First report of *Burkholderia pseudomallei* ST412 and ST734 clones harbouring *bla*OXA-57 but susceptible to imipenem in India

A. Amladi, N. K. Devanga Ragupathi, K. Vasudevan, M. Venkatesan, S. Anandan and B. Veeraraghavan

Department of Clinical Microbiology, Christian Medical College, Vellore, 632 004, Tamil Nadu, India

Abstract

Melioidosis caused by *Burkholderia pseudomallei* has become an important clinical threat, especially in Northern Australia and Southeast Asia. However, the genome information on this pathogen is limited. *B. pseudomallei* isolates identified from bloodstream infections from inpatients were subjected to whole-genome sequencing by IonTorrent PGM and MinION Oxford Nanopore sequencing technologies. Highly accurate complete genomes of two strains, VB3253 and VB2514, were obtained by a hybrid genome assembly method using both short and long DNA reads. Both isolates carried *bla*PenI and carbapenemase-encoding *bla*OXA-57 genes, although the isolates were susceptible to imipenem by E-test method with MIC 1 μg/mL. Multiple IS family transposases specific for all non-fermenting Gram-negative bacteria (NFGNBs)—especially IS3 and IS5, which facilitate mobilization of extended-spectrum β-lactamase (ESBL) and carbapenemase genes—were carried in these genomes. This further adds to the complexity of gene transmission. These IS families were identified only upon hybrid genome assembly and would otherwise be missed.

Keywords: *blaOXA-57*, *blapenI*, *Burkholderia pseudomallei*, imipenem, phylogeny, SNPs

Original Submission: 4 June 2019; **Revised Submission:** 3 October 2019; **Accepted:** 7 October 2019

Article published online: 15 October 2019

Corresponding author: V. Balaji.
E-mail: vbalaji@cmcvellore.ac.in

Introduction

Burkholderia pseudomallei is an important clinical pathogen which causes melioidosis. The disease has a broad spectrum of clinical presentations, ranging from a mild subclinical infection to severe septicaemic shock. The commonly recognized risk factors for melioidosis include diabetes mellitus (type 2), male gender, occupational exposure to soil and water, and renal impairment, among others [1]. Confirmed cases of *B. pseudomallei* infection in patients require a minimum of 6 months of antibiotic treatment and life-long follow-up [1,2].

Northern Australia and north-eastern Thailand are the hotspots; Southeast Asia has high mortality and relapse. The known endemic areas in Southeast Asia include the Indian subcontinent, southern China, Hong Kong, Malaysia, Cambodia and Taiwan, while the highest documented infection rate (20%) was observed in north-eastern Thailand [3]. Mortality rates for melioidosis range widely from 10% to 50% of infected individuals and with a recurrence of one in 16 patients [4].

The genome composition of *B. pseudomallei* is complex, with two different chromosomes of high GC content (about 68%), which makes it challenging to sequence this highly dynamic genome. It has about 2590 core genes in common with other those of other members of the *Burkholderia* genus [5,6]. Genome information on Indian *B. pseudomallei* strains is limited. The aim of the present study is to investigate the complete genome information of clinical *B. pseudomallei* isolates from hospitalized patients using hybrid assembly with IonTorrent and MinION sequencing platforms.

Methods

Strain isolation and characterization

Bacterial strains VB3253 and VB2514 were isolated from two patients diagnosed with septicaemic melioidosis. Blood samples were collected before the administration of antibiotics. As per institution protocol, blood samples for both cases were collected and sent to the microbiology laboratory in BacT/Alert bottles (Biomerieux, France). The bottles were loaded into the BacT/Alert modules. The blood culture VB3253 flagged positive in 1.05 days while VB2514 flagged positive in 2.28 days after loading. As per standard laboratory protocol, smears were made from the bottles which flagged positive. In both cases the smears showed Gram-negative bacilli. The blood culture broth was then subcultured onto sheep-blood agar and MacConkey agar. Appropriate biochemicals were also set up [7]. The plates showed growth of non-haemolytic grey colonies at 24 h on

https://doi.org/10.1016/j.nmni.2019.100613

sheep blood agar, which turned hazy β-haemolytic by 48 h. On MacConkey agar fine pale colonies formed at 24 h, and the colonies turned pink (lactose fermenting) by 48 h. The colonies were oxidase-positive and showed a 4 + reaction on agglutination with *B. pseudomallei*-specific antiserum raised in rabbits [8]. The preliminary screening media and other biochemicals confirmed the organism to be *B. pseudomallei*. A fresh subculture of both strains was then used for the molecular work-up.

