

The Use of Molecular Typing to Evaluate the Dissemination of Antimicrobial Resistance Among Gram-Negative Rods in Brazilian Hospitals

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Antimicrobial resistance has increased rapidly in Brazil and worldwide during the past few years, giving rise to a growing necessity for antimicrobial resistance surveillance programs. These programs have been instituted in order to monitor bacterial resistance in various regions, and to guide empirical antimicrobial therapy. We evaluated the use of molecular typing in multicenter surveillance programs. We also studied the dissemination modes of selected resistance profiles. Antimicrobial susceptibility to various antimicrobial agents was evaluated by the reference broth microdilution method. Bacterial isolates with selected susceptibility patterns were characterized by pulsed field-gel electrophoresis (PFGE). A total of 119 Gram-negative bacteria were molecularly typed, including 22 imipenem-resistant *Pseudomonas aeruginosa*, 26 ESBL-producing *Escherichia coli*, 27 ceftaxime-resistant-ESBL-producing *Klebsiella pneumoniae*, 33 *Enterobacter* spp., 8 *Citrobacter* spp., and 3 *S. marcescens* isolates resistant to ceftazidime. The isolates were from clinically apparent bacteremia of patients hospitalized in medical centers located in 13 cities of 11 Brazilian states. Our molecular typing results revealed a great genetic diversity among isolates of the same species. However, some major PFGE patterns were found in more than one isolate. All repeated PFGE patterns were detected in only 2 isolates, which were isolated within the same institutions or in different medical centers. We conclude that the ability to characterize organisms phenotypically and genotypically is a powerful epidemiologic tool and it provides unique information that is very important for multicenter surveillance programs.

Key Words: Molecular typing, pulsed-field gel electrophoresis, antimicrobial resistance, carbapenem-resistant *Pseudomonas aeruginosa*, clonal dissemination, Sentinela Program.

Antimicrobial resistance has become an important problem worldwide [1]. Bacterial resistance to antimicrobial agents has been emerging and rapidly disseminating among many nosocomial and community-acquired pathogens [2]. Control of antimicrobial resistance has become a strategic priority for hospitals

and health care providers on a global scale. As part of this effort, there are now several major programs that have been organized to conduct surveillance for antimicrobial resistance on both national and international levels [3-5].

Most surveillance programs use a conventional antimicrobial susceptibility testing method to determine phenotypic profiles. Although useful as a screening method for detecting certain resistance profiles and for selecting potentially useful therapeutic agents, conventional antimicrobial susceptibility testing methods are insensitive tools for tracing the spread of individual strains within a hospital or region. On the other hand, molecular typing methods provide powerful tools to track bacterial strains. Molecular typing contributes to the evaluation of nosocomial infection outbreaks,

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are probably producers of chromosomally derepressed *ampC* beta-lactamases, were characterized by macrorestriction analysis of chromosomal DNA using the pulsed field-gel electrophoresis (PFGE) technique [16]. Genomic DNA inserts were digested at 37°C, during an overnight period (12 to 16hs), with 10U/ml of *SpeI* enzyme (New England Biolabs, Inc., Beverly, MA, USA). Electrophoresis was performed in a CHEF-DRIII apparatus (Bio-Rad, Richmond, CA, USA), under the following conditions: 0.5 TBE, 1% agarose, 13°C, 200V. The electrophoresis ran for 21hs, with the switch interval ramped appropriately for the species being tested [16]. Photographs of ethidium bromide-stained gels were examined visually. Isolates were considered distinct strains whenever there were 3 or more different fragments or bands in the comparisons between PFGE profiles. Isolates were considered similar (subtypes of a major PFGE pattern) whenever there were 1 to 3 band differences between PFGE patterns. Isolates with the same PFGE profile were considered indistinguishable [16]. Major PFGE patterns were identified by a capital letter, while their subtypes were denominated with the same capital letter followed by a subscribed number (A_1 and A_2 for example).

Results

In general, molecular typing revealed considerable genetic diversity among isolates of the same species. However, some major PFGE patterns were found in more than one isolate. Isolates sharing identical or similar PFGE patterns are shown in Table 1. Repeated PFGE patterns were never detected in more than 2 isolates, which were isolated within the same institutions or from distinct medical centers.

