UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

FACULDADE DE FARMÁCIA

TRABALHO DE CONCLUSÃO DE CURSO

Avaliação do sistema MALDI-TOF MS como metodologia para a identificação e diferenciação de espécies do Complexo *Burkholderia cepacia*

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Trabalho de Conclusão do Curso, apresentado por Mayana Kieling Hernandez como requesito parcial para obtenção de GRAU DE FARMACÊUTICO.

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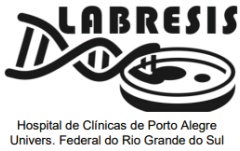

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"You must always remember this: Have courage and be kind"

Brittany Candau

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Summary

Resumo

As bactérias do Complexo *Burkholderia cepacia* (Bcc) estão comumente associadas à infecção no trato respiratório em pacientes com Fibrose Cística (CF). O tratamento das infecções por Bcc nesses pacientes é complexo e a infecção, em alguns casos, está relacionada a um mau prognóstico. De fato, a espécie *Burkholderia cenocepacia*, membro do Bcc, é frequentemente associada à pneumonia necrotizante. Portanto, a identificação precoce da *B. cenocepacia* pode favorecer o prognóstico dos pacientes com CF. No entanto, a identificação e diferenciação de membros do Bcc por métodos fenotípicos é difícil. Sendo assim, métodos moleculares são considerados o padrão ouro para a diferenciação dos membros, mas requerem um laboratório especializado e mão de obra qualificada. O uso da espectrometria de massa para a identificação de microrganismos como o sistema MALDI-TOF MS tem apresentado ótima acurácia para identificação das principais espécies bacterianas de importância clínica, embora a diferenciação de espécies de um mesmo complexo, como o Bcc, nem sempre seja possível. Tendo em vista a necessidade de agilidade diagnóstica, o objetivo deste estudo foi avaliar o desempenho do sistema MALDI-TOF MS como método de identificação da *Burkholderia cenocepacia* e a diferenciar de outras espécies do Bcc. Um total de 53 colônias sugestivas de Bcc foram analisadas pelo sistema MALDI-TOF usando dois protocolos de extração de proteínas: (A) Método direto: as colônias foram transferidas para uma placa de MALDI-TOF e fixadas com 1µL de ácido fórmico 70%; (B) Extração em tubo: as colônias foram transferidas para um microtubo e adicionadas 900 µL de etanol à 100%; após centrifugação o etanol foi removido e o pellet foi misturado com 25 µL de ácido fórmico 70% e 25 µL de acetonitrila 70%. Um volume de 1 µL da mistura foi transferido para a placa alvo do MALDI-TOF. Após a fixação das colônias na placa, em ambos os métodos, foi adicionado 1uL de ácido α-ciano-4-hidroxicinâmico e após submetidas à identificação no equipamento Microflex MALDI-TOF (Bruker ®). Paralelamente, todos os isolados foram submetidos ao diagnóstico molecular (PCR com *primer*s específicos) para identificação de espécies pertencentes ao Bcc e diferenciação de *B. cenocepacia* no genomovar IIIA ou IIIB. O MALDI-TOF foi capaz de identificar 100% (53/53) em nível de gênero e 94,34% (51/53) em nível de espécie, usando ambos os métodos de extração de proteína (A) e (B), de acordo com a PCR. Embora ambas as extrações tenham apresentado resultados confiáveis, o método (A) é mais rápido e requer menos reagentes. O sistema MALDI-TOF foi capaz de identificar corretamente 38 dos 40 isolados identificados como *B. cenocepacia* pela técnica de PCR. O sistema MALDI-TOF (Bruker®), devido a sua praticidade e custo baixo, pode ser usado como metodologia para diferenciar as espécies de Bcc.

Palavras-chave: Complexo *Burkholderia cepacia*; Fibrose cística; Reação em Cadeia da Polimerase (PCR); MALDI-TOF.

Tables

Table 1. Comparison of protein extraction protocols using the numerical score for identification of species of *Burkholderia* genus by MALDI-TOF (classification at the species level and genus level was considered for a score of ≥2.3; ≥ 2.0 and ≥ 1.7≤1.99, respectively).

