Original article

Presence of colistin- and tigecycline-resistant *Klebsiella pneumoniae* ST29 in municipal wastewater influents in Japan

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Abstract

The aim of this study was to investigate the presence of colistin- and/or tigecycline-resistant *Klebsiella* spp. in influents from four wastewater treatment plants (WWTPs) which partly reflect the gut microbiome of human populations. Colistin- and tigecycline-resistant *K. pneumoniae* isolates (K30/ST29) were detected four times from the WWTP A for three months. Disruptions of the *mgrB* and *ramR* genes by IS*Ec68* and IS*Kpn21*, respectively, were identified in those four isolates. They also shared the IncL/M 86,197-bp plasmids carrying a $bla_{\text{CTX-M-3}}$ and Tn*1548*-associated *armA* [IS*26*-*IntI1*-*dfrA12*-*gucF*-*aadA2*-*qacEΔ1*-*sul1*-IS*CR1*-IS*Ec28*-*armA*-IS*Ec29*-*msr*(E)-*mph*(E)-IS*26*]. Those isolates formed a distinct cluster within wgMLST clusters of ST29 K30 public reference strains of human origin, and were unique due to harboring Tn*21*-like mercury resistance operon transposons in addition to silver, copper, and arsenic resistance determinants. Five *K. pneumoniae* with different STs and 1 *K. quasipneumoniae*, exhibiting colistin resistance, were detected in WWTPs B, C and D. For those isolates, disruptions of *mgrB* by IS*Ec68* (3 isolates) or IS*Ecl1* (1 isolate), an insertion of IS*2* in the *mgrB* promoter region (1 isolate), and an inactivation of MgrB by nonsense mutation (1 isolate) were identified. Close monitoring of those *mcr*-negative colistin- and/or tigecycline-resistant bacteria in wastewater influents is imperative to avoid further limiting of treatment options.

Introduction

Colistin is considered the antimicrobial of last resort for treating infections with multidrug-resistant (MDR) Gram-negative bacteria, including carbapenemase-producing *Enterobacteriales*. Among those bacteria, *Klebsiella pneumoniae* is the most frequent pathogen with high mortality due to the virulence and the high level of antimicrobial resistance.[1] Unfortunately, colistin resistance in MDR *K. pneumoniae* has been increasingly reported from clinical settings worldwide.[2] Mutations/disruptions of the MgrB which acts as a negative regulator of the PhoP/PhoQ system, leading to the upregulation of the Pmr for LPS modification could be a common mechanism of colistin resistance in this organism including our previously reported colistin-resistant OXA-181 carbapenemase-producing *K. pneumoniae*.[3-5] Besides, acquisition of plasmid-mediated colistin resistance *mcr* genes encoding a phosphoethanolamine transferase has been recently reported in MDR carbapenemase-producing *K. pneumoniae*.[6] Though the *mcr* genes, and their variants, have been detected in members of *Enterobacteriales* from humans, animals, and the environments around the world, the prevalence of those genes in *K. pneumoniae* is much lower than that in *E. coli*.[7]

Tigecycline is also one of the last-resort antimicrobials for treating carbapenem-resistant *Enterobacteriales* (CRE). However, acquisition of tigecycline resistance has been increasingly reported among MDR *K. pneumoniae* human clinical isolates in recent years. In contrast, the tigecycline-resistant *K. pneumoniae* isolates have scarcely been reported in animals and environmental settings.[8-10] Tigecycline resistance in *K. pneumoniae* has mainly been attributed to overexpression of the AcrAB-TolC efflux system owing to the inactivation of the RamR negative regulator gene resulting in RamA upregulation.[10-12]

Combination of tigecycline with colistin has exhibited i*n vitro* and *in vivo* synergistic interactions against extended-spectrum β-lactamase (ESBL) producers and CRE including *K. pneumoniae* biofilm-forming isolates, making it an expected strategy to treat those CRE and other MDR bacterial infections.[13,14] Therefore, the acquisition of resistance to both tigecycline and colistin in MDR *K. pneumoniae* clinical isolates may severely limit therapeutic options though those such resistant isolates have rarely been reported so far in this organism.[15-17]

Wastewater influents may partly reflect the gut bacterial community of human populations.[18,19] Thus, it would be meaningful to investigate influents to know the presence of the colistin- and/or tigecycline-resistant *Enterobacteriales* originating from people in the community. However, there have been no previous reports with a specific focus on colistin- and/or tigecycline-resistant pathogens in untreated wastewater in Japan except our study, where we have demonstrated the presence of colistin resistance and the *mcr-1* gene in *E. coli* isolates that carry multiple virulence genes associated with avian pathogenic *E. coli* and neonatal meningitis-causing *E. coli*.[20] The aim of this study was the molecular characterization of *mcr*-negative colistin- and tigecycline-resistant CTX-M-3 ESBL-producing *K. pneumoniae* human epidemic ST29 clone detected repeatedly from influents of the Matsumoto City wastewater treatment plant (WWTP). Chromosome-mediated colistin resistance is not transferable like plasmid-mediated colistin resistance conferred by *mcr* genes. However, the occurrence of *mcr*-negative colistin-resistant isolates that have been reported to exhibit higher colistin MICs than *mcr*-positive ones[21] among *K. pneumoniae* epidemic clone could pose a great threat in clinical environments as well as human community. We also characterized *mcr*-negative colistin-resistant *Klebsiella* spp. detected from influents

of another three WWTPs.

Materials and methods

Wastewater influents

Nine crude influent samples were collected from municipal WWTP A serving 125,000 people in Matsumoto City, Nagano Prefecture, Japan during October and December 2017; one sample each was collected from three inlets A1, A2, and A3, once a month for 3 months. In addition, six influent samples comprising one sample each from two inlets B1 and B2 at WWTP B serving 162,000 people and inlets C1 and C2 at WWTP C serving 134,000 people in Nagano City, and inlets D1 and D2 at WWTP D serving 95,000 people in Azumino City collected during July and September 2018 were collected (Figure S1). Approximately 500 mL each of the influent samples taken into sterile glass bottles was transported rapidly under cooling conditions using cooler box with sufficient ice packs to our laboratory and were processed within 3 hours after obtaining the samples.