Seventeen different PFGE patterns were observed among the 19 imipenem-resistant *P. aeruginosa* strains. Two major PFGE patterns were observed in more than one sample (two samples each) (Figure 1). Two isolates with an identical PFGE pattern (called "B") were isolated in different institutions located in the same city (São Paulo), whereas samples with similar patterns (I_1 and I_2) were isolated from the same hospital.

Similarly, considerable genetic diversity was observed among both ESBL-producing *K. pneumoniae* and ceftoxitin-resistant-ESBL-producing *E. coli*. Three PFGE patterns were shared by more than one *K. pneumoniae* isolate and two PFGE patterns were shared by more than one *E. coli* isolate. Each of these repeated PFGE patterns was detected in two isolates from different institutions. In addition, three of these repeated patterns were shared by isolates from institutions located in different cities.

Three PFGE patterns were shared by more than one *E. cloacae* isolate and two were shared among *E. aerogenes* isolates (Table 1). Two pairs of clonally related isolates were collected from patients hospitalized in a medical center located in São Paulo city, whereas the other repeated pattern (two samples) was found in samples isolated from different cities (São Paulo and Curitiba, Figure 2). Both pairs of *E. aerogenes* isolates sharing identical PFGE patterns were collected from patients hospitalized in cities located 700 to 1000 km apart (Table 1 and Figure 3). Similarly, the clonally related *Citrobacter* spp. strains were isolated in Juiz de Fora and São Paulo, which are located approximately 400 km from each other (Table 1 and Figure 4).

Discussion

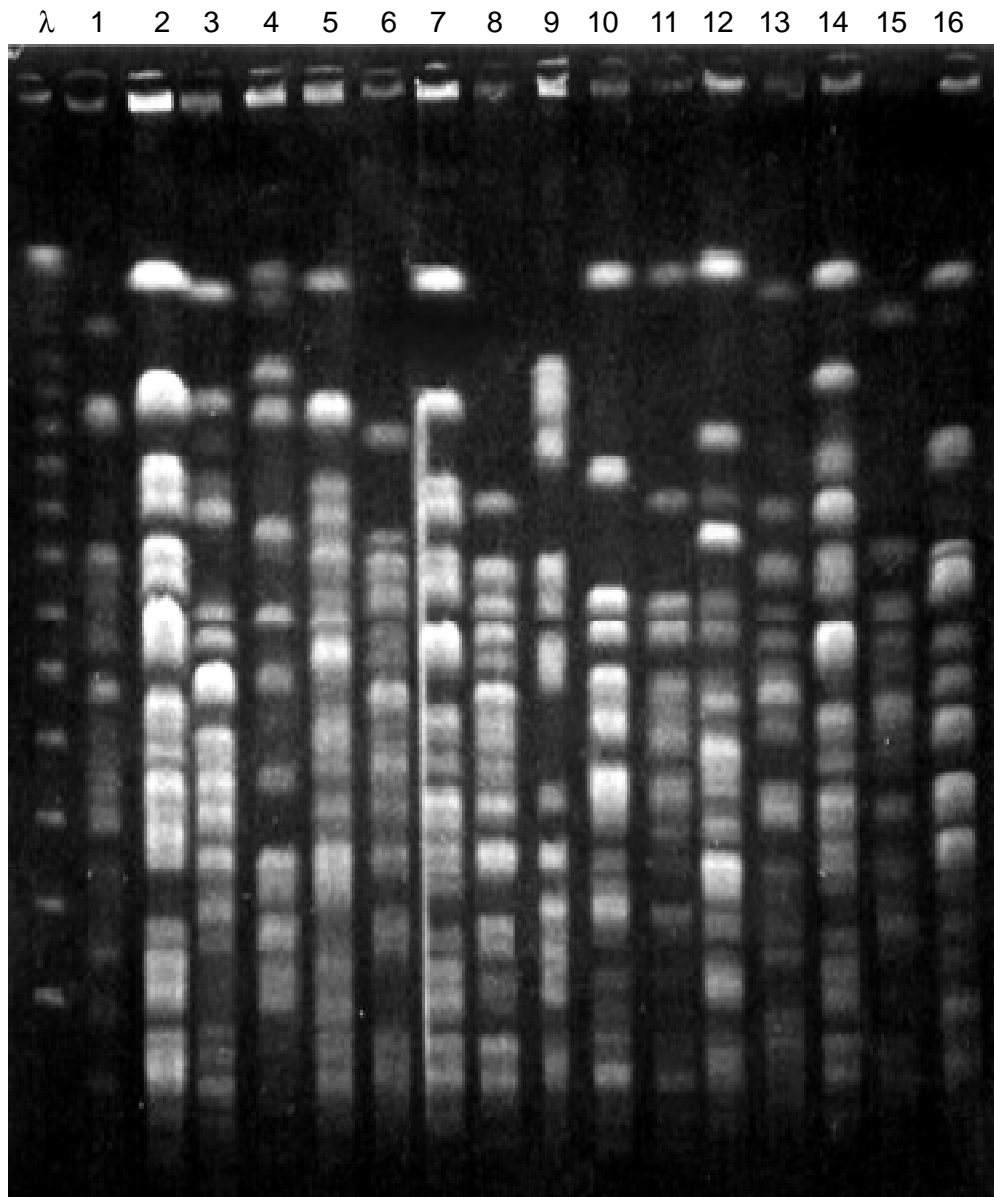
The molecular typing characterization of microorganisms to provide evidence of genetic relatedness is frequently used by physicians, microbiologists, and epidemiologists as an aid in the epidemiological investigation of infectious diseases [7]. A need to determine the relatedness of organisms may arise during an outbreak investigation in which a cluster of infections due to organisms of the same species and similar antimicrobial resistance profiles is identified, in order to determine clonal spread within a microenvironment, and to determine the source of infection [6].