Table 2. Comparison of identification by MALDI-TOF and the molecular technique (PCR) – (classification at the species level was considered for a *score* ≥ 2.0).

Title: Evaluation of MALDI-TOF MS system for the identification and differentiation of *Burkholderia cepacia* Complex species

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Abstract

Bacteria of the *Burkholderia cepacia* Complex (Bcc) are commonly associated with respiratory tract infection in patients with cystic fibrosis (CF). In fact, *Burkholderia cenocepacia*, a member of the Bcc, is commonly associated with necrotizing pneumonia in some CF patients. Therefore, an early identification of *B. cenocepacia* would favor the prognosis among CF patients. However, the identification of Bcc species is difficult to be achieved using phenotypic methods. Molecular methods are considered the gold standard for those differentiation, but those assays require a skilled labor, time to prepare and high costs. The use of mass spectrometry, such as MALDI-TOF MS system, for identification of microorganisms has been presented a very good accuracy. Although, the differentiation of species within the same complex, such as Bcc, may not always be achieved by MALDI-TOF. Due to the need for rapid diagnosis of Bcc species, the objective of this study was to evaluate the performance of MALDI-TOF MS system for identification of *Burkholderia cenocepacia* and differentiates from other Bcc species. A total of 53 colonies suggestive of Bcc were submitted to MALDI-TOF (Bruker®) system for identification and two protocols of protein extraction were compared (A) Direct Method and (B) Tube Extraction. In parallel, all isolates were subjected to molecular diagnosis (PCR with primers for recA gene) to identify species belonging to Bcc and to differentiate *B. cenocepacia* in genomovar IIIA or IIIB. MALDI-TOF was able to discriminate 100% (53/53) of the isolates to the gender level and 94.34% (50/53) to the species using either method, according to the PCR. Although both extractions presented reliable results, method (A) is faster and requires fewer reagents. Moreover, the MALDI-TOF system was able to identify 38 out of the 40 isolates identified by the molecular technique as *B. cenocepacia*. Due to the fact that MALDI-TOF (Bruker®) is a feasible technique and presents a low cost of reagents, it can be used for reliable identification of species of the Bcc complex.

Keywords: *Burkholderia cepacia* complex; MALDI-TOF MS; Diagnosis; Polymerase Chain Reaction.

Declarations

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Conflict of interest

The authors declare that they have no conflicts of interests.

Ethical approval

This cross-sectional and prospective study was approved by the Comitê de Ética em Pesquisa do HCPA (CAAE 23417419.7.0000.5327).

Consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and material

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request

Code availability

Not applicable

1. Introduction

Cystic fibrosis (CF) is an autosomal recessive genetic disease that affects several organs being mainly associated with chronic airway infection which can lead to intermittent pulmonary exacerbations [1]. It is estimated that 80-95% of CF patients may develop respiratory failure due to chronic bacterial infections [2]. The microorganisms commonly associated with this pathology in the respiratory system are: *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Haemophilus influenzae*, *Burkholderia cepacia* complex (Bcc) species, *Stenotrophomonas maltophilia*, *Achromobacter xylosoxidans*, among other pathogens [3]. Despite advances in the treatment of CF, infections due to bacteria belonging to Bcc still play an important role in the morbidity and mortality of these patients [3-4]. Airway infections due to Bcc are usually chronic, refractory to therapy due to resistance rates of Bcc isolates and related to a poor prognosis [5-6].

In 1992, a bacterium previously called *Pseudomonas cepacia* was reclassified as *Burkholderia cepacia* and a new genus was established [7]. Afterwards, with the improvement of molecular techniques, more species were included in this group of glucose non-fermenters bacilli. Therefore, bacteria biochemically identified as *B. cepacia* consist of at least five different genetically species, named genomovars [7-8]. Several species of this genus were grouped and identified as members of the *Burkholderia cepacia* Complex (Bcc), a very heterogeneous group of Gram-negative rods which is composed of 23 species. However, new members could often be included in the complex [9-12].

