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

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ABSTRACT

Japan is a representative country with a low prevalence rate of carbapenem-resistant *Pseudomonas aeruginosa* (CRPA). A comprehensive overview of the genotype and phenotype patterns of CRPA in the Asia-Pacific region is lacking. Herein, we conducted genome sequencing and standardized 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*. Only 4.1% of CRPA isolates carried carbapenemase: GES-5 (2), IMP-1 (5), IMP-10 (4), IMP-7 (3), IMP-34 (1), and IMP-98 (1). Regarding chromosomal

23 mutations, 87.1% of CRPA isolates possessed inactivating or other resistance mutations in *oprD*,
24 and 28.8% showed mutations in the regulatory genes (*mexR, nalC*, and *nalD*) for the MexAB-
25 OprM efflux pump. Additionally, 4.7% of CRPA isolates carried a known resistance mutation in
26 the PBP3-encoding gene *ftsI*. Of the 148 sequence types detected, ST274 (9.7%) was
27 predominant, followed by ST235 (7.6%) and ST253 (4.5%). The findings from this study and
28 other surveillance studies collectively demonstrate that CRPA exhibits marked genetic diversity
29 and that its multidrug resistance in Japan is less perceived than in other regions. This study
30 contributes a valuable dataset that addresses a gap in genotype/phenotype information
31 regarding CRPA in the Asia-Pacific region, where the epidemiological background markedly
32 differs between regions.
33

34 **INTRODUCTION**

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

44 elements (ICEs), after which it elicits high levels of resistance to multiple antimicrobials, thereby
45 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). A recent cohort study revealed that
50 patients infected with carbapenemase-producing CRPA have a 9% higher 30-day mortality rate
51 than patients infected with carbapenemase-nonproducing CRPA (7).

52 Carbapenem resistance of *P. aeruginosa* often involves inactivating mutation in several
53 chromosomal genes: inactivation of OprD porin leads to inefficient penetration of the outer
54 membrane by carbapenems (8), and inactivating mutation in two direct or one indirect
55 transcriptional regulators (repressors) MexR, NalD, and NalC induces increased production of
56 the MexAB-OprM efflux pump, thereby increasing β-lactam minimum inhibitory concentration
57 (MIC) (9–11). Furthermore, an experimental evolution study showed that nonsynonymous
58 substitutions in *ftsI*, an essential gene encoding PBP3, contribute to carbapenem resistance (12).
59 Notably, the extent to which these mutations have become prevalent in *P. aeruginosa* genomes
60 in clinical settings remains largely unknown.

61 Certain sequence types (STs) of *P. aeruginosa* are widely recognized as global high-risk
62 clones due to their high mortality rates and propensity to acquire multidrug resistance (MDR)
63 and to transmit worldwide (13). The top ten global high-risk clones are ST111, ST175, ST233,
64 ST235, ST244, ST277, ST298, ST308, ST357, and ST654 (13, 14). International consortiums have
65 made efforts to build a large *P. aeruginosa* dataset to obtain quantitative information for CRPA

66 prevalence, its susceptibility profiles, and the possession rate of carbapenemases (7, 15–17);
67 here, both class A group 2f β-lactamase and class B group 3 β-lactamase (so called metallo-β-
68 lactamases, MBLs) are included for simplicity. Globally disseminated carbapenemase types in *P.*
69 *aeruginosa* include VIM, IMP, NDM, GES, and KPC; however, the carbapenemase possession rate
70 and the relative frequencies of carbapenemase types differ between geographic regions (7, 16,
71 17). Notably, in the Asia-Pacific region, the Antimicrobial Testing Leadership and Surveillance
72 (ATLAS) program took the initiative of *P. aeruginosa* surveillance, revealing distinct patterns of
73 resistance rate and predominant β-lactamase levels in the *P. aeruginosa* population between 12
74 countries/regions, but the ATLAS did not cover genome surveillance (16).
75 Japan exhibits characteristic features in the epidemiology of antimicrobial-resistant
76 bacterial infection in the Asia-Pacific region. Regarding carbapenem resistance, the prevalence
77 of meropenem-resistant *E. coli* in bloodstream infections in Japan as of 2019 was 2 per 100,000
78 test patients, which is lower than, for example, 79 per 100,000 test patients observed in
79 Singapore, which is located in Southeast Asia, according to the WHO Global Antimicrobial
80 Resistance and Use Surveillance System (18). The established national phenotypic antimicrobial
81 resistance surveillance program—the Japan Nosocomial Infections Surveillance (JANIS) (19,
82 20)—reported that the carbapenem (meropenem) resistance rate of *P. aeruginosa* clinical
83 isolates in Japan, as of 2020, was 10.5%. This was rather low among representative countries
84 where antimicrobial resistance surveillance for *P. aeruginosa* was once or is currently being
85 performed; the resistance rate was 29.3% in India (ATLAS 2015–2019), 19.3% in China (CHINET
86 2020), 6.5% in Australia (ATLAS 2015–2019), 28.5% in Poland (EARS-net 2020), 15.7% in the
87 Czech Republic (EARS-net 2020), 13.9% in Germany (EARS-net 2020), 12.6% in France (EARS-net

88 2020), and 3.7% in Finland (EARS-net 2020) (16, 21, 22). Previous nationwide surveillance
89 studies of *P. aeruginosa* in Japan primarily focused on amikacin-nonsusceptible MDR *P.*
90 *aeruginosa* or isolates from respiratory system origin only (23–25). Therefore, a comprehensive
91 overview of the resistance phenotype, STs, carbapenemase presence, and chromosomal
92 resistance mutations of more common CRPA in Japanese clinical settings remains lacking.
93 To obtain an overview of both genomic features and resistance phenotypes of bacterial
94 pathogens in Japan, we initiated the national bacterial genome surveillance—Japan
95 Antimicrobial-Resistant Bacterial Surveillance (JARBS)—in 2019 (26). Through the JARBS-PA
96 project performed in 2019 and 2020, 382 meropemen-resistant CRPA isolates were collected
97 from 78 hospitals across Japan. Herein, we aimed to clarify the genotype and susceptibility
98 patterns of the CRPA isolates by conducting genome sequencing and wide-range MIC
99 remeasurements using the same susceptibility panel.
100
101 **RESULTS**
102 **Nationwide sampling.** From 2019 to 2020, more than 600 *P. aeruginosa* isolates were
103 transferred from Japanese hospitals to the Antimicrobial Resistance Research Center (AMR-RC)
104 at the National Institute of Infectious Diseases (NIID) based on the following criterion:
105 meropenem or imipenem MIC ≥8μg/ml in initial testing at the isolation site. All the collected
106 isolates were tested for antipseudomonal agent MICs using the Beckman MicroScan system at
107 AMR-RC. In total, 382 *P. aeruginosa* isolates showing meropenem MIC ≥8μg/ml in the tests at
108 AMR-RC were defined as CRPA and focused on in this study. The 382 CRPA isolates originated
109 from nonoverlapping patients in 78 hospitals (the number of collected isolates per island was as

110 follows: *n* = 30 from Hokkaido Island, *n* = 307 from Honshu Island, *n* = 7 from Shikoku Island, *n* =
111 32 from Kyusyu Island, and *n* = 6 from Okinawa Island). Source specimens were classified into
112 one of the following JANIS categories (19, 20): Oral/Endotracheal/Respiratory (*n* = 189),
113 Urinary/Genital (*n* = 76), Blood/Fluid (*n* = 40), Digestive (*n* = 27), and Other (*n* = 50). Table S1
114 provides more detailed source categories and metadata for each isolate.