On the other hand, epidemiological surveillance conducted over time requires the monitoring of clonal spread, and determination of the prevalence of strains within a population to monitor the emergence and

Table 1. Hospital location of isolates with repeated major PFGE patterns

Organism	Medical center #/ isolate #	Location	PFGE Pattern
<i>Pseudomonas aeruginosa</i>	1/90069	São Paulo	B
	4/96073	São Paulo	B
	8/103073	Campinas	I ₁
	8/103051	Campinas	I ₂
<i>E. coli</i>	9/115090	Florianópolis	G
	16/115002	Porto Alegre	G
	14/102002	Porto Alegre	L
	15/102039	Porto Alegre	L
<i>Klebsiella pneumoniae</i>	2/101056	São Paulo	B
	3/111056	São Paulo	B
	2/101082	São Paulo	C
	15/111078	Porto Alegre	C
	3/111042	São Paulo	F
	10/121042	Rio de Janeiro	F
<i>Enterobacter cloacae</i>	7/97073	São Paulo	B
	11/97091	Curitiba	B
	1/97004	São Paulo	C
	1/97054	São Paulo	C
	1/97068	São Paulo	E
	1/97063	São Paulo	E
<i>Enterobacter aerogenes</i>	3/106061	São Paulo	D
	9/106005	Florianópolis	D
	8/97048	Campinas	G
	16/97071	Porto Alegre	G
<i>Citrobacter</i> spp.	5/95069	Juiz de Fora	D ₁
	3/109044	São Paulo	D ₂

Figure 1. Pulsed-field gel electrophoresis patterns of imipenem-resistant *Pseudomonas aeruginosa* isolates (lane – PFGE pattern): 1 – A; 2 – B, 3 – C, 4 – D, 5 – E, 6 – F; 7 – B; 8 – G, 9 – H, 10 – I₁, 11 – I₂, 12 – J, 13 – K, 14 – L, 15 – M, 16 – Z (control). Identical strains (PFGE type B) are shown in lanes 2 and 7, similar PFGE patterns I₁ and I₂ are shown in lanes 10 and 11, and the remaining lanes represent strains with unique PFGE patterns. λ - lambda marker.