Detection and characterization of the colistin-resistant Klebsiella spp.

Isolation of colistin-resistant *Klebsiella* spp. was conducted in the same manner described previously by us, except for using MacConkey agar (Eiken Chemical Co., Tokyo, Japan) with 2mg/liter colistin.[20,22] From each influent sample, up to five colonies showing the typical *Klebsiella* morphological appearance were subjected to MALDI-TOF MS (Bruker Daltonics, Bremen, Germany) using score cutoffs of ≥2.000 for species-level identification. Co-presence of a large number of other Gram-negative bacteria showing

intrinsic resistance to colistin such as *Providencia rettgeri*, *Proteus mirabilis*, *Morganella morganii*, *Serratia marcescens*, etc. was observed. Furthermore, multiplex PCR-based identification targeting the core chromosomal class A β-lactamase gene was performed to differentiate *K. pneumoniae*, *K. quasipneumoniae* subsp. *quasipneumoniae*, *K. quasipneumoniae* subsp. *similipneumoniae*, and *K. variicola* based on the presence of bla_{SHV}, $bla_{OKP\text{-}A}$/$bla_{OKP\text{-}B}$, and bla_{LEN}, respectively.[23]

Multilocus sequence typing (MLST) for *Klebsiella* spp. isolates was conducted according to the Institute Pasteur *Klebsiella* MLST website (https://bigsdb.pasteur.fr/klebsiella/klebsiella.html).

For each influent sample from WWTP A, all *Klebsiella* colonies from A1-1 (October, 2017), A1-2 (November, 2017), A1-3 (December, 2017), and A2-1 (October, 2017) shared an identical ST29, $bla_{CTX\text{-}M\text{-}3}$ gene, and *armA*; thus, one isolate from each of four influent sample was selected to represent those isolates. *Klebsiella* colonies were not observed in the remaining five influents (A2-2, A2-3, A3-1, A3-2, and A3-3) from WWTP A. Only one or two *Klebsiella* colonies were detected in each influent sample B1 (isolates B1-1 and B1-2), C1 (isolate C1-1), and D1 (isolate D1-1) and D2 (isolates D2-1 and D2-2) from WWTPs B, C, and D, respectively, whereas they were not found in influent samples B2 and C2. In all, ten colistin-resistant *Klebsiella* spp. isolates were selected for further characterization.

Antimicrobial susceptibility testing

MIC determination was performed by the broth microdilution method recommended by the Clinical and Laboratory Standards Institute using dry plate DP31 (Eiken Chemical Co., Tokyo, Japan), and the results were interpreted according to CLSI M100-Ed30 guidelines except for tigecycline.[24] The MICs for

121 colistin and tigecycline were determined by in-house prepared panels according to the CLSI broth
122 microdilution method. The MIC values for tigecycline were interpreted and categorized according to
123 EUCAST Clinical Breakpoint Tables v8.1.

124 The MIC measurement was also performed for cefotaxime (2 mg/liter)-selected *E. coli* CSH-2
125 transconjugants derived from the conjugation with *Klebsiella* spp. from WWTP A.

126

127 *Analysis of resistance genes, mgrB, and ramR*

128 PCR detection of *mcr* genes (*mcr*-1 to -5) and *armA* was performed as described previously.[25,26] PCR
129 amplicons of structural genes of $bla_{\text{CTX-M-3}}$, *mgrB*
130 (5′-TTAAGAAGGCCGTGCTATCC-3′/5′-AAGGCGTTCATTCTACCACC-3′), and *ramR*
131 (5′-CACGGTTCATATCCTGACCA-3′/5′-CCRTCGACCTTAAACACGTC-3′) were sequenced as
132 described previously.[27-29]

133

134 *Whole-genome sequencing analysis of colistin- and tigecycline-resistant K. pneumoniae*

135 Whole-genome sequencing and *de novo* assembly of representative *K. pneumoniae* isolates (strains A1-1,
136 A1-3, and A2-1) from WWTP A, which were selected because they were human epidemic ST29 clone
137 and were resistant to both colistin and tigecycline was conducted as described previously.[20,22] The
138 assembled contigs/scaffolds were queried with ResFinder 3.1 and PlasmidFinder 2.0 available from the
139 Center for Genomic Epidemiology (CGE, http://www.genomicepidemiology.org) for antimicrobial
140 resistance gene identification and plasmid replicon typing, respectively. The capsular serotype and

141 virulence factors were analyzed using the BIGSdb-Kp database (http://bigsdb.web.pasteur.fr/klebsiella).
142 Average nucleotide identity based on MUMmer calculation (ANIm) of paired genomes was calculated
143 among *K. pneumoniae* strains from WWTP A, *K. pneumoniae* ATCC BAA-2146, *K. pneumoniae*
144 ATCC35657, *K. pneumoniae* KP-1, and *K. pneumoniae* INF249 using JSpeciesWS
145 (http://jspecies.ribohost.com/jspeciesws/). Single nucleotide polymorphism (SNP)-based phylogeny was
146 analyzed using CSI Phylogeny 1.4 (CGE). Annotation of scaffolds was performed by the DDBJ Fast
147 Annotation and Submission Tool (DFAST, https://dfast.nig.ac.jp/). Whole-genome multilocus sequence
148 typing (wgMLST) tree was constructed using PGAdb-builder (http://wgmlstdb.imst.nsysu.edu.tw). The
149 reference genomes of 19 ST29 K30 *K. pneumoniae* strains obtained from NCBI (Table S2) were included
150 in wgMLST. The phylogenetic tree was visualized using iTOL v5 (http://itol.embl.de/). An in-depth
151 exploration of drug-resistant plasmids, colicin plasmids, and virulence genes was performed manually on
152 WGS data.
153
154 *Accession number.*
155 The genomic sequencing data were deposited at NCBI under BioProject accession number
156 PRJNA647060; GenBank assembly accession numbers GCA_014596075.1, GCA_014596045.1, and
157 GCA_014595525.1 for *K. pneumoniae* A1-1, A1-3, and A2-1, respectively. Accession numbers of the
158 nucleotide sequences of *mgrB*, *ramR*, plasmids pA1-1, pA1-3, pA2-1, pColRNAI, and pCol440I are
159 shown in Table S1.
160