115 **Antimicrobial susceptibility profiles and STs of CRPA in Japan.** Table 1 summarizes the
116 susceptibilities for antipseudomonal agents. Although the collected CRPA isolates were often
117 nonsusceptible to antipseudomonal agents other than meropenem, the percentage of
118 susceptible isolates remained 52.9% for piperacillin-tazobactam, 26.4% for ciprofloxacin, and
119 88.0% for amikacin (Table 1). Among the CRPA isolates, 27.7% (106/382) were deemed difficult-
120 to-treat resistance *P. aeruginosa* (DTR-*P. aeruginosa*) in Tamma *et al.*'s definition (27), and
121 85.8% (328/382) were MDR in Magiorakos *et al.*'s definition (28). Only three CRPA isolates were
122 resistant to colistin; two of the three colistin-resistant isolates (Table S1) were however DTR-
123 *P.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 (29).
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,
131 suggesting that clonal expansion is not common. All the top ten global high-risk clones (13)

132 were detected, accounting for 17.0% of the total CRPA in Japan (Table S1). A recent study on
133 CRPA (7) proposed globally most common CGs (10 CGs in Fig. 1B). Those globally common CGs
134 comprised 34.8%. Despite being among the top two most common CGs in the USA and Central
135 South America, CG111 remains a minor CG in Japan (Fig. 1B). Surprisingly, the dominant clone
136 CG463 (ST463), prevalent in Chinese clinical settings (7, 30), was entirely absent in the 382 CRPA
137 isolates collected in this study.

138 The proportions of the top eight most common CGs in Japan are shown per phenotype
139 group in Fig. 1C. In DTR-*P. aeruginosa*, the proportion of two major CGs, CG274 and CG235,
140 increased from 9.7% (37/382) in total CRPA to 11.3% (12/106) and from 7.6% (29/382) to 13.2%
141 (14/106), respectively (Fig. 1C), suggesting their propensity to accumulate MDR. However, when
142 focused on amikacin-nonsusceptible DTR-P. *aeruginosa* isolates (n = 25), the proportion of
143 CG274 decreased to 4.0% (1/25), while the proportion of CG235 increased to 36% (9/25),
144 highlighting the difference in their potential to acquire amikacin resistance.

145 **Resistance mutations and carbapenemases in CRPA.** The molecular mechanisms of
146 meropenem resistance are primarily classified into four types: porin inactivation, efflux pump
147 overproduction, target alteration, and horizontal acquisition of β-lactamase (2). Those
148 mechanisms are respectively associated with inactivation of the *oprD* gene, inactivation of
149 either *mexR*, *nalC*, or *nalD* genes encoding transcriptional regulators (repressors) for the
150 MexAB-OprM efflux pump (9–11), nonsynonymous substitutions in *ftsI* (12), and acquisition of
151 plasmid or ICE-borne carbapenemase genes (4, 5). We searched for the presence of inactivating
152 mutations (frameshift mutations due to insertion/deletion, nonsense mutations, and
153 inactivation of start codon or stop codon) in *oprD*, *mexR*, *nalC*, and *nalD* as well as resistance

154 mutations registered in the NCBI AMRFinderPluS database, as these likely contribute to
155 increasing meropenem MIC.
156 Database searches revealed that only 4.2% (16/382) of CRPA in Japan possessed
157 acquired carbapenemases. Known carbapenemase and MBL detected were GES-5 (*n* = 2), IMP-1
158 (*n* = 6), IMP-10 (*n* = 4), IMP-7 (*n* = 3), and IMP-34 (*n* = 1). No OXA-type carbapenemases were
159 detected. ST274 CRPA isolates rarely (2.7%, 1/37) carried carbapenemase, contrasting with the
160 34.5% (10/29) possession rate of ST235 isolates. One ST235 isolate encoded a novel variant of
161 IMP-type MBL, which was assigned a new allele name IMP-98 by NCBI.
162 The prevalence of inactivating mutations or known nonsynonymous resistance
163 mutations in chromosomal genes in 382 CRPA isolates was 87.1% (333/382) for *oprD*, 14.1%
164 (54/382) for *mexR*, 9.1% (35/382) for *nalD*, and 6.3% (24/382) for *nalC*. Collectively, 28.8%
165 (110/382) of CRPA carried an inactivating mutation in at least one of the three repressors for
166 *mexAB*. The most common mutation type in *oprD* was IN/DELs causing frameshift (237/382),
167 followed by nonsense mutations (90/382); these include, for instance, W277stop (24/90),
168 W339stop (9/90), and W138stop (9/90). In addition, 4.7% (18/382) of CRPA isolates carried
169 known resistance mutations in *ftsI* (P527S, F533L, R504C, or V471G) (12).
170 A simultaneous occurrence of resistance mutations and/or carbapenemase was
171 observed (Fig. 2). While 26.4% (101/382) of CRPA isolates had an inactivating mutation in both
172 *oprD* and at least one regulator for *mexAB*, 4.5% (17/382) had both *oprD* and *ftsI* mutations,
173 with 3.1% (12/382) exhibiting the *oprD* mutation and carbapenemase. The acquisition of
174 carbapenemase alone occurred only in 0.7% (4/382) of the CRPA. Notably, 9.1% (35/382) did

175 not carry identifiable inactivating mutations in the four focused genes, known *ftsI* resistance
176 mutations, or carbapenemase.

