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1 **Diverse Mechanisms of Resistance in Carbapenem-Resistant Enterobacteriaceae at a**

2 **Health Care System in Silicon Valley, California**

3

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23 **Keywords:** carbapenem-resistant enterobacteriaceae, carbapenemase, porin, avibactam,

24 relebactam, vaborbactam

25 **Running title:** Resistance mechanism in CRE isolates

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31 **Abstract**

32 Carbapenem-resistant Enterobacteriaceae (CRE) are emerging as a major health threat in North
33 America. The mechanism of resistance to carbapenems has therapeutic and public health
34 implications. We comprehensively characterized the underlying mechanisms of carbapenem
35 resistance in CRE isolates recovered between 2013 and 2016 at a health system in Northern
36 California. Genotypic methods were used to detect carbapenemases and plasmid-encoded
37 cephalosporinases, and mass spectrometry was used to quantify relative porin levels for OmpC
38 and OmpF and their analogs. MICs for imipenem-relebactam, meropenem-vaborbactam,
39 ceftazidime-avibactam, and ceftolozane-tazobactam were measured. Whole genome sequencing
40 was used for strain typing. A carbapenemase gene encoding $bla_{\text{OXA-48 like}}$, bla_{NDM}, bla_{KPC}, bla_{SME},
41 bla_{IMP}, and bla_{VIM} was detected in 38.7% (24/62) of CRE isolates. Porin levels was down at least
42 2-fold in 91.9% (57/62) of isolates. Including carbapenemase genes and porin loss, the
43 mechanism of resistance was identified in 95.2% (59/62) of CRE isolates. Of the carbapenemase
44 gene-positive isolates, bla_{KPC} -positive isolates were 100% susceptible to ceftazidime-avibactam,
45 meropenem-vaborbactam, and imipenem-relebactam; $bla_{\text{OXA-48 like}}$-positive isolates were 100%
46 susceptible to ceftazidime-avibactam; and bla_{SME}-positive isolates were 100% susceptible to
47 meropenem-vaborbactam and ceftolozane-tazobactam. 100% (38/38), 92.1% (35/38), 89.5%
48 (34/38), and 31.6% (12/38) of carbapenemase gene-negative CRE isolates were susceptible to
49 ceftazidime-avibactam, meropenem-vaborbactam, imipenem-relebactam, and ceftolozane-
50 tazobactam, respectively. None of the CRE strains were genetically identical. In conclusion, at
51 this health system in Silicon Valley, carbapenemase-producing CRE occurred sporadically and
52 were mediated by diverse mechanisms. Nucleic acid testing for $bla_{\text{OXA-48 like}}$, bla_{NDM}, bla_{KPC},
53 bla_{IMP}, and bla_{VIM} was sufficient to distinguish between carbapenemase-producing and non-

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54 producing CRE and accurately predicted susceptibility to ceftazidime-avibactam, meropenem-
55 vaborbactam and imipenem-relebactam.
56

57 **Introduction**

58 Carbapenemase-producing carbapenem-resistant *Enterobacteriaceae* (CP-CRE) have
59 successfully spread worldwide over the recent decades (1, 2). In some regions, CP-CRE have
60 become endemic in hospital settings (2). The proportion of CRE in acute-care hospitals in the
61 U.S. has increased steadily (3, 4). Infection with CRE is associated with increased morbidity and
62 mortality (5-7). Thus, early diagnosis of CRE infection is essential for timely initiation of
63 targeted-antimicrobial therapy and early implementation of infection prevention precautions
64 aimed to prevent nosocomial spread (8, 9).

65 The main mechanisms of resistance to carbapenems in CRE include hydrolysis of carbapenems
66 by a plasmid-encoded carbapenemase, impaired outer membrane permeability due to inactivation
67 of particular porins (i.e., OmpC and OmpF in *E. coli* and their analogs) coupled with high-level
68 expression of cephalosporinases such as AmpC and/or extended spectrum β-lactamase (ESBL),
69 or a combination of these mechanisms (2, 10). Moreover, CRE commonly encode genetic
70 determinants of resistance to other classes of antibiotics rendering them pan-resistant (11). The
71 underlying mechanism of resistance in CRE has prognostic and therapeutic implications for the
72 newer β-lactam combination drugs that are approved by the FDA (e.g., ceftazidime-avibactam
73 and meropenem-vaborbactam), and for those in clinical trials (e.g., imipenem-relebactam) (7, 12-
74 14). For example, *in vitro* studies have shown the ceftazidime-avibactam combination to be
75 effective against isolates harboring serine carbapenemases such as class A β-lactamases
76 *Klebsiella pneumoniae* carbapenemase (KPC) and class D β-lactamases OXA-48 but not class B

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77 metallo β-lactamases (Verona integron encoded Metallo β-lactamse (VIM), IMP, or New Delhi
78 Metallo β-lactamase (NDM)) (12). However, metallo β-lactamase-producing CRE are
79 susceptible to avibactam when combined with aztreonam (13, 14). Thus, it is important to know
80 both the local prevalence of CRE resistance mechanisms, and the *in vitro* susceptibility to newer
81 β-lactam-β-lactamase inhibitor antibiotics.
82
83 Although the incidence of CRE has been lower on the West Coast of the United States compared
84 with Eastern States (4), region-specific data are not available and comprehensive phenotypic and
85 genotypic characterization of CRE isolates in Northern California has not been performed. We
86 have previously reported sporadic isolation of CP-CRE at our institution (15, 16). The aim of this
87 study was to comprehensively characterize the mechanism of carbapenem resistance in CRE
88 isolates at our institution in Silicon Valley over a four-year period and to correlate the underlying
89 mechanisms of resistance with susceptibility to newer β-lactam-β-lactamase inhibitor antibiotics.
90
91 **Materials and Methods**
92 **Ethics.** This study was approved by the Stanford University Internal Review Board.
93
94 **CRE isolates.** All consecutive CRE isolates were included from all clinical sources from patients
95 who received medical care at Stanford Health Care or Lucille Packard Children's Health
96 between January 2013 and December 2016. Both health systems consist of a tertiary academic
97 hospital and affiliated clinics but without skilled nursing facilities. Only the first CRE isolate was
98 included from each patient except for one patient with two strains on presentation. Isolates were
99 identified by biochemical testing and matrix-assisted laser desorption and ionization time-of-

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100 flight mass spectrometry (MALDI-TOF) (Bruker Daltonics, Bremen, Germany). Carbapenem
101 susceptibility testing was performed prospectively on MicroScan WalkAway plus System
102 (Beckman Coulter, San Diego, CA) for non-urinary isolates and Vitek 2 (bioMérieux, Durham,
103 NC) for urinary isolates. Imipenem and meropenem non-susceptible (i.e., intermediate or
104 resistant) isolates were confirmed using disk diffusion. CRE isolates were prospectively tested
105 for bla_{KPC}, bla_{NDM}, bla_{IMP}, bla_{VIM} and $bla_{OXA\text{-}48\ like}$ using a laboratory-developed PCR assay and
106 results were reported to providers. CRE was defined per the pre-2015 Center for Disease Control
107 (CDC) CRE surveillance definition (17) as nonsusceptibility to imipenem (i.e., MIC > 1 μg/mL)
108 or meropenem (i.e., MIC > 1 μg/mL), or doripenem (i.e., MIC > 1 μg/mL), and resistance to all
109 third generation cephalosporins tested except for *Serratia marcescens* expressing bla_{SME}, which
110 can be susceptible to third generation cephalosporins.
111
112 **Chart review.** Electronic medical records were reviewed to obtain demographics and clinical
113 characteristics of patients with CRE infection.
114
115 **Antibiotic susceptibility testing.** The following tests were performed retrospectively for
116 research purposes. Minimum inhibitory concentrations (MIC) for imipenem, meropenem, and
117 ertapenem were determined for urinary isolates using the MicroScan WalkAway plus System
118 (Beckman Coulter) for comparison to non-urine isolates. Susceptibility testing for ceftolozane-
119 tazobactam and ceftazidime-avibactam was performed by Etest (bioMérieux, Durham, NC) and
120 meropenem-vaborbactam was performed by MIC test strip (Liofilchem Diagnostici, Teramo,
121 Italy). Imipenem-relebactam was tested with microbroth dilution method. Relebactam was tested
122 at a fixed concentration of 4 μg/mL. Interpretation of antimicrobial susceptibility testing results

