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Usefulness of Pulsed Field Gel Electrophoresis Assay in the Molecular Epidemiological Study of Extended Spectrum Beta Lactamase Producers

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1. Introduction

A major problem in several health institutions, countries and regions is to categorically define or delineate the source or index case of any microbial organism/s during an outbreak of an infection. Understanding bacterial distribution and their relatedness is essential for determining the epidemiology of nosocomial infections and aiding in the design of rational pathogen control methods. The role of bacterial typing is to determine if epidemiologically identical or related isolates are also genetically related **[Singh A et al, 2006].** Based on phenotypic and genotypic typing methods, multi-drug resistant bacteria organisms such as extended spectrum beta lactamase (ESBL) enzyme producing pathogens e.g. *Escherichia coli* and *Klebsiella pneumoniae* can be traced to have been transferred from one hospital to another, from one country or region to another. Such information and knowledge have greatly assisted clinicians and health care policy makers to determine the best approach of stopping or eliminating such spreads and transfers of the pathogenic organisms involved in the infection.

As noted in the reviews by Singh A et al, the use of molecular methods for typing of nosocomial pathogen has assisted in efforts to obtain a more fundamental assessment of strain interrelationship **[Singh A et al, 2006].** Establishing clonality of pathogens can aid in the identification of the source (environmental or personnel) of organisms, distinguish infectious from non infectious strains, and distinguish relapse from reinfection. Many of the species that are key hospital-acquired causes of infection are also common commensal organisms, and therefore it is important to be able to determine whether the isolate recovered from the patient is a pathogenic strain that caused the infection or a commensal contaminant that likely is not the source of the infection. Likewise, it is important to know whether a second infection in a patient is due to reinfection by a strain distinct from that causing the initial infection or whether the infection is likely a relapse of the original infection. If the infection is due to relapse, this may be an indication that the initial

treatment regimen was not effective, and alternative therapy may be required **[Singh A et al, 2006].**

Gel electrophoresis and in particular Pulsed-Field-Gel-Electrophoresis (PFGE) is a tool that has made genotyping of bacterial isolates possible. The PFGE is a laboratory technique used for separation of large deoxyribonucleic acid (DNA) molecules if electric current that periodically changes direction is applied to it. The PFGE is the "gold standard "technique used in this discipline of molecular epidemiological studies and it is basically the comparison of large genomic DNA fragments after digestion with a restriction enzyme that cuts infrequently. Since the bacterial chromosome is typically a circular molecule, the digestion by the enzyme yields several linear large DNA molecules. Moving these large DNA molecules posed a problem but Schwartz and Cantor in 1984 introduced a voltage gradient that gave better resolution and the ability to move large molecules **[Schwartz DC & Cantor CR, 1984]**

Conventional agarose gel electrophoresis can only be used for the separation of DNA fragments that ranges between 20 – 25 base pair (kbp) by using specialized apparatus no matter how long it is run. The distance between DNA bands of a given length is determined by the percent agarose in the gel. The disadvantage of higher concentrations is the long run times (sometimes days). PFGE uses a special type of agarose that has a larger matrix pore sizes even at a higher percentages such as 1%. The most commonly utilized PFGE methods approaches include the contour-clamped homogenous electric field (CHEF) and field inversion gel electrophoresis (FIGE) **[Carle, G. F et al, 1986; Finney, M. 1993].** Field inversion gel electrophoresis utilizes a conventional electrophoresis chamber in which the orientation of the electric field is periodically inverted by 180^{o} and has an upper limit of resolution about 200kbp. CHEF uses a more complex electrophoresis chamber with multiple electrodes to achieve highly efficient electric field conditions for separation; typically the electrophoresis apparatus reorients the DNA molecules by switching the electric fields at 120^{o} angles. CHEF can separate even up to 2-3 Mbp.