177 IMP-98 differed from IMP-1 by only one site at the nucleotide level and likely originated
178 from the *bla*$_{IMP-1}$ gene cassette in the integron by C637T nonsynonymous substitution, leading
179 to P213S amino acid change (Fig. 3A). To deduce the origin of *bla*$_{IMP-98}$, we searched for the
180 *bla*$_{IMP-98}$-flanking sequences in the NCBI blast nr-database (2022.12.22). The top hit was an
181 integron gene cassette array found in a genome of an *E. coli* isolate from India (GenBank ID:
182 LC169568.1, Fig. 3B). This hit contains *bla*$_{IMP-1}$ instead of *bla*$_{IMP-98}$ in the gene cassette array. The
183 second hit was an integron region carrying *bla*$_{GES-1}$ found in the chromosome of the *P.*
184 *aeruginosa* ST308 isolate from Mexico (GenBank ID: CP012755.1). This region was a class 1
185 integron as the contig contained specific motifs of *attI1* (5′-
186 GTTATGGAGCAGCAACGATGTTACGCAGCAGGGCAGTCGCCCTAAAACAAAGTTAGGC-3′: 7-bp core
187 sites are underlined) (30). Thus, *bla*$_{IMP-98}$ might have originated from an ancestral integron like
188 that in LC169568.1.

189 To test whether IMP-98 is functional, *bla*$_{IMP-98}$ was cloned and introduced into the drug-
190 susceptible *E. coli* strain DH5α, after which the susceptibility for various β-lactams was
191 investigated (Table 2). MICs of β-lactams except for aztreonam were considerably higher against
192 *E. coli* DH5α carrying *bla*$_{IMP-98}$ than the control strain, confirming that IMP-98 retains the wide
193 substrate range of MBL.

194 **Other resistance determinants.** Among 46 amikacin-nonsusceptible CRPA isolates, 28.3%
195 (13/46) possessed known amikacin resistance genes *aac(6′)* variants, resistance mutations such
196 as *fusA1* (Q455R) and *amgS* (V121G) (31), or inactivating mutation in *mexZ* encoding a repressor

197 for the MexXY efflux pump contributing to aminoglycoside resistance (32) (Table S1). However,
198 73% (33/46) of amikacin-nonsusceptible CRPA did not possess identifiable amikacin resistance
199 genes or mutations. Among 281 ciprofloxacin nonsusceptible CRPA isolates, 74.3% (209/281)
200 possessed at least one of the quinolone resistance gene *crpP* (33) (166/281) and resistance
201 mutations in *gyrA* (T83I, D87N, or D87G; 99/281), *gyrB* (E468D, S466F, or S466Y; 13/281), or
202 *parE* (A473V; 4/281) (Table S1). Three colistin-resistant isolates did not possess any known
203 colistin resistance mutations or genes.
204
205 **DISCUSSION**
206 Before the global launch of national action plans on antimicrobial resistance, the endemic
207 spread of ST235 clones was occasionally reported in Japan (34–36). This was in part because the
208 Ministry of Health, Labor and Welfare of Japan monitors the occurrence of amikacin-
209 nonsusceptible, carbapenem and fluoroquinolone-resistant *P. aeruginosa* as a part of the
210 National Epidemiological Surveillance of Infectious Diseases program (37). However, national
211 surveillance of clinically more common CRPAs remained lacking until the present study. As a
212 country with a low prevalence rate of meropenem-resistant CRPA, Japan is expected to have a
213 low carbapenemase possession rate of *P. aeruginosa*. This study demonstrates that the
214 carbapenemase possession rate of meropenem-resistant CRPA in Japan is 4.2%, which is among
215 the lowest in the regions where surveillance studies have been performed to date: the
216 carbapenemase possession rate of meropenem-resistant CRPA was 32.9% in the Asia-Pacific
217 region in 2015–2019 (16), 1.9% for the USA in 2018–2019 (7), 69% for South and Central

218 America in 2018–2019 (7), 32% for China in 2018–2019 (7), and 21.9% for Europe in 2017–2019
219 (17).

220 The ciprofloxacin and amikacin susceptibility rates regarding CRPA were 26.4% and 88.0%,
221 respectively, in Japan. These values exceed 20.6% and 60.6% of pooled CRPA values reported in
222 the Asia-Pacific region (16), and are then equivalent to and higher than 26.2% and 63.4% of the
223 global CRPA population, respectively (17). Furthermore, the piperacillin-tazobactam
224 susceptibility rate in Japan was 52.9%, which contrasts with the 25.3% susceptibility rate in the
225 global CRPA population (17). This difference might reflect the difference in the carbapenemase
226 possession rate in CRPA between Japan and other regions. Therefore, in Japan, MDR is not
227 heavily perceived in the *P. aeruginosa* population in clinical settings compared with other Asia-
228 Pacific regions or the world average.

229 The most frequent ST in the meropenem-resistant CRPA in Japan was ST274 but not
230 ST235 (n = 37 vs. n = 29). ST274 and CG274 members have frequently been detected in clinical
231 settings worldwide (14) and are also known for chronic infection and intrabody evolution
232 involving MDR in patients with cystic fibrosis in Europe (38, 39). We do not know whether the
233 ST274 isolates collected in this study are associated with a chronic lung infection, but ST274 was
234 rather overrepresented in the "Oral/Endotracheal/Respiratory" source group (odds ratio = 1.84,
235 P = 0.054 in one-tailed Fisher's exact test). Since patients with cystic fibrosis are rare in Japan,
236 ST274 might generally be fitter in the respiratory systems than other STs.

237 Inactivating mutation in *oprD* was the most frequent meropenem-resistance mechanism
238 (found in 87.1%), as noted in previous genome surveillance performed in other regions; 69% of
239 meropemen-resistant CRPA possessed *oprD*-inactivating mutations and 22% possessed acquired

240 carbapenemase (7). We show that in Japan, not only *oprD*-inactivating mutations but also efflux
241 pump regulator inactivation and *ftsI* resistance mutations are more common carbapenem-
242 resistance mechanisms than the horizontal acquisition of carbapenemases. Therefore, future
243 surveillance of *P. aeruginosa* should prioritize the study of chromosomal mutations.

244 Since several countries/regions normally categorized in the Asia-Pacific region are
245 geographically isolated by the presence of sea, we expected to observe distinct features of the
246 CRPA genotype in Japan and other Asia-Pacific regions. The striking feature was the
247 predominance of CG463/ST463 in China (7) and its absence in Japan (Fig. 1B). Among the
248 carbapenemase types, IMP was the most frequent (87.5%; 14/16) in Japan. This trend contrasts
249 with the predominance of NDM in *P. aeruginosa* borne-carbapenemases in India (16), KPC in
250 China (7), and VIM in Europe, Africa/Middle East, and Latin America (17).

251 Surprisingly, 9.2% (35/382) of the CRPA isolates did not possess apparent inactivating
252 mutations, known *ftsI* resistance mutations, or carbapenemase. Furthermore, known resistance
253 mutations could not account for the mechanisms of amikacin nonsusceptibility in 73% of
254 amikacin-nonsusceptible isolates. These CRPA isolates must possess unreported resistance
255 genes or mutations in their genomes. A comparative genomics approach is required to clarify
256 this claim in the future to fully understand and control CRPA spread.

