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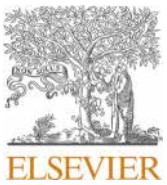

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Rapid confirmation of autochthonous origin in suspected cases of melioidosis from French overseas departments in the Caribbean and the Indian Ocean by PCR-high resolution melting (HRM) analysis

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ABSTRACT

Burkholderia pseudomallei, a soil-borne bacterium that causes melioidosis, endemic in South and Southeast Asia and northern Australia, is now emerging in new regions. Since the 1990s, cases have been reported in French overseas departments, including Martinique and Guadeloupe in the Caribbean, and Reunion Island and Mayotte in the Indian Ocean, suggesting a local presence of the bacterium. Our phylogenetic analysis of 111 *B. pseudomallei* genomes isolated worldwide, including three strains from Martinique, revealed distinct geography-specific clades for Africa, the Americas, Asia and Australasia. Single nucleotide polymorphisms (SNP) that define clade branches in the phylogeny were identified; we selected those specific to three regions relevant to the French overseas departments: the Indian Ocean, the Americas and a unique subset specific to Martinique. Three SNP markers (one per region) were used to develop a PCR-high resolution melting tool to discriminate between local and imported strains in each region. Blind tests on *B. pseudomallei* strains from French patients, from overseas departments and mainland France, were used for validation. Our method accurately predicted the geographic origin of the patient as recorded from the patient travel history and/or from the multilocus sequence typing data. This rapid typing method, which allows timely identification of local cases and targeted public health interventions, is particularly valuable in the French overseas departments where melioidosis is emerging and regulatory constraints limit the handling of *B. pseudomallei*. Although initially tailored to specific regions, this tool can be adapted for use in other areas to support local epidemiological surveillance of melioidosis.

1. Introduction

Melioidosis, caused by the bacterium *Burkholderia pseudomallei*, is a severe and challenging infection with a high mortality rate that affects both humans and animals (Currie, 2022; Meumann et al., 2023). Historically, melioidosis has been considered endemic to south-east Asia and northern Australia, where it is a major cause of sepsis and a leading cause of pneumonia (Currie, 2022; Meumann et al., 2023). However, its endemic range is expanding, with increasing numbers of cases reported in South Asia, Africa, the Americas, and in Australasia (Benoit et al., 2015b; Steinmetz et al., 2018; Warner and Currie, 2018). A 2016 modelling study estimated that there are 165,000 human melioidosis cases annually worldwide, resulting in over 89,000 deaths (Limmathurotsakul et al., 2016). These figures are likely to be underestimated due to limited access to diagnosis and low awareness among clinicians in certain regions. In addition to the 45 countries with documented cases, this study suggests that a further 34 countries, mainly in Africa, may harbour undiagnosed cases (Limmathurotsakul et al., 2016).

The French overseas departments, including French Guiana, Martinique, and Guadeloupe in the Americas, and Reunion Island and Mayotte in the Indian Ocean, are located in areas where melioidosis is emerging (Limmathurotsakul et al., 2016). Between 1993 and 2023, 25 human cases were reported in the French West Indies (Guadeloupe and Martinique), resulting in 12 deaths despite appropriate treatment

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(Meckenstock et al., 2012; Melot et al., 2020; Olive et al., 1995; Pasquier et al., 2019; Pérez et al., 1997; Théodose et al., 1999; unpublished data for five cases). In 2023, *B. pseudomallei* has also been isolated from a soil in Les Saintes, part of the Guadeloupe archipelago, confirming the endemicity of the bacteria (Gasqué et al. 2024). While no human cases have been officially diagnosed in French Guiana, cases have been reported in neighbouring Brazil, particularly in the state of Ceará (Benoit et al., 2015a; Rolim et al., 2018). *B. pseudomallei* has also been isolated from soil in the Brazilian states of Bahia, Ceará and Amapá, the latter bordering French Guiana (Benoit et al., 2015b; Sanchez-Villamil and Torres, 2018). In the islands of the southwestern Indian Ocean, some rare cases have been documented in Reunion Island and Mayotte (both French departments), as well as in Mauritius, the Seychelles, and Madagascar (Biscornet and Bibi, 2013; Borgherini et al., 2015; Issack et al., 2005; Rakotondrasoa et al., 2018; Renou et al., 2020).

The emergence of *B. pseudomallei* in new regions highlights the need to raise awareness of melioidosis as an emerging health risk among clinicians and local populations. As human infection typically occurs through contact with contaminated water or soil (Currie, 2022; Meumann et al., 2023), it is important to be able to distinguish between locally acquired cases (with possible evidence of a local contact with the bacterium) and those imported through international travel.

