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Hypermutable *Pseudomonas aeruginosa* in Cystic Fibrosis Patients from Two Brazilian Cities

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Hypermutable (HPM) strains of *Pseudomonas aeruginosa* have been found at high frequencies in cystic fibrosis (CF) patients in Europe. We report the results of testing for HPM frequencies, mutator genotype, and antimicrobial resistance of *P. aeruginosa* strains from Brazilian CF patients. A modified disk diffusion technique was used to quantify antibiotic-resistant subpopulations of an isolate, and estimations of the frequency of mutation to rifampin resistance were determined for 705 isolates from 149 patients attending clinics in two Brazilian cities. Mutations in the *mutS* gene were detected by sequencing assays. We found 194 (27.5%) HPM isolates in samples from 99 (66.4%) patients. Thirty-five HPM isolates (18.0%) from 31 (31.3%) patients exhibited a high increased spontaneous mutation rate compared with controls, and eight isolates from six patients displayed a defective *mutS* gene. The dominant HPM population was associated with very low antibiotic resistance levels, while HPM subpopulations were generally more resistant to antimicrobials. A relatively high prevalence of HPM *P. aeruginosa* in CF patients was associated with surprisingly low antibiotic resistance levels, in contrast to some earlier studies.

Pseudomonas aeruginosa is commonly associated with the chronic, progressive lung disease that is the leading cause of morbidity and mortality in cystic fibrosis (CF) patients (1). During the course of infection, isolates of *P. aeruginosa* undergo a series of profound genotypic and phenotypic changes to adapt to the CF lung environment, and they promote their survival by maximizing diversity in cell populations. Hypermutation (HPM) in specific regions of the genome is one of the mechanisms used for this purpose and may confer fitness benefits for colonization of anatomical niches (2). It has been shown that 37% to 54% of CF patients chronically infected with *P. aeruginosa* harbor isolates with a hypermutator phenotype, as defined by an increased spontaneous frequency of mutations (3–5). HPM strains usually exhibit alterations in genes participating in DNA error avoidance systems (6), and the majority of these strains from CF patients are deficient in the mismatch repair system (MRS), with the *mutS* gene being most frequently affected (3, 4, 7). The MRS is a major barrier to interspecies recombination events. Removal of this barrier also enhances the frequency of horizontal gene transfer, which is an important mechanism of acquired drug resistance in bacteria (8). Maciá and collaborators (4) proposed that the presence of subpopulations of resistant mutant colonies growing in the zone of inhibition of particular antibiotics could be used as a screening test to detect HPM.

An association between high antibiotic resistance rates of *P. aeruginosa* in CF patients and the presence of a high proportion of HPM strains has been repeatedly documented (3, 4, 7, 9), but there is no consensus as to whether there is a causative link between increased antimicrobial resistance and hypermutability.

We report here the results of a survey of *P. aeruginosa* isolates from CF patients in three CF reference centers in two Brazilian cities with respect to their mutator frequencies, mutator genotypes, and correlations with antimicrobial resistance.

MATERIALS AND METHODS

Patients and clinical samples. From 2002 to 2008, *P. aeruginosa* isolates representing different colony morphotypes (mucoid, nonmucoid, pigmented, and nonpigmented) were selected from sputum culture from 51 pediatric and 78 adult CF patients attending the CF Reference Center in Porto Alegre (CFRCPA) in southern Brazil and 14 pediatric and six adult CF patients attending two different CF Reference Centers in Rio de Janeiro (CFRCRJ). These cities are located 1,500 km apart and represent two of the main CF centers in Brazil. The inclusion criteria for patients were chronic pulmonary infection by *P. aeruginosa*, defined as the continuous presence of this microorganism in sputum over 1 year prior to the study or at least three *P. aeruginosa*-positive cultures all separated by more than 1 month during the study period. A series of isolates from individual patients collected on a regular basis were also evaluated. Isolates were identified by conventional standard tests and the API 20NE system (bioMérieux, Marcy l'Etoile, France).