257

258 **MATERIALS AND METHODS**

259 **Strains, media, and plasmids.** CRPA candidates participating in our genome surveillance
260 program (the Japan Antimicrobial-Resistant Bacterial Surveillance [JARBS]) and associated with
261 isolation source and date information were transferred from hospitals to the NIID AMR center.

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

267 *E. coli* strain DH5α [F^-, Φ80d*lacZ*ΔM15, Δ(*lacZYA-argF*)U169, *deoR*, *recA*1, *endA*1,
268 *hsdR*17(r_K^-, m_K^+), *phoA*, *supE*44, λ^-, *thi*-1, *gyrA*96, *relA*1] was used for DNA cloning and
269 functional analysis of bla_{IMP-98}. DH5α was cultured using BD Difco™ LB, Lennox Broth and agar
270 (Becton, Dickinson and Company). A gene cassette array of bla_{OXA-10}-*acc(6′)*-bla_{IMP-98} in *P.*
271 *aeruginosa* ST235 strain JBBCAEG-19-0032 was PCR-amplified using primers blaIMP_F: 5′-
272 TCGAGCTCGGTACCCCGCTACTTGAAGTGTTGACGC-3′ and blaIMP_R: 5′-
273 CTCTAGAGGATCCCCTTAGTTGCTTGGTTTTGATGGTTTTTTACTTTCG-3′, and KODone polymerase
274 (TOYOBO CO., LTD., Osaka, Japan). A low-copy number vector pMW218 (*nptII*, pSC101 replicon;
275 NIPPON GENE CO., LTD., Tokyo, Japan) was also PCR-amplified with primers pMW_mcs_F: 5′-
276 GGGTACCGAGCTCGAATTCGTA-3′ and pMW_mcs_R: 5′-GGGGATCCTCTAGAGTCGACCT-3′. The
277 two PCR products were joined using NEBuilder (New England Biolabs, Ipswich, MA, USA). The
278 resulting construct was named pHY1399. A segment on pHY1399 was PCR-amplified to delete
279 the bla_{OXA-10}-*acc(6′)-Ib* region using 5′ phosphorylated primers P_Integ_del_F: 5′P-
280 CATGGCACCTTCTTGGTGGCTAACG-3′, and P_Integ_del_R: 5′P-GAGCGAACACGCAGTGATGCC-3′,
281 then the purified PCR product was ligated using Ligation High ver.2 (Toyobo) and introduced into
282 DH5α. The resulting recombinant plasmid obtained from one transformant was named
283 pHY1401.

284 **Antimicrobial susceptibility test.** To obtain MICs of antipseudomonal agents, CRPA strains were
285 propagated on Drigalski agar and incubated for 1 day at 35°C. Then, a single colony was
286 inoculated into MicroScan Neg MIC NF-1J panel using Prompt Inoculation System D (Beckman
287 Coulter Inc., Brea, CA, USA). Inoculated panels were incubated at 35°C for 18 h in MicroScan
288 Walkway40 Plus (Beckman Coulter Inc.) or MicroScan Walkway DxM 1096 (Beckman Coulter
289 Inc.). To cover a therapeutically achievable range, the antimicrobial susceptibility testing was
290 designed to measure major antimicrobials using five dilutions (26). To obtain β-lactam MIC
291 against two *E. coli* strains DH5α (pMW218) and DH5α (pHY1401), the strains were first
292 propagated on LB agar containing kanamycin (25 μg/ml) and incubated at 37°C overnight, then
293 the colonies were diluted and inoculated into the Neg MIC EN-2J panel containing β-lactams
294 listed in Table 2 and incubated in MicroScan Walkway40 Plus (Beckman Coulter Inc.).
295 Breakpoint criteria for susceptible (S), intermediate resistance (I), and resistance (R) followed
296 the CLSI 2022 guidelines M100-Ed32. The definition of DTR-*P. aeruginosa* followed Tamma *et*
297 *al.*'s criteria (27): nonsusceptible to all the eight antimicrobials belonging to either five
298 antipseudomonal agent categories, and is based on the following MIC criteria:
299 piperacillin/tazobactam ≥32/4 μg/ml, ceftazidime ≥16 μg/ml, cefepime ≥16 μg/ml, meropenem
300 ≥4 μg/ml, imipenem ≥4 μg/ml, aztreonam ≥16 μg/ml, ciprofloxacin ≥1 μg/ml, and levofloxacin
301 ≥2 μg/ml (I or R in CLSI 2022). The definition of MDR was modified from Magiorakos *et al.*'s
302 criteria (28) as follows: nonsusceptible to at least one agent in at least three categories except
303 for polymyxin listed in Table 1.
304 **Genome sequencing.** *P. aeruginosa* strains were cultured in Muller–Hinton II broth overnight at
305 35°C. Cells were collected in 1.5 mL tubes and resuspended in a lysis solution containing

306 lysozyme and RNaseA, then incubated at 37°C for 1 h. After adding proteinase K and SDS, the
307 reaction mixture was incubated at 55°C overnight. Genomic DNA was purified from the cell
308 lysates using Agencourt AMPure XP beads (Beckman Coulter Inc.) following the protocol
309 recommended by the manufacturer. NGS libraries were constructed using Enzymatics 5× WGS
310 fragmentation mix and WGS ligase reagents (Qiagen, Hilden, Germany), and then libraries were
311 sequenced on HiSeq X Five platform in Macrogen Japan (Tokyo, Japan). Low-quality reads were
312 removed from the original reads using fastp (40), after which the trimmed reads were
313 assembled using Shovill pipeline (https://github.com/tseemann/shovill/tree/master) set to
314 default options. Genomic assemblies fulfilled L90 < 150.
315 **Data analysis.** The PubMLST ST number was assigned by searches against the PubMLST
316 database (29). When hit ST was not present in PubMLST, MLST profiles of the isolates were
317 submitted to PubMLST, after which new ST numbers (ST4091-ST4125) were assigned to the new
318 MLST profiles. The relationship between MLST profiles was represented as a minimum spanning
319 tree by GrapeTree software (41). Acquired antimicrobial resistance genes in draft genome
320 assemblies were searched for using AMRFinderPlus (database ver. 2023-04-17.1) with the "--
321 organism Pseudomonas_aeruginosa" option, otherwise default setting (42). The
322 presence/absence and integrity of specific chromosomal genes (frameshift mutations, nonsense
323 mutations, and gene fusions caused by substitutions at stop/start codon positions) were
324 determined by tblastn searches against an in-house generated nucleotide database using blast
325 v2.12.0 (43), and the following retrieval of hit sequences and inspection of the alignments. The
326 RefSeq/GenBank accession numbers of blast queries used are as follows: ExoU,
327 WP_034024595.1; ExoS, NP_252530.1; FtsI (PBP3), WP_003094139.1; MexR, NP_249115.1;

328 NalC, NP_252410.1; NalD, NP_252264.1; OprD, NP_249649.1; MexZ, WP_003088626.1. The
329 alignments were generated using MAFFT v 7.490 with the einsi option (44). The integron
330 integrase gene and *attC* were searched for using IntegronFinder 2.0.2 with the default
331 parameter setting (45).

