to zigzag through the thickness of the gel, and all lanes experience the same effects, so the bands remain straight (Steward *et al.,* 1988). As the molecules move down the gel, they are subjected to continual variations in field strength and reorientation angle, but to all lanes equally. However, the angle between the electric fields varies from the top of the gel (115°) to the bottom (approximately 165°) and hence molecules still do not move at a constant velocity over the length of the gel. TAFE technology, with regular and sharp separation of DNA bands, will be of special advantage in the study of genetics of many pathogenic protozoans, where such analysis was impossible before (Steward *et al.,* 1988). TAFE has been used for the separation of fragments up to 1,600 kilobase fragments.

2.3 Contour-Clamped Homogeneous Electric Fields (CHEF)

CHEF is the most widely used apparatus. The CHEF apparatus provides a more sophisticated solution to the distorting effects of both the edges of the chamber and the passive electrodes. CHEF has twenty-four point electrodes equally spaced around the hexagonal contour. In the CHEF system, there are no passive electrodes. All the electrodes are connected to the power supply via an external loop of resistors, all of which have the same resistance. This loop is responsible for setting the voltages of all the electrodes around the hexagonal contour to values appropriate to the generation of uniform fields in each of the alternate switching positions. The CHEF system sets the voltages at these 24 points. This apparatus produces electric fields that are sufficiently uniform so that all lanes of a gel run straight. CHEF uses an angle of reorientation of 120° with gradient of electro potential radiating from the positive to the negative pores. Molecules up to 7,000 KB can be separated by CHEF (Levene, 1992).

2.4 Orthogonal-Field Alternation Gel Electrophoresis (OFAGE)

In 1984, Carle and Olson reported similar apparatus that used two nonhomogeneous electric fields. The major drawbacks of these apparatuses were that because the electric fields were not uniform, and the angle between the electric field varied across the gel, DNA molecules migrated at different rates depending on their location in the gel. This is especially problematic in mammalian genome mapping, where a continuous distribution of fragment sizes is generated. Lane-to-lane comparisons and size estimations for digested genomic DNA are less straightforward when fewer discrete bands are being separated, as with the chromosomes of lower organisms like yeast. The angle between the electric fields varies from less than 180° and the more than 90°. DNA molecules from 1,000 to 2,000 KB can be separated in OFAGE (1 Chu *et al.,* 1986).

2.5 Rotating Gel Electrophoresis (RGE)

In England in 1987, Southern *et al.,* (1987) described a novel PFGE system that rotates the gel between two set angles while the electrodes are off. In RGE, the electric field is uniform and bands are straight because only one set of electrodes is used. RGE makes it easy to perform time and voltage ramping. It also enables users to study the effects of different angles, and even to vary these, during an experiment-angle ramping. RGE uses a single homogeneous field and changes the orientation of the electric field in relation to the gel by discontinuously and periodically rotating the gel. Switch times are too long in RGE. The DNA molecules migrate in straight lanes, due to the homogeneous fields, and DNA molecules from 50 KB to 6,000 KB can be separated by adjusting the frequency of the gel rotation. In addition, the angle of reorientation can be easily altered simply by changing the angle of rotation (Gardiner, 1991).

2.6 Programmable Autonomously Controlled Electrodes (PACE)

The PACE electrophoresis system offers precise control over all electric field parameters by the independent regulation of the voltages on 24 electrodes arranged in a closed contour. The flexibility of the PACE system derives from its ability to generate an unlimited number ofelectric fields of controlled homogeneity, voltage gradient, orientation and duration. The PACE system can perform all previous pulsed field switching regimens (i.e. FIGE, OFAGE, PHOGE, unidirectional pulsing), as well as generate voltage clamped homogeneous static fields. The PACE system separates DNA fragments from 100 bp to over 6 MB. The ability to alter the reorientation angle between the alternating fields permits an increased speed of separation for large DNA molecules. A computer-driven system known as PACE, designed by Lai *et al.* (1989) may be the ultimate PFGE device. It is an extremely useful tool for studying variables such as pulse time, temperature, agarose concentration, voltage and angles between fields affecting DNA migration in PFGE (Birren *et al.,* 1988).