B. pseudomallei strains possess highly recombinogenic genomes characterised by extensive mutation accumulation and lateral gene transfer (Chapple et al., 2016; Pearson et al., 2009). Comparative genomic analyses have identified geographically specific clades, including the Americas and Africa, highlighting the presence of distinct, locally established populations (Chewapreecha et al., 2017; Hall et al., 2019; Sarovich et al., 2016). Molecular techniques such as multilocus sequence typing (MLST), which analyses the sequences of seven conserved *B. pseudomallei* genes, are useful tools for determining the geographic origin of strains (Aziz et al., 2017). However, MLST has limitations in resolving fine-scale population structures (Sarovich et al., 2016) and sometimes shows poor discrimination due to homoplasy (Aziz et al., 2017). Whole genome analysis provides more comprehensive insights, and single nucleotide polymorphism (SNP) markers allow precise strain differentiation (Pearson et al., 2009).

PCR-HRM (Polymerase Chain Reaction - High Resolution Melting) is a method for identifying sequence variants, including single nucleotide polymorphisms (SNPs). The detection is achieved post-PCR by analysing the melting behaviour of the nucleic acids *via* the detection of the fluorescence signal of the double-stranded DNA intercalating dye and analysing the melting curves of the PCR products, which vary according to the nucleotide composition of the sequence (Reed et al., 2007). It does not require sequencing or the use of sequence-specific probes, making it a cost-effective and rapid approach to detecting genetic variation. For *B. mallei*, a species that diverged from *B. pseudomallei* (Godoy et al., 2003; Losada et al., 2010), a PCR-HRM scheme based on 15 SNPs (that subtypes the species into 3 lineages and 12 branches/sub-branches) has proven highly effective for geographic strain differentiation (Girault et al., 2018), a level of accuracy that MLST cannot achieve.

Our research focused on identifying phylogenetically informative SNPs markers that would allow the specific identification of strains from the Americas and Africa, and more specifically French overseas departments in the Caribbean and in the Indian Ocean. We then developed a PCR-HRM method that was tested and validated through the typing of strains from melioidosis cases diagnosed in metropolitan France in travellers returning from different countries, and in French overseas departments.

2. Materials and methods

2.1. Strains and DNA used in this study

Ten *B. pseudomallei* strains from the Collection of the Institut Pasteur (CIP) (Table 1) were cultured on non-selective agar plates at 37 °C for 48 h. The colonies were then suspended in sterile water, lysed at 100 °C for 10 min, and centrifuged at 13,000 rpm for 10 min. Supernatants from lysates were used for the evaluation of newly designed PCR primers chosen for the amplification of a target SNP fragment (see §2.2 and 2.3).

Table 1
Burkholderia pseudomallei strains (n = 10) from the Collection of the Institut Pasteur (CIP) used to evaluate newly designed PCR primers for SNP determination. The table lists the identifier, year, host, country and continent of isolation of each strain, together with its sequence type (ST) as determined by multilocus sequence typing (MLST). For each ST, the number of strains recorded in the PubMLST database (https://pubmlst.org/organisms/burkholderia-pseudomallei) and their geographical origin (country(ies) and date of isolation) are given. For new STs, information on the closest related STs is included.

Strain identifier	Year of isolation	Host	Country / Continent	ST	Number of strains registered in the PubMLST database for each ST (country, sample type, date)
CIP 52.238	1952	Human	Vietnam/ Asia	ST67	n = 1 (Singapore, human, 1988)
CIP 55.135	1955	Human	Vietnam/ Asia	ST99	n = 14 (Philippines; Thailand; Malaysia; Cambodia)
CIP 59.6	1959	Human	Unknown	ST2191	New ST; closely related to ST1573 (Vietnam, environment, 2016)
CIP 60.67	1953	Human	Vietnam/ Asia	ST288	n = 50 (Cambodia; Malaysia; Thailand; Vietnam)
CIP 60.68	1948	Human	Vietnam/ Asia	ST169	n = 10 (Australia; Cambodia; Malaysia; Thailand; Vietnam)
CIP 62.27	1962	Human	Unknown	ST67	n = 1 (Singapore, human, 1988)
CIP 68.2	1967	Animal	Malaysia/ Asia	ST661*	n = 1 (Malaysia, animal, 1967)
CIP 68.3	1967	Animal	Malaysia/ Asia	ST661*	n = 1 (Malaysia, animal, 1967)
CIP A202	1947	Human	Vietnam/ Asia	ST169	n = 10 (Australia; Cambodia; Malaysia; Thailand; Vietnam)
CIP A203	1947	Human	Vietnam/ Asia	ST56	n = 20 (Bangladesh, Burma, Cambodia, Malaysia, Thailand, Vietnam)

* CIP 68.3 strain is the only record for ST661 in the PubMLST database.

