Nationwide genome surveillance of carbapenem-resistant *Pseudomonas aeruginosa* in Japan

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ABSTRACT

Japan is a country with an approximate 10 % prevalence rate of carbapenem-resistant *Pseudomonas aeruginosa* (CRPA). Currently, a comprehensive overview of the genotype and phenotype patterns of CRPA in Japan is lacking. Herein, we conducted genome sequencing and quantitative antimicrobial susceptibility testing for 382 meropenem-resistant CRPA isolates that were collected from 78 hospitals across Japan from 2019 to 2020. CRPA exhibited susceptibility rates of 52.9%, 26.4%, and 88.0% against piperacillin-tazobactam, ciprofloxacin, and amikacin, respectively, whereas 27.7% of CRPA isolates were classified as difficult-to-treat resistance *P. aeruginosa*. Of the 148 sequence types detected, ST274 (9.7%) was predominant, followed by ST235 (7.6%). The proportion of urine isolates in ST235 was higher than that in other STs (*P* =

0.0056, chi-square test). Only 4.1% of CRPA isolates carried the carbapenemase genes: *bla*$_{GES}$ (2) and *bla*$_{IMP}$ (13). One ST235 isolate carried the novel *bla*$_{IMP}$ variant *bla*$_{IMP-98}$ in the chromosome. Regarding chromosomal mutations, 87.1% of CRPA isolates possessed inactivating or other resistance mutations in *oprD*, and 28.8% showed mutations in the regulatory genes (*mexR, nalC*, and *nalD*) for the MexAB-OprM efflux pump. Additionally, 4.7% of CRPA isolates carried a resistance mutation in the PBP3-encoding gene *ftsI*. The findings from this study and other surveillance studies collectively demonstrate that CRPA exhibits marked genetic diversity and that its multidrug resistance in Japan is less prevailed than in other regions. This study contributes a valuable dataset that addresses a gap in genotype/phenotype information regarding CRPA in the Asia–Pacific region, where the epidemiological background markedly differs between regions.

INTRODUCTION

Pseudomonas aeruginosa is a common opportunistic pathogen associated with pneumonia, urinary tract infections, and bacteremia in clinical settings worldwide. Currently, it ranks among the top five bacterial pathogens associated with human death globally (1). It also intrinsically shows reduced susceptibility to many antibiotics due to its possession of four types of RND family efflux pump, chromosomally encoded AmpC β-lactamase (also called *Pseudomonas*-derived class C β-lactamase: PDC), and other antimicrobial-inactivating enzymes (2, 3). Furthermore, *P. aeruginosa* can acquire resistance mutations at multiple genomic locations and antimicrobial resistance genes via mobile genetic elements, such as plasmids and

44 integrative and conjugative elements (ICEs), after which it elicits high levels of resistance to
45 multiple antimicrobials, thereby limiting treatment options for infected patients (3–5).
46 Carbapenems represent a crucial class of therapeutic options for treating *P. aeruginosa*
47 infections. In 2017, the World Health Organization (WHO) listed carbapenem-resistant *P.*
48 *aeruginosa* (CRPA) as one of the bacterial priority pathogens classified in "Priority 1: CRITICAL,"
49 for which new effective antibiotics are urgently required (6). Horizontally transferrable
50 carbapenemases represent a significant public health concern due to their insensitivity to the
51 conventionally used β-lactamase inhibitors (7). A recent cohort study demonstrated a 9% higher
52 30-day mortality rate among patients infected with carbapenemase-producing CRPA when
53 compared to those infected with carbapenemase-nonproducing CRPA (8). Currently, three
54 classes of β-lactamases, namely class A, class B (known as metallo-β-lactamases or MBLs), and
55 class D β-lactamases, are recognized as transferrable carbapenemase. Globally disseminated
56 carbapenemase types in *P. aeruginosa* include VIM, IMP, NDM, GES, and KPC (9). In addition to
57 the horizontal acquisition of carbapenemases, *P. aeruginosa* can develop carbapenem
58 resistance through the mutational inactivation of several chromosomally coded functions. These
59 include the inactivation of the OprD porin (10) and the inactivation of two direct or one indirect
60 transcriptional regulators (repressors, MexR, NalC, and NalD) of the MexAB-OprM efflux pump
61 (11–13). Furthermore, an experimental evolution study revealed that nonsynonymous
62 substitutions in *ftsI*, an essential gene encoding PBP3, contribute to carbapenem resistance (14).
63 Recently, international consortia have made efforts to build a large *P. aeruginosa* dataset
64 to obtain quantitative information for CRPA prevalence, its susceptibility profiles, and the
65 possession rate of carbapenemases (8, 15–17); Notably, in the Asia–Pacific region, the

66 Antimicrobial Testing Leadership and Surveillance (ATLAS) program took the initiative of *P.*
67 *aeruginosa* surveillance, revealing distinct patterns of resistance rate and predominant β-
68 lactamase levels in the *P. aeruginosa* population between 12 countries/regions, but the ATLAS
69 did not cover genome surveillance (16). Hence, information of the major types of resistance
70 mechanisms and dominant clones of *P. aeruginosa* in the Asia–Pacific region remain lacking.
71 Japan exhibits characteristic features in the epidemiology of antimicrobial-resistant
72 bacterial infection in the Asia–Pacific region. Regarding carbapenem resistance, the prevalence
73 of meropenem-resistant *E. coli* in bloodstream infections in Japan as of 2019 was 2 per 100,000
74 test patients, which is lower than, for example, 79 per 100,000 test patients observed in
75 Singapore, which is located in Southeast Asia, according to the WHO Global Antimicrobial
76 Resistance and Use Surveillance System (18). The established national phenotypic antimicrobial
77 resistance surveillance program—the Japan Nosocomial Infections Surveillance (JANIS) (19,
78 20)—reported that the carbapenem (meropenem) resistance rate of *P. aeruginosa* in
79 hospitalized patients in Japan, as of 2020, was 10.5%. This was average among representative
80 countries where antimicrobial resistance surveillance for *P. aeruginosa* was once or is currently
81 being performed; the resistance rate was 29.3% in India (ATLAS 2015–2019), 19.3% in China
82 (CHINET 2020), 6.5% in Australia (ATLAS 2015–2019), 28.5% in Poland (EARS-net 2020), 15.7% in
83 the Czech Republic (EARS-net 2020), 13.9% in Germany (EARS-net 2020), 12.6% in France (EARS-
84 net 2020), and 3.7% in Finland (EARS-net 2020) (16, 21, 22).
85 To obtain an overview of both genomic features and resistance phenotypes of bacterial
86 pathogens in Japan, we initiated the national bacterial genome surveillance—Japan
87 Antimicrobial-Resistant Bacterial Surveillance (JARBS)—in 2019 (23). Through the JARBS-PA

88 project performed in 2019 and 2020, 382 meropemen-resistant CRPA isolates were collected
89 from 78 hospitals across Japan. Herein, we report the genotype and susceptibility patterns of
90 the CRPA isolates in Japan based on genome surveillance and standardized MIC
91 remeasurements.
92
93 **RESULTS**
94 **Nationwide sampling.** From 2019 to 2020, more than 600 *P. aeruginosa* isolates were
95 transferred from Japanese hospitals to the Antimicrobial Resistance Research Center (AMR-RC)
96 at the National Institute of Infectious Diseases (NIID) based on the following criterion:
97 meropenem or imipenem MIC ≥8μg/ml in initial testing at the isolation site. All the collected
98 isolates were tested for antipseudomonal agent MICs using the Beckman MicroScan system at
99 AMR-RC. In total, 382 *P. aeruginosa* isolates showing meropenem MIC ≥8μg/ml in the tests at
100 AMR-RC were defined as CRPA and focused on in this study. The 382 CRPA isolates originated
101 from nonoverlapping patients in 78 hospitals (the number of collected isolates per island was as
102 follows: *n* = 30 from Hokkaido Island, *n* = 307 from Honshu Island, *n* = 7 from Shikoku Island, *n* =
103 32 from Kyusyu Island, and *n* = 6 from Okinawa Island). Source specimens were classified into
104 one of the following JANIS categories (19, 20): Oral/Endotracheal/Respiratory (*n* = 189),
105 Urinary/Genital (*n* = 76), Blood/Fluid (*n* = 40), Digestive (*n* = 27), and Other (*n* = 50). Table S1
106 provides more detailed source categories and metadata for each isolate. Draft genome
107 sequences of 382 CRPA strains were determined to identify the sequence type (ST), resistance
108 genes, and mutations.

