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Clinical and environmental isolates of *Burkholderia pseudomallei* from Brazil: Genotyping and detection of virulence gene

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ABSTRACT

Objective: To evaluate the genetic diversity of clinical and environmental isolates of *Burkholderia pseudomallei* (*B. pseudomallei*) recovered in Ceará, Brazil, and screen these isolates for the presence of type three secretion system virulence gene.

Methods: Nineteen *B. pseudomallei* isolates (9 from clinical cases and 10 from soils) were analyzed. Random amplified polymorphic DNA was performed with primers OPQ-2, OPQ-4 and OPQ-16 to evaluate the genetic diversity, and type three secretion system gene was detected through polymerase chain reaction.

Results: Random amplified polymorphic DNA showed a genetic relatedness of approximately 50% among the tested *B. pseudomallei* isolates, which were grouped into two clades, of which the biggest ones comprised 18/19 isolates for primer OPQ-2, and 17/19 isolates for primer OPQ-16. Primer OPQ-4 grouped the isolates into three clades comprising 1/19, 3/19 and 15/19 isolates. Additionally, type three secretion system gene was detected in all tested isolates.

Conclusions: This is an effort to type *B. pseudomallei* strains from Ceará, which is important for better understanding this pathogen, contributing for the epidemiological surveillance of melioidosis in this endemic region.

1. Introduction

Burkholderia pseudomallei (*B. pseudomallei*), a Gram-negative bacillus, is the agent of melioidosis, a tropical infectious disease, which is endemic to Southeast Asia and Northeast Australia. It is commonly found in soil and water, and it has been isolated from a variety of clinical samples in endemic areas [1,2]. Besides Southeast Asia and Northeast Australia, melioidosis has been reported in the South Pacific, Africa, India, the Middle East, Central America and South America [2,3]. In Brazil, it has been considered an emerging disease since 2003, when it was first diagnosed in the state of Ceará, in the Northeastern region, where over 25 cases have been reported [3].

The disease presents a wide variety of clinical manifestations, from subclinical forms to acute, sub-acute, chronic and overwhelming sepsis [4]. The lethality rate for melioidosis is high and depends on the country where it is diagnosed. In Thailand, it is around 40.0%, while in Northeast Australia it is around 10.0% [5]. On the other hand, in Brazil, more specifically in the state of Ceará, melioidosis has mostly occurred as severe infection, with a reported lethality rate of 73.3%, which can reach up to 100.0% among patients with septic melioidosis [6].

Based on the severity of the clinical manifestations of melioidosis in Ceará, Brazil, as shown by the high lethality rates of

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the disease in this region, it is important to further investigate the Brazilian isolates of *B. pseudomallei*, including genotyping and detection of an important virulence gene for this bacterial species. Several methods have been proposed for typing *B. pseudomallei*, including ribotyping, restriction fragment length polymorphism, pulsed-field gel electrophoresis, random amplified polymorphic DNA (RAPD) [7,8], multilocus sequence typing [7,9,10] and more recently, whole genome sequencing [10]. RAPD method is a polymerase chain reaction (PCR) -based technique that uses short arbitrary primers to amplify target DNA under low stringency conditions. This technique has been widely adopted for genotyping several microorganisms [7,8,11–13], and can potentially be used to assess the genetic diversity in whole genomes [8]. As for the virulence of *B. pseudomallei*, several factors have been reported, such as the production of exoenzymes and biofilms, and the presence of the type-three secretion system (TTSS). This molecular structure is responsible for the inoculation of bacterial compounds into host cells, promoting cell invasion [14].

Thus, this work aimed at evaluating, through RAPD-PCR, the genetic diversity of clinical and environmental isolates of *B. pseudomallei* recovered in the state of Ceará, Brazil, and screening these isolates for the presence of the TTSS virulence gene.