In addition, DNA from 11 *B. pseudomallei* strains isolated from human melioidosis cases diagnosed in France, including French overseas departments, was provided by the French Armed Forces Biomedical Research Institute (IRBA) (strains IRBA_Bpseu_014 to IRBA_Bpseu_024). These DNAs were used to blind test the ability of our newly designed primers (after validation using CIP strains) to predict the geographical origin of contamination in these human cases.

2.2. Whole genome phylogenetic analysis, SNPs selection and PCR primer design

Complete or draft genomes of *B. pseudomallei* strains (n = 111) were obtained from the PAthosystems Resource Integration Center (PATRIC) (https://www.bv-brc.org, accessed 28 August 2021) (Wattam et al., 2014) and the European Nucleotide Archive (ENA) (https://www.ebi.ac.uk/ena/browser/home, accessed 28 August 2021). All genomes from the Americas (n = 47), Africa (n = 15), and Europe (n = 8) were selected, as well as a selection of genomes from Asia (n = 24) and Australasia (n = 17) that are commonly used in *B. pseudomallei* genome studies (Chewapreecha et al., 2017; Sarovich et al., 2016). The selected

B. pseudomallei genome sequences included 88 human strains, 8 animal strains, 11 environmental strains and 4 strains of undetermined origin except for their geographical location (**Supplementary data S1**). Strain K96243 (isolated in 1996 from a diabetic patient with fatal septicemic melioidosis in Thailand; accession no. SRR1614021) served as a reference genome for all the genomic SNP analyses (Johnson et al., 2015), comprising chromosome_1 (4.07 Mb) and chromosome_2 (3.17 Mb).

Whole genome analysis was performed using BioNumerics 7.6.3 (Applied Maths, BioMérieux, Marcy-l'Étoile, France). Reads in FASTQ format (.fq files) were directly imported into BioNumerics, while complete genomes in FASTA format (.fna files) were first artificially fragmented into 250 bp reads using Art-Illumina tools (Huang et al., 2012), with parameters set to 50× coverage. Reads from all genomes were aligned to the *B. pseudomallei* K96243 reference genome using the BWA algorithm in BioNumerics, with an identity threshold of 90 %. SNPs were identified using the BioNumerics wgSNP module and filtered using the following parameters: minimum coverage of 20×, minimum distance between SNPs of 40 bases, and removal of ambiguous and unreliable bases. The resulting SNP matrix was used to construct a neighbour-joining tree with bootstrap resampling ($n = 1000$) then refined through a parsimony analysis using the BioNumerics software.

Phylogenetically informative SNPs, *i.e.*, unique to each group of interest, were identified from the SNP matrix, and selected SNPs met the following criteria: (i) an exact match of the 40 bases flanking the SNP across all genomes in the study, (ii) a purine-to-pyrimidine (A/G to C/T) transition to differentiate strains within the groups of interest, and (iii) a GC content of the 81 bp fragment ≤65 %.

Primers for amplification of SNP-containing fragments were designed either manually or using the software Primer3Plus (Untergasser et al., 2007) (http://www.bioinformatics.nl/primer3plus) or RealTimeDesign (https://www.biosearchtech.com/). Primer design criteria were a length of 18–20 bases, GC content ≤65 % and a hybridization temperature between 55 and 65 °C. If no suitable primers were found within the 81 bp region surrounding the SNP, the search was extended beyond this region under the same criteria that all strains had identical sequences except at the SNP site. Self-hybridized hairpins were checked using the ThermoFisher Multiple Primer Analyzer (https://www.thermofisher.com).

2.3. PCR-HRM analysis

PCR-HRM analysis was performed on a ViiA7™ Real-Time PCR instrument (Life Technologies). Reaction mixtures consisted of 5 µL of DNA, 0.4 µM of each primer, 10 µL of LightCycler® 480 HRM Master Mix (Roche Diagnostics) and 2.5 mM of $MgCl_2$ to a final volume of 20 µL.

PCR thermocycling conditions were as follows: an initial denaturation step at 95 °C for 10 min, followed by 40 cycles consisting of 10 s at 95 °C (denaturation), 10 s at 60 °C (hybridization), and 20 s at 72 °C (elongation). High-resolution melting assays were performed as the final step immediately after the PCR amplification cycles. PCR products were heated to 95 °C for 30 s, cooled to 65 °C for 1 min and then heated from 65 °C to 99.9 °C for 30 s at a rate of 0.025 °C/s with 25 fluorescence acquisitions per °C. The acquired HRM data (melting temperatures (Tm) and melting curves) were analyzed using the QuantStudio Real Time PCR software (version 1.7.2).

