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PII: S2213-7165(25)00157-2
DOI: <https://doi.org/10.1016/j.jgar.2025.07.002>
Reference: JGAR 2611

To appear in: *Journal of Global Antimicrobial Resistance*

Received date: 19 February 2025
Revised date: 30 May 2025
Accepted date: 2 July 2025

Please cite this article as: Lisa GÖPEL , TRUONG Nhat My , Kaan KOCER , Thi Anh Mai PHAM , Le Thi Kieu LINH , Bui Tien SY , Leo HUBER , Tran Thanh TUNG , NGUYEN Trong The , LE Huu Song , Sébastien BOUTIN , Thirumalaisamy P. VELAVAN , Dennis NURJADI , Genomic characterisation of carbapenem-resistant *Pseudomonas aeruginosa* from ICU admission screening in Hanoi, Vietnam, 2023., *Journal of Global Antimicrobial Resistance* (2025), doi: <https://doi.org/10.1016/j.jgar.2025.07.002>

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Highlights:

- High prevalence of metallo-beta-lactamase producers of carbapenem-resistant *P. aeruginosa*, mainly NDM-1
- High prevalence of resistance towards cefiderocol
- Predominance of ST₃₀₈ carbapenem-resistant *P. aeruginosa* in Northern Vietnam

Genomic characterisation of carbapenem-resistant *Pseudomonas aeruginosa* from ICU admission screening in Hanoi, Vietnam, 2023.

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Keywords: carbapenem-resistant *Pseudomonas aeruginosa*, Vietnam, admission screening, genomic investigation, metallo- β -lactamase, NDM

Abstract (250 words)

Objectives: Vietnam is among the countries most affected by antimicrobial resistance (AMR) in the Asia-Pacific. While multidrug-resistant (MDR) Enterobacterales have been extensively studied, genomic data on MDR *Pseudomonas aeruginosa* in Vietnam remains scarce. To address this, we characterized 20 carbapenem-resistant *P. aeruginosa* (CRPA) isolates from rectal colonization of ICU patients.

Methods: Screening for CRPA was conducted using a selective chromogenic medium (mSuperCARBA). Species identification was achieved through MALDI-TOF mass spectrometry, while antimicrobial susceptibility testing (AST) was performed using the Vitek®2 system and broth microdilution. Whole-genome sequencing (WGS) was performed using the Illumina NextSeq platform.

Results: Twenty CRPA isolates were collected from rectal swabs of 691 patients admitted to the ICUs of the 108 Military Central Hospital in Hanoi, Vietnam, between July 1, 2023, and October 31, 2023. The predominant multilocus sequence type (MLST) was ST308, accounting for 50% (10/20) of the isolates. Notably, 70% (14/20) of the CRPA isolates harboured genes encoding metallo- β -lactamases (MBL), with *bla*_{NDM-1} being the most prevalent (86%, 12/14), followed by *bla*_{IMP-26} (14%, 2/14). Low susceptibility was observed for ceftazidime-avibactam (15%, 3/20) and ceftolozane-tazobactam (10%, 2/20), while cefiderocol resistance was observed in 50% (10/20) of isolates. Colistin demonstrated the most favourable susceptibility profile, with 90% (18/20) of isolates remaining susceptible.

Conclusion: A significant proportion of CRPA isolates in our study were MBL producers, with high levels of resistance to novel β -lactams and β -lactam/ β -lactamase inhibitor combinations. These findings underscore the urgent need for effective infection prevention and control strategies to mitigate the further spread of MBL-producing CRPA.