Within the Bcc, the species *Burkholderia cenocepacia* stands out, due to its intraspecific diversity which lead to distinct genomovars: IIIA; IIIB; IIIC; IIID. The interest to differentiate the *B. cenocepacia* is associated to the clinical status of CF patients infected with different genomovars. As the genomovar IIIA can cause a necrotizing pulmonary infection, known as “cepacia syndrome”, with a high mortality rate [4, 9, 13]. Moreover, some strains of the Bcc may present a high degree of transmissibility among CF patients [14-15]. Thus, a few reference centers for treatment of CF patients have established a physical barrier to prevent contagious among CF patients who are not colonized. Hence, the laboratories that attend CF centers must be able to identify the Bcc isolates using reliable techniques [16].

Differentiation of species of the Bcc cannot be achieved using traditional phenotypic methods in the clinical microbiology laboratories. Currently, the main discriminatory methodologies include molecular techniques such as the Polymerase Chain Reaction (PCR) with primers for the rec-A gene locus [17] or sequencing of the 16S rDNA. Although these methods are more accurate than the phenotypic identification; they are expensive and require specialized professionals as well as proper equipment [18]. In addition, they may have a limited capacity to differentiate all Bcc species since the variation in nucleotide sequences among Bcc genes may not be discriminatory [17, 19].

A technology that allows culture-dependent identification of microorganisms with a fast, accurate, practical and low-cost of reagents is the MALDI-TOF MS (Matrix-Assisted Laser Desorption-Ionization Time of Flight - Mass Spectrometry). MALDI-TOF technology is based on evaluation of the protein profile (generated by ionization of the molecules) of a bacterium which is compared with a database of a standard protein of bacterial profiles. MALDI-TOF is very robust which allows the identification of bacteria, such as of glucose non-fermenters bacilli, with very good accuracy. The

performance of MALDI-TOF to different Bcc species is not yet well established [18]. In fact, the MALDI-TOF MS may not be able to identify intraspecific differences, as *Burkholderia cenocepacia* and its genomovars [10]. Thus, the objective of this study was to evaluate the performance of the MALDI-TOF MS system for the identification of *Burkholderia cenocepacia* and differentiation from other species of the *Burkholderia cepacia* Complex.

2. Materials and methods

2.1 Bacterial isolates

A total of 53 colonies suggestive of Bcc from were obtained from routine sputum or oropharyngeal swab culture of CF patients attended at "Hospital de Clínicas de Porto Alegre (HCPA)" in southern Brazil. All colonies were obtained from *Burkholderia cepacia* Selective Agar (BCSA - Remel, KS, USA), incubated for 24h-72h at 32ºC ± 2ºC. MALDI-TOF and PCR techniques were performed at "Laboratório de Pesquisa em Resistência Bacteriana (LABRESIS)" of HCPA.

2.2 MALDI-TOF MS

Prior to the identification in MALDI-TOF, two different methods of protein extraction were performed in duplicate: (A) Direct Method and (B) Tube Extraction method. In method (A), 1uL of 70% Formic Acid was added later to the fixation of the colonies in each target. After evaporation of the Formic Acid, 1uL of HCCA (α-cyano-4-hydroxycinnamic acid, Bruker Daltonik, Bremen, Germany) was pipetted and the sample was submitted to identification in MALDI-TOF Microflex LT 4.0® (Bruker Daltonik, Bremen, Germany). In Tube Extraction (B), the bacterial mass was transferred to a microtube which was added of 900µL of 100% Ethanol. Afterwards this microtube was centrifuged and the bacterial pellet was added of 25 µL of 70% Formic Acid and 30% Acetonitrile. A volume of 1 µL of the extraction supernatant was placed on the target plate and, after evaporation, added 1 µL of HCCA and submitted for MALDI-TOF in the same equipment as described above. Identification to species and genus levels were considered satisfactory for a score of ≥ 2.0 and ≤1.99 to 1.70, respectively. All unsatisfactory results were re-analyzed.

2.3 Extraction of DNA

All 53 colonies were submitted to a molecular technique (PCR) which was considered the reference method for identification of Bcc species. The DNA of bacterial colonies was extracted by thermal lysis as follows: two or three colonies were suspended in 600µL of TE buffer (10 mM Tris-HCl (pH 8.0); 0.1 mM EDTA) and subjected to heat for 10 min at 100 ° C followed by cooling to -20°C (for 20 min). The aliquots were centrifuged for three minutes at 16,000 rpm and the supernatant (DNA) was stored at -20ºC in a microtube.

