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**MOLECULAR IDENTIFICATION OF *BURKHOLDERIA CEPACIA* COMPLEX AND SPECIES DISTRIBUTION AMONG CYSTIC FIBROSIS PATIENTS SEEN AT THE REFERENCE CENTER IN SOUTHERN BRAZIL****IDENTIFICAÇÃO MOLECULAR DO COMPLEXO *BURKHOLDERIA CEPACIA* E DISTRIBUIÇÃO POR ESPÉCIE ENTRE PACIENTES COM FIBROSE CÍSTICA EM UM CENTRO DE REFERÊNCIA NO SUL DO BRASIL**

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**ABSTRACT**

**Background:** *Burkholderia cepacia* complex (Bcc) infections in cystic fibrosis (CF) patients are associated with decline in lung function and reduced survival. The potential transmissibility of Bcc among CF patients has been reported, indicating that strict segregation of CF patients with Bcc is crucial.

**Aims:** To standardize the PCR-RFLP (polymerase chain reaction-restriction fragment length polymorphism) assay in order to identify Bcc species and to establish the prevalence of Bcc species and their susceptibility profile among CF patients seen at the Hospital de Clínicas de Porto Alegre (HCPA).

**Methods:** The classification of the clinical isolates recovered from respiratory tract specimens of CF patients as Bcc was achieved using the API-20NE<sup>®</sup> phenotypic commercial system. The identification of the Bcc species was performed using PCR-RFLP. The antimicrobial disc diffusion susceptibility testing was performed according to the CLSI (2006).

**Results:** API-20NE<sup>®</sup> was able to identify Bcc isolates (244 specimens), such as *B. cepacia*, indicating that it was not able to distinguish among the Bcc species. The PCR-RFLP molecular method discriminated the eight reference Bcc species, thus validating the method for clinical isolates. Bcc prevalence determined by PCR-RFLP was 10.6% (26/244). The molecular analysis identified *B. cenocepacia* in 53.8% (14/26) of infected patients, *B. multivorans* in 15.4% (4/26), and *B. vietnamiensis* and *B. ambifaria* in 7.7% (2/26). The antibiotic resistance profile was variable among Bcc species.

**Conclusions:** The PCR-RFLP method was validated for the identification of Bcc species. *B. cenocepacia* proved to be the most prevalent species among the CF patients seen at the HCPA.

**Keywords:** *Burkholderia cepacia*; cystic fibrosis; PCR-RFLP; *recA* gene.

**RESUMO**

**Introdução:** Infecções por bactérias do complexo *Burkholderia cepacia* (CBC) em pacientes com fibrose cística (FC) estão associadas a declínio da função pulmonar e diminuição da sobrevida. O potencial de transmissibilidade de CBC entre pacientes com FC é uma realidade, tornando-se importante a estrita segregação dos pacientes infectados.

**Objetivos:** Padronizar a técnica de PCR-RFLP (reação em cadeia da polimerase seguida de clivagem com enzimas de restrição) para diferenciação das espécies de CBC e estabelecer a prevalência dessas espécies e seus perfis de sensibilidade em pacientes com FC atendidos no Hospital de Clínicas de Porto Alegre (HCPA).

**Métodos:** A identificação dos isolados clínicos do trato respiratório de pacientes com FC como CBC foi feita pelo sistema de identificação fenotípica comercial API-20NE<sup>®</sup>. A diferenciação das espécies de CBC foi realizada por PCR-RFLP, e o teste de suscetibilidade aos antimicrobianos por disco-difusão foi realizado de acordo com o CLSI (2006).

**Resultados:** O sistema API-20NE<sup>®</sup> identificou todos os isolados do CBC (244 amostras) como *B. cepacia*, indicando claramente que não distingue as espécies do complexo. O método molecular de PCR-RFLP discriminou as oito espécies de referência de CBC, validando o método para isolados clínicos. A prevalência de CBC por PCR-RFLP foi de 10,6% (26/244). A análise molecular apontou *B. cenocepacia* colonizando em 53,8% (14/26) dos pacientes infectados, *B. multivorans* em 15,4% (4/26) e *B. vietnamiensis* e *B. ambifaria* em 7,7% (2/26). O perfil de resistência entre as espécies de CBC para os antibióticos testados foi variado.

**Conclusão:** Foi validada a aplicação do método molecular PCR-RFLP para identificar espécies de CBC, e *B. cenocepacia* foi a espécie mais prevalente entre os pacientes fibrocísticos atendidos no HCPA.

