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Application of Molecular Typing Methods to the Study of Medically Relevant Gram-Positive Cocci

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1. Introduction

The development of molecular genotyping methods has been a landmark in the possibility of classifying microorganisms below the species level. The ability to differentiate efficiently related bacterial isolates is essential for the control of infectious diseases and has become a necessary technology for clinical microbiology laboratories.

Strain typing is an integral part of epidemiological investigations of bacterial infections. Typing methods fall into two broad categories: phenotypic and genotypic methods. Phenotypic methods are those that characterize the products of gene expression in order to differentiate strains. Properties such as biochemical profiles, antimicrobial susceptibility profiles, bacteriophage types, and antigens present on the cell surface are examples of phenotypic methods that can be used for typing isolates. Since they involve gene expressions, these properties have a tendency to vary, based on changes in growth conditions and growth phase, being often difficult to detect.

Methods for distinguishing among bacterial strains have profoundly changed over the last years mainly due to the introduction of molecular technology. Genotypic strain typing methods are based on the analysis of differences in the chromosomal and extrachromosomal nucleic acid sequences between strains. Molecular epidemiology of infectious diseases integrates practices and principles of molecular biology with those of epidemiology (Tenover et al. 1997).

Investigations of presumed outbreaks of bacterial infections in hospitals often require strain typing data to identify outbreak-related strains and to distinguish epidemic from endemic or sporadic isolates.

All typing systems can be characterized in terms of typeability, reproducibility, discriminatory power, ease of performance, and ease of interpretation. For each isolate, the system should provide an interpretable result, preferably based on objective criteria. Ideally, results should be reproducible from day to day and from laboratory to laboratory and should allow differentiation of unrelated strains. Additionally, the method should be standardized and if possible should be technically simple, cost-effective, and rapid (van Belkum, et al. 2007).

The results of bacterial strain typing have many different applications including outbreak investigation and surveillance in clinical care settings and public health investigations and also within other contexts such as food and pharmaceutical industries and environmental analysis.

The aim of this chapter is to provide an overview of the methods available for analyzing bacterial isolates, focusing on those methods employed for typing *Streptococcus pneumoniae* and *Staphylococcus aureus*. Different molecular approaches have been used to better understand the epidemiology of these medically relevant gram-positive cocci (Willems. et al. 2011).

2. Genotypic methods

The application of molecular biology tools to infectious disease epidemiology is perhaps just as revolutionary in advancing knowledge and concepts in epidemiology. Genotypic typing methods assess genome variation in bacterial isolates.

The advantages of nucleic acid-based typing systems lie in that they are less likely to be affected by growth conditions or the laboratory manipulations to which organisms are subjected. Undoubtedly, genetic materials undergo changes due to natural or artificial selective pressures, but this mechanism is exactly the basis for their typeability.

Compared to the classical phenotypic typing techniques, genotypic typing techniques have several advantages such as general applicability and a high discriminatory power.

A molecular technique must take into consideration the relative accumulation of variation (short or long term) of a targeted set of genes in a pathogen. Nearly all the typing systems can be grouped into variants of just three basic analytical procedures: (i) PCR, (ii) the use of restriction enzymes, and (iii) nucleic acid sequencing. These procedures allow for the use of common equipment and standard reagents to analyze many different types of infectious agents. In addition, genotypic characterization of pathogens facilitates standardization of information storage and data analyses, interpretation, and communication, which are all amenable to computer-assisted manipulations.

2.1 PCR-based typing methods

In the last years, a number of PCR-based strategies have been developed for use as typing tools. PCR can be readily performed with commercially available supplies and there is little variation in the reagents and equipment needed to perform PCR assays from different microorganisms. The major advantages of PCR-based techniques are speed and simplicity.

2.1.1 Repetitive element sequence-based PCR (rep-PCR)

A variety of repetitive DNA sequence elements have been identified in bacterial pathogens, which have been exploited to develop strategies for bacterial typing. Rep-PCR is a simple PCR-based technique that targets multiple copies of repetitive elements in the bacterial genome to generate DNA fingerprints (Versalovic et al. 1991). Primers designed to anneal in the outward direction, near the end of these repetitive elements bind to multiple non-coding, repetitive sequences interspersed throughout the bacterial genome. Multiple DNA

fragments between those sites (interrepeat fragments) are amplified. Since the number and location of the repetitive elements are variable, the sizes and number of effectively amplified fragments vary depending on the strain (Figure 1).

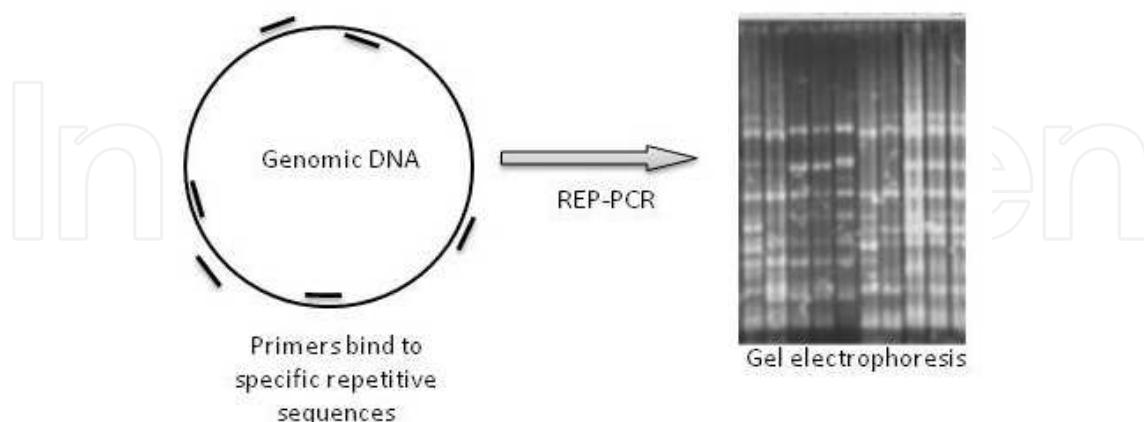


Fig. 1. Schematic representation of REP-PCR assay. On the right BOX-PCR patterns of *S. pneumoniae* isolates using BOXAR1 primer.

Two different Rep-PCR have been used for typing enteric bacteria: a 38-bp repetitive extragenic palindromic element (REP) and a 126-bp enterobacterial repetitive intergenic consensus (ERIC) sequence (Versalovic et al. 1991), whose function has not yet been elucidated.

A BOX repetitive element is a highly conserved repeated DNA element that has been identified in the *Streptococcus pneumoniae* (pneumococcus) chromosome. Although the function of this element has not yet been completely understood, it has been demonstrated that the presence of a BOX element is associated with variation in colony opacity of the pneumococcus (Saluja & Weiser 1995). BOX-PCR has been effectively used for typing *S. pneumoniae* as well as other bacterial species (van Belkum et al. 1996).

Several genetic elements have been used for developing Rep-PCR to type *Staphylococcus aureus*. The element IS256 occurs in the genome either independently or as part of the composite transposon Tn4001, IS256 insertion position is strain-specific and spaced close enough to allow amplification of polymorphic inter-IS256 element sequences (Deplano et al. 1997). Another element used for this methodology is RW3A, a repetitive sequence initially found in *Mycoplasma pneumoniae*, which also generates strain-specific DNA fragments when *S. aureus* DNA is used as template (van der Zee et al. 1999).

