Duration of Carbapenemase-Producing *Enterobacteriaceae* Carriage in Hospital Patients

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To determine the duration of carbapenemase-producing *Enterobacteriaceae* (CPE) carriage, we studied 21 CPE carriers for ≈1 year. Mean carriage duration was 86 days; probability of decolonization in 1 year was 98.5%, suggesting that CPE-carriers' status can be reviewed yearly. Prolonged carriage was associated with use of antimicrobial drugs.

Rapid global dissemination of carbapenemase-producing *Enterobacteriaceae* (CPE) poses a public health threat (*1*). To prevent the spread of CPE in healthcare settings, international guidelines advocate for early identification, isolation, and contact precautions (*2*,*3*). To provide information helpful for the design of rational infection control policies, we estimated CPE carriage duration in a hospital cohort and identified risk factors for prolonged carriage.

The Study

During October 2016–February 2018, we conducted a prospective cohort study involving CPE carriers from 2 tertiary care centers in Singapore. CPE carriers were identified by routine collection of rectal swab samples in accordance with local infection control policies. We included patients who were >21 years of age and had the capacity to provide consent (Appendix Figure 1, https://wwwnc.cdc.gov/EID/article/26/9/19-0592-App1.pdf). We retrieved from medical records of enrolled patients the latest dates of CPE-negative rectal swab samples before the first positive sample. We collected fecal samples from participants at the time of enrollment, weekly for 4 weeks, monthly for 5 months, and bimonthly for 6 months. We recorded demographic characteristics, healthcare contact history, and medication history.

The fecal samples were inoculated onto selective chromogenic agar (CHROMID CARBA SMART; bioMérieux, https://www.biomerieux-diagnostics.com), and species identification was performed with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (Bruker, https://www.bruker.com). Antimicrobial susceptibility testing was performed by using VITEK-2 (bioMérieux). All *Enterobacteriaceae* isolates with a MIC of ≥2 mg/L for meropenem or ≥1.0 mg/L for ertapenem underwent PCR to detect bla_{NDM-1}, bla_{KPC}, bla_{OXA-48}, bla_{IMI-1}, and bla_{IMP} genes (*4*). All CPE isolates and fecal DNA underwent sequencing on an Illumina HiSeq 4000 sequencer (https://www.illumina.com). We used the Shannon diversity index to measure α-diversity for fecal microbial communities (https://cran.r-project.org/web/packages/vegan/vegan.pdf).

We analyzed data by using Bayesian multistate Markov models to account for interval censoring (Appendix). First, we estimated the overall transmission rates by considering patients to be in either CPE colonized or noncolonized states. Second, we considered CPE colonization on the species level and included as separate states carbapenemase-producing (CP)–*Escherichia coli* (CP-EC) colonized, CP–*Klebsiella pneumoniae*

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(CP-KP) colonized, and CP-EC/KP co-colonized (Appendix Figure 2). All analyses were performed by using R version 3.4.4 (https://www.R-project.org) and RStan (http://mc-stan.org).

We enrolled 21 patients (Table). Mean (± SD) follow-up period was 294 (± 77) days, and each participant provided 12 (± 1.5) samples. Throughout follow-up, 15 (71.4%) participants carried >1 species of CPE, and only 3 (14.3%) carried >1 type of carbapenemase gene (χ^2 of difference in proportions = 14.8, simulated p = 0.0005) (Appendix Table 1). The most common species carried were *K. pneumonia*e (18 [85.7%]) and *E. coli* (16 [76.2%]). The most frequently observed carbapenemase genes were bla_{OXA-48} (11 [52.4%]) and bla_{KPC} (8 [38.1%]). We obtained 76 CP-KP isolates from the samples; the most common sequence type (25 [32.9%]) was 307. Among the 83 CP-EC isolates, the most common sequence type (22 [26.5%]) was 131. Sample positivity was continuous until clearance for most (17 [81.0%]) of the 21 participants. For 4 participants, negative samples were followed by positive samples; the longest period was 3 negative samples over 3 consecutive weeks (Appendix Figure 5).

The estimated mean duration of CPE carriage was 86 (95% credible interval [CrI] 60– 122) days. The probability of decolonization in 1 year was 98.5% (95% CrI 95.0%–99.8%), assuming a constant decolonization rate within the time interval. The longest observed carriage duration was 387 days. We performed a sensitivity analysis that included 16 participants who became decolonized during follow-up (i.e., the last sample collected was negative for CPE). This analysis gave a mean carriage time of 77 (95% CrI 53–108) days and a 98.8% (95% CrI 96.5%–99.9%) probability of decolonization within 1 year.