Background

The emergence of antimicrobial resistance (AMR) poses significant challenges to global health, particularly in low- and middle-income countries. Resistance to third-generation cephalosporins and carbapenems in Gram-negative bacilli, including *Pseudomonas aeruginosa*, has prompted the WHO to classify these pathogens as critical priority targets for research and development. [1]

P. aeruginosa, a major cause of healthcare-associated infections, exhibits intrinsic resistance mechanisms and the ability to acquire additional resistance determinants, such as carbapenem resistance, further restricting treatment options. Vietnam is among the countries most severely affected by AMR in the Asia-Pacific region. While genomic studies on multidrug-resistant organisms in Vietnam have largely focused on *Klebsiella pneumoniae* and *Escherichia coli*, *P. aeruginosa* has received comparatively less attention, despite its clinical significance. In a pilot study on carbapenem-resistant Gram-negative bacteria conducted from 1st July to 30th October 2023, we found that 3% (21/691) of patients admitted to the ICU were colonized with carbapenem-resistant *P. aeruginosa* (CRPA). It should be noted that 76% (16/21) of these isolates were found to be carbapenemase producers, which is a surprisingly high percentage given that carbapenem resistance in *P. aeruginosa* is usually mediated by non-carbapenemase mechanisms.[2]

Colonization by *P. aeruginosa*, both perianal and in the respiratory, has been reported to be a risk factor for acquiring *P. aeruginosa* ICU pneumonia with a hazard ratio of 4.4; 95% CI, 1.7–11.6.[3] These infections are associated with substantial morbidity, mortality, and increased healthcare costs, compounded by limited treatment options due to AMR. Given that rectal colonization with *P. aeruginosa* may enhance the risk of acquiring subsequent infections during hospitalization, it is crucial to characterize CRPA strains to understand their molecular characteristics.

To address this, we conducted whole-genome sequencing (WGS) and extended antimicrobial susceptibility testing (AST) on CRPA isolates identified in our previous study. These comprehensive analyses aim to elucidate the molecular epidemiology of CRPA in Vietnam, providing valuable insights that can inform strategies to prevent infections, optimize treatment, and ultimately improve patient outcomes.

Methodology

Collection of carbapenem-resistant P. aeruginosa isolates

In a prospective cohort study, we conducted admission screening for patients admitted to the ICUs between 1st July to 30th October 2023 at the infectious diseases department of the 108 Military Central Hospital in Hanoi, Vietnam. The inclusion criteria were as follows: admission to the ICU, informed consent, age over 18 years, and screening for multidrug-resistant Gram-negative bacteria (MDRGN) within 48 hours post admission. A total of 691 patients were included in the study.

Microbiological procedure

Rectal swabs were collected using eSwabs (Copan, Italy) for carbapenem-resistant Gram-negative bacteria screening in the microbiological laboratory. Briefly, 10 µl of the Amies medium was inoculated on CHROMagar mSuperCARBA plates and incubated overnight to select for carbapenem-resistant Gram-negative bacilli. Plates were inspected for microbial growth, and colonies growing on the selective medium were identified using mass spectrometry MALDI-TOF (Vitek MS, BioMérieux) in the microbiological laboratory in Vietnam. AST was performed using the Micronaut AST panel for MDRGN (Bruker, Germany). Antibiotics included in the test panel were amikacin, cefepime, ceftazidime, ceftazidime-avibactam, ceftolozane-tazobactam, ciprofloxacin, colistin, imipenem, levofloxacin, meropenem, piperacillin, and piperacillin-tazobactam. *E. coli* ATCC®25922 was used as a quality control strain. AST for aztreonam/avibactam was performed using the MIC gradient strip method (MAST diagnostics, Germany). Cefiderocol AST was performed using the reference method with iron-depleted cation-adjusted Mueller-Hinton broth (CAMHB), as published.[4] To determine the contribution of the metallo-β-lactamase production on the cefiderocol susceptibility, the AST was repeated using iron-depleted CAMHB supplemented with 100 mg/L pyridine-2,6-dicarboxylic acid (DPA). *P. aeruginosa* ATCC®27853 was used as the quality control strain for cefiderocol testing. The antibiotic susceptibility was interpreted using the EUCAST clinical breakpoints version 14.0. In accordance with the definitions published by Magiorakos *et al.* in 2012, multidrug resistance (MDR) was defined as nonsusceptibility to one or more antimicrobial agents in at least three antimicrobial categories. Extensively drug-resistant (XDR) bacteria were defined as exhibiting susceptibility to ≤ 2 antimicrobial categories.[5] Cefepime-taniborbactam resistance was tested using the broth microdilution method with CAMHB as 2-fold dilutions of cefepime (MedchemExpress, USA) (range 0.25-128 mg/L) in combination with taniborbactam fixed at 4 mg/L (MedchemExpress, USA). Results were interpreted according to the EUCAST breakpoints for cefepime (≤ 8 mg/L: intermediate; > 8 mg/L: resistant).