332 **Data availability.** Raw sequence reads generated in this study are deposited under DDBJ
333 Sequence Read Archive (DRA) accession number DRA017464. Draft genome assemblies are
334 available from Zenodo repository [DOI: 10.5281/zenodo.10081563] (46). PubMLST allele
335 profiles of 382 isolates and sequence alignments of *oprD*, *mexR*, *mexZ*, *nalC*, *nalD*, and *ftsI* are
336 available from Zenodo repository [DOI: 10.5281/zenodo.10081526] (47). The nucleotide
337 sequence of bla_{IMP-98} is deposited under GenBank/DDBJ/EMBL accession number LC740578.1.
338

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353 and K.Y.; Data Curation, H.Y., N.K., and K.Y.; Methodology, Y.S.; Visualization, H.Y.; Writing -
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356 final manuscript.

357 **ETHICAL STATEMENT**

358 This study (JARBS) was reviewed and approved by the Institutional Review Board of the
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360 approved by Ministry of Health, Labor and Welfare of Japan (approval number 1553).

361

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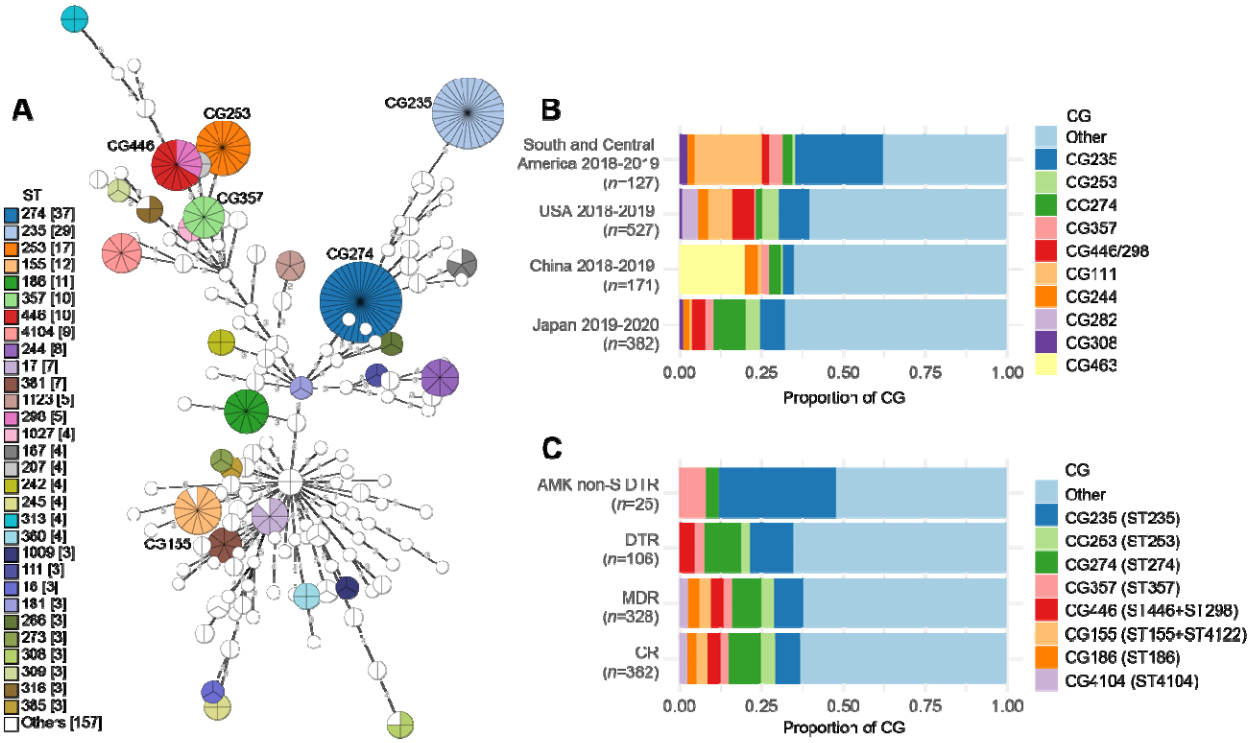
531 2019–2020. Zenodo doi: 10.5281/zenodo.10081526 Accessed Nov 13 2023.
532
533 **SUPPLEMENTAL MATERIAL**
534 **Table S1**. Susceptibility profile, genotype, and isolation source of 382 CRPA isolates.
535
536 **FIGURE LEGENDS**

537



538 **FIG 1** (A) Minimum spanning tree of 382 meropenem-resistant CRPA isolates based on MLST

539 profiles. Each node represents ST, otherwise clonal group (CG). STs with one allele difference

540 were collapsed into one node as CG. Node size is proportional to the number of isolates. The

541 number on the branch denotes the number of different alleles at the 7 MLST loci between two

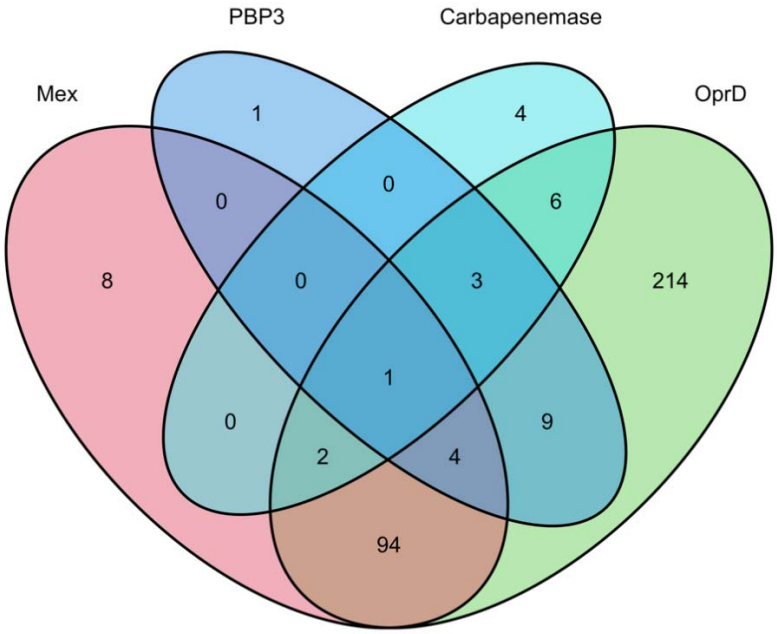
542 nodes. (B) Proportions of 10 globally most common CGs in Japan and other regions. Proportion

543 of CGs in the USA, China, and South and Central America are based on Reyes *et al.*'s report (7).