Results

Detection of colistin-resistant Klebsiella spp. isolates and their MICs of antimicrobials

A total of ten colistin-resistant *Klebsiella* spp. isolates were included in this study; strains A1-1 (October 2017), A1-2 (November 2017) and A1-3 (December 2017) from inlet A1 and A2-1 (October 2017) from inlet A2 of WWTP A, strains B1-1 and B1-2 (July 2018) from inlet B1 of WWTP B, strain C1-1 (August 2018) from inlet C1 of WWTP C, strains D1-1 (September 2018) from inlet D1 and strains D2-1 and D-2 (September 2018) from inlet D2 of WWTP D. Nine isolates were identified as *K. pneumoniae*, while the remaining one was identified as *Klebsiella quasipneumoniae* subsp. *similipneumoniae* based on the presence of the $bla_{\text{OKP-B-4}}$ gene confirmed by PCR and sequencing (Tables 1 and 2). All strains that were negative for *mcr* genes exhibited high colistin MICs of 32 - >128 mg/liter, where CLSI counts ≥4 mg/liter as resistance. *K. pneumoniae* strains A1-1, A1-2, A1-3, and A2-1 were resistant to extended-spectrum cephalosporins except for ceftazidime, aminoglycosides, sulfamethoxazole-trimethoprim, and fosfomycin. *K. pneumoniae* strain B1-1 and *K. quasipneumoniae* subsp. *similipneumoniae* strain B1-2, *K. pneumoniae* strain C1-1, and *K. pneumoniae* strains D1-1, D2-1, and D2-2 were susceptible to β-lactams, aminoglycosides, and sulfamethoxazole-trimethoprim. Of note, all four *K. pneumoniae* strains A1-1, A1-2, A1-3, and A2-1 showed resistance to tigecycline with MIC value 4 mg/liter.

Genetic relatedness of K. pneumoniae strains from WWTP A

The colistin- and tigecycline-resistant *K. pneumoniae* strains A1-1, A1-2, A1-3, and A2-1, belonging

to ST29 harbored *bla*$_{\text{CTX-M-3}}$ and *armA*. Those strains shared insertion of IS*Ec68* elements (1,197 bp) belonging to the IS*5* family within *mgrB* coding sequence (Table 2). Also, *ramR* disruption by the insertion of an IS*Kpn21* (2,278 bp) belonging to the IS*NCY* family was shared by those four strains.

The WGS assembly of *K. pneumoniae* A1-1, A1-3, and A2-1 contained 210, 204, and 201 contigs, with a total length of 5,709,680 bp, 5,716,245 bp, and 5,611,001 bp, respectively with an average GC content of 57.0%. *K. pneumoniae* strain A1-1 showed the highest ANIm value of 99.99% with strains A1-3 and A2-1, which were higher than those values of 99.25, 99.30, 99.83, and 99.92% with *K. pneumoniae* strains ATCC BAA-2146, ATCC35657, KP-1, and INF249, respectively. The SNP-based analysis revealed that strains A1-1, A1-3, and A2-1, differing from each other by 8 and 22 SNPs, were clustered together. In contrast, there were 380 to 20436 SNP differences between strain A1-1 and the above four *K. pneumoniae* strains (Figure 1A). *K. pneumoniae* A1-1, A1-3, and A2-1 belonged to capsular serotype K30 with the wzc-903 and wzi-85 alleles and shared antimicrobial resistance genes *bla*$_{\text{SHV-187}}$, *bla*$_{\text{TEM-1}}$, *oqxA*, *oqxB*, *fosA, aadA2*, *aac(3)-IId*, *msrE, mphE, sul1*, *sul2*, and *dfrA12* in addition to *bla*$_{\text{CTX-M-3}}$ and *armA* (Table 3). They were negative for *tet*(A), *tet*(M), and *tet*(X) genes.

The *bla*$_{\text{CTX-M-3}}$ gene was co-transferred with *armA* by conjugation, and both genes were found to be located on IncL/M plasmids pA1-1 (strain A1-1), pA1-2 (strain A1-2), and pA2-1 (strain A2-1). *E. coli* CSH-2 transconjugants showed resistance to cefotaxime (MIC 4-8 mg/liter), amikacin (MIC >32 mg/liter) and sulfamethoxazole/trimethoprim (MIC >38/2 mg/liter), while they were susceptible to minocycline and fosfomycin. The complete sequence of circular plasmids pA1-1, pA1-2, and pA2-1, determined by gap-closing PCR and Sanger sequencing were 86,197 bp in length, with a GC content of 50.8%, where

the nucleotide sequence of pA1-1 differed one and five nucleotides from pA1-2 and pA2-1, respectively. The plasmids were predicted to harbor 103 protein-coding genes, where two fragments of 26,279-bp Tn*3* family transposase-flanking sequence and the 3,681-bp IS*Ecp1* (IRL)-*bla*$_{\text{CTX-M-3}}$-*orf477*-*ΔmucA* (127-bp spacer between IS*Ecp1* and *bla*$_{\text{CTX-M-3}}$) were inserted separately into the IncL/M plasmid backbone (Figure 1B). The 26,279-bp fragment contained IS*26*-flanked composite transposon Tn*1548* consisting of IS*26*-*IntI1*-*dfrA12*-*gucF*-*aadA2*-*qacEΔ1*-*sul1*-IS*CR1*-IS*Ec28*-*armA*-IS*Ec29*-*msr*(E)-*mph*(E)-*orf1*-*orf2*-IS *26*, and 6,693-bp *aac(3)-IId*-IS*Cfr1*-*bla*$_{\text{TEM-1}}$. These plasmids pA1-1, pA1-2, and pA2-1 exhibited 99.98% nucleotide identity with the pCTX-M3 (AF550415) harbored by a *Citrobacter freundii* clinical isolate in Poland.