Whole genome sequencing and bioinformatics analysis

DNA extraction was performed using the Qiagen DNeasy Blood and Tissue Kit (Qiagen GmbH, Hilden, Germany) following manufacturer's instructions. The genomic DNA was used as input for library construction using the Ligation Sequencing Kit V14 (Oxford Nanopore Technology, Oxford, United

Kingdom) and Illumina DNA Prep Kit (Illumina Inc., San Diego, CA) for long and short read sequencing, respectively. The long-read sequencing was performed on a MinION™ Mk1B (R10.4.1) sequencer. Data acquisition and basecalling was performed using Dorado (0.7.0) super-accurate mode. Paired-end library (2 x 50bp) for short-read sequencing was sequenced on Illumina Nextseq2000 platform.

QC and assembly

Raw reads from Illumina sequencing were curated using fastp[6] (v0.23.1 with parameters -q=30 and -l=45) while the raw reads from Nanopore sequencing were curated using filtlong (<https://github.com/rrwick/Filtlong>, v0.2.1 with the parameters: --min-length 2500, --keep_percent 95). The library with only short read available were assembled with SPAdes 4.0.0 (with the option —careful and—only-assembler). Draft genomes was curated by removing <500 bp contigs and/or coverage < 10×. For the isolate PA-05, curated reads from long and short reads sequencing were combined to create an hybrid assembly using Unicycler (v0.5.0)[7] and polished using Polypolish (v0.5.0).[8] The quality of the final draft was quality-controlled using Quast (v5.0.2)[9] (Supplementary dataset). Species identification of each draft genome was done using mash (sub-command screen)[10] by screening each draft genome against a database composed of a representative genome of each species present in the Microbial Genomes resource (<https://www.ncbi.nlm.nih.gov/genome/microbes/>). The complete draft genomes were processed through available databases using Abricate (<https://github.com/tseemann/abicate>) to identify antimicrobial resistance (NCBI [11], CARD [12], ARG-ANNOT [13], ResFinder [14] databases) and plasmid type (PlasmidFinder [15] database) to determine the plasmid Inc type (Supplementary dataset). Mutation associated with colistin resistance were investigated using Snippy (v 4.6.0, <https://github.com/tseemann/snippy>) using the reference strain PAO1 as all strains resistant to colistin belong to Clade 1. All clade 1 genome were mapped and compared to extract SNPs unique to resistant strains as potentially related to colistin resistance.

Core genome calculation

Core genome was evaluated using Roary (v 3.13.0)[16] using genes present in more than 99% of the population as core genome. In total, 5129 genes were conserved for a total length of 5007792 bp. The core genome alignment was used to create the phylogenetic tree using Raxml (v8.2.12) with the model GTRGAMMA and 100 permutations. The same approach was used to compare our isolates from ST308 and ST233 to publicly available genome from NCBI microbial resources with a geographical location in the metadata. All publicly available genomes used in the comparison are presented in the supplementary dataset. Clonal groups were defined by isolates sharing more than 99.99% Average Nucleotide Identity (ANI) calculated using ANIclustermap (<https://github.com/moshi4/ANIclustermap>) as described previously.[17]

Statistical analysis

Descriptive statistics were performed using R 4.3.3.

Data availability

The draft genomes presented in this study can be found in the NCBI Genbank repositories under the Bioproject PRJNA1118572. The accession number(s) can be found in the Supplementary dataset.

