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Research paper

Emergence of distinct *Burkholderia pseudomallei* lineages in Brazil: Phenotypic, genotypic, and phylogenetic agreement

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ABSTRACT

Burkholderia pseudomallei is a Gram-negative facultative intracellular bacterium that causes melioidosis, a neglected tropical disease that spreads beyond traditional endemic regions. This study examined *B. pseudomallei* isolates obtained from five melioidosis cases in Piauí, Brazil, using phenotypic, molecular, and genomic methods. Isolates were identified as *B. pseudomallei* using VITEK 2 system and MALDI-TOF mass spectrometry. Conventional PCR targeting the *orf11*, *burk17,* and *burk475* gene regions confirmed species-level identification, with sequences showing 98.78–100% identity to GenBank *B. pseudomallei* references. Whole-genome sequencing and multi-locus sequence typing (MLST) revealed two novel sequence types; ST2226 (four isolates), clustering closely with strains from Ceará (ST1455 and ST1458), ST2234 (one isolate), related to previously reported ST95 and ST1355 strains, previously detected in Brazil. Phylogenetic analysis based on single nucleotide polymorphism (SNPs) and concatenated MLST data showed clear genetic distinction between the two lineages, suggesting regional spread or shared ancestry. These findings confirm the presence of at least two distinct genetic *B. pseudomallei* lineages in Piauí, providing insights into bacterial diversity and regional epidemiology in Brazil and reinforcing the importance of molecular surveillance and improved diagnostic capacity for melioidosis.

1. Introduction

Burkholderia pseudomallei is a Gram-negative, facultative intracellular bacterium found in the rhizosphere of tropical soils. It causes melioidosis, an opportunistic disease affecting humans and animals, transmitted through contact with contaminated soil and water (Chen et al., 2015). Bacteria can enter the body *via* inhalation of aerosols, ingestion, or skin exposure through wounds. Melioidosis is most prevalent in Southeast Asia and Australia, with an estimated 165,000 cases and 89,000 deaths annually (Limmathurotsakul, 2016). Although historically associated with these regions, cases have been reported in other continents, suggesting a broader geographical distribution (Wiersinga et al., 2018). Limmathurotsakul et al. identified 34 countries in which melioidosis may be endemic but underreported, including 24 in Africa and three in the Middle East. Melioidosis remains underreported in the Americas; however, recent studies have indicated an increasing number of cases in tropical and subtropical regions. Over the past five years, research has demonstrated the environmental presence of *B. pseudomallei* and an increase in clinical cases in several American countries, suggesting a significant underestimation of the disease burden (Limmathurotsakul, 2016; Birnie et al., 2019).

Between 1962 and 2015, 37 suspected melioidosis cases, possibly originating in South America, were reported in Brazil, Colombia, Venezuela, and Ecuador. Brazil accounted for most cases (67%), with Ceará in Northeastern Brazil accounting for 91% of cases (Rolim et al., 2005; Brilhante et al., 2012). Ceará was the first state to document cases of melioidosis in 2003, as four brothers contracted an acute infection after recreational exposure to a dam, and three died within a week

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(Rolim et al., 2005; Virginio et al., 2006). Additionally, other states have reported the isolation of *B. pseudomallei*, including Piauí (Velôso et al., 2023), Sergipe (Morais et al., 2024), Amapá (Sousa et al., 2015), Mato Grosso (Barth et al., 2007), and the Federal District (Vale and Matias, 2022).

Clinical diagnosis is challenging because of varying disease manifestations, ranging from localized infections to severe systemic conditions, and often mimic other diseases. Early detection depends on clinical suspicion based on the patient's medical history and risk factors and specific laboratory tests. Timely and accurate diagnosis is critical for effective treatment and improved patient outcomes (Currie, 2015; Wiersinga et al., 2012). Laboratory diagnoses rely on the detection and identification of *B. pseudomallei (*Wiersinga et al., *2018;* Currie, *2015)*. Although bacterial cultures remain the gold standard, they have several limitations, including low sensitivity (approximately 60%) and prolonged response times (Perumal Samy et al., 2017). Treatment typically involves antimicrobials, such as sulfamethoxazole, trimethoprim, meropenem, and ceftazidime (Lim et al., 2022; Dance, 2014; Pitman et al., 2015). However, *B. pseudomallei*'s intrinsic resistance to several antibiotics, including penicillin, streptomycin, aminoglycosides, cephalosporins, polymyxins, quinolones, and macrolides, limits therapeutic options (Estes et al., 2010). Advanced diagnostic techniques, such as mass spectrometry (MALDI-TOF), polymerase chain reaction (PCR), and other molecular approaches, are increasingly used for faster and more accurate strain identification. (Lau et al., 2015; Merritt et al., 2006).

The *B. pseudomallei* genome (7.25 Mb) comprises two chromosomes. The larger chromosome (4.07 Mb) encodes essential growth and virulence factors (toxins, proteases, adhesins, efflux pumps, polysaccharide capsules, and secretory systems). The smaller chromosome (3.17 Mb) aids in environmental adaptation (Holden et al., 2004). Comparative genomic analyses revealed distinct geographical clades, including those in the Americas and Africa, indicating the presence of locally established populations (Chewapreecha et al., 2017; Hall et al., 2019; Sarovich et al., 2016; Gasqué et al., 2024). Molecular approaches, such as multilocus sequence typing (MLST), which examines seven conserved *B. pseudomallei* genes, are valuable for determining strain origin (Aziz et al., 2017). Whole-genome analysis provides a more detailed perspective (Gasqué et al., 2024; Pearson et al., 2009). This study aimed to characterize five clinical isolates of *B. pseudomallei* using diagnostic tools, such as MALDI-TOF mass spectrometry, and molecular analyses, including phylogenetic analysis.

