BRAZILIAN JOURNAL OF MICROBIOLOGY

http://www.bjmicrobiol.com.br/

Bacterial, Fungal and Virus Molecular Biology

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*

ARTICLE INFO

Article history:
Received 27 March 2017
Accepted 3 July 2017
Available online 4 November 2017
Associate Editor: Cristiano Gallina Moreira

Keywords:
Cystic fibrosis
Nonfermenting Gram-negative bacteria
Identification scheme
Diagnosis

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).
https://doi.org/10.1016/j.bjm.2017.07.002

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-

Table 1 – PCR conditions used in the present study.

Primer Target	Primers	Annealing temperature (°C)	Product size (bp)[a]	References
B. cepacia (Genomovar I)	BCRG11 BCRG12	62	492	Mahenthiralingam et al.[22]
B. multivorans (Genomovar II)	BCRBM1 BCRBM2	62	714	Mahenthiralingam et al.[22]
B. cenocepacia (Genomovar III-A)	BCRG3A1 BCRG3A2	62	378	Mahenthiralingam et al.[22]
B. cenocepacia (Genomovar III-B)	BCRG3B1 BCRG3B2	60	781	Mahenthiralingam et al.[22]
B. stabilis (Genomovar IV)	BCRG41 BCRG42	64	647	Mahenthiralingam et al.[22]
B. vietnamiensis (Genomovar V)	BCRBV1 BCRBV2	62	378	Mahenthiralingam et al.[22]
B. dolosa (Genomovar VI)	G6N BCR1	67	135	Vermis et al.[23]
B. ambifaria (genomovar VII)	BCRGC1 BCRGC2	62	810	Coenye et al.[24]
B. anthina (genomovar VIII)	BCRG81 BCRG82	61	473	Vandamme et al.[25]
Complexo *B. cepacia* – Gene *recA*	BCR1 BCR2	58	1043	Mahenthiralingam et al.[22]
A. xylosoxidans	AX-F1 AX-B1	56	163	Liu et al.[26]
S. maltophilia	SM1 SM4	58	531	Whitby et al.[27]
P. sputorum	spuF spur	63	813	Coenye et al.[28]
Genus *Ralstonia*	RalGS-F RalGS-R	58	546	Coenye et al.[29]
Genus *Cupriavidus*	ral2f ral2r	58–61	187	Barrett and Parker[30]

[a] Size of amplified product by PCR.

Table 2 – Comparison of the primers and genus/species proposed by the literature and proposed by the present study.

Primers	Genus/species		Targets	Proposed modifications[a]
	Literature	The present study		
BCR1/BCR2	Bcc bacteria	Bcc bacteria		AT 55 °C and 56 °C
AX-F1/AX-B1	*A. xylodoxidans*	*Achromobacter* sp.		NMT
SM-1/SM4	*S. maltophilia*	*S. maltophilia*		NMT
spuF/spuR	*P. sputorum*	*Pandoraea* sp. + *Ralstonia* sp.		NMT
RalGS-F/RalGS-R	*Ralstonia* sp.	*Ralstonia* sp. + *Cupriavidus* sp.		NMT
ral2f/ral2r	*Cupriavidus* sp.	*Cupriavidus* sp. + *Ralstonia* sp. + *Pandoraea* sp. + *Achromobacter* sp.		AT 62 °C

[a] According to identification carried out with the reference strains and clinical isolates. AT, annealing temperature; NMT, no modification to previously proposed temperature.

ical Sciences of the State University of Campinas. Informed consent was obtained from all participants included in the study. The following tests were carried out for screening of the NFGNB isolates: macroscopic characteristics in Mueller-Hinton agar supplemented with 5% sheep's blood, MacConkey agar (Oxoid, Basingstoke, Hampshire, UK) and *B. cepacia* Selective Agar (BCSA – Oxoid, Basingstoke, Hampshire, UK); Gram morphology; oxidation/fermentation of glucose and xylose (Difco, Franklin Lakes, New Jersey, USA); and production of oxidase, as previously described.[31]

