

Pseudomonas aeruginosa clonal dissemination in Brazilian intensive care units

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OBJECTIVE. Investigate clonal dissemination of nosocomial multidrug-resistant *Pseudomonas aeruginosa* isolates within and between Brazilian intensive care units, which participated in the MYSTIC Program Brazil 2002.

METHODS. Thirty-six *P. aeruginosa* isolates resistant to meropenem or imipenem plus at least two of the following drugs: ciprofloxacin, cefepime, ceftazidime or piperacillin/tazobactam were isolated during 2002 at 4 centres in São Paulo and 1 centre in Brasília. Chromosomal restriction fragments obtained with *SpeI* were separated by pulsed-field gel electrophoresis (PFGE). Electrophoretic patterns were analyzed with GelCompar II v. 2.5.

RESULTS. Five major clones were identified (A, B, C, D, G). Clone A was constituted by 8 isolates with indistinguishable PFGE pattern present in 2 centres. Clone B was constituted by 4 indistinguishable isolates predominant in centre 6. Clone C had 3 indistinguishable isolates, with closely related clones (C1-3). Also, Clone D had 3 indistinguishable isolates, with closely related (D1) and possibly related (D2/D3) clones. Clones C and D were present in centre 1. Clone G was constituted by 2 indistinguishable isolates and was present in centre 7. Finally, 8 isolates were unique. Isolates from Centre 4 were unique.

CONCLUSIONS. Clonal dissemination was detected within (clones A, B, C, D, and G) and between centres (clone A). These findings are important when analyzing surveillance data, since susceptibility rates may be significantly affected by the dissemination of a resistant clone.

Key words: Pulsed-field gel electrophoresis. *Pseudomonas aeruginosa*. Multi-drug resistance.

Diseminación clonal de *Pseudomonas aeruginosa* en unidades de cuidados intensivos brasileñas

OBJETIVO. Investigar la diseminación clonal de cepas nosocomiales multirresistentes de *Pseudomonas aeruginosa* en y entre unidades de cuidados intensivos brasileñas participantes en el MYSTIC Program Brazil 2002.

MÉTODOS. Durante 2002, se aislaron en cuatro hospitales de São Paulo y en un hospital de Brasília 36 cepas de *P. aeruginosa* resistentes a meropenem o imipenem y a al menos dos de los siguientes: ciprofloxacino, cefepima, ceftazidima o piperacilina/tazobactam. Los fragmentos de restricción cromosómica obtenidos mediante *SpeI* se separaron con electroforesis en campo pulsado (PFGE) y se analizaron mediante GelCompar II v.2.5.

RESULTADOS. Se identificaron cinco clones principales (A, B, C, D y G). El clon A constaba de ocho cepas con un patrón PFGE indistinguible, aisladas en dos centros. El clon B estaba formado por cuatro cepas indistinguibles, predominantes en el centro 6. El clon C estaba formado por tres cepas indistinguibles con clones estrechamente relacionados (C1-3). Además, el clon D estaba formado por tres cepas indistinguibles con clones estrechamente relacionados (D1) o posiblemente relacionados (D2/D3). Los clones C y D se detectaron en el centro 1. El clon G estaba formado por dos cepas indistinguibles y se observó en el centro 7. Finalmente, ocho cepas fueron específicas. Las cepas del centro 4 fueron específicas.

CONCLUSIONES. Se detectó diseminación clonal en los propios centros (clones A, B, C, D y G) y entre centros (clon A). Estos hallazgos son importantes para evaluar los datos de vigilancia epidemiológica pues la diseminación de un clon resistente puede influir de manera significativa en las tasas de sensibilidad.

Palabras clave: Electroforesis sobre el gel con campo pulsado. *Pseudomonas aeruginosa*. Resistencia a múltiples fármacos.

Introduction

Overuse and misuse of antimicrobials has contributed to the emergence of multidrug-resistant (MDR) bacteria, especially in intensive care units. Among MDR bacteria,

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P. aeruginosa is the second most frequent pathogen associated with pneumonia in hospitalized patients in North America¹ and the most frequent in South America². In addition to its relevance as a pathogen, it can acquire resistance to all available antibacterial agents normally used in therapy. *Pseudomonas aeruginosa* has inherent resistance to many drug classes, can acquire resistance to all relevant treatments via mutations, and can harbour integrons with multiple resistance genes, as those coding for metallo-beta-lactamases, which can cleave the most active antimicrobial agents against *P. aeruginosa* and *Enterobacteriaceae*: the carbapenems³.