For each selected SNP, synthetic DNA oligonucleotides corresponding to the amplified fragment with either the C/G or A/T substitution (Eurofins, Germany) were synthesised and used as controls. Those controls, corresponding to the two possible nucleotides for each SNP, hence two possible melting curves, served as comparison when testing a sample.

2.4. Multilocus sequence typing (MLST) analysis

MLST was performed on all lysates of CIP strains and on all DNA of IRBA clinical strains. The 7 MLST genes (*ace, gltB, gmhD, lepA, lipA, nark, ndh*) were amplified according to the method described by (Godoy et al., 2003). The amplicons were sequenced by Eurofins in Germany. The resulting sequences were submitted to the *B. pseudomallei* PubMLST database for MLST profiling (https://pubmlst.org/, accessed 17 February 2023) (Jolley et al., 2018).

For the *B. pseudomallei* genome sequences obtained from the PATRIC and ENA databases, the 7 MLST genes were extracted *in silico* using the MLST plugin in BioNumerics (version 7.6.3).

3. Results

3.1. Phylogenetic analysis and identification of clusters

Analysis of the 111 selected *B. pseudomallei* genomes identified 61,052 SNPs that met our criteria. Phylogenetic analysis of these SNPs identified several major clades corresponding to different geographical regions. Strains from Africa, the Americas, Asia and Australasia each formed distinct clades named after their respective continents. In particular, Asian strains clustered into 2 separate clades. Similarly, African strains grouped into 2 clades: the Indian Ocean clade, which included strains from Kenya, Madagascar and Mauritius, and the continental Africa clade, which included strains from Burkina Faso, Chad, Gabon, Madagascar and Nigeria (Fig. 1).

Of the 111 genomes included in the phylogeny, 12 were found in geographic clades different from their presumed origin. Notably, eight genomes originating from European patients clustered in Asia ($n = 7$) or the Americas ($n = 1$) clades, which is consistent with the absence of *B. pseudomallei* in Europe (Table 2). An *in silico* MLST analysis assigned these 12 genomes to eight different STs (**Supplementary data S1,** Table 2). The geographic origins attributed to these STs (gathered from records in the PubMLST database) were mostly consistent with the results of our phylogenetic analysis. The seven genomes from Europe that clustered in the Asia clade in our phylogeny were assigned to ST414, ST158 and ST376. In the PubMLST database, these STs are associated with environmental strains from Asia (ST414 and ST158 in Thailand; ST376 in Thailand and Laos), strongly supporting their Asian origin. The eighth genome from Europe, that clustered in the Americas clade in our phylogeny, was assigned to ST436. In the PubMLST database, this ST is associated with clinical strains from Australia (n = 1) and the USA with a travel history to Guatemala and Panama ($n = 2$). Two genomes from American patients, each clustering in one of the Asia clades in our phylogeny, were assigned to ST17 and ST426. These STs are associated with clinical strains from Asia (ST17 in Thailand and Malaysia; ST426 in Malaysia). A genome from a Kenyan patient that clustered in the Asia clade, and a genome from a Californian patient that clustered in the Australasia clade were assigned to ST18 and ST880, respectively. These were the only records for these STs in the PubMLST database.

3.2. Selection of SNPs for geographic differentiation by PCR-HRM analysis

To identify strains from emerging regions in Africa and the Americas, particularly the French overseas departments in the Caribbean and the Indian Ocean, we compared genomic data to find geographically specific SNPs. We focused on strains from Martinique (part of the Americas clade) and the Indian Ocean (part of the Africa clade) (Fig. 1).

We identified a single SNP common to all strains from the Americas and 45 SNPs common to all Martinique strains. We could not find any SNPs common to all African strains nor to all continental African strains, but identified six SNPs common to all the Indian Ocean strains (**Supplementary Data S2**). Due to the criteria we applied to the primer design, particularly in terms of GC content and hybridisation temperatures, only 34 SNPs met our standards: the America (1 SNP), Indian Ocean (2 SNPs) and Martinique (31 SNPs) (**Supplementary Data S2**).