K. pneumoniae A1-1, A1-3, and A2-1 carried the chromosomal resistance genes *bla*$_{\text{SHV-187}}$, *oqxABR*, *fosA,* and *sul2*. The IS*CR2*-*sul2* was located in 15-kb integrative element GI*sul2*, which also carried arsenate/arsenite resistance genes *arsBCHR*. Those three strains had an amino acid substitution Gly256Arg in PmrB.

Genes for virulence and heavy metal resistance in K. pneumoniae A1-1, A1-3, and A2-1

The *K. pneumoniae* A1-1, A1-3, and A2-1 harbored chromosomally located virulence operons *entCEBAH*, and *ybtA*-*irp2*-*irp1*-*ybtUTE*-*fyuA* and *ybtPQXS* encoding biosynthetic and transport proteins of enterobactin, and yersiniabactin, respectively (Table 3). The type 3 fimbrial operon *mrkABCDF* associated with biofilm formation and *uge* encoding UDP galacturonate 4-epimerase were also identified. They harbored ColRNAI plasmid (pColRNAI, 9,295 bp) carrying *ccl* and *cim* encoding cloacin protein

and cloacin immunity protein, respectively. In addition, strains A1-1 and A1-3 harbored Col440I plasmid (pCol440I, 3,549 bp) carrying klebicin B structural gene and klebicin B immunity gene.

Multiple heavy metal resistance genes conferring silver, copper, mercury, and arsenic resistance were identified in *K. pneumoniae* A1-1, A1-3, and A2-1 (Table 3 and Figure 2). The *sil* operon, *silCFBAP* and *silRSE* encoding silver-efflux pump was located adjacent to the *pco* operon, *pcoABCDRSE* responsible for copper detoxification. The *mer* operon, *merRTPCADE* involved in mercurial resistance was associated with a Tn*21*-like transposon. The *ars* operon, *arsRDABC* encoding arsenite-efflux pump was identified, which was flanked by two insertion sequence families IS*Kpn21* (upstream) and IS*3* (downstream).

K. pneumoniae strains A1-1, A1-3, and A2-1 formed a distinct cluster within wgMLST clusters of ST29 K30 strains of human origin (Figure 2). Analysis of virulence-associated genes revealed that those three strains were unique relative to public reference strains of human origin because they harbored Tn*21*-like mercury resistance operon transposons in addition to silver, copper, and arsenic resistance determinants (Figure 2).

Genetic characteristics of Klebsiella spp. strains from WWTPs B, C, and D

The colistin-resistant *K. pneumoniae* B1-1 and D2-1 were assigned to novel sequence types, ST3410 (allelic profile 182-3-2-219-6-4-4), a double-locus variant of ST29 and ST3440 (allelic profile 2-6-1-5-363-1-6), respectively (Table 2). The remaining colistin-resistant *K. quasipneumoniae* subsp. *similipneumoniae* B1-2, *K. pneumoniae* C1-1, D1-1, and D2-2 belonged respectively to STs 1803, 872, 941, and 36. The disruption of the *mgrB* gene by an insertion sequence IS*Ec68* (1,197 bp) was identified

241 in *K. pneumoniae* C1-1, D1-1, and D2-1. In *K. pneumoniae* B1-1, *mgrB* disruption by insertion of IS*Ecl1*
242 (1,336 bp) belonging to the IS*3* family was identified. *K. quasipneumoniae* subsp. *similipneumoniae* B1-2
243 had a nonsense mutation leading to a premature stop codon in the *mgrB* gene. *K. pneumoniae* D2-2 had a
244 1,331-bp IS*2* element belonging to the IS*3* family at position -4 (upstream of the start codon) of the *mgrB*
245 gene.
246
247 **Discussion**
248 Colistin and tigecycline are regarded as a last-resort approach to face the challenges of MDR
249 Gram-negative bacteria.[30] In Japan, there have been very few reports on colistin- or tigecycline-resistant *K.*
250 *pneumoniae* derived from human clinical isolates and companion animals.[5,31,32] Of note, co-resistance to
251 colistin and tigecycline has been found in human and canine *K. pneumoniae* isolates belonging to human
252 epidemic lineages.[10,33] In this study, the emergence of colistin- and tigecycline-resistant isolates was
253 confirmed in wastewater influents of WWTP A. Those three ST29 K30 *K. pneumoniae* strains A1-1,
254 A1-3, and A2-1 were found to be highly homogeneous irrespective of the sampling time and inlet. *K.*
255 *pneumoniae* A1-2, belonging to ST29 also harbored *bla*$_{\text{CTX-M-3}}$ and *armA*, and altered *mgrB* and *ramR*,
256 suggesting a high genetic relatedness to strains A1-1, A1-3, and A2-1. Thus, recurrent recoveries of
257 colistin- and tigecycline-resistant CTX-M-3-producing *K. pneumoniae* ST29 for at least 3 months indicate
258 that they could persist and spread in communities linked to the sewage system and could be shed
259 continuously to the system. Alternatively, the possibility that these strains may be resident organisms in
260 the sewer system cannot be excluded. We performed the exploration of heavy metal resistance genes