The Meropenem Yearly Susceptibility Test Information Collection (MYSTIC) is a global, annual and multi-centre surveillance study that compares the activity of several broad-spectrum antimicrobial agents against bacteria isolated from inpatients at specialized hospital units that are carbapenem users. During the 2002 Brazilian Program's edition, elevated resistance rates in *P. aeruginosa* were observed to all antimicrobials. As previously described, high resistance level to carbapenems in *P. aeruginosa* correlates with clonal dissemination⁴. This study aimed at investigating intra and inter-centre clonal dissemination of multi-resistant *P. aeruginosa* responsible for infections in Brazilian intensive care units and to analyse if the high resistance level observed correlates with clonal dissemination.

Methods

Bacterial isolates

Thirty-six multiresistant *P. aeruginosa* isolates collected during the Brazil MYSTIC Program 2002 from five Intensive Care Units in two Brazilian cities were selected. All the isolates were resistant to meropenem or imipenem plus at least two of the following drugs: ciprofloxacin, cefepime or ceftazidime, piperacillin/tazobactam.

Four centres were located in São Paulo (centres # 1 / 4 / 6 / 7) and one in Brasília (# 5). Only one isolate per patient was included in the study. The isolates were obtained from different clinical specimens, including urine, respiratory tract secretions, blood specimens and catheter tips.

Identification and susceptibility testing procedures

Bacterial isolates were identified with the GNI VITEK system card (BioMérieux, Inc., Hazelwood, Missouri, USA) and conventional biochemical tests as described elsewhere⁵. Susceptibility testing was determined by agar diffusion with Etest (AB BIODISK, Solna, Sweden) according to the manufacturer's procedures. Interpretative criteria used were those described in NCCLS document M100-S14⁶.

Phenotypic metallo-beta-lactamase production was investigated by E-test methodology (AB Biodisk, Solna, Sweden), with strips containing imipenem and imipenem plus EDTA. An imipenem/imipenem + EDTA ratio of greater than or equal to eight between the two results was considered as presence of metallo-beta-lactamase. Results were reported as positive (Ratio ≥ 8), negative (Ratio < 8), and non-determinable if dilutions did not allow interpretation as above.

Genotyping

Evaluation of chromosomal polymorphisms was performed by pulsed-field gel electrophoresis (PFGE) as described by Denton et al⁷ with minor modifications. Each plug was digested with 10U of *Spe*I restriction endonuclease (Invitrogen, Carlsbad, CA) at 37 °C for 12 h. Electrophoresis was performed by 1% PFGE agarose gel run on

CHEF-DR III system (Bio-Rad Laboratories, Richmond, CA) over 22 h at 14 °C with 5 to 35 s of linear ramping at 6 V/cm. Electrophoretic patterns were analysed with GelCompar II v. 2.5 (Applied Maths, Kortrijk, Belgium) using the interpretative criteria described by Tenover et al⁸.

Results

Twenty-two distinct PFGE patterns were observed among 36 isolates. Indistinguishable isolates were interpreted as having the same clonal origin. Five major clones were identified, named A, B, C, D and G (fig. 1). These clones were composed by the following isolates:

Eight indistinguishable isolates (14, 25, 26, 41, 42, 48, 62 and 68) belonged to clone A, four isolates (10, 30, 39 and 50) to clone B, three isolates (78, 80 and 101) to clone C, three isolates (6, 8 and 73) to clone D, and two (isolates number 108N and 203N) belonged to clone G.

Furthermore, seven isolates closely related to the above-mentioned clones A, C and D were identified and named A1, A2, A3, C1, C2, C3, and D1. Also, two isolates possibly related to clone D were identified and named respectively D2 and D3. Eight isolates (11, 63, 79, 46AE, 48AE, 53AE, B15 and B19) were unique.