Identification of clinical isolates was performed by PCR using primers of genus/species according to proposed modifications by this study (Table 2). All clinical isolates identified as belonging to Bcc were selected to perform restriction fragment length polymorphism analysis (PCR-RFLP) in order to identify the species of Bcc. Amplicons generated by PCR using the primers BCR1 and BCR2 were digested with *Hae*III restriction endonuclease in accordance with the manufacturer's instructions (Thermo Fisher Scientific, Waltham, Massachusetts, USA).[32] Generated restriction fragments were analyzed by agarose gel electrophoresis (2%). Molecular size markers were used (100 bp ladder, Thermo Fisher Scientific, Waltham, Massachusetts, USA). After electrophoresis, the gels were stained with ethidium bromide and visualized and

Table 3 – Results of amplification of all NFGNB using proposed modifications.

Bacteria tested	Genus/species Primers					
	Bcc BCR1/BCR2	*P. sputorum* spuF/spuR	*Ralstonia* sp. RalGS-F/RalGS-R	*A. xylosoxidans* AX-F1/AX-B1	*S. maltophilia* SM-1/SM4	*Cupriavidus* sp. ral2f/ral2r
Reference strains						
A. denitrificans LMG 1231	Negative	Negative	Negative	**Positive**	Negative	**Positive**[a]
A. piechaudii LMG 1873	Negative	Negative	Negative	**Positive**	Negative	**Positive**[a]
A. xylosoxidans LMG 1863	Negative	Negative	Negative	**Positive**	Negative	**Positive**[a]
A. dolens LMG	Negative	Negative	Negative	**Positive**	Negative	**Positive**[a]
A. insuavis	Negative	Negative	Negative	**Positive**	Negative	**Positive**[a]
A. mucicolens	Negative	Negative	Negative	**Positive**	Negative	**Positive**[a]
A. ruhlandii	Negative	Negative	Negative	**Positive**	Negative	**Positive**[a]
C. gillardi LMG 5886	Negative	Negative	**Positive**[a]	Negative	Negative	**Positive**
P. norimbergensis LMG 18379	Negative	**Positive**	Negative	Negative	Negative	**Positive**[a]
P. pnomenusa LMG 18087	Negative	**Positive**	Negative	Negative	Negative	**Positive**[a]
P. pulmonicula LMG 18106	Negative	**Positive**	Negative	Negative	Negative	**Positive**[a]
P. apista LMG 16407	Negative	**Positive**	Negative	Negative	Negative	**Positive**[a]
R. picketti LMG 5942	Negative	**Positive**[a]	**Positive**	Negative	Negative	**Positive**[a]
S. maltophilia LMG 958	Negative	Negative	Negative	Negative	**Positive**	Negative
B. cepacia 1254	**Positive**	Negative	Negative	Negative	Negative	Negative
B. multivorans 788	**Positive**	Negative	Negative	Negative	Negative	Negative
B. cenocepacia LMG 21462	**Positive**	Negative	Negative	Negative	Negative	Negative
B. cenocepacia 818 Gen. IIIA	**Positive**	Negative	Negative	Negative	Negative	Negative
B. cenocepacia 17604 Gen. IIIA	**Positive**	Negative	Negative	Negative	Negative	Negative
B. cenocepacia 842 Gen. IIIB	**Positive**	Negative	Negative	Negative	Negative	Negative
B. cenocepacia 805 Gen. IIIB	**Positive**	Negative	Negative	Negative	Negative	Negative
B. stabilis 790	**Positive**	Negative	Negative	Negative	Negative	Negative
B. stabilis 825	**Positive**	Negative	Negative	Negative	Negative	Negative
B. vietnamiensis LMG 10929	**Positive**	Negative	Negative	Negative	Negative	Negative
B. vietnamiensis 1109	**Positive**	Negative	Negative	Negative	Negative	Negative
B. dolosa LMG-18943	**Positive**	Negative	Negative	Negative	Negative	Negative
B. ambifaria LMG 19182	**Positive**	Negative	Negative	Negative	Negative	Negative
B. ambifaria ATCC-53266	**Positive**	Negative	Negative	Negative	Negative	Negative
B. ambifaria AMMD	**Positive**	Negative	Negative	Negative	Negative	Negative
B. anthina LMG-20980	**Positive**	Negative	Negative	Negative	Negative	Negative
B. anthina LMG-16670	**Positive**	Negative	Negative	Negative	Negative	Negative
B. pyrrocinia ATCC-39227	**Positive**	Negative	Negative	Negative	Negative	Negative
B. pyrrocinia LMG-14191	**Positive**	Negative	Negative	Negative	Negative	Negative
Isolates						
Bcc ($n = 91$)	**Positive**	Negative	Negative	Negative	Negative	Negative
Achromobacter sp. ($n = 85$)	Negative	Negative	Negative	**Positive**	Negative	**Positive**[a]
S. maltophilia ($n = 12$)	Negative	Negative	Negative	Negative	**Positive**	Negative
Ralstonia sp. ($n = 10$)	Negative	**Positive**[a]	**Positive**	Negative	Negative	**Positive**[a]
Pandoraea sp. ($n = 2$)	Negative	**Positive**	Negative	Negative	Negative	**Positive**[a]
Cupriavidus sp. ($n = 1$)	Negative	Negative	**Positive**[a]	Negative	Negative	**Positive**