2. Materials and methods

2.1. Isolated microorganisms

The 19 strains of *B. pseudomallei* included in this study belong to the bacterial collection of the Laboratory of Emerging and Reemerging Pathogens of the Postgraduate Program in Medical Microbiology of the Federal University of Ceará, Brazil. The tested bacterial isolates included 9 isolates recovered from naturally occurring clinical cases of melioidosis, in the state of Ceará [4], and 10 isolates recovered from soils [15]. All procedures involving laboratory manipulation of the evaluated *B. pseudomallei* isolates were performed in a Biosafety Level 3 laboratory. To confirm the identification of the clinical and environmental strains of *B. pseudomallei*, they were first identified through the automated VITEK2 system (BioMérieux, affiliate in Brazil). Molecular identification was carried out with the PCR technique, through amplification of the specific 16S−23S spacer region of *B. pseudomallei*, as previously described [16]. Additionally, the nearly complete *16S rRNA* gene was amplified by PCR using the primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1525R (5′-AAGGAGGTGATCCAGCC-3′) [17], and sequenced with the DYEnamic ET terminators cycle sequencing kit (GE Healthcare Life Sciences) [18–20]. The *16S rRNA* gene sequences obtained were compared to those previously deposited in the GenBank database (www.ncbi.nlm.nih.gov/Genbank/index.html) using the Basic Local Alignment Search Tool [21], which allowed the identification of the strains.

2.2. RAPD

The optimized technique for RAPD-PCR was performed according to Leelayuwat *et al.* (2000) [8], with three primers: OPQ-2 (5′-TCTGCTGGTC-3′), OPQ-4 (5′-AGTGCGCTGA-3′) and OPQ-16 (5′-AGTGCAGCCA-3′). This involved initial denaturation at 94 °C for 5 min, followed by 35 cycles with a denaturation step at 94 °C for 1 min, an annealing step at 37 °C for 1 min, and an extension step at 72 °C for 2 min, followed by a final extension at 72 °C for 10 min. The obtained band patterns were visualized through gel electrophoresis and were analyzed with the software GelAnalyzer 2010a™. Then, a binary matrix was generated, according to the presence (1) or absence (0) of bands, the dice similarity coefficient was measured and a dendrogram was obtained through the use of the Unweighted Pair Group Method with Arithmetic Average, through the software PyElph 1.4™. Epidemiological data, including patients, clinical data (occurrence of sepsis and clinical outcome) and the geographical distribution of cases of melioidosis were also analyzed, linking them to the obtained clades [8].

2.3. Detection of the TTSS gene

The primers BPTTSF (5′-CTTCAATCTGCTCTTTCCGTT-3′) and BPTTSR (5′-CAGGACGGTTTCGGACGAA-3) (Invitrogen, Carlsbad, USA) were used for amplification of the *TTSS* gene. The gene was amplified from 100 ng of total DNA with the above primers. The PCR was performed according to the previously described protocol [22]. The initial denaturation occurred at 95 °C for 1 min, followed by 35 cycles at 95 °C for 30 s, 60 °C for 30 s, 72 °C for 30 s and a final extension at 72 °C for 2 min. The amplification of the *TTSS* gene resulted in fragments of approximately 500 bp, which were visualized through gel electrophoresis, using a molecular weight marker of 100 bp [23].

3. Results

The RAPD-PCR yielded DNA band sizes ranging from 281 bp to 1 595 bp and 14 RAPD patterns for primer OPQ-2 (Figure 1). As for OPQ-4, a band size range of 512–1 226 bp was observed, yielding 12 RAPD-patterns (Figure 2), while amplification with OPQ-16 yielded band sizes ranging from 142 bp to 1 596 bp, and 17 RAPD patterns (Figure 3).

The three primers were used to construct individual dendrograms. OPQ-2 primer showed 51.4% genetic relatedness between the 19 isolates, with two major clades, one containing 18 isolates and the other formed by one ungrouped isolate. Two subclades contained two and four environmental isolates with the same band pattern (100% relatedness), while one subclade contained two clinical isolates with identical band patterns (Figure 1). Moreover, OPQ-4 primer showed 50% genetic relatedness between all tested isolates, with three clades, one containing three isolates with the same RAPD pattern, the other formed by one ungrouped isolate and the biggest clade containing 15/19 isolates. One subclade within this clade contained six isolates with the same band pattern, of which five were recovered from environmental sources (Figure 2). As for OPQ-16, a 56.7% genetic relatedness was observed between the 19 isolates, forming two clades, one containing two clinical isolates and the other formed by 17 isolates. Two subclades within this clade contained two environmental isolates with the same RAPD pattern and one subclade was formed by three isolates recovered from lethal cases of melioidosis (Figure 3).