**Palavras-chave:** *Burkholderia cepacia*; fibrose cística; PCR-RFLP; Gene *recA*.

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Cystic fibrosis (CF) is the most common lethal genetic disease among Caucasians (1,2). CF causes a systemic disease which affects mainly the respiratory tract leading to chronic

infection of the airways. There is a variety of bacterial species involved in the CF respiratory infection, but *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Burkholderia cepacia*

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complex (Bcc) are the main microorganisms associated with airway infections (3,4). Bcc consists of a group of phenotypically and phylogenetically closely related but distinct bacterial species (previously termed genomovars) as follows: *Burkholderia cepacia* (genomovar I), *Burkholderia multivorans* (genomovar II), *Burkholderia cenocepacia* (genomovar III with subgroups IIIA, IIIB, IIIC and IIID), *Burkholderia stabilis* (genomovar IV), *Burkholderia vietnamiensis* (genomovar V), *Burkholderia dolosa* (genomovar VI), *Burkholderia ambifaria* (genomovar VII), *Burkholderia anthina* (genomovar VIII), *Burkholderia pyrrocinia* (genomovar IX), and *Burkholderia ubonensis* (genomovar X). A novel species is under process of being formally designated according to multilocus sequence typing (5).

Bcc infections are usually associated with a decline in lung function, which may reduce the survival of CF patients. Such infections may also increase morbidity and mortality following lung transplantation (6-8). Patients infected with Bcc may develop a severe condition characterized by a fulminating pneumonic illness sometimes associated with septicemia, known as "cepacia syndrome", which is related to high mortality (9). Although Bcc is found in soil and water, patient-to-patient spread among CF patients has been reported for all the Bcc species; hence, the importance of a strict segregation of infected patients (10-12). Intrinsic and acquired antibiotic resistance is considered to be a characteristic of Bcc. All Bcc strains are resistant to polymyxin B, and there are only a few antimicrobials effective against Bcc infections (13,14). Although *B. cenocepacia* has been considered the most common species, Bcc species prevalence may vary among different CF centers (15-17).

Based on the importance of patient segregation and on the different prognosis of infected CF patients, the identification of Bcc and its species has been considered very important (18). The identification of Bcc is problematic and misidentification is common as Bcc is easily confused with other non-fermentative Gram-negative bacteria (4). Furthermore, because of the taxonomic complexity of these bacteria and the similarity of the species, the identification of individual Bcc species based on phenotypic methods is often a challenge (13,19). The molecular procedure polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) based on the *recA* gene has been described as one of the most reliable methods for the identification of Bcc species (20-22).

The aims of the present study were to validate the PCR-RFLP and to establish the prevalence of Bcc species and their susceptibility profile among CF patients seen at a university hospital in southern Brazil (Hospital de Clínicas de Porto Alegre - HCPA).

## METHODS

### Study population

To analyze the prevalence of the species, we considered all patients seen at the HCPA from February to December 2006. We studied 1,238 isolates from 244 patients (one to 20 for each patient). Isolates that met the inclusion criteria (80 isolates) were selected for the study.

### Inclusion criteria

All bacteria isolated from respiratory material of CF patients that grew in *B. cepacia* selective agar medium (BCSA-Oxoid<sup>®</sup>) supplemented with *Burkholderia cepacia* Selective Supplement (Oxoid<sup>®</sup>) which proved to be resistant to polymyxin (disc with 300U) and were negative for pyrrolidonyl arylamidase (PYR) were selected for the PCR-RFLP.

### Bacterial reference strains

Strains belonging to the different Bcc genomovars I to VIII used to validate the PCR-RFLP technique were obtained from the Health Protection Agency, London, UK.

### Phenotypic identification

A total of 77 isolates that met the inclusion criteria were also submitted to phenotypic identification using the API-20NE system (Biomerieux<sup>®</sup>) in accordance with the manufacturer's protocol. We considered as unequivocal identification by the API system all results showing 99.9% of likelihood percentage. Moreover, we considered the identification of *B. cepacia* by the API system as comparable to the identification of Bcc by PCR.

### Preparation of template DNA from bacterial cultures

Fresh cultures of the bacterial isolates were suspended in 500 µL of TE buffer, heated to 80 °C for 20 minutes and immediately frozen. After 20 minutes, the suspension was thawed and centrifuged at 11,000 g for 4 minutes. Supernatants containing the bacterial genomic DNA were collected and stored at 2 to 8 °C. The presence of DNA in the supernatants was confirmed by electrophoresis in 0.7% (wt/vol) agarose gels (Life Technologies GIBCO BRL Products<sup>®</sup>) and the quantity and purity of DNA were assessed at 260 and 280 nm.