Medical center 1 2 2 2 3 4 4 5 7 8 8 8 10 10 10 2

Figure 2. Pulsed-field gel electrophoresis patterns of *Enterobacter cloacae* isolates (lane – PFGE pattern): 1 – A; 2 – B; 3 – C; 4 – C; 5 – D; 6 – E; 7 – E; 8 – F; 9 – Z (control). Identical strains are shown in lanes 3 and 4 (PFGE pattern C) and in lanes 6 and 7 (PFGE pattern E), respectively. The remaining lanes represent strains with unique PFGE patterns. λ - lambda marker.

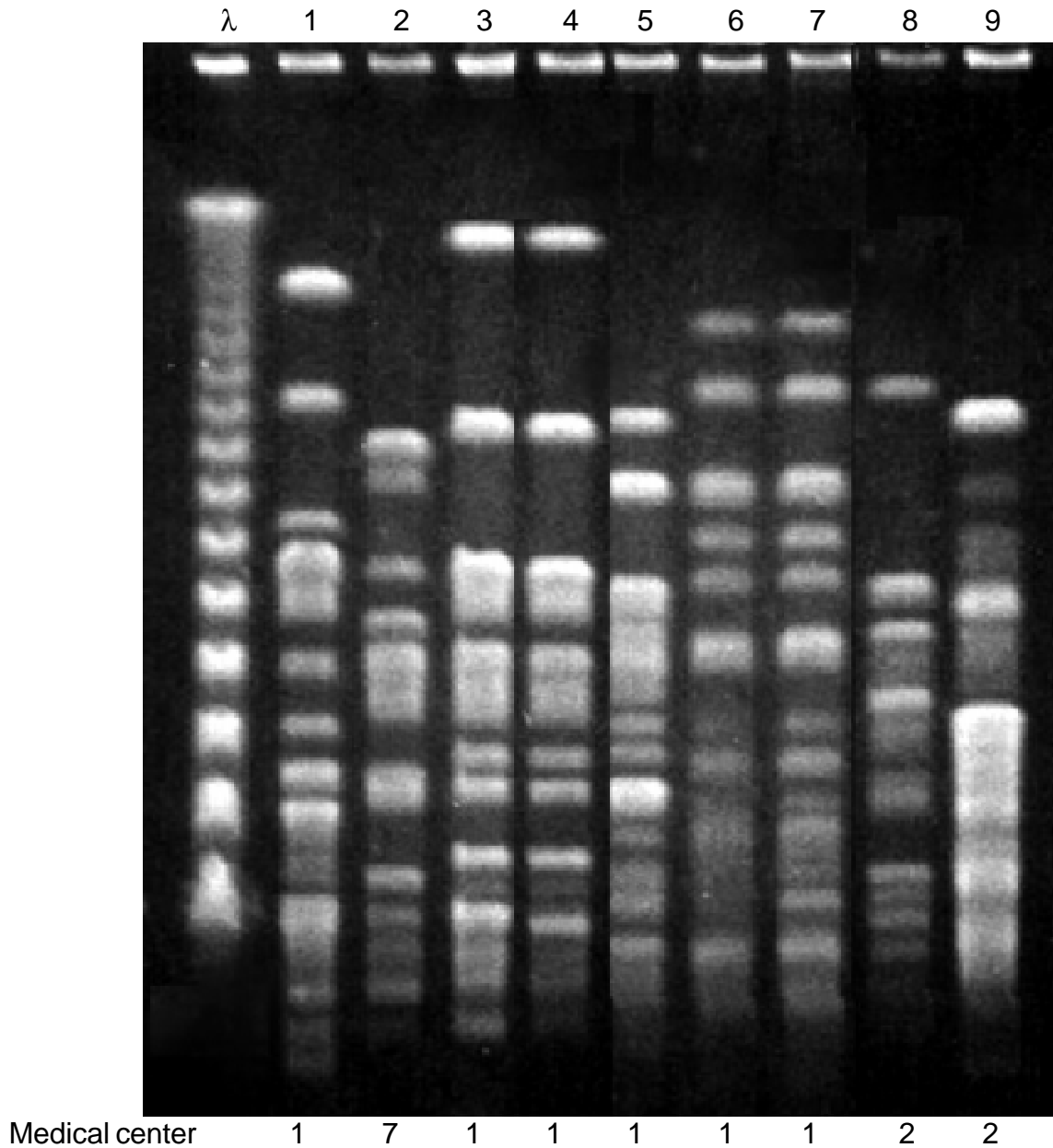


Figure 3. Pulsed-field gel electrophoresis patterns of *Enterobacter aerogenes* (lane – PFGE patterns): 1 – A; 2 – B; 3 – C; 4 – D; 5 – D; 6 – E; 7 – F; 8 – G; 9 – G; 10 – H. Identical strains (PFGE type D and type G) are shown in lanes 4 and 5 and 8 and 9, respectively (D e G). The remaining lanes represent strains with unique PFGE patterns. λ - lambda marker.