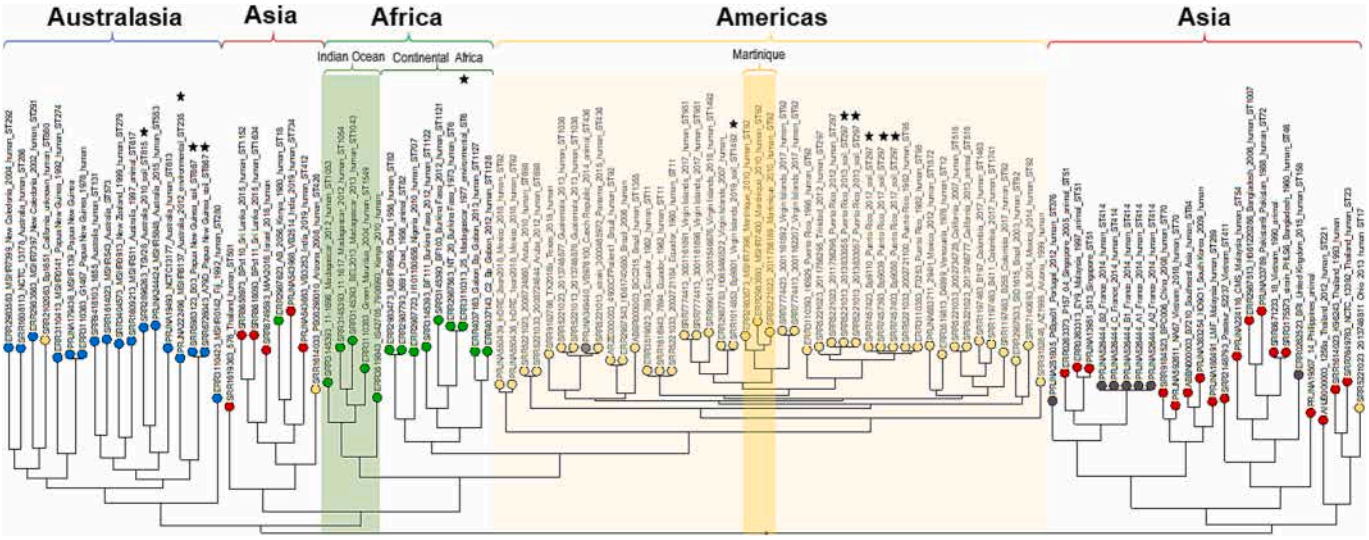

Fig. 1. Neighbour-joiningSNP-based phylogeny of 111 published *B. pseudomallei* genomes. Sequences were aligned to the *B. pseudomallei* K96243 reference genome with 90 % identity using the BWA algorithm implemented in BioNumerics. The tree was generated under the following conditions: 20× coverage, no ambiguous or unreliable bases, and a minimum SNP distance of 40 bp. Strain identifiers include database accession number, country of diagnosis (which may differ from the contamination site), year o f diagnosis and sequence type. Strains are color-coded according to their geographical origin: Australasia (blue), Africa (green), Americas (yellow), Asia (red), and Europe (grey). Environmental strains are indicated by a black star. Groups selected for PCR-HRM development are shaded according to their continent. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 2
List of strains (from 111 genomes analyzed) that showed differences between their country of isolation and the geographical clustering after phylogenetical analysis (as seen in Fig. 1). The sequence type (ST) of each strain was determined by *in silico* MLST based on whole genome data. The table also shows the number of strains (and associated information) for each same ST, as recorded in the *B. pseudomallei* PubMLST database.

Strain identifier	Continent/ country of isolation	Prediction of origin by phylogeny (continent)	ST	Number of strains registered in the PubMLST database for each ST (country, travel history if known)
AB_2056	Africa/ Kenya	Asia	ST18	n = 1 (Kenya*)
PB08298010	Americas/ Arizona	Asia	ST426	n = 2 (USA*, Malaysia)
Bp1651	Americas/ California	Australasia	ST880	n = 1 (USA* with travel history in Australia)
2013746811	Americas/ Ohio	Asia	ST17	n = 7 (USA*, Malaysia/Thailand)
A1 A2 B1 B2 C	Europe/ France	Asia	ST414	n = 20 (France*, Thailand, Singapore, Malaysia)
BRI	Europe/ United Kingdom	Asia	ST158	*n* = 3 (United Kingdom*, Thailand)
PtBps01	Europe/ Portugal	Asia	ST376	*n* = 30 (Portugal*, Thailand, Laos, Malaysia, Australia)
VB976100	Europe/ Czech Republic	Americas	ST436	n = 4 (Czech Republic*, USA with travel history to Guatemala or Panama, Australia)

* An asterisk near a specific country of origin indicates the strains from our list that are recorded in the PubMLST database and their origin.