2.1.2 Randomly Amplified Polymorphic DNA-PCR (RAPD-PCR) or Arbitrarily-Primed PCR (AP-PCR)

Randomly Amplified Polymorphic DNA-PCR (RAPD-PCR), also referred to as Arbitrarily-Primed PCR (AP-PCR), is a variation of the PCR technique employing a single, generally short primer, that is not targeted to amplify a specific bacterial DNA sequence. Low annealing temperatures are used during amplification, allowing imperfect hybridization at multiple chromosomal locations. When the primer binds in two sites on opposite strands, at the proper orientation and with sufficient affinity to allow the initiation of polymerization,

the amplification of the fragment between those sites will occur. The amplified products will be various different-sized fragments that can be resolved by conventional agarose gel electrophoresis (Figure 2).

Although the method is much faster than many other typing methods, it is much more susceptible to technical variation. Slight variations in the reaction conditions or the reagents can lead to difficulty in result reproducibility and in the band patterns generated. Therefore, trying to make comparisons among potential outbreak strains can be very problematic (van Belkum et al. 1995). When RAPD-PCR is tightly controlled, it can provide a high level of discrimination, especially when multiple amplifications with different primers are performed.

PCR-based typing methods are simplest and rapid genotyping methods, but is remarkable for its susceptibility to minor variations in experimental conditions.

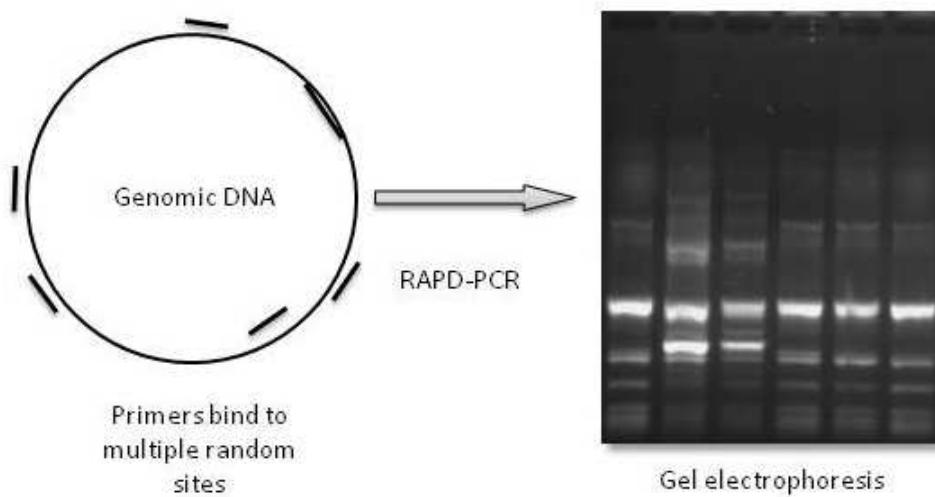


Fig. 2. Schematic representation of RAPD-PCR assay. On the right RAPD-PCR patterns of *Enterococcus faecalis* isolates using D8635 primer.

2.2 Based on enzymatic restriction of chromosomal DNA

2.2.1 Restriction endonuclease analysis of chromosomal DNA by hybridization with a nucleic acid probe (Southern blotting)

Following digestion with high frequency restriction endonucleases, chromosomal DNA is separated into different-sized fragments by conventional agarose gel electrophoresis, but this type of polymorphism is difficult to interpret due to the high number of fragments generated. However, interpretation of these polymorphisms can be facilitated by a Southern blot hybridization technique. By this methodology, fragments are separated by electrophoresis and transferred to a nitrocellulose or nylon membrane and hybridized using specific chemically or radioactively-labeled probes (Figure 3). DNA probes are designed for specific sequences that are found in multiple copies and in different positions of the chromosome. One of the most frequently used probes is ribosomal RNA (16s rRNA) because most species have more than one chromosomal rRNA operon distributed around the chromosome. This particular technique is denominated ribotyping. In recent years, the use

of this technique has declined, mainly due to its limited discriminatory power compared to other techniques. Several DNA probes have been employed for the study of methicillin-resistant *S. aureus* (MRSA) outbreaks, including various insertion sequences, such as *IS431*, *IS256* and *mecA* gene.

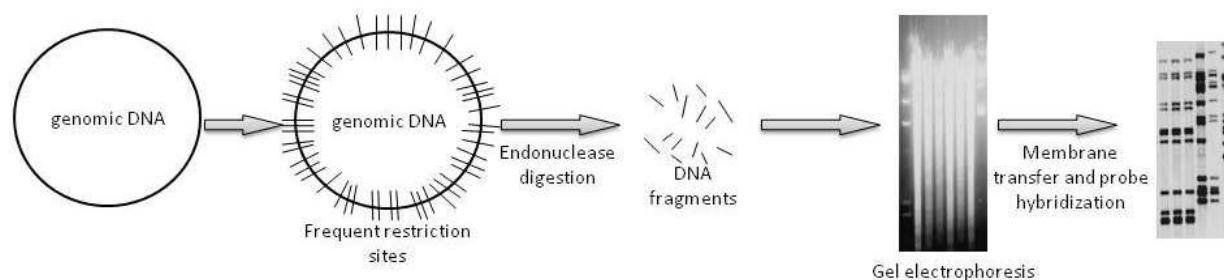


Fig. 3. Schematic representation of restriction endonuclease analysis by Southern-blotting assay. On the right *IS6110*-restriction fragment length of *Mycobacterium tuberculosis* isolates.

2.2.2 Pulsed-field gel electrophoresis (PFGE) of chromosomal DNA

Pulsed-field gel electrophoresis is based on the digestion of bacterial DNA with restriction endonucleases that recognize few sites along the chromosome, generating large DNA fragments (30-800 Kb) that cannot be effectively separated by conventional electrophoresis. The basis for PFGE separation is the size-dependent time-associated reorientation of DNA migration achieved by periodic switching of the electric field in different directions. The DNA fragments will form a distinctive pattern of bands in the gel, which can be analyzed visually and electronically (Figure 4 A). Bacterial isolates with identical or very similar band patterns are more likely to be related genetically than bacterial isolates with more divergent band patterns.

This technique is laborious and includes several steps, requires good standardization and takes at least two days for obtention of results. Procedures will differ to some extent depending on the organism that is being analyzed.

Regarding DNA preparation, PFGE requires intact DNA for restriction endonuclease treatment. The risk of mechanical breakage to DNA molecules during the extraction procedure is avoided by embedding intact organisms into agarose plugs where cells are enzymatically lysed and cellular proteins digested. After endonuclease treatment, the agarose plugs containing the digested DNA are then submitted to PFGE (Figure 4B). The choice of the restriction enzyme for DNA digestion and pulse-time switching parameters for PFGE are critical variables for the obtention of restriction profiles to show well-resolved fragments.

Recent protocols can be completed in as little as two days through shortcuts such as the direct addition of lytic enzymes to the agarose mixture before the blocks are cast and also high temperature short-term washes which facilitate the extraction of unwanted compounds (Goering 2010; Halpin et al. 2010).

Isolates with identical PFGE patterns were considered to represent the same epidemiological type. Isolates differing by one genetic event were considered epidemiologically-related subtypes, expecting that a single genetic event could occur in the chromosome of an

organism as it moved from patient to patient. Isolates differing by two genetic events were also deemed to be potentially related, while three or more chromosomal differences were thought to represent an epidemiologically-significant difference (unrelated isolates). Van Belkum suggested a more conservative approach where only nosocomial isolates differing by a single genetic event (up to four differences in the PFGE restriction fragment pattern) were considered related subtypes. The terminology within both proposed formats was left intentionally vague, understanding that molecular typing is only one component of epidemiological evaluation which must include other available clinical data for accurate analysis (Tenover et al. 1995; van Belkum et al. 2007; Goering 2010).