109 **Antimicrobial susceptibility profiles and STs of CRPA in Japan.** Table 1 summarizes the
110 susceptibilities for antipseudomonal agents. Although the collected CRPA isolates were often
111 nonsusceptible to antipseudomonal agents other than meropenem, the percentage of
112 susceptible isolates remained 52.9% for piperacillin-tazobactam, 26.4% for ciprofloxacin, and
113 88.0% for amikacin based on breakpoints in CLSI M100-ed 32 in 2022 (Table 1). When focused
114 on isolates from urine (CLSI M100-ed 33 in 2023), the proportion of amikacin-susceptible
115 isolates was decreased to 75%.

116 Susceptibility data for individual isolates are provided in Table S1. Among the CRPA
117 isolates, 27.7% (106/382) were deemed difficult-to-treat resistance *P. aeruginosa* (DTR-*P.*
118 *aeruginosa*) in Tamma *et al*.'s definition (24), and 85.8% (328/382) were MDR in Magiorakos *et*
119 *al*.'s definition (25). The susceptibility profiles of DTR strains are summarized in Table S2. Based
120 on the CLSI M100-ed 32 breakpoints, tobramycin (80.2%) exhibited the highest susceptibility
121 rate of DTR isolates among the three aminoglycosides analyzed. Only three CRPA isolates were
122 resistant to colistin; two of the three colistin-resistant isolates (Table S1) were however DTR-*P.*
123 *aeruginosa* and elicited resistance to at least one agent from each of the seven antimicrobial
124 agent categories listed in Table 1.

125 The MLST profiles of 382 CRPA isolates were investigated in the PubMLST database (26).
126 Including newly assigned ST (ST4091–ST4125), 148 STs were identified (Fig. 1A). The most
127 frequently encountered clonal group (CG) was CG274 (comprising only ST274: type III secretion
128 system effector protein genotype *exoS*$^{+}$/*exoU*$^{-}$, 9.7%), followed by CG235 (ST235: *exoS*$^{-}$/*exoU*$^{+}$,
129 7.6%), CG253 (ST253: *exoS*$^{-}$/*exoU*$^{+}$, 4.5%), and CG155 (ST155 and ST4122: *exoS*$^{+}$/*exoU*$^{-}$, 3.4%).
130 Many minor STs, each contributing about 1%, occupied 49.0% of the total CRPA in Japan. We

131 further investigated the occurrence of nearly identical isolates among different patients in our
132 dataset by analyzing Mash distances between draft genomes (27). Among the 382 isolates
133 examined, 7.6% (29/382) showed a Mash distance of $<10^{-5}$ to at least one other isolate, which we
134 defined as nearly identical. We extended this analysis to an external dataset used in Reyes *et al*'s
135 CRPA surveillance across various regions (8). In the USA, the proportion of nearly identical CRPA
136 isolates was 0.6% (4/527), in China it was 19.2% (33/171), in Singapore it was 4.9% (2/41), and in
137 Central South America it was 3.5 % (6/171). Consequently, the contribution of outbreaks to the
138 CRPA dataset in Japan is lower than in China but higher than in the USA.

139 Furthermore, we identified all the top ten global high-risk clones (ST111, ST175, ST233,
140 ST235, ST244, ST277, ST298, ST308, ST357, and ST654) (28), which collectively accounted for
141 17.0% of the total CRPA isolates in Japan (Table S1). Reyes *et al* (8) proposed globally most
142 common CGs (10 CGs in Fig. 1B). Those globally common CGs comprised 34.8%. Despite being
143 among the top two most common CGs in the USA and Central South America, CG111 remains a
144 minor CG in Japan (Fig. 1B). Surprisingly, the dominant clone CG463 (ST463), prevalent in
145 Chinese clinical settings (7, 30), was entirely absent in the 382 CRPA isolates collected in this
146 study. The characteristic features of two major CG/STs in Japan, ST274 and ST235 are described
147 in more detail with genotype information later in the result section.

148 **Resistance mutations and carbapenemases in CRPA.** The molecular mechanisms of
149 meropenem resistance are primarily classified into four types: porin inactivation, efflux pump
150 overproduction, target alteration, and horizontal acquisition of β-lactamase (2). Those
151 mechanisms are respectively associated with inactivation of the *oprD* gene, inactivation of
152 either *mexR*, *nalC*, or *nalD* genes encoding transcriptional regulators (repressors) for the

153 MexAB-OprM efflux pump (11–13), nonsynonymous substitutions in *ftsI* (14), and acquisition of
154 plasmid or ICE-borne carbapenemase genes (4, 5). We searched for the presence of inactivating
155 mutations (frameshift mutations due to insertion/deletion, nonsense mutations, and
156 inactivation of start codon or stop codon) in *oprD*, *mexR*, *nalC*, and *nalD* as well as resistance
157 mutations registered in the NCBI AMRFinderPluS database, as these likely contribute to
158 increasing meropenem MIC.

159 Database searches revealed that only 4.2% (16/382) of CRPA in Japan possessed
160 acquired carbapenemases. Known carbapenemases detected were GES-5 (*n* = 2), IMP-1 (*n* = 6),
161 IMP-10 (*n* = 4), IMP-7 (*n* = 3), and IMP-34 (*n* = 1). No OXA-type carbapenemases were detected.
162 ST274 CRPA isolates rarely (2.7%, 1/37) carried carbapenemase, contrasting with the 34.5%
163 (10/29) possession rate of ST235 isolates. One ST235 isolate encoded a novel variant of IMP-
164 type MBL, which was assigned a new allele name IMP-98 by NCBI.

165 The prevalence of inactivating mutations or known nonsynonymous resistance
166 mutations in chromosomal genes in 382 CRPA isolates was 87.1% (333/382) for *oprD*, 14.1%
167 (54/382) for *mexR*, 9.1% (35/382) for *nalD*, and 6.3% (24/382) for *nalC*. Collectively, 28.8%
168 (110/382) of CRPA carried an inactivating mutation in at least one of the three repressors for
169 *mexAB*. The most common mutation type in *oprD* was IN/DELs causing frameshift (237/382),
170 followed by nonsense mutations (90/382); these include, for instance, W277stop (24/90),
171 W339stop (9/90), and W138stop (9/90). In addition, 4.7% (18/382) of CRPA isolates carried
172 known resistance mutations in *ftsI* (P527S, F533L, R504C, or V471G) (14).

173 A simultaneous occurrence of resistance mutations and/or carbapenemase was
174 observed (Fig. 2). While 26.4% (101/382) of CRPA isolates had an inactivating mutation in both

175 *oprD* and at least one regulator for *mexAB*, 4.5% (17/382) had both *oprD* and *ftsI* mutations,
176 with 3.1% (12/382) exhibiting the *oprD* mutation and carbapenemase. The acquisition of
177 carbapenemase alone occurred only in 0.7% (4/382) of the CRPA. Notably, 9.1% (35/382) did
178 not carry identifiable inactivating mutations in the four focused genes, known *ftsI* resistance
179 mutations, or carbapenemase.

180 ***bla*$_{\text{IMP-98}}$.** IMP-98 differed from IMP-1 by only one site at the nucleotide level and likely
181 originated from the *bla*$_{\text{IMP-1}}$ gene by C637T nonsynonymous substitution, leading to P213S
182 amino acid change (Fig. 3A). To deduce the origin of the *bla*$_{\text{IMP-98}}$ region, we determined
183 complete genome sequence of ST235 strain, JBBCAEG-19-0032, carrying *bla*$_{\text{IMP-98}}$ using long
184 reads. This revealed that strain JBBCAEG-19-0032 genome consists of one chromosome, and
185 *bla*$_{\text{IMP-98}}$ is embedded in a class 1 integron as the upstream region of *bla*$_{\text{IMP-98}}$ contained specific
186 motifs of *attI1* (5'-
187 GTTATGGAGCAGCAACGATGTTACGCAGCAGGGCAGTCGCCCTAAAACAAAGTTAGGC-3': 7-bp core
188 sites are underlined) (29).