[a] Unexpected results. Gen., genomovar.

photographed with the AlphaImager System® (Alpha Innotech, San Leandro, California, USA). All reference strains of Bcc were used as positive controls.

PCRs carried out for primers as shown in Table 2 allowed the recognition only of the genus for most NFGNB, except for primer pair SM-1/SM-4, which was specific to the genus and species for *S. maltophilia*.

Primers BCR1/BCR2 and SM-1/SM-4, used to identify Bcc isolates and *S. maltophilia*, respectively, generated amplicons of the expected size and showed high specificity and sensitivity. Primers BCR1/BCR2 showed positive results not only for *B. cepacia*, *B. multivorans*, *B. cenocepacia* IIIA and IIIB, *B. stabilis* and *B. vietnamiensis* as presented by Mahenthiralingam et al.,[22] but also for *B. dolosa*, *B. ambifaria*, *B. anthina*, and *B. pyrrocinia* (Table 3).

According to Liu et al.,[26] the primer pair used to identify *A. xylosoxidans* (AX-F1/AX-B1) showed 97% specificity. In this study, this primer pair generated amplicons only for *Achromobacter* genus. However, it generated amplicons for *A. xylosoxidans*, and for all isolates of *A. piechaudii*, *A. denitrificans*, *A. dolens*, *A. insuavis*, *A. mucicolens*, and *A. ruhlandii* (Table 3). According to Coenye et al.,[28] the primer pair spuF/spuR was effective for identification of *P. sputorum* and genomospecies 2 and 3. In the present study, these primers generated amplicons for all species of *Pandoraea* tested, but they also generated amplicons for *Ralstonia* sp. The primer pair RalGS-F/RalGS-R

provided amplicons for *Ralstonia* sp., but they also generated amplicons for *Cupriavidus* sp. The primer pair ral2f/ral2r, which is specific for *Cupriavidus*, also provided amplicons for three other bacteria genera: *Achromobacter*, *Pandoraea* and *Ralstonia* (Table 3). It was necessary to perform PCR using both primer pairs spuF/SpuR and RalGS-F/RalGS-R to allow identification of these microorganisms. If both primer pairs generate amplicons, the isolate should be identified as belonging to the genus *Ralstonia*. If there are amplicons only with the pair of primers spuF/spuR, the isolate should be identified as belonging to the *Pandoraea* genus. If after conducting PCR for the genera *Pandoraea* and *Ralstonia*, only amplicons from the primer pair RalGS-F/RalGS-R are generated, the *Cupriavidus* genus is suspected. Therefore, it is also necessary to obtain amplicons with primer pair ral2f/ral2r to confirm the identification as *Cupriavidus*.