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123 was done according to Clinical and Laboratory Standards Institute (CLSI) criteria (18) except for
124 imipenem-relebactam and meropenem-vaborbactam which were interpreted using imipenem
125 CLSI MIC breakpoints and package insert, respectively (18).
126
127 **Genotypic β -lactamase testing.** Isolates were screened for plasmid-encoded ESBL and AmpC
128 cephalosporinases using the Check-Points CT 103 XL Check-MDR assay (Wageningen, The
129 Netherlands) per the package instruction. The Check-Points assay detects the following ESBLs:
130 $bla_{\text{CTX-M-1 group}}$, $bla_{\text{CTX-M-1-like}}$, $bla_{\text{CTX-M-15-like}}$, $bla_{\text{CTX-M-32-like}}$, $bla_{\text{CTX-M-2 group}}$, $bla_{\text{CTX-M-8, \&-25 group}}$,
131 $bla_{\text{CTX-M-9 group}}$, $bla_{\text{TEM-types}}$, $bla_{\text{SHV-types}}$, bla_{VEB}, bla_{PER}, bla_{BEL}, bla_{GES}; and the following AmpCs:
132 $bla_{\text{CMY I/MOX}}$, bla_{ACC}, bla_{DHA}, $bla_{\text{ACT/MIR}}$, $bla_{\text{CMY II}}$, bla_{FOX}. Detection of carbapenemase genes
133 was carried out using the Xpert Carba-R cartridge (Cepheid, Sunnyvale, CA), which detects
134 bla_{KPC}, bla_{NDM}, bla_{IMP}, bla_{VIM} and $bla_{\text{OXA-48 like}}$; Check-Points assay which detects additionally
135 $bla_{\text{OXA-23 like}}$, $bla_{\text{OXA-58 like}}$, bla_{SPM}, bla_{GES}, and bla_{GIM}; and three lab-developed multiplexed PCR
136 assays which detect bla_{SME}, bla_{SIM}, bla_{SPM}, bla_{GES}, bla_{IMI}, $bla_{\text{NMC-A}}$, and bla_{GIM} (Table 1). DNA
137 was extracted by boiling a bacterial colony in molecular-grade water for 10 min. PCR reactions
138 consisted of 2 µL of forward and reverse primer to achieve 0.5 µM, 5 µL of 2× FastStart SYBR
139 Green Master mix (Roche Applied Science, Indianapolis, IN), and 3 µL of DNA extract. The
140 reactions were run on a Rotor-Gene 6000 real-time cycler (Qiagen, Germantown, MD) with
141 following cycling parameters: 95°C for 5 min and 40 cycles of 95°C for 15 sec, 60°C for 30 sec,
142 and 72°C for 30 sec, followed by melting with ramping from 60°C to 95°C in 0.2°C increments.
143 Melting curve analysis was performed to identify the amplicons (Table S1). Positive controls for
144 each carbapenemase included bla_{SME}-positive *S. marcescens* MBRL055 and bla_{IMI}-positive
145 *Enterobacter cloacae* MBRL1077 provided by the Mayo Clinic (Rochester, MN); bla_{SIM}-

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146 positive *Acinetobacter baumannii* YMC 03/9/T104 provided by Yonsei University College of
147 Medicine (Seoul, South Korea); bla_{GIM}-positive *E. cloacae* M15 provided by Heinrich Heine
148 University Düsseldorf (Düsseldorf, Germany); $bla_{\text{NMC-A}}$-positive *E. cloacae* and bla_{GES}-positive
149 *A. baumannii* provided by JMI Laboratories (North Liberty, IA); and 5 bla_{GES}-positive and 5
150 bla_{SPM}-positive *Pseudomonas aeruginosa* isolates provided by Merck (Schaumburg, IL).
151

152 **Carbapenemase activity.** CRE isolates were tested for carbapenemase activity using the
153 modified carbapenem inactivation method (mCIM) as previously described (19). Isolates with
154 indeterminate mCIM result were tested for carbapenemase activity (i.e., imipenem degradation)
155 with MALDI-TOF (Bruker Daltonics) as previously described (20).
156

157 **Porin protein expression.** Levels of OmpC and OmpF in *E. coli* and their analogs in other
158 species were measured using mass spectrometry (MS). Isolates were cultured overnight in 20 mL
159 of LB broth shaking at 250 revolutions per min at 37°C. Bacterial pellets were washed in
160 sodium phosphate buffer (SPB) and resuspended in 0.5 mL of SPB and transferred to O-ring
161 tubes containing 0.2 mL of 0.1-mm zirconia/silica beads. Bacteria were mechanically disrupted
162 with three 0.5-min pulses at 2,500 oscillations per min in a Mini-BeadBeater-1 (BioSpec
163 Products, Bartlesville, OK) with 1-min intervals on ice. The lysates were sedimented two times
164 for 10 min at 1,500 × g to remove cellular debris. To enrich for membrane proteins, the
165 supernatants were sedimented two times for 30 min each at 21,000 × g and the second pellet was
166 resuspended in 45 µL of SPB. Protein concentrations were measured using the Quick Start™
167 Bradford Protein Assay (Bio Rad, Hercules, CA) and 20 µg was separated on a 10% SDS-PAGE
168 gel. Gels were stained with Coomassie Brilliant Blue R-250 and protein bands with molecular

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169 weight between 31 and 40 kDa were cut and digested with in-gel tryptic digestion kit (Thermo
170 Scientific, Waltham, MA) per the package insert. Samples were concentrated in thermo savant
171 iss110 speedvac system (Thermo Scientific) and resuspended in 20 µL of 0.1% formic acid in
172 LC-MS grade water. Tryptic peptides (2 µL for each sample) were injected with a nanoAcquity
173 sample manager (Waters, Milford, MA), trapped for 1 min at 15 µL/min on a Symmetry trap
174 column (Waters), and separated on a 1.7 µm particle size BEH C18 column (Waters) by reversed
175 phase LC using a nanoAcquity binary solvent manager (Waters). A 30 min linear acetonitrile
176 gradient (3–35%) was applied. Peptides were ionized by nano-ESI using a pico-emitter tip (New
177 Objective, Woburn, MA) and analyzed by an Impact HD UHR-QTOF mass spectrometer
178 (Bruker Daltonics) in data-dependent acquisition mode. The acquisition parameters and batch
179 processing conditions used for DDA have been previously reported (21). Data was analyzed in
180 PreView (Protein Metrics, San Carlos, CA) using the SwissProt FASTA database entries for
181 *Enterobacteriaceae* (www.uniprot.org) to determine the dominant post-translational
182 modifications and mass calibration parameters. A more specific search was carried out in Byonic
183 (Protein Metrics, San Carlos, CA) using the TrEMBL database filtered for the taxonomy of the
184 particular organism under study. MS and MS/MS tolerances were respectively set to 10 and 30
185 ppm. The main modifications considered were cysteine trioxidation, methionine oxidation and
186 N-Term acetylation. The protein false detection rate was set to 1% and all matches with less than
187 2 unique peptides were discarded. The resulting protein lists were then compiled with an R script
188 (http://www.R-project.org/) to classify the identified porin variants based on homology into
189 OmpC (OmpK36 used for *K. pneumoniae*) and OmpF (OmpK35 for *K. pneumoniae*) categories.
190 The total intensity of all the MS/MS spectra contributing to peptide identification for each
191 category was summed. Fold change in relative porin expression was determined by calculating

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192 the ratio of each porin in CRE isolates to averaged expression in four pan-sensitive strains of the
193 same species.
194
195 **Porin RNA expression.** Porin RNA expression was performed on the 39 CRE isolates recovered
196 between 2013 and 2015 excluding *S. marcescens* isolates and one *E. cloacae* complex. CRE
197 isolates were cultured in Mueller Hinton broth in the presence of a carbapenem (either
198 meropenem 2 μg/mL or imipenem 2 μg/mL and if necessary ertapenem 1μg/mL) at a starting
199 density 1×10^5 CFU/mL and harvested at 1×10^8 CFU/mL. RNAprotect Bacteria Reagent (Qiagen)
200 was added to cultures at a ratio 3:1 and incubated at ambient temperature for 5 min. RNA was
201 extracted from bacterial pellets and DNase-treated using RNA Extraction Kit and RNase-free
202 DNase Kit (Qiagen), respectively. cDNA was constructed using the QuantiTect Reverse
203 Transcription Kit (Qiagen). An identical reaction not treated with reverse transcriptase was
204 included to control for genomic DNA carryover. Quantitative reverse transcription-PCR (qRT-
205 PCR) was performed for porin genes (*ompC* and *ompF* in *E. coli* and their analogs in other
206 species) and the housekeeping gene *rpoB*. qRT-PCR primers are shown in Table S2. Expression
207 profiling of *ompF* analog in *E. cloacae* and *Citrobacter freundii* was not performed due to lack
208 of PCR primers. PCR reactions were carried out in 10 μL containing of 0.5 μM of each primer,
209 1× FastStart SYBR Green Master mix, and 3 μL of cDNA. Amplification conditions were as
210 described above. The specificity of PCR products was confirmed by melting point analysis. The
211 cDNA copy number of each gene was extrapolated from a standard curve prepared using serial
212 10-fold dilution of genomic DNA from the respective species. Expression of porin genes was
213 normalized to *rpoB* in the same sample. Fold change in porin expression was determined by
214 calculating the ratio of normalized porin expression in CRE isolates to a pan-sensitive control

