bla$_{GES}$ carrying *Pseudomonas aeruginosa* isolates from a public hospital in Rio de Janeiro, Brazil

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Previous analysis of *Pseudomonas aeruginosa* class-1 integrons from Rio de Janeiro, Brazil, revealed the *bla*$_{GES}$ gene in one isolate. We screened isolates of two widespread PFGE genotypes, A and B, at a public hospital in Rio, for the presence of *bla*$_{GES}$. The gene was detected in all seven *P. aeruginosa* isolates belonging to genotype B. Three of the seven genotype-B isolates were resistant to amikacin, aztreonam, ceftazidime, cefepime, ciprofloxacin, gentamicin, imipenem, meropenem, piperacillin-tazobactam and ticarcillin-clavulanic acid. The other four isolates were resistant to all these agents, except gentamicin, imipenem, meropenem and piperacillin-tazobactam. A synergistic effect between ceftazidime and imipenem or clavulanic acid suggested the production of GES-type ESBL.

Key Words: *Pseudomonas aeruginosa*, *bla*$_{GES}$, antibiotics, public hospital, Rio de Janeiro, Brazil.

Extended spectrum beta-lactamases (ESBLs) are enzymes with the ability to inactivate extended-spectrum cephalosporins and monobactams [1]. Various ESBL-types have been found in *Pseudomonas aeruginosa*, but new types are still emerging, including an extended spectrum beta-lactamase, GES [2]. Clinical laboratory detection of ESBL producing *P. aeruginosa* is important, because ESBL may confer resistance to ceftazidime (CAZ), an antimicrobial agent widely prescribed for *P. aeruginosa* infections [3].

GES-1 beta-lactamase was first detected in a *Klebsiella pneumoniae* isolate obtained in France in 1998 [2] from a child transferred from Cayenne, French Guiana. The gene, *bla*$_{GES-1}$, conferred an extended-spectrum cephalosporin resistance profile, including clavulanic acid (CA), tazobactam and imipenem (IPM) [2]. The *bla*$_{GES-1}$ gene was subsequently detected in *P. aeruginosa* from France, [4] and in *K. pneumoniae* from Portugal [5], isolated between 1999 and 2001. A GES-1 producing *P. aeruginosa* isolate was also detected in São Paulo, Brazil in the SENTRY surveillance program [6]. A new variant, GES-2, was described in *P. aeruginosa* from South Africa, isolated in 2000 [7], with the ability to confer intermediate resistance to IPM. Two other ESBLs, IBC-1 and IBC-2, were included in the group of GES-type beta-lactamases due similarity in the amino acid sequences. IBC-1 was described in an *Enterobacter cloacae* clinical isolate and IBC-2 in a *P. aeruginosa* clinical isolate, both obtained in Greece between 1998 and 2000 [8-10]. A double-disk synergy test with CAZ and IPM was able to identify 16 of 19 CAZ-resistant, bla$_{IBC-1}$-carrying *E. cloacae* isolates [9]. The *bla*$_{GES}$ and *bla*$_{IBC}$ genes were found as gene cassettes integrated into class-1 integrons. Two other types of the GES enzyme were described (GES-3 and GES-4) in *Klebsiella pneumoniae* isolates from Japan [11, 12], also encoded by gene cassettes inserted into class-1 integrons located in plasmids.

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Between 1999 and 2000, we detected two multidrug-resistant pulsed-field gel electrophoresis (PFGE) *P. aeruginosa* genotypes, named A and B, among 115 clinical isolates obtained at Hospital Universitário Clementino Fraga Filho (HUCFF), a public teaching hospital in Rio de Janeiro city, Brazil [13]. We subsequently detected the SPM metallo-beta-lactamase gene, and we studied class-1 integrons in these isolates (submitted for publication). The analyses of class-1 integrons revealed the *bla*$_{GES}$ gene in one genotype B isolate with a sequence identical to that reported for *bla*$_{GES-1}$ (GenBank accession number AF355189). We screened all isolates belonging to genotype A and B from HUCFF for the presence of the *bla*$_{GES}$ gene and evaluated the detection of synergistic effects between CAZ and IPM or CA as a screening test for GES production.