Funding

The study was funded through grants from the PAN-ASEAN Coalition for Epidemic and Outbreak Preparedness (PACE-UP; DAAD Project ID: 57592343)

Ethics

The study was approved by Institutional Review Board of the 108 Military Central Hospital, Hanoi, Vietnam (108MCH/RES/AMR-HAI-V-D2-05-07-2023)

Results

From July 1st to October 31st, 2023, a total of 691 patients admitted to the ICUs were screened for MDRGN. Rectal colonisation with carbapenem-resistant *P. aeruginosa* (CRPA) was detected in 3% (21/691 patients). All isolates were non-duplicates, and 20 patients' isolates were characterised by short-read genome sequencing due to the non-recovery of one isolate. Clinical data were available for 19 patients, and the median age was 61 (range: 20 to 83 years old). 35% (7/20) of the patients were females, and four patients had an infection (three airways and one blood infection). Unfortunately, the infection isolates were not available for genotyping and therefore could not be included in the analysis. Five patients tested positive for MDRGN at admission; the other colonization was detected during the weekly follow-up.

Molecular epidemiology

ST308 was the predominant MLST (50%, 10/20), followed by ST155, ST233 and ST235 with two isolates each. Other detected MLST were ST179, ST1228, ST381 and ST645. All isolates were found to harbour a *bla*_{OXA-50-like} gene in addition to a variant of *bla*_{PDC}. Furthermore, all ST308 isolates were positive for *bla*_{PME-1}. Overall, 75% (15/20) of CRPA collected in this study harboured at least one carbapenemase-encoding gene. The most prevalent carbapenemase was NDM-1 (60%, 12/20), of which one isolate exhibited an additional *bla*_{KPC-2} carbapenemase gene (isolate PA-05). Two isolates harboured *bla*_{IMP-26} (both ST235), and one isolate harboured *bla*_{KPC-2} only (PA-01, ST179). Two isolates harboured a *bla*_{OXA-10} gene (both ST155) and all ST308 and ST233 CRPA harboured *bla*_{NDM-1}. Two potential transmission clusters were identified, based on the ANI (cut of 99.99%) (Figure 1).

We compare our genomes from ST308 and ST233 to the publicly available genome associated with a geographical location (figure 2, Table S1). We observed that the acquisition of *bla*_{NDM-1} in ST308 is contained in phylogenetically related subclade isolated in Asia, Africa and Europe indicating a probable acquisition of the plasmid by a common ancestor (figure 2A). The acquisition of *bla*_{NDM-1} in ST233 was rare and only one other isolate from Myanmar carried the genes and was not related to our isolates (figure 2B).

Antibiotic susceptibility

All isolates were classified as XDR-*P. aeruginosa*. The majority of isolates exhibited resistance to antipseudomonal antibiotics currently in clinical use, including carbapenem with two exceptions observed for PA-07 (meropenem susceptible and imipenem susceptible at higher exposure) and PA-08 (meropenem susceptible at higher exposure and imipenem resistant). Those two isolates as well as PA-01 and PA-17 presented a specific mutation in the loop L7-short of the *oprD* gene (V359L), which has been described to confer resistance to carbapenems, especially imipenem.[18] We also detected mutation in the PBP3 (*ftsI* R504C) gene involved in β -lactam resistance.[19] With regard to the newer β -lactams

and β -lactam- β -lactamase inhibitor combinations, only 15% (3/20) demonstrated susceptibility to ceftazidime-avibactam, and only 10% (2/20) were susceptible to ceftolozane-tazobactam. Resistance to these substances correlated with the presence of MBL-encoding genes (*bla*_{IMP26} and *bla*_{NDM-1}). Interestingly, three ceftazidime-avibactam-resistant isolates, PA-06, PA-15 and PA-17 were non-MBL producers. On the other hand, all strains producing NDM-1 or IMP-26 were indeed resistant to aztreonam-avibactam.