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215 strain of the same species. Each experiment was performed in triplicate, and results were
216 presented as mean value of three experiments. *E. coli* ATCC 25922, *E. cloacae* ATCC 13047, *E.*
217 *aerogenes* ATCC 13048, *K. pneumoniae* ATCC13883, and *C. freundii* ATCC 8454 were used as
218 negative controls and *K. pneumoniae* isolate #404 (22) was used as a positive control for porin
219 down-regulation.
220
221 **Whole genome sequencing.** Genomic DNA was extracted from bacterial cultures with the
222 Gentra Puregene Yeast/Bact. Kit (Qiagen) per the manufacturer's instructions. Dual-indexed
223 sequencing libraries were prepared using the Nextera XT Sample Prep Kit (Illumina, San Diego
224 CA). Libraries were subjected to 101bp paired-end sequencing on the Illumina HiSeq 4000
225 platform, to approximately 100× coverage per strain. Sequencing data was demultiplexed by
226 unique indices. Read quality was assessed using FastQC v0.11.4 (23). Reads were deduplicated
227 using SuperDeduper v1.4 with the start location in the read at 5 bp (-s 5) and length 50 (-l 50)
228 (24). Deduplicated reads were then trimmed using TrimGalore v0.4.4, a wrapper for CutAdapt,
229 with a minimum quality score of 30 for trimming (-q 30), minimum read length of 50 (--length
230 50) and the "--nextera" flag. Preprocessed reads for each isolate were aligned to the RefSeq
231 reference genome for the respective species using the Burrows-Wheeler Aligner (BWA) v0.7.10
232 with default parameters. Pileup files were generated using Samtools v1.5 (25), and Varscan
233 v2.3.9 was used to identify single nucleotide variants (SNVs) with at least 40× coverage (--min-
234 coverage 40), 90% frequency (--min-var-freq 0.9), and base quality of at least 20 to support a
235 base call (--min-avg-qual 20), with the strand filter parameter turned off (--strand-filter 0). (26).
236 Varscan output was parsed with custom scripts to generate a consensus sequence for each
237 sample, requiring at least 0.9 frequency to support a SNP or reference base call. SNVs between

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238 strain pairs were counted using custom scripts. To build phylogenetic trees, core genome
239 positions were identified between all strains of a given species. Core genome positions are
240 defined as genome positions where a base call can be made for each input genome. To permit
241 multiple sequence alignment, 30,000 SNV positions from each core genome set were randomly
242 subsampled and concatenated into a FASTA file using custom scripts. Multiple sequence
243 alignment was performed with MUSCLE v3.8.31, and phylogenetic trees were computed from
244 the resulting alignments using FastTree v2.1.7 (27, 28). Trees were visualized with FigTree
245 v1.4.3 (http://tree.bio.ed.ac.uk/software/figtree/). An isolate of *E. coli* that was sequenced in two
246 separate runs was analyzed with this pipeline and shown to yield zero SNVs, as one would
247 expect for an identical strain. Genome sequences for CRE isolates were deposited in the NCBI
248 BioSample database (accession numbers SAMN08623777- SAMN08623838)
249 (https://www.ncbi.nlm.nih.gov/Traces/study/?acc=SRP133707).
250
251 **Statistical Analysis.** Fisher's exact test was used to compare differences in proportions.
252 Statistical analysis was done with the software GraphPad Prism 5.0, San Diego, CA.
253
254
255 **Results**
256 **CRE rates**
257 Between 2013 and 2016, out of 19,271 non-duplicate *Enterobacteriaceae* cultures with antibiotic
258 susceptibility results, 62 (0.32%) CRE isolates from 61 patients were identified (Table 1).
259 Demographic and clinical characteristics of patients with CRE isolates are shown in Table 2.
260 Annual CRE rates between 2013 and 2016 did not vary significantly (0.22%, 0.39%, 0.38%, and
261 0.32%, respectively) (Table 1). CRE species included *Klebsiella pneumoniae* (n=19),

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262 *Enterobacter cloacae complex* (n=14), *Escherichia coli* (n=11), *Enterobacter aerogenes* (n=8),
263 *Serratia marcescens* (n=7), and *Citrobacter freundii complex* (n=3). Carbapenem MICs for CRE
264 isolates ranged from ≤0.5 to >4 µg/mL (interquartile range [IQR], 4 to >4) for ertapenem, ≤1 to
265 >8 µg/mL (IQR, ≤1 to >8) for imipenem, and ≤1 to >8 µg/mL (IQR, ≤1 to >8) for meropenem.
266

267 **Genotypic carbapenemase testing**

268 CRE isolates were genotypically tested for previously characterized plasmid-encoded and
269 chromosomally-encoded carbapenemases using the Xpert Carba-R cartridge, Check-Points
270 microarray, and a lab-developed multiplexed, real-time PCR assay. A single carbapenemase gene
271 was detected in 38.7% (24/62) of CRE isolates which consisted of *bla*$_{\text{OXA-48 like}}$ (n=6), *bla*$_{\text{NDM}}$
272 (n=5), *bla*$_{\text{KPC}}$ (n=5), *bla*$_{\text{SME}}$ (n=5), *bla*$_{\text{IMP}}$ (n=2), and *bla*$_{\text{VIM}}$ (n=1) (Figure 1 and Table S3). The
273 bacterial species encoding carbapenemase genes included *E. coli*, *E. cloacae* complex, *K.*
274 *pneumoniae* and *S. marcescens*. The remaining 61.3% (38/62) of CRE isolates were negative for
275 *bla*$_{\text{KPC}}$, *bla*$_{\text{NDM}}$, *bla*$_{\text{IMP}}$, *bla*$_{\text{VIM}}$ and *bla*$_{\text{OXA-48 like}}$, *bla*$_{\text{SME}}$, *bla*$_{\text{SIM}}$, *bla*$_{\text{SPM}}$, *bla*$_{\text{GES}}$, *bla*$_{\text{IMI}}$, *bla*$_{\text{NMC-A}}$,
276 and *bla*$_{\text{GIM}}$. The species in this group included *C. freundii* complex, *E. coli*, *E. cloacae* complex,
277 *E. aerogenes*, *K. pneumoniae* and *S. marcescens*. Annual non-CP-CRE rates between 2013 and
278 2016 did not vary significantly (63.6%, 50.0%, 61.1%, and 68.4%, respectively). The "SPACE"
279 organisms that are likely to carry chromosomal AmpC such as *Serratia*, *Citrobacter*, and
280 *Enterobacter*, made up 63.2% (24/38) of CRE isolates lacking a carbapenemase gene compared
281 with 33.3% (8/24, p=0.04) of isolates harboring a carbapenemase gene. ESBL and plasmid-
282 encoded AmpC cephalosporinases were detected with the Check-Points assay in 75.0% (18/24)
283 and 20.8% (5/24), respectively, of carbapenemase gene-positive and 34.2% (13/38, P=0.004) and
284 28.9% (11/38, p=0.6), respectively, of carbapenemase gene-negative isolates. Either an ESBL or

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285 plasmid-encoded AmpC gene was detected in 79.2% (19/24) of carbapenemase gene-positive
286 and 60.5% (23/38, p=0.2) of carbapenemase gene-negative isolates. All 5 carbapenemase gene-
287 positive CRE isolates without an ESBL or AmpC gene were bla_{SME}-positive *S. marcescens*
288 isolates. Compared with carbapenemase gene-negative CRE, a higher proportion of
289 carbapenemase gene-positive CRE showed an elevated imipenem and meropenem MIC >8
290 (15.8% vs. 45.8%, p<0.02; 10.5% vs. 58.3%, p<0.001, respectively) (Figure 2).
291

292 **Phenotypic carbapenemase testing**

293 To determine whether carbapenemase gene-negative CRE isolates display carbapenemase
294 activity (presumably due to previously uncharacterized carbapenemases), we performed a
295 modified carbapenem inactivation method (mCIM) on all CRE isolates (19). While 100%
296 (24/24) of carbapenemase gene-positive CRE isolates were mCIM-positive, 86.8% (33/38) of
297 carbapenemase gene-negative CRE isolates were mCIM-negative, and the remaining 13.2%
298 (5/38) were mCIM-intermediate. The mCIM-indeterminate isolates were further tested with a
299 MALDI-TOF-based carbapenemase activity assay (20). All 5 mCIM-indeterminate isolates were
300 confirmed carbapenemase-negative while all carbapenemase gene-positive CRE isolates
301 evaluated tested positive for imipenem hydrolysis.
302

303 **Porin expression**

304 To determine whether porin expression is lower in CRE isolates without a carbapenemase gene,
305 a novel mass spectrometry-based assay was employed to measure porin proteins in all 62 CRE
306 isolates irrespective of their species. As shown in Figure 3B, relative porin levels of OmpC and
307 OmpF and their analogs was down 2-fold or greater in 54.2% (13/24) and 83.3% (20/24) of