Table 1 shows the minimum inhibitory concentration (MIC) range for imipenem, meropenem, cefepime, ceftazidime, ciprofloxacin, piperacillin/tazobactam, gentamicin, and tobramycin and the results of the metallo-beta-lactamase

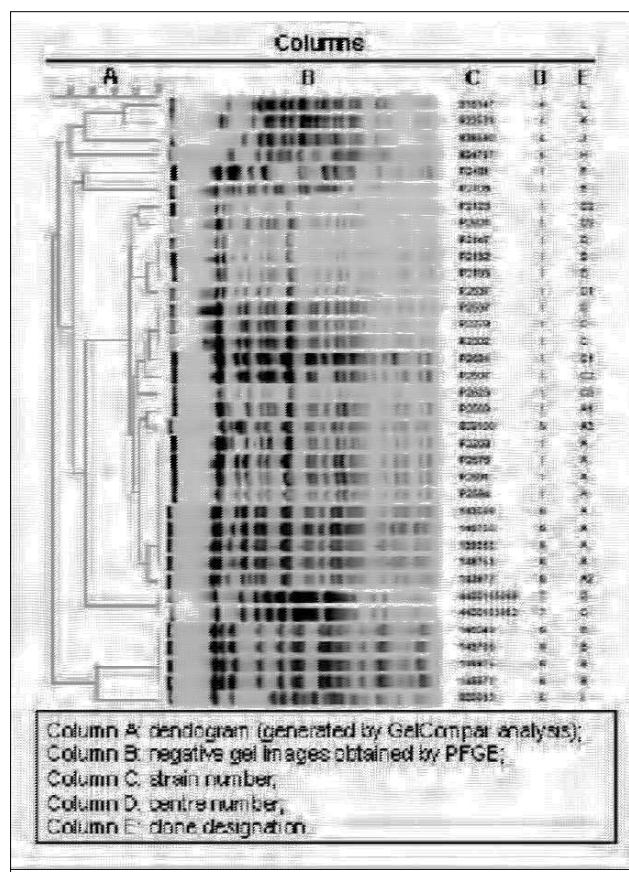


Figure 1. Clonal analysis (gel compar analysis).

TABLE 1. Major clones of *Pseudomonas aeruginosa*, amount of isolates per clones (n), occurrence per centre, MIC range for various antimicrobials and metallo-beta-lactamase production by Etest methodology

Clones	n	Centres	MIC range (µg/ml)								Metallo-beta-lactamase
			IMI	MEM	CPM	CAZ	CIP	PTZ	GEN	TOB	
A (incl. A1, A2, A3)	11	1, 5, 6	> 32	32-> 32	> 256	> 256	> 32	256-> 256	> 256	256-> 256	Positive
B	4	6	> 32	> 32	16-32	16-24	> 32	24-32	> 256	> 256	Positive
C (incl. C1, C2, C3)	6	1	> 32	> 32	> 256	> 256	> 32	48-> 256	> 256	> 256	Positive
D (incl. D1, D2, D3)	6	1	32->32	16-> 32	16-> 256	32-> 256	> 32	> 256	> 256	256-> 256	Negative*
G	2	7	> 32	> 32	> 256	> 256	> 32	> 256	> 256	> 256	Positive

*Except for clone D3 = Positive for metallo-beta-lactamase production.

IMI: imipenem; MEM: meropenem; CPM: cefepime; CAZ: ceftazidime; CIP: ciprofloxacin; PTZ: piperacillin/tazobactam; GEN: gentamicin; TOB: tobramycin.

TABLE 2. Non-related clones of *Pseudomonas aeruginosa* (single isolates), occurrence per centre, MIC values for various antimicrobials and metallo-beta-lactamase production by Etest methodology

Clones	Centres	MIC (µg/ml)								Metallo-beta-lactamase
		IMI	MEM	CPM	CAZ	CIP	PTZ	GEN	TOB	
E	1	> 32	> 32	24	> 256	> 32	64	> 256	> 256	Positive
F	1	32	32	16	> 256	> 32	> 256	> 256	> 256	Negative
H	5	> 32	> 32	24	96	> 32	> 256	16	12	Positive
I	5	> 32	> 32	> 256	> 256	> 32	64	> 256	> 256	Non-determinable
J	4	> 32	> 32	96	48	> 32	> 256	> 256	> 256	Positive
K	4	> 32	> 32	12	16	> 32	> 256	> 256	> 256	Negative
L	4	> 32	> 32	64	> 256	> 32	> 256	> 256	> 256	Positive

IMI: imipenem; MEM: meropenem; CPM: cefepime; CAZ: ceftazidime; CIP: ciprofloxacin; PTZ: piperacillin/tazobactam; GEN: gentamicin; TOB: tobramycin.

mass production test for each of the major clones (i.e. with more than one isolate per group) as determined by Etest methodology, as well as the amount of isolates per clone and their occurrence per centre. Table 2 shows the MIC for the same antimicrobials and the metallo-beta-lactamase production assay for each non-related clone (i.e. with single isolates), as well as their occurrence per centre.