2. Material and methods

2.1. Sampling

The study samples were obtained from five cases of laboratory-confirmed melioidosis diagnosed between 2019 and 2023 at a public hospital in Piauí, Brazil (Velôso et al., 2023). The inclusion criteria were as follows; (i) confirmation of infection with *B. pseudomallei via* culture of clinical samples and (ii) availability of biological material for further analysis. The patients presented with various clinical symptoms, including pneumonia, sepsis, liver abscesses, and osteomyelitis, which are often associated with comorbidities, such as diabetes mellitus and chronic kidney disease. Five *B. pseudomallei* isolates were selected from this cohort for phenotypic, genotypic, and phylogenetic characterization based on culture viability. The isolates, which originated from blood, bronchoalveolar lavage, or liver abscess aspirates, were cultured on blood agar, chocolate agar, MacConkey agar, BHI broth, or thioglycolate broth and incubated at 37 ± 2 °C. Growth was assessed at 24, 48, and 72 h. The bacterial colonies were suspended in 3 mL of 0.45% NaCl, mixed, and adjusted to a McFarland turbidity of 0.5–0.63 using the Densi-CHEK® plus equipment. This device is intended for use with the VITEK2® system (bioMérieux) to measure the optical density of a microorganism suspension, with the results obtained from the reading range in McFarland units ranging from 0.0 to 4.0 (bioMérieux, 2016). The suspension was then applied to an identification card for Gram-negative bacteria and analyzed by the VITEK2® system (bioMérieux).

2.2. Identification using MALDI-TOF mass spectrometry

Bacterial colonies were inactivated with ethanol, and protein profiles were obtained and analyzed using a MALDI Biotyper® (Bruker Daltonics, GmbH) (Sauer et al., 2008; Freiwald and Sauer, 2009). Following washing and centrifugation, proteins were extracted using formic acid and acetonitrile, applied to a MALDI target, overlaid with an alpha-cyano-4-hydroxycinnamic acid (CHCA) matrix solution, and analyzed using a MALDI Biotyper® Sirius One mass spectrometer (Bruker Daltonik, GmbH) (Bier et al., 2017). Identification was performed using Revision K of the MBT Compass Library (April 2022), containing 11,897 reference spectra for 4274 species of microorganisms (Bruker Daltonik, GmbH). To ensure reliable analysis, additional reference spectra were uploaded for *Burkholderia mallei* (33 isolates) and *B. pseudomallei* (20 isolates) from the database of highly pathogenic bacteria established at the Robert Koch Institute, Berlin, Germany (RKI) and available on the ZENODO web platform (Lasch et al., 2025). The creation and identification of the main spectral profiles (MSPs) were performed using the standard MALDI Biotyper® methods provided in the MALDI Biotyper® Compass Explorer 4.1 software (Bruker Daltonik, GmbH).

2.3. Antibiotic susceptibility

The antimicrobial susceptibility test was performed based on the disk-diffusion methodology (Kirby-Bauer technique), which consists of a phenotypic method that uses paper disks impregnated with predefined concentrations of antimicrobials. These disks are placed on the surface of agar (Mueller-Hinton) in a Petri dish previously inoculated with a microorganism, which was made into a suspension with a standardized inoculum. Subsequently, the plate is placed in an incubator at a standardized temperature and time. After the disks diffuse into the agar, inhibition zones are formed, and the diameter formed is measured to determine the susceptibility category by comparing it with the cut-off points established in guidelines by reference institutions, such as CLSI (Clinical and Laboratory Standards Institute) and EUCAST (European Committee on Antimicrobial Susceptibility Testing) (Rivera et al., 2023).

Antimicrobial susceptibility testing was performed using standardized antibiotics for *B. pseudomallei*, including sulfamethoxazole/trimethoprim (DME-23.75/1.25 μg), meropenem (DME-10 μg), ceftazidime (DME-10 μg), and amoxicillin/clavulanic acid (DME-20/10 μg), in accordance with the BrCAST/EUCAST guidelines.

2.4. PCR analysis

Genomic DNA was isolated from bacterial colonies using an adapted protocol (van Embden et al., 1993). *Escherichia coli* DNA was included as a negative control for DNA extraction, and sterile DNase- and RNase-free water was used for PCR reactions. Additionally, an environmental *B. pseudomallei* and a laboratory strain of *B. mallei* (BAC 86/19) (PAP et al., 2023) were used as controls. Conventional PCR specifically was used to specifically target *B. pseudomallei* (*orf11*) and both species (*bm17* and *burk475*) (Table 1). PCR reactions were performed in 20 μL using the ready-to-use GoTaq Colorless Master Mix (2×) (Promega, USA), with 0.25 μM of each primer, and 500 ng of template DNA. Amplification was conducted using an automated thermal cycler (Veriti 96-well Fast Thermal Cycler, Applied Biosystems) with the following thermal cycles: for the *orf11* target: 95 °C for 10 min, followed by 35 cycles of 95 °C for 30 s, 60 °C for 1 min, 72 °C for 1 min, with a final extension at 72 °C for 7 min; for the *burk475* and *bm17* targets: 95 °C for 15 min, followed by 35 cycles of 95 °C for 1 min, 60 °C for 1 min, 72 °C for 1 min, and a final extension at 72 °C for 7 min. Amplified products were analyzed by agarose gel electrophoresis and confirmed by DNA sequencing.

Table 1
Primers used in detection of *B. mallei* and *B. pseudomallei*.

PCR target	Sequence (5` to 3`)	Amplicon size (pb)	Target species	Reference
orf11	F: ATCGCCAAATGCCGGGTTTC R: GTGCATCCATTCATCAAAG	706	*B. pseudomallei*	(Aung et al., 2023)
bm17 – MLVA marker	F: TATACGCGAGGTTATAACGGATG R: CTTTCTGCTTTTCTAACGTTTCC	281 321	*B. mallei* *B. pseudomallei*	(Yen et al., 2009)
burk475 – Type IV secretion protein Rhs	F: AATTGGTCGCCGTGATAGAC R: TCGGCTCGATGGAAAGTAAC	475	*B. mallei* *B. pseudomallei*	(Fonseca Júnior et al., 2021)

The obtained sequences were analyzed using BioEdit software (Hall, 1999; Alzohairy, 2011) to generate consensus sequences, which were then subjected to a homology search by comparison with the NCBI database (http://www.ncbi.nlm.nih.gov/BLAST/) using the BLASTn program.