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308 carbapenemase gene-positive and 71.1% (27/38, p=0.3) and 81.6% (31/38, p=1.0) of
309 carbapenemase gene-negative CRE isolates, respectively (Figure 3B and Table S3). The
310 expression of either OmpC or OmpF and their analogs was decreased in 91.7% (22/24) of
311 carbapenemase gene-positive CRE isolates compared with 92.1% (35/38, p=1.0) of
312 carbapenemase gene-negative CRE isolates. The two carbapenemase gene-positive isolates with
313 normal porin levels were both bla_{SME}-positive *S. marcescens* (CRE35 and 49); three
314 carbapenemase gene-negative CRE isolates with normal porin levels were two strains of *E.*
315 *cloacae complex* (CRE71 and 81), and one *C. freundii complex* (CRE21).
316 We also performed quantitative reverse transcription-PCR (qRT-PCR) on 39 CRE isolates
317 recovered between 2013 and 2015 to measure porin mRNA transcripts in CRE isolates. The
318 expression of *ompC* and *ompF* and their analogs was downregulated 2-fold or more in 11.8%
319 (2/17) and 26.7% (4/15) of carbapenemase gene-positive CRE isolates compared with 45.5%
320 (10/22, p=0.04) and 64.7% (11/17, p=0.04) of carbapenemase gene-negative CRE isolates,
321 respectively. In carbapenemase gene-negative CRE isolates, 63.6% (14/22) showed
322 downregulation of either *ompC* or *ompF* and their analogs compared with 29.4% (5/17) in
323 carbapenemase gene-positive CRE (p=0.05) (Figure 3A and Table S3).
324

325 **Susceptibility of CRE to newer β-lactam/β-lactamases inhibitors**

326 In vitro studies have shown predictable susceptibility of CP-CRE to newer β-lactam/β-lactamase
327 inhibitor combinations such as imipenem-relebactam, meropenem-vaborbactam, and
328 ceftazidime-avibactam, depending on the molecular class of the carbapenemases they carry (12-
329 14). We therefore investigated the susceptibility of CRE isolates to newer β-lactam/β-lactamase
330 inhibitor combination drugs. Among carbapenemase-positive CRE isolates, 41.7% (10/24),

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331 58.3% (14/24) and 66.7% (16/24) were susceptible to imipenem-relebactam, meropenem-
332 vaborbactam, and ceftazidime-avibactam, respectively (Figure 4 and Table S3). Isolates that
333 remained resistant to imipenem in the presence of relebactam were positive for class A serine β-
334 lactamase (*bla*$_{SME}$), class B metallo β-lactamases B (i.e., *bla*$_{NDM}$ and *bla*$_{VIM}$), and class D serine
335 β-lactamase (i.e, *bla*$_{OXA-48\ like}$). Isolates that remained non-susceptible to meropenem in the
336 presence of vaborbactam were positive for class B metallo β-lactamases B (i.e., *bla*$_{NDM,}$ *bla*$_{IMP}$,
337 and *bla*$_{VIM}$) and class D serine β-lactamase (i.e, *bla*$_{OXA-48\ like).}$ All 8 ceftazidime-avibactam-
338 resistant isolates were positive for class B metallo β-lactamases (i.e., *bla*$_{NDM}$, *bla*$_{IMP}$, and *bla*$_{VIM}$)
339 (Figure 4 and Table S3). Among carbapenemase gene-negative CRE isolates, 89.5% (34/38),
340 92.1% (35/38) and 100% (38/38) were susceptible to imipenem-relebactam, meropenem-
341 vaborbactam and ceftazidime-avibactam, respectively (Figure 4 and Table S3). Three isolates
342 that were non-susceptible to both imipenem-relebactam and meropenem-vaborbactam included
343 *E. aerogenes* (CRE09), *E. coli* (CRE15) and *K. pneumoniae* (CRE25) (Table S3). Average
344 relative porin levels were lower in these isolates compared with imipenem-relebactam and
345 meropenem-vaborbactam susceptible isolates that were non-susceptible to the carbapenem alone
346 (0.01 and 0.002 vs. 1.25 and 0.41 for OmpC and OmpF and their analogs, respectively). A *S.*
347 *marcescens* CRE isolate (CRE05) was non-susceptible to imipenem-relebactam (Table S3).
348 We also investigated the susceptibility of CRE isolates to ceftolozane-tazobactam. Among
349 carbapenemase gene-positive CRE, 20.8% (5/24) were susceptible and 16.7% (4/24) were
350 intermediate to ceftolozane-tazobactam (Figure 4 and Table S3). All 5 susceptible isolates were
351 *bla*$_{SME}$-positive *S. marcescens*. The 4 intermediate isolates consisted of a *bla*$_{OXA-48\ like}$-positive *K.*
352 *pneumoniae*, a *bla*$_{OXA-48\ like}$-positive *E. coli*, a *bla*$_{KPC}$-positive *K. pneumoniae* and a *bla*$_{KPC}$-
353 positive *E. cloacae complex.* Among carbapenemase gene-negative isolates, 31.6% (12/38) were

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354 susceptible and 15.8% (6/38) were intermediate to ceftolozane-tazobactam. There was no
355 evidence of correlation between resistance to ceftolozane-tazobactam and lower porin protein
356 levels (95.0% vs. 88.9%; p=0.3).
357

358 **Molecular epidemiology**

359 Whole genome sequencing was performed to investigate clonality of CRE isolates recovered
360 between 2013 and 2016. Whole genome sequences were obtained for all CRE isolates excluding
361 CRE30, which yielded no interpretable results. The only two CRE strains with temporal
362 association were non-CP-CRE *K. pneumoniae* CRE24 and CRE25 which were isolated on
363 12/28/2014 and 12/31/2014, respectively, from patients on two different medical wards.
364 Phylogenetic trees constructed based on whole genome sequence analysis are shown in Figure 5.
365 We did not find any identical CRE strains among *C. freundii complex*, *E. cloacae complex*, *E.*
366 *aerogenes*, *E. coli*, *K. pneumoniae*, and *S. marcescens* isolates (Figure 5). The number of single
367 nucleotide variants (SNVs) for closely related strains is shown in Table 4. Phylogenetic distance
368 between strains within a species do not indicate any transmission events, as no identical strains
369 were observed in our data set. Even the most closely related CRE strains on the phylogenetic tree
370 (non-CP-CRE *K. pneumoniae* CRE04 isolated on 4/16/2013 and CRE24 isolated on 12/28/2014)
371 had 5 SNVs. The only two CRE strains with temporal association (CRE24 and CRE25) had 10
372 SNVs.
373

374 **Discussion**

375 Compared to national CRE rates of 4.2% or 1.4%, based on 2011 data submitted to National
376 Healthcare Safety Network and 2010 data submitted to Surveillance Network-USA (4),

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377 respectively, the CRE rate of 0.32% between 2013 and 2016 at our healthcare system serving the
378 Silicon Valley area was 4 to 13-fold lower, respectively. Although surveillance data in Northern
379 California is lacking, our CRE rate was also lower than that reported by an academic health
380 system in Los Angeles, California (0.32% vs. 0.73%) (29). The reason for a lower CRE rate at
381 our institution could be due to differences in patient population (i.e., less health-care exposure),
382 differences in infection control and prevention practices (i.e., isolation of patients with CP-CRE),
383 absence of skilled nursing facilities and long-term-acute-care hospitals from our health system
384 (30), and the definition used to define CRE. The second difference seems to be critical as CDC
385 reported at least one CRE infection in 3.9% of short-stay hospitals compared with 17.8% of
386 long-term-acute-care hospitals (4). Furthermore, in this study we applied the pre-2015 CDC CRE
387 surveillance definition, which excludes ertapenem. Inclusion of ertapenem would have increased
388 our CRE rate (17). While the rate of health care-associated infection caused by carbapenem-
389 nonsusceptible *Enterobacteriaceae* has increased in the U.S between 2001 and 2011 (4), the
390 annual CRE rates at our institution did not vary significantly over a four-year study period from
391 2013 to 2016.
392
393 The CP-CRE rate of 38.7% among all CRE at our institution is comparable to 47.9% reported for
394 metropolitan areas in 7 U.S. states, including Oregon and Colorado, but much lower than 81.7%
395 reported by an academic health system in Los Angeles (31). Unlike all other North American
396 institutions where KPC predominates as the most common carbapenemase among CP-CRE
397 isolates (7, 29, 31-33), at our institution CP-CRE were evenly caused by KPC (20.8%), OXA-48
398 like (25.0%), NDM (20.8%), and SME (20.8%), and less commonly by IMP (8.3%) and VIM
399 (4.2%). The reason for non-predominance of KPC at our health system may be in part due to the