Nine isolates (45%, 9/20) were found to be resistant to cefiderocol (MIC range 4 mg/L to 16 mg/L), and all nine cefiderocol-resistant isolates were identified as either NDM-1 (77.8%, 7/9) or IMP-26 (22.2%, 2/9) producers. NDM-1-producing cefiderocol-resistant CRPA had higher cefiderocol MICs (4 to 16 mg/L) compared to IMP-26 producers (4 to 8 mg/L). The addition of 100 ng/mL of dipicolinic acid, a zinc chelating agent, which can inhibit the activity of metallo- β -lactamases [20], resulted in a reduction of all cefiderocol MICs to levels below the clinical breakpoint for resistance (< 2 mg/L). Hence, suggesting that the reduced susceptibility towards cefiderocol was mediated by the metallo- β -lactamase activity. Colistin demonstrated the most favourable susceptibility profile, with 90% (18/20) of the CRPA strains remaining susceptible to colistin despite exhibiting an XDR phenotype. Genomic analyses of the two resistant isolates identified 3385 SNPs present in at least one of the isolates and absent from all the sensitive isolates. However, mutations in genes previously described in colistin-resistant isolates such as *lptD*, *pmrE*, *cprS*, *lpxA*, *lpxB*, and *lpxO1* [21] were also found, suggesting other mechanisms may be involved (Supplementary dataset). Interestingly, we observed that the ST308 lineage presents the mutation V15I in the gene *pmrB*, which is classified in the database to be leading to colistin resistance but is actually also present in colistin-susceptible strains such as the lab strain PA14 [22].

Sixteen isolates (80%, 16/20) were resistant to the novel β -lactam/ β -lactamase inhibitor combination of cefepime-taniborbactam. As expected, the combination was ineffective against the two *bla*_{IMP26}-carrying isolates. Among the five carbapenemase-negative isolates, four remained susceptible to the combination cefepime-taniborbactam. The MIC for cefepime of isolate PA-08 (positive for *bla*_{OXA-10}) was reduced by taniborbactam, though not to levels of susceptibility. In the 12 isolates harbouring *bla*_{NDM-1}, taniborbactam did not restore susceptibility to cefepime.

Discussion

In this study, we performed genotyping of CRPA isolated from rectal swabs obtained from patients admitted to the ICU in Hanoi, Vietnam. *P. aeruginosa* is considered a particularly problematic pathogen, which is very difficult to treat due to the intrinsic resistances to many antibiotics, limiting the usage of many β -lactam substances. Antibiotic resistance evolution in *P. aeruginosa* is often linked with antibiotic usage and exposure [23]. In Vietnam, antibiotic consumption in both clinical settings and the agricultural sector ranks among the highest in Southeast Asia, potentially influencing the resistance profiles of bacterial species colonizing the human gut. When the gut barrier is compromised or the normal microbiota is disrupted, these colonizing bacteria may translocate and lead to systemic infections [24, 25].

The prevalence of CRPA colonization is relatively low, with a rate of 3% in our study, in comparison to data on colonization with carbapenem-resistant Enterobacterales in the similar setting.[26] This finding remains a cause for concern, as our research indicates that the majority of carbapenem resistance is mediated by MBLs, particularly NDM. While infections due to carbapenem resistance due to porin mutations or upregulation of efflux pumps can usually be treated with newer β -lactam/ β -lactamase inhibitor combinations, NDM enzyme can completely or partially degrade most β -lactams and β -lactamase inhibitor in clinical use [27]. We observed a dominant establishment of the NDM-carrying ST308 lineage, which is a known high-risk clone as well as ST233, and ST235 [28]. Indeed all ST308 and ST233 clones in our study harboured *bla*_{NDM-1}. One isolate even harboured two carbapenemases, *bla*_{NDM-1} and *bla*_{KPC-2}, simultaneously, as previously reported [2]. As a successful high-risk clone, the detection of ST308 *P. aeruginosa* is often linked with outbreak events in Europe [29]. However the European ST308 *P. aeruginosa* mostly harbour the IMP-type and VIM-type carbapenemases but rarely the NDM-type carbapenemase. In contrast, the emergence of ST308 *P. aeruginosa* harbouring *bla*_{NDM-1} gene has been reported on numerous occasion in Singapore [30] and in some European countries such as Greece [31]. An analysis of the publicly available genome of ST308 showed that the acquisition of *bla*_{NDM-1} is contained in phylogenetically related clones isolated in Asia, Africa and Europe indicating a probable acquisition of the plasmid by a common ancestor in this sub-clade (Figure 2). Regarding ST233, while occurrence on NDM producing ST233 in Egypt has been reported [32], only one publicly available genome from Myanmar was carrying *bla*_{NDM-1} and is phylogenetically really distant from our isolates [33]. Another high-risk clone, ST235, has been identified as one of the predominant carbapenem-resistant *Pseudomonas aeruginosa* (CRPA) clones circulating in Southeast Asia, including countries such as Thailand and Vietnam [34-36]. Although this clone was also detected in our study, the findings suggest a potential shift in the molecular epidemiology toward the emergence and dominance of NDM-producing CRPA clones. The predominance of ST235 CRPA in Southeast Asia has been reported in several studies [36]. however, most are based on data collected prior to 2020, which may no