2.4 Polimerase Chain Reaction (PCR)

PCR was performed in duplicate. Initially, a PCR with BCR1 and BCR2 primers was used to generate amplicons of interest to another two PCR. In order to confirm that the isolates belonged to the Bcc, a second PCR with primers REC-IN5 and BCRBM2 was performed using the amplicons of the first PCR reaction. Finally, a third PCR, also using the initially amplicons was performed with primers BCRG3A1 and BCRG3A2 for identify genomovar IIIA and BCRG3B1 and BCRG3B2 for genomovar IIIB [17].

For the first PCR, the mix was composed of 5uL of 10x buffer, of 2.5uL of $MgCl_2$ and of 5uL dNTP at a concentration of 2.5 mM. A volume of 20 picomoles of each primer (BCR1 and BCR2) was added in a final volume of 1.4uL with 25.7uL of water for PCR and 0.4uL of *Taq* Platinum DNA polymerase (Thermo Fisher Scientific, MA, USA). The amplification program used an initial denaturation for 5 min at 95 °C, 35 cycles of 45 seconds at 95 °C, 45 seconds at 58 °C and 90 seconds at 72 °C. A final extension of 10 minutes at 72 °C was used at the end of the cycles.

The amplicons from the first PCR reaction were subjected to two other PCR reactions, as mentioned above. The second PCR reaction (for identification of species of the Bcc) used 5.0µL of 10x buffer solution, 1.5µL of 50 mM $MgCl_2$ and 4.0µL of dNTP mixture at a concentration of 2.5mM. The primers *REC-IN5* and *BCRBM2* were added at a concentration of 10µM with 37.3µL of water for PCR, 0.2µL of Platinum *Taq* DNA polymerase and 2.0µL of the product of the first reaction. The program on the thermal cycler comprised 4 stages: Stage 1 - 5 cycles of 30 seconds at 94 ° C, 45 seconds at 67 ° C, 1 minute at 72 ° C; Stage 2 - 5 cycles of 30 seconds at 94 ° C, 45 seconds at 65 ° C, 1 minute at 72 ° C; Stage 3 - 15 cycles of 30 seconds at 94 ° C, 45 seconds at 63 ° C, 1 minute at 72 ° C; Final extension - 5 minutes at 72 ° C.

The third PCR reaction (for *Burkholderia cenocepacia* genomovars IIIA and IIIB) used 2.5uL of the 10x buffer solution, 0.75uL of the 50mM $MgCl_2$ and 2.0uL of the dNTP mixture. A volume of 1.0uL of each set of BCRG3A1 and BCRG3A2 primers was added at a concentration of 10uM, with 14.65uL of water for PCR, 0.1uL of Platinum *Taq* DNA polymerase and 3.0uL of the product of the first reaction. The thermocycler program was the same as in the second reaction.

The detection of the PCR reaction products was performed by visual inspection in an electrophoresis agarose gel. The amplicon of the second reaction (*primer*s *REC-IN5* and *BCRBM2*) was a 620bp DNA fragment which confirmed that the species belonged to the *Burkholderia cepacia* Complex. The amplicon of the third reaction (*primer*s BCRG3A1 and BCRG3A2) was a DNA fragment of 380bp corresponding to *Burkholderia cenocepacia* genomovar IIIA and a DNA fragment of 780bp corresponding to *B. cenocepacia* genomovar IIIB [17].

The samples that presented questionable characteristics regarding the discrimination of the species had their amplification product sent to a sequencing by Sanger.