3.2.1. *Evaluation of selected SNPs on CIP B. pseudomallei strains*

Primers were designed to target SNPs specific to the Americas and Indian Ocean clades and the Martinique subgroup. PCR amplification was first tested using one CIP *B. pseudomallei* strain (CIP 52.238) from Asia. It was then validated against synthetic DNA oligonucleotides before being tested on nine supplementary *B. pseudomallei* strains from the CIP, including seven strains from Asia, and two strains of unknown origin (CIP 59.6 and CIP 62.27) (Table 1). The PCR products showed distinct melting peaks with Tm values specific to each variant group, correlating with purine/pyrimidine transitions (Table 3).

All 10 CIP strains were classified as not belonging to the Americas or Indian Ocean clades, nor to the Martinique subgroup (Fig. 2). These results were consistent with the geographical origins inferred from our MLST data, *i.e.* the origin of strains sharing those same STs (as recorded in the *B. pseudomallei* PubMLST database) originated from Asia or Australasia (Table 1**)**. A new allelic combination was identified for the CIP 59.6 strain (ST2191), which is closely related to ST1573 from an environmental strain in Vietnam.

3.2.2. *Evaluation of the three selected SNPs on clinical strains isolated from French patients (IRBA)*

The three selected SNPs, specific for the Americas clade, its Martinique subgroup and the Indian Ocean clade, were blindly tested by PCR-HRM on DNA from 11 *B. pseudomallei* strains isolated from French patients, including from French overseas departments (Table 4). Six strains isolated from patients in Martinique and Guadeloupe (IRBA_Bpseu_016 to IRBA_Bpseu_021) were classified as belonging to both the Americas clade and the Martinique subgroup. All were ST92, a ST associated with clinical strains from Brazil ($n = 5$), Puerto Rico ($n = 1$), and people who had travelled to Martinique (1 Swiss) and Mexico (2 Americans).

Strain IRBA_Bpseu_014 was classified as belonging to the Indian Ocean clade by PCR-HRM and identified as ST1043, previously found in a Belgian patient who had travelled to Madagascar. Strain IRBA_Bpseu_015 was classified as not belonging to the Americas nor the Indian Ocean clades by PCR-HRM and identified as ST349, previously found in a Spanish patient who had travelled to West Africa and in a patient from Martinique, which may be IRBA_Bpseu_015 strain.

The remaining three strains (IRBA_Bpseu_022 to IRBA_Bpseu_024),

Table 3
Characteristics of the selected SNPs for the Americas clade, Martinique subgroup and Indian Ocean clade, together with the corresponding PCR primers. The SNP positions correspond to their positions in the *B. pseudomallei* K96243 reference genome. Primer sequences, amplicon sizes, target-specific SNPs and non-target SNPs are given. SNP: single nucleotide polymorphism; Tm: melting temperature.

Clade/subgroup	SNP position on reference K96243 strain	Forward primer 5′-3’	Reverse primer 5′-3’	Amplicon size (pb)	Target group		Non-target	
					SNP	Tm (°C)	SNP	Tm (°C)
Americas	1,004,059	AACCCTTGACGAGCGTGAT	CGCAAGGTCGAGAAGATGG	110	A	79.8	G	80.2
Martinique	219,918	GTTCGGCTCGATGGTGAT	CGAGCAGATGGAAGAGCAG	101	C	82.6	T	82.2
Indian Ocean	920,813	GATTTCTGCGACAAGCTCAA	AGAATCCAGCGGATCTTGGT	109	A	81.1	G	81.5

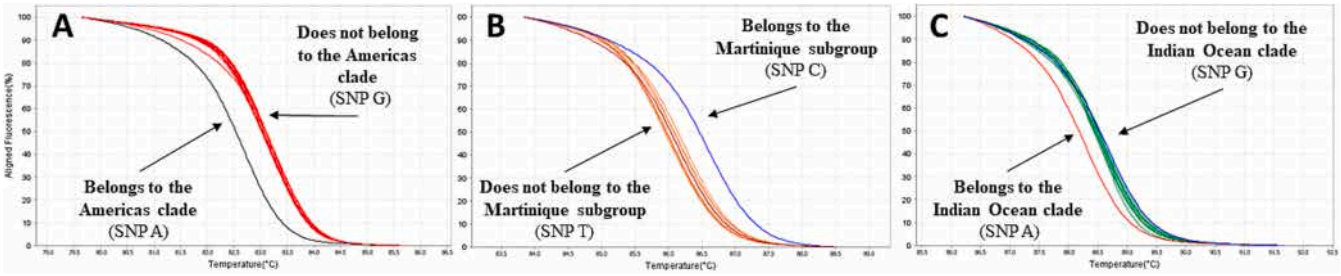

Fig. 2. Normalized melting curves for the (A) Americas clade, (B) Martinique subgroup and (C) Indian Ocean clade. For each marker, the melting curves indicate the geographic origin of the SNP based on the amplified fragment compared to the oligonucleotide controls (target and non-target). Each marker was tested on the 10 *B. pseudomallei* strains from the Collection of the Institut Pasteur (CIP) listed in Table 1. None of these strains originated from the Americas or the Indian Ocean.