longer accurately represent the current epidemiological landscape. Continued surveillance is crucial to track the emergence and dissemination of NDM-1-producing CRPA in the region.

The predominance of NDM-1-producing CRPA was unexpected since *bla*_{NDM}-harbouring *P. aeruginosa* are relatively rare [37]. Genomic analysis of our strains did not suggest that the predominance of NDM-producing *P. aeruginosa* came from a single clonal lineage, suggesting that NDM-producing *P. aeruginosa* is already circulating in the community in Vietnam. NDM-producing *P. aeruginosa* has been reported in Asia, such as in Singapore [30, 38], and Malaysia [39], but is considered rare in other region. However recently, several cases have been reported in Spain [40, 41], the UK [42], and the Netherlands [41, 43] to name a few, mostly linked with Ukrainian patients with war injuries.

There are only limited molecular epidemiology studies on CRPA in Vietnam. Previous studies conducted in 2011-2015 in Hanoi, Vietnam, suggested the abundance of IMP-producing CRPA [44]. In their study, Tran *et al.* did not detect NDM-producing CRPA. In contrast, CRPA harbouring the *bla*_{IMP-26} gene was rare in our study. The most probable explanation is a shift in the molecular epidemiology of CRPA in Vietnam due to different sample collecting periods, which is supported by the fact that the ST308 and ST233 were not detected in the previous studies [44]. Nevertheless, the current high rate of NDM producers is alarming since NDM remains a major clinical challenge with limited treatment options. Although novel β -lactams, such as cefiderocol, may be effective against NDM producers, 50% of the CRPA isolated in our study were resistant towards cefiderocol. This resistance mechanism is likely linked to the NDM activity since NDM inhibiting Zn^{2+} chelator lowers the cefiderocol MIC to susceptible levels. Further, NDM is still a major challenge for β -lactamase inhibitors since novel β -lactamase inhibitors cannot effectively inhibit NDM in *P. aeruginosa* [45]. Taniborbactam is a novel β -lactamase inhibitor, currently in phase 3 clinical trial and has been shown to inhibit NDM activity in Enterobacterales [46]. However, all of our NDM-producing *P. aeruginosa* were resistant to cefepime/taniborbactam. For this study, we interpreted the AST results for cefepime/taniborbactam according to the EUCAST breakpoints (MIC value of ≥ 8 mg/L is considered resistant). Notably, there are slight discrepancies when using CLSI breakpoints (MIC value of ≥ 16 mg/L is considered resistant). If CLSI breakpoints had been used, all of our IMP- and NDM-producing strains would have remained resistant, while the remaining strains would have been classified as intermediate or susceptible.

Of the last-resort antibiotics tested, colistin exhibited the most favourable susceptibility profile. This finding was unexpected given the high usage of colistin in Vietnam and the numerous reports of a significant colistin resistance rates in carbapenem-resistant Enterobacterales [47-49]. Consequently, it was anticipated that CRPA would show similarly high rates of colistin resistance due to colistin selection pressure. A potential limitation for the colistin AST is the detection of heteroresistance. In this study, we used a commercially available broth microdilution panel, approved for *in vitro* diagnostics, which may

not be able to detect colistin heteroresistance [50]. Caution should be exercised when promoting colistin use due to its toxicity and the potential for resistance to emerge rapidly following exposure[21].