189 A comprehensive analysis of the *bla*$_{\text{IMP-98}}$ context using the ISFinder database (30)
190 revealed that the class 1 integron is situated within a Tn*402*-related transposon, which is further
191 nested within a Tn*As3*-related transposon. The archetype Tn*As3* was originally identified in the
192 genus *Aeromonas* (Fig. 3B) (31, 32). However, the integron structure differs between Tn*As3* and
193 the Tn*As3*-related transposon in JBBCAEG-19-0032 in the gene cassette array composition and
194 the absence of conserved *attC*/*attI* motifs of class 1 integron in Tn*As3* (Fig. 3C).

195 Both transposon units in JBBCAEG-19-0032 possess intact terminal inverted repeats and
196 are flanked by 5-bp target site duplication (TSD): CCCTG for the Tn*402*-related transposon and

197 TTATA for the Tn*As3*-related transposon, indicating their insertions through transposition events.
198 Further comparison of the JBBCAEG-19-0032 chromosome with the PAO1 chromosome
199 revealed that the Tn*As3*-related transposon is nested within a genomic island containing distant
200 homologs of core genes of a recently defined mobile DNA element, the strand-biased
201 circularizing integrative element (SE) (33–35) (Fig. 3BD). The products of the SE core genes in
202 JBBCAEG-19-0032 (IntA: encoding tyrosine recombinase, Tfp: tyrosine recombinase fold protein,
203 IntB: large tyrosine recombinase, Srap: SE-associated recombination auxiliary protein) exhibited
204 low identities (22.6%–28.5%) in BLASTp comparisons to homologs of a characterized SE, SE-
205 6945, from the genus *Vibrio* (Fig. 3D) (33). However, IntA and IntB homologs of JBBCAEG-19-
206 0032 possess the catalytic RHRY motif of the tyrosine recombinase (36)(Supplemental material
207 Fig. S1), whereas Tfp and Srap homologs share secondary structures with known *Vibrio*
208 homologs (Fig. S2 and Fig. S3). The *in silico* removal of a 37,833-bp segment from the JBBCAEG-
209 19-0032 chromosome, with a putative 6-bp footprint normally formed on the right side of SE
210 after SE integration (33–35), regenerated an intact form of the PA2049-equivalent gene
211 encoding a hypothetical protein (Fig. 3E). This suggests that the 38-kb region was inserted via
212 site-specific recombination. Hence, we designated the 37.8-kb insert as putative SE, SE-
213 PaeJB0032. A BLASTn search against the NCBI-nr/nt database (2024-02-07) and a database
214 containing 382 Japanese CRPA strains using the left end (*intA* region) of the SE as a query did
215 not yield significant hits, indicating that SE-PaeJB0032 may have recently integrated into the *P.*
216 *aeruginosa* genome.
217 To test whether IMP-98 is functional, $bla_{\text{IMP-98}}$ was cloned and introduced into the drug-
218 susceptible *E. coli* strain DH5α, after which the susceptibility for various β-lactams was

219 investigated (Table 2). MICs of β-lactams except for aztreonam were considerably higher against
220 *E. coli* DH5α carrying bla_{IMP-98} than the control strain, confirming that IMP-98 retains the wide
221 substrate range of MBL.

222 **Other resistance determinants.** Among 46 amikacin-nonsusceptible CRPA isolates, 54.3%
223 (25/46) possessed known amikacin resistance genes *aac(6')* variants, resistance mutations such
224 as *fusA1* (Q455R) and *amgS* (V121G) (37), or inactivating mutation in *mexZ* encoding a repressor
225 for the MexXY efflux pump contributing to aminoglycoside resistance (38) (Table S1). However,
226 45.7% (21/46) of amikacin-nonsusceptible CRPA did not possess identifiable amikacin resistance
227 genes or mutations. Among 281 ciprofloxacin nonsusceptible CRPA isolates, 76.9% (216/281)
228 possessed at least one of the quinolone resistance gene *crpP* (39) (164/281) and resistance
229 mutations in *gyrA* (T83I, D87N, or D87G; 122/281), *gyrB* (E468D, S466F, or S466Y; 16/281), or
230 *parC* (S87L, S87W; 46/281), *parE* (A473V; 6/281) (Table S1). Three colistin-resistant isolates did
231 not possess any known colistin resistance mutations or genes.

232 **Features of two major STs in Japan.** Specifically, we aim to determine whether notable
233 phenotypic or genotypic features are associated with ST274 (n = 37) and ST235 (n = 29). Table 3
234 presents the observation frequencies of four source groups in ST274 and ST235, as well as other
235 STs. The relative frequencies among the three ST groups differed significantly ($P = 2.5 \times 10^{-2}$, chi-
236 square test for Table 3, df = 6). The proportion of isolates liked to the
237 Oral/Endotracheal/Respiratory category was higher in ST274 (64.8%, 24/37) compared to the
238 rest (48.1%, 166/345), however, this difference was not significant ($P = 7.8 \times 10^{-2}$, chi-square
239 test, df = 1). A significant difference was observed in the proportion of isolates in the

240 Urinary/Genital category between ST235 (41.3%, 12/29) and the rest (18.1%, 64/353) (P = 5.6 x
241 10^{-3}), suggesting that ST235 may have a competitive advantage over other STs in urine.
242 Table 4 provides a summary of resistance phenotypes, genes, and mutations observed in
243 the two major STs. Regarding nonsusceptibility, different proportions were observed among the
244 three ST groups for piperacillin-tazobactam, ceftadizime, ciprofloxacin, and amikacin (Table 3).
245 Notably, the proportion of amikacin-nonsusceptible isolates differed significantly between
246 ST235 (62.0%, 18/29) and the rest (7.9%, 28/353) ($P < 2.2 \times 10^{-16}$, chi-square test, df = 1).
247 The proportion of DTR isolates also varied among STs ($P = 4.2 \times 10^{-2}$, adjusted P-value in
248 Table 3). Particularly, the proportion of DTR isolates in ST235 (48.0%, 14/29) significantly
249 differed from the rest (26.1%, 92/352) ($P = 1.8 \times 10^{-2}$). This trend was not significant in ST274
250 (32.4% in ST274 vs 27.2% in other STs) (P = 0.6).
251 The possession rates of chromosomal mutations in *oprD*, *mexAB* regulator genes, and
252 *ftsI* did not differ among STs (Table 4). However, the possession rates of carbapenemase genes
253 bla_{IMP} and bla_{GES} differed significantly among STs. The possession rates of bla_{IMP} and bla_{GES} in
254 ST235 were significantly higher than that in other STs: 34.5% in ST235 vs 1.7% in other STs (P =
255 1.4×10^{-15}). This trend was also observed for the possession rate of amikacin resistance genes:
256 58.6% in ST235 vs 2.0% in other STs ($P < 2.2 \times 10^{-16}$). These characteristic features of ST235 were
257 absent in ST274.
258 Fluoroquinolone resistance determinants can be categorized into chromosomal
259 resistance mutations (*gyrA*, *gryB*, *parC*, *parD*), and transferrable quinolone resistance gene *crpP*
260 (39). Although both types appeared at different proportions among STs (Table 4), the
261 distribution trend differed between the two types. The proportion of isolates carrying *gyr* or *par*

262 mutations was higher in the two major STs (75.4%, 49/65) than in others (28.5%, 90/316) (P =
263 5.6×10^{-15}), while the proportion of isolates carrying *crpP* was higher in nonST274 and
264 nonST235 (63.0%, 199/316) than the two STs (34.8%, 23/66) ($P = 4.6 \times 10^{-5}$). Surprisingly, *crpP*
265 was present in 57% (58/101) of ciprofloxacin-susceptible isolates. Thus, its significance should
266 be interpreted with caution. These observations validate the notion that ST235 exhibits an
267 extraordinary resistance phenotype among STs, functioning as an epidemic high-risk clone (28).
268