2.5. Whole-genome sequencing

DNA libraries were prepared using the NEBNext Ultra II DNA Library Kit (New England Biolabs, USA) and sequenced on a NovaSeq6000 (Illumina, San Diego, CA, USA) with the NovaSeq6000 S4 Reagent Kit v1.5 and 300 paired-end sequencing cycles. Reads were quality checked using FastQC (v0.11.9), trimmed using Trimmomatic (v0.39; Phred >28, length > 28, and adapter removal), and assembled using SPAdes (v3.15.5). Clustering was performed using RagTag (v2.1.0) and the *B. mallei* ATCC23344 genome (GCF_033956065.1_ASM3395606v1). Synteny analysis (ACT v18.0.0) was used to identify the syntenic regions, which were extracted and analyzed using BLASTx (v2.12.0) against RefSEq. (v222). The identified genes were examined using BLASTn, and the top 100 matches were reviewed. Contigs specific to *B. mallei* and *B. pseudomallei* were reanalyzed using BLASTn against the reference genomes of these bacterial strains (strain K96243, GCA_000959285.1_ASM75612v1) (Luz et al., 2024).

2.6. MLST analysis

The seven MLST genes for *B. pseudomallei* were extracted *in silico* from genomic sequences obtained from the PATRIC (https://www.bv-brc.org/, accessed December 1, 2024), NCBI (https://www.ncbi.nlm.nih.gov/, accessed December 1, 2024), and ENA (https://www.ebi.ac.uk/ena/browser/home, accessed December 1, 2024) databases using the MLST plugin in BioNumerics (v7.6.3). Allelic assignments were determined by querying the sequences of each locus in the PubMLST online database for *B. pseudomallei*. The allelic profile was used to determine sequence type (ST). The new allelic sequences are available on the PubMLST website for *B. pseudomallei*. All records associated with the Americas were selected from the PubMLST database (accessed January 2025) and a cluster analysis was conducted in BioNumerics using the categorical parameter and *Neighbor-Joining* method, including STs of the five strains used in this study.

2.7. SNP selection and PCR primer design

All complete or draft genomes of the Americas (n = 108) were retrieved from PATRIC (https://www.bv-brc.org/, accessed December 1, 2024), NCBI (https://www.ncbi.nlm.nih.gov/, accessed December 1, 2024), and ENA (https://www.ebi.ac.uk/ena/browser/home, accessed December 1, 2024) and supplemented by the five genomes sequenced. The dataset included the genomes of strains isolated from 79 humans, five animals, 23 environmental samples, and one strain of unknown origin. Whole-genome SNP analysis was performed (Gasqué et al., 2024), with the Thai strain K96243 (SRR1614021) serving as an outgroup. Reads were aligned to K96243 using BioNumerics software (v7.6.3) with a 90% identity threshold. SNPs were obtained using the wgSNP module and filtered for ≥20× coverage with ambiguous/low-quality bases being removed. A neighbor-joining tree (1000 bootstraps) was constructed and refined using parsimony analysis.

3. Results

3.1. Phenotypic characterization

Five strains were successfully isolated from patients with melioidosis: CH581-001R0001 (BP03), CH581-001R0002 (BP04), CH581-001R0003 (BP06), CH581-001R0004 (BP07), and CH581-001R0005 (BP08) (Table 2). All the strains were grown on blood, MacConkey agar, and chocolate agar. BP03, BP04, and BP08 were obtained from blood cultures; BP06 from bronchial lavage; and BP07 and BP08 from liver abscess samples.

All isolates were phenotypically identified using the VITEK 2 automated system, which provided a high level of confidence in strain identification (98–99%). Antimicrobial susceptibility testing showed consistently large inhibition zones for meropenem (28–30 mm), ceftazidime (46–51 mm), and amoxicillin/clavulanate (48–50 mm), indicating preserved susceptibility according to the BrCast-Eucast breakpoints. Contrastingly, the results for sulfamethoxazole/trimethoprim were more variable (15–50 mm); strains BP03, BP06, BP07, and BP08 displayed inhibition zones of 45–50 mm, consistent with susceptibility, whereas strain BP04 showed a markedly smaller zone (15 mm), indicating reduced susceptibility or resistance.

Table 2
Data on *B. pseudomallei* strains.

Strain ID	Culture Media	Biological Sample	IVITEK2 Identification (CI%)	Susceptibility (AST)
BP03	BA MAC CHOC	Blood	*B. pseudomallei* (99%)	MEM (30 mm) SXT (45 mm) CAZ (50 mm) AMC (50 mm)
BP04	BA MAC CHOC	Blood	*B. pseudomallei* (99%)	MEM (29 mm) SXT (15 mm) CAZ (49 mm) AMC (50 mm)
BP06	BA MAC CHOC	Bronchial lavage	*B. pseudomallei* (99%)	MEM (28 mm) SXT (50 mm) CAZ (51 mm) AMC (49 mm)
BP07	BA MAC CHOC	Liver abscess	*B. pseudomallei* (99%)	MEM (28 mm) SXT (48 mm) CAZ (48 mm) AMC (50 mm)
BP08	BA MAC CHOC	Blood Liver abscess	*B. pseudomallei* (98%)	MEM (29 mm) SXT (49 mm) CAZ (46 mm) AMC (48 mm)

BA, blood agar; MAC, MacConkey agar; CHOC: Chocolate agar; AST: Antimicrobial susceptibility test; MEM: Meropenem, SXT: Sulfamethoxazole/trimethoprim, CAZ: Ceftazidime, AMC: Amoxicillin/Clavulanic acid. Breakpoints (BrCAST/EUCAST - 2025): MEM (Susceptible ≥24 mm, Resistant <24 mm), SXT (Susceptible ≥50 mm, Resistant <17), CAZ (Susceptible ≥50 mm, Resistant <18 mm), AMC (Susceptible ≥50 mm, Resistant <22 mm).