As time-fixed covariates, we analyzed age, co-colonization with other multidrug-resistant organisms, presence of a urinary catheter, antimicrobial drug use during follow-up, Charlson Comorbidity Index score, and readmission; as a time-varying covariate, we used the Shannon Diversity Index score to explore the covariates' association with decolonization (Appendix Figure 3). The only factor associated with prolonged CPE carriage was antimicrobial drug use during the follow-up period (hazard ratio 0.48, 95% CrI 0.20–0.93). The rate of decolonization for CP-EC was lower than that for CP-KP (0.018 [95% CrI 0.007–0.031] per day vs. 0.030 [95% CrI 0.016–0.049] per day) (Appendix Table 2, Figure 4).

Conclusions

CPE infections are typically preceded by asymptomatic carriage, especially in vulnerable patients such as those who are immunocompromised and critically ill (*5*). To prevent transmission, active surveillance to identify CPE carriers is essential but may be associated with a high cost:benefit ratio if implemented without knowledge of the natural history of CPE carriage.

Previously reported CPE carriage durations vary widely; median durations range from 43 to 387 days (*5*–*7*). These variations probably result from differences in follow-up schedules, microbiological and molecular methods used to identify CPE, and criteria to define clearance. Studies that reported longer carriage duration tended to adopt an opportunistic sampling strategy and considered both clinical and fecal samples to determine carriage (*7*). Opportunistic sampling may lead to selection bias because patients with more healthcare contacts would have more samples collected. Infrequent and inconsistent sampling is more likely to misclassify recolonization from a new transmission event as continuous colonization, resulting in perceived longer duration of carriage.

Of note, the participants carried more species of CPE than types of carbapenemase genes. Although this observation may be the result of new acquisition events, it is more parsimoniously explained by active interspecies horizontal gene transfer, especially

Table. Demographics for 21 participants in study of duration of carbapenemase-producing *Enterobacteriaceae* carriage in hospital patients, Singapore*

Characteristic	No. (%)
Sex	
M	15 (71.4)
F	6 (28.6)
Ethnicity	
Chinese	15 (71.4)
Malay	3 (14.3)
Indian	2 (9.5)
Other	1 (4.8)
Ambulatory status	
Independently performs ADL	12 (57.1)
Requires assistance in ADL	4 (19.0)
Wheelchair bound	3 (14.3)
Bed bound	2 (9.5)
Recent surgery†	15 (71.4)
Colonization or infection with another MDRO in the year preceding enrollment	4 (19.0)
Hospitalization in past year	11 (52.4)
Antibiotic intake during follow-up period	13 (61.9)
Readmission during follow-up period	10 (47.6)
Recent overseas travel	13 (61.9)

*Median age (interquartile range) was 60.0 (50.0–69.0) y; median Charlson Morbidity Index (interquartile range) 3.0 (2.0–5.0). ADL, activities of daily living; MDRO, multidrug-resistant organisms, including methicillin-resistant *Staphylococcus aureus*, vancomycin-resistant *Enterococcus*, carbapenem-resistant *Enterobacteriaceae*, carbapenem-resistant *Acinetobacter baumanii*, and carbapenem-resistant *Pseudomonas aeruginosa*, recorded from surveillance and clinical cultures taken in the year before study enrollment.
†Gastrointestinal surgeries (n = 7), skin and soft tissue surgeries (n = 4), neurosurgery (n = 2), removal of Tenckhoff catheter (n = 1), and urologic procedure (n = 1).

in a low-transmission setting such as Singapore. Differential rates of clearance of CP-KP and CP-EC can be related to colonizing affinity of the species and fitness cost of carbapenemase genes, which vary widely among different species (*8*). Further studies incorporating between-host and within-host transmission dynamics of resistance may shed light on the roles of bacterial clones and plasmids in spreading and maintaining resistance.

Our study has limitations. First, because the participants were screened after hospital admission, the time of initial colonization could not be confidently determined. However, our multistate Markov models assume a constant rate of decolonization. Our sensitivity analysis used the latest CPE-negative swab samples, so carriage duration estimates were similar to those calculated without the last known CPE-negative swab samples, suggesting that our modeling assumptions were reasonable. Second, our sample size was small and drawn from a single population, and the extent to which our findings can be extrapolated to other populations is uncertain. However, our study was rigorously conducted in terms of frequency of fecal sample collection, duration of follow-up, and number of participants in a nonoutbreak setting. The use of multistate models has been shown to preserve power with modest sample size given more frequent follow-ups (*9*).