269 **DISCUSSION**

270 Since the adoption of a global action plan on antimicrobial resistance at the WHO
271 conference, regional surveillance on antimicrobial-resistant bacterial pathogens has
272 commenced worldwide (8, 15–17, 23). However, in the case of CRPA, most surveillance projects
273 have not provided both MIC values of antimicrobial agents and genome sequences for the
274 collected strains simultaneously, limiting data interpretation and reuse. Furthermore,
275 comprehensive surveillance in a single country with a 10% prevalence rate of CRPA, such as
276 Japan, has been lacking until this study. This study was aimed at addressing these gaps.
277 The ciprofloxacin and amikacin susceptibility rates regarding CRPA were 26.4% and 88.0%,
278 respectively, in Japan. These values exceed 20.6% and 60.6% of pooled CRPA values reported in
279 the Asia–Pacific region (CLSI breakpoint criteria before 2023) (16), and are then equivalent to
280 and higher than 26.2% and 63.4% of the global CRPA population, respectively (17).
281 Furthermore, the piperacillin-tazobactam susceptibility rate in Japan was 52.9%, which contrasts
282 with the 25.3% susceptibility rate in the global CRPA population (17). This difference might
283 reflect the difference in the carbapenemase possession rate in CRPA between Japan and other

284 regions. Therefore, in Japan, MDR has not yet heavily prevailed in the *P. aeruginosa* population
285 in clinical settings compared with other Asia–Pacific regions or the world average. Notably,
286 fluoroquinolone resistance among CRPA is a global problem and is common even in non-high-
287 risk clones.

288 Since several countries/regions normally categorized in the Asia–Pacific region are
289 geographically isolated by the presence of sea, we expected to observe distinct features of the
290 CRPA genotype in Japan and other Asia–Pacific regions. The striking feature was the
291 predominance of CG463/ST463 in China (8) and its absence in Japan (Fig. 1B). Among the
292 carbapenemase types, IMP was the most frequent (87.5%; 14/16) in Japan. This trend contrasts
293 with the predominance of NDM in *P. aeruginosa* borne-carbapenemases in India (16), KPC in
294 China (8), and VIM in Europe, Africa/Middle East, and Latin America (17). As a country with an
295 average prevalence rate of meropenem-resistant CRPA, Japan was expected to have a low
296 carbapenemase possession rate among *P. aeruginosa* strains. This study reveals that the
297 carbapenemase possession rate in Japan is 4.2%, which is among the lowest compared to other
298 regions where surveillance studies have been conducted. For instance, the carbapenemase
299 possession rate of meropenem-resistant CRPA was 32.9% in the Asia–Pacific region from 2015
300 to 2019 (16), 1.9% for the USA in 2018–2019 (8), 69% for South and Central America in 2018–
301 2019 (8), 32% for China in 2018–2019 (8), 57% in Singapore–Australia in 2018–2019 (8), and
302 21.9% for Europe in 2017–2019 (17).

303 The prevalence rate of carbapenemase is influenced by genetic, demographic, and
304 environmental factors. The genetic factors include the type of STs constituting the *P. aeruginosa*
305 population. As shown in Table 5, only so-called high-risk clones, represented by ST235, seem to

306 have a trend toward capturing transferrable carbapenemase. This could be associated with the
307 number of integrons possessed by the STs, as most reported MBL genes other than NDM are
308 present as integron gene cassettes (28). In Japan, the proportion of high-risk clones is 17%,
309 which is lower than that in China (30%, including KPC-producing ST463) and Singapore (60% in
310 Reyes's surveillance (8)). Therefore, potential carbapenemase gene recipients might be fewer in
311 Japan than in other regions. The demographic factor includes the low migration rate of
312 carbapenemase-producing CRPA to hospitals in Japan from high-carbapenemase prevalence
313 countries/regions. However, this factor is challenging to evaluate based on publicly available
314 information currently. Environmental factors include the hospital's environmental condition that
315 can prevent outbreaks and the resulting clonal expansion of carbapenemase-producing CRPA
316 within the country. The low occurrence of outbreaks is supported by the low frequency (7.6%)
317 of nearly identical isolates in the 382 CRPA dataset, as opposed to 19.2% in the Chinese CRPA
318 dataset. Previously, VIM-producing CRPA isolates were found in hospitals in Japan (40–42);
319 however, they were not detected in the 78 hospitals participating in the JARBS from 2019 to
320 2020. Furthermore, according to the National Epidemiological Surveillance of Infectious
321 Diseases by the Ministry of Health, Labor, and Welfare, the number of amikacin-nonsusceptible,
322 carbapenem, and fluoroquinolone-resistant *P. aeruginosa* has been decreasing over a decade in
323 Japan (43). Therefore, it is speculated that hospital environments are being managed to reduce
324 outbreaks and horizontal transmission of carbapenemases in Japan.

325 The complete sequence determination of a $bla_{\text{IMP-98}}$-carrying strain revealed that the *bla*-
326 containing-integron was embedded in a Tn*3*-family transposon of *Aeromonas* origin and nested
327 in a putative SE region in the chromosome, rather than in the plasmid. Previous studies have

328 identified *Pseudomonas* as one of the top five representative host genera of SEs; however, to
329 date, only one terminus-delineated SE from *P. aeruginosa*, SE-PaeBT2436, carrying the *tmexCD-*
330 *topJ* cluster conferring tetracycline and tigecycline resistance (34). Since homologs of SE core
331 genes could not be detected by nucleotide-based search in either the current NCBI database or
332 the CRPA dataset, the SE-PaeJB0032 type might have formed only recently, potentially capturing
333 $bla_{\text{IMP-98}}$, in other taxa such as *Aeromonas*, and then jumping into the chromosome of unique
334 ST235 clone.

335 This surveillance revealed that two STs, ST274 and ST235, members of the top 10
336 globally most common CG in CRPA, were also the most common in Japan. ST274 and CG274
337 members have frequently been detected in clinical settings worldwide (44) and are known for
338 chronic infection and intrabody evolution involving MDR in patients with cystic fibrosis in
339 Europe (45, 46). Whether the ST274 isolates collected in this study are associated with a chronic
340 lung infection remains unknown, but the "Oral/Endotracheal/Respiratory" source category was
341 most frequent in ST274 (Table 4). However, the proportion of this category did not significantly
342 differ from other STs (P = 0.078). Conversely, the other major ST, ST235, has more striking
343 features than ST274 in isolation source, nonsusceptibility, and resistance gene possession rate
344 (Table 3, Table 4). ST235 was proposed as an epidemic high-risk clone due to its worldwide
345 occurrence with an extremely drug-resistant phenotype (28, 44). However, statistical data
346 supporting this notion in a single surveillance has been lacking. Our data demonstrate that
347 ST235 has a trend to acquire resistance genes and resistance phenotypes more frequently than
348 other STs. The association of ST235 with urine is a novel finding, but the underlying mechanisms
349 remain to be investigated.

350 Inactivating mutations in *oprD* were the most frequent meropenem resistance
351 mechanism (found in 87.1%), as noted in previous genome surveillance performed in other
352 regions, where 69% of meropenem-resistant CRPA possessed *oprD*-inactivating mutations and
353 22% possessed acquired carbapenemase (8). We show that in Japan, not only *oprD*-inactivating
354 mutations but also efflux pump regulator inactivation and *ftsI* resistance mutations are more
355 common carbapenem resistance mechanisms than the horizontal acquisition of
356 carbapenemases. Therefore, future surveillance of *P. aeruginosa* should prioritize the study of
357 chromosomal mutations.
358 Surprisingly, 9.2% (35/382) of the CRPA isolates did not possess apparent inactivating
359 mutations, known *ftsI* resistance mutations, or carbapenemase. A recent multicenter study (9)
360 reported that 14% of modified carbapenemase-inactivation method (mCIM)-positive CRPA,
361 constituting 4.6% of the total examined CRPA, did not possess known carbapenemases.
362 Therefore, a fraction of the 9.2% of Japanese CRPA without a known resistance gene or
363 mutation may harbor enzymes capable of partially degrading meropenem. The potentially
364 relevant enzymes include variants of β-lactamase such as PDC and OXA (detected variants are
365 listed in Table S1), as well as Zn^{2+}-dependent imipenemase (47). However, pinpointing the
366 causal loci of meropenem resistance in the 9.2% of Japanese CRPA is challenging with current
367 knowledge, as these variants have been poorly characterized to date. Furthermore, known
368 resistance mutations could not account for the mechanisms of amikacin nonsusceptibility in
369 45.7% of amikacin-nonsusceptible isolates. These CRPA isolates must possess unreported
370 resistance genes or mutations in their genomes. A comparative genomics approach is required
371 to clarify this claim in the future to fully understand and control CRPA spread.