### PCR analysis

A PCR with a specific primer for the *recA* gene was used to identify the Bcc. The Bcc *recA* gene (1,043 bp) was amplified using primers BCR1 (5'TGACCGCCGAGAAGAGCAA3') and BCR2 (5'CTCTTCTTCGTCCATCGCCTC3'). Approximately 20 ng of DNA was transferred to a tube with 50 µL of reaction mix containing 1 U Taq DNA polymerase, 250 µM (each) deoxynu-

cleoside triphosphate, 1.5 mM MgCl<sub>2</sub>, and 1X PCR buffer. Approximately 20 pmol of each primer was added to reaction mix plus DNA and amplification was carried out using a Thermo Techne TC-300 Barloworld Scientific. Specimens were initially heated at 96 °C for 3 minutes before amplification using 35 cycles consisting of 1 minute of denaturation at 96 °C, 1 minute of annealing at 56 °C, and 1.5 minute of extension at 72 °C. PCR was completed with a final extension step at 72 °C for 10 minutes.

#### RFLP analysis of the *Bcc recA* gene

*Bcc recA* amplicons were digested with *Hae*III (Amersham-Pharmacia Biotech, St. Albans, England) and *Mn*I (New England Biolabs Inc., Hitchin, England) restriction endonucleases. Amplicons (5 µL) were mixed with the endonuclease and its appropriate enzyme buffer, in accordance with the manufacturer's instructions and incubated at 37 °C for 2 hours (22).

#### Detection of PCR and RFLP products

PCR-amplified products and the restriction fragments were analyzed by electrophoresis in 2% (wt/vol) agarose gels. Molecular size markers (100-bp ladder; Life Technologies GIBCO BRL Products®) were used in all gels. DNA products were stained with ethidium bromide and viewed under UV light.

#### Antibiotic resistance

The disc diffusion susceptibility testing was performed to compare the results among the different species of *B. cepacia*. The technique was performed in accordance with the Clinical and Laboratory Standards Institute (CLSI) (23) and the results were classified as "Resistant", "Intermediate" and "Susceptible" according to the CLSI criteria used for *P. aeruginosa* for the following antibiotics (Oxoid®): amikacin, aztreonam, cefepime, ciprofloxacin, doxycycline, chloramphenicol, gentamicin, imipenem, ticarcillin-clavulanate, tobramycin, and piperacillin-tazobactam. The antibiotics ceftazidime, meropenem, and trimethoprim-sulfamethoxazole were classified according to the criteria of *B. cepacia* (23).

#### Statistical analysis

Comparison of the antibiotic resistance among genomovars was performed using the Fisher's exact test. The SPSS 12.0 was used for statistical analysis. A P-value ≤ 0.05 was considered statistically significant for the majority of the comparisons. The Bonferroni correction (i.e., P < 0.0036) was also used for a few comparisons due to the small numbers of isolates.

## RESULTS

#### Phenotypic analysis

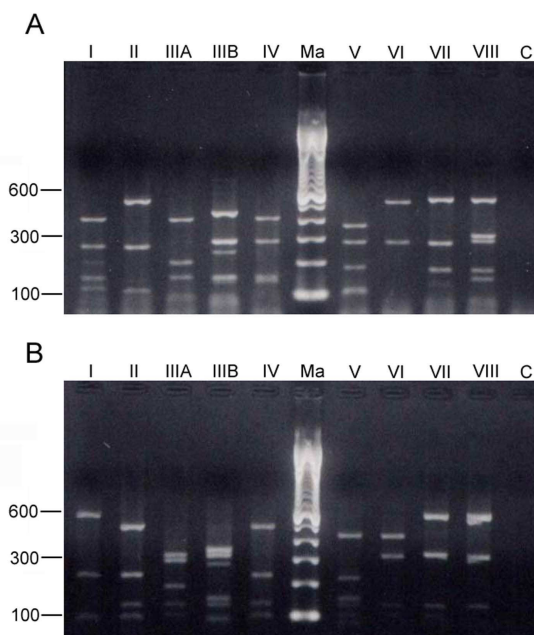
Of the 77 isolates submitted to API-20NE identification, 37 were identified as *Burkholderia cepacia*, two were *Pseudomonas fluorescens*, two were *Oligella* sp, one was *Alcaligenes xylosoxidans*, and one was *Bordetella bronchiseptica*. The API-20NE was not able to identify 34 isolates.