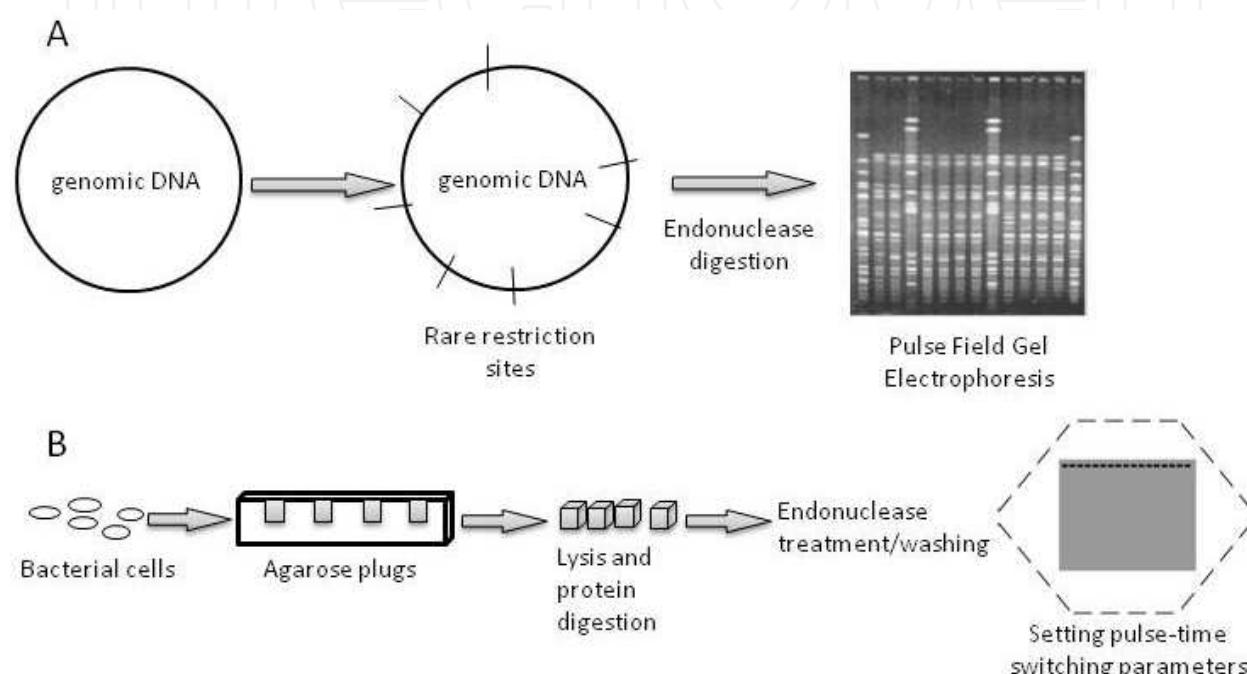


Fig. 4. A. Schematic representation of pulse field gel electrophoresis. On the right PFGE of *Sma*I-digested genomic DNA of *S. aureus* isolates. B. Sequence of steps involved in PFGE.

Furthermore, isolates with more uniform PFGE profiles require more conservative interpretation. The fact that two strains share the same pattern does not prove that they are epidemiologically related. The establishment of an epidemiologic relationship depends on the frequency with which the "indistinguishable" pattern is seen among epidemiologically-unrelated isolates and correlation with clinical and epidemiological information. If common contact between two patients with strains having the same pulsed-field gel electrophoresis (PFGE) type can be established, the chances are greater that an epidemiologic link could be ascribed. Thus, the greatest power of PFGE typing lies in showing strain dissimilarity rather than in proving similarity or relatedness. These considerations must be taken into account for banding pattern analysis from other molecular typing methods.

In some instances, initial unsatisfactory PFGE results may be aided by the use of an alternative restriction enzyme (Kam et al. 2008; Bosch et al.) or, in more difficult situations, the use of one or more additional typing methods (van Belkum et al. 2007).

The intra- and interlaboratory reproducibility of this method depends on understanding and controlling variables (Cookson et al. 1996; van Belkum et al. 1998; te Witt et al. 2010).

This success is due to an emphasis on standardized quality control especially in major areas of potential PFGE variability such as DNA sample preparation, choice of restriction enzyme, and electrophoresis conditions.

PFGE has been applied to a wide range of microorganisms and has remarkable discriminatory power and reproducibility. It is currently considered the strain typing method of choice for many commonly encountered pathogens. However, one of the main notable limitations is the need for specialized and relatively expensive equipment.

2.3 DNA sequencing-based methods

Genotyping methods based on DNA sequencing discriminate among bacterial strains directly from polymorphisms in their DNA considering the original sequence of nucleotides.

2.3.1 Single-locus sequence typing

Sequencing of a single genetic locus has been used for epidemiological studies of many bacterial species, yielding valuable typing results. In this approach, it is essential to select highly variable gene sequences. Valuable typing results have been obtained for *S. pyogenes* by DNA sequencing of 150 nucleotides coding for the N-terminal end of M protein (*emm* typing) (Beall et al. 2000). Another example is spa typing for *S. aureus* that consists in sequencing of the X region of the protein A gene (*spa* typing). This technique is widely used for subtyping methicillin-resistant *S. aureus* (MRSA) strains (Shopsin, 1999, 2000; Shopsin & Kreiswirth 2001; Harmsen et al. 2003), (Figure 5).

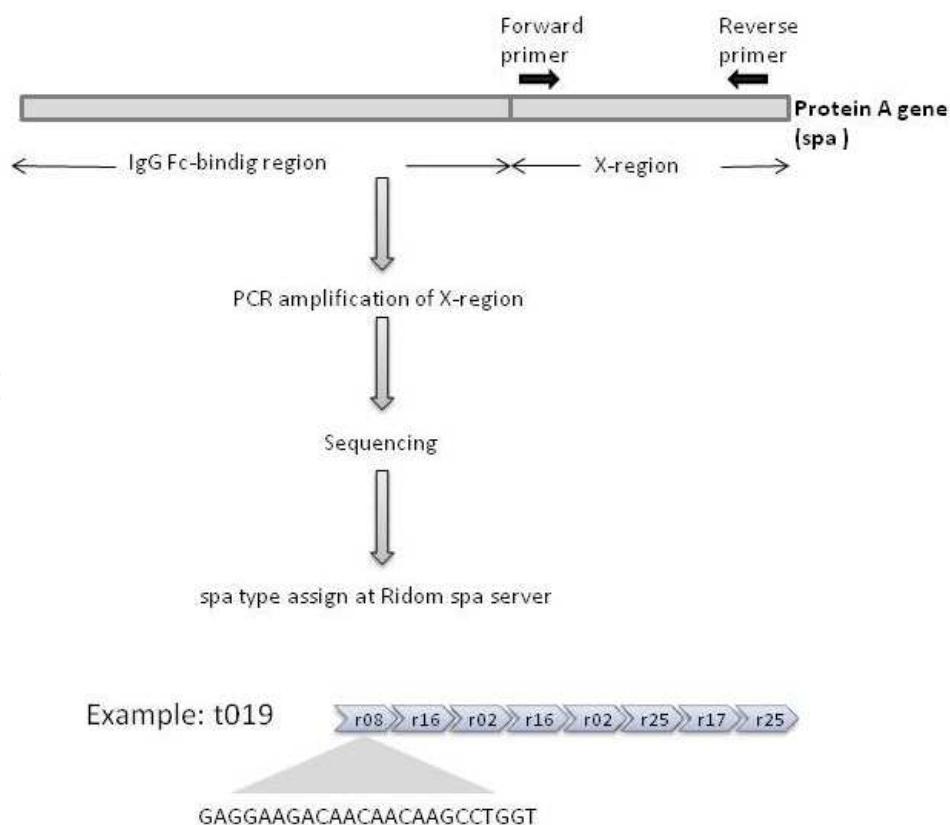


Fig. 5. Sequence of steps involved in *spa* typing.

