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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 was 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

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