3.2. Mass spectrometry

Representative MALDI-TOF mass spectra of the five isolates show peptide-protein profiles in the 2000–20,000 Da range (Fig. 1). The x-axis represents the mass-to-charge ratio (m/z), and the y-axis represents the relative signal intensity. It is observed that approximately in the region of 2000 to 5000 m/z there are multiple peaks of low to medium intensity, where smaller protein fragments possibly occur. This profile is similar among the samples, suggesting a probable taxonomic proximity. However, in the region of 5000 to 9000 m/z, more intense peaks are

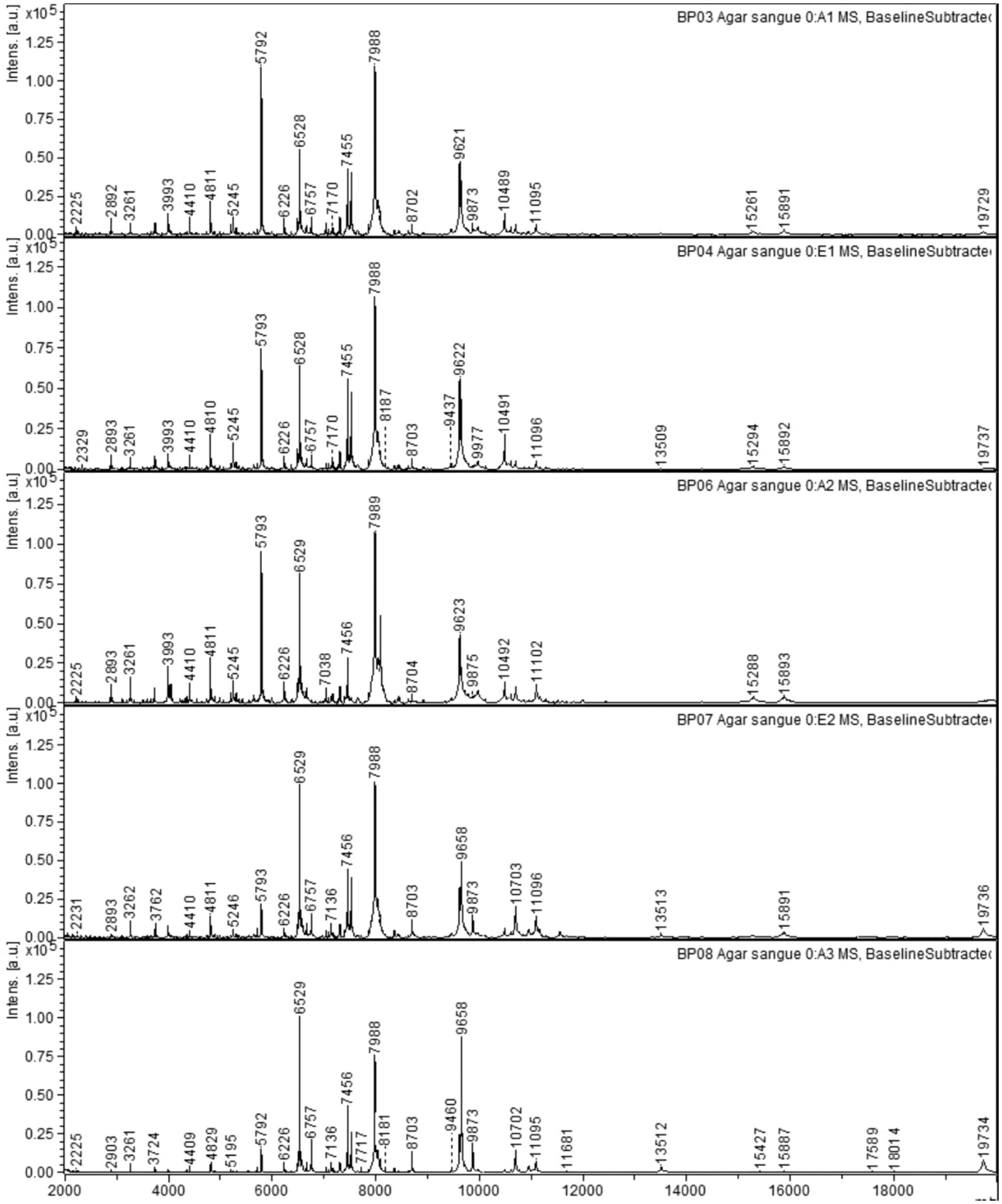

Fig. 1. MALDI-TOF mass spectra of *B. pseudomallei* isolates. Colonies grown on blood agar were picked and cellular proteins are extracted using a solution of ethanol and formic acid. The samples are applied to a CHCA matrix for ionization and mass spectra are acquired in linear TOF mode over the mass range of 2–2000 Da. The Y- and X-axis shows signal intensity (in arbitrary units) and mass-to-charge ratio (m/z), respectively.

present, representing excellent reproducibility among the samples and demonstrating more significantly and consistently that the isolates belong to the same species.

All isolates were identified as *B. pseudomallei* by MALDI-TOF Biotyper® analysis (Table 3). No species other than *B. pseudomallei* appeared in the two highest-ranking hits despite the relatively low score values obtained for the five isolates, averaging approximately 2.00. According to the manufacturer's guidelines, this method generally only supports genus-level identification. This discrepancy may be owing to strain-specific variability, differences in cultivation conditions, or variations in mass spectral acquisition methods, all of which could reduce the similarity between the isolates and reference spectra available in the database.