Interpreting DNA fragment patterns generated by PFGE and relating or associating them into epidemiologically useful information for typing nosocomial pathogens, the clinical microbiologist or researcher must understand how to compare PFGE patterns and how random genetic events can alter these patterns. Ideally, the PFGE isolates representing an outbreak strain will be indistinguishable from each other and distinctly different from those of epidemiologically unrelated strains. If this occurs, the outbreak is relatively easy to identify. A random genetic activity such as mutation in a DNA can occur and when this happens, it will change the restriction fragment profile obtained during the course of the outbreak **[Hall LMC, 1994; Quintiliani R., Jr., & P. Courvalin, 1996; Thal LA et al, 1997]** These random variations in the fingerprints will depend on the organism and the time period of the outbreak

The aim of this study is to demonstrate the usefulness of PFGE techniques as a tool to be used in identifying outbreaks of bacterial infection and hence can be used as a tool for infection control measures in a hospital. Also to determine its importance in delineating the clonal relatedness or diversity of bacterial strains isolated from several regional hospitals in Trinidad and Tobago. The PFGE has been shown to be useful in the determination of the sources, clonal relatedness and spread of bacterial isolates in hospitals and countries where the isolates have been recovered or encountered.

2. Materials & methods

More than 230 strains of *Klebsiella pneumoniae* and *Escherichia coli* obtained routinely from three major regional hospitals in Trinidad and Tobago were used for this study. These non consecutive bacterial isolates were identified using standard microbiological methods as had been previously reported **[Akpaka PE & Swanston WH; 2008].** The initial screening for ESBL production by these pathogens using MIC values at concentrations and breakpoints recommended by the CLSI for ESBL screening **[CLSI 2010]** were performed with the automated micro dilution MicroScan WalkAway-96 System (Siemens, USA). Structured standardized questionnaire was used to extract epidemiological information from hospital records of the patients yielding these isolates. Such information included bio data, gender, hospital facilities where the patients were attended to, clinical signs and symptoms, diagnosis, other forms of investigations and treatments, treatment failures and complications.

2.1 Confirmation of ESBL phenotypes

In accordance with the protocols from the manufacturer to phenotypically determine the ESBL production by bacterial isolates, the E-test strips (AB Biodisk, Solna Sweden), a very sensitive and convenient assay to use was employed to confirm ESBL production in the isolates. The control strain for all the phenotypic testing were *E. coli* ATCC 25922 (negative control) and *K. pneumoniae* ATCC 700603 (ESBL positive).

2.2 Multiplex PCR amplification

The detection of gene sequences coding for the TEM, SHV, and CTX-M enzymes were carried out using multiplex PCR as previously described with some modifications **[Monstein HJ et al, 2007].** The cycling conditions used in the PCR assays were as previously described **[Paterson DL et al, 2003; Boyd DA et al, 2004].** The oligonucleotide primer sets specific for the SHV, TEM and CTX-M genes and the cycling conditions used in the PCR assays were as described previously and are depicted in the Table 1 below.

