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A practical molecular identification of nonfermenting Gram-negative bacteria from cystic fibrosis

Carolina Paulino da Costa Capizzani[a], *Natália Candido Caçador*[a], *Elizabeth Andrade Marques*[b], *Carlos Emílio Levy*[c], *Ludmilla Tonani*[a], *Lidia Alice Gomes Monteiro Marin Torres*[d], *Ana Lúcia da Costa Darini*[a,*]

[a] *Universidade de São Paulo, Faculdade de Ciências Farmacêuticas de Ribeirão Preto, Departamento de Análises Clínicas, Toxicológicas e Bromatológicas, Ribeirão Preto, SP, Brazil*
[b] *Universidade Estadual do Rio de Janeiro, Faculdade de Ciências Médicas, Departamento de Microbiologia, Imunologia e Parasitologia, Rio de Janeiro, RJ, Brazil*
[c] *Universidade Estadual de Campinas, Faculdade de Ciências Médicas, Departamento de Patologia Clínica, Campinas, SP, Brazil*
[d] *Universidade de São Paulo, Faculdade de Medicina de Ribeirão Preto, Departamento de Puericultura e Pediatria, Serviço de Referência de Fibrose Cística do Hospital das Clínicas, Ribeirão Preto, SP, Brazil*

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ABSTRACT

Identification of nonfermenting Gram-negative bacteria (NFGNB) of cystic fibrosis patients is hard and misidentification could affect clinical outcome. This study aimed to propose a scheme using polymerase chain reaction to identify NFGNB. This scheme leads to reliable identification within 3 days in an economically viable manner when compared to other methods.

* *Corresponding author at*: Faculdade de Ciências Farmacêuticas de Ribeirão Preto, Universidade de São Paulo, Avenida do Café, s/nº, Ribeirão Preto, SP, Brazil.
E-mail: aldarini@usp.br (A.L. Darini).
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Chronic respiratory tract infection is responsible for high morbidity and mortality in cystic fibrosis (CF) patients and is frequently associated with nonfermenting Gram-negative bacteria (NFGNB).[1] The microbiology of CF lung disease has changed substantially in recent decades, and now includes novel NFGNB such as *Burkholderia cepacia* complex (Bcc), *Achromobacter xylosoxidans* and *Stenotrophomonas maltophilia*, as well as several species of *Ralstonia*, *Cupriavidus* and *Pandoraea*.[1,2] Most exhibit high resistance to antimicrobials, which makes treatment problematic, and have the potential for interpatient transmission, leading some healthcare facilities to strongly recommend patient segregation.[1,3,4]

Chronic infections, especially by Bcc species, may result in accelerated decline of pulmonary function.[5] This complex comprises at least 20 species, and although most are potentially capable of causing infections, *Burkholderia cenocepacia* and *Burkholderia multivorans* are considered the most prevalent, and *B. cenocepacia* is related to highly transmissible and virulent clonal lineages.[6] Some are associated with cepacia syndrome, a necrotizing pneumonia that leads to rapid deterioration of lung function, bacteremia and increased mortality.[5,7,8] *A. xylosoxidans*, which is considered the most common species within the genus *Achromobacter*, has been shown to cause a level of inflammation similar to *Pseudomonas aeruginosa* in chronically infected CF patients and a greater decline in lung function in these patients compared to non-infected patients.[9] Chronic pulmonary infection with *S. maltophilia* has been shown to be associated with an increased risk of pulmonary exacerbations, which leads to increased risk of lung transplantation or death in individuals with CF.[10] The large number of pili/fimbriae synthesized by *S. maltophilia*, which are associated with adhesion and biofilm formation, may contribute to the maintenance of this bacteria in lung infections, which shows why this microorganism is persistent and difficult to eradicate.[11] Since the consequence of these pathogens in the CF lung can be very serious, their correct identification is extremely important for a more efficient treatment.