Our systematic sampling and robust methods for identifying CPE enabled us to closely follow participants' carriage status. Using combined detection methods of culture on carbapenem-resistance selective media, antibiotic susceptibility testing, and PCR, we found that 4 (19.0%) patients had intervening negative samples, 1–3 weeks apart. This finding suggests that a patient should have ≥2 negative samples 4–6 weeks apart before considering CPE to be eliminated. The finding that the probability of decolonization is 98.5% in 1 year suggests that a policy of reviewing CPE carrier status yearly may be appropriate for this population. Further health economics analysis is needed to make institution-specific recommendations for rescreening frequency. Given the finding that antimicrobial drug use was the most important factor associated with prolonged CPE carriage, use of antimicrobial drugs in these patients should be avoided if not clinically indicated.

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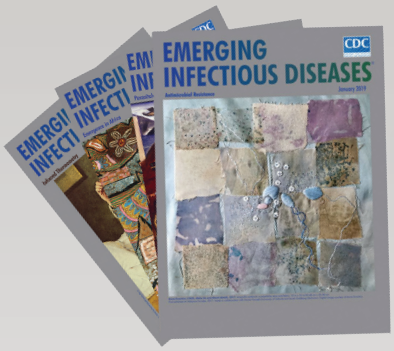

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Appendix

Supplemental Material 1: Methodology

Microbiology

Microbiological cultures to identify CPE were performed via direct inoculation of stool samples onto selective and indicative agar, chromID® CARBA SMART Agar (CARB/OXA, Biomerieux). After overnight incubation, colonies were identified at the species level with matrix assisted laser desorption ionization-time of flight-mass spectrometry (MALDI-ToF-MS, Bruker Daltonics GmHB, Bremen, Germany). Phenotypic antimicrobial susceptibility testing was performed using VITEK-2 (bioMérieux Vitek, Inc., Hazelwood, MO). All *Enterobacteriaceae* isolates with a MIC to meropenem ≥2mg/L, or Ertapenem MIC ≥1.0mg/L, underwent polymerase chain reaction (PCR) to test for the presence of blaNDM-1, blaKPC (blaKPC-2 to blaKPC-13), blaOXA48, blaIMI-1 and blaIMP carbapenemase genes, as previously reported. (Teo *et al*, Singapore J Med Microbiol. 2013)

Genomic analysis

Library preparation for DNA from CPE isolates was performed using the NEBNext® Ultra DNA Library Prep Kit for Illumina®. Sequencing with 2x151bp reads was performed using the Illumina HiSeq 4000 sequencer.

Raw FASTQ reads were processed using standard in-house pipelines. Briefly, MLST and antibiotic resistance genes were called directly from raw reads as well as from de novo assemblies; discrepancies between these were resolved with manual examination of both types of data. For analysis of raw reads, MLST and antibiotic resistance genes were called directly from the FASTQ files using SRST2 (version 0.1.8) (Inouye *et al*, Genome Med. 2014) with default settings using the ARGAnnot database provided with the SRST2 distribution for resistance

genes. De novo assemblies were performed using the Velvet assembler (version 1.2.10) (Zerbino *et al*, Genome Res. 2008) with parameters optimized by the Velvet Optimiser script packaged with the velvet distribution, scaffolded with Opera (version 1.4.1) (Gao *et al*, J Comput Biol. 2011), and finished with FinIS (version 0.3) (Gao *et al*, Algorithms in Bioinformatics 2012). Genomes were annotated with Prokka (version 1.11) (Seemann *et al*, Bioinformatics. 2014). For analysis of de novo assemblies, resistance genes were called using BLASTN with a minimum identity of 90% over 90% of the gene length required for calling a gene present, using the same ARGAnnot database as used by SRST2. MLST calls were made by using a custom BLASTN-based MLST caller. The MLST databases were downloaded using the SRST2 helper scripts from https://pubmlst.org.

Shotgun metagenomics

DNA from stool samples was extracted using PowerSoil® DNA Isolation Kit (12888, MOBIO Laboratories) with modifications to the manufacturer's protocol. To avoid spin filter clogging, we extended the centrifugation to twice the original duration and solutions C2, C3 and C4 were doubled in volume. DNA was eluted in 80µL of Solution C6. Concentration of DNA was determined by Qubit dsDNA BR assay (Q32853, Thermo Fisher Scientific). For the library construction, 50ng of DNA was re-suspended in a total volume of 50µL and was sheared using Adaptive Focused Acoustics (Covaris) with the following parameters; Duty Factor: 30%, Peak Incident Power (PIP): 450, 200 cycles per burst, Treatment Time: 240s. Sheared DNA was cleaned up with 1.5× Agencourt AMPure XP beads (A63882, Beckman Coulter). Gene Read DNA Library I Core Kit (180434, Qiagen) was used for end-repair, A-addition and adaptor ligation. Custom barcode adapters (HPLC purified, double stranded, 1st strand: 5′ P-GATCGGAAGAGCACACGTCT; 2nd strand: 5′ ACACTCTTTCCCTACACGACGCTCTTCCGATCT) were used in replacement of Gene Read Adaptor I Set for library preparation. Library was cleaned up twice using 1.5× Agencourt AMPure XP beads (A63882, Beckman Coulter). Enrichment was carried out with indexed-primers according to an adapted protocol from Multiplexing Sample Preparation Oligonucleotide kit (Illumina). We polled the enriched libraries in equi-molarity and sequenced them on an Illumina HiSeq sequencing instrument to generate 2 × 101 bp reads, yielding 17,744 million paired-end reads in total and 49 million paired-end reads on average per library.