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400 geographic location of our institution in the Silicon Valley where high-tech industry draws
401 people from major cities around the globe where different plasmid-encoded carbapenemases are
402 endemic. Another important contributor is the fact that nosocomial transmission of KPC did not
403 occur at our institution during the study period. In fact, whole genome sequence-based strain
404 typing showed that all plasmid-encoded CP-CRE were distinct strains and therefore had occurred
405 sporadically. As discussed above, absence of skilled nursing facilities and long-term-acute-care
406 hospitals from our health system may have contributed to lack of CRE transmission (30).
407
408 A major strength of study is that we employed comprehensive phenotypic and genotypic
409 analyses to determine the molecular basis of carbapenem resistance in CRE isolates recovered
410 longitudinally at our institution. Importantly, concordance between carbapenemase activity and
411 carbapenemase gene detection was 100%, which indicates genotypic testing would be sufficient
412 to detect all plasmid-mediated (i.e., bla_{KPC}, $bla_{OXA\text{-}48\ like}$, bla_{NDM}, bla_{IMP}, and bla_{VIM}) and
413 chromosomally-encoded (i.e., bla_{SME}) CP-CRE at our institution. Although FDA-cleared
414 commercial assays currently do not detect bla_{SME} (34), this carbapenemase should be suspected
415 in carbapenem-resistant carbapenemase-producing *S. marcescens* isolates that are negative for
416 bla_{KPC}, $bla_{OXA\text{-}48\ like}$, bla_{NDM}, bla_{IMP}, and bla_{VIM} (35). Resistance in non-CP-CRE is mediated
417 through high-level expression of cephalosporinases such as AmpCs and ESBLs coupled with
418 porin inactivation (2, 10). Using a novel mass spectrometry assay, we showed porin levels of
419 either OmpC or OmpF and their analogs was down in 92.1% of non-CP-CRE isolates. Although
420 only 60.5% of non-CP-CRE encoded an ESBL or AmpC, respectively, the Check-Points assay
421 does not detect chromosomally-encoded AmpCs present in the "SPACE" organisms (i.e.,
422 *Serratia*, *Citrobacter*, and *Enterobacter*), which accounted for 63.2% of non-CP-CRE isolates in

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423 this study. Overall, between carbapenemase and porin loss detection, we could account for
424 mechanism of resistance in 95.2% (59/62) of CRE isolates in this study. The mechanism of
425 resistance in unaccounted isolates may include efflux pump and target modification (36, 37).
426
427 The antibiotic susceptibility findings from this study are consistent with prior studies showing
428 the underlying mechanism of carbapenem resistance predicts *in vitro* susceptibility to newly-
429 developed β-lactam-β-lactamase inhibitor combinations, three of which (i.e., ceftazidime-
430 avibactam, meropenem-vaborbactam, and ceftolozane-tazobactam) are FDA cleared and
431 commercially available (12-14, 38). Susceptibility of CP-CRE isolates to ceftazidime-avibactam,
432 meropenem-vaborbactam, and imipenem-relebactam was dependent on the molecular class of
433 carbapenemase they encoded such that 100% of isolates encoding bla_{KPC} were susceptible to
434 ceftazidime-avibactam, meropenem-vaborbactam and imipenem-relebactam; 100% of isolates
435 encoding $bla_{OXA\text{-}48\ like}$ were susceptible to ceftazidime-avibactam but not to meropenem-
436 vaborbactam and imipenem-relebactam; and 100% of isolates encoding metallo β-lactamases
437 (i.e., bla_{NDM}, bla_{IMP}, and bla_{VIM}) were non-susceptible to ceftazidime-avibactam, meropenem-
438 vaborbactam, and imipenem-relebactam, excluding one bla_{IMP}-positive isolate that was
439 susceptible to imipenem alone. In non-CP-CRE isolates, 100%, 92.1%, and 89.5% were
440 susceptible to ceftazidime-avibactam, meropenem-vaborbactam, and imipenem-relebactam,
441 respectively. Susceptibility of non-CP-CRE isolates to imipenem-relebactam is consistent with
442 findings by Livermore and colleagues but not by Lapuebla and colleagues (14, 39). This
443 discrepancy could be due to extend of porin inactivation among non-CP-CRE isolates in
444 different studies given that we showed isolates with resistance to imipenem-relebactam had
445 nearly undetectable porins. Overall, our findings indicate nucleic acid testing for bla_{KPC}, $bla_{OXA\text{-}}$

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446 $_{48\ \mathrm{like}}$, *bla*$_{NDM}$, *bla*$_{IMP}$, and *bla*$_{VIM}$ is sufficient to distinguish between CP-CRE and non-CP-CRE
447 and to accurately predict susceptibility to ceftazidime-avibactam, meropenem-vaborbactam, and
448 imipenem-relebactam at our institution. Although not developed for treatment of CP-CRE, 100%
449 of *S. marcescens* encoding *bla*$_{SME}$ were susceptible to ceftolozane-tazobactam while the rest of
450 CP-CRE were resistant or intermediate. Further, 31.6% of non-CP-CRE were susceptible to
451 ceftolozane-tazobactam, however, genotypic cephalosporinase testing could not identify this
452 group.
453
454 Although our findings are informative for management of patients with CRE infection, this study
455 has several limitations. First, this was a single-center study. Given that patient population,
456 medical management, and infection control practices vary between health systems, our findings
457 might not be generalizable. Thus, a multicenter study in our geographic region is needed to
458 confirm our findings. Second, despite including all CRE isolates at our institution over a 4-year
459 period, the total number of CRE isolates was relatively small. However, this reflects natural
460 epidemiology of CRE at our institution. Studies with large number of CRE isolates are needed to
461 confirm our findings. Third, we compared the susceptibility of CRE isolates to four β-lactam
462 combination drugs using three different susceptibility testing methods. Although our findings
463 were consistent with prior reports, using a single susceptibility testing method for all four drugs
464 might have allowed for more accurate comparison between drugs.
465
466 In conclusion, comprehensive phenotypic and genotypic characterization of CRE isolates
467 longitudinally at a health system in Silicon Valley identified diverse resistance mechanisms
468 including representation of all plasmid-encoded carbapenemases. On demand nucleic acid testing

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469 was able to accurately distinguish between CP-CRE and non-CP-CRE and predict *in vitro*
470 susceptibility to ceftazidime-avibactam, meropenem-vaborbactam, and imipenem-relebactam.
471

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477

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616 **Figure Legends**

617 **Figure 1. Carbapenemase genes detected in CRE isolates annually from 2013 to 2016.**

618 Carbapenemase genes tested include *bla*KPC, *bla*NDM, *bla*IMP, *bla*VIM, *bla*OXA-48 like, *bla*SME, *bla*SIM,

619 *bla*SPM, *bla*GES, *bla*IMI, *bla*NMC-A, and *bla*GIM.

620

621 **Figure 2. Distribution of imipenem and meropenem MICs in CRE isolates with and**

622 **without a carbapenemase gene.** Bars show percentage of imipenem (A) and meropenem (B)

623 MICs for carbapenemase gene-negative (CARBase gene -; green bars) and carbapenemase gene-

624 positive (CARBase gene +; blue bars) CRE isolates.

625

626 **Figure 3. Porin protein and mRNA levels in CRE isolates with and without a**

627 **carbapenemase gene.** Bars show percentage of carbapenemase gene-positive (CARBase gene +)

628 and gene-negative (CARBase gene -) CRE isolates with relative protein (A) and porin mRNA

629 (B) down 2-fold or more compared with susceptible isolates.

630

631 **Figure 4. Susceptibility of CRE isolates to imipenem-relebactam, meropenem-**

632 **vaborbactam, ceftazidime-avibactam, and ceftolozane-tazobactam.** Bars show percent

633 susceptibility of carbapenemase gene-positive (CARBase gene +) and gene-negative (CARBase

634 gene -) CRE to (A) imipenem-relebactam, (C) meropenem-vaborbactam, (E) ceftazidime-

635 avibactam, and (G) ceftolozane-tazobactam. Graphs on the right (B, D, F, H) show fraction of

636 carbapemase genes detected in carbapenemase gene-positive isolates that are susceptible,

637 intermediate, or resistant to the respective antibiotic combination.

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639 **Figure 5. Phylogenetic tree for CRE isolates based on whole genome sequencing.**

640 Phylogenetic trees were computed from multiple sequence alignments of concatenated SNVs on

641 a per-species basis. Scale bars show evolutionary distances.

