Title: The surveillance of colistin-resistance and mobilized colistin resistance genes in multi-drug resistant Enterobacteriaceae isolated in Japan

Running Title: Surveillance of colistin-resistant Enterobacteriaceae

Authors:

Yasuhide Kawamoto[1], Norihito Kaku[1,2,3], Norihiko Akamatsu[1], Kei Sakamoto[1,2]
Kosuke Kosai[1,2], Yoshitomo Morinaga[1,2,4], Norio Ohmagari[5], Koichi Izumikawa[6],
Yoshihiro Yamamoto[7], Hiroshige Mikamo[8], Mitsuo Kaku[9], Kazunori Oishi[10,11],
Katsunori Yanagihara[1,2]

Affiliations:

[1]Department of Laboratory Medicine, Nagasaki University Hospital, Japan.
[2]Department of Laboratory Medicine, Nagasaki University Graduate School of Biomedical Sciences, Japan.
[3]Division of Pulmonary and Critical Care Medicine, Department of Internal Medicine, University of Michigan, Japan.

[4]Department of Microbiology, Graduate School of Medicine and Pharmaceutical Sciences, University of Toyama,

[5]Disease Control and Prevention Center, National Center for Global Health and Medicine, Japan.

[6]Department of Infectious Diseases, Nagasaki University Graduate School of Biomedical Sciences, Japan.

[7]Department of Clinical Infectious Diseases, Graduate School of Medicine and Pharmaceutical Sciences for Research, University of Toyama, Japan.

[8]Department of Clinical Infectious Diseases, Aichi Medical University Graduate School of Medicine, Japan.

[9]Department of Infection Control and Laboratory Diagnostics, Tohoku University Graduate School of Medicine, Japan

[10]Infectious Disease Surveillance Center, National Institute of Infectious Diseases Correspondence, Japan

[11]Toyama Institute of Health, Japan.

Address for correspondence:

Norihito Kaku, Department of Laboratory Medicine, Nagasaki University Graduate School of Biomedical Sciences, 1-7-1 Sakamoto, Nagasaki, Nagasaki 852-8501, Japan; email: norihitk@gmail.com; tel/fax: +81-95-819-7413

Abstract

Background. The plasmid-mediated bacterial colistin-resistant gene, *mcr*, is of global concern in clinical health care. However, there are few reports of surveillance for *mcr* in Japan. This study aimed to study the prevalence of colistin resistance by identifying nine *mcr* genes in ESBL-producing Enterobacteriaceae and CRE isolates in Japan.

Methods. We collected 273 ESBL and CRE clinical isolates from the patients in five tertiary hospitals between August 2016 to March 2017. MIC of colistin was measured using the microdilution method. PCR was performed to detect *mcr-1* to *mcr-9* genes in all strains. Additionally, if we identified a *mcr*-gene that had not been reported from patients in Japan, we performed a WGS analysis.

Results. The rate of colistin resistance was 7.7% in all strains. The rate of colistin resistance in the CRE strains was higher than that in the ESBL-producing strains (20.4% versus 1.1%). The *mcr-5* and *mcr-9* gene were detected in one ESBL-producing

E. coli strain (1/273, 0.37%) and three CRE strains (3/273, 1.1%), respectively. Since the ESBL-producing *E. coli* strain was the first clinical strain with *mcr-5* in Japan, whole-genome sequencing analysis was performed for the strain. The sequence type of the *mcr-5* positive strain was ST1642 and it carried two distinct plasmids, ESBL gene-carrying pN-ES-6-1, and *mcr-5.1*-carrying pN-ES-6-2.

Conclusions. We showed that the frequency of colistin resistance and *mcr*-positive strains is not high in Japan. Since the MIC for colistin was low in the *mcr-5.1* and *mcr-9* gene-positive strain, continuous monitoring of *mcr* genes is necessary.

Keywords. *mcr-5*; *mcr-9*; colistin; Enterobacteriaceae; Surveillance; Japan

Introduction

The emergence and spread of antimicrobial resistance are a cause of global concern. In Japan, a previous study reported that ESBL-producing *Escherichia coli* (*E. coli*) and *Klebsiella pneumoniae* (*K. pneumoniae*) strains are spreading, accounting for 23.0% of *E. coli* and 10.7% of *K. pneumoniae* infections from 2014 to 2015 [1]. Because of the distribution of fluoroquinolone resistance in ESBL-producing *E. coli*, the clinical use of carbapenems is increasing [2, 3]. The increased use of carbapenems induces carbapenem-resistant Enterobacteriaceae (CRE), therefore, colistin is becoming an important alternative to carbapenems[4]. In recent years, carbapenem-resistant Enterobacteriaceae (CRE) has become a serious problem worldwide. Some CREs have multidrug resistance against fluoroquinolone as well as beta-lactam [5]. Therefore, colistin, which belongs to the family of polymyxins and has broad-spectrum activity against gram-negative bacteria, is an important antibiotic in the treatment of CRE and ESBL-producing Enterobacteriaceae [6].