3.3. Molecular characterization

A set of primers were used to amplify their retrospective targets in bacterial isolates obtained from clinical samples of the patients studied. Specific primers for *B. pseudomallei* were used, targeting a 706 bp fragment of the Type III Secretion System (TTS1) of the open reading frame (*orf*11), which can differentiate *Burkholderia* species, particularly *B. mallei* and *B. thailandensis* (Aung et al., 2023). The *bm*17 primer set was originally developed to enable use in multilocus variable number tandem repeat (MLVA) analysis, allowing genotyping of *B. mallei* and *B. pseudomallei*, the aforementioned target (*bm*17) provides discriminatory power to differentiate *B. mallei* from closely related species, it can amplify a 281 bp fragment for *B. mallei* or a 321 bp fragment for *B. pseudomallei* (Yen et al., 2009) The 281 bp product was successfully obtained from the DNA of a Brazilian strain of *B. mallei* isolated from a human patient, similarly, a 321 bp amplicon was generated from the DNA of *B. pseudomallei* isolated from a Brazilian human case of melioidosis (Moriya et al., 2025). We used a set of primers to amplify a 475 bp fragment targeting the *burk*475 gene of the *Rhs* protein of the type IV secretion system corresponding to the species *B. pseudomallei* and *B. mallei* (Fonseca Júnior et al., 2021). We decided to use this primer combination to optimize detection accuracy, minimizing false negatives and improving species differentiation.

All samples tested positive for *B. pseudomallei* using conventional PCR (Fig. 2). In the aforementioned agarose gel image, the expressive amplifications with the formation of well-defined bands corresponding to the clinical isolates from the patients (B03, B04, B06, B07, and B08) are notable, correlated to the target genes (*orf*11–706 bp - *B. pseudomallei*; *bm*17–281 bp - *B. mallei* and 321 bp - *B. pseudomallei*; and *burk*475–475 bp - *B. mallei* and *B. pseudomallei*). Therefore, consistent amplifications obtained from the clinical samples are observed, with the positive and negative controls having their functionalities well preserved during the reactions, ensuring excellent amplification stability. Thus, the combination of the three targets determines a better strategy to allow for robustness in diagnosis, reliability of results, and certainty regarding the differentiation of closely related species.

Amplicon sequencing confirmed the PCR results, demonstrating high identity in all samples when sequence analysis was performed for the three *B. pseudomallei* targets (Table 4). The *orf*11 gene showed identity results ranging from 99.85% (B03 and B08) to 100% (B04, B06, and B07), thus verifying a homogeneity that confirms the identification of the isolates for *B. pseudomallei*, since the *orf*11 gene is a highly conserved marker in this species. A minimal difference of 0.15% was observed between B03 and B08; this is possibly associated with point nucleotide substitutions, which do not have a functional impact, being compatible with the genetic variation that occurs naturally within the species. Regarding the *bm*17 gene (MLVA), there is a high range of variation among the samples, ranging from 98.78% (B06) to 99.15% (B08). Sample B06 shows the lowest identity value for this target, thus possibly resulting in greater divergence regarding the number of repetitions. Regarding the *burk*475 gene, excellent conservation is observed, with 100% identity in samples B04, B06, B07, and B08, and 99.34% for B03.

Table 3
MALDI Biotyper® identification of *B. pseudomallei* isolates from melioidosis cases in the state of Piauí, Brazil (2019–2023).

Strain ID	Replicated number	First Identification	Log Score	Second Identification	Log Score
BP03	1	*B. pseudomallei* A-269 – RKI	2.07	*B. pseudomallei* 0304448 – RKI	2.03
	2	*B. pseudomallei* A-269 – RKI	2.11	*B. pseudomallei* 0304448 – RKI	2.07
	3	*B. pseudomallei* A-269 - RKI	2.05	*B. pseudomallei* 0304448 – RKI	2.04
BP04	1	*B. pseudomallei* PITT 521 – RKI	1.97	*B. pseudomallei* A-269 – RKI	1.96
	2	*B. pseudomallei* A335 – RKI	2.04	*B. pseudomallei* A-269 – RKI	2.01
	3	*B. pseudomallei* 0304448 - RKI	2.11	*B. pseudomallei* A335 – RKI	2.10
BP06	1	*B. pseudomallei* A-269 – RKI	2.09	*B. pseudomallei* A335 – RKI	2.01
	2	*B. pseudomallei* A-269 – RKI	2.10	*B. pseudomallei* A335 – RKI	2.01
	3	*B. pseudomallei* A-269 – RKI	2.19	*B. pseudomallei* A335 – RKI	2.14
BP07	1	*B. pseudomallei* A-269 - RKI	1.65	*B. pseudomallei* A335 – RKI	1.60
	2	*B. pseudomallei* A335 – RKI	1.79	*B. pseudomallei* A-269 – RKI	1.75
	3	*B. pseudomallei* A335 – RKI	1.90	*B. pseudomallei* A-269 – RKI	1.88
BP08	1	*B. pseudomallei* A335 – RKI	1.72	*B. pseudomallei* A-269 – RKI	1.69
	2	*B. pseudomallei* A335 – RKI	1.94	*B. pseudomallei* A-269 – RKI	1.90
	3	*B. pseudomallei* A335 – RKI	1.65	*B. pseudomallei* A-269 – RKI	1.63

3.4. Phylogenetic analysis and MLST analysis

In silico analysis of the five genomes revealed novel sequence types (ST) in these strains. Four strains (BP03, BP04, BP07, and BP08) shared the same allelic profile, comprising an unreported combination of known alleles (*ace1, gltB1, gmhD13, lepA1, lipA1, nark6,* and *ndh1*), corresponding to ST2226. Conversely, BP06, which harbors a novel *lepA* sequence (*ace1, gltB1, gmhD2, lepA127, lipA5, nark1,* and *ndh1*), was assigned to ST2234 and showed a close relationship with ST95, a sequence type previously described in Brazil. Phylogenetic analysis based on concatenated MLST sequences revealed that all isolates from Piauí with the ST2226 clustered into a distinct branch, closely related to two strains from the neighboring state of Ceará (CEMM05–6-101 [ST1458] and CEMM05–5-066 [ST1455]). Contrastingly, BP06 (ST2234) formed a separate and distant branch, clustering with strains from Ceará belonging to ST95 and ST1355 (Fig. 3).