3. Results

3.1 Identification by MALDI-TOF MS: comparison of the two extraction protocols

A total of 53 colonies suggestive of Bcc in the BCSA medium were submitted to identification by the MALDI-TOF system. Regarding the protocols of protein exposure, both extractions, (A) and (B), presented excellent results to distinguish the isolates at genus and species level. Both methods together identified at genus and species level, respectively, 100% (53/53) and 96.22% (51/53). Moreover, 98.11% (52/53) of the isolates achieved an identification score greater than 2.0 in at least one of the protocols of extraction. These difference is attributed to an isolate that presented an unsatisfactory identification by MALDI-TOF to specie level as follows: according to extraction protocol A as *"B. pyrrocinia"* (score 1.99) and according to protocol B as "Member of *Burkholderia cepacia* Complex" (score 2.03). The bacteria was submitted to Sanger sequencing as described below. In addiction one isolate presented score <2.0 for both protocols (score of 1.94 using protocol A and score 1.72 using protocol B); according to MALDI-TOF cut-off, this isolate was identified as *Burkholderia* genus level (Table 1).

When considering the efficiency of identification according to score of MALDI-TOF, the tube extraction (protocol B) proved to present better results than the direct method of extraction (protocol A). In fact, the extraction protocol B presented 60.3% (32/53) of score ≥ 2.3, while the extraction protocol A presented only 35.8% (19/53) of score ≥ 2.3 (Table 1). The average score of the identifications was 2.23 for the direct method (A) and 2.27 for tube extraction (B) (p-0.04686). In addition, one isolate (1.9% - 1/53) present divergent results in the extraction protocols; was describe as "*B. cepacia"* by direct extraction (score 2.11) and the tube extraction identified as "*B. cenocepacia"* with score of 2.47.

3.2 Comparison of the MALDI-TOF identification with the molecular reference method (PCR)

The PCR techniques were able to classify 98.11% (52/53) of the isolates to the Bcc complex with the *B. cenocepacia* being the most common species (75.5% (40/53)). Only one isolate (1.9% (1/53)) did not belong to Bcc, according to PCR results (Table 2). This isolate was identified as *B. gladioli* by the MALDI-TOF MS system.

One isolate classified only as a member of Bcc by PCR, with a score greater than 2.0 in protocol B, had its amplicon submitted to Sanger sequencing in order to confirm the species identification. The result obtained by sequencing was compared with the GenBank database using the *National Center for Biotechnology Information Computer Blast* program (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The closer match observed with 100% identity in an overlap of 604 nucleotides occurred with the registration under the name "*B. contaminans* strain UFLA02-28 RecA (recA) gene, partial cds". Thus, this isolate was considered as *B. contaminans*, and consequently a member of the Bcc.

The PCR for the differentiation of *B. cenocepacia* genomovars indicated that 75% (30/40) of the isolates corresponded to the IIIB genomovar and 25% (10/40) to the IIIA genomovar.

In fact, one isolate previously identified in the MALDI-TOF system as *B. cepacia* (score according protocols A and B were 2.36 and 2.30, respectively), was identified as *B. cenocepacia* IIIB by PCR. Based on cut-off 2.00 at MALDI-TOF to considerer satisfactory identification at species level, one isolated presented a score lower than 2.00, and was considered as *Burkholderia* genus level. Thus, 95.0% (38/40) of the *B. cenocepacia* isolate had an agreement between MALDI-TOF and PCR. In addition, all species previously classified as belonging to Bcc and which were not *B. cenocepacia*, did not present any amplification product in the specific PCR for genomovar differentiation. In total, 94.34% (50/53) of the *Burkholdeira* isolates were distinct by the species level with a score ≥ 2.0, considering either methods (A) and (B).

4. Discussion

This study aimed to evaluate the performance of the MALDI-TOF system for the identification and differentiation of *Burkholderia cenocepacia* species from other Bcc members. We have also compared different protein extraction protocols to be used prior to the MALDI-TOF analysis. The average score of MALDI-TOF identification of direct method (protocol A) and tube extractions (protocol B) were 2.23 and 2.27, respectively. This indicated that both extraction methods presented an excellent performance to distinguish the *Burkholderia* isolates to species level.

Although the identification based on extraction B presented a higher *score* when compared to extraction A, latter protocols can properly be used as an extraction method with security to realize the identification without loss of quality [20]. In fact, the direct extraction still presents faster results and reduced costs when compared to the tube extraction technique [20-22]. Other authors have also reported the efficiency of the direct extraction for other glucose non-fermenters bacilli [18, 23-27]. We would suggest to use the direct method in the routine of MALDI-TOF identification and to use the tube extraction only when the results of the direct methods present lower score as the tube extraction protocol, because it allows a greater exposure of proteins and a better yield of identification.