544 (C) Proportion of eight most common CGs in four phenotype groups in Japan. AMK non-S DTR,

545 AMK nonsusceptible DTR-*P. aeruginosa*.

546



547

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

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

550 having an acquired carbapenemase, a point mutation registered in AMRFinderPlus (42), or

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

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

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

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

555

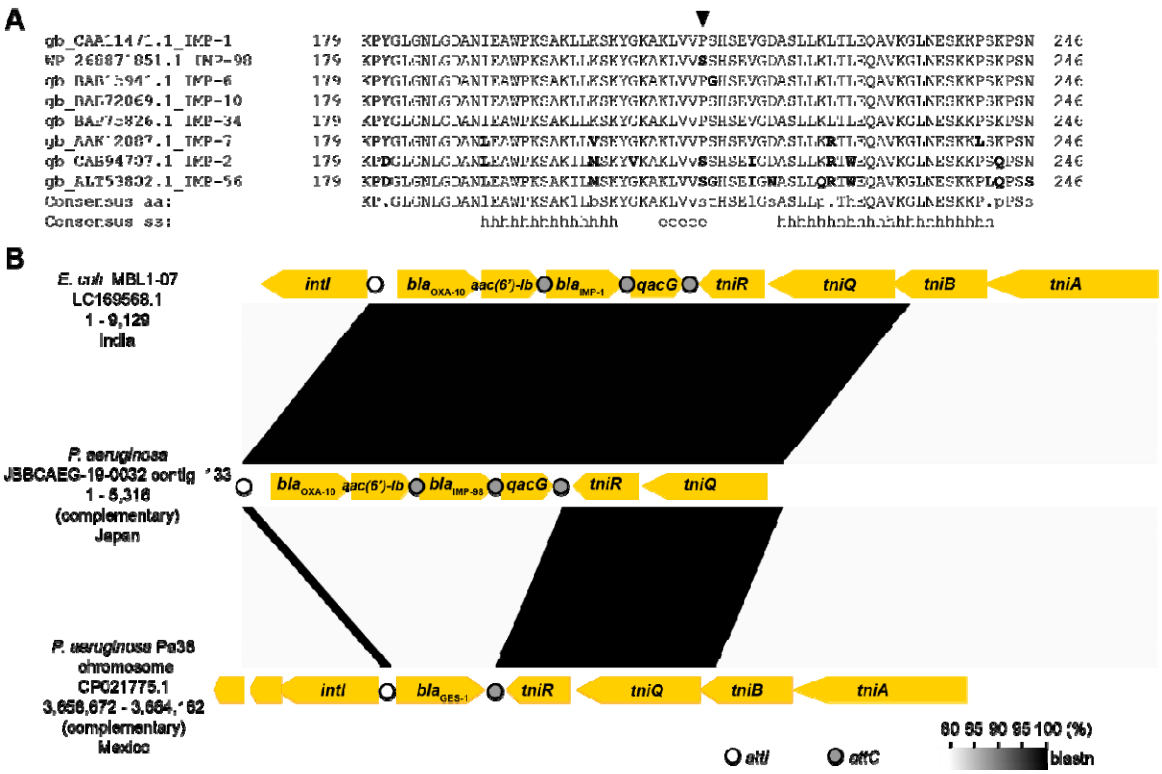


FIG 3 (A) Alignment of carboxyl-terminal regions of IMP variants. H, helix; e, sheet. IMP-98 differs from IMP-1 only at P213 indicated by an arrowhead. (B) Structure of class 1 integrons of *P. aeruginosa* ST235 strain JBBCAEG-19-0032, *E. coli* strain MBL1-07, and *P. aeruginosa* ST308 strain Pa38. The *tniABQR* genes are homologues of Tn*402* family transposon genes. bla_{OXA-10} and *aac(6')-Ib* overlapped by 23 bp. *intI* and *attC* were identified by IntegronFinder2.0 (45).

TABLES

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

Antimicrobial agents	Percentage of susceptibility group (number of isolates)		
	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	52.9 (202)	34.8 (133)	22.8 (87)
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	63.4 (242)	22.3 (85)	14.4 (55)
Tobramycin	88.7 (339)	2.1 (8)	9.2 (35)
Amikacin	88.0 (336)	4.2 (16)	7.1 (27)
Polymyxin			
Colistin	-	99.2 (379)	0.8 (3)

566 [a] Criteria for S/I/R follows CLSI M100-Ed32 (2022).