2.3.2 Multi-locus sequence typing (MLST)

MLST is a genotyping method based on the measurement of DNA sequence variation in a set of housekeeping genes (usually seven genes) whose sequences are constrained because of the essential function of the proteins they encode. This method was proposed in 1998 as a general approach to provide accurate, portable data that were appropriate for the epidemiological investigation of bacterial pathogens and which also reflected their evolutionary and population biology (Maiden et al. 1998).

MLST schemes have been developed for several species and databases containing the allelic profiles of a great number of strain types with corresponding clinical information that can be readily consulted over the Internet (<http://www.mlst.net/> and <http://pubmlst.org/>), (Aanensen & Spratt 2005). Additional information such as date, place of isolation and antibiotype is included in the database when a strain is deposited so this database is continuously expanding as new STs are identified and additional nucleotide sequence data are deposited.

Internal fragments of the seven housekeeping genes are amplified by PCR from chromosomal DNA using the primer pairs described in the web site. The amplified fragments are directly sequenced in each direction. The sequences at each of the seven *loci* are then compared with all the known alleles at that *locus*, and a number representing a previously described allele (or a new one) is assigned to the *locus*. For a given isolate, alleles present at each gene position are combined into an allelic profile and assigned a sequence type (ST) designation (Maiden et al. 1998). Relationships among isolates are assessed by comparisons of allelic profiles: closely related isolates have identical STs, or STs that differ at a few *loci*, whereas unrelated isolates have unrelated STs (Figure 6).

A number of clustering algorithms have been employed to analyze the data in the MLST scheme, including UPGMA (unweighted pair group method with arithmetic mean) and eBURST analyses (Feil et al. 2004).

The original conception of MLST used the allele number as the primary unit of analysis (Enright & Spratt 1998; Maiden et al. 1998) which was appropriate for organisms where horizontal genetic exchange is common. However, MLST data can also be interpreted by tree-building approaches that use nucleotide substitutions rather than allelic changes as the unit of analysis; this is more pertinent to bacteria where mutational change predominates over genetic exchange in the evolution of variants.

An important advantage of MLST is that results are unambiguous and easily and unequivocally exchangeable, much more so than images of agarose gel electrophoresis patterns. MLST drawbacks are practical, including limited accessibility and high cost. It is a relatively expensive technique available for the characterization of bacterial isolates, mainly in reference or research laboratories. However, MLST is increasingly applied as an informative typing tool that enables international comparison of isolates. It has been applied to problems as diverse as the emergence of antibiotic-resistant variants (Crisostomo et al. 2001; Enright et al. 2002), the association of particular genotypes with virulence (Brueggemann et al. 2003) or antigenic characteristics (Meats et al. 2003) and also the global spread of disease caused by novel variants (Albarracin Orio et al. 2008). In addition to these medically-motivated epidemiological analyses, MLST data have been exploited in evolutionary and population analyses (Jolley et al. 2000) that estimate recombination and

mutation rates (Feil et al. 2001) and in investigation of the evolutionary relationships among bacteria that are classified as belonging to the same genus (Godoy et al. 2003).

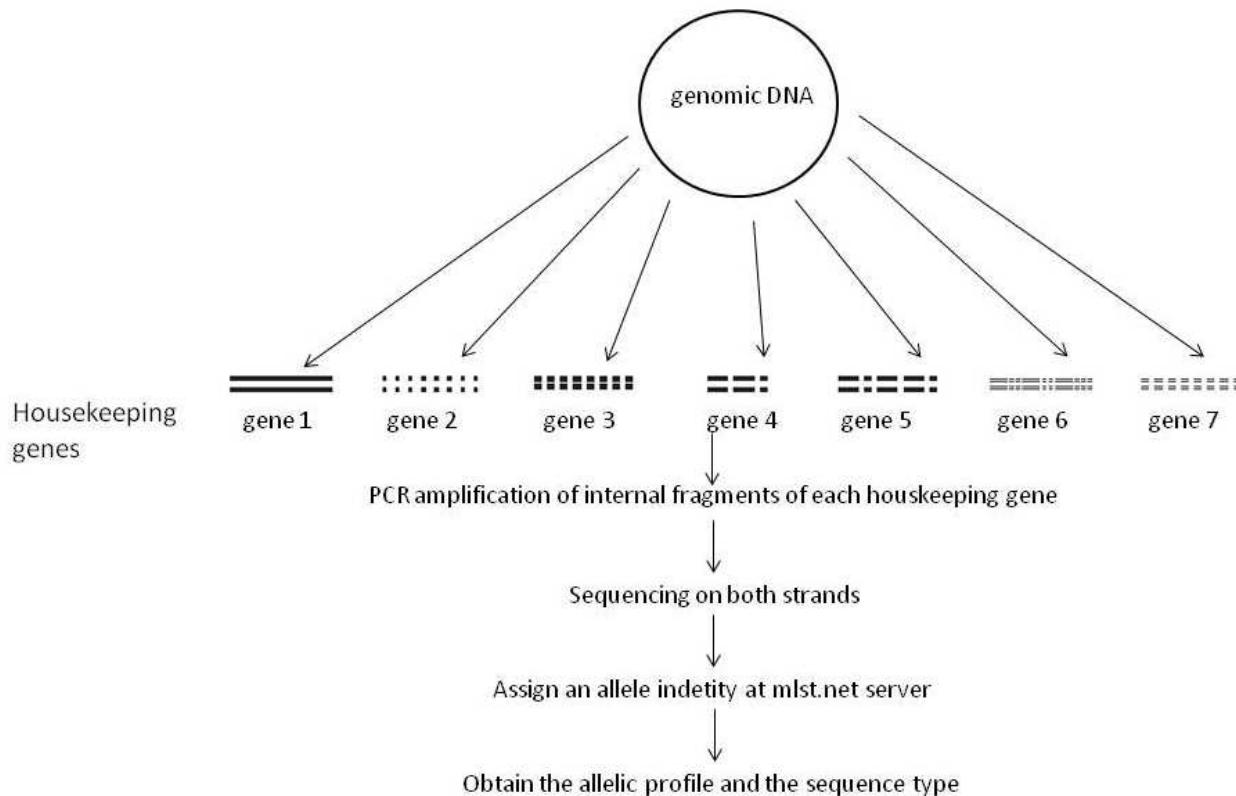


Fig. 6. Sequence of steps involved in MLST scheme. Adapted from Vazquez et al. 2004.

2.4 Analysis of results obtained by molecular epidemiology

Comparison and interpretation of raw data generated by molecular typing methods, such as gel electrophoresis band patterns, sequence alignments, or hybridization matrix patterns could be performed by visual analysis when there are few strains. However, if the analysis includes many strains, the comparison turns out to be very difficult. Therefore computer programs have become indispensable in molecular epidemiological investigations.