Antimicrobial susceptibility testing was carried out using commercially available E-tests (ceftazidime, imipenem and trimethoprim–sulfamethoxazole, Biomerieux, France) and the Clinical and Laboratory Standard Institute (CLSI) guidelines were used for interpretation of the MIC values obtained [9].

Genome sequencing

Genome sequencing and assembly. B. pseudomallei genomic DNA was extracted using the QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany). Whole-genome sequencing (WGS) was performed in an IonTorrent™ Personal Genome Machine™ (PGM) (Life Technologies, Carlsbad, CA, USA) with 400-bp read chemistry as per manufacturer's instructions. *De novo* assembly was performed using raw reads with an Assembler SPAdes v.5.0.0.0 embedded in a Torrent Suite Server v.5.0.3.

Long-read sequencing was performed in a MinION Oxford nanopore sequencer as per manufacturer's instructions (Oxford Nanopore Technologies, Oxford, UK) with a 1D sequencing method in a FLO-MIN106 R9 flow cell. The Fast5 files were base-called with Albacore 2.0.1 (https://nanoporetech.com/about-us/news/new-basecaller-now-performs-raw-basecalling-improved-sequencing-accuracy). Canu 1.7 [10] was employed for error correction and genome assembly. Nanopolish 0.10.1 was used for polishing the contigs after *de novo* assembly (https://github.com/jts/nanopolish).

Hybrid assembly using IonTorrent and MinION reads. Hybrid assembly using both IonTorrent and MinION reads was performed to decrease the number of indels due to long-read sequencing. Unicycler (v0.4.6) was employed to overlay the accurate short-read sequences over the long reads to achieve complete genomes [11]. Contigs obtained were polished with multiple rounds of Pilon [12] to reduce the base-level errors.

Genome annotation and multilocus sequence typing (MLST) analysis

Genomes were annotated using PATRIC, the bacterial bioinformatics database and analysis resource (http://www.patricbrc.org) and NCBI Prokaryotic Genomes Automatic Annotation Pipeline (PGAAP, http://www.ncbi.nlm.nih.gov/genomes/static/Pipeline.html). Sequence types were analysed using MLST 1.8 (https://cge.cbs.dtu.dk//services/MLST/).

Phylogenomic analysis

Complete genomes of global *B. pseudomallei* isolates were downloaded from the NCBI public database. *B. pseudomallei* genome contigs were mapped to the reference genome K96243 (Acc. No. BX571965-BX571966) using Snippy v4.3.5 (https://github.com/tseemann/snippy). Single-nucleotide polymorphisms (SNPs) were called using default parameters. The core-genome SNPs obtained were aligned for all isolates to infer a phylogeny.

The phylogenetic tree was constructed by the maximum-likelihood method using FastTree v2.1.10 [13]. FastTree was run using the generalized time-reversible (GTR) model of nucleotide evolution and incorporated the CAT approximation for identifying the evolutionary rate. Bootstrapping was performed by feeding 1000 resampled alignments generated in SEQBOOT v3.69 (http://evolution.genetics.washington.edu/phylip/doc/seqboot.html) into FastTree using the -n option.

Results

Antimicrobial susceptibility testing

Both isolates were found to be susceptible to ceftazidime, imipenem and trimethoprim–sulfamethoxazole by MIC; VB3253: 1 μg/mL, 0.25 μg/mL and 0.125 μg/mL, and VB2514: 1 μg/mL, 1 μg/mL and 0.19 μg/mL, respectively.

Treatment and outcome

The patient infected with VB3253 was treated with meropenem intravenously (1 g over 3 h in 100 mL saline) for 4 weeks along with a trimethoprim–sulfamethoxazole tablet (160:800 mg). The patient with VB2514 was treated with meropenem 2 g intravenously and trimethoprim–sulfamethoxazole (160:800 mg) for 28 days. Both patients responded positively to the antibiotic administration and were discharged after recovery.