Overall, the three primers showed that most environmental isolates are closely grouped. However, only two pairs of environmental isolates (CEMM 03-6-046, CEMM 03-6-047, CEMM 03-6-045 and CEMM 03-6-048) presented the same RAPD

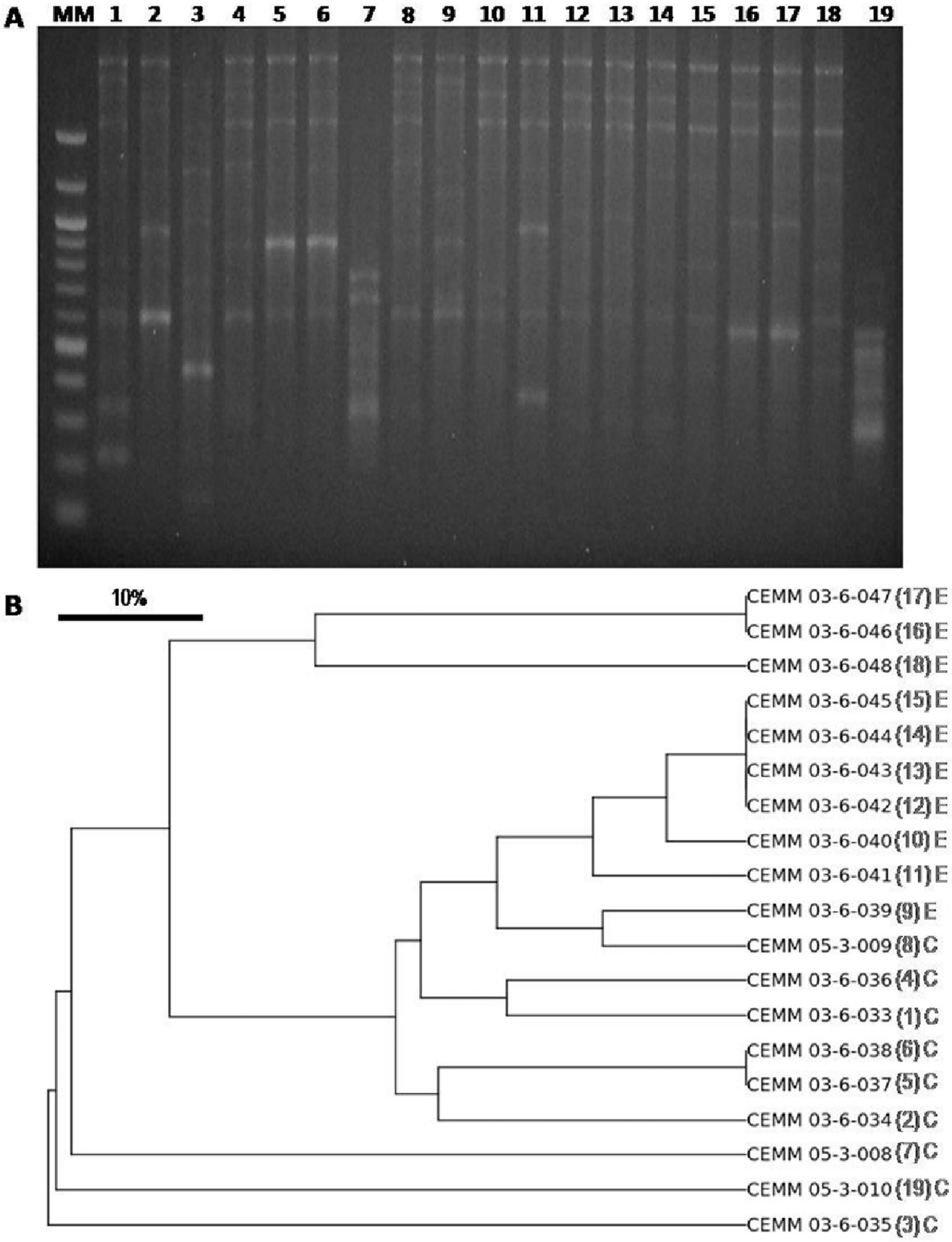

Figure 1. RAPD-PCR results for primer OPQ-2.
A) RAPD-PCR patterns of clinical (n = 9) and environmental (n = 10) *B. pseudomallei* isolates from Ceará, obtained with the arbitrary primer OPQ-2. MM: 100 bp molecular marker; lanes 1–19: analyzed isolates. The identification of the isolates is described in the dendrogram. B) Dendrogram constructed from the analysis of the gel obtained through RAPD-PCR. Numbers in parenthesis refer to the band patterns of each isolate presented in the agarose gel. E indicates environmental isolates, C indicates clinical isolates. Dendrogram generated by PyElph 1.4™.