#### PCR analysis

PCR primers BCR1 and BCR2 amplified a single 1kb-amplicon from 66/80 isolates that met the inclusion criteria.

#### PCR-RFLP for *Bcc* species

The analysis of RFLP patterns generated by digestion with *Hae*III and *Mn*I was capable of discriminating all eight species of the reference strains, including the subgroups IIIA and IIIB. Therefore, we considered that this method was validated to be used for the isolates included in this study. It is worth noting, however, that RFLP by *Mn*I was not able to distinguish *Burkholderia ambifaria* (VII) from *Burkholderia anthina* (VIII). These species were discriminated only by *Hae*III PCR-RFLP (Figure 1).



**Figure 1** - RFLP analysis of the *recA* gene amplified from strains of *B. cepacia* (I), *B. multivorans* (II), *B. cenocepacia* IIIA, *B. cenocepacia* IIIB, *B. stabilis* (IV), *B. vietnamiensis* (V), *B. dolosa* (VI), *B. ambifaria* (VII) and *B. anthina* (VIII). **A:** *Hae*III RFLP analysis. **B:** *Mn*I RFLP analysis. Ma: molecular size marker (100-bp ladder). C: negative control.

### Correlation of phenotypic and genotypic identification

Of the 37 isolates identified as *B. cepacia* by API-20NE, 34 were confirmed by PCR. Two isolates, from the same patient, were identified as *Pseudomonas fluorescens* by API-20NE and proved to be *B. vietnamiensis* by the molecular method. The bacteria identified by API-20NE as *Oligella sp.*, *Alcaligenes xylooxidans* and *Bordetella bronchiseptica* were negative for Bcc by PCR. Three isolates were identified by API-20NE as belonging to Bcc and were negative by PCR.

### Prevalence of Bcc genomovars among CF isolates

Of the 66 isolates which were PCR positive for Bcc, 40 were identified as *B. cenocepacia* (31 IIIA and 9 IIIB), 12 as *B. multivorans*, four as *B. vietnamiensis*, and six as *B. anthina*. We were not able to identify the species of four isolates by PCR-RFLP (Table 1).

**Table 1** - Distribution of species among 66 Bcc isolates recovered from 26 CF patients (PCR-RFLP method).

Genomovar	Isolates <sup>a</sup> (%)	Patients <sup>b</sup> (%)
<i>B. cepacia</i>	0	0
<i>B. multivorans</i>	12 (18.1)	4 (15.4)
<i>B. cenocepacia</i>	40 (60.6)	16 (61.5)
Subgroup IIIA	31 (77.5)	12 (75.0)
Subgroup IIIB	9 (22.5)	6 (37.5)
<i>B. stabilis</i>	0	0
<i>B. vietnamiensis</i>	4 (6.1)	2 (7.7)
<i>B. dolosa</i>	0	0
<i>B. ambifaria</i>	6 (9.1)	2 (7.7)
<i>B. anthina</i>	0	0
<i>B. pyrrocinia</i>	0	0
<i>B. ubonensis</i>	0	0
Not determined by PCR	4 (6.1)	2 (7.7)

<sup>a</sup> Number of isolates belonging to a specific species (as percentage of the 66 isolates examined).

<sup>b</sup> Number of patients infected with a specific Bcc species (as percentage of the 26 patients examined). The sum of the percentage of patients infected with the different genomovars IIIA and IIIB exceeds 100% since two patients were infected with both genomovars.

The prevalence of Bcc among CF patients was established as we were able to identify 34 patients with isolates that met the inclusion criteria. Among these, eight patients had isolates that were negative for Bcc by PCR and two patients had isolates that were positive for Bcc but were not identified to species level by PCR-RFLP. Therefore, only 24 patients had isolates that were identified to Bcc species level. The species distribution among these 24 patients was as follows: 14 patients were colonized with *B. ceno-*

*cepacia* (10 patients with Bcc IIIA and four with Bcc IIIB), four patients were positive for *B. multivorans*, two for *B. vietnamiensis*, two for *B. ambifaria*, and two patients were positive for both *B. cenocepacia* IIIA and IIIB in different isolates (Table 1).

Considering the total population of CF patients which had respiratory material submitted for bacteriological culture (244 patients), the prevalence of Bcc in CF was only 10.6% (26/244).