Genome length, CDS and ST types

The VB3253 and VB2514 genomes were obtained with 30x and 40x coverage, respectively. Each genome had two chromosomes. VB3253 had a genome size of 4 017 865 bp and 3 162 636 bp, while VB2514 had chromosomes of 3 987 244 bp and 3 116 035 bp. MLST 1.8 revealed the ST of two pathogens to be ST412 and ST734 for VB3253 and VB2514, respectively. Both the genomes VB3253 and VB2514 were submitted to GenBank under the accession numbers CP040531-CP040532 and CP040551-CP040552, respectively.

Antimicrobial resistance (AMR) genes

Both the isolates had *bla*OXA-57 and *bla*PenI β-lactamases with multiple IS family transposases (Fig. 1). Though the isolates

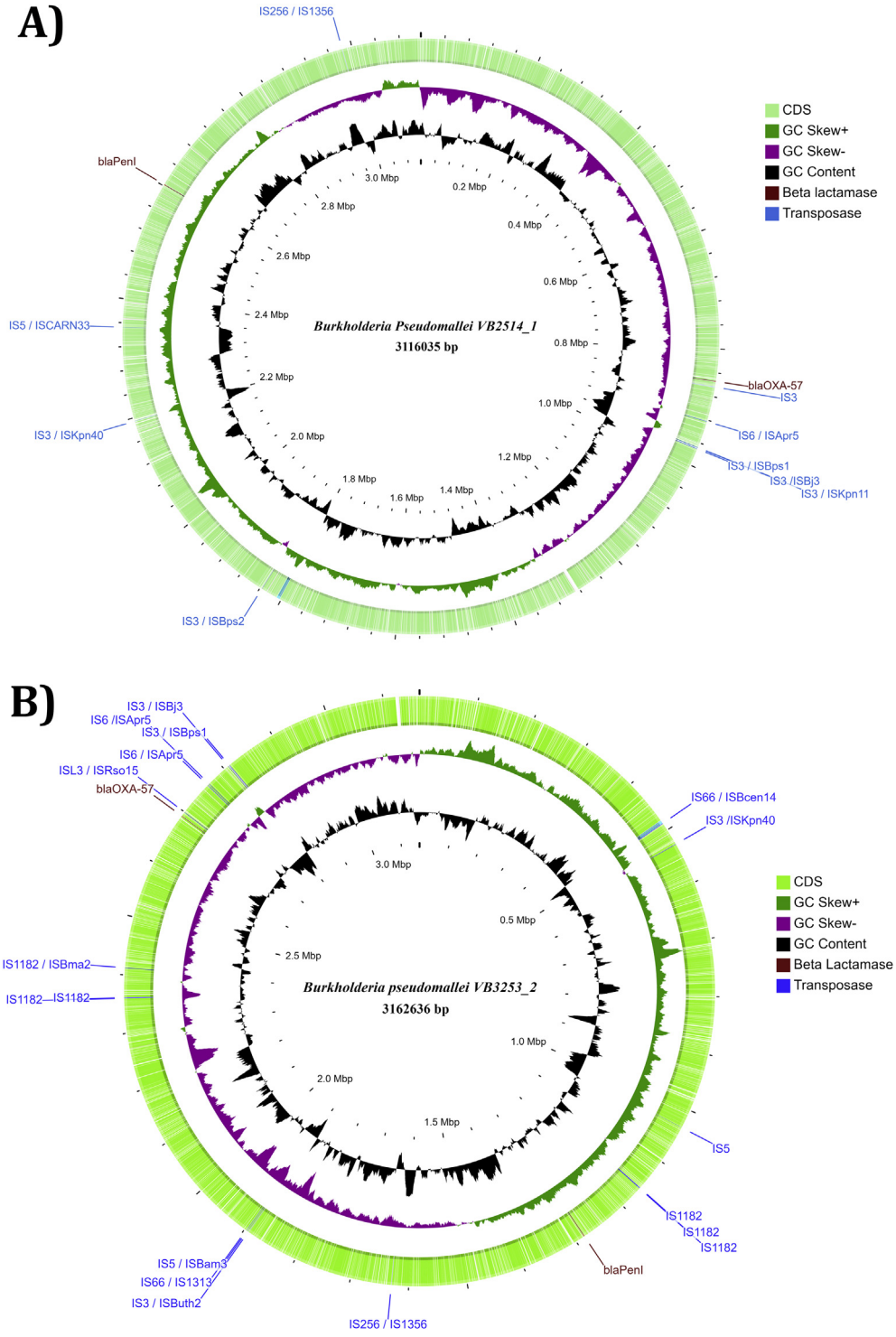

FIG. 1. The β-lactamase genes *bla*OXA-57 (responsible for carbapenemase) and *bla*PenI (responsible for penicillin resistance) from VB2514 (A) and VB3253 (B), along with multiple family transposases.

belong to different sequence types, the genetic arrangements of the genomes remain almost same.