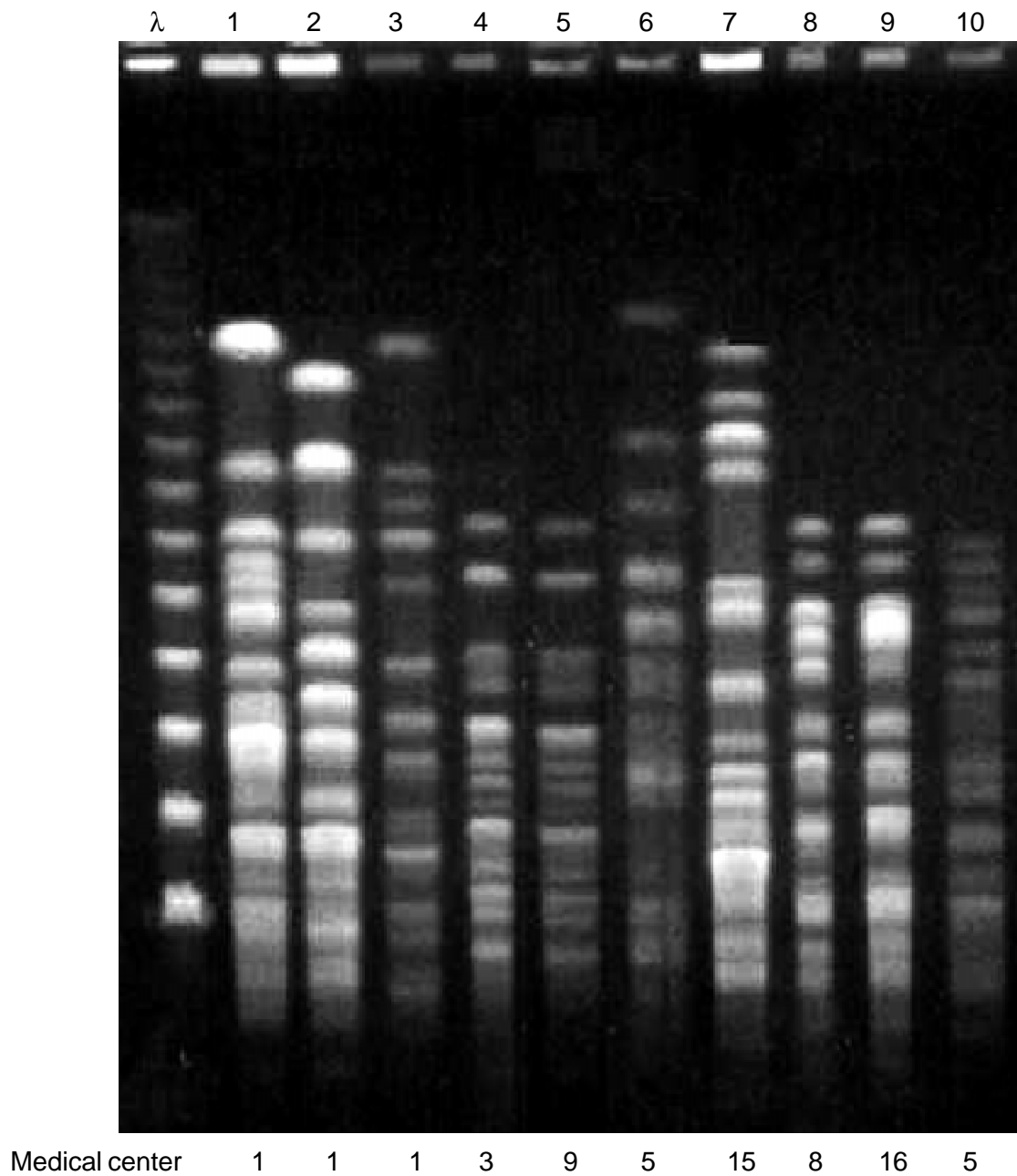