Analysis of 105 *B. pseudomallei* genomes from the Americas using the Thai strain K96243 as the reference outgroup identified 51,823 SNPs. Four strains (BP03, BP04, BP07, and BP08) clustered within a distinct node, distant from the other Brazilian isolates and positioned close to strains isolated from Ceara, including one human and one environmental strain (CEMM05–6-104 and CEMM03–6-039). The fifth strain (CH581-001R0003BP06) did not cluster with any other isolates; however, it was positioned outside the main branch encompassing most Brazilian strains (Fig. 4).

4. Discussion

This study conducted phenotypic and molecular methods of five human strains of melioidosis recently diagnosed and for the first time in Piaui, in which human cases are mostly diagnosed in the neighboring state of Ceará. All isolates were identified as *B. pseudomallei* using MALDI-TOF MS, with scores ranging from 1.65 to 2.19 and scores <2.0,

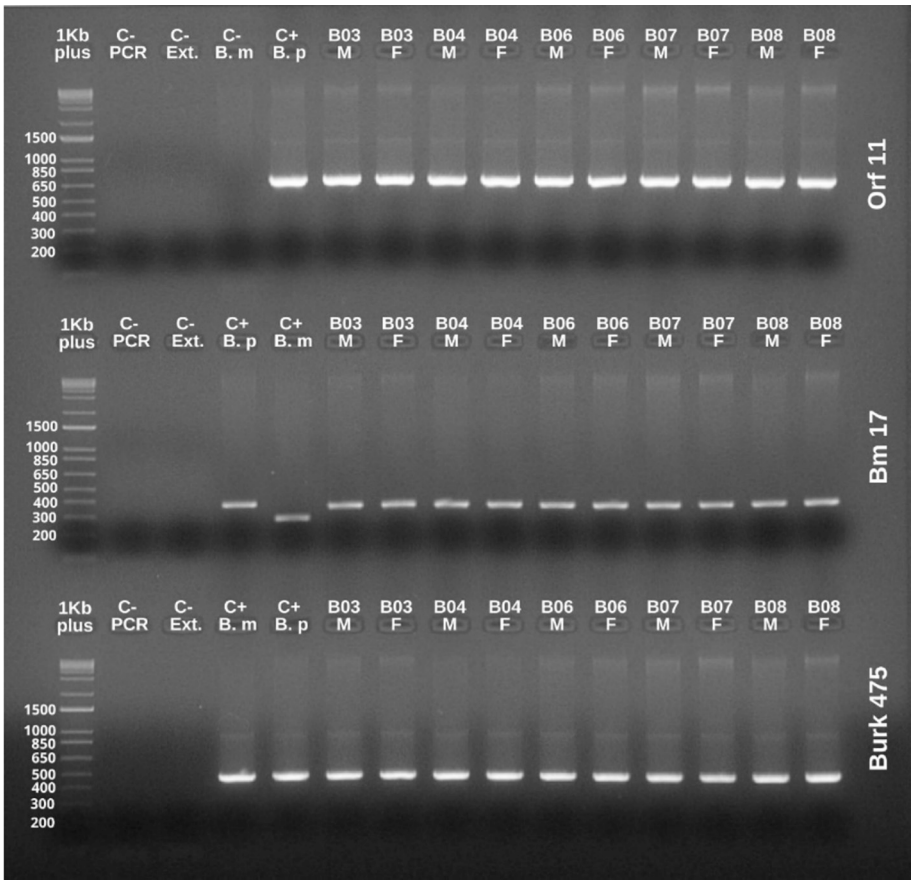

Fig. 2. PCR detection of *Burkholderia pseudomallei* from DNA extracted from clinical isolates (B03, B04, B06, B07, and B08) from melioidosis patients in Piauí, Brazil. Amplicons: *orf11* (706 bp-*B. pseudomallei*), *bm17* (281 bp-*B. mallei* and 321 bp-*B. pseudomallei*), and *burk475* (475 bp-*B. mallei* and *B. pseudomallei*). 1 Kb plus: molecular marker (Thermo Fisher, USA), C- PCR: PCR negative control, C- Ext.: DNA extraction negative control, C- B.m: *B. mallei* negative control, C+ B.m: *B. mallei* positive control, C+ B.p: *B. pseudomallei* positive control.

which are considered less reliable for species-level identification. However, *B. pseudomallei* was consistently ranked as primary or secondary. MALDI-TOF MS can facilitate rapid identification if the databases are expanded to include South American strains.

Previous studies (Li et al., 2019; Gassiep et al., 2019) have demonstrated the effectiveness of MALDI-TOF as a rapid non-sequencing approach for identifying *B. pseudomallei*. However, this species are excluded in the standard databases of widely used systems, such as MALDI Biotyper® (Bruker Daltonik GmbH) and VITEK MS (bioMérieux), leading to misidentification or uclassified results. Several studies have validated the MALDI Biotyper® for *B. pseudomallei* identification (Cox et al., 2014; Suttisunhakul et al., 2017), whereas fewer evaluations exist for VITEK MS. For example, Watthanaworawit et al. (Watthanaworawit et al., 2021) successfully identified 25 isolates in triplicate; 76% were correctly identified in all three replicates, 12% in two replicates and 12% in one replicate. Overall, 95% of spectra showed ≥99.9% identity.

A significant limitation is the scarcity of spectra in Latin America. There is an abundance of spectra in Asia and Oceania, in which melioidosis is endemic. Incorporating spectra from Brazilian strains into reference databases may improve diagnostic accuracy and reliability. Our profiles address this issue by providing data from Latin America.

The conventional PCR-based molecular identification corroborated previous reports with the specific amplification of three *B. pseudomallei*-associated gene targets (*orf11*, *burk17*, *burk475*) (Aung et al., 2023; Yen et al., 2009; Fonseca Júnior et al., 2021). Amplicon sequencing revealed a high degree of identity (98.78–100%) with the GenBank-deposited sequences from *B. pseudomallei*, confirming the identity of the strains and supporting the reliability of the PCR systems used. These results further validate the specificity of the primers and effectiveness of PCR for species identification, which remains the gold standard for laboratory diagnosis of melioidosis in countries with limited infrastructure for bacterial identification (Orababa et al., 2023; Zakharova et al., 2018).