372 Limitations of this research include the inability to evaluate whether CRPA isolates are
373 associated with colonization or infection (the cause of disease) in patients when collecting
374 isolates from hospitals, and the lack of investigation into the effectiveness of new antimicrobial
375 agents such as cefiderocol, ceftolozane-tazobactam, and ceftazidime-avibactam against CRPA
376 isolates carrying chromosomal resistance mutations and carbapenemases. Further surveillance
377 studies will require new study designs to address these limitations.

378 **Conclusions**

379 The first nationwide surveillance for CRPA in Japan demonstrates Japan to be a low-prevalence
380 country for carbapenemase-producing CRPA. Although the CRPA largely consists of many minor
381 STs in Japan, the presence of globally common CG/ST and all top 10 high-risk clones was noted.
382 This underscores the need for continued and comprehensive surveillance. A high-risk clone
383 ST235 has a distinguished feature among STs to become DTR relatively easily and capture
384 carbapenemase and aminoglycoside resistance genes. The carbapenem resistance mechanisms
385 of CRPA cannot be fully explained by known resistance genes and mutations. The dataset built
386 in this study provides the national epidemiological features and serves as a foundation for
387 future studies on global epidemiology, therapeutic options, and infection control.

388

389 **MATERIALS AND METHODS**

390 **Strains, media, and plasmids.** CRPA candidates participating in our genome surveillance
391 program (the Japan Antimicrobial-Resistant Bacterial Surveillance [JARBS]) and associated with
392 isolation source and date information were transferred from hospitals to the NIID AMR center.
393 As part of this program, hospitals were requested to transfer all isolates showing an imipenem

394 or meropenem MIC ≥8 in the initial test, limited to one isolate per patient. A total of 668 viable
395 isolates were received at the AMR-RC between 2019 and 2020. Since the JARBS-PA project
396 commenced in mid-2019, the dataset primarily consists of isolates identified in 2020.
397 Subsequently, 273 isolates exhibiting a meropenem MIC < 8 upon remeasurements at AMR-RC
398 were excluded from the dataset to align with the definition of CRPA used in other surveillance
399 studies (8, 16, 17). Additionally, three isolates lacked linked isolation source data, and 10
400 isolates with poor draft genome assembly quality (L90 > 150) were excluded. The final CRPA
401 dataset comprises 382 isolates with linked isolation source data.

402 Single colony isolation was performed if necessary, using BD BBL™ BTB Lactose-
403 Contained Agar Medium (also called Drigalski agar) (Becton, Dickinson and Company, Franklin
404 Lakes, NJ, USA). BD BBL™ Muller–Hinton II (Cation adjusted) Broth (Becton, Dickinson and
405 Company) was used to culture *P. aeruginosa* strains in liquid. Table S1 contains the *P.*
406 *aeruginosa* strains and associated metadata.

407 *E. coli* strain DH5α [F^-, Φ80d*lacZ*ΔM15, Δ(*lacZYA-argF*)U169, *deoR*, *recA*1, *endA*1,
408 *hsdR*17(r_K^-, m_K^+), *phoA*, *supE*44, λ^-, *thi*-1, *gyrA*96, *relA*1] was used for DNA cloning and
409 functional analysis of bla_{IMP-98}. DH5α was cultured using BD Difco™ LB, Lennox Broth and agar
410 (Becton, Dickinson and Company). A gene cassette array of bla_{OXA-10}-*acc(6')*-bla_{IMP-98} in *P.*
411 *aeruginosa* ST235 strain JBBCAEG-19-0032 was PCR-amplified using primers blaIMP_F: 5'-
412 TCGAGCTCGGTACCCCGCTACTTGAAGTGTTGACGC-3' and blaIMP_R: 5'-
413 CTCTAGAGGATCCCCTTAGTTGCTTGGTTTTGATGGTTTTTTACTTTCG-3', and KODone polymerase
414 (TOYOBO CO., LTD., Osaka, Japan). A low-copy number vector pMW218 (*nptII*, pSC101 replicon;
415 NIPPON GENE CO., LTD., Tokyo, Japan) was also PCR-amplified with primers pMW_mcs_F: 5'-

416 GGGTACCGAGCTCGAATTCGTA-3′ and pMW_mcs_R: 5′-GGGGATCCTCTAGAGTCGACCT-3′. The two
417 PCR products were joined using NEBuilder (New England Biolabs, Ipswich, MA, USA). The
418 resulting construct was named pHY1399. A segment on pHY1399 was PCR-amplified to delete
419 the *bla*$_{OXA-10}$-*acc(6′)-Ib* region using 5′ phosphorylated primers P_Integ_del_F: 5′P-
420 CATGGCACCTTCTTGGTGGCTAACG-3′, and P_Integ_del_R: 5′P-GAGCGAACACGCAGTGATGCC-3′,
421 then the purified PCR product was ligated using Ligation High ver.2 (Toyobo) and introduced into
422 DH5α. The resulting recombinant plasmid obtained from one transformant was named
423 pHY1401.
424 **Antimicrobial susceptibility test.** To obtain MICs of antipseudomonal agents, CRPA strains were
425 propagated on Drigalski agar and incubated for 1 day at 35°C. Then, a single colony was
426 inoculated into MicroScan Neg MIC NF-1J panel using Prompt Inoculation System D (Beckman
427 Coulter Inc., Brea, CA, USA). Inoculated panels were incubated at 35°C for 18 h in MicroScan
428 Walkway40 Plus (Beckman Coulter Inc.) or MicroScan Walkway DxM 1096 (Beckman Coulter
429 Inc.). To cover a therapeutically achievable range, the antimicrobial susceptibility testing was
430 designed to measure major antimicrobials using four dilutions for amikacin and colistin, and five
431 dilutions for other antimicrobials.
432 To obtain β-lactam MIC against two *E. coli* strains DH5α (pMW218) and DH5α
433 (pHY1401), the strains were first propagated on LB agar containing kanamycin (25 μg/ml) and
434 incubated at 37°C overnight, then the colonies were diluted and inoculated into the Neg MIC
435 EN-2J panel containing β-lactams listed in Table 2 and incubated in MicroScan Walkway40 Plus
436 (Beckman Coulter Inc.). Breakpoint criteria for susceptible (S), intermediate resistance (I), and
437 resistance (R) followed the CLSI 2022 guidelines M100-Ed32. The definition of DTR-*P.*