Computer programs that compare band sequences or patterns employ clustering algorithms that can generate dendograms or trees illustrating the arrangement of the clusters produced. For pattern recognition, such as electrophoretic banding patterns or hybridization matrices, additional programs are needed to capture, digitize, and normalize the patterns.

There are different commercially available platforms for databasing and gel analysis that have been developed for computer-assisted analysis such as BioNumerics, GelCompar (Applied Maths, Sint-Martens-Latem, Belgium), Diversity Database Fingerprinting Software (Bio-Rad Laboratories, Hercules, Ca). Treecon (Van de Peer and De Wachter 1994).

3. Epidemiologic applications of bacterial typing techniques

A more comprehensive knowledge of the evolution and the epidemiology of bacterial pathogens had been obtained by combination of genetic, phenotypic, spatial and temporal data.

Multiple techniques have been developed to assess genomic differences among different isolates or clones of the same species, such as PCR-based methods and PFGE. These methods present portability problems and limited comprehension of the processes by which variation occurs. However, DNA sequence-based techniques generate portable differentiation in bacterial populations that can be used to understand their phylogenetic history. Extensive genomic and phenotypic diversity exists within populations of microbial pathogens of the same species. This diversity reflects the evolutionary divergence arising from mutations and gene flux. These distinctive characters are scored by typing systems which are designed to optimize discrimination between epidemiologically related and unrelated isolates of the pathogen of interest (Maslow and Mulligan 1996; Struelens 1996).

Epidemiologic typing systems can be used for outbreak investigations to confirm and delineate the transmission patterns of one or more epidemic clone(s), to test hypotheses about the sources and transmission vehicles of these clones and to monitor the reservoirs of epidemic organisms. Typing also contributes to epidemiologic surveillance and evaluation of control measures by documenting the prevalence over time and the circulation of epidemic clones in infected populations. Clearly, different requirements will be needed for these distinct applications (Maslow and Mulligan 1996; Struelens 1996).

Typing can be undertaken at two different levels, depending on the situation: i) short term or local epidemiology, when organisms are recovered in a defined setting over a short period of time, which is used to study nosocomial outbreaks, local transmission and carriage, and the relationship between isolates associated with carriage and infection in a given geographic area, ii) long term or global epidemiology, when strains are recovered from one geographic area related to those isolated worldwide or strains recovered at different times.

Local epidemiology is applied to study outbreaks with the aim to characterize that the increase in incidence of infection is caused by enhanced transmission of a specific strain. In this framework, typing methods are applied to investigate the sources of contamination and the route of transmission. Accurate application of bacterial typing will support appropriate control measures designed to contain or interrupt the outbreak and prevent further spread of disease. Typing may also be used for isolates cultured from the same patient over time to help define whether a second episode of infection is due to relapse or reinfection. PCR fingerprinting is the simplest and most rapid genotypic method for local application; however, PCR typing is very susceptible to minor variations in experimental conditions and reagents. Therefore, the method is more appropriate for the comparison of a limited number of samples processed simultaneously and run on one gel. By contrast, PFGE has good reproducibility and is highly discriminatory. Therefore, PFGE is considered the current gold standard for outbreak and local epidemiology studies.

At a different level, collaborative studies have been performed to define major internationally disseminated bacterial clones of important human pathogens. Currently, MLST in combination with PFGE is the most appropriate strategy for long term epidemiology and have reached useful conclusions from infectious disease surveillance data. The evaluation of global population genetic structure, genetic evolution, genetic diversity and pathogenicity has been successfully developed within this framework.

For eukaryotes, clones are genetically identical organisms. However, in bacterial epidemiology, the clone concept is of an even more pragmatic nature, denoting isolates

obtained during real outbreaks with common features (e.g. multiple antibiotic-resistant isolates) from different geographic locations, the so-called epidemic clones.

The threshold of marker similarity used for definition of a clone need to be adjusted to the species studied, the typing system used, the environmental selective pressure and the time and space scale of the study (Tibayrenc 1995; Struelens 1996). Mutation rate and gene flux vary between species, pathovars and environments. In vivo micro-evolution of most pathogens remains poorly understood. Subclonal evolution and emergence of variants that occur in individual hosts or during prolonged transmission can be recognized by several high resolution molecular typing systems, like, for instance, macrorestriction analysis by pulsed-field gel electrophoresis (Struelens 1996).

4. Strategies applied for surveillance and typing of relevant gram-positive pathogens

4.1 Methicillin-resistant *Staphylococcus aureus*

Staphylococcus aureus is recognized as one of the most important human pathogens. It has shown great ability to acquire resistance to different antimicrobial agents. The first isolation of methicillin-resistant *S. aureus* (MRSA) was reported in 1960 and since then, the prevalence of this pathogen has increased.

Methicillin resistance is conferred by the *mecA* gene which codes for an additional penicillin-binding protein named PBP 2a; this protein has reduced affinity to β-lactam agents. This gene is located in a mobile genetic element of variable size known as staphylococcal cassette chromosome *mec* (SCC*mec*). So far, eight types and several subtypes of SCC*mec* have been characterized (Deurenberg & Stobberingh 2008; Chambers & Deleo 2009).

The incidence of MRSA varies geographically throughout the world. MRSA has emerged as an important pathogen among hospitalized patients. Most hospital-acquired infections caused by methicillin-resistant *Staphylococcus aureus* (HA-MRSA) are associated with a relatively small number of epidemic clones that spread over different continents. According to the Sistema Informático de Resistencia (Asociación Argentina de Microbiología, Buenos Aires, Argentina), MRSA strains are among the most prevalent nosocomial pathogens (<http://www.aam.org.ar>) in Argentina, whereas the Brazilian clone, the pediatric clone and the Cordobés clone have been found to be the main clones associated with HA-MRSA infections (Corso et al. 1998; Sola et al. 2002; Gardella et al. 2005).

However, since 1990, MRSA has been recognized as a cause of infections in people without established risk factors for HA-MRSA, such as recent hospitalization, surgery, residence in a long-term care facility, receipt of dialysis, or presence of invasive medical devices (Fridkin et al. 2005; Chambers & Deleo 2009). These infections are thought to be acquired in the community and are referred to as community-associated MRSA infections (CA-MRSA). This term has also been used to refer to MRSA strains with bacteriological characteristics considered typical of isolates recovered from patients with CA-MRSA infections (Salgado et al. 2003). HA-MRSA strains are generally resistant to antibiotics other than β-lactams, whereas typical CA-MRSA strains are only resistant to methicillin. HA-MRSA isolates frequently harbor SCC*mec* types-I, II and III whereas CA-MRSA strains carry types IV and V (Ma et al. 2002; Naimi et al. 2003).

The Panton-Valentine leukocidin (PVL) toxin has been described as a genetic marker of CA-MRSA isolates, rarely identified in HA-MRSA isolates (Ma et al. 2002; Naimi et al. 2003). Several studies have demonstrated that the presence of PVL genes is associated with *S. aureus* recovered from patients suffering from primary skin infections (Lina et al. 1999), severe necrotizing pneumonia, and increased complications of hematogenous osteomyelitis; however, the role of PVL in the pathogenesis of *S. aureus* infections has not yet been fully elucidated.