Amplification with specific primers for bacterial species from Bcc did not produce nonspecific reactions to other bacterial genera that were not from Bcc. Primers used to identify the genomovars *B. cenocepacia* (III-A), *B. cenocepacia* (III-B), *B. stabilis* (IV), *B. dolosa* (VI) and *B. anthina* (VIII) generated amplicons of the expected size with high specificity. However, the primers used for identification of *B. anthina* (VIII) provided amplicons for strain *B. anthina* (VIII BC LMG-16670), but not *B. anthina* (VII BC AMMD). The primer pairs recommended for identification of *B. cepacia* (I), *B. multivorans* (II) and *B. vietnamiensis* (V) allowed amplification of fragments of the same size as other standard strains, different from what was expected: primers BCRG11/BCRG12 were considered effective for identification of *B. cepacia*, but also provided amplicons for *B. cenocepacia* (IIIA, 818 BC), *B. pyrrocinia* (IX, ATCC 39227) and *B. pyrrocinia* (LMG 14191); primers BCRBM1/BCRBM2 were considered effective for identification of *B. multivorans* (II, 788 BC) but also produced amplicons for *B. cenocepacia* (IIIB, 842 BC) and *B. stabilis* (IV BC 790); and primers BCRBV1/BCRBV2 were considered effective for the identification of *B. vietnamiensis* (V BC 1109) but also produced amplicons for *B. multivorans* (II, 788 BC), *B. cenocepacia* (IIIB, 842 BC), *B. stabilis* (IV, 790 BC), *B. anthina* (VIII BC 16670) and *B. pyrrocinia* (IX, BC 39227). The primer pair recommended for *B. ambifaria* (VII) did not work at any annealing temperature (data not shown).

All PCRs were repeated at least five times to ensure reproducibility under the conditions described by the authors (Table 1) and under the proposed conditions for annealing temperature (Table 2).

Analysis of PCR-RFLP patterns generated by digestion with the restriction enzyme *Hae*III was able to discriminate all genomovars, except genomovars I and IIIA, which were differentiated by PCR. This analysis was also able to differentiate subgroups IIIA and IIIB of *B. cenocepacia*. Due to the high cost of sequencing, PCR-RFLP may be considered an option for clinical laboratories, especially because of its ability to identify the species *B. multivorans* and *B. cenocepacia*, which are linked to worse clinical prognoses than other species.[4]