261 because the relationship between heavy metal- and antimicrobial resistance has been documented
262 previously.[34,35] Strains A1-1, A1-3, and A2-1 were characterized by possession of diverse repertoires of
263 heavy metal resistance genes including Tn*21*-like mercury resistance transposons in addition to harbor
264 biofilm-associated genes, bacteriocins, and antimicrobial resistance determinants. This combination of
265 genes would likely improve survival in untreated sewage and wastewater contaminated with high levels of
266 heavy metals.[36] The proliferation of antimicrobial resistance might be facilitated through the coselection of
267 antimicrobial and heavy-metal resistance genes in metal-contaminated environments.[35,37]

268 The inactivation of the *mgrB* gene is mediated by diverse mechanisms, where *mgrB* disruption by IS
269 elements such as IS*5*-like, IS*10R*, IS*903*, IS*Kpn13*, IS*Kpn14*, IS*Kpn26*, IS*Kpn28*, and IS*1R* is most
270 frequently identified.[38] In this study, the insertional inactivation of *mgrB* gene by an insertion sequence
271 IS*Ec68* was identified in all four *K. pneumoniae* strains A1-1, A1-2, A1-3 and A2-1 from WWTP A as
272 well as in *K. pneumoniae* strains C1-1 from WWTP C, and D1-1 and D2-1 from WWTP D. Of note, in
273 six of those seven strains from different lineages of STs 29, 872 and 3440, the IS*Ec68* insertion had
274 occurred at nucleotide position 75 of the *mgrB* sequence, suggesting a hotspot's position for the
275 integration of IS*5* family members including IS*Ec68* as described previously.[3,38] IS*Ec68* insertion at
276 nucleotide position 126 occurring in strain D1-1 in this study has less frequently been recognized in
277 colistin-resistant *K. pneumoniae*.[38] The findings not described previously, that is, inactivation of *mgrB*
278 gene by IS*Ecl1* insertion in *K. pneumoniae* B1-1, premature termination due to nonsense mutation in *K.*
279 *quasipneumoniae* subsp. *similipneumoniae* B1-2, and IS*2* insertions upstream of the start codon of *mgrB*
280 in *K. pneumoniae* D2-2, may also confer the colistin resistance in those strains. Previous studies revealed

281 the involvement of IS elements IS*10* or IS*Kpn1*8 in *ramR* disruption leading to tigecycline resistance in *K.*
282 *pneumoniae*,[10-12] and the possible role of IS*Kpn21* in tigecycline resistance via insertional inactivation of
283 RamR was identified in *K. pneumoniae* A1-1, A1-2, A1-3 and A2-1 from WWTP A. It is worrisome that
284 *mgrB* inactivation would occur easily without requiring a significant fitness cost *in vitro* and *in vivo* and
285 without a loss of virulence in *K. pneumoniae*, and is stably retained even in the absence of antimicrobial
286 pressure.[39,40] Additionally, the *mgrB* inactivation has shown enhanced *K. pneumoniae* virulence by
287 decreasing antimicrobial peptide susceptibility and attenuating early host defense response activation.[41]
288 Also, mutated *ramR* has easily occurred with lower fitness costs, and the rapid emergence of *ramR*
289 inactivation via IS*Kpn18* insertion has been recognized.[12,42] There are several reports on colistin-resistant
290 *K. pneumoniae* isolates from sewage, but these studies focus on resistance incurred by *mcr-1*.[43,44] In this
291 study, colistin-resistant *K. pneumoniae* isolates recovered from WWTP influents did not possess *mcr*,
292 while harboring inactivated *mgrB*. Though such chromosome-mediated colistin resistance is not
293 transferable, *mcr*-negative colistin-resistant isolates have been reported to exhibit higher colistin MICs
294 than *mcr*-positive isolates.[21]

295 Increasing occurrence of the combined sewer overflows in urban areas due to unusually heavy rainfall,
296 leading to people being exposed to unrecognized antimicrobial-resistant pathogenic bacteria in the
297 untreated wastewater flood have raised serious public health concerns. This study confirmed the repeated
298 detection of *mcr*-negative colistin- and tigecycline-resistant CTX-M-3-producing human
299 infection-associated ST29 *K. pneumoniae* in influents, suggesting its constant presence in people in the
300 community, or it may be resident in the sewage drainage system. Some potential risk could be considered

for this finding; risk for horizontal transfer of *bla*$_{\text{CTX-M-3}}$ and *armA* genes from this clone to other pathogenic Gram-negative bacteria via conjugative plasmids in sewage and wastewater environments, and elevated risk of community people or pets to be exposed to this clone via contaminated environmental surfaces, leading its intrusion into clinical settings. This study highlights the importance of close monitoring of the epidemiology of those *mcr*-negative colistin- and/or tigecycline-resistant bacteria in the wastewater influents to avoid further limiting of treatment options.

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Disclosure Statement

No competing financial interests exist.

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Supplementary Material

Supplementary tables S1 and S2, and figure S1

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TABLE 1 MICs of antimicrobials for four colistin and tigecycline-resistant *K. pneumoniae* strains recovered from WWTP A influents

Antimicrobial agents	MIC (mg/L)[a]							
	Inlet A1						Inlet A2	
	K. pneumoniae A1-1 (October, 2017)		*K. pneumoniae* A1-2 (November, 2017)		*K. pneumoniae* A1-3 (December, 2017)		*K. pneumoniae* A2-1 (October, 2017)	
Piperacillin	>64	R	>64	R	>64	R	>64	R
Ampicillin/sulbactam	>16/8	R	>16/8	R	>16/8	R	>16/8	R
Cefazolin	>16	R	>16	R	>16	R	>16	R
Cefotiam	>16	–	>16	–	>16	–	>16	–
Cefotaxime	32	R	32	R	32	R	>32	R
Ceftazidime	4	S	2	S	2	S	2	S
Cefpodoxime	>4	R	>4	R	>4	R	>4	R
Cefepime	8	SDD	16	R	16	R	16	R
Flomoxef	≤0.5	–	1	–	≤0.5	–	≤0.5	–
Aztreonam	8	I	8	I	8	I	8	I
Imipenem	≤0.25	S	≤0.25	S	≤0.25	S	≤0.25	S
Meropenem	≤0.25	S	≤0.25	S	≤0.25	S	≤0.25	S
Ertapenem	0.5	S	0.5	S	0.5	S	0.5	S
Amikacin	>32	R	>32	R	>32	R	>32	R
Gentamicin	>8	R	>8	R	>8	R	>8	R
Minocycline	>8	R	8	R	8	R	8	R
Levofloxacin	0.5	S	1	I	1	I	1	I
Sulfamethoxazole/trimethoprim	>38/2	R	>38/2	R	>38/2	R	>38/2	R
Fosfomycin	>128	R	128	I	128	I	>128	I
Colistin	64	R	>128	R	64	R	64	R
Tigecycline[b]	4	R	4	R	4	R	4	R