### Longitudinal distribution among CF patients

We were able to evaluate 26 patients with more than one isolate and 24 of them proved to be colonized with the same Bcc species. It is worth noting that two patients had isolates belonging to the genomovar III (*B. cenocepacia*) which were subdivided into types IIIA and IIIB. Isolates from two patients were not identified to species level.

### Antibiotic resistance profile

We compared each species with all the other species identified in this study according to their antibiotic resistance. *B. multivorans* proved to be more resistant to cefepime (P = 0.005), piperacillin-tazobactam (P = 0.012), chloramphenicol (P = 0.003), and imipenem (P < 0.001). Conversely, all *B. multivorans* were susceptible to doxycycline (P = 0.029). *B. cenocepacia* IIIA was more susceptible than the other species to cefepime (P = 0.005), doxycycline (P = 0.029), trimethoprim-sulfamethoxazole (P = 0.040), piperacillin-tazobactam (P = 0.012), chloramphenicol (P = 0.003), and imipenem (P < 0.001). *B. cenocepacia* IIIB and *B. vietnamiensis* did not display statistical difference of antibiotic profile in relation to the others. *B. ambifaria* proved to be more resistance to chloramphenicol (P = 0.003) (Table 2).

## DISCUSSION

Bcc infection may be associated with an accelerated decline in pulmonary function and, therefore, it is extremely important to identify Bcc accurately in the CF pulmonary material. As Bcc may be transmissible among CF patients, it seems obvious that it is important to segregate Bcc-positive from Bcc-negative CF patients, considering their expected prognosis. Moreover, it has been described that *B. cenocepacia* is linked to an even more rapid progression of lung failure in CF patients and this leads to a need for species identification (10,11,24).

Bcc has a complex taxonomic organization and its identification is a challenge for microbiology laboratories. In the present study, 77/80 isolates were submitted to both API-20NE and PCR-RFLP identification and only 34 (44%) were identified as belonging to Bcc (or *B. cepacia* in

the case of API-20NE) by both methods. According to these results, the API-20NE system proved to be relatively reliable for Bcc identifica-

tion (considering the identification of *B. cepacia* as Bcc). However, it was not able to distinguish among Bcc species.

**Table 2** - Percentage of antibiotic resistance among the main Bcc species (genomovars) from CF patients.

	II	IIIA	IIIB	V	VII	P-value
AMK	10(100%)	31(100%)	8(100%)	3(75%)	6(100%)	0.068
ATM	7(87.5%)	4(25%)	3(50%)	1(33.3%)	2(40%)	0.053
FEP	8(80%) <sup>†</sup>	8(25.8%) <sup>†</sup>	5(62.5%)	0	3(50%)	0.005
CAZ	4(40%)	2(6.5%)	1(11.1%)	0	0	0.075
CIP	6(60%)	16(51.6%)	4(50%)	2(66.7%)	5(83.3%)	0.712
DOX	0 <sup>†</sup>	14(45.2%) <sup>†</sup>	3(37.5%)	0	1(16.7%)	0.029
CHL	3(100%) <sup>†</sup>	5(22.7%) <sup>†</sup>	1(25%)	2(66.7%)	3(100%) <sup>†</sup>	0.003
GEN	10(100%)	30(96.8%)	8(100%)	4(100%)	5(100%)	1.000
IPM	5(100%) <sup>†</sup>	2(10%) <sup>†</sup>	2(50%)	0	1(50%)	<0.001
MEM	4(44.4%)	4(12.9%)	2(22.2%)	0	1(16.7%)	0.265
SXT	7(77.8%)	7(30.4%) <sup>†</sup>	5(71.4%)	1(50%)	4(80%)	0.040
TIM	10(100%)	23(74.2%)	7(87.5%)	3(75%)	6(100%)	0.260
TOB	10(100%)	29(96.7%)	8(100%)	3(75%)	6(100%)	0.301
TZP	7(70%) <sup>†</sup>	4(13.3%) <sup>†</sup>	2(25%)	1(25%)	1(25%)	0.012

II: *B. multivorans*, IIIA: *B. cenocepacia* IIIA, IIIB: *B. cenocepacia* IIIB, IV: *B. stabilis*, V: *B. vietnamiensis*, VI: *B. dolosa*, VII: *B. ambifaria*, VIII: *B. anthina*, AMK: Amikacin; ATM: Aztreonam; FEP: Cefepime; CAZ: Ceftazidime; CIP: Ciprofloxacin; DOX: Doxycycline; CHL: Chloramphenicol; GEN: Gentamicin; IPM: Imipenem; MEM: Meropenem; SXT: Trimethoprim-Sulfamethoxazole; TIM: Ticarcillin-Clavulanate; TOB: Tobramycin; TZP: Piperacillin-Tazobactam P < 0.05 <sup>†</sup> P < 0.0036