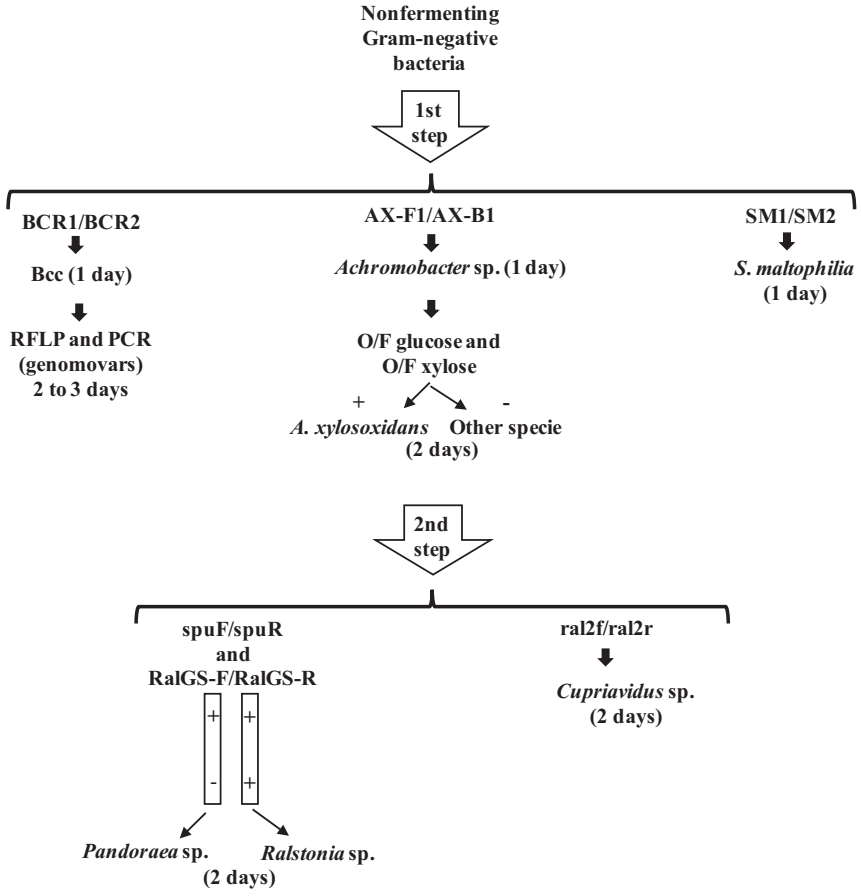

Fig. 1 – Proposed scheme for molecular identification of NFGNB isolated from patients with CF. Bcc, *Burkholderia cepacia* complex; RFLP, restriction fragment length polymorphism; PCR, polymerase chain reaction; O/F, oxidation/fermentation.

Based on the results of the identification of reference strains and clinical isolates, the present study proposed a PCR scheme for identification of emerging NFGNB isolated from CF patients (Fig. 1). The proposed scheme suggested that the first primers to be tested must have the highest specificity and first target the most frequent NFGNB with greater clinical impact.

The first step proposed was using the primer pairs BCR1/BCR2, AX-F1/AX-F2 and SM1/SM2 for Bcc, *Achromobacter* sp. and *S. maltophilia*, respectively. Within a shorter time frame compared with traditional tests, this approach allows the adoption of a segregation policy for individuals infected with Bcc. When amplification for Bcc occurs, PCR-RFLP and PCR with the primers BCRG3A1/BCRG3A2, which is used to identify *B. cenocepacia* (IIIA), should be performed to identify Bcc genomovars, since only PCR-RFLP was not able to discriminate *B. cepacia* from *B. cenocepacia* (IIIA).

The second step proposed was using the primer pairs spuF/spuR, RalGS-F/RalGSR and ral2f/ral2r to differentiate *Pandoraea*, *Ralstonia* and *Cupriavidus*, which were effectively identified by this scheme.

This scheme leads to reliable identification of all these microorganisms within three days, and this rapid identification allows for early antibacterial therapy and segregation. Comparative outcome studies are needed before conclusions about the relative virulence of specific strains can be drawn, and the first step is accurate identification in the laboratory. Furthermore, this scheme is more economically viable when compared to other methods based on sequencing. Therefore, this scheme can provide early bacterial diagnosis in CF patients, which can assist in increasing life expectancy.

Conflicts of interest

The authors declare that they have no conflict of interest.

Acknowledgments

We wish to thank Joseane Cristina Ferreira and Rubens Eduardo Silva for technical assistance. We would like to thank Roberto Martinez and the Hospital das Clínicas da Faculdade de Medicina de Ribeirão Preto for bacterial isolates. This work was supported by the São Paulo Research Foundation (FAPESP 2014/14494-8), the National Council for Scientific and Technological Development (CNPq 473053/2012-8), and the Coordination for the Improvement of Higher Education Personnel program (CAPES). We also thank the Departamento de Microbiologia e Imunologia of Universidade do Estado do Rio de Janeiro and the Departamento de Patologia Clínica of Faculdade de Ciências Médicas of Universidade de Campinas for all reference strains.