567

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

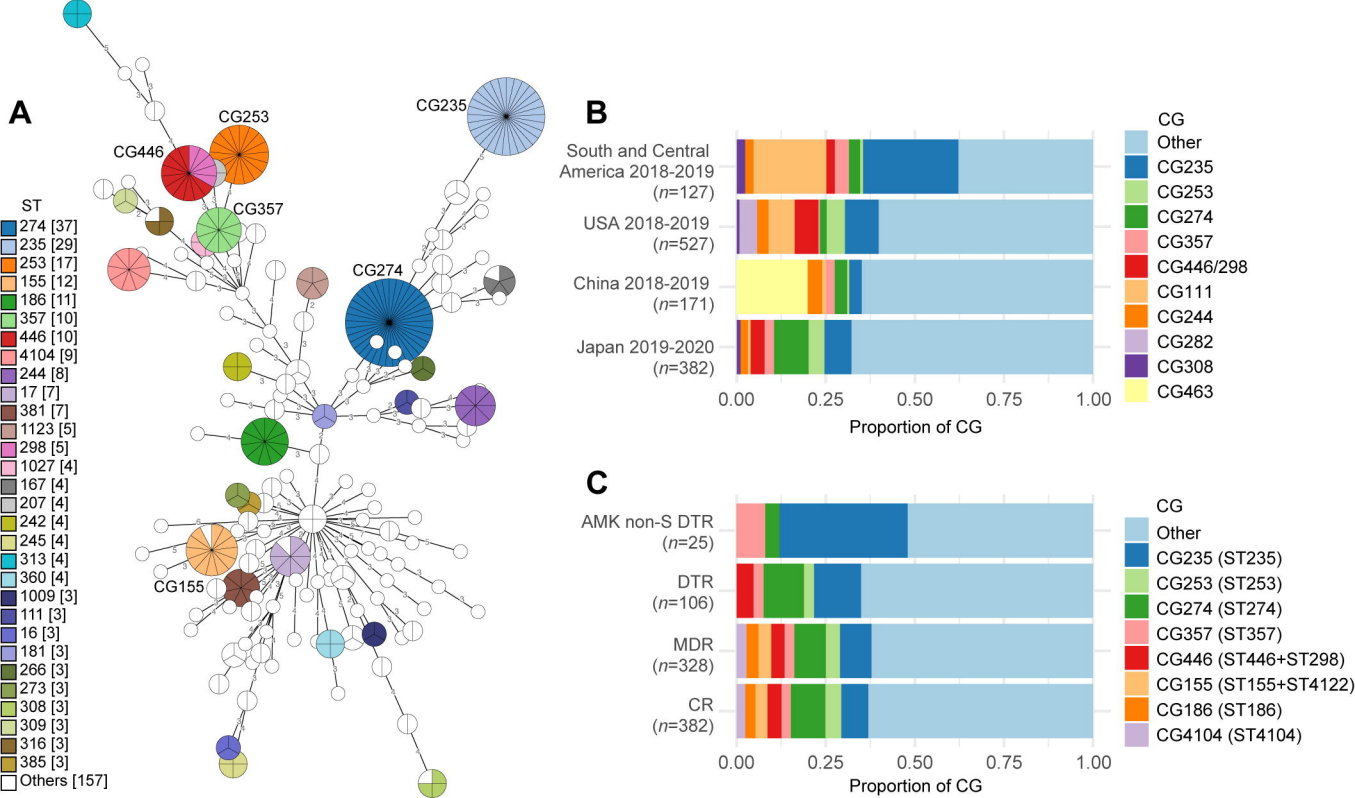
Antimicrobials	DH5α(pMW218)	DH5α(pHY1401)[a]
Ampicillin	≤4	>16
Ampicillin-sulbactam	≤4/2	>16/8
Amoxicillin-clavulanate	≤8/4	>16/8
Piperacillin	≤4	32
Piperacilin-tazobactam	≤4	8
Cefazolin	≤1	>16
Cefotiam	≤8	16
Cefaclor	≤8	>16
Cefmetazole	≤4	>32
Cefotaxime	≤0.5	>2
Ceftriaxone	≤0.5	>2
Ceftazidime	≤1	>8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

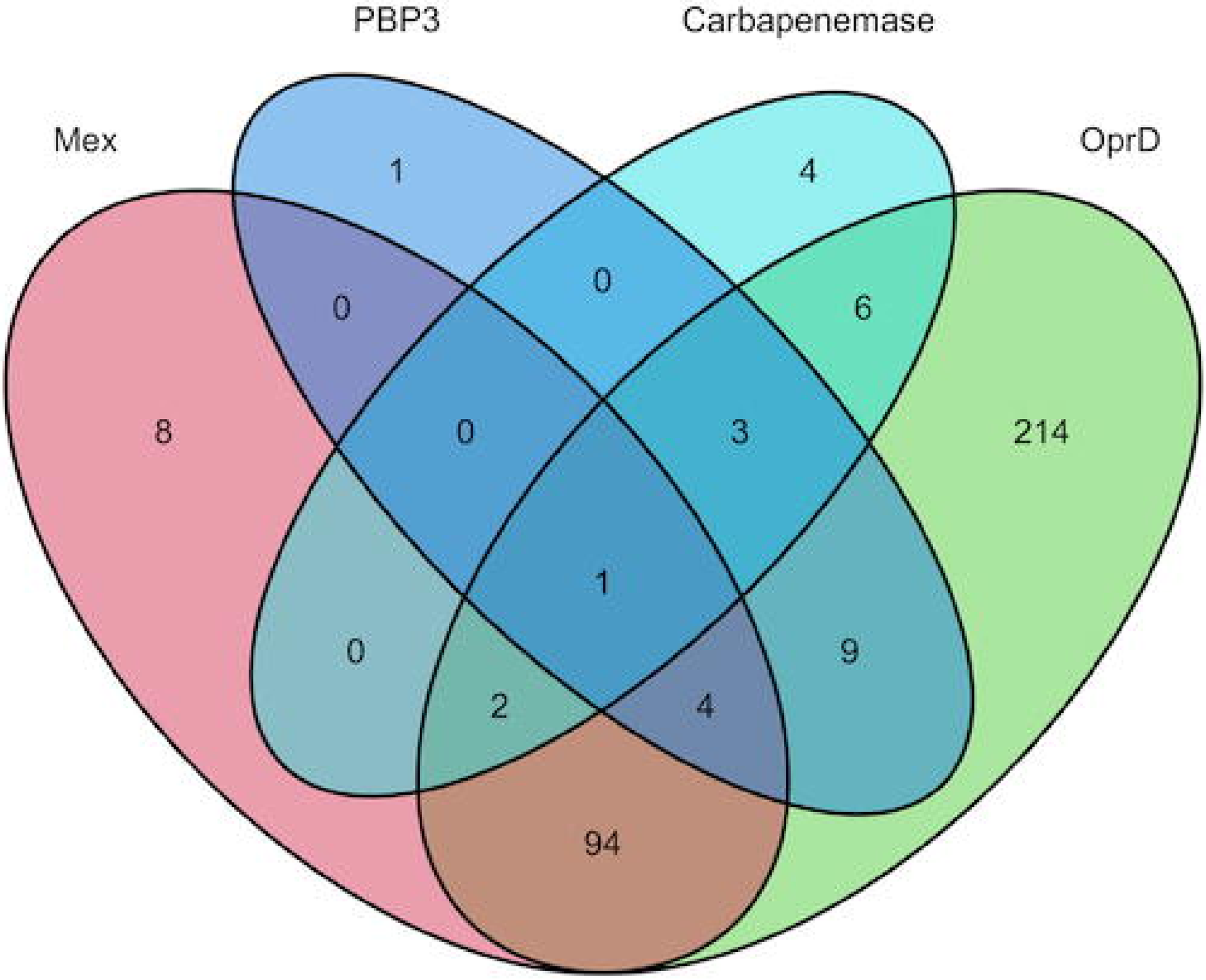
Meropenem	≤0.25	2
Ertapenem	≤0.25	>1
Flomoxef	≤8	32
Aztreonam	≤1	≤1