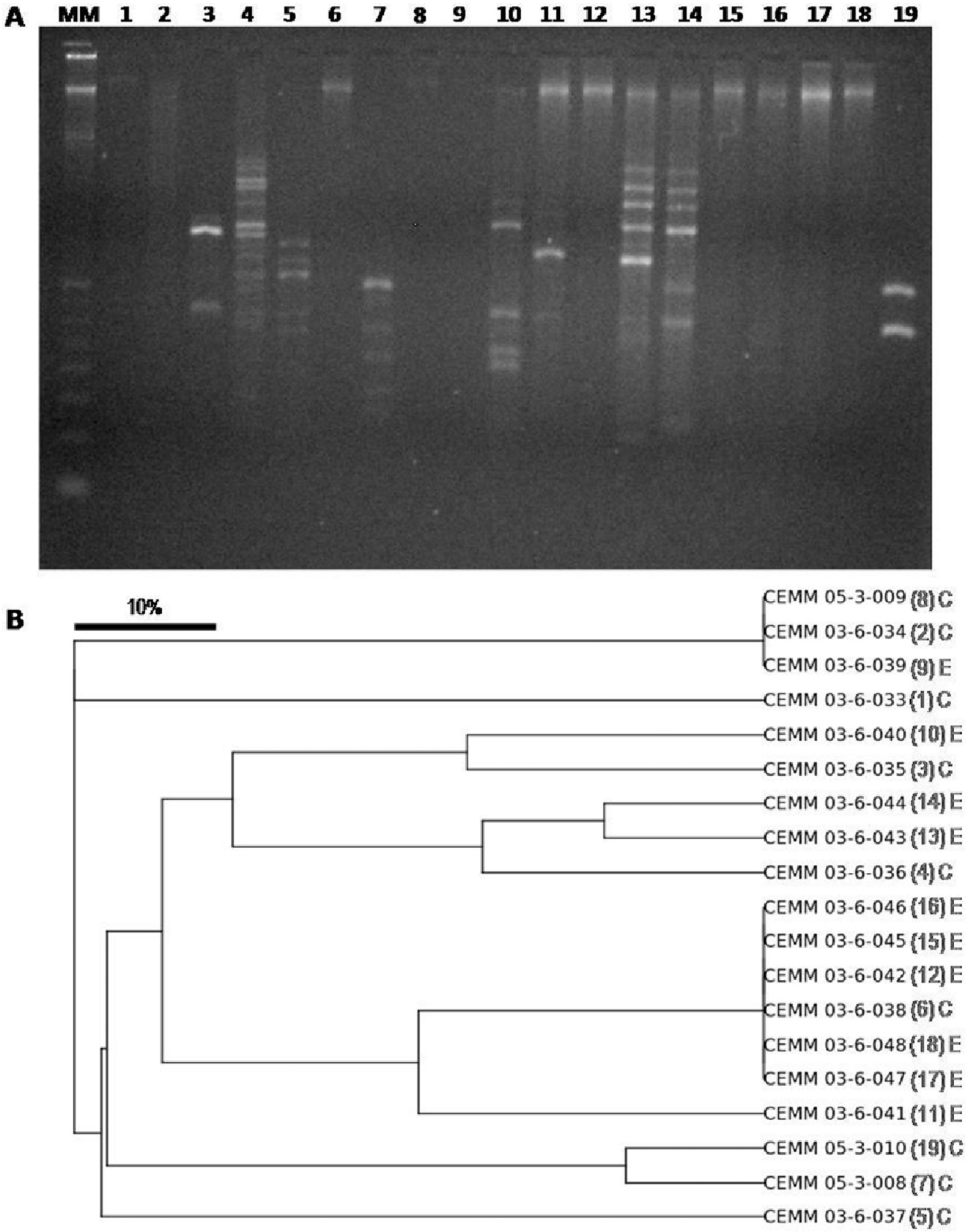

Figure 2. RAPD-PCR results for primer OPQ-4.
A) RAPD-PCR patterns of clinical (n = 9) and environmental (n = 10) *B. pseudomallei* isolates from Ceará, obtained with the arbitrary primer OPQ-4. MM: 100 bp molecular marker; lanes 1–19: analyzed isolates. The identification of the isolates is described in the dendrogram. B) Dendrogram constructed from the analysis of the gel obtained through RAPD-PCR. Numbers in parenthesis refer to the band patterns of each isolate presented in the agarose gel. E indicates environmental isolates, C indicates clinical isolates. Dendrogram generated by PyElph 1.4™.