Our study has several limitations. First, it was conducted at a single center, which limits its generalisability. However, given the scarcity of WGS data on *P. aeruginosa* from Vietnam, we believe our findings provide valuable insights into the molecular epidemiology of *P. aeruginosa* in the country. We encourage surveillance efforts that incorporate genomic analyses in regions with a high burden of AMR and limited resources, as these efforts ' can offer valuable insights into the population dynamics and evolution of AMR. Such data are essential for developing targeted interventions to mitigate AMR in these contexts. Furthermore, given the emergence of resistance to novel agents, our study emphasises the importance of performing AST prior to using novel antimicrobial agents to treat infections.

Funding

The study was funded through grants from the PAN-ASEAN Coalition for Epidemic and Outbreak Preparedness (PACE-UP; DAAD Project ID: 57592343)

Transparency declaration

DN received speakers honoraria from Cepheid and Shionogi outside the scope of this work and has participated in the advisory board of Shionogi. All other authors no conflicts of interest.

Acknowledgment/Use of generative AI

During the revision process, DeepL Write was used to check for spelling and grammar errors and to improve the overall flow of the text. The authors conducted a final review to ensure that the intended meaning of the sentences remained unchanged

Figure 1. Phylogeny of carbapenem-resistant *Pseudomonas aeruginosa* isolates from Hanoi, Vietnam. MLST is provided with a color-code of the tip of the tree while the clonal relationship defined as an ANI > 99.99% is displayed as a star. A summary of selected β -lactamase genes and phenotypic resistance of antibiotics is displayed respectively in the left and right heatmaps. The black square indicate the presence and the grey square indicate the absence of the gene or the specific mutations while the resistance status is shown as a color-code based on the antimicrobial susceptibility testing, classified according to EUCAST; S – Susceptible (green), I – Susceptible, increased exposure (orange); R – Resistant (dark red).