2.7 Pulsed-Homogeneous Orthogonal Field Gel Electrophoresis (PHOGE)

The major difference between this instrument and other systems with homogeneous electric fields is that the field reorientation angle is 90°. PHOGE uses a 90° reorientation angle, but the DNA molecules undergo four reorientations per cycle instead of two. The DNA lanes in PHOGE do not run straight, a phenomenon, which has been described for gel runs involving multiple electric fields in this manner. This system separates DNA fragments of up to 1 Mb (Ziegler and Vols, 1992).

3. The Basic Components of PFGE System.

The basic components of a PFGE system consist of a gel box with some means of temperature regulation, a switching unit for controlling the electric fields, a cooler and a power supply (Basim et al 1999).

3.1 Gel Box

The basic design of PFGE boxes consists of an immobilized gel within an array of electrodes and a means of circulating the electrophoresis buffer. Voltage gradients of 10 volts/cm are commonly used in PFGE. Voltage gradients as high as 15 volts/cm have been used in field inversion separations of cosmid clones (Lai *et al.* (1989). The temperature of the buffer is controlled by a heat-exchange mechanism. Generally, the buffer is re-circulated throughout the gel box using inlet and outlet ports (Carle *et al.,* 1986).

3.2 High Voltage Power Supply

Precise control of the electric field gradient is necessary to obtain consistent PFGE separations. The output ratings of the power supply should therefore be high enough to meet both the voltage and current requirements of the gel box. A typical PFGE gel box has electrodes that are 25 to 50 cm apart. To achieve the commonly used range of voltage gradients of 1.5 to 15 volts/cm requires a power supply with a maximum voltage rating of 750 volts. The current drawn at this voltage in most PFGE boxes is about 0.5 amperes at 14°C using 0.5x TBE (1x TBE is 89 mM Tris pH: 7. 89 mM Boric acid and 2 mM EDTA) as running buffer.

3.3 *Switch Unit*

The ability to reproducibly control the switch interval is critical for the separation. The limited speed at which relays can switch will not accommodate the fast switching necessary for the PFGE separation of small DNA molecules (2-50 kb). A computer usually controls the relays. To overcome the drawbacks of electromechanical relays, high-voltage solid-state electronics has supplanted electromechanical relays in recently designed commercial PFGE systems. These switching units are commonly based on the use of metal oxide semiconductor field effect transistors (MOSFETs) in both switching and electrode voltage control circuits. These designs offer the advantages of improved reliability, the capability of high-speed switching (0.1 ms) and ample voltage (750 V) and current (0.5 amperes) ratings

(Lai *et al.,* 1989). These apparatuses have the ability to control the reorientation angles between electric fields. However, these instruments cannot provide fast enough switching for the improvement of the separation of DNA molecules smaller than 50 KB.

3.4 Cooler

Buffer recirculation is an important factor, as it eliminates temperature variations within the gel so as to alleviate buffer breakdown due to electrolysis. DNA molecule migration is sensitive to temperature, and thus a uniform temperature across the gel is needed to ensure even migration in each of the lanes. Buffer is re-circulated through the gel chamber by a reciprocating solenoid pump at a rate of about 450 ml/min. The buffer is chilled in its reservoir tank by cold water circulated through a glass tubing heat exchanger. Buffer temperature is thus maintained at 14 - 15^0C) throughout a typical run.

4. Factors Affecting PFGE.

PFGE separations are sensitive to a variety of different molecular and environmental variables. The principle significant variables are the molecular properties of the DNA, the pulse time, the electrical field shape, the electrical field strength, the gel composition, the sample concentration and the temperature.