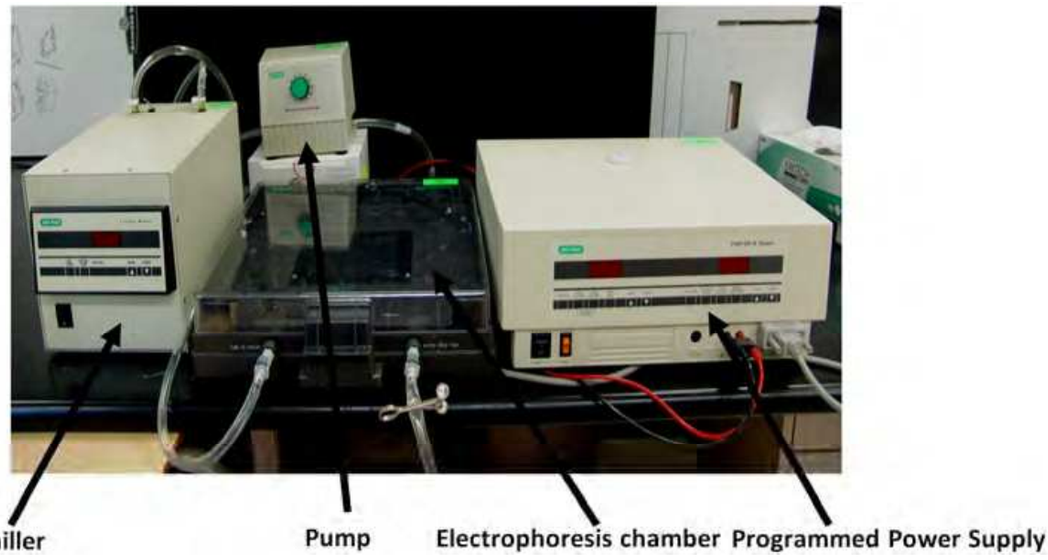
Gene	Primer	bp Sizes	Reference
*bla*SHV	5′-ATG CGT TAT ATT CGC CTG TG-3′	747-bp	Paterson DL *et al*
	5′-TGC TTT GTT ATT CGG GCC AA-3′		
*bla*TEM	5′-TCG CCG CAT ACA CTA TTC TCA GAA TGA-3′	445-bp	Boyd DA *et al*
	5′-ACG CTC ACC GGC TCC AGA TTT AT-3′		
*bla*CTX-M	5′-ATG TGC AGY ACC AGT AAR GTK ATG GC-3′	593-bp	Boyd DA *et al*
	5′- TGG GTR AAR TAR GTS ACC AGA AYCAGC GG-3′		

Table 1. Showing primers used for amplifications of the genes in ESBL producers

A Multiplex PCR method previously described **[Woodford et al, 2006]** for detection of *bla*CTX-M alleles was used to identify the CTX-M phylogenetic group of positive isolates. All PCR reactions were carried out using 2µl bacterial cell suspension (density of 70%T in Vitek Colorimeter) as the DNA template. Respective genes were detected by the size separation PCR amplicons by agarose gel electrophoresis.

2.3 DNA Electrophoresis

The molecular genotyping method employed to compare the DNA of the ESBL producing isolates was the PFGE. This was carried out as previously described **[Kaufmann ME, 1998]** with some modifications. Briefly, the bacterial isolate suspensions were embedded in agarose plugs. The cells were lysed and the proteins digested. The plugs were washed to remove cellular debris and they were sectioned. Restriction analysis of chromosomal DNA with *Xba*1 (New England BioLabs, Beverly, MA) was carried out, and separation of the DNA was performed using 1% pulsed-field gel agarose (Bio-Rad Laboratories, La Jolla, CA). The pulsed-field gel electrophoresis was performed using a contour-clamped homogeneous electric field apparatus set (CHEF DRIII, Bio-Rad Hercules, CA, USA) as in Figure 1 below.



The different components of the CHEF system are indicated as electrophoresis chamber, chiller, pump, and the programmed power supply in the above figure. Alternating the electric field between spatially distinct pairs of electrodes causes large and small DNA fragments to re-orient and move at different speeds through the pores in an Agarose gel

Fig. 1. Picture of CHEF DRIII, Bio-Rad Hercules, CA, USA used for the Pulsed field gel Electrophoresis for the microbial agent DNA separation technique.

The gels were stained and images captured on the Gel Doc imaging system using Quantity One software version 4.4.1 (Bio-Rad Laboratories, Hercules CA, USA), Figure 2 below. After viewing the banding patterns, the results were compared and analyzed by manual visualization from a computer monitor following previously established criteria **[Tenover FC et al, 1995]** so as to determine potential outbreak patterns or spread of the infections from one patient to another or hospital facility to another.