Reads were processed with an in-house shotgun metagenomics data analysis pipeline (https://github.com/CSB5/shotgun-metagenomics-pipeline). Read quality trimming was performed using famas (https://github.com/andreas-wilm/famas, v0.10,–no-order-check), and bacterial reads were identified by mapping to the human reference genome hg19 using bwa-mem (v0.7.9a, default parameters).

Microbial community taxonomic profiles were obtained using MetaPhlAn (v2.0, default parameters, relative abundance >0.01%) which provides relative abundances of bacteria, fungi and viruses at different taxonomic levels. The Shannon diversity index was computed from species-level taxonomic profiles using the function diversity from the R library vegan. The detection of antibiotic resistance genes was performed using SRST2 (v0.1.4, fraction of gene covered >99%) using the predefined ARGAnnot database. (Appendix Table 1, Figures 1,2)

To estimate rates of colonization and duration of carriage, we modeled colonization and carriage dynamics using multi-state Markov models. First, we considered CPE to be a homogeneous bacterial group and at any sampling time point patients belonged to one of two states: “non-colonised” or “colonised “. Secondly, we considered CPE by species with patients being “not colonized by any CPE,” “colonized by CP-*E. coli*,” “colonized by CP-*K. pneumoniae*” or colonised by “both CP-*E. coli* and *K. pneumoniae*,” resulting in a four-state Markov model. In either model, transitions from one state to another was governed by a $K \times K$ intensity matrix Q (where *K* is the number of states). For $r \neq s$, the rate of transition from state r to state s, $q_{rs} = Q[r, s]$ was modeled by the linear equation:

$$\log\left(q_{rs}\right) = \beta_{0+} \sum_{i=1}^{D} \beta_i \times X_i$$

where $\{X_1, X_2, \ldots, X_D\}$ is a set of covariates.

For the two-state homogeneous CPE model we included age, taking antibiotics during follow-up, Charlson comorbidity index, co-colonization with other multidrug resistant organisms, infection with CPE, readmission, and presence of urinary catheter as time-fixed covariates, and Shannon diversity index as a time-varying covariate.

We implemented the models in STAN modeling language within the R environment. We used N~(0,1) as prior distributions for parameters estimates, while posterior distributions were

sampled using the Hamiltonian Markov Chain Monte Carlo method. The prior is a generic weakly informative prior as recommended by Rstan which contains enough information to regularize i.e., rule out unreasonable values but is not so narrow as to rule out probable values (Gelman, Github. 2019, https://github.com/stan-dev/stan/wiki/Prior-Choice-Recommendations). Posterior distributions were sampled from 4 chains run over 20,000 iterations (including 10,000 burn-in) and we assessed model convergence using the Gelman-Rubin convergence diagnostic statistic and through visualization of trace plots. (Appendix Table 2).