The spectrum of disease caused by CA-MRSA appears to be similar to that of methicillin-susceptible *Staphylococcus aureus* (MSSA) in the community. Skin and soft tissue infections (SSTIs), specifically furuncles (abscessed hair follicles or "boils"), carbuncles (coalesced masses of furuncles), and abscesses, are the most frequently reported clinical manifestations (Fergie & Purcell 2001; Baggett et al. 2003; Fridkin et al. 2005). Less commonly, MRSA has been associated with severe and invasive staphylococcal infections in the community, including necrotizing pneumonia, bacteremia, osteomyelitis, toxic shock syndrome, and meningitis (Deurenberg & Stobberingh 2008). The rapid emergence of these infections has been one of the most unexpected events in bacterial infectious diseases in the recent years.

Distinct genetic lineages associated with CA-MRSA infections have been determined by typing and their geographic dissemination evaluated in different countries. In Latin America, CA-MRSA has been described several times (Ma et al. 2005; Ribeiro et al. 2005; Alvarez et al. 2006; Gardella et al. 2008).

Typing of MRSA strains is necessary for proper epidemiological investigations of sources and modes of transmission of these strains in hospitals, and the design of appropriate control measures and the application of different typing methods have contributed to understanding the emergence of MRSA in the community. Phenotyping methods generally have limited discriminatory power and poor typeability; therefore, a number of molecular techniques have been developed for *S. aureus* typing, namely restriction fragment length polymorphism (RFLP) analysis techniques, including ribotyping and Southern blot analysis with probes for mobile elements present in multiple copies in the staphylococcal genome, like insertion sequences (IS256, IS257, IS431 and IS1181) and transposons (Tn554 and Tn4001) (Wei et al. 1992; Tenover et al. 1994; Kreiswirth et al. 1995).

Among PCR methods, rep-PCR and RAPD-PCR analysis were found to be epidemiologically useful, but interlaboratory studies showed that reproducibility is an important drawback for these techniques (Saulnier et al. 1993; van Belkum et al. 1995; Deplano et al. 1997; van der Zee et al. 1999). Pulsed-field gel electrophoresis (PFGE) analysis is an accurate and discriminating method which is now used as the reference method for *S. aureus* typing in some reference centers (Bannerman et al. 1995). However, PFGE analysis is costly and technically demanding and still requires interlaboratory standardization (Cookson et al. 1996). PFGE proved to be a highly discriminatory and sensitive technique in microepidemiological (local or short term) and macroepidemiological (national, continental, or long term) surveys (Struelens et al. 1993; McDougal et al. 2003). Nevertheless, some authors have argued that the stabilities of PFGE markers may be insufficient for the reliable application of PFGE to long-term or macroepidemiological studies (Blanc et al. 2002).

Sequence-based methods such as multilocus sequence typing (MLST) has proved to be adequate for long-term global epidemiology and the study of the recent evolution of *S. aureus*

(Enright, 2000, 2002). Another useful technique is the Staphylococcal cassette chromosome *mec* (SCC*mec*) typing, based on the molecular characterization by multiplex PCR of the mobile genetic element carrying the methicillin-resistant gene (*mecA*) (Oliveira and de Lencastre 2002). The combination of the MLST type and the SCC*mec* type, defined as the "clonal type," is now used for the international nomenclature of MRSA clones (Enright et al. 2002).

Moreover, single-locus DNA sequencing of repeat regions of the *coa* (coagulase) gene and the *spa* gene (protein A), respectively, could be used for reliable and accurate MRSA typing (Shopsin, 1999 2000; Tang et al. 2000; Shopsin & Kreiswirth 2001; Harmsen et al. 2003). Spa typing is especially interesting for rapid typing of MRSA in a hospital setting since it offers higher resolution than *coa* typing (Shopsin et al. 2000). The repeat region of the *spa* gene is subject to spontaneous mutations, as well as to loss and gain of repeats. Repeats are assigned an alpha-numerical code, and the spa type is deduced from the order of specific repeats. There is a good correlation between clonal groupings determined by MLST and the respective spa types (Harmsen et al. 2003).

We performed different studies to characterize MRSA clones within diverse scenarios of Argentina. In 2005, we demonstrated the replacement of the multiresistant MRSA "Brazilian" clone (SCC*mec* III, ST239) by the "Cordobes" clone (SCC*mec* I, ST5), a MRSA clone susceptible to rifampin, minocycline and trimethoprim/sulfamethoxazole in two university hospitals. Isolates were characterized by using RAPD-PCR and PFGE and SCC*mec* typing (Gardella et al. 2005).

Later, we analyzed community-associated methicillin-resistant *Staphylococcus aureus* (CA-MRSA) isolates recovered from patients suffering from different types of infections. All CA-MRSA isolates carried SCC*mec* type IV. Four major clones were detected in Argentina by PFGE. The largest cluster was named CAA clone: Pulsotype A, spa type 311, ST 5, LPV (+) (Gardella et al. 2008) and two isolates of this clone were recovered from two cases of acute bacterial meningitis (von Specht et al. 2006), (Figure 7).

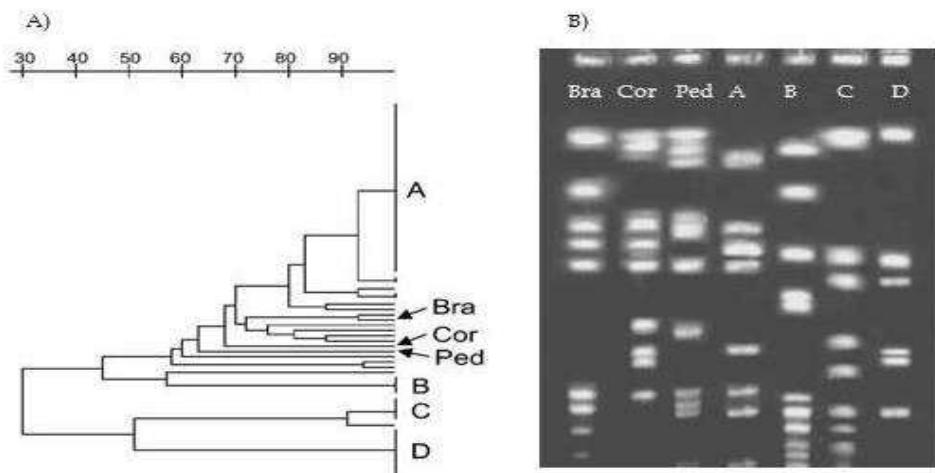


Fig. 7. (A) Dendrogram of pulsed-field electrophoresis banding pattern of CA-MRSA isolates and the 3 clonal types most prevalent in Argentinean hospitals: Brazilian clone (Bra), Cordobes clone (Cor), Pediatric clone (Ped). Similarity coefficient was calculated by using Dice coefficient, and cluster analysis was performed by the unweighted pair-group method. Four major pulsotypes were coded from A, B, C and D and representative HA-MRSA strains of prevalent clones in Argentina.

We also characterized CA-MRSA strains isolated from skin and soft-tissue infections in isolates recovered from Uruguay in 2005. In that study, we identified three major groups of CA-MRSA strains (1, 2, and 4) that were defined according to phenotypic and genotypic characteristics. The most frequent group, G1, showed a PFGE pattern identical to that of CA-MRSA strains previously isolated in Uruguay and Brazil; these strains are still producing SSTI, illustrating the stability of this emergent pathogen over time, as well as its excellent adaptation to the community environment (Pardo et al. 2009).