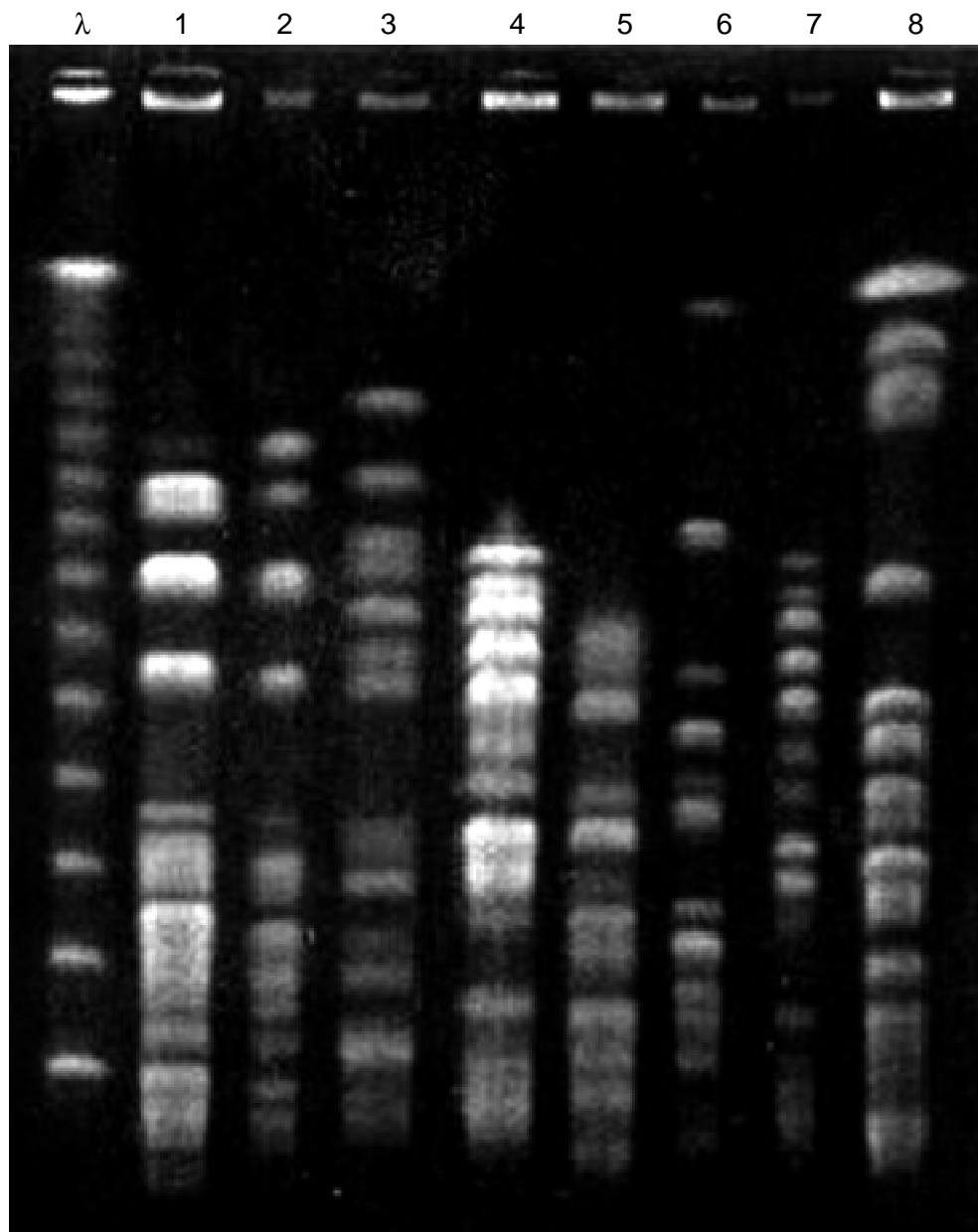