438 *aeruginosa* followed Tamma *et al.*'s criteria (24): nonsusceptible to all the eight antimicrobials
439 belonging to either five antipseudomonal agent categories, and is based on the following MIC
440 criteria: piperacillin/tazobactam ≥32/4 μg/ml, ceftazidime ≥16 μg/ml, cefepime ≥16 μg/ml,
441 meropenem ≥4 μg/ml, imipenem ≥4 μg/ml, aztreonam ≥16 μg/ml, ciprofloxacin ≥1 μg/ml, and
442 levofloxacin ≥2 μg/ml (I or R in CLSI 2022). The definition of MDR was modified from
443 Magiorakos *et al.*'s criteria (25) as follows: nonsusceptible (CLSI 2022) to at least one agent in at
444 least three categories except for polymyxin listed in Table 1.
445 Notably, the equivalence of the Beckman MicroScan panel system to the broth dilution
446 method per antimicrobial, and received the following 510(k) numbers from The United States
447 Food and Drug Administration: imipenem, K162740; meropenem, K192355; doripenem,
448 K101425; levofloxacin, K193358; ciprofloxacin, K193536; ceftazidime, K202343; piperacillin-
449 tazobactam, K955910; amikacin, K862140; tobramycin, K862140; gentamycin, K862140;
450 aztreonam, K863776; cefepime, K962150; and colistin, N62-160/S1. Essential agreement
451 between the MicroScan Walk away method and the reference method for *P. aeruginosa* was
452 reported to be 91.3%–96.8% (48–51)
453 **Genome sequencing.** Draft genome was determined for all 382 CRPA isolates. For this purpose,
454 *P. aeruginosa* strains were cultured in Muller–Hinton II broth overnight at 35°C. Cells were
455 collected in 1.5 mL tubes and resuspended in a lysis solution containing lysozyme and RNaseA,
456 then incubated at 37°C for 1 h. After adding proteinase K and SDS, the reaction mixture was
457 incubated at 55°C overnight. Genomic DNA was purified from the cell lysates using Agencourt
458 AMPure XP beads (Beckman Coulter Inc.) following the protocol recommended by the
459 manufacturer. NGS libraries were constructed using Enzymatics 5× WGS fragmentation mix and

460 WGS ligase reagents (Qiagen, Hilden, Germany), and then libraries were sequenced on HiSeq X
461 Five platform in Macrogen Japan (Tokyo, Japan). Low-quality reads were removed from the
462 original reads using fastp (52), after which the trimmed reads were assembled using Shovill
463 pipeline (https://github.com/tseemann/shovill/tree/master) set to default options. Draft
464 genome assemblies fulfilled L90 < 150.

465 To determine the complete genome sequence of strain JBBCAEG-19-0032, genomic DNA
466 was extracted using the Monarch HMW DNA Extraction Kit for Tissues (New England Biolabs,
467 Ipswitch, MA, USA). Long-read sequencing was performed on the GridION platform (Oxford
468 Nanopore Technologies, Oxford, UK). The library was prepared using the Rapid Barcoding Kit,
469 and sequencing was performed using the R9.4.1 flow cell (Oxford Nanopore Technologies).
470 Basecalling was performed using the high-accuracy base calling model of Guppy v5.0.12 (Oxford
471 Nanopore Technologies). The complete sequence of JBBCAEG-19-0032 was obtained through
472 long-read-only assembly using Flye v. 2.9-b1768, set to the "--nano-raw" option (53), followed
473 by polishing with Illumina reads using Pilon v. 1.22 (54).

474 **Data analysis.** The PubMLST ST number was assigned by searches against the PubMLST
475 database (26). When hit ST was not present in PubMLST, MLST profiles of the isolates were
476 submitted to PubMLST, after which new ST numbers (ST4091-ST4125) were assigned to the new
477 MLST profiles. The relationship between MLST profiles was represented as a minimum spanning
478 tree by GrapeTree software (55). Acquired antimicrobial resistance genes in draft genome
479 assemblies were searched for using AMRFinderPlus (database ver. 2023-04-17.1) with the "--
480 organism Pseudomonas_aeruginosa" option, otherwise default setting (56). The
481 presence/absence and integrity of specific chromosomal genes (frameshift mutations, nonsense

482 mutations, and gene fusions caused by substitutions at stop/start codon positions) were
483 determined by tblastn searches against an in-house generated nucleotide database using blast
484 v2.12.0 (57), and the following retrieval of hit sequences and inspection of the alignments. The
485 RefSeq/GenBank accession numbers of blast queries used are as follows: ExoU,
486 WP_034024595.1; ExoS, NP_252530.1; FtsI (PBP3), WP_003094139.1; MexR, NP_249115.1;
487 NalC, NP_252410.1; NalD, NP_252264.1; OprD, NP_249649.1; MexZ, WP_003088626.1. The
488 alignments were generated using MAFFT v 7.490 with the einsi option (58). The integron
489 integrase gene and *attC* were searched for using IntegronFinder 2.0.2 with the default
490 parameter setting (59). Secondary structure predictions for SE-associated proteins were
491 conducted using PROMAL3D (60). Gene synteny was visualized using
492 GenomeMatcher(61).
493 The genetic distance between two draft genomes was evaluated based on Mash
494 distance (27) using Mash's "mash dist" function with default parameter setting (k =21, s = 1000).
495 Importantly, Mash distance serves as an approximation of 1- average nucleotide identity (ANI)
496 (27). When required, we used Shovill genome assemblies of global CRPA isolates as external
497 data (BioProject accession: PRJNA824880) (8). The Mash distance matrix of the 382 Japanese
498 CRPA isolates and subsets from Reyes *et al*'s CRPA isolates were provided in the Zenodo
499 repository.
500 **Data availability.** Raw sequence reads generated in this study are deposited under DDBJ
501 Sequence Read Archive accession number DRA017464 and DRA017994. Draft genome
502 assemblies and assembly quality statistics are available from Zenodo repository [DOI:
503 10.5281/zenodo.10693593] (62). PubMLST allele profiles of 382 isolates and sequence

504 alignments of *oprD*, *mexR*, *mexZ*, *nalC*, *nalD*, *ftsI*, and Mash distance matrices are available from
505 Zenodo repository [DOI: 10.5281/zenodo.10693794] (63). The nucleotide sequence of $bla_{\text{IMP-98}}$
506 is deposited under GenBank/DDBJ/EMBL accession number LC740578.1.
507 Complete sequence of strain JBBCAEG-19-0032 is deposited under GenBank/DDBJ/EMBL
508 accession number AP029374.
509

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528 **ETHICAL STATEMENT**

529 This study (JARBS) was reviewed and approved by the Institutional Review Board of the
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532

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746

747 **SUPPLEMENTAL MATERIAL**

748 **Table S1**. Susceptibility profile, genotype, and isolation source of 382 CRPA isolates.

749 **Table S2**. Summary of susceptibility profile of DTR isolates.

750 **FIG S1.** Secondary structure alignment of IntA (JBP_33480 product) and IntB (JBP_33500
751 product) of JBBCAEG-19-0032 with known tyrosine recombinases. R-H-R-Y motif was highlighted
752 by yellow background. The alignment was generated using PROMAL3D. e: sheet; h, helix.

753 **FIG S2.** Secondary structure alignment of JBP_33490 product of JBBCAEG-19-0032 with known
754 Tfp homologs. The alignment was generated using PROMAL3D. e: sheet; h, helix.

755 **FIG S3.** Secondary structure alignment of JBP_33510 of JBBCAEG-19-0032 with known Srap
756 homologs. The alignment was generated using PROMAL3D. e: sheet; h, helix.