During the 2008 school- year period we conducted the first epidemiological study of *S. aureus* carriage in Argentina. Carriage was investigated in all children attending the last year of kindergarten in a city of Buenos Aires province, Argentina. Of 316 healthy children, 31.0% carried *S. aureus*, including 14 MRSA carriers (4.4%). All MRSA isolates carried the SCCmec type IV cassette. Eight of those 14 carriers were closely related to the CAA clone, which was responsible for the most severe community-acquired MRSA infections caused in our country (PFGE A, SCCmec IV, spa t311, ST5), (Gardella et al.).

Our results should serve as a warning for the health system since the main clone circulating in the community presents epidemic characteristics and also possesses a genetic background (ST5) of demonstrated plasticity and efficiency to be established as prevalent in the hospital environment.

4.2 Molecular epidemiology of *Streptococcus pneumoniae*

Streptococcus pneumoniae is a human pathogen of increasing clinical relevance causing important diseases such as meningitis, pneumonia, bacteremia and otitis media. Surveillance has become progressively more important because of the worldwide distribution of penicillin-resistant and multidrug-resistant pneumococci clones in the last 15 years.

S. pneumoniae with resistance to one or more antibiotics has been isolated since 1990. Penicillin is the drug of choice for the treatment of pneumococcal infections. The resistance mechanism includes the modification of penicillin-binding proteins (PBP), which in general is associated to cephalosporin resistance.

The clinical relevance of multiple antibiotic-resistant pneumococcal strains has led to the creation of a network. The Pneumococcal Molecular Epidemiology Network (PMEN) was established in 1997 with the aim of global surveillance of antibiotic-resistant *S.pneumoniae* and the standardization of nomenclature and classification of resistant clones (<http://www.sph.emory.edu/PMEN/index.html>). The PMEN also includes major invasive antibiotic-susceptible clones that have a wide geographic spread. Up to date, there are currently 43 clones described by the PMEN. Of those, three penicillin- resistant and two penicillin- susceptible PMEN clones have been detected in Argentina <http://www.sph.emory.edu/PMEN/> (Zemlickova et al. 2005). Clones to be included in the Network have to be subjected to PFGE, MLST and PBP fingerprinting to confirm that they differ from previously accepted ones. PBP fingerprinting is a typing technique that includes PCR amplification of the *pbp1a*, *pbp2b* and *pbp2x* genes with previously described primers (Gherardi et al. 2000). Amplified genes are digested with *HaeIII+DdeI* (*pbp1a*) and *HaeIII+RsaI* (*pbp2b* and *pbp2x*) restriction enzymes and electrophoresed on 3% gels. This technique has been used in many reports over the last 20 years to study the molecular

epidemiology of *S. pneumoniae* resistance to β -lactams (Zhang et al. 1990; Munoz et al. 1991). For macrolide-resistant strains, tests for *erm* and *mef* genes have to be performed (<http://www.sph.emory.edu/PMEN>). The PMEN network has included BOX-PCR typing in the guidelines for the recognition of pneumococcal clones (McGee et al. 2001).

In addition, the use of modern typing methods, mainly MLST, has greatly helped track the geographical spread of specific *S. pneumoniae* strains and follow the dynamics of microbial populations over time. The application of all these techniques have shown that the spread of multiresistant international clones defined by the PMEN is the main cause of increase in pneumococcal resistance to β -lactams and other drugs (Munoz et al. 1991; Klugman 2002; Smith et al. 2006; Sadowy et al. 2007; Soriano et al. 2008). In pneumococcus, each serotype may typically be made up of a number of clones, which are not closely related and are not equivalent in terms of antibiotic resistance.

Furthermore, molecular methods showed that the evolution of penicillin-resistance and multiresistance is a phenomenon in which the acquisition and/or alteration of molecular targets is mainly a consequence of intergenic change and that *S. pneumoniae* diversity has largely been driven by recombination (Hermans et al. 1997; Enright & Spratt 1998; Enright et al. 1999; Descheemaeker et al. 2000; McGee et al. 2001).

As multiresistance has increased the difficulties of treating this serious bacterial infection, prevention through vaccination has become even more important. There are at least 91 known pneumococcus capsular types, with 23 capsular types included in the current pneumococcal polysaccharide (adult) vaccine and 13 types included in the current conjugate (child) vaccine. To overcome serotype specificity of actual vaccines, upcoming pneumococcal vaccines should offer a different approach to the prevention of pneumococcal disease and the decrease in carriage. Several proteins have been identified as possible candidates to develop more appropriate vaccines. One of them, the pneumococcal surface protein A (PspA), is a surface virulence factor, antigenically variable yet cross-reactive that interferes with complement-mediated clearance of pneumococci (McDaniel et al. 1991; Tu et al. 1999). Since 1993, six Latin-American countries have been participating in an epidemiological surveillance study conducted by the Pan American Health Organization (PAHO) in order to determine the relative prevalence of capsular types and antimicrobial resistant patterns of *S. pneumoniae* causing invasive infections in children <5 years of age. One of these studies showed that, the prevalence of penicillin resistant *S. pneumoniae* (PRSP) in Argentina was 24.4%, which was significantly associated with the expansion of serotype 14 clone that had been previously described in Europe expressing serotype 9V (Rossi et al. 1998). A similar situation was encountered in Uruguay in the same period (Camou et al. 1998).

Ongoing surveillance programs for invasive pneumococcal disease also monitor the appropriateness of existing vaccine formulations and provide valuable information on which to base the formulation and application of new vaccines that are currently under development. In this framework, from 1993 to 2000, with the participation of the Argentinean *Streptococcus pneumoniae* Working Group, 1293 invasive isolates were studied to determine capsular type distribution and antimicrobial susceptibility. We selected a sample of 149 strains, having the same serotype distribution as in the total collection, in order to characterize the distribution of PspA variants among Argentinean invasive isolates recovered from children less than 6 years of age. The genetic relatedness among the isolates of the major serotypes was also evaluated by BOX-PCR because it is a quick molecular

method that is suitable for investigation of genetic relatedness of pneumococcal strains and provides results whose interpretation is relatively unambiguous (Hermans et al. 1995; van Belkum et al. 1996). This study provided epidemiological information about the PspA family distribution and the genetic diversity of Argentinean *S. pneumoniae* isolates and informed of the potential coverage of a PspA- based vaccine. It was the first insight into diversity of PspA within strains circulating in Argentina (Mollerach et al. 2004). Family 1 PspA was detected in 54.4% of the isolates, 41.6% of which were family 2 and 4.0% expressed both family 1 and family 2 PspAs. This observation indicates that a PspA vaccine containing only family 1 and family 2 PspAs should be able to cover the bulk of the strains in this region. Box typing revealed the Argentinian strains were from at least 10 clonally related groups.

In some cases, a strong association between one PspA type and a certain capsular type was found. For example, serotype 1 and 5 and the majority of isolates of penicillin-susceptible serotype 14 isolates exhibited PspA family 1. On the other hand, serotypes 7 F, serotype14 PRSP and the majority of type 9V isolates were assigned to PspA family 2. BOX-PCR analysis revealed genetic homogeneity of serotype 14 PRSP and serotype 5 isolates. Antibiotype suggests correlation with the Spain^{9V-3} clone and Colombia⁵⁻¹⁹ clone, respectively. These clones had been previously described in the region (Gamboa et al. 2002; Brandileone et al. 2004; Zemlickova et al. 2005).

Nowadays, the sequencing of DNA allows to compare the results between laboratories and to obtain a global look for the situation of the circulating multidrug-resistant clones. This effort includes a database that contains information concerning the clones that are currently widespread in different parts of the world (<http://spneumoniae.mlst.net>).