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642 **Table 1. Annual CRE rates at Stanford Health Care**

Species	No. of CRE/CRE + non-CRE isolates (%)				
	2013	2014	2015	2016	2013-16
Citrobacter freundii complex	0/120 (0)	2/81 (2.5)	0/93 (0)	1/108 (0.9)	3/402 (0.7)
Citrobacter koseri	0/68 (0)	0/54 (0)	0/71 (0)	0/74 (0)	0/267 (0)
Enterobacter aerogenes	2/122 (1.6)	1/92 (1.1)	4/104 (3.8)	1/115 (0.9)	8/433 (1.8)
Enterobacter cloacae complex	3/240 (1.3)	0/226 (0)	3/239 (1.3)	8/289 (2.8)	14/994 (1.4)
Escherichia coli	1/3117 (0)	4/2080 (0.2)	2/3008 (0.1)	4/3834 (0.1)	11/12039 (0.1)
Klebsiella oxytoca	0/167 (0)	0/129 (0)	0/127 (0)	0/160 (0)	0/583 (0)
Klebsiella pneumoniae	4/631 (0.6)	7/503 (1.4)	7/565 (1.2)	1/824 (0.1)	19/2523 (0.8)
Morganella morganii	0/58 (0)	0/47 (0)	0/60 (0)	0/66 (0)	0/231 (0)
Proteus mirabilis	0/245 (0)	0/178 (0)	0/288 (0)	0/306 (0)	0/1017 (0)
Proteus vulgaris	0/16 (0)	0/13 (0)	0/11 (0)	0/12 (0)	0/52 (0)
Salmonella enterica	0/56 (0)	0/34 (0)	0/29 (0)	0/37 (0)	0/156 (0)
Serratia marcescens	1/161 (0.6)	0/113 (0)	2/157 (1.3)	4/143 (2.8)	7/574 (1.2)
All species	11/5001 (0.2)	14/3550 (0.4)	18/4752 (0.4)	19/5968 (0.3)	62/19271 (0.3)

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644 **Table 2. Demographics and clinical characteristics of patients with CRE infection**

Characteristic	No. (%) (n=61)
Female	25 (41)
Age - Median (range)	56 (1-86)
Inpatient	34 (55.7)
Comorbidities	
Immunosuppression	33 (54)
Solid-tumor malignancy	13 (21.3)
Solid organ transplant	11 (18)
Hematological-malignancy	8 (13.1)
HSCT	1 (1.6)
HIV-AIDS	0 (0)
Diabetes	12 (19.6)
Congestive Heart Failure	10 (16.4)
Cirrhosis	4 (6.5)
Structural lung disease	4 (6.5)
Major surgery within 30 days	4 (6.5)
Prematurity	1 (1.6)
No comorbidities	7 (11.5)
Specimen source	
Urinary tract	24 (39.3)
Blood	12 (19.7)
Intra-abdominal	8 (13.1)
Lower respiratory tract	7 (11.5)
Osteoarticular	4 (6.5)
Biliary tract	2 (3.3)
Skin-soft tissue	2 (3.3)
Genital	1 (1.6)
Rrespiratory sinus	1 (1.6)

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646 HSCT, Hematopoietic Stem Cell Transplant

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650 **Table 3. Antimicrobial susceptibility of CRE isolates**

Antimicrobial Agent	No. (%) of susceptible isolates								
	All CRE (n=62)	Non-CP-CRE (n=38)	CP-CRE (n=24)						
			All CP-CRE	*bla* $_{OXa\text{-}48\ like}$ (n=6)	*bla* $_{KPC}$ (n=5)	*bla* $_{NDM}$ (n=5)	*bla* $_{IMP}$ (n=2)	*bla* $_{VIM}$ (n=1)	*bla* $_{SME}$ (n=5)
Carbapenems									
Imipenem	21 (33.9)	16 (42.1)	5 (20.8)	3 (50)	0 (0)	0 (0)	2 (100)	0 (0)	0 (0)
Meropenem	21 (33.9)	17 (44.7)	4 (16.7)	4 (66.7)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Ertapenem	4 (6.5)	3 (7.9)	1 (4.2)	1 (16.7)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Monobactams									
Aztreonam	8 (12.9)	3 (7.9)	5 (20.8)	0 (0)	0 (0)	1 (20)	0 (0)	1 (100)	3 (60)
Cephems									
Ceftriaxone	5 (8.1)	0 (0)	5 (20.8)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	5 (100)
Ceftazidime	7 (11.3)	2 (5.3)	5 (20.8)	1 (16.7)	0 (0)	0 (0)	0 (0)	0 (0)	4 (80)
Cefepime	25 (40.3)	18 (47.4)	7 (29.2)	1 (16.7)	1 (20)	0 (0)	0 (0)	0 (0)	5 (100)
β-lactamase inhibitor combinations									
Piperacillin-tazobactam	12 (19.4)	8 (21.1)	4 (16.7)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	4 (80)
Ceftolozane-tazobactam	17 (27.4)	12 (31.6)	5 (20.8)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	5 (100)
Ceftazidime-avibactam	54 (87.1)	38 (100)	16 (66.7)	6 (100)	5 (100)	0 (0)	0 (0)	0 (0)	5 (100)
Imipenem-relebactam	44 (71)	34 (89.5)	10 (41.7)	3 (50)	5 (100)	0 (0)	2 (100)	0 (0)	0 (0)
Meropenem-vaborbactam	49 (79)	35 (92.1)	14(60)	4 (66.7)	5 (100)	0 (0)	0 (0)	0 (0)	5 (100)
Fluoroquinolones									
Ciprofloxacin	33 (53.2)	24 (63.2)	9 (37.5)	0 (0)	3 (60)	0 (0)	0 (0)	1 (100)	5 (100)
Levofloxacin	35 (56.5)	26 (68.4)	9 (37.5)	0 (0)	3 (60)	0 (0)	0 (0)	1 (100)	5 (100)
Aminoglycosides									
Gentamicin	41 (66.1)	33 (86.8)	8 (33.3)	1 (16.7)	2 (40)	0 (0)	0 (0)	0 (0)	5 (100)
Tobramycin	36 (58.1)	27 (71.1)	9 (37.5)	2 (33.3)	1 (20)	0 (0)	1 (50)	0 (0)	5 (100)
Amikacin	50 (80.6)	35 (92.11)	15 (62.5)	5 (83.3)	3 (60)	0 (0)	1 (50)	1 (100)	5 (100)
Tigecycline	54 (87.1)	34 (89.5)	20 (83.3)	4 (66.7)	5 (100)	5 (100)	0 (0)	1 (100)	5 (100)
Trimethoprim/Sulfamethoxazole	35 (56.5)	27 (71.1)	8 (33.3)	0 (0)	1 (20)	1 (20)	1 (50)	0 (0)	5 (100)

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652 non-CP, non-carbapenemase producing; CP, carbapenemase producing

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661 **Table 4. Single nucleotide variants (SNVs) of related CRE strains**

Isolate ID No.	Species	Collection Date	Carbapenemase gene	SNVs
CRE50	*E. coli*	2/1/2016	Negative	136
CRE75		8/16/2016	Negative	
CRE17	*E. coli*	10/15/2014	bla_{NDM}	1527
CRE87		11/13/2016	bla_{NDM}	
CRE04	*K. pneumoniae*	4/6/2013	Negative	5
CRE24		12/28/2014	Negative	
CRE04	*K. pneumoniae*	4/6/2013	Negative	7
CRE25		12/31/2014	Negative	
CRE24	*K. pneumoniae*	12/28/2014	Negative	10
CRE25		12/31/2014	Negative	
CRE08	*K. pneumoniae*	8/2/2013	$bla_{OXA\text{-}48\ like}$	1907
CRE18		10/19/2014	bla_{NDM}	
CRE22	*K. pneumoniae*	11/19/2014	Negative	163
CRE43		12/24/2015	bla_{KPC}	
CRE35	*S. marcescens*	9/2/2015	bla_{SME}	30
CRE49		1/27/2016	bla_{SME}	
CRE35	*S. marcescens*	9/2/2015	bla_{SME}	40
CRE94		12/5/2016	bla_{SME}	
CRE49	*S. marcescens*	1/27/2016	bla_{SME}	40
CRE94		12/5/2016	bla_{SME}	
CRE39	*E. aerogenes*	10/30/2015	Negative	1258
CRE41		11/18/2015	Negative	
CRE09	*E. aerogenes*	8/23/2013	Negative	176
CRE77		8/30/2016	Negative	
CRE54	*E. cloacae*	3/1/2016	Negative	55
CRE71		8/25/2016	Negative	