Table 4
PCR-HRM and MLST results of 11 *B. pseudomallei* strains isolated from human clinical cases diagnosed in France. DNA samples were provided by the Biomedical Research Institute of the French Armed Forces (IRBA). Three SNP-based markers were tested for the Americas and the Indian Ocean clades, and for the Martinique subgroup (part of the Americas). The “origin group” of these strains, determined by PCR-HRM, and their sequence types (STs), identified by sequencing, are given. In addition, the number of strains with the same or similar STs and related information are provided as recorded in the *B. pseudomallei* PubMLST database (https://pubmlst.org/organisms/burkholderia-pseudomallei).

Strain identifier	Country of travel or residence	Year of isolation	Origin group as determined by PCR-HRM			ST	Number of strains registered in the PubMLST database for each ST (origin, travel history if known)
			Americas	Martinique	Indian Ocean		
IRBA_Bpseu_014	Martinique (residence)	Before 2012	no	no	yes	ST1043	n = 2 (Belgium with travel history to Madagascar)
IRBA_Bpseu_015	Martinique (residence)	Before 2012	no	no	no	ST349	n = 2 (Martinique; Spain with travel history to West Africa)
IRBA_Bpseu_016	Guadeloupe (residence)	Before 2012	yes	yes	no	ST92	*n* = 9 (Switzerland with travel history to Martinique; Puerto Rico; USA-Mexico; Brazil)
IRBA_Bpseu_017	Martinique (residence)	Before 2012	yes	yes	no	ST92	
IRBA_Bpseu_018	Guadeloupe (residence)	Before 2012	yes	yes	no	ST92	
IRBA_Bpseu_019	Martinique (residence)	Before 2012	yes	yes	no	ST92	
IRBA_Bpseu_020	Martinique (residence)	Before 2012	yes	yes	no	ST92	
IRBA_Bpseu_021	Martinique (residence)	2018	yes	yes	no	ST92	
IRBA_Bpseu_022	Malaysia (travel)	2017	no	no	no	ST834	n = 2 (Cambodia; Thailand)
IRBA_Bpseu_023	Laos/Thailand (travel)	2016	no	no	no	ST2210*	29 closely related STs (including environmental strains from Thailand and Australia)
IRBA_Bpseu_024	Ivory Coast (travel)	2017	no	no	no	ST2211*	2 closely related STs: ST1121 (Burkina Faso) and ST1454 (Brazil)

* New ST described in this study.

from patients who had travelled to Asia or Africa, were classified by PCR-HRM as not belonging to the Americas nor the Indian Ocean clades (Table 4). Strain IRBA_Bpseu_022 was assigned to ST834, which was recorded in Asia (Cambodia and Thailand). Strain IRBA_Bpseu_023, from a patient who travelled to Thailand and Laos, had a new ST, ST2210, which is closely related to 29 known STs, including those from environmental strains in Thailand and Australia. Strain IRBA_Bpseu_024, from a patient who travelled to the Ivory Coast, had a new ST, ST2211, which is related to two STs previously identified in Burkina Faso (ST1121) and Brazil (ST1454).

4. Discussion

The emergence of *B. pseudomallei* in new regions and the geographic clustering of isolates (Chewapreecha et al., 2017; Gee et al., 2017; Petras et al., 2023; Sarovich et al., 2016; Savelkoel et al., 2023) inspired us to develop a tool to identify geographically specific SNPs in genomes from Africa and the Americas. Our goal was to confirm the autochthony of

melioidosis cases diagnosed in French overseas departments located in the Indian Ocean (Africa) and the Caribbean (the Americas).

Our comparative analysis of *B. pseudomallei* genomes from several regions, including all available genomes from Africa and the Americas, confirmed that distinct genetic clades correspond to specific geographic origins (Chewapreecha et al., 2017; Hall et al., 2019; Sarovich et al., 2016). In our phylogeny, some genomes clustered within geographic clades that were different from their country of isolation. However, the STs assigned to these genomes had previous records in countries/regions in agreement with our geographic phylogenetic clustering. This discrepancy between the country of isolation and the origin of the STs assigned to the genomes is likely due to bacterial contamination that occurred during international travel to melioidosis endemic areas (Le Tohic et al., 2019; Norman and Chen, 2023). However, patients' travel history is not always available or recorded.