[a]S, susceptible; I, intermediate; R, resistant; SSD, susceptible-dose dependent (CLSI M100-Ed30).

[b]Interpretive categories from EUCAST Clinical Breakpoint Tables v8.1 was used.

TABLE 2 Characteristics and mutations in *mgrB* and/or *ramR* of ten colistin- and/or tigecycline-resistant *Klebsiella* spp.

WWTP	Inlet	*Klebsiela* spp.	MLST	MIC (mg/liter)		*mgrB*			*ramR*		
				Colistin	Tigecycline	Mutation	Nucleotide position	Accession no.	Mutation	Nucleotide position	Accession no.
A	A1	*K. pneumoniae* A1-1	ST29	64	4	insertion of IS*Ec68* (FW)[a]	+75	LC506383	insertion of IS*Kpn21* (FW)	+19	LC506434
		K. pneumoniae A1-2	ST29	>128	4	insertion of IS*Ec68* (FW)	+75		insertion of IS*Kpn21* (FW)	+19	
		K. pneumoniae A1-3	ST29	64	4	insertion of IS*Ec68* (FW)	+75		insertion of IS*Kpn21* (FW)	+19	
	A2	*K. pneumoniae* A2-1	ST29	64	4	insertion of IS*Ec68* (FW)	+75		insertion of IS*Kpn21* (FW)	+19	
B	B1	*K. pneumoniae* B1-1	ST3401[b]	64	0.25	insertion of IS*Ecl1* (RV)	+137	LC504251	Ile141Thr[c]	+422	LC533900
		K. quasipneumoniae subsp. *similipneumoniae* B1-2	ST1803	64	1	premature stop codon (TGG→TAG)	+17	LC504200	Ala6Val[d]	+17	
C	C1	*K. pneumoniae* C1-1	ST872	>128	1	insertion of IS*Ec68* (FW)	+75	LC505034	–[e]	–	
D	D1	*K. pneumoniae* D1-1	ST941	>128	1	insertion of IS*Ec68* (RV)	+126	LC505455	–	–	
	D2	*K. pneumoniae* D2-1	ST3440[b]	64	1	insertion of IS*Ec68* (RV)	+75	LC505456	–	–	
		K. pneumoniae D2-2	ST36	32	0.5	insertion of IS*2* in the promoter region (FW)	-4	LC505457	–	–	

[a]FW, transposase gene is integrated in the same orientation as *mgrB* or *ramR* gene; RV, transposase gene is integrated in the reverse orientation to *mgrB* or *ramR* gene.

[b]Sequence types newly assigned in this study.

[c]Amino acid substitution found regardless of tigecycline susceptibility status.

[d]Amino acid substitution compared to the sequences of *K. quasipneumoniae* subsp. *similipneumoniae* strain ATCC 700603 (CP029597).

[e]No detected substitution.

TABLE 3 WGS-based analysis of colistin and tigecycline-resistant *K. pneumoniae* A1-1, A1-3, and A2-1 from municipal WWTP A

Genetic characteristics		Inlet A1		Inlet A2
		K. pneumoniae A1-1	*K. pneumoniae* A1-3	*K. pneumoniae* A2-1
Sequence type		ST29		
Serotype		K30		
Antimicrobial resistance genes in:	Chromosome	$bla_{SHV-187}$, *oqxA*, *oqxB*, *fosA, sul2*		
	Plasmid	$bla_{CTX-M-3}$, bla_{TEM-1}, *armA*, *aadA2, aac(3)-IId, msrE, mphE, sul1, dfrA12*		
Virulence-associated genes in:	Chromosome	*entCEBAH, ybtPQXS, mrkABCDF, uge*		
	Plasmid	*ccl*		
Heavy metal resistance genes		*silCFBAP*, *silRSE*, *pcoABCDRSE*, *merRTPCADE*, *arsRDABC*		
Plasmid replicon types		IncL/M, IncFIB, IncFII, ColRNAI, Col440I		IncL/M, IncFIB, ColRNAI

entCEBAH, enterobactin biosynthesis; *ybtPQXS*, yersiniabactin biosynthesis; *mrkABCDF,* type 3 fimbriae synthesis; *uge*, UDP galacturonate 4-epimerase; *ccl*, cloacin; *silCFBAP*, silver resistance; *silRSE*, silver resistance; *pcoABCDRSE,* copper resistance; *merRTPCADE*, mercury resistance; *arsRDABC*, arsenic resistance.