Currently, only a limited number of *B. pseudomallei* strains from the Americas have been typed using MLST (Gee et al., 2021) and deposited in the PubMLST database. Of these, 39 originated from patients in Ceará between 2003 and 2017, whereas the geographic origin was not specified for the other patients. In total, 15 STs have been reported in this region, of which ST92 and ST95 are the most common.

This study identified two novel STs; ST2226 in four patients from Piauí and ST2234 in another patient. Both STs were closely related to STs previously described in Ceará. These findings suggest the existence of at least two circulating lineages in Piauí, which could reflect a common origin or cross-border dissemination from Ceará. The close relationship between ST2226 and the Ceará strains (ST1455 and ST1458) supports this hypothesis, indicating a potential regional spread of the bacteria, a process that remains poorly documented in South America. The discovery of new STs is significant, as it enriches the global MLST database and enhances the molecular surveillance of *B. pseudomallei*. Because of the remarkable genetic diversity of this pathogen, even within restricted geographic areas, such data are essential for a better understanding of its evolutionary dynamics.

Phylogenetic analyses based on MLST and SNPs revealed new information on the genetic diversity of *B. pseudomallei* in Northeastern Brazil. Four analyzed strains (BP03, BP04, BP07, and BP08) clustered together, suggesting a common source of contamination, whereas BP06

Table 4
BLASTn analysis of PCR amplicon sequences for the three targets.

Sample ID	PCR target	Best hit	*E*-value	Identity
B03	*orf11*	*Burkholderia pseudomallei* strain 2,013,833,057 chromosome 2, complete sequence	0.0	99.85%
	bm17 - MLVA marker	*Burkholderia pseudomallei* strain 3,000,047,530 chromosome 2, complete sequence	$8{,}00^{-147}$	98.99%
	burk475 - Type IV secretion protein Rhs	*Burkholderia pseudomallei* strain 2,011,756,296 chromosome 2 sequence	0.0	99.34%
B04	*orf11*	*Burkholderia pseudomallei* strain 2,013,833,057 chromosome 2, complete sequence	0.0	100%
	bm17 - MLVA marker	*Burkholderia pseudomallei* strain 3,000,047,530 chromosome 2, complete sequence	$4{,}00^{-170}$	99.12%
	burk475 - Type IV secretion protein Rhs	*Burkholderia pseudomallei* strain 2,011,756,296 chromosome 2 sequence	0.0	100.00%
B06	*orf11*	*Burkholderia pseudomallei* BPC006 chromosome II, complete sequence	0.0	100.00%
	bm17 - MLVA marker	*Burkholderia pseudomallei* strain VB976100 chromosome 2, complete sequence	$2{,}00^{-162}$	98.78%
	burk475 - Type IV secretion protein Rhs	*Burkholderia pseudomallei* strain 3,000,015,486 chromosome 2, complete sequence	0.0	100.00%
B07	*orf11*	*Burkholderia pseudomallei* 576 chromosome 2, complete sequence	0.0	100.00%
	bm17 - MLVA marker	*Burkholderia pseudomallei* strain 3,000,047,530 chromosome 2, complete sequence	$7{,}00^{-163}$	99.08%
	burk475 - Type IV secretion protein Rhs	*Burkholderia pseudomallei* strain 2,011,756,296 chromosome 2 sequence	0.0	100.00%
B08	*orf11*	*Burkholderia pseudomallei* 576 chromosome 2, complete sequence	0.0	99.85%
	bm17 - MLVA marker	*Burkholderia pseudomallei* strain 3,000,047,530 chromosome 2, complete sequence	$7{,}00^{-178}$	99.15%
	burk475 - Type IV secretion protein Rhs	*Burkholderia pseudomallei* strain 2,011,756,296 chromosome 2 sequence	0.0	100.00%

clustered on a distinct branch. However, no epidemiological links were identified among these cases. According to available clinical and surveillance data, the patients did not share familial relationships, common residences, or known exposure settings. Additionally, no relevant travel history was documented, suggesting a shared source of infection. Therefore, the observed genetic clustering may reflect the regional circulation of related lineages, rather than a point-source outbreak. A documented travel history was identified for patient BP04, who had traveled to Fortaleza (Ceará) for liver transplantation before diagnosis. No epidemiological links were identified between the other patients or between them and the sixth reported case. Although Ceará is recognized as an endemic area for melioidosis in Brazil and geographically adjacent to Piauí, the available data do not support inference of direct transmission events or a common source of infection.

Previous genomic studies have characterized *B. pseudomallei* in Northeastern Brazil and its neighboring countries. Gee et al. (Gee et al., 2021) sequenced 37 isolates from Ceará and Alagoas (2003–2015) and identified 14 STs, including four previously known (ST92, ST95, ST297, and ST1355) and 10 new (ST1454–ST1463). ST95 was the most frequent, and included clinical and environmental isolates from multiple municipalities. SNP analysis revealed that distinct genotypes coexisted, even within a single outbreak, highlighting regional diversity. STs found in Ceará have been reported in Puerto Rico, Mexico, and the USA, indicating their wide distribution in the Western Hemisphere.

Phenotypic (VITEK 2), spectrometric (MALDI-TOF), and molecular (PCR and WGS) methodologies effectively identified and characterized the pathogen, increasing diagnostic efficiency in reference laboratories and highlighting the importance of maintaining laboratory surveillance of its phenotypic and genomic aspects to strengthen the tracking of infection sources and possible routes of dissemination. Therefore, seeking integrated public health actions, laboratory diagnosis, and training of health professionals for early detection and adequate management of the disease. The study findings broaden our understanding of the diversity of *B. pseudomallei* in Brazil and demonstrate the presence of genetically distinct lineages in Piauí.

Ethical aspects

The study was approved by the Research Project Evaluation Committee (CAPP) and Research Ethics Committee (CEP) under CAAE Opinion No. 33127120.0.0000.8050. The study was conducted in accordance with the ethical guidelines. Written informed consent for the use of clinical data and biological materials in further research was obtained from all patients or their legal representatives.