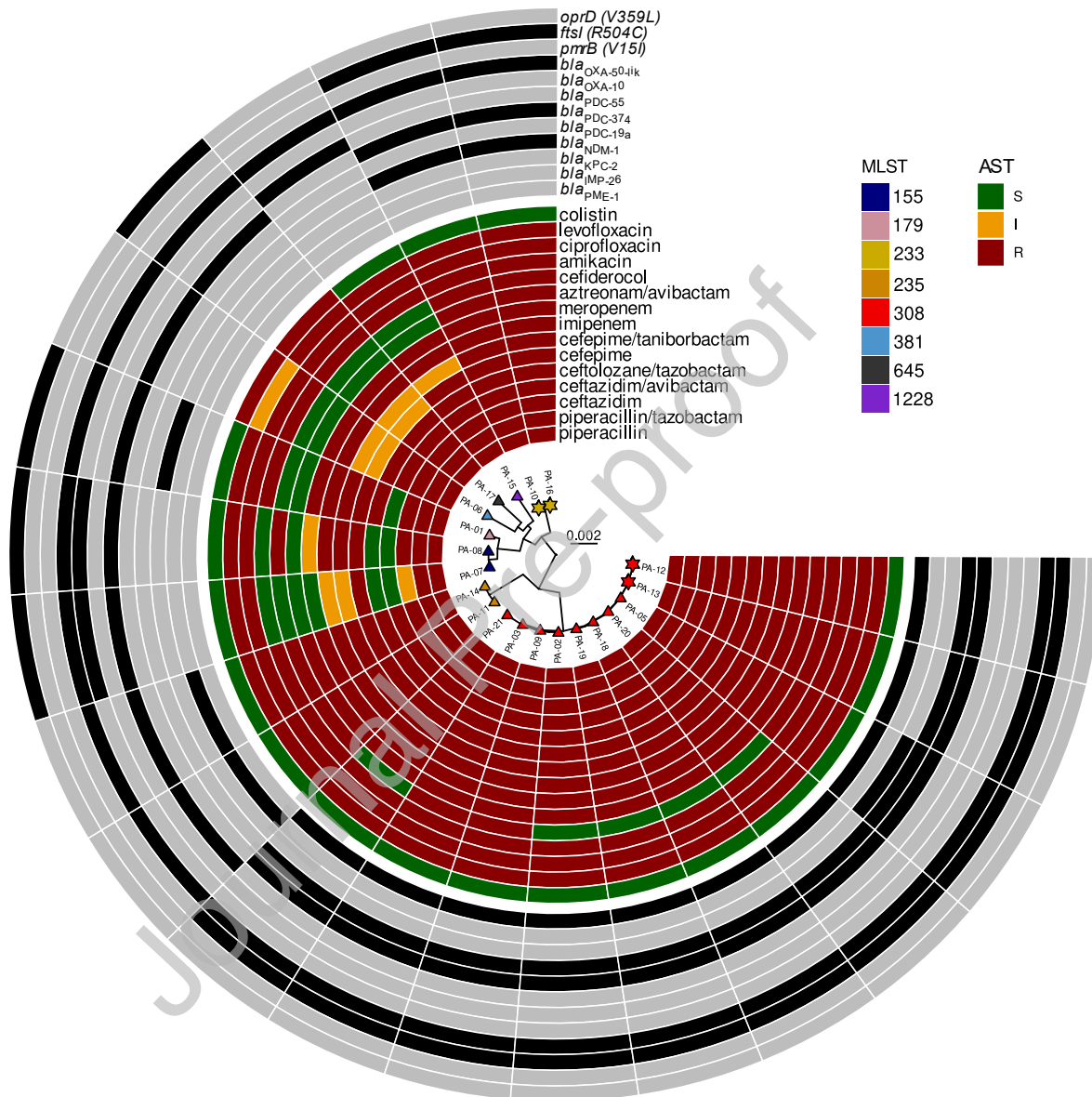
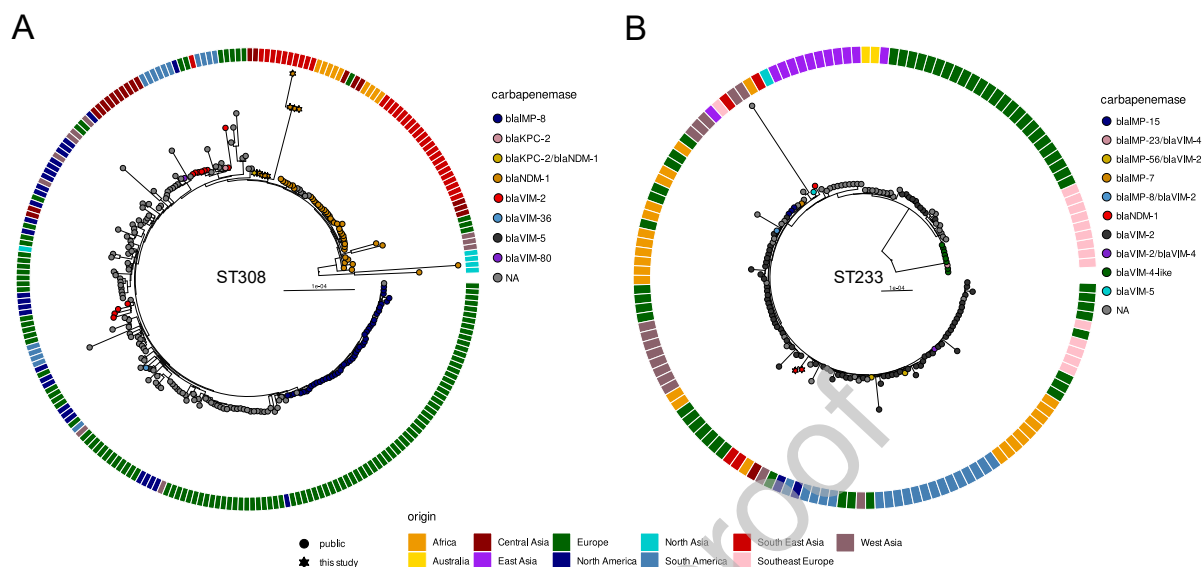


Figure 2. Maximum-likelihood phylogenetic tree with local and public *P. aeruginosa* ST308 and ST233 genomes. Continent of origin is provided with a color-code on the outer ring. Isolates from our study are represented by a star while the public isolate are represented by a dot at the tip of the tree. The tips are also color-coded based on the carbapenemase genes found in the genomes.



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