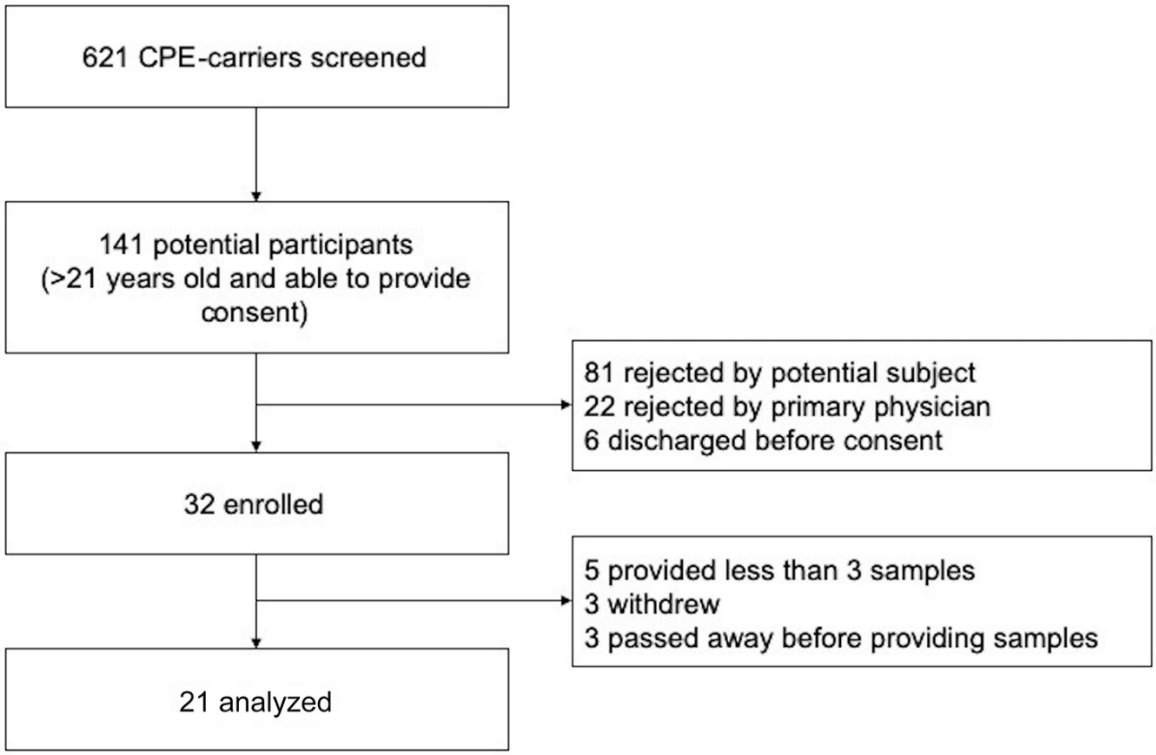
Appendix Table 1. Carbapenamase producing *Enterobacteriaceae* and associated plasmids carried by 21 participants throughout the study period

CPE species	No. (%)
Klebsiella pneumoniae (%)	18 (85.7)
Eschericia coli (%)	16 (76.2)
Enterobacter spp (%)	5 (23.8)
Citrobacter spp (%)	3 (14.3)
Co-colonization by >1 CPE species during follow up	15 (71.4)
Types of carbapenem-resistance genes	
OXA-48 (%)	11 (52.4)
KPC (%)	8 (38.1)
NDM-1 (%)	3 (14.3)
IMP (%)	2 (9.5)
Co-colonization by >1 type of plasmid during follow up	3 (14.3)

*Percentages refer to proportion of participants carrying the bacterial species or carbapenem-resistance genes. A single participant may carry more than one type of bacterial species or carbapenem-resistance genes during the observational period.

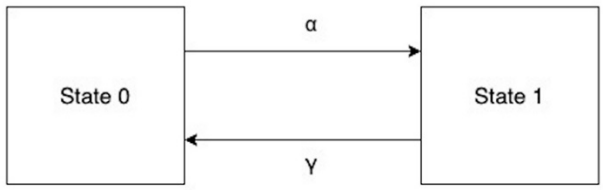
Appendix Table 2. Rates of the four-state multistate model with CP-*E. coli*, CP-*Klebsiella*, carrying both CP-*E. coli* and CP-*Klebsiella* (Both), CPE-non-carrying state (None)

State transitions	Event /day	95% CrI
CP-*E. coli* to none	0.018	0.007 – 0.031
CP-*Klebsiella* to none	0.030	0.016 – 0.050
CP-*E. coli* to both	0.041	0.018 – 0.078
CP-*Klebsiella* to both	0.045	0.019 – 0.087
Both to CP-*E. coli*	0.037	0.014 – 0.076
Both to CP-*Klebsiella*	0.040	0.018 – 0.073



Appendix Figure 1. Recruitment flow diagram.

Multistate model 1:
Two states with carbapenemase-producing Enterobacteriaceae (CPE) carrying state and CPE-non-carrying state