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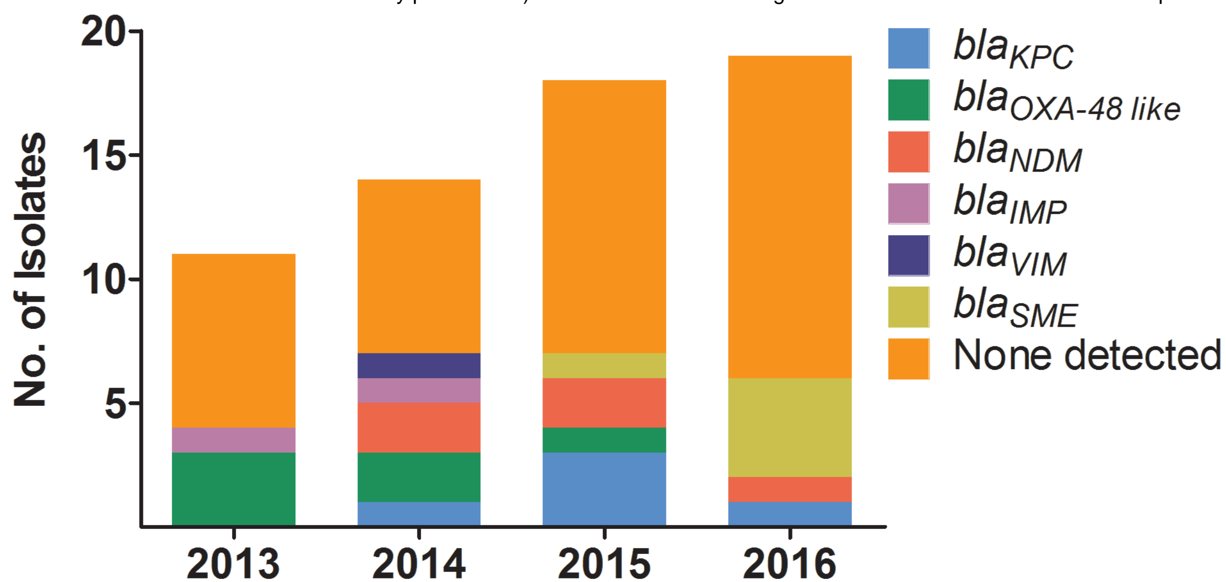

Figure 1. Carbapenemase genes detected in CRE isolates annually from 2013 to 2016.

Carbapenemase genes tested include *bla*$_{\mathrm{KPC}}$, *bla*$_{\mathrm{NDM}}$, *bla*$_{\mathrm{IMP}}$, *bla*$_{\mathrm{VIM}}$, *bla*$_{\mathrm{OXA\text{-}48\ like}}$, *bla*$_{\mathrm{SME}}$, *bla*$_{\mathrm{SIM}}$, *bla*$_{\mathrm{SPM}}$, *bla*$_{\mathrm{GES}}$, *bla*$_{\mathrm{IMI}}$, *bla*$_{\mathrm{NMC\text{-}A}}$, and *bla*$_{\mathrm{GIM}}$.

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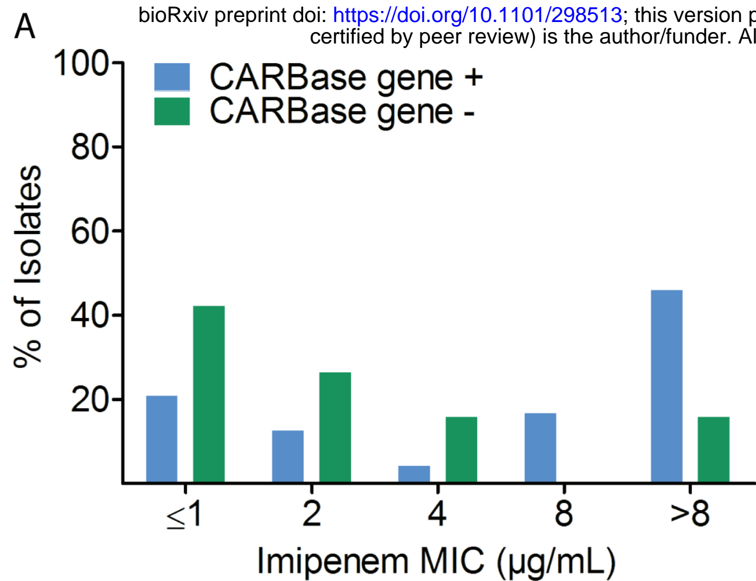

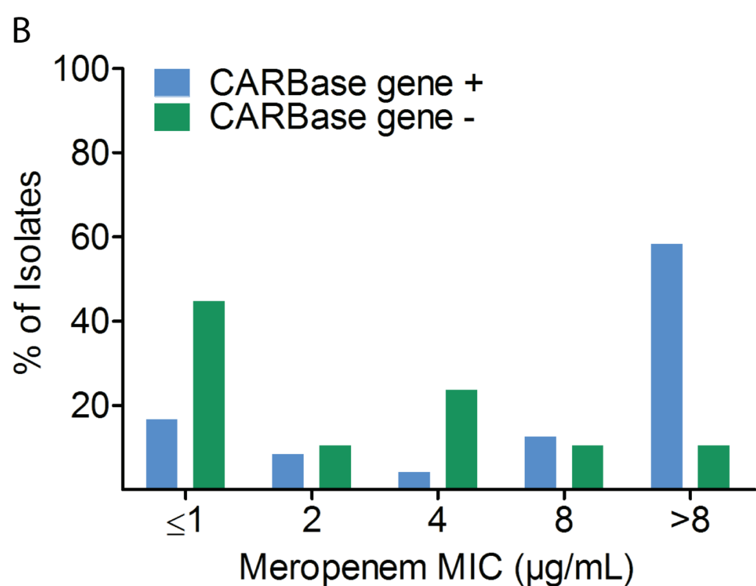

Figure 2. Distribution of imipenem and meropenem MICs in CRE isolates with and without a carbapenemase gene. Bars show percentage of imipenem (A) and meropenem (B) MICs for carbapenemase gene-negative (CARBase gene -; green bars) and carbapenemase gene-positive (CARBase gene +; blue bars) CRE isolates.

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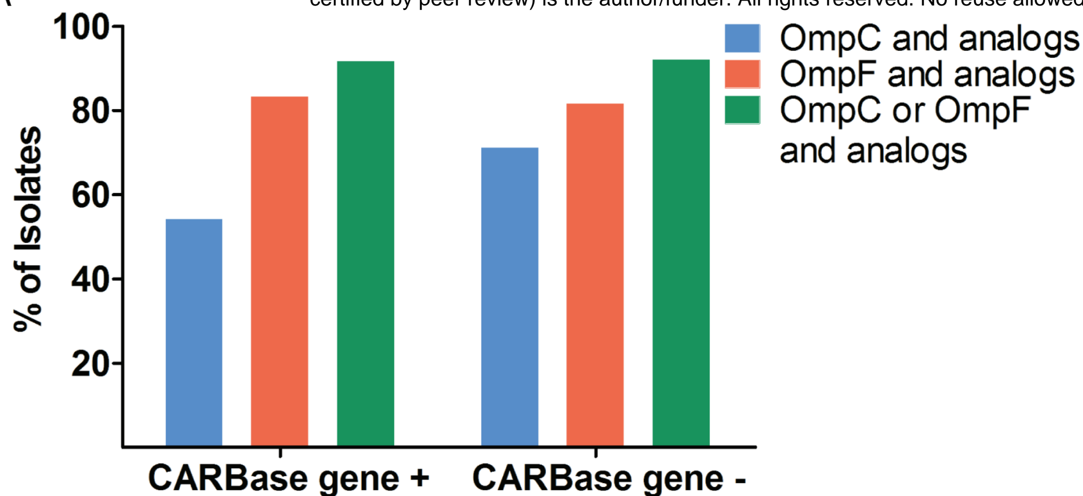
A



B

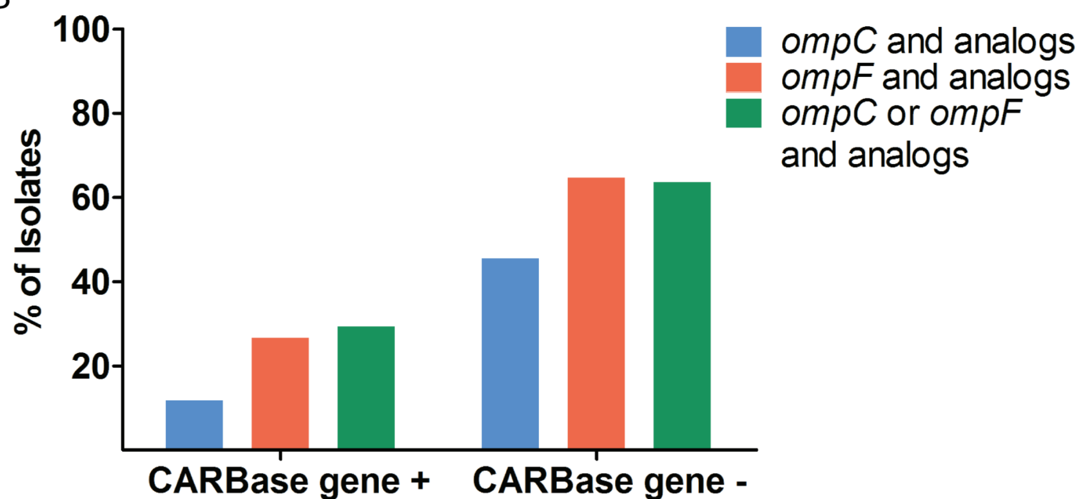


Figure 3. Porin protein and mRNA levels in CRE isolates with and without a carbapenemase gene. Bars show percentage of carbapenemase gene-positive (CARBase gene +) and gene-negative (CARBase gene -) CRE isolates with relative protein (A) and porin mRNA (B) down 2-fold or more compared with susceptible isolates.