The established criteria or guidelines proposed by Tenover *et al*. were used for the interpretation of PFGE **[Tenover FC et al, 1995].** With these guidelines, a banding pattern difference of up to three fragments could have occurred due to a single genetic event and thus these isolates are classified as highly related, differences of four to six restriction fragments are likely due to two genetic events, and differences of equal to or greater than

seven restriction fragments are due to three or more genetic events. Isolates that differ by three fragments in PFGE analysis may represent epidemiologically related subtypes of the same strain. Conversely, isolates differing in the positions of more than three restriction fragments may represent a more tenuous epidemiologic relation. Some studies using PFGE and other typing methods indicate that single genetic events, such as those that may alter or create a new restriction endonuclease site or DNA insertions/deletions associated with plasmids, bacteriophages, or insertion sequences, can occur unpredictably even within the time span of a well-defined outbreak (1 to 3 months) **[Arbeit RD et al 1990; Sader HS et al 1993; Tenover FC et al 1995].** With the detection of two genetic variation events by differences in fragment patterns compared to the outbreak strain, the determination of relatedness to an outbreak falls into a gray zone. The results may indicate that these isolates are related (especially if isolates were collected over a long period of time, such as 3 to 6 months), but there is also a possibility that strains are unrelated and not part of the outbreak, hence demonstrating the usefulness of PFGE techniques as a tool in infection control measures in a hospital. PFGE results should always be considered in conjunction with the epidemiologic information and data. The bacterial isolates may also show some degree of clonal relatedness or diversity, thus helping in the determination of the sources, clonal relatedness and spread of the bacterial isolates in hospitals and countries where the isolates have been encountered.

The Gel Doc image system captures picture of the stained gel with the bands and this is transmitted to a computer and monitor for better visualization and analysis

Fig. 2. Picture of Gel Doc (Bio-Rad Hercules, CA, USA) imaging system used to visually analyze images captured after staining the bands formed in the gels.

3. Results

3.1 Bacterial isolates

One hundred and ninety-eight bacterial isolates comprising 120 *K. pneumoniae* and 78 *E. coli* isolates from patients with ages ranging from 2 days old to 85 years had higher MIC values from the E-test assays and thus fulfilled the criteria for further molecular characterization. More than 70% of the isolates were recovered from female patients. The isolates involved in urinary tract infections were 60%. Skin and soft tissue isolates and infections contributed 30% of the isolates and the rest of the isolates were either from respiratory tract system (5%), blood streams (4%) or central nervous systems (1%) respectively. Most of these ESBL isolates were recovered from adult patients admitted into the medical (48%) and surgical (35%) facilities of the hospital. The rest were from patients seen in the paediatrics wards (9%), Obstetrics and gynaecology ward (5%), and intensive care units (3%).

3.2 Multiplex PCR gene detection

The multiplex PCR assay detected the 100% *bla*TEM genes, 25% *bla* SHV and 52% *bla* CTX-M genes among the *E. coli* isolates. Similarly, 94% *bla*TEM, 42% *bla*SHV and 70% *bla*CTX-M genes in the *K. pneumoniae* isolates were detected. All CTX-M genes were identified as alleles belonging to the phylogenetic group I.

3.3 Pulsed-field gel electrophoresis

The PFGE picture of all the isolates used for this study is partly represented in Figure 3 below. As depicted in the figure, the PFGE typing of the ESBL-producing isolates revealed various different and diverse DNA banding profiles among the *E. coli* and *K. pneumoniae* isolates. Bacteriophage lambda ladder PFGE marker (New England Biolabs) is all depicted on the lanes λ. The *E. coli* isolates are demonstrated on lanes 1 – 6, 7 – 12 while lanes 13 – 18 and 19 – 24 shows the *K. pneumonia* isolates. Note that except for lanes 4 and 7, all the lanes containing the isolates have significantly divergent banding patterns. From these results therefore, one would interpret the data as stating that all the *E. coli* or *K. pneumoniae* isolates are distinguishable by the PFGE and divergent from each other (>7 band difference).