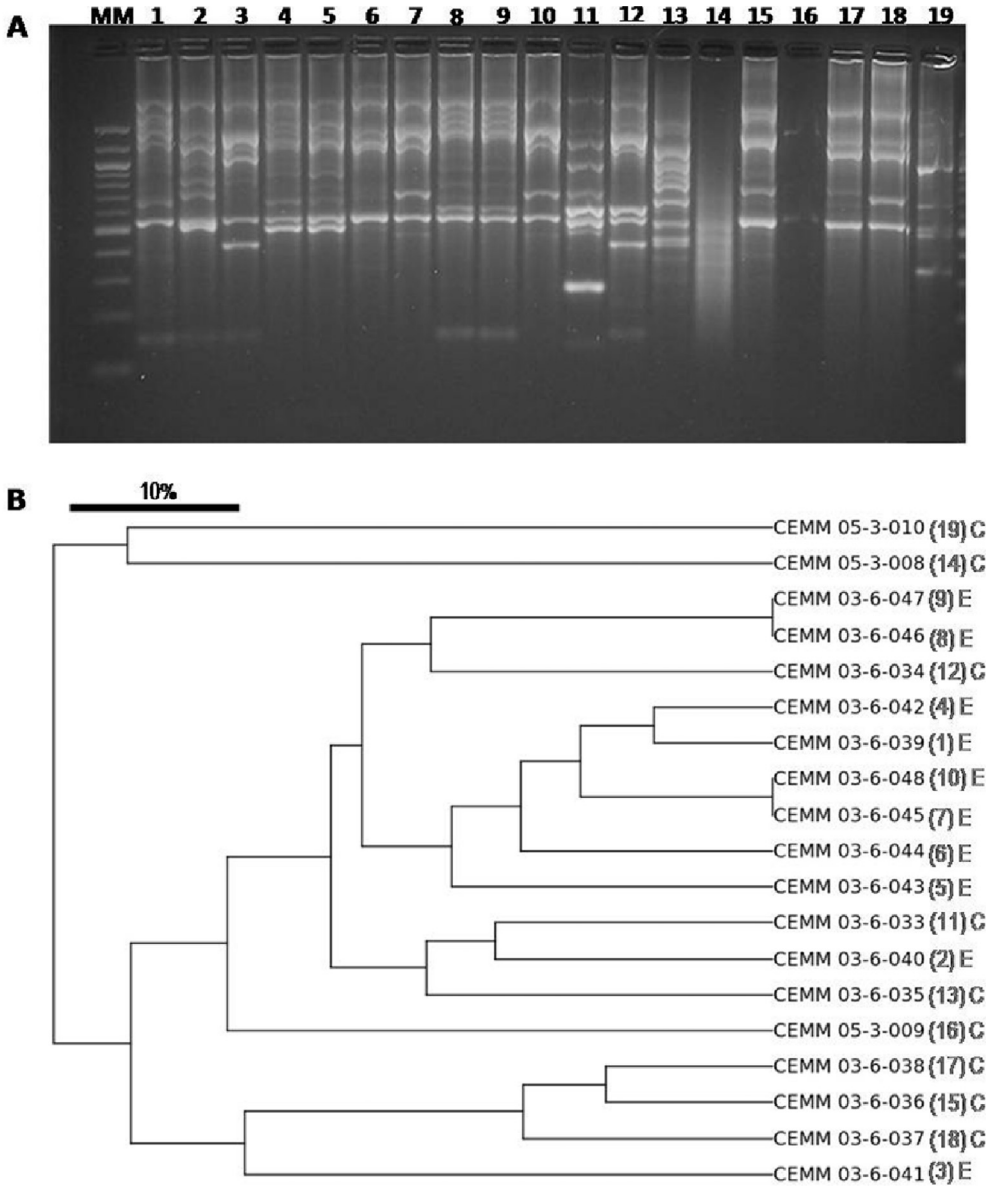

Figure 3. RAPD-PCR results for primer OPQ-16.
A) RAPD-PCR patterns of clinical (n = 9) and environmental (n = 10) *B. pseudomallei* isolates from Ceará, obtained with the arbitrary primer OPQ-16. MM: 100 bp molecular marker; lanes 1–19: analyzed isolates. The identification of the isolates is described in the dendrogram. B) Dendrogram constructed from the analysis of the gel obtained through RAPD-PCR. Numbers in parenthesis refer to the band patterns of each isolate presented in the agarose gel. E indicates environmental isolates, C indicates clinical isolates. Dendrogram generated by PyElph 1.4™.