REFERENCES

1. Schaffer K. Epidemiology of infection and current guidelines for infection prevention in cystic fibrosis patients. *J Hosp Infect*. 2015;89(4):309–313.
2. LiPuma JJ, Rathinavelu S, Foster BK, et al. In vitro activities of a novel nanoemulsion against *Burkholderia* and other multidrug-resistant cystic fibrosis-associated bacterial species. *Antimicrob Agents Chemother*. 2009;53(1):249–255.
3. LiPuma JJ. The changing microbial epidemiology in cystic fibrosis. *Clin Microbiol Rev*. 2010;23(2):299–323.
4. Bittar F, Rolain J-M. Detection and accurate identification of new or emerging bacteria in cystic fibrosis patients. *Clin Microbiol Infect*. 2010;16(7):809–820.
5. Zlosnik JEA, Speert DP. The role of mucoidy in virulence of bacteria from the *Burkholderia cepacia* complex: a systematic proteomic and transcriptomic analysis. *J Infect Dis*. 2010;202(5):770–781.
6. Vasiljevic ZV, Novovic K, Kojic M, et al. *Burkholderia cepacia* complex in Serbian patients with cystic fibrosis: prevalence and molecular epidemiology. *Eur J Clin Microbiol Infect Dis*. 2016;35(8):1277–1284.
7. Shommu NS, Vogel HJ, Storey DG. Potential of metabolomics to reveal *Burkholderia cepacia* complex pathogenesis and antibiotic resistance. *Front Microbiol*. 2015;6:1–6.
8. Folescu T, da Costa C, Cohen R, da Conceição Neto OC, Albano R, Marques E. *Burkholderia cepacia* complex: clinical course in cystic fibrosis patients. *BMC Pulm Med*. 2015;15:158.
9. Rønne Hansen C, Pressler T, Høiby N, Gormsen M. Chronic infection with *Achromobacter xylosoxidans* in cystic fibrosis patients; a retrospective case control study. *J Cyst Fibros*. 2006;5(4):245–251.
10. Wettlaufer J, Klingel M, Yau Y, et al. Longitudinal study of *Stenotrophomonas maltophilia* antibody levels and outcomes in cystic fibrosis patients. *J Cyst Fibros*. 2016, http://dx.doi.org/10.1016/j.jcf.2016.06.007.
11. Crossman LC, Gould VC, Dow JM, et al. The complete genome, comparative and functional analysis of *Stenotrophomonas maltophilia* reveals an organism heavily shielded by drug resistance determinants. *Genome Biol*. 2008;9(4):R74.
12. Cloud JL, Harmsen D, Iwen PC, et al. Comparison of traditional phenotypic identification methods with partial 5′ 16S rRNA gene sequencing for species-level identification of nonfermenting Gram-negative bacilli. *J Clin Microbiol*. 2010;48(4):1442–1444.
13. Ferroni A, Sermet-Gaudelus I, Abachin E, et al. Use of 16S rRNA gene sequencing for identification of nonfermenting Gram-negative bacilli recovered from patients attending a single cystic fibrosis center. *J Clin Microbiol*. 2002;40(10):3793–3797.
14. Otto-Karg I, Jandl S, Muller T, et al. Validation of Vitek 2 nonfermenting Gram-negative cards and Vitek 2 version 4.02 software for identification and antimicrobial susceptibility testing of nonfermenting Gram-negative rods from patients with cystic fibrosis. *J Clin Microbiol*. 2009;47(10):3283–3288.
15. Caballero Jde D, del Campo R, Tato M, et al. Microbiological diagnostic procedures for respiratory cystic fibrosis samples in Spain: towards standard of care practices. *BMC Microbiol*. 2014;14(1):335.
16. Porte L, García P, Braun S, et al. Head-to-head comparison of Microflex LT and Vitek MS systems for routine identification of microorganisms by MALDI-TOF mass spectrometry in Chile. *PLoS ONE*. 2017;12(5):e0177929.
17. Patel R. MALDI-TOF MS for the diagnosis of infectious diseases. *Clin Chem*. 2015;61(1):100–111.
18. Almuzara M, Barberis C, Traglia G, Famiglietti A, Ramirez MS, Vay C. Evaluation of matrix-assisted laser desorption ionization-time-of-flight mass spectrometry for species identification of nonfermenting Gram-negative bacilli. *J Microbiol Methods*. 2015;112:24–27.
19. Manji R, Bythrow M, Branda JA, et al. Multi-center evaluation of the VITEK® MS system for mass spectrometric identification of non-Enterobacteriaceae Gram-negative bacilli. *Eur J Clin Microbiol Infect Dis*. 2014;33(3):337–346.