Phylogenomic relationship between clinical strains and global isolates

Core-genome-based phylogenetic analysis of the clinical strains and the global isolates revealed similarity between Indian and Sri Lankan strains (Fig. 2). The next closely related strains were from Taiwan, Malaysia, Australia and the U.S. population.

Discussion

Melioidosis has emerged as an important cause of mortality in the last 3 decades, especially in Southeast Asia and Northern Australia [14,15]. Timely diagnosis of melioidosis is critical to avoid fatalities. Identification based on clinical presentation is often challenging, and the disease is popularly known as the 'great imitator' [4,8]. Rapid molecular techniques are crucial for accurate and quick identification of *Burkholderia* spp [16]. Accordingly, isolates were subjected to whole-genome sequencing to understand their genetic background. Both the isolates from this study were carrying *bla*OXA-57 gene, class D β-lactamase. Interestingly, a study reported that >90% of *bla*OXA-57-carrying *B. pseudomallei* isolates were phenotypically susceptible to imipenem [17]. This is in line with the observation of this study, as the isolates were susceptible to imipenem with MIC <1 μg/mL. The isolates were also susceptible to ceftazidime (1 μg/mL) and trimethoprim–sulfamethoxazole (0.125 μg/mL and 0.19 μg/mL for VB3253 and VB2514 isolates, respectively).