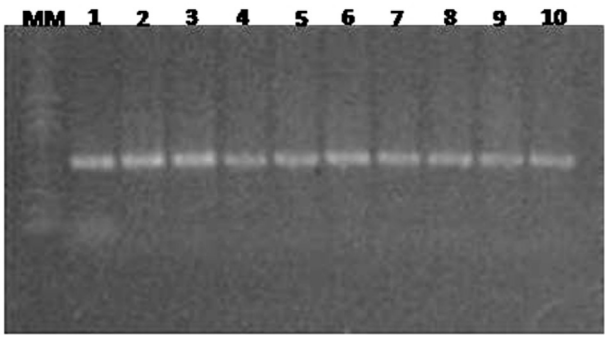

Figure 4. Representative agarose gel for detection of *TTSS* gene in 10 isolates of *B. pseudomallei*.
MM: molecular marker. Lanes 1–10: isolates of *B. pseudomallei* from Ceará.

pattern for at least two of the three tested primers (Figures 1–3). As for the clinical isolates, none presented identical RAPD patterns for more than one primer.

Finally, the *TTSS* gene was detected in all 19 strains, which showed a single band of approximately 500 bp (Figure 4).

4. Discussion

The RAPD-PCR method is a PCR-based technique that has been widely used for genotyping several microorganisms and assessing microbial genetic diversity [7,8,11,12]. Historically, this methodology has been associated with poor reproducibility, but it becomes more reliable if the PCR conditions are optimized [8]. For *B. pseudomallei* and other bacteria, RAPD-PCR has been used to evaluate the genetic diversity of isolates from a given region, with the advantage of being a cheap, fast and more sensitive typing method, which is useful for addressing

epidemiological problems, such as microbial clonality during infection outbreaks and mechanisms of disease transmission [7,8,11,12]. However, an important drawback for the use of RAPD-PCR for genotyping bacterial strains is the fact that the obtained results are not reliably comparable with those results obtained by other institutions. For this purpose, more elaborate and expensive techniques must be used, such as multilocus sequence typing [7,9,10], and whole-genome sequencing [10].

In the present research, the RAPD-PCR was applied to assess the genetic diversity of *B. pseudomallei* isolates from Ceará, Brazil. This was an effort to type strains of *B. pseudomallei* from Northeastern Brazil. The primers OPQ-2, OPQ-4 and OPQ-16 were chosen because they have been shown to yield multiple variable bands on agarose gel electrophoresis [8]. The three tested primers revealed similar genetic relatedness between the evaluated isolates, which varied from 50.0% to 56.7%. OPQ-16 primer yielded 17 different RAPD patterns, and it was more discriminatory than OPQ-2 and OPQ-4, which yielded 14 and 12 patterns, respectively.

Most of the environmental isolates were grouped by the three used primers in clades with genetic relatedness ranging from 50% to 75%, but only one pair of isolates (CEMM 06-3-046 and CEMM 06-3-047) presented 100% genetic relatedness for the three tested primers, which implies that they may be clones of one single strain. Even though the environmental isolates were all recovered from the municipality of Tejuçuoca, Ceará, where the first three cases of melioidosis were reported [4], these isolates were not closely related to those from the local clinical cases of the disease (CEMM 06-3-033, CEMM 06-3-034 and CEMM 06-3-035). In addition, these three isolates from the outbreak of melioidosis in Tejuçuoca were not closely grouped by any of the used primers. Considering that RAPD-PCR is a sensitive technique to detect genetic heterogeneity, these observations suggest that *B. pseudomallei* from Brazil is genetically diverse, even within a limited area, which may be associated with the natural colonization of Brazilian soils, with this bacterial pathogen, as previously proposed [2], leading to the exchange of genetic material between environmental strains, increasing the population diversity. Moreover, clinical and environmental isolates were grouped together, following no apparent pattern, after amplification with the three primers, corroborating the findings of Chen *et al.* (2015) [24], who demonstrated the epidemiological relationship between clinical and environmental strains.