Material and Methods

Bacterial isolates

All *P. aeruginosa* genotype A and B isolates from HUCFF were included in our study. Antimicrobial susceptibility of the 25 isolates belonging to genotype A (18) and B (7), based on disk-diffusion [14], revealed universal susceptibility only to polymyxin. Resistance rates to other antimicrobial agents were: 100% (CAZ, cefepime, ciprofloxacin and ticarcillin-CA), 96% (gentamicin, meropenem and piperacillin-tazobactam), 92% (amikacin and IPM) and 76% (aztreonam). Three of the seven genotype-B isolates were resistant to all the antimicrobial agents tested. The four other isolates were resistant to all of the agents, except gentamicin, IPM, meropenem and piperacillin-tazobactam.

Evaluation of antimicrobial synergy

A phenotypic test to evaluate possible synergistic effects with CAZ was performed by the addition of IPM (0.1μg and 1μg) or CA (10 μg) to CAZ (30μg)-containing disks placed on Mueller-Hinton agar medium inoculated with 0.5 McFarland

density standard bacterial suspensions. The amount of IPM used in this test was previously determined to be sub-inhibitory for the growth of *P. aeruginosa* ATCC 27853. An enhancement of the CAZ inhibition zone (around 5 mm or more) in the presence of IPM or CA was interpreted as presumptive evidence for GES.

PCR screening for bla$_{GES}$ beta-lactamase

Chromosomal DNA was obtained from bacterial suspensions grown overnight in Luria Bertani broth with shaking, suspended in 100 mL of sterile water, and boiled for 10 min. After boiling the bacterial cells, PCR was performed on total DNA using GES-1A and GES-1B primers for the *bla*$_{GES-1}$ gene [2] to screen all the isolates. The cycling parameters were 94ºC for 2 min, followed by 30 cycles of denaturation at 94ºC for 1 min, annealing at 50ºC for 1 min and extension at 72ºC for 1 min, with a final 4 min extension step at 72ºC. PCR products were visualized by electrophoresis at 90V for 2 h in 1.5% agarose gel stained with 1% ethidium bromide, visualized by UV transillumination, and photographed.

Results and Discussion

All genotype B isolates were positive for the *bla*$_{GES}$ gene by PCR, while all genotype A isolates were negative. A synergistic effect between CAZ and IPM was observed for four of the seven genotype-B isolates, tested by the addition of 0.1μg IPM to CAZ-containing disks (Figure 1). No synergistic effect was observed after the addition of 1μg IPM to CAZ disks. The test for synergy between CAZ and CA was positive for only three genotype-B isolates, including two with positive synergy to CAZ and IPM (Table 1). Genotype A isolates did not show any positive synergism.

GES is another example of a class-A ESBL in *P. aeruginosa*. The production of GES beta-lactamase has been associated with extended-spectrum cephalosporin resistance antagonized by the addition of various beta-lactams, including IPM [2, 9]. We found a positive synergism between CAZ and IPM for the *bla*$_{GES}$ gene-carrying isolates, reinforcing the utility of synergy tests to detect ESBL in *P. aeruginosa*. However, difficulties for this detection stem from several factors: false-negative results due to naturally-occurring beta-lactamases, such as over-expressed AmpC, the simultaneous presence of metallo-beta-lactamases or oxacillinases, relative resistance to inhibition by clavulanate, and combined resistance mechanisms, such as impermeability and efflux [15]. This is the first report describing a test for synergism in *P. aeruginosa* by addition of beta-lactamase inhibitors to cephalosporin-containing disks, as opposed to disk approximation or minimal inhibitory concentration (MIC) determination. This method could have an advantage over double-disk tests since interpretation of results appears to be easier.

GES-1 and IBC-1 are inhibited by IPM at relatively low concentrations, suggesting high affinity for the antibiotic [2, 9]. However, we only detected a synergistic effect when 0.1μg IPM was added to CAZ-containing disks. The addition of 1μg IPM to CAZ disks possibly induced AmpC-type beta-lactamase production by the *P. aeruginosa* isolate, which could have masked the inhibitory effect upon GES-1 ESBL. Consequently, the synergy tests may be useful as an initial screening for *bla*$_{GES-1}$ producing *P. aeruginosa* isolates; but their efficiency still needs to be determined in order to substitute PCR methods to identify GES-type ESBL-producing isolates. Nucleotide sequence analysis of PCR products is still the only acceptable way to accurately discriminate between ESBL genes of the same family [15].