In the framework of PAHO in Latin American countries, surveillance data revealed that penicillin-nonsusceptible *S. pneumoniae* (PNSP) type 6B increased from 15.8 % in the period between 1993-1997, to 67.3 % in 1998-2002 ($p<0.001$). Serotype 6 ranks fourth among capsular types causing invasive diseases in Argentinean patients under 6 years of age, and it has been included in the heptavalent conjugate polysaccharide vaccine licensed in Argentina in 2001 and also in the 13-valent introduced in 2010 (Organización Panamericana de la Salud 2007; Ruvinsky et al. 2008). This serotype is a frequent cause of invasive diseases (Riedel et al. 2007; Gabastou et al. 2008; Darabi et al.). We characterized the population of penicillin non-susceptible *S. pneumoniae* type 6B strains isolated from pediatric patients in Argentina between 1993-2002 with the use of molecular typing methods including BOX-PCR, PFGE and MLST (Bonofiglio et al. 2011) (Figure 8). The results of the study showed that the increase in penicillin resistance in serotype 6B may be partly explained by the entrance of the Poland^{6B-20} clone, which is a PMEN clone not previously described in Argentina. Our findings showed that the Poland^{6B-20} clone established in 1999; and the use of BOX-PCR and PFGE subtypes suggested that horizontal transfer or differentiation events had occurred after the common lineage became established. Dissemination of this clone could be traced through demographic data, as isolates representative of the clone had been recovered in different regions of Argentina and its expansion is also responsible for the emergence of erythromycin-resistance in *S. pneumoniae* serotype 6B. The pneumococcal MLST database currently contains information of 81 strains of the Poland^{6B-20} clone.

Other similar studies were carried out by Sadowy et al, who analyzed isolates recovered in Poland in the period 2003-2005 using serotyping, MLST and sequencing of *murM* and *pspA* alleles. They demonstrated that the vast majority of the isolates (90.7%) belong to

international multiresistant clones whereas, the Spain 9V -ST156 clonal complex being the most prevalent. Moreover, this clone has evolved rapidly, as demonstrated by the observed number of STs, the use of another approach of MLST (multiple locus variable-number-tandem repeat analysis) and the polymorphism of *pbp* and *pspA* genes (coding for penicillin-binding proteins and the pneumococcal surface protein A, respectively) (Sadowy et al.).

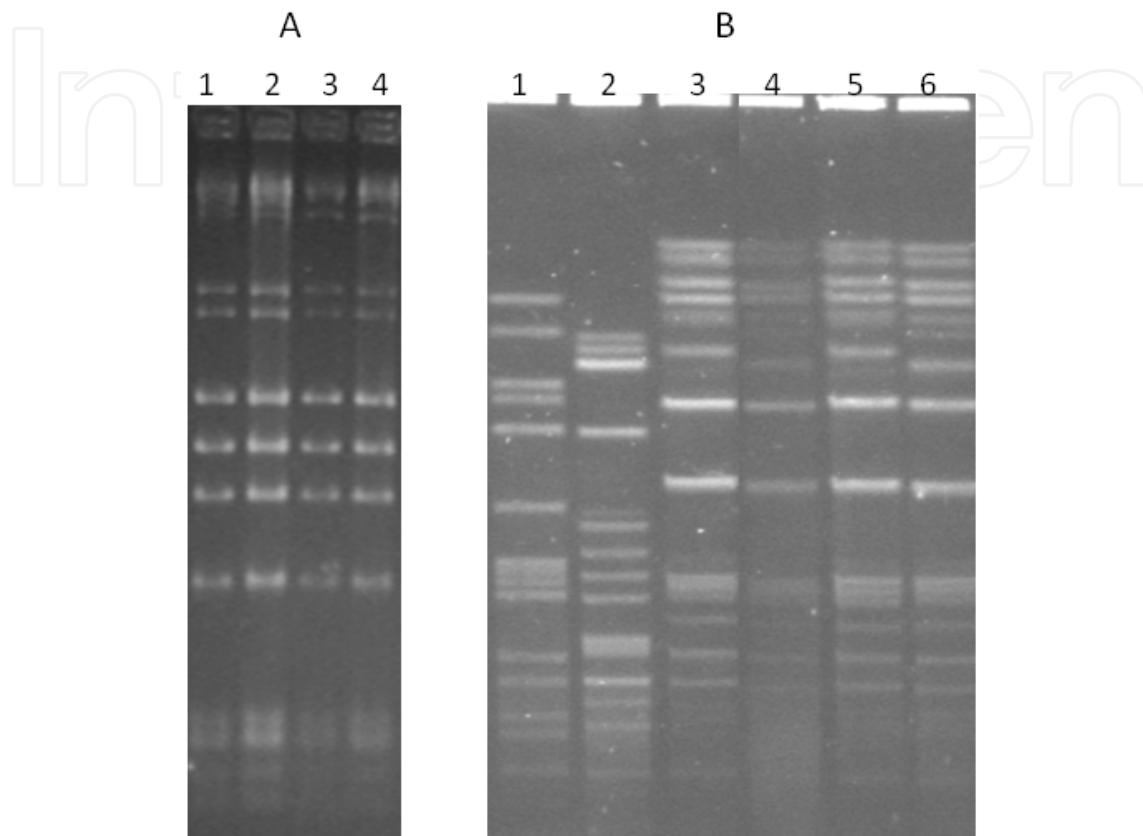


Fig. 8. A) BOX-PCR patterns of 4 Argentinean isolates belonging to Poland^{6B}-20 clone. B). Pulse field gel electrophoresis of *Sma*I digested DNA of *S. pneumoniae*. Lane1: *S.pneumoniae* R6, lane2: *S.pneumoniae* 6B (1994), lanes3-6: *S.pneumoniae* isolates belonging to Poland^{6B}-20 clone recovered in Argentina (2000).

The relationship between PspA and ST was also explored (Qian et al.). This author analyzed 171 invasive *S. pneumoniae* isolates from Chinese children in 11 hospitals between 2006 and 2008. He found that Family 1 and family 2 PspAs were prevalent and that strains with the same ST always presented the same PspA family.

5. Conclusion

This chapter has reviewed some of the most popular molecular methods for the epidemiological typing of two medically relevant gram-positive cocci, discussing their principles, strengths and weaknesses. We have described several examples of our recent work showing the application of molecular typing techniques to the study of two relevant pathogens. The examples we have considered herein include a relative clonal species such as *S.aureus*, and on the other hand, a pathogen showing a high recombination rate, such as *S. pneumoniae*.

Molecular epidemiology has enormous potential in understanding the evolution of bacterial populations and can help establish appropriate control measures and interventions, including the use of vaccines, therapeutics, public health actions and ongoing pathogen surveillance.

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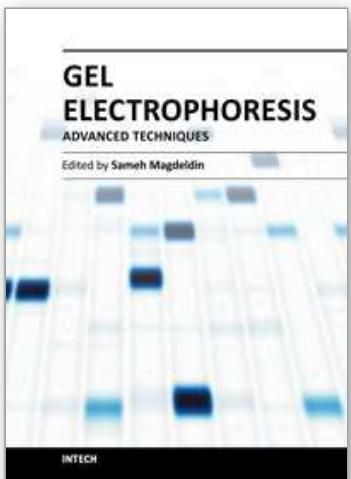
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As a basic concept, gel electrophoresis is a biotechnology technique in which macromolecules such as DNA, RNA or protein are fractionated according to their physical properties such as molecular weight or charge. These molecules are forced through 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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