Previous researches with *B. pseudomallei* have tried to associate RAPD patterns with clinical and epidemiological data, but only one study reported the association of certain RAPD patterns with the occurrence of septic melioidosis, with no association with its lethality rate [8]. In the present study, the primer OPQ-16 yielded one subclade composed by three clinical isolates from lethal cases of septic melioidosis (CEMM 03-6-036, CEMM 03-6-037 and CEMM 03-6-038). However, the other two primers did not group these isolates in the same subclade. In addition, these isolates were recovered from patients with comorbidities, such as chronic obstructive pulmonary disease and cranioencephalic trauma [4], which may imply that isolate lethality was, at least in part, associated with the host condition, not only with strain-specific features.

As for the detection of the *TTSS* virulence gene, all tested isolates from Ceará were positive, corroborating the findings of Smith-Vaughan *et al.* (2003) [22] who detected this gene in 116 clinical and environmental isolates of *B. pseudomallei*, but not in other common environmental species of *Burkholderia*, including *Burkholderia thailandensis* and *Burkholderia cepacia*. In order to better analyze the virulence of *B. pseudomallei* isolates from Ceará, Brazil, the expression of TTSS may be evaluated, as it has been shown that a *B. pseudomallei* mutant strain lacking this molecular structure is less virulent for murine models of infection [14]. In addition, this PCR reaction for the detection of a specific gene cluster of the TTSS is highly useful for the identification of *B. pseudomallei* and its detection in environmental samples [22–25].

In conclusion, the present research showed that *B. pseudomallei* from Ceará is genetically diverse, and that genetic relatedness exists between clinical and environmental isolates, corroborating the idea that this bacterium is acquired from the environment. In addition, all clinical and environmental isolates have the type-three secretion system virulence gene in their genome, suggesting that they have the potential to express this important virulence gene. Finally, this was an effort to type clinical and environmental *B. pseudomallei* isolates from Ceará, Brazil, which is important for better understanding this emerging pathogen, contributing for the epidemiological surveillance of melioidosis in this endemic region.

Conflict of interest statement

We declare that we have no conflicts of interest.

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References

[1] Benoit TJ, Blaney DD, Gee JE, Elrod MG, Hoffmaster AR, Doker TJ, et al. Melioidosis cases and selected reports of occupational exposures to *Burkholderia pseudomallei* – United States, 2008–2013. *Cent Dis Cont Prev* 2015; **64**(5): 1-9.

[2] Limmathurotsakul D, Golding N, Dance DAB, Messina JP, Pigott DM, Moyes CL, et al. Predicted global distribution of *Burkholderia pseudomallei* and burden of melioidosis. *Nat Microbiol* 2016; **1**(1): 1-5.

[3] Benoit TJ, Blaney DD, Doker TJ, Gee JE, Elrod MG, Rolim DB, et al. A review of melioidosis cases in the Americas. *Am J Trop Med Hyg* 2015; **93**(6): 1134-1139.

[4] Churuangsuk C, Chusri S, Hortiwakul T, Charernmak B, Silpapojakul K. Characteristics, clinical outcomes and factors influencing mortality of patients with melioidosis in southern Thailand: a 10-year retrospective study. *Asian Pac J Trop Med* 2016; **9**(3): 256-260.

[5] Chewapreecha C, Holden MTG, Vehkala M, Välimäki N, Yang Z, Harris SR, et al. Global and regional dissemination and evolution of *Burkholderia pseudomallei*. *Nat Microbiol* 2017; **2**: 16263.

[6] Brilhante RSN, Bandeira TJPG, Cordeiro RA, Grangeiro TB, Lima RAC, Ribeiro JF, et al. Clinical-epidemiological features of 13 cases of melioidosis in Brazil. *J Clin Microbiol* 2012; **50**(10): 3349-3352.

[7] Antonov VA, Tkachenko GA, Altukhova VV, Savchenko SS, Zinchenko OV, Viktorov DV, et al. Molecular identification and typing of *Burkholderia pseudomallei* and *Burkholderia mallei*: When is enough enough? *Trans R Soc Trop Med Hyg* 2008; **102**(1): S134-S139.