Supplementary Table S1. GenBank accession numbers of the nucleotide sequences of plasmids pA1-1, pA1-3, pA2-1, pColRNAI, pCol440I, *mgrB*, and *ramR*

Gene name	*Klebsiela* spp.	GenBank accession number
pA1-1	*K. pneumoniae* A1-1	LC505604
pA1-3	*K. pneumoniae* A1-3	LC508263
pA2-1	*K. pneumoniae* A2-3	LC508722
pColRNAI	*K. pneumoniae* A1-1	LC505458
pCol440I		LC505603
mgrB	*K. pneumoniae* A1-2	LC571522
ramR		LC571523
mgrB	*K. pneumoniae* B1-1	LC504251
ramR		LC533900
mgrB	*K. quasipneumoniae* subsp. *similipneumoniae* B1-2	LC504200
ramR		LC569723
mgrB	*K. pneumoniae* C1-1	LC505034
ramR		LC569862
mgrB	*K. pneumoniae* D1-1	LC505455
ramR		LC569863
mgrB	*K. pneumoniae* D2-1	LC505456
ramR		LC569864
mgrB	*K. pneumoniae* D2-2	LC505457
ramR		LC569865

Supplementary Table S2. List of 19 ST29 K30 *K. pneumoniae* reference genomes included in wgMLST

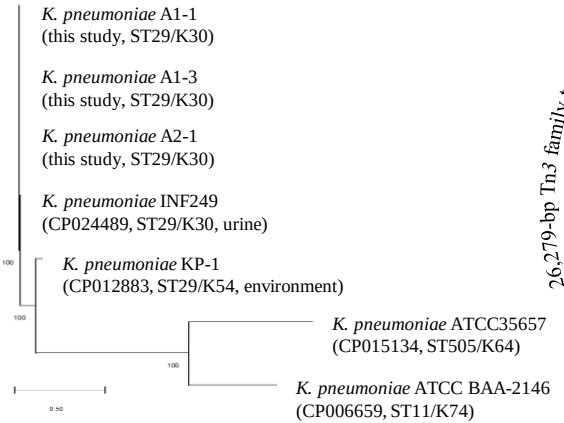
GenBank assembly accession	BioProject	strain	country
GCA_000694555	PRJNA234265	MGH66	USA
GCA_000785625	PRJNA259998	XDR	China
GCA_001546515	PRJNA272089	MJR8396D	USA
GCA_002752775	PRJNA351909	INF249	Australia
GCA_002752815		INF322	Australia
GCA_003227595	PRJNA431724	DG6106	Italy
GCA_900084725	PRJEB5065	k283	UK
GCA_900085565		k2328	UK
GCA_900086335		k1604	UK
GCA_900173625	PRJEB18814	VRCO0412	UK
GCA_900173655		VRCO0413	UK
GCA_900173675		VRCO0415	UK
GCA_900180815	PRJEB6574	3189STDY5864831	Pakistan
GCA_900501625	PRJEB10018	EuSCAPE_RS001	Serbia
GCA_900505215		EuSCAPE_RS036	Serbia
GCA_900507205		EuSCAPE_RS121	Serbia
GCA_900512465		EuSCAPE_DE041	Germany
GCA_900513895		EuSCAPE_ME009	Montenegro
GCA_900607415	PRJEB28660	kpneu003	Switzerland

Figure legends

Fig. 1. SNP-based phylogeny of ST29 K30 *K. pneumoniae* strains A1-1 (GCA_014596075.1), A1-3 (GCA_014596045.1), and A2-1 (GCA_014595525.1), and circular genetic map of the IncL/M plasmid pA1-1 (LC505604) harbored by strain A1-1. (A) A phylogenetic tree was constructed using the distance matrix of SNP differences between each pair of genomes among *K. pneumoniae* strains A1-1, A1-3, and A2-1, ST11 K74 *K. pneumoniae* ATCC BAA-2146 (CP006659), ST505 K64 *K. pneumoniae* ATCC35657 (CP015134), ST29 K54 *K. pneumoniae* KP-1 (CP012883) and ST29 K30 *K. pneumoniae* INF249 (CP024489). (B) The antimicrobial resistance genes and mobile elements are shown in red and blue, respectively. The *tra/trb* conjugation genes are shown in green. The arrows show the direction of transcription.

Fig. 2. ST29 K30 *K. pneumoniae* wgMLST tree showing phylogenetic relationships of the strains A1-1, A1-3, and A2-1 in this study compared with other 19 public reference strains (Table S2). Presence (gray) or absence (white) of heavy metal resistance genes among strains is shown.

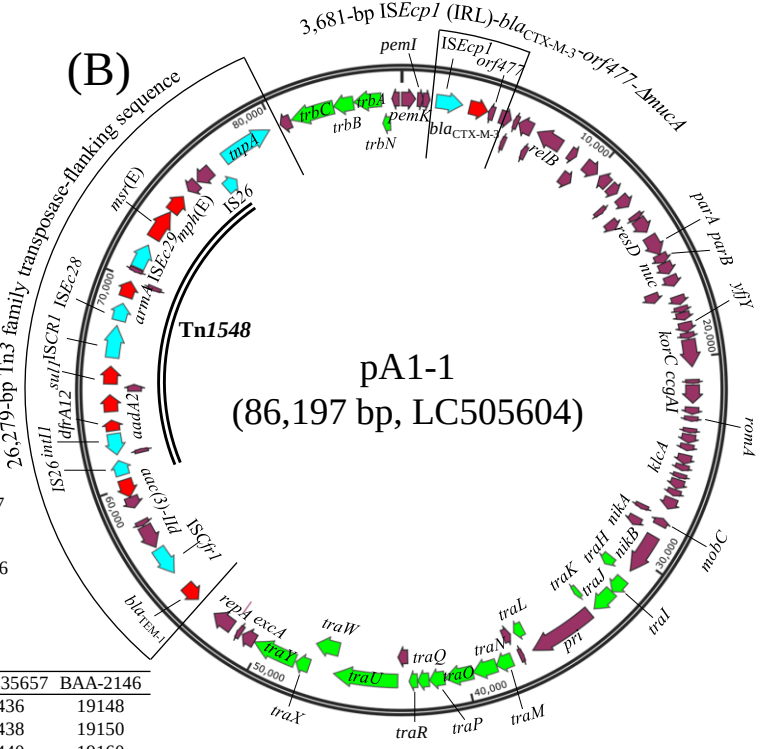
(A)