There was no major clonal similarity or relatedness of either the *K. pneumoniae* or *E. coli* producing ESBL isolates regardless of which hospital facility the patient was admitted to or specimen the bacterial pathogen was recovered from. In addition, one could notice that from the Figure 2, the bands of the DNA were not separated (i.e. no resolution) for isolates in lanes 4 and 7. This phenomenon is called smearing and occurs when there is a contamination of the nucleases (agarose plug, buffer or reagents), or use of abnormal temperature and concentration of the buffers or wrong conditions which may all affect the enzyme. All these were the case with these isolates because when the tests were repeated with only the two isolates the bands were fully separated.

4. Discussions

The chromosomal DNA is the most fundamental component of identity of the cell and therefore represents a preferred method to assess the relatedness of the strains. And the PFGE method is the gold standard for now in assessing this property of the microbial agents

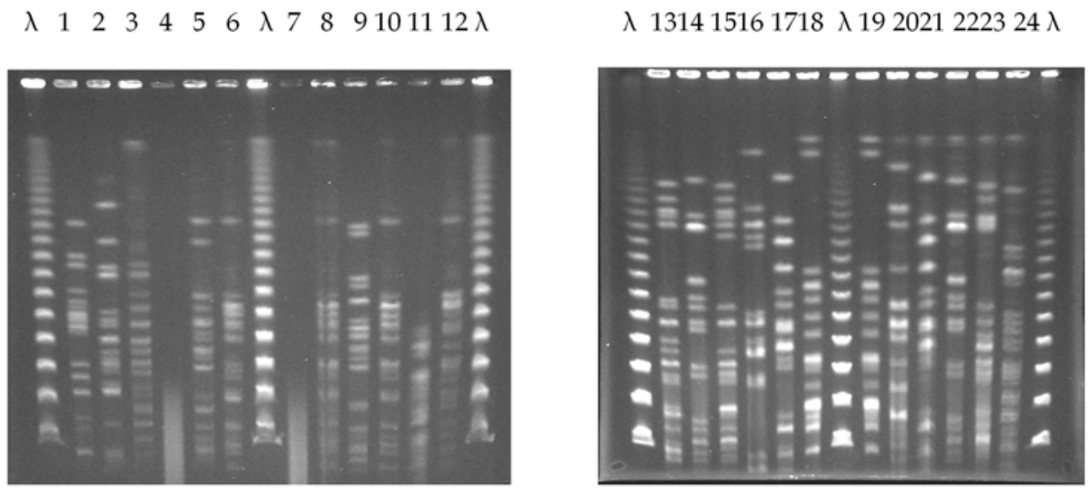


Figure 3 Picture depicting patterns generated by PFGE of *xba*1-digested chromosomal DNA obtained from *bla* TEM, SHV and CTX-M genes produced by *Escherichia coli* and *Klebsiella pneumoniae* isolates. Lane λ, bacteriophage lambda ladder PFGE marker (New England Biolabs), lanes 1 – 6, 7 – 12 *E. coli* isolates and lanes 13 – 18 and 19 – 24, *K. pneumonia* isolates. Smearing phenomenon occurred in lanes 4 and 7 hence the DNA particles were not completely separated or resolved.

Fig. 3. PFGE picture of *Escherichia coli* and *Klebsiella pneumoniae* ESBL producers.

including bacterial cells. All state public health laboratories in the USA as well as Centers for Disease Control and Prevention (CDC) perform molecular epidemiology testing using the PFGE. The PFGE assay can adequately be used to type several organisms including the ones involved in nosocomial infections or pathogens associated with food-borne diseases. PFGE is one of the most reproducible and highly discriminatory typing methods that is available and is a method of choice for many epidemiologic evaluations.

The PFGE typing method used in this study to characterize the ESBL-producing *Klebsiella pneumoniae* and *Escherichia coli* isolates showed various DNA banding profiles. These banding profiles were in no way similar or related to each other indicating their independent origin. This clonal diversity detected among these ESBL-producing isolates suggests that most of the strains have been unable to be maintained or spread in the different wards or facilities of the hospitals from where the bacterial isolates used in this present study were recovered from. This observation may challenge the many conventional thoughts about the nosocomial epidemiology of antibiotic resistance in the hospitals where the isolates were recovered in Trinidad and Tobago. But it is clearly obvious from the PFGE picture that the isolates were in no way closely related as there were different band patterns produced after restriction by the same enzymes under the same physical conditions.