All seven genotype-B isolates from Rio carried the *bla*$_{GES-1}$ gene, and four of these isolates revealed a synergistic effect between IPM and CAZ, evidenced by an increase of at least five mm in the inhibition zone obtained with a 30 μg CAZ disk, compared to that obtained with a 30 μg CAZ disk with 0.1 μg IPM added. Genotype B was the second-most-prevalent genotype detected in HUCFF in 1999-2000, showing a multidrug-resistance profile, including extended-spectrum cephalosporins and carbapenems. We found that genotype B isolates produced a gene coding an emerging ESBL. The identification of the *bla*$_{GES}$ gene in *P. aeruginosa* isolates from Brazil demonstrates that a new type of beta-lactamase has emerged on three continents within only four years. Rio de Janeiro city and São Paulo are located at least 3,000 km from the other places locations. Whether this gene was introduced into Brazil or if it appeared as a result of independent selection by antibiotic pressure within Brazil is yet to be determined. These observations are important, since confronting *P. aeruginosa* with multiple resistance mechanisms is becoming a challenge.

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Figure 1. Synergy test for *bla*$_{GES}$ carrying a *Pseudomonas aeruginosa* isolate (A) and not carrying the gene isolate (B). Arrows indicate a positive synergistic effect. CAZ: ceftazidime; IPM: imipenem; CA: clavulanic acid. Top left: CAZ; top center: CAZ plus 10µg clavulanic acid; top right: CAZ plus 1µg IPM; bottom left: CAZ plus 0.1µg IPM; bottom center: blank disk with 0.1µg IPM; bottom right: blank disk with 1µg IPM.

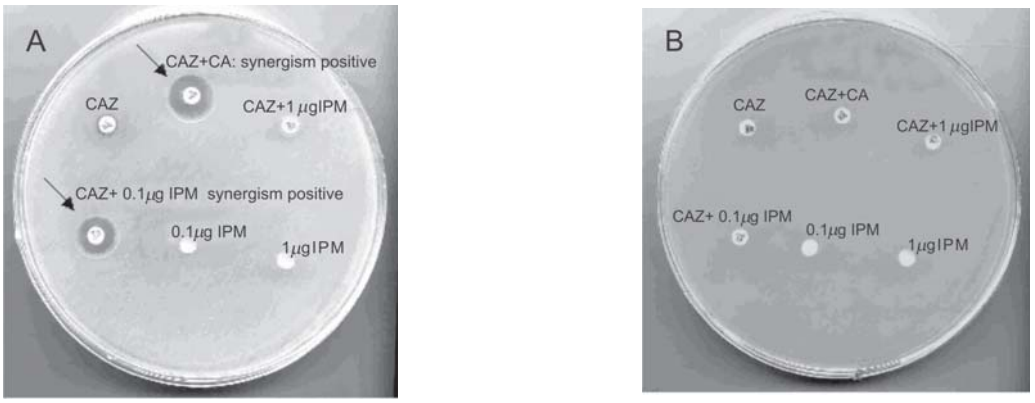

Table 1. Characteristics of *bla*$_{GES}$ carrying *Pseudomonas aeruginosa* isolates belonging to PFGE genotype B

Isolate code	Resistance profile[a]	Synergistic effect with CAZ	
		IPM (0.1µg)	Clavulanic acid (10µg)
PHU99-04	AMK ATM CAZ FEP CIP GEN IPM MEM TZP TIM	+	+
PHU99-16	AMK ATM CAZ FEP CIP GEN MEM TZP TIM	+	+
PHU99-159	AMK ATM CAZ FEP CIP GEN IPM MEM TIM	+	-
PHU99-52	AMK ATM CAZ FEP CIP GEN TZP TIM	+	-
PHU99-29	AMK ATM CAZ FEP CIP GEN IPM MEM TZP TIM	-	-
PHU99-37	AMK ATM CAZ FEP CIP GEN IPM MEM TZP TIM	-	+
PHU99-49	AMK ATM CAZ FEP CIP IPM MEM TZP TIM	-	-

[a]IPM: imipenem; AMK: amikacin; ATM: aztreonam; CAZ: ceftazidime; CIP: ciprofloxacin; FEP: cefepime; GEN: gentamicin; MEM: meropenem; TIM: ticarcillin-clavulanic acid; TZP: piperacillin-tazobactam.

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