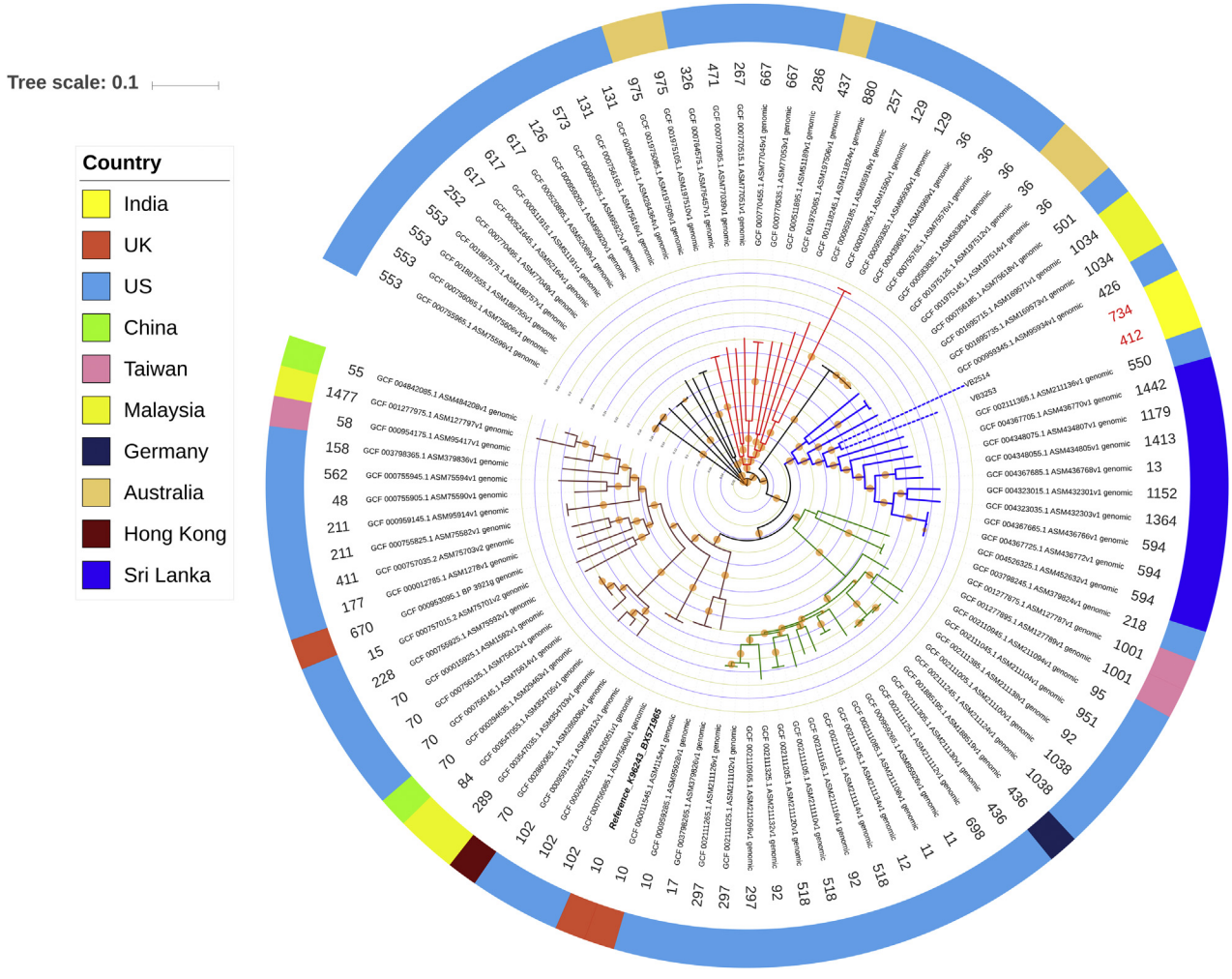

FIG. 2. Phylogenomic relationships among clinical *Burkholderia pseudomallei* strains based on core-genome-based single-nucleotide polymorphisms (SNPs) revealing the evolution of Indian strains in comparison to the global microbial population. The outer ring represents the country of isolation and the inner ring represents the sequence types. Indian strains VB3253 and VB2514 group with the Sri Lankan clade. Phylogeny was calculated using FastTree 2.1.10 with the maximum-likelihood method, and metadata were mapped using iTOL v4.

The *B. pseudomallei* genome is usually made up of two chromosomes of 4 Mb and 3.17 Mb. Of these, the larger one carries core genes and the smaller one carries accessory genes, including AMR genes [18]. Genomes of *B. pseudomallei* are known for their high rate of evolution and diverse clonality due to large gene acquisition rates [19,20]. Previous studies from India have reported the globally prevalent STs from Singapore (ST51), China (ST51, ST1099), Thailand (ST51, ST99, ST375, ST228, ST300), Malaysia (ST51, ST99), Burma (ST51), Bangladesh (ST56), Cambodia (ST56), Vietnam (ST56), Philippines (ST99) and Sri Lanka (ST1364), along with multiple novel sequence types [20].

Although multiple STs have previously been reported from India, as evident from the PubMLST database, this is the first report of both ST412 and ST734 from India. ST734 was known as an endemic clone in Australia, reported consistently between 1999 and 2018 from both human and environmental sources. ST412 had previously been reported only once in Thailand in 1966 from the environment (PubMLST). The ST734 and ST412 strains appeared to be tri-loci variants, with allele numbers *gltB* 2, 4, *gmhD* 3, 10, and *lepA* 2, 1, respectively.

The clonality comparison based on core-genome SNPs showed that Indian isolates were similar to Sri Lankan isolates. The near-neighbour clones were from Taiwan, Malaysia and Australia, including U.S. travel isolates. However, the sequence types varied between the clones, indicating rapid evolution and genome plasticity. Genomic information from this study concurs with that from a previous study which showed variation among *B. pseudomallei* isolates in both systemic and localized infections as a possible evolution over a short period of time [20]. Results from this study necessitate thorough surveillance to understand the rapidly evolving genetic characters of *B. pseudomallei*.

Conclusion

This study reveals the presence of *bla*OXA-57-carrying *B. pseudomallei* genomes. Their comparison with the global isolates reveals that the clones are similar to Sri Lankan clones. There is a threat of acquisition of AMR and other genes for fitness as well as spreading of the resistance gene to other NFGNBs due the high plasticity of the genome. Multiple IS family transposases specific for all NFGNBs, especially IS3 and IS5 (which facilitates mobilization of ESBL and carbapenemase genes) were carried in these genomes. This further adds to the complexity of gene transmission. These IS families were identified only upon hybrid genome assembly, and would otherwise have been missed. Findings from this study necessitate continuous monitoring to track AMR gene acquisition and spread among hospital-acquired infections.

Transparency declaration

The authors declare that they have no conflict of interests.