The cut-off point for the precise species identification by MALDI-TOF is a widely discussed topic. Some authors suggest that a score ≥ 2.3 is more reliable for species identification [28-31]. In this study, 60.3% (32/53) of isolates presented a score ≥2.3 by method B, which is directly associated with a higher protein exposure compared to method A. The cut-off used in this article was ≥ 2.0 to distinguished the isolates to species level and we observed 94,34% (50/53) of agreement between the methodologies. Other researchers have suggested a different cut-off point for identification; as Gautam and collaborators in 2017 [27] suggested that a score ≥ 1.9 was sufficient to discriminate Bcc species. If considering the cut-off point of Gautam et al. [27], the use of MALDI-TOF system in our study presented 96.22% (51/53) of its identifications compatible with PCR results.

One isolate presented the following result for protocol (A) and (B), respectively: "*B. pyrrocinia*" (s*core* 1.99) and "Member of the *Burkholderia cepacia* Complex" (*score* 2.03). Due to the discrepancy in the identification by MALDITOF in one isolate, its amplicons were forwarded for the Sanger method.

The result of the sequencing was blasted to the genbank which indicated that the isolate was closely related to *B. contaminans*. Another discrepant identification by MALDI-TOF technology was the identification of *B. cepacia,* with a higher score for both protocol extraction, and in the molecular assay, this isolate was confirmed as *B. cenocepacia* IIIB.

Some species may present higher rates of incorrect identification by MALDI-TOF, such as *B. contaminans* and *B. cepacia* [25, 32]. The incorrect identifications are due to the formation of similar or almost equal spectra, due to the high phenotypic and genotypic similarity among Bcc species [10,12]. Furthermore, some of these failures may be associated with a lack of spectra such as the case of newly cataloged bacteria. Therefore, the constant updating and expansion of databases and software can minimize problems of identification in some microorganisms using MALDI-TOF [25, 33-35]. Fehlberg et al. [25] reported that MALDI-TOF was not able to differentiate any *B. contaminans* isolates tested, in addition, to presented unsatisfactory identification in 77.7% of *B. cepacia* isolates. Wong et al [35] also did not find agreement in the identification of these isolates.

It is estimated that about 30% of CF patients will be colonized by Bcc and most infections are caused by *B. cenocepacia* and *B. multivorans* [36]. Those two species together correspond to 85-97% of the CF airway infection cases of Bcc, although other members of the complex also be associated with chronic infections [4]. Our epidemiological data are similar to another study conducted earlier in the same institution [36] which indicated that 75.5% (40/53) of the isolates of Bcc corresponded to *B. cenocepacia*. As Lutz et. al [36] we also found a prevalence of *B. cenocepacia* IIIB species when compared to B. *cenocepacia* IIIA.

Although all species of the Bcc may be related to a poor prognosis in the CF community, patients colonized with *B. cenocepacia* may present an increased chance of drastic reduction in lung functions. Such severity can be explained by the survival of *B.cenocepacia* or, even, by a different virulence profile, which can directly reflect on the condition of chronic pulmonary infection in individuals [4]. Reports in the literature demonstrate that 66.6% of patients with *B. cenocepacia* survive for only 5 years after the acquisition of this microorganism. This is worrisome, as CF patients with *Pseudomonas aeruginosa* have a survival rate greater than 85% [4].

Molecular techniques have been used as reference methods for bacteria identification, but these techniques require a highly qualified workforce and demand time to perform. Despite the high cost required for acquisition of MALDI-TOF equipment; the reagents needed for identification used in this system are usually with a very low cost (approximately USD 0.2 per isolate). Moreover, the MALDI-TOF allows very fast identification (approximately 3 minutes) of bacteria and presents high accuracy.

We conclude that clinical microbiology laboratories that already have MALDI-TOF Microflex LT 4.0® (Bruker Daltonik, Bremen, Germany) and attend Reference Centers for the treatment of CF patients can use this technique to identify Bcc species.