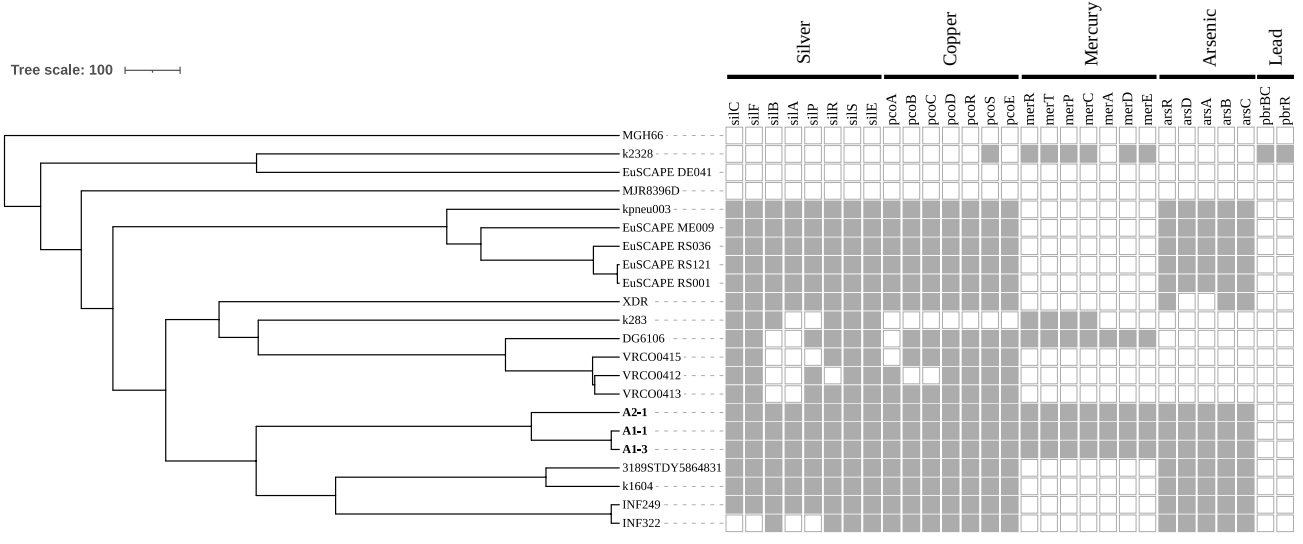


Matrix of SNP pair counts

	A1-1	A1-3	A2-1	INF249	KP-1	ATCC35657	BAA-2146
A1-1	0	8	22	380	4661	20436	19148
A1-3	8	0	22	382	4663	20438	19150
A2-1	22	22	0	368	4649	20440	19160
INF249	380	382	368	0	4627	20447	19184
KP-1	4661	4663	4649	4627	0	20449	18982
ATCC35657	20436	20438	20440	20447	20449	0	19397
BAA-2146	19148	19150	19160	19184	18982	19397	0

(B)




Tree scale: 100
Silver
Copper
Mercury
Arsenic
Lead
silC
silF
silB
silA
silP
silR
silS
silE
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pcoB
pcoC
pcoD
pcoR
pcoS
pcoE
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MJR8396D
kpneu003
EuSCAPE ME009
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EuSCAPE RS121
EuSCAPE RS001
XDR
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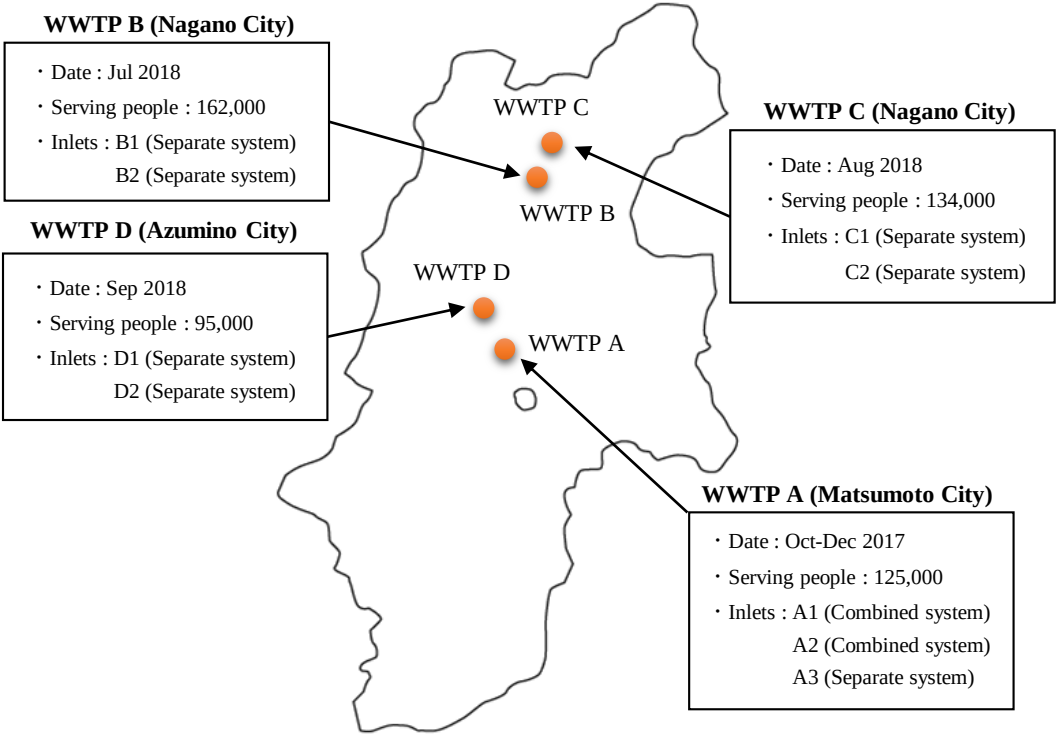
k1604
INF249
INF322



Supplementary Figure S1 Geographical locations of 4 wastewater treatment plants (WWTPs) A, B, C, and D in Nagano Prefecture, Japan