757

758 **FIGURE LEGENDS**

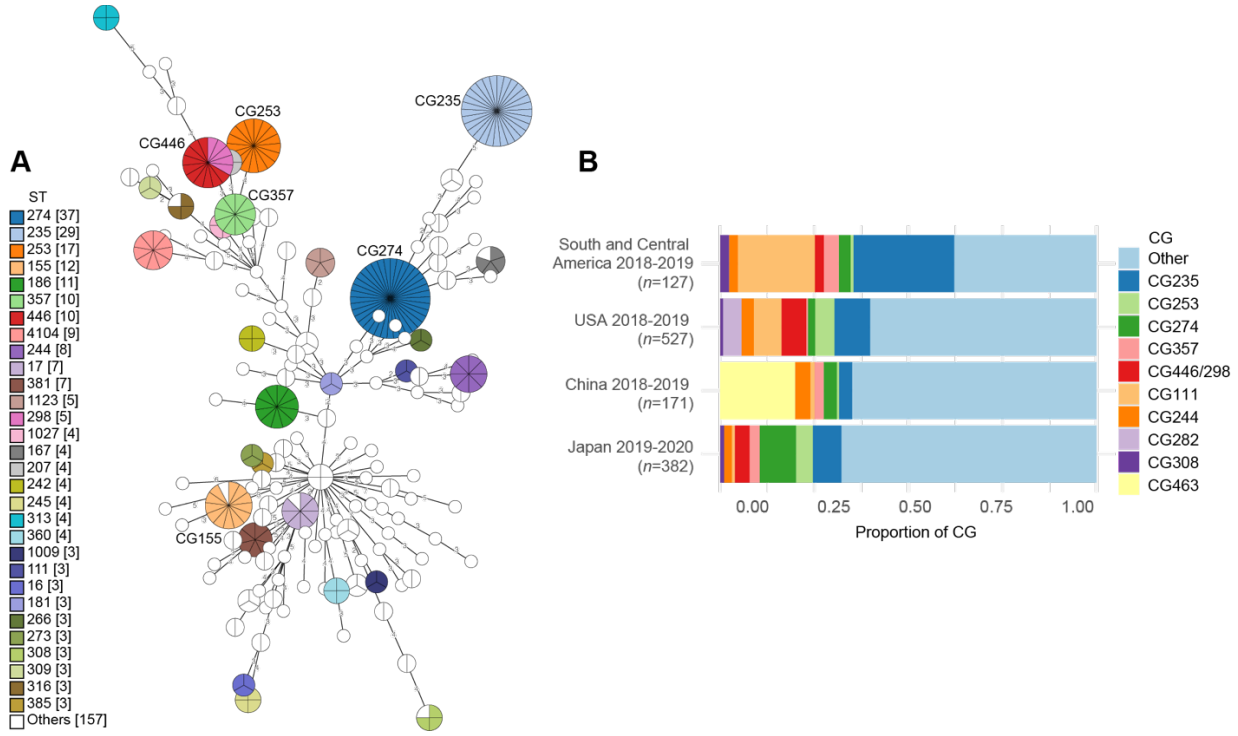
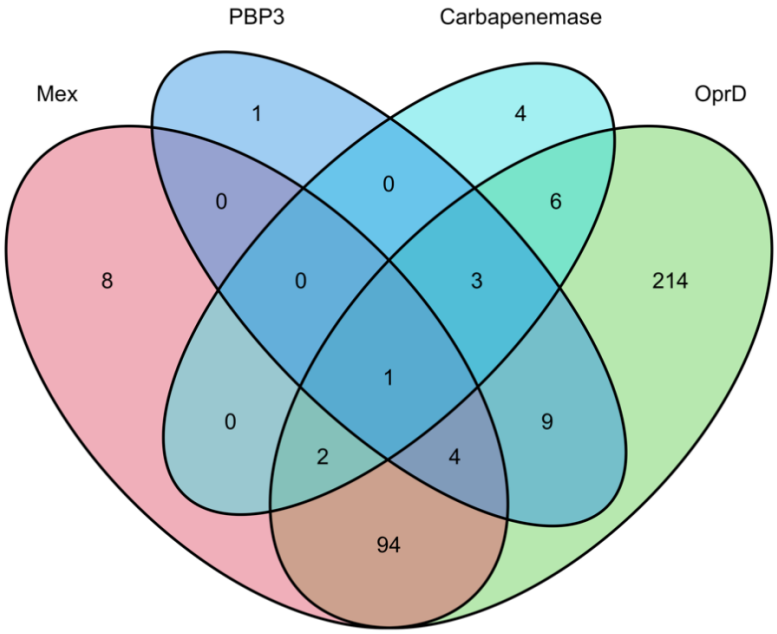


FIG 1 (A) Minimum spanning tree of 382 meropenem-resistant CRPA isolates based on MLST profiles. Each node represents sequence type (ST), otherwise clonal group (CG). STs with one allele difference were collapsed into one node as CG. Node size is proportional to the number of isolates. The number on the branch denotes the number of different alleles at the 7 MLST loci between two nodes. (B) Proportions of 10 globally most common CGs in Japan and other regions. Proportion of CGs in the USA, China, and South and Central America are based on Reyes *et al.*'s report (8).



768

769 **FIG 2.** Venn diagram showing the simultaneous occurrence of resistance mutations in

770 meropenem-resistant *P. aeruginosa*. Numbers in circles represent the numbers of isolates

771 having an acquired carbapenemase, a point mutation registered in AMRFinderPlus (56), or

772 inactivating mutations. The "Mex" category indicates the presence of a mutation in at least one

773 of three loci (*mexR*, *nalC,* and *nalD*) encoding a negative regulator for the *mexAB* operon. The

774 "PBP3" category indicates the presence of a mutation in *ftsI*. The acquired

775 carbapenemase/MBLs detected are GES-5, IMP-1, IMP-10, IMP-7, IMP-34, and IMP-98.

776

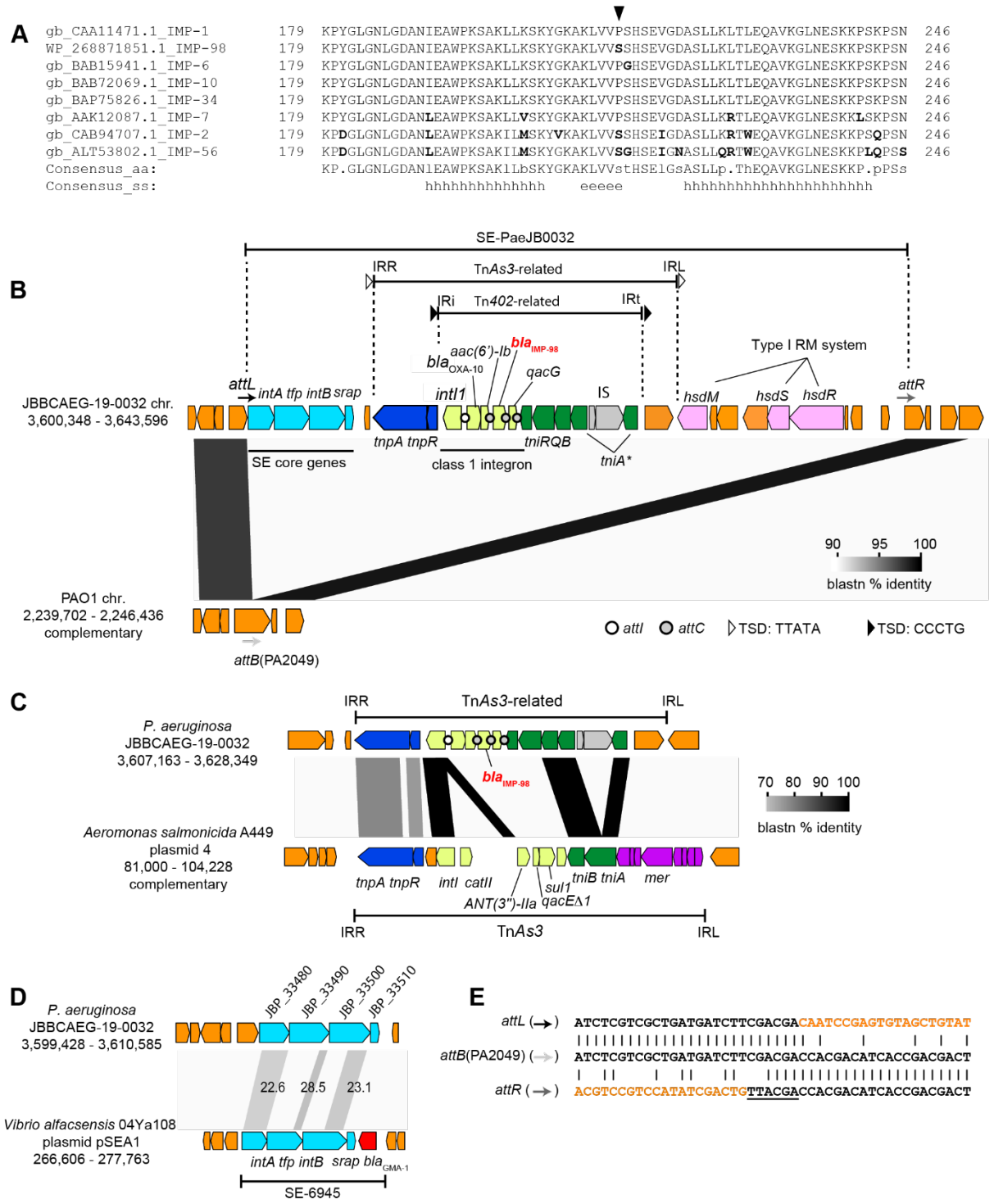


FIG 3 (A) Alignment of carboxyl-terminal regions of IMP variants. H, helix; e, sheet. IMP-98 differs from IMP-1 only at P213 indicated by an arrowhead. (B) Genetic context of *bla*$_{\text{IMP-98}}$. *tni* genes in green are associated with the transposition of the Tn*402*-related element. *tniA**