20. Garibyan L, Nidhi A. Research techniques made simple: polymerase chain reaction (PCR). *J Invest Dermatol*. 2013;133(3):392–395.
21. Rossolini GM. Molecular cloning and expression in *Escherichia coli* of the *Salmonella typhi* gene cluster coding for type 1 fimbrial. *Biol Salmonella*. 1993:409–413.
22. Mahenthiralingam E, Bischof J, Byrne SK, et al. DNA-based diagnostic approaches for identification of *Burkholderia cepacia* complex, *Burkholderia vietnamiensis*, *Burkholderia multivorans*, *Burkholderia stabilis*, and *Burkholderia cepacia* genomovars I and III. *J Clin Microbiol*. 2000;38(9):3165–3173.
23. Vermis K, Coenye T, LiPuma JJ, Mahenthiralingam E, Nelis HJ, Vandamme P. Proposal to accommodate *Burkholderia cepacia* genomovar VI as *Burkholderia dolosa* sp. nov. *Int J Syst Evol Microbiol*. 2004;54(3):689–691.
24. Coenye T, Mahenthiralingam E, Henry D, et al. *Burkholderia ambifaria* sp. nov., a novel member of the *Burkholderia cepacia* complex including biocontrol and cystic fibrosis-related isolates. *Int J Syst Evol Microbiol*. 2001;51(4):1481–1490.
25. Vandamme P, Henry D, Coenye T, et al. *Burkholderia anthina* sp. nov. and *Burkholderia pyrrocinia*, two additional *Burkholderia cepacia* complex bacteria, may confound results of new molecular diagnostic tools. *FEMS Immunol Med Microbiol*. 2002;33(2):143–149.
26. Liu L, Coenye T, Burns JL, Whitby PW, Stull TL, LiPuma JJ. Ribosomal DNA-directed PCR for identification of *Achromobacter* (Alcaligenes) xylosoxidans recovered from sputum samples from cystic fibrosis patients. *J Clin Microbiol*. 2002;40(4):1210–1213.
27. Whitby PW, Carter KB, Burns JL, Royall JA, LiPuma JJ, Stull TL. Identification and detection of *Stenotrophomonas maltophilia* by rRNA-directed PCR. *J Clin Microbiol*. 2000;38(12): 4305–4309.
28. Coenye T, Liu L, Vandamme P, John J. Identification of Pandoraea species by 16S ribosomal DNA-based PCR assays. *J Clin Microbiol*. 2001;39(12):4452–4455.
29. Coenye T, Spilker T, Reik R, Vandamme P, Lipuma JJ. Use of PCR analyses to define the distribution of Ralstonia species recovered from patients with cystic fibrosis. *J Clin Microbiol*. 2005;43(7):3463–3466.
30. Barrett CF, Parker MA. Coexistence of *Burkholderia*, *Cupriavidus*, and *Rhizobium* sp. nodule bacteria on two *Mimosa* spp. in Costa Rica. *Appl Environ Microbiol*. 2006;72(2):1198–1206.
31. Leão RS, Pereira RHV, Ferreira AG, Lima AN, Albano RM, Marques EA. First report of *Paenibacillus cineris* from a patient with cystic fibrosis. *Diagn Microbiol Infect Dis*. 2010;66(1):101–103.
32. McDowell A, Mahenthiralingam E, Moore JE, et al. PCR-based detection and identification of *Burkholderia cepacia* complex pathogens in sputum from cystic fibrosis patients. *J Clin Microbiol*. 2001;39(12):4247–4255.