Susceptibility testing and detection of HPM isolates. Inhibition zone diameters were determined for ceftazidime (CAZ, 30 μg), ciprofloxacin (CIP, 30 μg), imipenem (IPM, 10 μg), meropenem (MEM, 10 μg), and tobramycin (TOB, 10 μg) (Oxoid, Basingstoke, United Kingdom) using the disk diffusion method according to the CLSI (10). Hypermutable isolates were detected as described by Maciá et al. (4). Briefly, the presence or absence of resistant subpopulations within the inhibition zones, and

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TABLE 1 Hypermutable, frequency of mutation, and *mut*S mutation prevalences of *Pseudomonas aeruginosa* isolates from Brazilian CF patients

Characteristic	No. (%) positive					
	At CFRCPA		At CFRCRJ		Total	
	Isolates	Patients	Isolates	Patients	Isolates	Patients
HPM	151 (28.7)	82 (63.6)	43 (24.0)	17 (85.0)	194 (27.5)	99 (66.4)
Non-HPM	375 (71.3)	47 (42.0)	136 (76.0)	3 (15)	511 (72.5)	50 (33.5)
SIS*f*	9 (6.0)	7 (8.5)	3 (7.0)	2 (11.8)	12 (6.2)	9 (9.1)
WIS*f*	21 (13.9)	20 (24.4)	2 (4.7)	2 (11.8)	23 (11.9)	22 (22.2)
NIS*f*	121 (80.1)	55 (67.1)	38 (88.4)	13 (76.4)	159 (81.9)	68 (68.7)
mutS mutation	5 (55.6)	4 (57.1)	3 (100)	2 (100)	8 (66.7)	6 (66.7)

HPM, hypermutable isolates; SIS*f*, strong increased spontaneous frequency of mutation; WIS*f*, weak increased spontaneous frequency of mutation; NIS*f*, non increased spontaneous frequency of mutation; CFRCPA, Cystic Fibrosis Reference Center in Porto Alegre; CFRCRJ, Cystic Fibrosis Reference Center in Rio de Janeiro.

the smallest inhibition zone diameters were recorded after 12 h. Isolates showing a reduced inhibition zone diameter of ≥5 mm for at least three antibiotics, with the exception of TOB, for which the presence of any mutant colony was considered, were defined as HPM. Colonies of this subpopulation were retested after growth in antibiotic-free medium to confirm the stability of the resistance phenotype and to exclude the possibility that they were a consequence of antibiotic inactivation during the incubation period. *P. aeruginosa* PAO1 and *P. aeruginosa* ATCC 27853 were used as quality controls for the procedure.

Phenotypic determination of mutation frequency. Isolates classified as HPM as described above were analyzed by the mutation frequency estimation method of Oliver et al. (3). Briefly, independent triplicate 10-ml Mueller-Hinton broth (MHB) overnight cultures were pelleted and resuspended in 1 ml MHB, and serial 10-fold dilutions were prepared in sterile saline. Volumes of 100 μl of each dilution were plated on Mueller-Hinton agar (MHA) with and without 300 μg of rifampin/ml. After 36 h of incubation, colonies were counted and the mean frequency of mutants was estimated. A positive HPM control strain (RH04000003-2) was kindly supplied by Joanne L. Fothergill, University of Liverpool, United Kingdom.

Isolates were classified according to the frequency of mutation (*f*) proposed by Ciofu and collaborators (5) in three categories: strongly increased spontaneous frequency of mutation (SIS*f*) when *f* was $\geq 2 \times 10^{-7}$, weakly increased spontaneous frequency of mutation (WIS*f*) when *f* was $< 2 \times 10^{-7}$ and $\geq 2 \times 10^{-8}$, and nonincreased spontaneous frequency of mutation (NIS*f*) when *f* was $< 2 \times 10^{-8}$.

Genotypic determination of *mutS* gene mutation. The isolates classified as SIS*f* were analyzed by sequencing of the *mutS* gene as described by Kenna et al. (11). DNA was amplified in an Eppendorf MasterCycler Gradient thermal cycler (Eppendorf, Inc., Hamburg, Germany). PAO1 was used as a positive control for PCR, and the negative control consisted of a PCR mix without DNA. PCR products were purified using the ExoSAP-IT purification kit (GE Healthcare, Piscataway, NJ).

PCR products were sequenced using both forward and reverse primers in a Beckman Coulter CEQ 8000 system (Beckman, High Wycombe, United Kingdom) and ABI-PRISM 3100 genetic analyzer (Applied Biosystems, Foster City, CA). Sequencing data were collected using the software Data Collection v1.0.1 (Applied Biosystems). DNA sequences were analyzed using BioNumerics software (Applied Maths, St. Marten-Latem, Belgium). Sequences were compared to the published PAO1 genome sequence (GenBank accession no. NC002516 or Pseudomonas Genome Database V2) in BioEdit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html).