782 indicates disrupted *tniA*. *tnpA* and *tnpR* genes are associated with the transposition of the
783 Tn*As3*-related transposon. The left four genes in the insertion in JBBCAEG-19-0032 are SE core
784 genes: *intA*, encoding tyrosine recombinase; *tfp*, tyrosine recombinase fold protein; *intB*, large
785 tyrosine recombinase; *srap*, SE-associated recombination auxiliary protein (34). bla_{OXA-10} and
786 *aac(6')-Ib* in integon overlapped by 23 bp. *intI* and *attC* were identified by IntegronFinder2.0
787 (59). (C) Comparison between Tn*As3* and Tn*As3*-realted elements in the JBBCAEG-19-0032
788 chromosome. Integron-associated genes are shown in light green. *attI* and *attC* sites were not
789 found in Tn*As3. mer* genes in purple are mercury resistance genes.
790 (D) Similarity of SE core genes between SE-PaeJB0032 and SE-6945 (33). The number indicates
791 percentage identity in BLASTp. (E) Comparison of border regions between SE-PaeJB0032 and
792 chromosome (*attL*, *attR*) with unoccupied integration site (*attB*) in PAO1. Sequences in orange
793 are putative SE regions. The underlined sequence is a putative 6-bp footprint generated by SE
794 integration (33, 34). Sequences are obtained from the following accession numbers: JBBCAEG-
795 19-0032 chromosome, AP029374; PAO1 chromosome, NC_002516.2; pSEA1, AP024167; Tn*As3*,
796 CP00654.1.
797

798 **TABLES**

799 **TABLE 1.** Antimicrobial susceptibilities of 382 meropenem-resistant CRPA isolates.

Antimicrobial agents	Percentage of susceptibility group (number of isolates)[a]		
	S	I	R
Antipseudomonal carbapenems			
Imipenem	1.0 (4)	2.9 (11)	96.1 (367)
Meropenem	0.0 (0)	0.0 (0)	100 (382)
Doripenem	10.7 (41)	36.6 (136)	53.7 (205)
Antipseudomonal cephalosporins			
Ceftazidime	53.1 (203)	12.8 (49)	34.0 (130)
Cefepime	47.4 (181)	27.2 (104)	25.4 (97)
Antipseudomonal penicillin + inhibitor			
Piperacillin-tazobactam (CLSI 2022)	52.9 (202)	24.3 (93)	22.8 (87)
Piperacillin-tazobactam (CLSI 2023)	52.9 (202)	11.0 (42)	35.3 (138)
Monobactam			
Aztreonam	18.3 (70)	29.8 (114)	51.8 (198)
Antipseudomonal fluoroquinolones			
Levofloxacin	27.5 (105)	20.7 (79)	51.8 (198)
Ciprofloxacin	26.4 (101)	35.6 (136)	38.0 (145)
Aminoglycosides			
Gentamycin (CLSI 2022)	63.4 (242)	22.3 (85)	14.4 (55)
Tobramycin (CLSI 2022)	88.7 (339)	2.1 (8)	9.2 (35)
Tobramycin (CLSI 2023)	51.6 (197)	31.7 (121)	16.8 (64)
Amikacin (CLSI 2022)	88.0 (336)	4.2 (16)	7.1 (27)
Amikacin (CLSI 2023)[b]	75.0 (54)	12.5 (9)	12.5 (9)
Polymyxin			
Colistin	-	99.2 (379)	0.8 (3)

800 [a] Unless mentioned, criteria for S/I/R are identical between CLSI M100-Ed32 (2022) and M100-

801 Ed33 (2023).

802 [b] Amikacin MIC against urine-associated isolates only (*n* = 72, linked to the source categories:

803 urine, urine collected by catheter, urine obtained from indwelling catheter, catheterized urine,

804 and urethral discharge in Table S1).

805

806

807 **TABLE 2.** β-lactam MICs against *E. coli* strain carrying bla_{IMP-98}.

Antimicrobials	DH5α(pMW218)	DH5α(pHY1401)[a]
Ampicillin	≤4	>16
Ampicillin-sulbactam	≤4/2	>16/8
Amoxicillin-clavulanate	≤8/4	>16/8
Piperacillin	≤4	32
Piperacilin-tazobactam	≤4	8
Cefazolin	≤1	>16
Cefotiam	≤8	16
Cefaclor	≤8	>16
Cefmetazole	≤4	>32
Cefotaxime	≤0.5	>2
Ceftriaxone	≤0.5	>2
Ceftazidime	≤1	>8
Cefpodoxime	≤1	>4
Cefditoren	≤1	>2
Cefoperazone-sulbactam	≤8/4	>32/16
Cefepime	≤1	8
Imipenem	≤0.5	2
Meropenem	≤0.25	2
Ertapenem	≤0.25	>1
Flomoxef	≤8	32
Aztreonam	≤1	≤1

808 [a] pHY1401 is a pMW218 derivative carrying bla_{IMP-98} and the putative promoter region of class 1

809 integron in strain JBBCAEG-19-0032.

810
811

812 **Table 3.** Isolation sources observed in two major STs.

Source category group	ST274	ST235	Other STs
Oral/Endotracheal/Respiratory	23	9	157
Urinary/Genital	4	12	60
Blood/Fluids	1	3	36
Other sources	9	5	63
Total	37	29	316

813 [a] More specific source categories are shown in Table S1.

814

815

816 **Table 4.** Observation frequency of specific resistance phenotype, genes, or mutations in two

817 major ST groups.

Phenotype or genotype	Percentage of relevant isolates in ST (number of isolates)			Adjusted *P*-value[a]
	ST274 (total 37)	ST235 (total 29)	Other STs (total 316)	
Non-susceptibilities[b]				
Piperacillin-tazobactam	70.2 (26)	58.6 (17)	45.9 (145)	0.02309
Ceftazidime	32.4 (12)	65.5 (19)	44.3 (140)	0.04234
Ciprofloxacin	70.2 (26)	93.1 (27)	72.1 (228)	0.05972
Amikacin	13.5 (5)	62.0 (18)	7.2 (23)	0.00035
DTR	32.4 (12)	48.2 (14)	25.3 (80)	0.04235
Resistance genes/mutations				
Carbapenem				
bla_{IMP}, or bla_{GES}	2.7 (1)	34.5 (10)	1.6 (5)	0.00035
oprD[c]	89.1 (33)	96.6 (28)	86.1 (272)	0.35682
mexR, *nalC,* or *nalD*[c]	27.0 (10)	27.6 (8)	28.4 (90)	1.0
ftsI[c]	5.4 (2)	10.3 (3)	4.1 (13)	0.37338
Fluoroquinolone				
gyrA, *gryB*, *parD,* or *parC*[c]	59.5 (22)	93.1 (27)	28.5 (90)	0.00035
crpP	32.4 (12)	37.9 (11)	63.0 (199)	0.00084
Amikacin				
acc(6')-1b, *aac(6')-II*, *aac(6')-31, aacA8*, *aac(6')-Ia*, *aac(6')-Ib3*, *aph(3')-XV*, or *aac(6')-Iae*	0 (0)	58.6 (17)	2.2 (7)	0.00035

818 [a] Chi-square test *P*-value after multiple testing corrections (false discovery rate) by Benjamini &

819 Hochberg method. The independence of two factors (phenotype/genotype and ST) was

820 evaluated by using a 2 by 3 contingency table (df = 2) where the rows are the count of qualified

821 isolates and the count of nonqualified isolates. DTR: difficult-to-treat resistance.

822 [b] Breakpoint criteria follows CLSI M100-ed 32 (2022).

823 [c] Acquired mutation in conserved genes.

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