[8] Leelayuwat C, Romphruk A, Lulitanond A, Trakulsomboon S, Thamlikitkul V. Genotype analysis of *Burkholderia pseudomallei* using randomly amplified polymorphic DNA (RAPD): indicative of genetic differences amongst environmental and clinical isolates. *Acta Trop* 2000; **77**(2): 229-237.

[9] Tellapragada C, Kamthan A, Shaw T, Ke V, Kumar S, Bhat V, et al. Unravelling the molecular epidemiology and genetic diversity among *Burkholderia pseudomallei* isolates from South India using multi-locus sequence typing. *PLoS One* 2016; **11**(12). e0168331.

[10] Price EP, Sarovich DS, Viberg L, Mayo M, Kaestli M, Tuanyok A, et al. Whole-genome sequencing of *Burkholderia pseudomallei* isolates from an unusual melioidosis case identifies a polyclonal infection with the same multilocus sequence type. *J Clin Microbiol* 2015; **53**(1): 282-286.

[11] Kumar S, Tripathi VR, Garg SK. Antibiotic resistance and genetic diversity in water-borne *Enterobacteriaceae* isolates from recreational and drinking water sources. *Int J Env Sci Technol* 2013; **10**(4): 789-798.

[12] Tissera K, Liyanapathirana V, Dissanayake N, Pinto V, Ekanayake A, Tennakoon M, et al. Spread of resistant Gram negatives in a Sri Lankan intensive care unit. *BMC Infect Dis* 2017; **17**(1): 490.

[13] Sermswan RW, Royros P, Khakhum N, Wongratanacheewin S, Tuanyok A. Direct detection of *Burkholderia pseudomallei* and biological factors in soil. *Trans R Soc Trop Med Hyg* 2015; **109**(7): 462-468.

[14] Vander Broek CW, Stevens JM. Type III secretion in the melioidosis pathogen *Burkholderia pseudomallei*. *Front Cell Infect Microbiol* 2017; **7**(255): 1-17.

[15] Rolim DB, Rocha MFG, Brilhante RSN, Cordeiro RA, Leitão-Júnior NP, Inglis TJJ, et al. Environmental isolates of *Burkholderia pseudomallei* in Ceará state, Northeastern Brazil. *Appl Environ Microbiol* 2009; **75**(4): 1215-1218.

[16] Merritt A, Inglis TJJ, Chidlow G, Harnett G. PCR-based identification of *Burkholderia pseudomallei*. *Rev Inst Med Trop* 2009; **48**(5): 239-244.

[17] Weisburg WG, Barns SM, Pelletier DA, Lane DJ. 16S ribosomal DNA amplification for phylogenetic study. *J Bacteriol* 1991; **173**(2): 697-703.

[18] Ewing B, Hillier L, Wendl MC, Green P. Base-calling of automated sequencer traces using Phred. I. Accuracy assessment. *Gen Res* 1998; **8**(3): 175-185.

[19] Ewing B, Green P. Base-calling of automated sequencer traces using Phred. II. Error probabilities. *Gen Res* 1998; **8**(3): 186-194.

[20] Gordon D, Abajian C, Green P. Consed: a graphical tool for sequence finishing. *Gen Res* 1998; **8**(3): 195-202.

[21] Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. *J Mol Biol* 1990; **215**(3): 403-410.

[22] Smith-Vaughan HC, Gal D, Lawrie PM, Winstanley C, Sriprakash KS, Currie BJ. Ubiquity of putative type III secretion genes among clinical and environmental *Burkholderia pseudomallei* isolates in Northern Australia. *J Clin Microbiol* 2003; **41**(2): 883-885.

[23] Gal D, Mayo M, Spencer E, Cheng AC, Currie BJ. Short report: application of a polymerase chain reaction to detect *Burkholderia pseudomallei* in clinical specimens from patients with suspected melioidosis. *Am J Trop Med Hyg* 2005; **73**(6): 1162-1164.

[24] Chen PS, Chen YS, Lin HH, Liu PJ, Ni WF, Hsueh PT, et al. Airborne transmission of melioidosis to humans from environmental aerosols contaminated with *B pseudomallei*. *PLoS Negl Trop Dis* 2015; **9**(6), e0003834.

[25] Lau SK, Sridhar S, Ho CC, Chow WN, Lee KC, Lam CW, et al. Laboratory diagnosis of melioidosis: past, present and future. *Exp Biol Med* 2015; **240**(6): 742-751.