Metallo-beta-lactamase detection. PCR was performed to detect the more prevalent metallo-beta-lactamases genes $bla_{\text{SPM-1}}$, $bla_{\text{IMP-1}}$, and $bla_{\text{VIM-2}}$ as described by Gales et al. (12) and Senda et al. (13) for all isolates with reduced susceptibility to imipenem and/or meropenem. *P. aeruginosa* SPM-1 (48-1997A), IMP-1 (PSA 319), and VIM-2 (AG-2) strains were used as positive controls for $bla_{\text{SPM-1}}$, $bla_{\text{IMP-1}}$, and $bla_{\text{VIM-2}}$, respectively, and the negative control consisted of PCR mix without DNA.

Ethical aspects. The bacterial isolates were obtained from clinical specimens sent for routine culture in the Microbiology Unit of Hospital de Clínicas de Porto Alegre and Laboratório de Bacteriologia of Hospital Universitário Pedro Ernesto. The information was compiled in such a way as to respect the privacy of patients; written informed consent for participation in the study was obtained from participants or, where participants were children, from a parent or guardian. This study was approved by the Ethics Committee in Research of the Hospital de Clínicas de Porto Alegre (project number 06-406) and by the Committee on Ethical Practice of the Hospital Universitário Pedro Ernesto (approval 1.118-CEP-HUPE).

RESULTS

In total, 526 isolates of *P. aeruginosa* from 129 CF patients (mean, 4 isolates/patient) from CFRCPA and 179 isolates from 20 CF patients (mean, 8 isolates/patient) from CFRCRJ were studied. One hundred fifty-one (28.7%) isolates from 82 (63.6%) CFRCPA patients and 43 (24.0%) isolates from 17 (85.0%) CFRCRJ patients were classified as HPM. Therefore, 194 (27.5%) isolates from 99 (66.4%) patients attending the three centers in two Brazilian cities proved to be HPM. Most HPM isolates were obtained from pediatric patients (13/17 patients at CFRCRJ and 43/82 patients at CFRCPA).

Classification of HPM isolates according to the frequency of mutation (*f*) revealed 35 (18.0%) isolates with an increased spontaneous mutation rate (SIS*f* or WIS*f*) from 31 (31.3%) CF patients. A total of 30 (19.9%) isolates were from 27 (32.9%) patients and five (11.6%) were from four (23.5%) patients with increased spontaneous mutation rates attending CFRCPA and CFRCRJ, respectively (Table 1).

Sequencing of the *mutS* gene from isolates classified as SIS*f* showed several synonymous (silent) substitutions in one or more loci of this gene, as well as mutations responsible for amino acid changes, due to base substitution. Alignment studies of the *mutS* gene revealed alterations in amino acids in five isolates from four patients and in three isolates from two patients attending CFRCPA and CFRCRJ, respectively (Table 1). The *mutS* gene mutation positions were variable for each isolate and corresponded to different nucleotide and amino acid changes (see Table S1 in the supplemental material). It is noteworthy that 6 of the 8 SIS*f* isolates (from 6 different patients) with *mutS* mutations belonged to different clones according to pulsed-field gel electrophoresis (PFGE) molecular typing.

The susceptibility profile of the dominant (unselected) population from HPM isolates for CAZ, CIP, IMI, MEM, and TOB in patients attending CFRCPA and CFRCRJ revealed very low resis-

TABLE 2 Antimicrobial resistance of dominant (unselected) population versus subpopulation from hypermutable *P. aeruginosa* isolates from CF patients

Antibiotic[a]	No. (%) of resistant isolates in population			
	CFRCPA ($n = 151$)		CFRCRJ ($n = 43$)	
	Dominant population	Subpopulation	Dominant population	Subpopulation
CAZ	13 (8.6)	45 (29.8)[b]	2 (4.6)	28 (65.1)[b]
CIP	23 (15.2)	44 (29.1)[b]	0	1 (2.3)
IMP	8 (5.3)	89 (58.9)[b]	0	13 (30.2)[b]
MEM	4 (2.6)	34 (22.5)[b]	0	7 (16.2)[b]
TOB	35 (23.2)	52 (34.4)[b]	0	2 (4.6)

[a] CAZ, ceftazidime; CIP, ciprofloxacin; IMP, imipenem; MEM, meropenem; TOB, tobramycin; CFRCPA, Cystic Fibrosis Reference Center in Porto Alegre; CFRCRJ, Cystic Fibrosis Reference Center in Rio de Janeiro.
[b] Fisher's exact test, $P \leq 0.05$.

tance to all antibiotics tested, and this was particularly striking at CFRCRJ, where resistance only to CAZ was observed (4.6%) (Table 2). However, a comparison of the profile of susceptibility to these antibiotics in the dominant population with that in the subpopulation selected by the Maciá test showed the latter to be markedly more resistant to all antibiotics tested (Table 2).