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CRediT authorship contribution statement

Dilbert Silva Velôso: Writing – original draft, Supervision, Methodology, Investigation, Conceptualization. **Flábio Ribeiro de Araújo:** Writing – original draft, Methodology, Formal analysis. **Lenita Ramires dos Santos:** Methodology. **Paula Adas Pereira Suniga:** Methodology. **Jéssica Cristine Kuramoto Moriya:** Methodology. **Newton Valério Verbisck:** Methodology. **Mégane Gasqué:** Methodology, Formal analysis. **Karine Laroucau:** Writing – original draft, Methodology, Formal analysis. **Elba Regina Sampaio Lemos:** Writing – review & editing, Visualization, Validation. **Marco Aurelio Horta:** Writing – review & editing, Visualization, Validation, Supervision, Project administration, Funding acquisition, Conceptualization.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work, no generative AI or AI-assisted technologies were used. All content was drafted, reviewed, and edited solely by the authors, who take full responsibility for the accuracy and integrity of the manuscript.

Declaration of competing interest

All authors declare no conflicts of interest. No financial or personal relationships with other organizations or individuals that could inappropriately influence this work were involved.

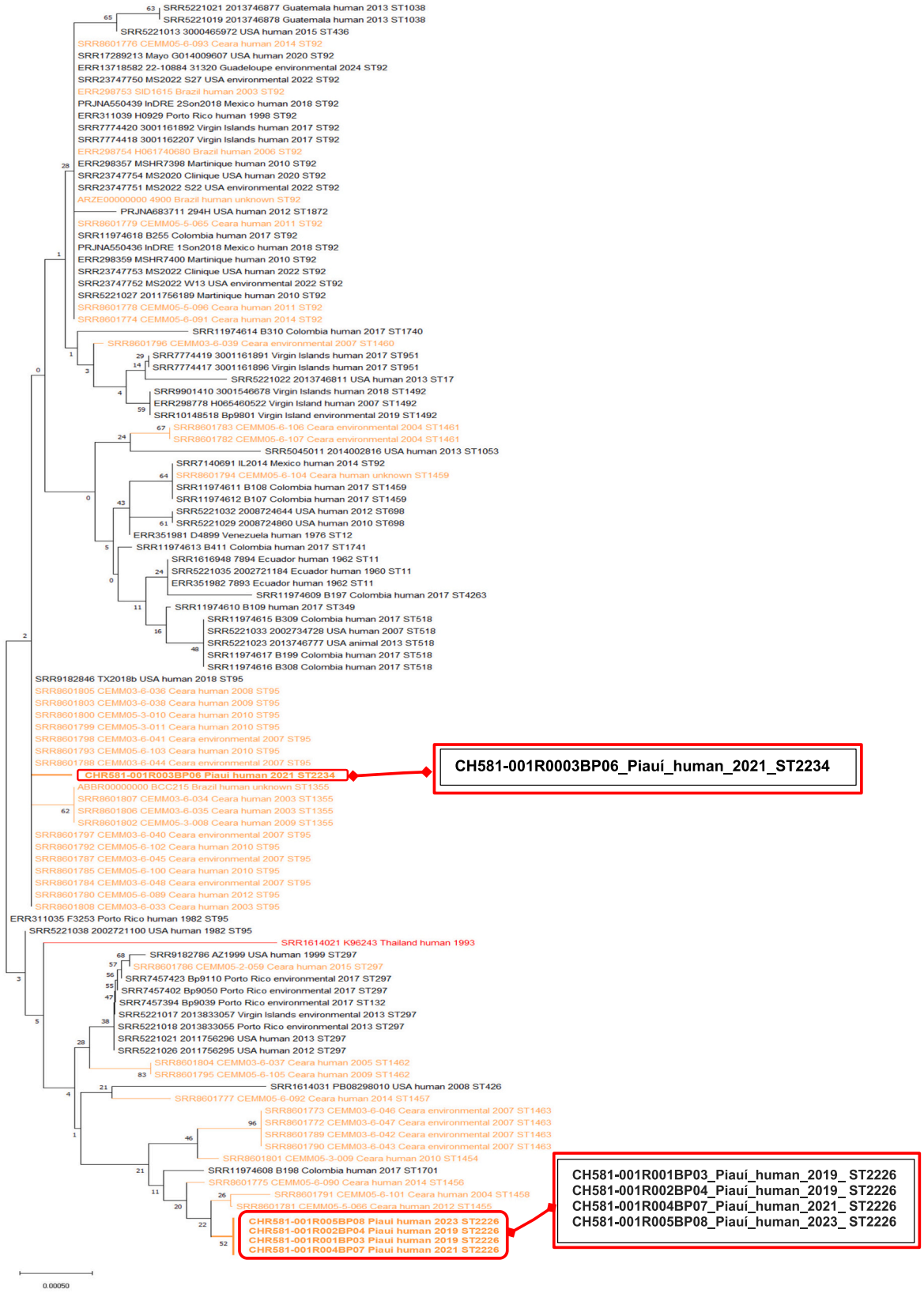

Fig. 3. *Neighbor-joining* MLST-based phylogeny of 104 published *B. pseudomallei* strains from the Americas. Sequences are aligned using MEGA11. One strain (SRR9182786_AZ1999) is excluded because of an incomplete profile (absence of the *ace* gene). Strain identifiers include database accession number, country of diagnosis (which may differ from the contamination site), year of diagnosis, and sequence type.

Fig. 4. *Neighbor-joining* SNP-based phylogeny of 105 published *B. pseudomallei* genomes. Sequences are aligned to the *B. pseudomallei* K96243 reference genome with 90% identity using the BWA algorithm in BioNumerics. The tree is constructed with a minimum coverage of 20×, excluding ambiguous or unreliable bases. Strain identifiers include the database accession number, strain name, country of diagnosis (which may differ from the location of contamination), year of diagnosis, and sequence type. Strains are color-coded by geographic origin: orange for those from Brazil, yellow for other strains from the Americas, and red for the reference strain from Thailand. Strains are indicated by a red arrow. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Data availability

Data will be made available on request.

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