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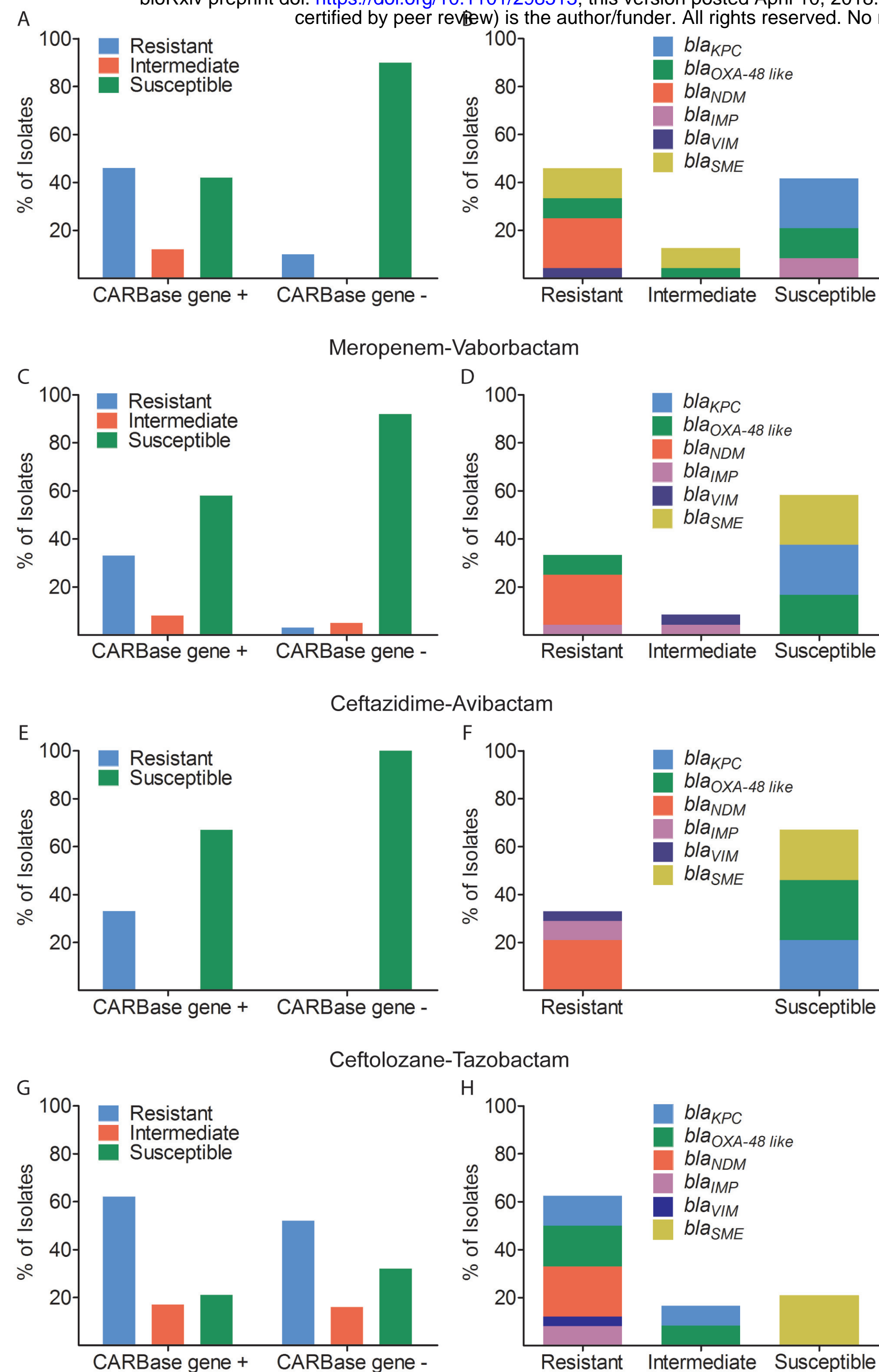

Figure 4. Susceptibility of CRE isolates to imipenem-relebactam, meropenem-vaborbactam, ceftazidime-avibactam, and ceftolozane-tazobactam. Bars show percent susceptibility of carbapenemase gene-positive (CARBase gene +) and gene-negative (CARBase gene -) CRE to (A) imipenem-relebactam, (C) meropenem-vaborbactam, (E) ceftazidime-avibactam, and (G) ceftolozane-tazobactam. Graphs on the right (B, D, F, H) show fraction of carbapemase genes detected in carbapenemase gene-positive isolates that are susceptible, intermediate, or resistant to the respective antibiotic combination.

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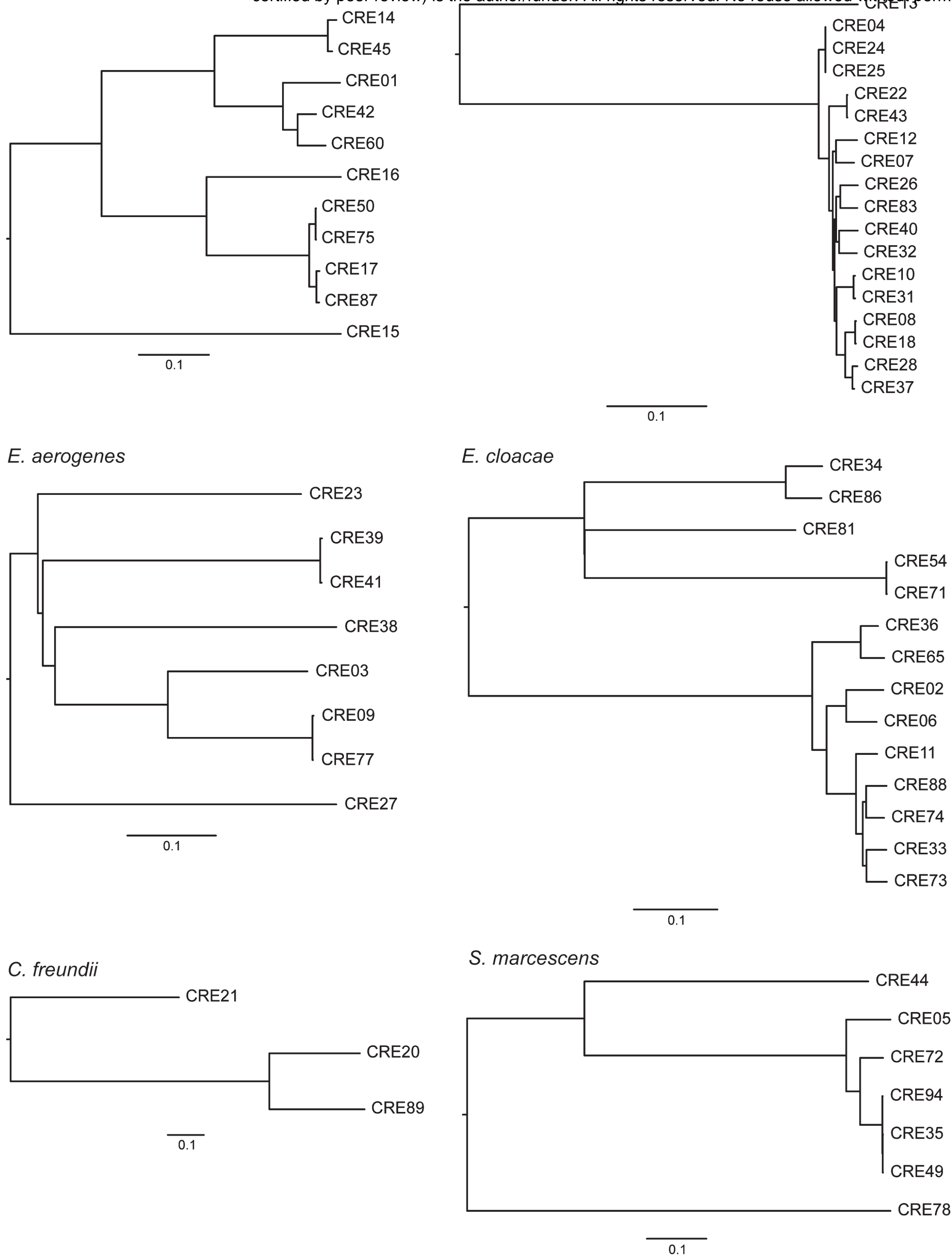

Figure 5. Phylogenetic tree for CRE isolates based on whole genome sequencing.

Phylogenetic trees were computed from multiple sequence alignments of concatenated SNVs on a per-species basis. Scale bars show evolutionary distances.