We found 78 (14.8%) *P. aeruginosa* strains from 28 (21.7%) patients attending CFRCPA and six (3.3%) isolates from four (20%) patients attending CFRCRJ, totaling 84 (11.9%) isolates from 32 (21.4%) patients, to be resistant to imipenem and/or meropenem. Ten of these isolates from seven CFRCPA patients were HPM. All carbapenem-resistant isolates were subjected to PCR for $bla_{\text{SPM-1}}$, $bla_{\text{IMP-1}}$, and $bla_{\text{VIM-2}}$ genes, and only one isolate (non-HPM) harbored the $bla_{\text{SPM-1}}$ gene.

The presence or absence of the mucoid phenotype was recorded at first isolation from the patient, and this phenotype was observed in 58 (38.4%) of HPM isolates from 34 (41.5%) of CFRCPA patients, while 190 (50.7%) of non-HPM isolates from 69 (61.6%) of CFRCPA patients exhibited this phenotype. For CFRCRJ patients, 19 (44.1%) HPM isolates from 11 (55%) patients were mucoid, and so were 28 (20.6%) of non-HPM isolates from 16 (80%) of CFRCRJ patients. There was no statistical correlation between HPM and mucoid phenotype ($P = 0.131$).

DISCUSSION

Over the last decade, several *in vitro* and *in vivo* experiments have shown that mutator phenotypes (HPM) confer an evolutionary advantage to bacteria exposed to new, stressful, or fluctuating environments, by the increase of adaptive mutations (14). These mutators have been observed particularly in *P. aeruginosa* from CF patients with significantly worse lung function (15). This study found a lower prevalence (27.5%) of HPM isolates among CF Brazilian patients than among CF patients in Spain (37%) (4). The higher prevalence of HPM in the latter appeared to be associated with *P. aeruginosa* with high antibiotic resistance rates recovered from the chronic stage of pulmonary infection in these patients (3, 4, 7, 9). In contrast to the Spanish study, we found a relatively high prevalence of HPM (24.0%) in patients attending the two centers in Rio de Janeiro, but antimicrobial resistance rates were very low or nonexistent. CF patients attending a larger clinic in Porto Alegre showed a higher prevalence of HPM (28.7%), but antimicrobial resistance was more common. A poor correlation between resistance and hypermutable phenotype was found in pediatric patients (15), and as most of the Brazilian CF patients included in this study were children and harbored mainly strains with antibiotic-susceptible phenotypes, one might expect a lower prevalence of HPM. It is noteworthy that the subpopulations of our HPM isolates generally proved to be more resistant to all antibiotics tested than the dominant (unselected) population, which is consistent with the results of others studies (3, 4, 9). Molecular typing by PFGE of isolates from different CF patients proved that HPM isolates belonged to different clones (data not shown).

In keeping with results obtained by Maciá et al. (9), we did not find a statistically significant association between expression of mucoid phenotype and HPM status.

Metallo-beta-lactamases (MBL) have been reported with increasing frequency worldwide, and $bla_{\text{SPM-1}}$ and $bla_{\text{IMP-1}}$ genes have been documented to be more frequent among Brazilian *P. aeruginosa* isolates (12, 16, 17). Nevertheless, we found an MBL gene ($bla_{\text{SPM-1}}$) in only one carbapenem-resistant CF isolate. Reports of MBL among CF *P. aeruginosa* are sparse but indicate a low prevalence of these carbapenemases (18, 19). This supports the view that carbapenem resistance mechanisms in CF *P. aeruginosa* are not solely due to widespread metallo beta-lactamases.

We were able to detect mutations in the *mutS* gene in HPM CF isolates with strong increased spontaneous mutation rates, confirming that the hypermutation phenomenon is usually associated with mismatch repair mutations, as previously described (7, 9, 11). Nevertheless, it has been suggested that other genes may also be associated with HPM (11).

In conclusion, we were able to demonstrate a relatively high prevalence of HPM isolates in Brazilian CF patients, although most of these isolates had very low antibiotic resistance levels, particularly those from Rio de Janeiro. In addition, the mutations found in the *mutS* gene support the view that hypermutation in *P. aeruginosa* is usually associated with mismatch repair mutations.

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We have no competing interests to declare.

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