References

[1] Cheng AC, Currie BJ. Melioidosis: epidemiology, pathophysiology, and management. Clin Microbiol Rev 2005;18(2):383–416.
[2] Dance D. Treatment and prophylaxis of melioidosis. Int J Antimicrob Agents 2014;43(4):310–8.
[3] Currie BJ, Dance DA, Cheng AC. The global distribution of *Burkholderia pseudomallei* and melioidosis: an update. Trans R Soc Trop Med Hyg 2008;102(Suppl. 1):S1–4.
[4] Wiersinga WJ, Currie BJ, Peacock SJ. Melioidosis. N Engl J Med 2012;367(11):1035–44.
[5] Tumapa S, Holden MTG, Vesaratchavest M, Wuthiekanun V, Limmathurotsakul D, Chierakul W, et al. *Burkholderia pseudomallei* genome plasticity associated with genomic island variation. BMC Genomics 2008;9:190.
[6] Sim SH, Yu Y, Lin CH, Karuturi RK, Wuthiekanun V, Tuanyok A, et al. The core and accessory genomes of *Burkholderia pseudomallei*: implications for human melioidosis. PLoS Pathog 2008;4(10):e1000178.
[7] Abbott SL, Cheung WKW, Janda JM. The genus *Aeromonas*: biochemical characteristics, atypical reactions, and phenotypic identification schemes. J Clin Microbiol 2003;41:2348–57.
[8] Jesudason MV, Balaji V, Sirisinha S, Sridharan G. Rapid identification of *Burkholderia pseudomallei* in blood culture supernatants by a coagglutination assay. Clin Microbiol Inf 2005;11(11):930–3.
[9] CLSI. Performance standards for antimicrobial susceptibility testing. 29th ed. Wayne, PA: Clinical and Laboratory Standards Institute; 2019. CLSI supplement M100.
[10] Koren S, Walenz BP, Berlin K, Miller JR, Bergman NH, Phillippy AM. Canu: scalable and accurate long-read assembly via adaptive k-mer weighting and repeat separation. Genome Res 2017;27(5):722–36.
[11] Wick RR, Judd LM, Gorrie CL, Holt KE. Unicycler: resolving bacterial genome assemblies from short and long sequencing reads. PLoS Comput Biol 2017;13:e1005595.
[12] Walker BJ, Abeel T, Shea T, Priest M, Abouelliel A, Sakthikumar S, et al. Pilon: an integrated tool for comprehensive microbial variant detection and genome assembly improvement. PLoS One 2014;9:e112963.
[13] Price MN, Dehal PS, Arkin AP. FastTree 2–approximately maximum-likelihood trees for large alignments. PLoS One 2010;5(3):e9490.
[14] Limmathurotsakul D, Peacock SJ. Melioidosis: a clinical overview. Br Med Bull 2011;99:125–39.
[15] Gopalakrishnan R, Sureshkumar D, Thirunarayan MA, Ramasubramanian V. Melioidosis: an emerging infection in India. J Assoc Phys India 2013;61:612–4.
[16] Naveen Kumar DR, Veeraraghavan B. Accurate identification and epidemiological characterization of *Burkholderia cepacia* complex: an update. Ann Clin Microbiol Antimicrob 2019;18(1):7.
[17] Panya M, Thirat S, Wanram S, Panomket P, Nilsakul J. Prevalence of blaPenA and blaOXA in *Burkholderia pseudomallei* Isolated from patients at Sappasitthiprasong Hospital and their susceptibility to ceftazidime and carbapenems. J Med Assoc Thai 2016;99:12.

[18] Holden MT, Titball RW, Peacock SJ, Cerdeno-Tarraga AM, Atkins T, Crossman LC, et al. Genomic plasticity of the causative agent of melioidosis, *Burkholderia pseudomallei*. Proc Natl Acad Sci USA 2004;101:14240–5.

[19] Price EP, Sarovich DS, Mayo M, Tuanyok A, Drees KP, Kaestli M, et al. Within-host evolution of *Burkholderia pseudomallei* over a twelve-year chronic carriage infection. mBio 2013;4. pii: e00388-13.

[20] Balaji V, Perumalla S, Perumal R, Inbanathan FY, Sekar SK, Paul MM, et al. Multi locus sequence typing of *Burkholderia pseudomallei* isolates from India unveils molecular diversity and confers regional association in Southeast Asia. PLoS Negl Trop Dis 2018;12(6): e0006558.