4.1. Pulse Time:

In PFGE, DNA is subjected alternately to two electrical fields at different angles for a time called the pulse time. The molecules must presumably change direction prior to net translational motion. Each time the field is switched; larger molecules take longer to change direction and have less time to move during each pulse, so they migrate slower than smaller molecules. Molecules so small that their reorientation time is short compared to the pulse time will spend most of the pulse duration in conventional electrophoretic motion where size resolution is quite limited. As a result of this, resolution in PFGE is likely to be optimal for molecules with reorientation times comparable to the pulse time. At applied field strengths of about 10 V/cm, 0.1 s pulse times resolve DNA optimally in the 5-kb size range, while pulse times of 1,000 s at 3 V/cm are used to resolve 3-7 Mb molecules. Pulse times are selected so that DNA molecules of a targeted size spend most of the duration of the pulse reorienting rather than moving through the gel, which accounts for the long periods of time, usually days or weeks, needed to fractionate large DNA molecules. The chromosomal DNA molecules of*Saccharomyces cerevisiae* in the 10 MB range requires longer electrophoresis of approximately one week (Cantor *et al.*, 1988).

4.2.Electrical Field Shape.

A number of different electrical field configurations were employed in early PFGE experiments. It was apparent that certain aspects at the field shape were critical in achieving high-resolution PFGE separations. Electrical field strength can be adjusted to tune the size range of effective PFGE resolution. The resolution of PFGE is affected by the number and configuration of the electrodes used, because these alter the shape of the applied electrical fields. The most critical variable appears to be the angle between the alternate electrical fields. The most effective electrode configurations yield angles of more than 110^o. A continually increasing angle between the fields produces band sharpening that greatly enhances the resolution. The angle between the alternate fields is always greater than 90^0 where good resolution is observed. In cases of excellent resolution, field angles typically range from 120^0 to 150^0. In contrast, where poor resolution was seen, the field angles ranged typically from110^0 to 150^0 (Cantor *et al.,* 1988). Angles of 90^0 or smaller are not effective, probably because the DNA molecules easily become oriented midway between the two applied fields. Angles larger than 90^0 are more effective (Cantor *et al.*, 1988). While more complete studies on optimum angles are needed, it is clear that angles in the range of 120^0-150^0 provide very high resolution (Cantor *et al.*, 1988). Field strengths that decrease or angles that increase, progressively along the direction of the net DNA motion produce band sharpening because the molecules at the front of each DNA zone always migrate more slowly than those at the rear.

4.3. Electrical Field Strength.

Electrophoretic mobility is defined as the velocity per unit field. In most ordinary electrophoresis, the mobility is independent of field strength. This independence is expected if the properties of the molecules are not directly altered by a separation process. The field strength affects mobility in two ways. The mobilities of 100-500 KB DNA show an approximately linear dependence on field strength. The field strength affects the DNA size of the transition between the two zones of resolution (Cantor *et al.*, 1988).

4.4. Reorientation Angle.

The widening of the reorientation angle should yield sharper bands and better resolution. The separation of yeast chromosomes is nearly identical for those chromosomes separated with reorientation angles of 110^0 and 165^0. However, when reorientation angles from 105^0 to 165^0 are used to separate molecules in the size range of *S.cerevisiae* (200-3,000 kb), there is a 4-fold difference among the DNA velocities observed with these different angles (Lai *et al.*, 1989). The increase in mobility obtained with smaller reorientation angles is even more pronounced when separating larger molecules. Most commercially available pulsed-field gel boxes use a fixed angle of 120^0 between the alternating fields.

4.5. Voltage.

As with switch time, the choice of the voltage used in PFGE must also be varied with the size of the DNA to be separated. While voltage gradients of 6-10 V/cm can be used to separate molecules up to 1 Mb, resolving molecules larger than this in pulsed field gels requires a reduction in voltage gradient (Birren *et al.*, 1988). Separation of chromosomes from the yeast *S. pombe* (3 Mb, 5 Mb and 6 Mb) requires that voltage gradients do not exceed 2 V/cm. The even larger chromosomes of *N. crassa* (larger than 12 Mb) were separated at 1.5 V/cm (Orcbach *et al.*, 1988). The practical effect is to increase the run times for larger DNA molecules. Thus, electrophoretic separation of *N. crassa* chromosomes required up to 7 days. When the voltage gradient is reduced to separate large DNAs, switching intervals must be lengthened (Orcbach *et al.*, 1988).