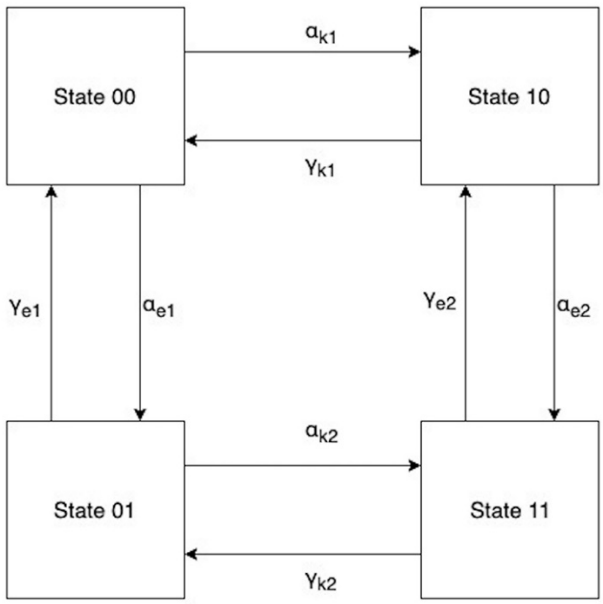


Legend

State 0: Not colonized with CPE
State 1: Colonized with CPE

α: Colonization rate
γ: Decolonization rate

Multistate model 2:
Four states with CP-*E coli* and CP-*Klebsiella* carrying states and CPE non-carrying state

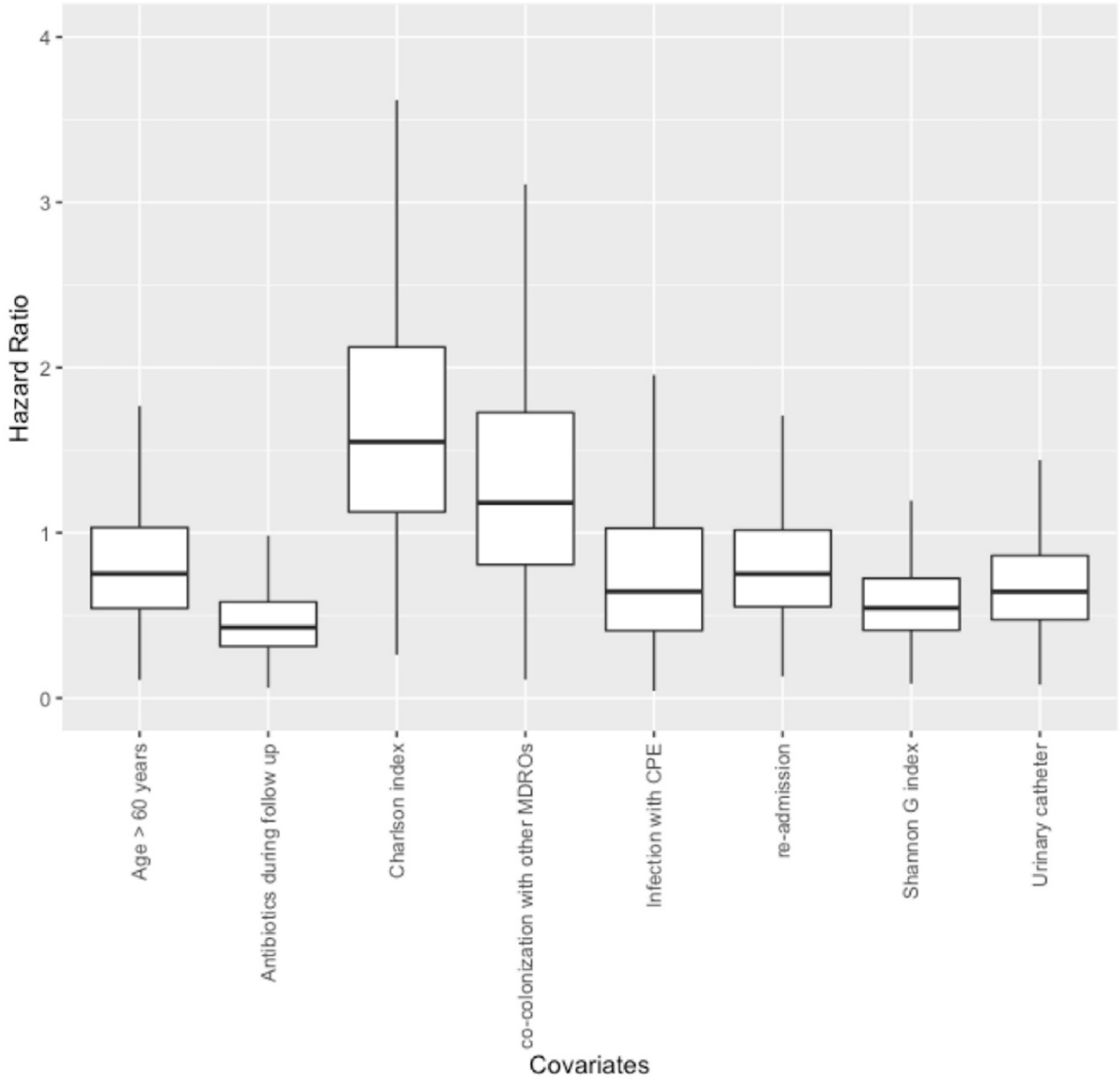


Legend

State 00: Not colonized with CPE
State 01: Colonized with CP-*E coli*
State 10: Colonized with CP-*Klebsiella*
State 11: Colonized with CP-*E coli* and CP-*Klebsiella*