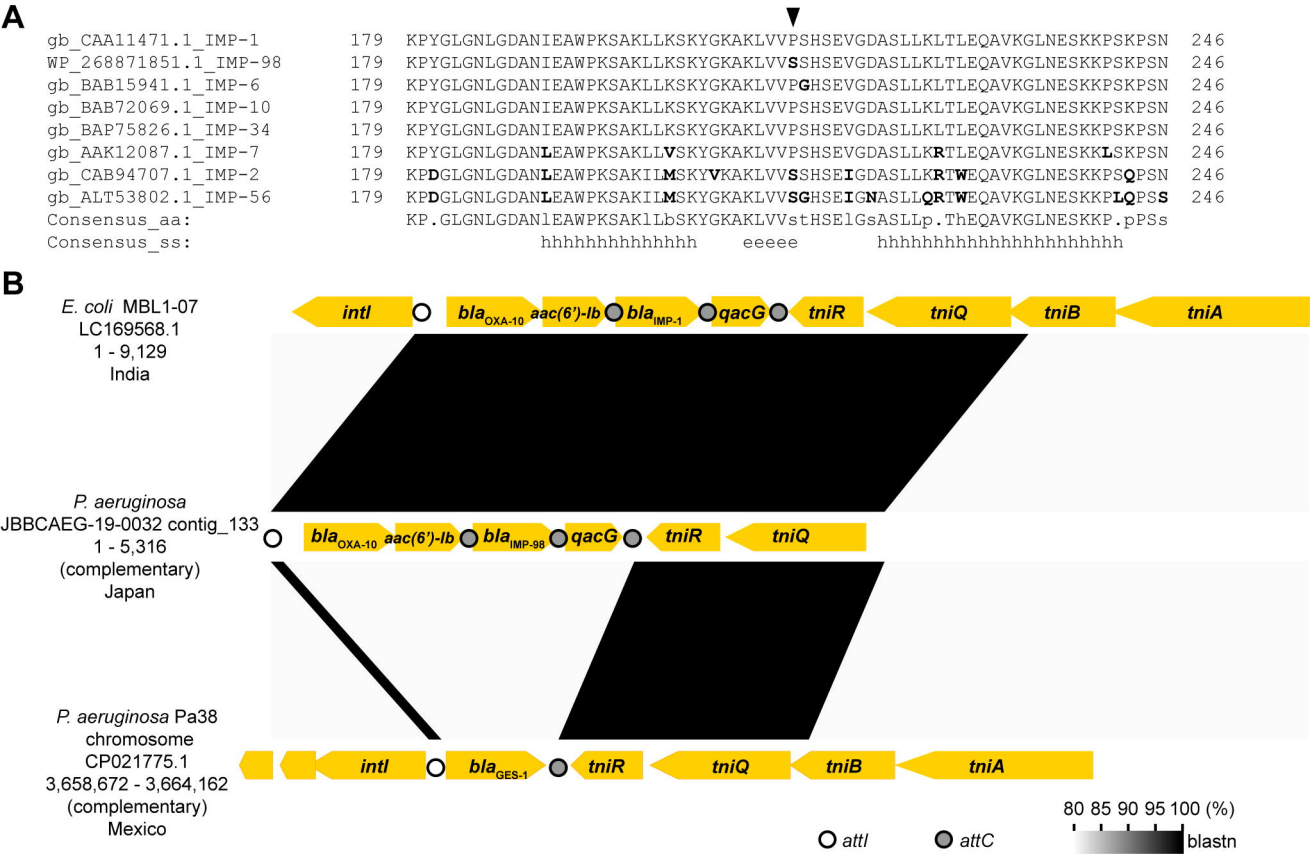
569 [a] pHY1401 is a pMW218 derivative carrying *bla*$_{IMP-98}$ and the putative promoter region of class 1

570 integron in strain JBBCAEG-19-0032.

571


A
CG253
CG235
CG446
CG357
CG274
CG155
ST
274 [37]
235 [29]
253 [17]
155 [12]
186 [11]
357 [10]
446 [10]
4104 [9]
244 [8]
17 [7]
381 [7]
1123 [5]
298 [5]
1027 [4]
167 [4]
207 [4]
242 [4]
245 [4]
313 [4]
360 [4]
1009 [3]
111 [3]
16 [3]
181 [3]
266 [3]
273 [3]
308 [3]
309 [3]
316 [3]
385 [3]
Others [157]
B
South and Central America 2018-2019 (n=127)
USA 2018-2019 (n=527)
China 2018-2019 (n=171)
Japan 2019-2020 (n=382)
0.00
0.25
0.50
0.75
1.00
Proportion of CG
CG
Other
CG235
CG253
CG274
CG357
CG446/298
CG111
CG244
CG282
CG308
CG463
C
AMK non-S DTR (n=25)
DTR (n=106)
MDR (n=328)
CR (n=382)
0.00
0.25
0.50
0.75
1.00
Proportion of CG
CG
Other
CG235 (ST235)
CG253 (ST253)
CG274 (ST274)
CG357 (ST357)
CG446 (ST446+ST298)
CG155 (ST155+ST4122)
CG186 (ST186)
CG4104 (ST4104)


PBP3
Carbapenemase
Mex
OprD
1
4
0
0
6
8
0
3
214
1
0
9
2
4
94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gb_CAA11471.1_IMP-1 179 KPYGLGNLGDANIEAWPKSAKLLKSKYGKAKLVVPSHSEVGDASLLKLTLEQAVKGLNESKKPSKPSN 246
WP_268871851.1_IMP-98 179 KPYGLGNLGDANIEAWPKSAKLLKSKYGKAKLVVSSHSEVGDASLLKLTLEQAVKGLNESKKPSKPSN 246
gb_BAB15941.1_IMP-6 179 KPYGLGNLGDANIEAWPKSAKLLKSKYGKAKLVVPGHSEVGDASLLKLTLEQAVKGLNESKKPSKPSN 246
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Consensus_aa: KP.GLGNLGDANlEAWPKSAKlLbSKYGKAKLVVstHSElGsASLLp.ThEQAVKGLNESKKP.pPSs
Consensus_ss: hhhhhhhhhhhhhh eeeee hhhhhhhhhhhhhhhhhhhhhh
B
E. coli MBL1-07
LC169568.1
1 - 9,129
India
intI
bla OXA-10
aac(6')-Ib
bla IMP-1
qacG
tniR
tniQ
tniB
tniA
P. aeruginosa
JBBCAEG-19-0032 contig_133
1 - 5,316
(complementary)
Japan
bla OXA-10
aac(6')-Ib
bla IMP-98
qacG
tniR
tniQ
P. aeruginosa Pa38
chromosome
CP021775.1
3,658,672 - 3,664,162
(complementary)
Mexico
intI
bla GES-1
tniR
tniQ
tniB
tniA
80 85 90 95 100 (%)
attI
attC
blastn