4.6 Temperature.

In conventional gel electrophoresis, DNA molecules were run at room temperature but, in PFGE, DNA is run at a low temperature (between 14^0C and 15^0C).Temperature has a dramatic effect on DNA mobility in PFGE. Temperatures between 14^0C and 22^0C are generally regarded as the best compromise between speed and resolution while gels can be run at room temperature, it is usually necessary to circulate the buffer through a heat exchanger to dissipate the heat generated by the voltage gradients used during most pulsed field runs (Cantor *et al.*, 1988). The velocity of lambda DNA at 34^0C is twice that at 4^0C. However, gels run at temperatures as high as 34^0C show diminished resolution (Lai *et al.*, 1989).

4.7. Switch interval.

The single most important determinant of mobility in PFGE is the interval at which the direction of the electric field is switched. If the switching interval is increased beyond the time required for a fragment to reorient, then the fragment will spend a large portion of the gel run migrating, as in conventional electrophoresis, with a resulting loss in resolution. The choice of an appropriate switching interval for PFGE must reflect the size range of the fragments to be resolved. Birren *et al.* (1988) have measured the velocity of DNA molecules from 50 to 1,000 KB in PFGE with switch times of from 5 to 300 s. The highest resolution for molecules of a given size is obtained by using the shortest switch intervals, which permit separation of the complete size range of the fragments.

4.8. Agarose Concentration

The agarose concentration will affect the separation obtained with PFGE. Faster DNA migration occurs in gels of lower agarose concentration. The DNA monomer (48.5 kb) migrates 50% faster in PFGE of 0.6% agarose compared to a 1% gel. The DNA bands which are

quite diffuse in the 0.7% gel become increasingly sharp as the agarose concentration is raised in 1.4% and 1.8% gels over identical times. The distance migrated by the identical samples demonstrated the decrease in velocity as the agarose concentration is increased (Birren *et al.*, 1988).

4.9. Restriction Enzymes.

The ability of restriction enzymes (REs) to cut DNA at a specific sequence of bases has greatly stimulated the growth of recombinant DNA technology. Over 1,900 REs are known, and of there 275 are available from companies based around the world (Bhagwat, 1992). The common restriction enzymes, EcoR I and Hind III, digest bacterial and mammalian DNA to fragments averaging approximately 4 KB in sizes much too small for PFGE. For this reason, it is advisable to use enzymes, which have relatively few sites and give larger fragments from the target DNA in PFGE (Bhagwat, 1992). Any enzyme producing a large number of small fragments (smaller than 10 kb) is unlikely to be useful for PFGE and mapping. A major factor in selecting suitable restriction enzymes is the base composition (%G+C content) of the target DNA. Analysis by PFGE and rare-restriction enzymes have been useful for obtaining relevant information on genome size, characterizing various strains at the DNA level, following the genetic history of a particular strain, constructing physical and genetic maps of bacterial chromosomes and studying chromosomal dynamics among bacteria (Grouthues and Tmmler, 1991). Enzymes that recognize sequences larger than 6 bp are potentially useful in genome mapping because they generate large fragments (Bhagwat, 1992).

There are nine restriction enzymes commercially available with an 8-bp recognition sequence. Of these, Not I, Sfi I, Srf I, Ase I, Pac I, and Swa I are rare-cutters in genomes with a G+C content of about 35-55%. Below and above these margins the number of fragments becomes too small and too large, respectively. Pac I (AATTAATT), Swa I (ATTTAAAT), Pme I (GTTTAAAC) and Sse 83871 (CCTGCAGG) are new on the market. On the other hand, Pac I and Swa I enzymes should be useful especially for genomes with a G+C contents in the range of 45-65% (Burmeister, 1992). The 4-bp pair sequence CTAG seems to be selected against in most bacterial genomes. This tetranucleotide is part of the recognition sequence of Spe I (A/CTAGT), Xba I (T/CTAGA), Nhe I (G/CTAGC) and Avr II (C/CTAGG). CTAG is found infrequently in most prokaryotes and restriction endonucleases that include this sequence in their recognition sequence cut bacterial genomes infrequently (Burmeister, 1992). PFGE of large fragments of DNA generated using infrequently cutting Swa I and Pac I restriction endonucleases were used in genome analysis of *Xanthomonas axonopodis pv.vesicatoria*, a causal agent of leaf spot disease of pepper and tomato, and optimal conditions for digestion in the genome analysis were determined (HacÝoÛlu *et al.*, 19).