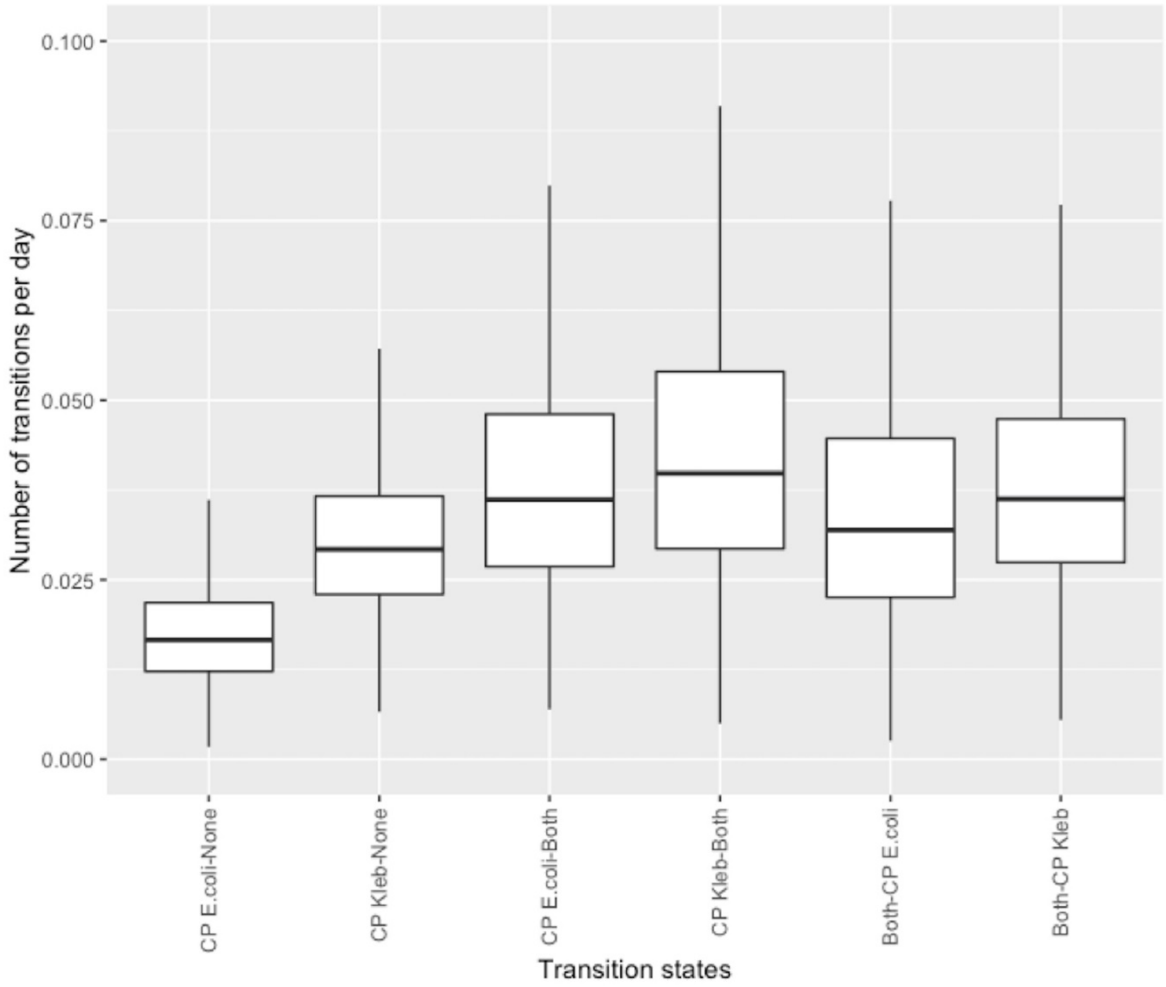
α_k: Colonization rate
γ_k: Decolonization rate
α_e: Colonization rate of CP-*E coli*
γ_e: Decolonization rate of CP-*Klebsiella*