For 28/36 (77.8%) isolates a positive metallo-beta-lactamase production assay was observed. Among the major clones, all but clone D presumably produced metallo-beta-lactamases. Three isolates in clone D, one closely related isolate (clone D1), and one possibly related isolate (clone D2) were not metallo-beta-lactamase producers. However, another possibly related isolate (clone D3) was detected as positive for the production of metallo-beta-lactamase. Among the non-related isolates (i.e. clones with single isolates), 4 tested positive and 2 tested negative for metallo-beta-lactamase production. One isolate (clone I) had a non-determinable result for metallo-beta-lactamase production.

Discussion

Multidrug-resistant *P. aeruginosa* isolates are usually reported to be responsible for nosocomial infections outbreaks, mainly in intensive care units (ICUs). In this study, 36 multidrug-resistant isolates of *P. aeruginosa* isolates were clustered in five major genotypes. The presence of a metallo-beta-lactamase was inferred in 28 (77.8%) out of the 36 isolates. No other test, neither phenotypic nor genotypic, to detect the presence of metallo-beta-lactamase was performed in the isolates. Thus, it is only possible to

infer the presence of these enzymes, since confirmatory tests would be necessary for precisely determining their production.

MIC results have shown that antimicrobial susceptibility phenotypes did not differ significantly within major clones. Furthermore, metallo-beta-lactamase production results among major clones (A, B, C, D and G) were also consistent. All isolates of clones A, B, C, and G tested positive for metallo-beta-lactamase production. Three isolates of clone D and two of its related clones (D1 and D2) tested negative for the production of metallo-beta-lactamase. However, clone D3 tested positive for metallo-beta-lactamase, suggesting a possible non-chromosomal acquisition of this enzyme. Isolates of one of these genotypes (clone A) were identified in two different hospitals in São Paulo, respectively centres 1 and 6, around five kilometres apart from each other. In three centres (1, 5 and 6) closely related isolates (clones A1, A2 and A3) were also detected. Interestingly, centre 5 is located in Brasília, situated more than 1,000 kilometres away from centres 1 and 6. However, these institutions are public teaching hospitals and might, in some instances, share patients and professionals along a certain period of time.

Our findings indicate the occurrence of inter-hospital spread of a *P. aeruginosa* genotype, probably due to transfers of infected patients, share of health care workers, and exchange of medical equipments among institutions. Another possible explanation for the fact might lie on contaminated commercial products commonly used by hospitals, although this is less probable and has not been investigated. The detection of clonal dissemination among different hospitals indicates inadequate nosocomial infection control practices. Those inadequacies might be due to non-adherence to infection control protocols, possibly as-

sociated with elevated workload or incorrect disinfection of medical devices. Nevertheless, it should be noted that only non-related MDR *P. aeruginosa* isolates were recovered from centre 4, a private reference institution located in São Paulo. This finding differs from those of Pellegrino et al⁹ who reported clonal dissemination between private and public institutions in Rio de Janeiro.

Many strategies have been proposed to control the emergence of antimicrobial resistance through prescription control: antibiotic cycling, antibiotic formulary restriction, educational programs, prior approval programs, and computer-assisted management¹⁰. These strategies, although useful in many situations, may sometimes be wrongly applied if used solely and as a consequence of surveillance programmes. Prescription control measures are not the methods of choice to uphold emergence of resistance due to clonal dissemination, since the problem usually lies on better performance of infection control practices.

Additionally, the use of certain antimicrobials has been previously linked to the emergence of resistant pathogens, as is the case for ciprofloxacin and MDR *P. aeruginosa*¹¹. On the other hand, apparently this has not been the case for the carbapenems and MDR *P. aeruginosa* as shown by Jones et al⁴ with the MYSTIC program data. In this situation, the observed high resistance rates to carbapenems were correlated to the persistence of specific clonal spreads. Thus, any analysis for investigating correlation between antimicrobial use and resistance should consider a possible biased result due to clonal dissemination and, preferably, should include a method to identify and exclude related clones from the correlation analysis.

In conclusion, surveillance programme's data may be affected in their susceptibility rates due to clonal dissemination, as was the case of *P. aeruginosa* collected during the MYSTIC Program Brazil 2002. Molecular epidemiology should complement data generated by these programmes in order to refine data analysis. Ultimately this

should maximise the identification of inadequate infection control practices and should improve the prevention of resistance emergence.

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