5. PFGE Analyses and Interpretation

No matter how simple or sophisticated, the value of any data-generating process ultimately resides in the degree to which the information produced can be meaningfully understood and analyzed. In terms of this review, the potentially “meaningful” purpose behind the PFGE method is the comparison of two or more bacterial isolates to obtain a sense of genomic relatedness, usually in an epidemiological context. While visual observation of PFGE banding patterns may be adequate for an initial impression, its usefulness becomes increasingly limited the greater the physical distance between the patterns being compared (i.e., not in close proximity on a gel or on different gels). Thus, commercially available programs have been developed for computer-assisted analysis (CAA) such as BioNumerics and GelCompar (Applied Maths, Sint-Martens-Latem, Belgium) and Diversity Database Fingerprinting Software (Bio-Rad Laboratories, Hercules, Ca). (Goering**,** 2010)

Regardless of the developer, CAA programs tend to be expensive with potentially steep learning curves. Overall, they serve to produce a numerical representation of PFGE banding pattern relatedness (i.e., based on the number and position of bands). For example, the commonly used Dice coefficient calculates similarity as a function of the number of common bands in two

PFGE patterns divided by the band totals for the two isolates (i.e., $_{SD} = 2N_{AB}/N_A + N_B$) (Vauterin and Vauterin, 2006). Thus, two patterns of 20 bands with a four-band difference (18 bands in common) would have a similarity by Dice coefficient of 0.9. However, despite their apparent sophistication, it is important to underscore the fact that CAA programs require significant input by the user with a far from automatic output. Proper comparison of intra- and inter-gel lanes (normalization) as well as comparison of data between different laboratories or over multi-year time periods requires that every gel contain the same specific PFGE reference pattern (i.e., the size standard) placed in uniform fashion in several gel lanes, which the program is directed to identify. At a minimum, this would mean standards placed in the outermost and middle gel lanes although, in practice, the more standard lanes present the greater the precision of the normalization. Thus, the investigator must weigh the balance between the number of samples analyzed and the degree of normalization precision. Beyond this, it should be emphasized that CAA programs are not capable of acting independently to accurately identify all bands in a PFGE pattern. Artifacts (e.g., an occasional brightly fluorescing spot) are frequently misidentified as bands and even optimized settings cannot insure the detection of subtle pattern differences the human eye may detect. Therefore, the end user must remain the final judge of CAA band assignments (Cardinali and Martini, 1999,Rementeria et al., 2001 and Van Belkum et al., 2007). In addition, whether visual or computer assisted, PFGE analysis is constrained by the limitations of electrophoretic resolution such as co-migration of fragments differing less than 5–10% in size (Struelens et al., 2001 and Goering, 2004) and difficult visualization of fragments <50 KB in size.

6. Applications of PFGE

The advent of PFGE techniques for the resolution of large DNA molecules has provided a new analysis approach for bacterial genomes (Dempsey et al., 1991). The PFGE of DNA fragments obtained using different enzymes is a powerful technique for quick resolution of the bacterial genome into a small number of large fragments. PFGE separated genomic DNA fragments obtained by using restriction endonucleases produce a discrete pattern of bands useful for the fingerprinting and physical mapping of the chromosome (Correia et al., 1994) as well as useful to establish the degree of relatedness among different strains of the same species (Correia et al., 1994). PFGE has proved to be an efficient method for genome size estimation and the construction of chromosomal maps, as well as being useful for the characterization of bacterial species (Basim et al., 1999; Churin et al., 1995; Roussel et al., 1994). PFGE technology has proven invaluable for the accurate estimation of genome size and in the construction of physical maps of a diverse range of prokaryotic organisms (Bourke et al., 1995; Pyle et al., 1990). This technique is a powerful tool for genome characterization and has led to the construction of the physical map of more than 180 bacterial chromosomes (Bourgeois et al., 1995). PFGE will greatly facilitate the precise selection of large DNA fragments for cloning. REs which are specific for cutting infrequently occurring sequences are used to create large DNA fragments which are then separated by PFGE. By blotting and hybridization the fragments containing the desired gene are determined. This region is recovered from the gel and cloned (Gardiner, 1991; Ziegler and Vols, 1992). This powerful molecular tool allows for easy isolation of the individual restriction fragments for further restriction mapping, gene insertion and functional gene mapping (Smith et al., 1988). The application of PFGE in molecular epidemiological studies of someclinical bacteria is discussed below