Figure 4. Pulsed-field gel electrophoresis patterns of *Citrobacter* spp. (lane – PFGE pattern): 1 – A; 2 – B; 3 – C; 4 – D₁; 5 – E; 6 – F; 7 – D₂; 8 – Z (control S). Similar strains (PFGE patterns D₁ and D₂) are shown in lanes 4 and 7. The remaining lanes represent strains with unique PFGE patterns λ - lambda marker.



Medical center	1	1	3	5	14	8	3	2
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dissemination of specific pathogens, especially MDR bacteria [10,17]. In any surveillance program the phenotypic characterization of microorganisms and the identification of clusters of isolates of certain species and of resistance phenotypes is the primary role of the microbiology laboratory. Consequently, the surveillance laboratory serves as an "early warning system," alerting the submitting institution of a potential problem with resistant organisms in the patient population [8]. In most of the existing antimicrobial resistance surveillance programs, whether they are local, regional, national, or international in scope, the process does not go much beyond phenotypic characterization and reporting. However, to be of maximum service to individual hospitals, regions, or countries, the surveillance laboratory must go one step further and provide a rapid assessment of microbial clonality by molecular typing [8]. The Sentinela Program is one of the rare examples of regional surveillance programs that use molecular typing characterization of microorganisms to evaluate the dissemination of resistance mechanisms. We have clearly demonstrated clonal dissemination of MDR strains among Brazilian hospitals.

The rapid emergence and dissemination of drug resistance among bacteria has provoked a call for control of these pathogens as a strategic priority for hospitals on a global scale [18], and the finding of clones within a phenotypically identical cluster may have a direct impact on the method of intervention [19]. If the clustered isolates are genotypically different, then the clustering may be due to chance alone, to grouping together of several highly susceptible patients, or to excessive drug pressure, resulting in the selection of a resistant phenotype within a group of unrelated isolates. Clonal dissemination of a resistant strain illustrates the need for more extensive investigations to identify the mechanism of spread and for renewed attention to infection control efforts.

The identification of an endemic resistance problem that is due to the occurrence of multiple clusters of organisms requires a composite approach involving antimicrobial restrictions and focused use of barrier infection-control precautions [19]. Determination of the resistance genotype provides insight into the mechanism of resistance and it may be necessary in

order to understand whether a resistance problem is due to the transmission of resistance genes from organism to organism (controlled by antimicrobial restrictions) or through the transmission of a single resistant strain (controlled by infection-control precautions). Identification of a subpopulation of organisms that are first-step mutants in a multistep pathway leading to high-level resistance (e.g., fluoroquinolone resistance) may trigger strategies that can both prevent the development of high-level resistance and improve therapy overall [20].

On a broader scale, identification of resistant clones with extensive geographic distributions may provide insight into strain virulence and pathogenesis and also may result in public health interventions, such as vaccination and antimicrobial restrictions aimed at reducing the spread of the pathogen and the resistance problem. These considerations provide the rationale for the comprehensive molecular typing program that is an integral part of the SENTRY Antimicrobial Surveillance Program [8]. Each year hundreds of organisms are molecularly characterized, the information is reported to the individual participating centers, and the aggregated data are used to help describe the epidemiology of MDR pathogens [10,11,21,22].

Our results are an overview of molecular typing characterization of organisms obtained during the course of an antimicrobial resistance surveillance program. In order to provide a comprehensive molecular characterization service, the organisms themselves must be readily available. This illustrates the need for a central reference laboratory to serve as a repository of organisms, as well as a standardized source for all microbial characterizations. The ability to utilize molecular typing methods to follow the geographic spread of specific clones provides new information and offers additional opportunities for research that may clarify issues of pathogenesis and epidemiology.

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