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Pulsed-Field Gel Electrophoresis as a molecular tool for characterizing genomes of certain food-borne bacterial isolates - A Review

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ABSTRACT

The evolutionary transition from phenotypic to molecular analysis of infectious disease in bacterial epidemiology led to the search for suitable approaches to ascertain genomic relatedness or heterogeneity between bacterial clinical isolates. Pulsed-field gel electrophoresis (PFGE) technique was developed for separating and analyzing long DNA fragments of several megabases in alternating electric field. Comparison of electrophoresis profiles of restriction enzyme-digested genomic DNA from bacterial isolates has proved to be a useful epidemiological tool for genetic discrimination of bacterial strains, detection of genetic relatedness, to locate the source of outbreak and to monitor the spread of the microorganisms in endemic zones. PFGE is considered as a gold standard method for typing of bacterial isolates because of the remarkable endurance of this technique as a typing method for the last 20 years in molecular epidemiology. In this current review the pros and cons of PFGE use in current molecular microbiological research are explored in the context of determination of genome organization of certain food-borne bacterial isolates causing infectious diseases in human beings.

Keywords: PFGE; Epidemiology; Molecular typing; Restriction enzyme; Genome diversity

1. INTRODUCTION

In recent years much of the rapid progress that is being made in molecular biology depends upon the ability to separate, determine size and visualize DNA molecules. Gel electrophoresis (Lai *et al.*, 1989) is one of the most commonly used separation techniques in the molecular biology laboratory. In conventional gel electrophoresis DNA molecules are separated in a gel matrix (i.e. agarose or polyacrylamide) under a static electric field. DNA fragments from 100 to 200 bp up to 50 kb are routinely separated by this technique. In 1982 Schwartz *et al.*, (Schwartz *et al.*, 1982) introduced the concept that DNA molecules larger than 50 kb can be separated by using two alternating electric fields employed in Pulsed field gel electrophoresis (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 because of the magnitude of 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 (Gardiner, 1991) 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. PFGE provides the means for the routine separation of fragments exceeding 6,000 kb (Gardiner, 1991; Steward *et al.,* 1988; Maloy *et al.,* 1994; Kaufmann and Pitt, 1994).

Therefore, PFGE separates DNAs from a few kilobase (kb) to over 10 megabase pairs (Mb) (Levene, 1992). The combined use of PFGE and restriction endonuclease digestion facilitated the determination of genome sizes as well as is the construction of physical maps of large numbers of bacterial isolates. The 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 initial step in cloning. The introduction of PFGE techniques for separating large DNA molecules has had a stimulating effect on the study of chromosomal DNA molecules, genome structure and diversity. In this review, the use of PFGE in molecular microbiological analysis and the detection of genomic heterogeneity and relatedness of certain pathogenic food-borne bacterial isolates are discussed.

2. PFGE TYPES

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.

2. 1. Field-Inversion Gel Electrophoresis (FIGE)

In 1986, Carle, Frank and Olson developed a simpler system, FIGE, in which the two fields were placed 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. FIGE is very popular for smaller fragment separations and 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. 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. TAFE has been used for the separation of fragments up to 1,600 kilobase fragments.

2. 3. Contour-Clamped Homogeneous Electric Fields (CHEF)

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. CHEF uses an angle of reorientation of 120° with gradiations of electropotential 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)

A similar apparatus that used two nonhomogeneous electric fields was reported by Carle and Olson (Carle and Olson, 1984) in 1984. DNA molecules from 1,000 to 2,000 kb can be separated in OFAGE (Carle and Olson, 1984; Chu *et* al., 1986).

2. 5. Rotating Gel Electrophoresis (RGE)

In England in 1987, Southern (Southern *et al.*, 1987) described a novel RGE 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. 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. The DNA molecules migrate [illegible] 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; Ziegler and Vols, 1992).

2. 6. Programmable Autonomously-Controlled Electrodes (PACE)

The PACE system can perform all previous pulsed-field switching regimens (i.e. FIGE, OFAGE, PHOGE, unidirectional pulsing), as well [illegible] 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.,* (Lai *et al.*, 198[illegible]) is considered to 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 gel boxes 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. This system separates DNA fragments of up to 1 Mb (Ziegler and Vols, 1992).

3. PFGE APPLICATIONS

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).

4. ROLE OF PFGE IN THE STUDY OF GENOMES OF SELECTED PATHOGENIC BACTERIAL ISOLATES

4. 1. *Shigella spp.*

Shigella spp. is one of the most prevalent food- and water-borne pathogen which 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).

4. 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).

4. 3. *Vibrio* [illegible]

Vibrio cholerae is a gram negative bacterium which lives freely in aquatic environment and causes cholera (Singh *et al.*, 2001). Cholera is endemic in many parts of the world, especially the countries which lack proper sanitation managements. In Malaysia cholera outbreaks due [illegible] 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 [illegible] 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 *ctx*A and *tcp*A.

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 current 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).

4. 4. *Escherechia coli*

STEC (Shiga toxin-producing *Escherichia coli*) consists of a group of food- and water-borne 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). Occassionally 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).

5. DISCUSSION AND CONCLUSION

PFGE has been used effectively as a molecular subtyping tool in outbreak investigations and surveillance and has been used to infer genetic relatedness between isolates of bacterial species (Shere *et al.,* 1998; Swaminathan *et al*., 2001). PFGE has been used for over a decade in epidemiologic studies of many eukaryotic and prokaryotic organisms and has proved to be a robust typing method for investigations of food-borne outbreaks and for hospital epidemiology. Alternative promising molecular methodologies for greater precision as well as higher efficiency for molecular epidemiologic analyses of microbial pathogens include fluorescent amplified fragment length polymorphisms (Zhao *et al.*, 2000) and DNA microarrays (Call *et al.*, 2001; Salazar and Caetano-Anolle ´s, 1996). According to a report, for proper estimation of genetic relatedness between different isolates six or more restriction enzymes would be needed for investigating proper epidemiologic relationships using PFGE method (Davis *et al.*, 2003).

The aim of this present review is to demonstrate the usefulness of PFGE techniques as a tool to be used in identifying source, clonal relatedness and spread of bacterial isolates in hospitals and countries where the isolates have been recovered or encountered. Future applications for PFGE techniques may include protein separations and nucleic acid sequencing and studies of DNA topology. This review will help current molecular biologists to assess the present status of this valuable technique so that further modifications of this method may provide an indispensable technology for genome study of organisms.

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