The smearing phenomenon whereby the DNA particles were not completely separated or resolved that occurred and was observed in lanes 4 and 7 in Figure 2 highlights some of the drawbacks to using the PFGE method in studying molecular studies. Once the experimental or laboratory errors are eliminated, results obtained are perfect. Again, an arguement can be made or put forward that the procedures takes several days to be completed. The PFGE process can take less than 48 hours to complete. More time is expended in recovering the bacterial isolates in pure cultures from the clinical specimen because this is the time required for incubation and identification of the bacterial isolate. Thus the turnaround time tends to

be long and this can also be a point against the use of PFGE. But despite the longer turnaround time PFGE method in performing molecular epidemiology of bacterial isolates still remains a gold standard for now.

Using the questionnaire to retrospectively review the laboratory and medical records, it was observed that these isolates did not significantly share the same patient demographics and occurrence periods. Despite being isolated mostly from urine of patients admitted in the medical and surgical facilities of the hospitals sharing significant patient demographics and isolate characteristics yet the ESBL enzymes differed. This clearly indicated that most ESBL-producing isolates were not sporadic but that multiple clones were widespread in the hospitals. The occurrence of these ESBL producing pathogens were definitely not from spread from one patient to another or from one ward to another. It must probably therefore be as a result of antibiotic use pressure stemming from the use and overuse of antibiotics such as third generation cephalosporins in these facilities and hospitals as already has been reported in the country **[Pinto Pereira LM et al, 2004; Akpaka PE et al, 2010].** This therefore calls for a need for continuous and active surveillance measures; and effective infection controls practices, most especially antibiotic stewardship which is nonexistent in these hospitals.

This is the first study to report bla*TEM*, bla*SHV* and bla*CTX-M* in the country that reveals that phylogenetic group 1 is the predominant CTX-M types prevalent in the hospitals. This study clearly indicated that CTX-M, mainly CTX-M-1 for ESBL-producing *E. coli* and *K. pneumoniae* was highly prevalent and probably endemic in Trinidad & Tobago. Most ESBL producers were resistant to oxyiminocephalosporins and other non-beta-lactam agents at high levels and exhibited a high rate of the MDR phenotype. The spread of ESBL-producing bacteria appeared to be polyclonal, and none of the major epidemic strains were identified.

5. Conclusions

In summary, this study reports the first extensive study regarding the prevalence and molecular characterization of ESBL genes and the epidemiology of ESBL-producing *E. coli* and ESBL-producing *K. pneumoniae* isolates causing infections in Trinidad and Tobago that was specifically and clearly delineated by the use of the PFGE method.

6. Acknowledgement

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Conflicts of Interests: None to declare

7. References

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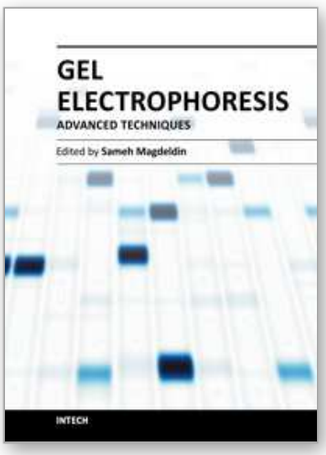

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As a basic concept, gel electrophoresis is a biotechnology technique in which macromolecules such as DNA, RNA or protein are fractionated according to their physical properties such as molecular weight or charge. These molecules are forced through a porous gel matrix under electric field enabling uncounted applications and uses. Delivered between your hands, a second book of this Gel electrophoresis series (Gel Electrophoresis- Advanced Techniques) covers a part, but not all, applications of this versatile technique in both medical and life science fields. We try to keep the contents of the book crisp and comprehensive, and hope that it will receive overwhelming interest and deliver benefits and valuable information to the readers.

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