6.1. Shigella spp.

Shigella spp. is one of the most prevalent food- and water-borne pathogen that is consistently associated with dysentery and persistent diarrhea (Ke et al., 2011). Shigellosis, the disease caused by Shigella, kills an estimated 1.1 million people per year worldwide, 60% of them children under the age of 5 (Weissman et al., 1975), and can result in reduced growth in children who survive. Shigella species appear highly adaptable to selective pressure and have developed resistance to a number of antimicrobials with patterns of

resistance varying temporally and geographically with antimicrobial usage patterns (Rowe-Magnus and Mazel, 2002; Goh et al., 2010; Cambray et al., 2010; Fluit and Schmitz,2004). Resistant clones of Shigella have emerged in Argentina (White et al., 2001; Madiyarov et al., 2010; WHO 1987). Shigella flexneri serotype X variant, which emerged in China in 2001, has rapidly spread, including through Argentina according to recent report (Talukder et al., 2006), undergoing frequent serotype switching and acquiring resistance to multiple antimicrobials in the process (Nastasi et al., 1993). Recently study conducted in Argentina it was detected clusters of shigellosis of public health importance, which have been confirmed by PFGE as consisting of closely related clones, and informed local public health efforts (Vinas et al., 2013). Thus PFGE proved to be a useful tool for surveillance of the disease in an area. In a previous study based on PFGE indicated the evolutionary aspects where the type 7 and type 1 isolates of S. dysenteriae were probably evolved from a same precursor, while the type 2 and S. flexneri type 2a were probably evolved and diversified from a common progenitor (Pal et al., 2013). PFGE analysis of certain strains of Shigella isolates in Bangladesh showed that S. sonnei biotype a strain was genetically more diverse than biotype strains of other Shigell isolates, and revealed that strains having different integron patterns belonged to different clusters Ud-Din et al., 2013). This finding is congruent with a previous study (Ranjbar et al., 2007).

6.2. Salmonella spp.

Salmonella enteritidis remains a significant pathogen and a substantial threat to the food supply. It also represents one of the most genetically homogeneous serotypes of Salmonella, and certain clonal lineages remain intractable to differentiation by commonly used conventional subtyping methods (Fitzgerald et al., 2007; Sukhnanand et al., 2005; McQuiston et al., 2008; Xi et al., 2008; Hudson et al., 2001; Olsen et al., 1994; Zheng et al., 2007; Wise et al., 2009; Cebula et al., 2005). The unusual genetic homogeneity observed among certain lineages of S. Enteritidis strains remains intriguing. Recent population genetic studies suggest that most S. Enteritidis strains belong to a single multilocus genotype (Botteldoorn et al., 2010; Liu et al., 2011; Olson et al., 2007). A subpopulation of this clone was shown to associate more frequently with egg-related salmonellosis and clinical illness (Botteldoorn et al., 2010). In a study it was described the natural genetic variation within S. Enteritidis isolates associated with a widespread egg contamination event and retaining PFGE pattern JEGX01.0004 and analyzed the comparative evolutionary genetics within this important foodborne pathogen and several of its closest relatives. Based on both PCR and sequencing evidence, numerous studies have found little genetic variation within S. Enteritidis (Olson et al., 2007; Guard et al., 2011; Shah et al., 2012; Tankouo-Sandjong et al., 2012). In a recent report on genomic diversity estimation for the S. Enteritidis PFGE Pattern JEGX01.0004 showed consistency with other diversity comparisons described between two S. Enteritidis isolates of phage type 13 (Guard et al., 2011). This variation was observed both as SNP variation among 366 genes as well as the presence and absence of numerous phages and plasmids among these close relatives. This genetic variability was used to define the most variable genes and to assess population and phylogenetic evolutionary patterns for these important foodborne pathogens. This report on comparative genomics approach allowed investigators to cluster clinical isolates within the context of their environmental source and farm isolates (Allard et al., 2013). In one finding based on the results obtained by PFGE, MLVA, PCR, and sequencing, the Salmonella monophasic strains seemed to have maintained great homogeneity over the years. Another study carried out with some Salmonella (4,5,12 :-) isolates from the United States and Spain concluded that this strain most likely represents multiple clones with distinct geographical distributions that emerged through independent deletion events (Soyer et al., 2009). This hypothesis was supported by another study and expanded the information given by Soyer et al., (Soyer et al., 2009) about Spanish monophasic strains, as they studied a larger number of strains and sequenced the fragments flanking the fljAB deletions (Laorden et al., 2010).