SNP selection for our PCR-HRM tool required targeting SNPs within short, conserved nucleotidic regions across strains. After analysing all available *B. pseudomallei* genomes for the Americas and Africa, we identified only one SNP specific to American strains and none specific to African strains. However, when examining specific subclades within the African clade, SNPs specific to the Indian Ocean clade were identified, but none specific to the continental Africa clade. This may be due to the limited number of African isolates, particularly from the Indian Ocean region, where the few available genomes appear to be relatively conserved. In contrast, although also limited in number, continental African strains appear to be more heterogeneous, probably due to multiple introduction events, as shown in a recent study of nine Nigerian environmental strains (Savelkoel et al., 2023). In a phylogenetic analysis, they all clustered within the continental Africa clade, but did not form a homogeneous group. These findings highlight the need for additional field and clinical studies, increased genotyping efforts, and more accurate mapping of the distribution of environmental *B. pseudomallei* in these emerging regions. Finer-scale studies at local and national levels will be required to identify SNP markers specific to the continental Africa clade.

We developed three SNP markers for the Americas clade, its Martinique subgroup and the Indian Ocean clade. We tested these markers on 11 strains from patients diagnosed in France, including in the French overseas departments. Three strains from patients who had travelled to Asia or Africa were classified by PCR-HRM as not belonging to the Americas and Indian Ocean clades. Six strains isolated from Martinique ($n = 4$) and Guadeloupe ($n = 2$), all ST92, were classified as belonging to the Americas clade and the Martinique subgroup. The identification of the Martinique subgroup in our phylogeny was based on three previously published genomes from Martinique. The ability to identify strains from neighbouring Guadeloupe using the same "Martinique" SNP marker suggests that this marker is suitable for the French Caribbean region. ST92 is not specific to the French Caribbean, having previously been found in South America, suggesting that our SNP marker provides better resolution than MLST. One strain isolated from Martinique was classified by PCR-HRM as belonging to the Indian Ocean clade, consistent with the MLST result (ST1043, previously recorded in two patients who had travelled to Madagascar). Unfortunately, no travel history was available for this patient from Martinique.

In regions where melioidosis is emerging, it is essential to isolate *B. pseudomallei* strains and collect genomic data, especially from autochthonous patients.

Our rapid typing tool, suitable for both DNA and lysates, allows clinicians in the French overseas departments to quickly determine whether a case of melioidosis is due to local contamination without the need for sequencing. The direct application of our markers to clinical samples of *B. pseudomallei* without prior isolation has yet to be validated, but the direct PCR-HRM typing of *B. mallei* from equine samples (DNA extraction from nasal and pus swabs) is encouraging (Koirala and Maharjan, 2022).

This rapid typing tool is particularly critical in light of the current French regulations governing the handling of *B. pseudomallei* strains and DNA. In France, including its overseas departments, such material can only be handled by a limited number of authorised laboratories in mainland France. As a result, hospitals are required to either transfer the strains to these laboratories or destroy them within one month. The high cost of shipping hazardous materials from overseas often leads to the destruction of strains, resulting in the loss of valuable associated data, including genomic and antibiotic susceptibly information. The use of our rapid typing tool now provides an alternative.

Future strains isolated from the Indian Ocean and the Americas will require systematic sequencing and monitoring of our selected SNPs to ensure that they remain geographically specific. Notably, our 'Americas' SNP marker was tested *in silico* against the genomes of environmental strains recently isolated in Mississippi, USA (Petras et al., 2023) and was identified in these genomes (data not shown), confirming that our selected SNP marker is conserved among locally established strains in the Americas.

Rapid strain characterization in emerging regions will improve local risk assessment and support public health investigations. Continued strain isolation and genomic analysis will be essential to study global strain diversity and validate SNP markers, potentially extending SNP tracking to other regions of interest.

CRediT authorship contribution statement

Mégane Gasqué: Validation, Investigation, Formal analysis, Conceptualization, Writing – review & editing, Writing – original draft. **Vanina Guernier-Cambert:** Validation, Supervision, Writing – review & editing. **Guillaume Girault:** Methodology, Writing – review & editing. **Jules Terret:** Investigation, Writing – review & editing. **Fabienne Neulat-Ripoll:** Resources, Writing – review & editing. **Emma Rochelle-Newall:** Validation, Supervision, Writing – review & editing. **Karine Laroucau:** Validation, Supervision, Conceptualization, Writing – review & editing, Writing – original draft.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.meegid.2024.105711.

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