Acknowledgments

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7. Attachment I

Table 1. Comparison of protein extraction protocols using the numerical score for identification of species of *Burkholderia* genus by MALDI-TOF (classification at the species level and genus level was considered for a score of ≥2.3; ≥ 2.0 and ≥ 1.7≤1.99, respectively).

	***Score* of Direct Method**			***Score* of Tube Extraction**		
	Protocol A			**Protocol B**		
Species (n)	**1.94–1.99**	**2.0–2.29**	**2.3–2.38**	**1.72–1.99**	**2.0–2.29**	**2.3–2.44**
B. cenocepacia (39)	2	21	15	1	13	25*
B. cepacia (5)	0	4*	2	0	3	2
B. vietnamiensis (5)	0	4	1	0	2	3
B. gladioli (1)	0	0	1	0	0	1
B. multivorans (1)	0	1	0	0	0	1
B. lata (1)	0	1	0	0	1	0
Unsatisfactory identification (1)**	1	0	0	0	1	0

* One isolate was identified according to protocol A as "*B. cepacia"* (score 2.11) and according to protocol B as "*B. cenocepacia"* (score 2.47).

** This isolate presented an unsatisfactory identification by MALDI-TOF to specie level as follows: according to extraction protocol A as "*B. pyrrocinia*" (score 1.99) and according to protocol B as "Member of the *Burkholderia cepacia* Complex" (score 2.03).

Table 2. Comparison of identification by MALDI-TOF and the molecular technique (PCR) – (classification at the species level was considered for a score ≥ 2.0).

Identification by PCR (n)	Identification by MALDI-TOF MS* (n)	Agreement	Sanger Sequencing
Positive for Bcc			
B. cenocepacia IIIA (10)	*B. cenocepacia* (39)	95.0% (38/40)	-
B. cenocepacia IIIB (30)			-
Non-*B.cenocepacia* (12)	*B. cepacia* (5)	-	-
	B. vietnamiensis (5)	-	-
	B. multivorans (1)	-	-
	B. lata (1)	-	-
	Unsatisfactory identification (1)*	-	*B. contaminans*
Negative for Bcc			
	B. gladioli (1)	-	-

* This isolate presented an unsatisfactory identification by MALDI-TOF to specie level as follows: according to extraction protocol A as "*B. pyrrocinia*" (*score* 1.99) and according to protocol B as "Member of the *Burkholderia cepacia* Complex" (*score* 2.03).

8. Attachment II

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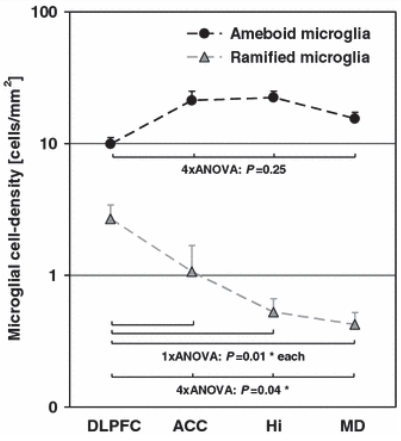

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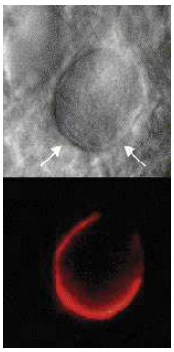

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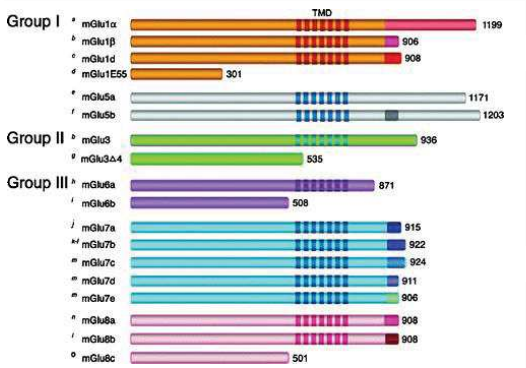

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List of Repositories

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- Research involving Human Participants and/or Animals
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• This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of University B (Date.../No. ...).

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- Informed consent was obtained from legal guardians.
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- Verbal informed consent was obtained prior tothe interview.
- The patient has consented to the submission of the case report for submission to the journal.

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- The participant has consented to the submission of the case report to the journal.
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