6.3. Vibrio spp.

Vibrio cholera is a Gram-negativebacterium that lives freely in aquatic environment and causes cholera (Singh et al., 2001). Cholera is endemic in many parts of the world, especially the countries that lack proper sanitation managements. In Malaysia cholera outbreaks due to the V. cholerae O1 serotype which occurs periodically (Vadivelu et al., 2000). The ratio of distribution of V. cholerae O139 to O1 serogroups isolated from seafood from 1998 to 1999 was 14:1. Non-O1/non- O139 V. cholerae is also frequently isolated from seafood and water sources but has not been implicated in any major outbreaks (Elhadi et al., 2004; Chen et al., 2004). Although non-O1/non-O139 V. cholerae is not associated with any major outbreak, it has been reported to be responsible for sporadic cases of diarrhea (Nandi et al., 2000; Rivera et al., 2001; Faruque et al., 2004). The well-known genes associated with colonization are ctxA and tcpA. These genes are commonly found in O1 and O139 serogroups. Olivier et al., (Olivier et al.,2007) had reported that accessory toxins such as hemolysin and multifunctional autoprocessing RTX toxin in El Tor .

V. cholerae are involved in prolonged colonization without cholera toxin (CT) or toxin-coregulated pili (TCP). As these accessory virulence genes are commonly found in all serogroups of V. cholerae, it is of interest to investigate the involvement of these accessory virulence genes for prolonged colonization in other serogroups of V. cholerae. Molecular subtyping of pathogen is important for tracing a new or previously found virulent or multidrug-resistant clone. Genomic variation and epidemiological study for different serogroups of V. cholerae have been carried out using many DNA-fingerprinting tools. PFGE is the most common subtyping tool to define strains from outbreaks and from sporadic cases of cholera as it has the highest discriminatory ability (Chen et al., 2004). A combination approach of PFGE and MLVA analysis may yield more information about the clonality of bacterial pathogens. PFGE is the most commonly used subtyping method to determine the epidemiological relatedness of the strains. In a study, the 23 O1 strains were subtyped into 18 pulsotypes (Teh et al., 2010). However, as different PFGE conditions were used by different researchers in the region, direct comparison was difficult. Adoption of a standardized PFGE protocol such as the PulseNet PFGE protocol proposed by CDC PulseNet, USA would greatly enhance interlaboratory comparison and improve tracking of *V. cholerae* strains among the endemic countries in the region (Teh et al., 2010). In another study combination of both PFGE and MLVA approaches for molecular typing to examine the bacterial genome by different criteria resulted from each individual assay. Similar results were observed not only in PFGE analysis but also in MLVA, though the components of some of the minor clusters differed. Furthermore, even the oldest El Tor variants studied had already showed some genetic diversity and were divided into different minor clusters. These results suggested that El Tor variants were related to various types of typical El Tor strains rather than classical type strains, and that the El Tor variant epidemic was likely to be caused by simultaneous or sequential emergence and expansion of multiclones, and not by the prevalence of a certain single clone (Morita et al., 2010).