Due to taxonomic complexity and high phenotypic similarity between these NFGNB, accurate identification represents a challenge for conventional microbiology. Conventional phenotypic methods including observation of colony morphology on media, analysis of manual biochemical reactions, and the use of automated and nonautomated commercially available biochemical panels are not suitable for CF isolates identification. NFGNB often present colonies of atypical appearance and lack key metabolic characteristics, which impairs the identification.[12,13] Automated systems, as Vitek® 2 (bioMérieux), lead to inaccurate identification of NFGNB due to their phenotypic variations and slower growth rates.[14] Moreover, commercial phenotypic databases are often outdated and lack current taxonomy.[12] Misidentification of NFGNB seriously compromises infection control measures and confounds efforts to more clearly understand the epidemiology and natural history of infection in CF.[4,15]

Currently, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) is used in clinical microbiology laboratories for identification of CF bacterial species. It presents low cost per sample, and is considered to be a faster, and more reliable alternative than polymerase chain reaction (PCR) for these microorganisms identification.[15] However, the high cost of the equipment may be considered a limitation for some laboratories.[16] In addition, many recent studies report difficulty in identifying microorganisms at the species level, due to great variations of protein spectra in different strains belonging to the same species.[17–19]

PCR is considered a simple and highly sensitive technique that produces results quickly, and it is economically viable when compared to sequencing methods.[20] The objective of the present study was to propose a scheme using PCR to identify NFGNB, based on the results of identification of reference strains and clinical isolates from CF patients.

The following reference strains were used in this study: *A. denitrificans* LMG 1231, *A. piechaudii* LMG 1873, *A. xylosoxidans* LMG 1863, *A. dolens* LMG, *A. insuavis*, *A. mucicolens*, *A. ruhlandii*, *C. gillardi* LMG 5886, *P. norimbergensis* LMG 18379, *P. pnomenusa* LMG 18087, *P. pulmonicula* LMG 18106, *P. apista* LMG 16407, *R. picketti* LMG 5942, *S. maltophilia* LMG 958, *B. cepacia* 1254, *B. multivorans* 788, *B. cenocepacia* LMG 21462, *B. cenocepacia* 818 Genomovar IIIA, *B. cenocepacia* 17604 Genomovar IIIA, *B. cenocepacia* 842 Genomovar IIIB, *B. cenocepacia* 805 Genomovar IIIB, *B. stabilis* 790, *B. stabilis* 825, *B. vietnamiensis* LMG 10929, *B. vietnamiensis* 1109, *B. dolosa* LMG-18943, *B. ambifaria* LMG 19182, *B. ambifaria* ATCC-53266, *B. ambifaria* AMMD, *B. anthina* LMG-20980, *B. anthina* LMG-16670, *B. pyrrocinia* ATCC-39227 and *B. pyrrocinia* LMG-14191. All of them were tested with each pair of primers shown in Table 1 in order to evaluate the specificity of PCR assays.

The extraction of genomic DNA was performed using the method described by Gianni-Rossolini.[21] PCR was conducted in a volume of 25 μL adding 2.5 μL of 10× concentrated PCR buffer solution, 2 mM magnesium chloride ($MgCl_2$), 0.625 U Taq DNA polymerase (Thermo Fisher Scientific, Waltham, Massachusetts, USA), 0.2 mM each of the 4 nucleotides (dNTP – Eppendorf, Hamburg, Germany), 20 pmol of the primers for amplification of genes, 60 ng of DNA and ultrapure water (Sigma–Aldrich, St. Louis, Missouri, USA). Amplification was carried out with the Mastercycler Gradient Thermal Cycler (Eppendorf, Hamburg, Germany). Cycling conditions for amplification were used according to the referenced articles (Table 1).

PCR was first performed according to Table 1. Modifications were proposed when nonspecific reactions were obtained or products were not amplified for the purpose of each primer (Table 2).

This study included 201 clinical isolates of NFGNB previously identified by the Vitek® 2 Compact system (bioMérieux, Marcy l'Etoile, France). The isolates were obtained from July 2011 to September 2014 from patients with CF who were treated at two locations: 44 patients from Hospital das Clínicas da Faculdade de Medicina de Ribeirão Preto – Universidade de São Paulo (HCFMRP-USP), and 56 patients from Hospital de Clínicas da Faculdade de Ciências Médicas – Universidade Estadual de Campinas (HCFCM-UNICAMP). The study was approved by the Committee on Ethical Practice of the Faculty of Pharmaceutical Sciences of Ribeirão Preto, University of São Paulo by number 210, with agreement by the Clinical Hospital of the Ribeirão Preto Medical School of the University of São Paulo and the Clinical Hospital of the School of Med-