Conversely, this phenotypic method was not capable of discriminating 34 (44%) of the isolates. Therefore, a variety of Bcc isolates (as established by PCR) was not identified by the API system. As the molecular identification by PCR is superior to the biochemical identification procedures, this commercial phenotypic identification does not seem to be suitable for identification, especially when it provide negative results for *B. cepacia*. Hence, in order to identify the species (or genomovars) within the Bcc complex, a molecular method needs to be used (19,25).

PCR Bcc investigation is based on the polymorphism of the *recA* gene. This gene encodes the *recA*, a multifunctional protein responsible for DNA repair. The *recA* specific primers, used in the PCR method, are highly specific for Bcc members (22). In the present study, we initially used the amplification of the *recA* gene with primers BCR1 and BCR2 to classify Bcc isolates. Further, RFLP was performed with restriction endonucleases *HaeIII* and *MnlI* in order to identify the specific species/genomovar. The PCR-RFLP procedure is fast, sensitive, specific, reproducible, and appears to be a more reliable tool to discriminate Bcc species (25). In the present study, this technique was capable of identifying 66 isolates as belonging to Bcc and it discriminated its species from I to VIII for 93.9% of the Bcc positive isolates. One reason that could explain the fact that a few species could not be identified by PCR-RFLP is that there are other

Bcc species which were not evaluated in our study. Another reason is that there is no consistent data in the literature regarding small variations in the PCR-RFLP patterns (26,27).

During the study period, 10.6% (26/244) of CF patients that met the inclusion criteria were colonized with Bcc. Molecular analysis of the *recA* gene showed that *B. cenocepacia* followed by *B. multivorans* were the most common Bcc species in our CF population and all patients were colonized with only a single species. This predominance of *B. cenocepacia* and *B. multivorans* in CF patients has also been reported in most countries. In Australia and New Zealand, *B. cenocepacia* and *B. multivorans* were more frequently encountered and accounted for a total of 75% of the isolates. *B. cenocepacia* accounts for 45.7% and *B. multivorans* for 29.3%, followed by *B. cepacia* (11.2%) (28).

The prevalence of *B. cenocepacia* found in our CF center was also observed in a variety of other countries. *B. cenocepacia* is prevalent in CF centers in Portugal (15), Italy (16,29,30), Canada (31), Ireland (32), and Czech Republic (33). The Bcc species distribution in French CF centers is slightly different from all these countries, where *B. multivorans* is the most prevalent (51%), followed by *B. cenocepacia* (45%) (17).

We understand that only a few antibiotics are standardized to be tested for Bcc. However, in our study, we evaluated a variety of antibiotics in order to obtain a comparison of results among all species. We found that the antimicrobial re-



sistance to the antibiotics tested varied greatly among Bcc species. As previously mentioned, we had to use a P value adjusted to a small number of isolates ( $P < 0.0036$ ). Therefore, *B. multivorans* was more resistant to chloramphenicol and imipenem; similarly, *B. ambifaria* was found to be markedly resistant to chloramphenicol. Conversely, *B. cenocepacia* IIIA was less resistant to these antibiotics. Among the antibiotics standardized for Bcc (ceftazidime, meropenem and trimethoprim-sulfamethoxazole), only trimethoprim-sulfamethoxazole showed statistical significance ( $P < 0.05$ ) to be more effective for *B. cenocepacia* IIIA. We understand that, regardless the antibiotics approved by the CLSI, it may be important to evaluate other antibiotics for Bcc as the clinical treatment of the CF patients frequently requires different antibiotics from those standardized by the CLSI.

The present study demonstrates the application of a molecular method based on the polymorphism of the *recA* gene to identify Bcc species. It also provides an overview of the prevalence, distribution, and bacterial resistance of Bcc species at a CF center in southern Brazil. Our results confirmed that *B. cenocepacia* is the most prevalent species among CF patients.

In conclusion, the phenotypic methods based on biochemical analysis proved not to be appropriate for the identification of Bcc. Therefore, the use of a molecular technique is crucial for microbiology laboratories that process specimens from CF patients. As we have demonstrated, PCR-RFLP showed to be an unequivocal method for this purpose.

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