Appendix Figure 2. Multistate Markov models for the analysis of CPE carriage

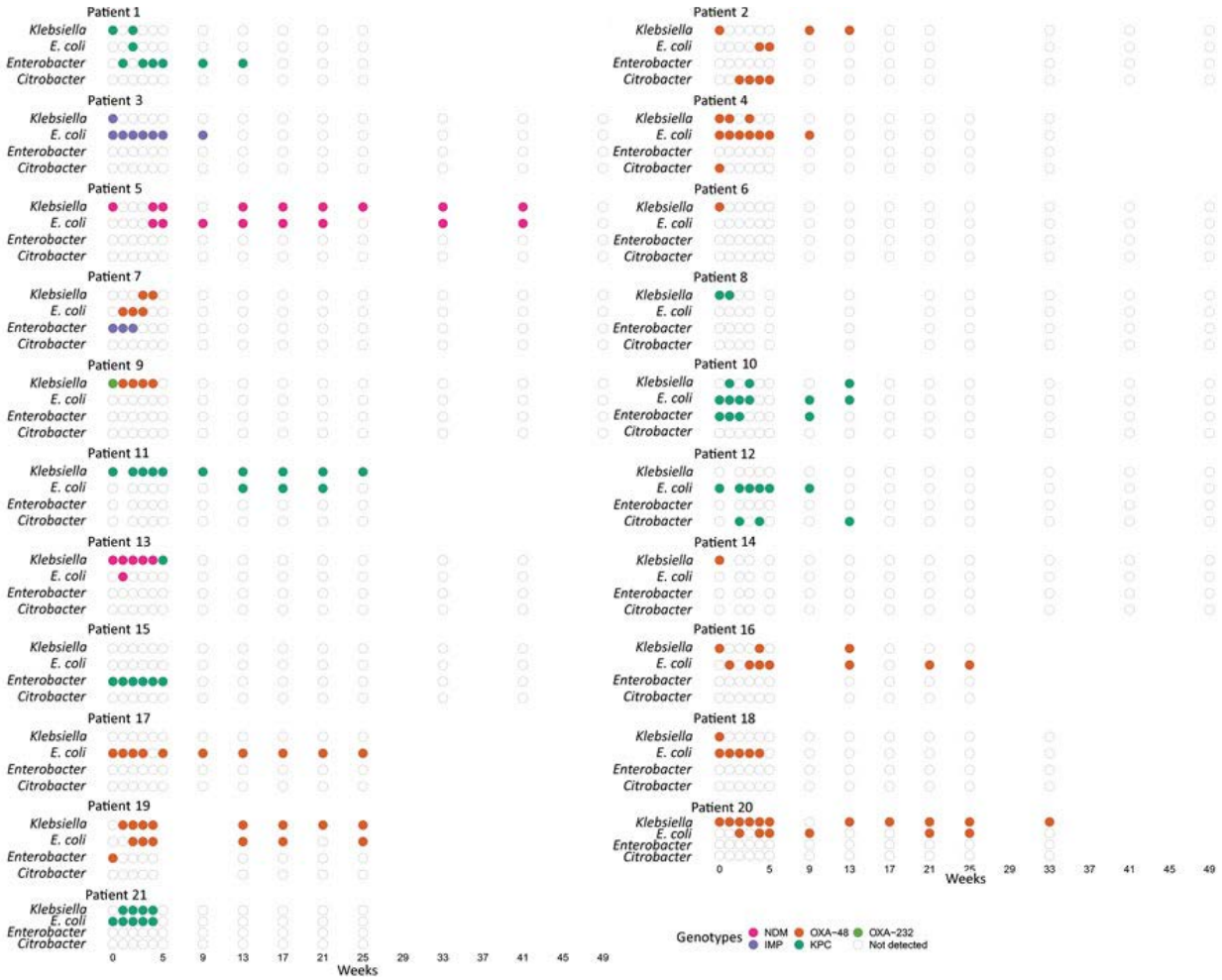


Appendix Figure 3. Associations of potential risk factors of CPE colonization and decolonization rate.

MDRO: Multidrug-resistant organisms including methicillin-resistant *Staphylococcus aureus*, vancomycin-resistant *Enterococcus*, carbapenem-resistant *Enterobacteriaceae*, carbapenem-resistant *Acinetobacter baumanii* and carbapenem-resistant *Pseudomonas aeruginosa*.



Appendix Figure 4. Rates of the four-state multistate model where CP-*E. coli and* CP-*Klebsiella pneumoniae* are separate states.



Appendix Figure 5. Types of carbapenemase-resistant *Enterobacteriaceae* and plasmid colonization for participants in study of duration of carbapenemase-producing *Enterobacteriaceae* carriage in hospital patients, Singapore. Microbiological outcomes of fecal samples from each participant are shown. Each column of dots represents 1 fecal sample. Each dot represents an *Enterobacteriaceae* species, including *Klebsiella spp., Escherichia coli, Enterobacter spp.*, *and Citrobacter spp.*, from the fecal samples carrying carbapenemase-resistant *Enterobacteriaceae* genes. Missing data are shown by the absence of dots on a particular week.