6.4. Escherechia coli

STEC (Shiga toxin-producing *Escherichia coli*) consists of a group of food- and waterborne pathogens that are known to cause human gastrointestinal diseases with a wide range of clinical spectra starting from watery and bloody diarrhea to hemorrhagic colitis (Gyles, 2007; Karmali, 2009). Occasionally disease symptoms result in the life-threatening, hemolytic uremic syndrome (HUS). Shiga toxins (Stx1 and Stx2) are the key virulence factors contributing to the development of HUS. Although more than 200 different serotypes of STEC have been isolated, O157:H7 has been the serotype most commonly associated with HUS in North America. Recent epidemiological studies have reported additional non-O157 serogroups, including O26, O45, O91, O103, O104, O111, O113, O121, and O145, among STEC strains that were linked to severe human disease in the United States, Europe and countries of Latin America (Brooks et al., 2005; Bettelheim, 2007; Caprioli et al., 2005; Mathusa et al., 2010; Beutin and Martin, 2012). PFGE, the

current gold standard molecular method, for assessing STEC O157 genetic diversity (Swaminathan et al., 2001), primarily detects insertions and/or deletions within genomic regions specific to STEC O157 (Kudva et al., 2002). In one study it is reported that the PFGE based diversity pattern surpassed polymorphism-derived genotype diversity overall, although the PFGE polymorphisms are known to change between subcultures of the same strain of STEC O157:H7 (Iguchi et al., 2002) and that plasmid DNA migration within PFGE can be unpredictable (Barrett et al., 2006). This group identified ten different PFGE patterns in two or more strains with different polymorphism genotypes with 42 polymorphism-derived genotypes, which have immediate potential to resolve genetically distinct STEC O157 strains comprising an outbreak investigation that may be indistinguishable by PFGE. They suggested future studies should be conducted that compare STEC O157 diversity assessed with the polymorphism-derived genotypes and PFGE using outbreak samples (Clawson et al., 2009). In a recent study conducted on extended-spectrum beta-lactamase (ESBL)-producing *E. coli* from hospitals in Bangladesh phenotypic and molecular characterization of isolates using PFGE-typing revealed 26 different pulsotypes, but identical pulsotype showed 6 isolates of serotype O25:H4. Thus PFGE profile analysis showed heterogeneity among majority of isolates except for a few that could be clustered into a single PFGE type (Lina et al., 2014)

Conclusion

After more than thirty years since its development and first use as a typing method, PFGE remains the most commonly employed means of assessing epidemiological relationships for most clinically relevant bacteria. This longevity as a “gold standard” is due to a number of factors. Despite the fact that every genetic change and macro-restriction fragment are not detected, the sum of the visible fragment sizes for the average bacterial PFGE pattern represents greater than 90% of the total genome. Thus, first and foremost, PFGE provides a highly visual sense of global chromosomal monitoring. For example, in well characterized MRSA lineages such as USA300, loss of the staphylococcal chromosomal cassette encoding methicillin resistance (SCC*mec*) or its adjacent arginine catabolic mobile element (ACME) can be recognized by changes in PFGE patterns (Goering et al., 2007). In addition, PFGE analysis is accomplished with standard methods varying little for different organisms other than the initial choice of Gram-negative vs. Gram-positive protocol and appropriate restriction enzyme (usually SmaI and XbaI for gram positives and gram negatives, respectively). Nevertheless, PFGE is a third-generation molecular typing approach which, along with a variety of other microbiology-related assays, is moving toward analysis based on DNA sequence (Van Belkum et al., 2007). Recent years have seen great advances in bacterial total genome sequencing and bioinformatics analysis. From an epidemiological standpoint, these fourth-generation methods have necessarily concentrated on the development of informative single or multi-locus strategies (Van Belkum et al., 2007 and Foley et al., 2009). These approaches and others yet to be described hold great promise as they are being developed and validated, clearly pointing toward the future of molecular epidemiology. However, for all of the reasons discussed here, PFGE remains broadly applicable with an enormous data and user base that it is reasonable to believe will continue to sustain it as a meaningful approach to molecular typing for years to come.

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