The major colistin resistance mechanisms are as follows: alteration of the LPS moiety resulting in a reduced net negative charge of LPS, increased drug efflux, overexpression of outer membrane protein (OprH), and the formation of capsules (*siaD*, *ompA*, *cps*) [7, 8]. Since these resistance mechanisms are intrinsic, mutational, and adaptive, colistin

78 resistance is unlikely to spread from cell to cell through the delivery of plasmids like
79 ESBL and carbapenemase-producing Enterobacteriaceae (CPE). However, in 2016, the
80 first plasmid-mediated colistin resistance gene, *mcr*, was identified in animals in China
81 [9]. Thereafter, *mcr*-positive Enterobacteriaceae have been identified in healthy people
82 and patients all over the world [10]. In Japan, although there have been some reports on
83 *mcr*-positive *E. coli* in animals and food sources [11-13], there are few studies in
84 humans [14]. In addition, the percentage of colistin resistance in Japan remains
85 unknown because the MIC of colistin in Enterobacteriaceae including ESBL and CRE
86 has not been evaluated.
87 The purpose of this study was to clarify the prevalence of colistin resistance and nine
88 *mcr* genes in ESBL-producing Enterobacteriaceae and CRE isolated from patients in
89 tertiary hospitals in Japan. Additionally, if we identify *mcr*-gene that had not been
90 reported from patients in Japan, we performed a WGS analysis for the strain.
91
92 **Materials and methods**
93 *Strains*
94 A total of 273 different clinical ESBL-producing Enterobacteriaceae and CRE isolates
95 were collected between August 2016 and March 2017 from five tertiary hospitals

96 representing the Western, Eastern, and Central regions of Japan. Only one isolate per
97 patient was included in this study. *Proteus* spp. and *Providencia rettgeri* were excluded
98 from the analysis because they are intrinsically resistant to colistin. A total of 180
99 ESBL-producing strains and 93 CRE strains were collected during the study period. The
100 ESBL-producing strains were *E. coli* (81.7%), *K. pneumoniae* (15.6%), and *Klebsiella*
101 *oxytoca* (2.8%). The CRE strains were *Klebsiella aerogenes* (45.2%), *Enterobacter*
102 *cloacae* complex (38.7%), *K. pneumoniae* (11.8%), *E. coli* (2.2%), and *Citrobacter* spp.
103 (2.2%). The strains were stored in a Microbank tube and placed at -80°C.
104
105 *Analysis of strains*
106 Bacteria were identified using MALDI-TOF MS (Bruker Daltonics GmbH, Bremen,
107 Germany). ESBL production was detected using the BD Phoenix system NMIC-207
108 panel (Becton Dickinson, Holdrege, USA) according to the manufacturer's instructions.
109 Carbapenemase genes were evaluated by Xpert Carba-R assay (Cepheid, Sunnyvale,
110 USA) according to the manufacturer's instructions. Broth microdilution MIC testing
111 was performed with colistin, piperacillin, ceftazidime, ceftriaxone, cefpodoxime,
112 cefepime, cefmetazole, aztreonam, piperacillin-tazobactam, ampicillin-sulbactam,
113 imipenem, meropenem, gentamicin, amikacin, minocycline, ciprofloxacin, levofloxacin,

and sulfamethoxazole-trimethoprim by a manual assay using the MIC panels (Eiken Chemical Co., Ltd, Tokyo, Japan). Susceptibility was determined according to the CLSI M100-S25 except for colistin[15], which was interpreted according to the EUCAST version 9.0 (MIC for susceptible, ≤ 2 mg/L; MIC for resistant, >2 mg/L)[16].

Analysis of mcr genes

PCR was performed to detect *mcr-1* to *mcr-9* genes in all strains using previously reported primers[17, 18]. DNA was extracted using the boiling method with minor modifications [19]. PCR amplification about *mcr-1* to *mcr-5* was performed under the following conditions: 15 min at 94°C, 25 cycles of 30 s at 94°C, 30 s at 58°C, 60 s at 72°C, and 10 min at 72°C for the final extension [17]. PCR amplification about *mcr-6* to *mcr-9* was performed under the following conditions: 3 min at 95°C, 30 cycles of 30 s at 95°C, 30 s at 55°C, 60 s at 72°C, and 10 min at 72°C for the final extension[18].

Whole-genome sequencing (WGS)

For one *mcr-5*-positive strain, WGS was performed according to the following procedure. DNA was extracted using the Quick-DNA^{TM} Fecal/Soil Microbe Miniprep kit according to the manufacturer's instructions (Zymo Research, CA, USA). Whole-

genome sequencing was performed using NextSeq 500 (Illumina Inc. San Diego CA USA) and GridION X5 (Oxford Nanopore Technologies, Oxford, UK). The *de novo* hybrid assembly of both short-reads (NextSeq 500) and long-reads (GridION X5) was performed using Unicycler v0.4.7 under conservative conditions. CheckM v1.0.12 was used to assess the quality of assembled genomes. The allele sequences and sequence types (STs) were determined according to the *E. coli* database (http://mlst.warwick.ac.uk/mlst/dbs/Ecoli). In the plasmid analysis, Prokka v1.13 was used for genome annotation. Antimicrobial resistance genes were identified using ResFinder v3.2 (https://cge.cbs.dtu.dk/services/ResFinder/). The bacterial insertion sequence was detected using the IS Finder database (https://isfinder.biotoul.fr). PlasmidFinder v2.0.1 was used to determine plasmid incompatibility (Inc) groups. Importing Prokka's annotation result and drawing the plasmid map with Snap Gene v4.3.10 GSL Biotech.

Phylogenetic analysis and genetic environment

The *mcr-5*-carrying plasmid sequencing from *Salmonella enterica* (Gene accession no. NC_003277.2), *Salmonella enterica* (Gene accession no. LC488708.01), *Escherichia coli* (Gene accession no. BENI01000099.1), pN-ES-6-2 *Escherichia coli,* and

Salmonella enterica (Gene accession no. KY807921.1) were downloaded from GenBank database. Phylogenetic tree and genetic environment were constructed using CLC Genomics Workbench version 21.0.3.

Data availability

Raw data were generated at Nagasaki University Hospital. Derived data supporting the findings of this study are available from the corresponding author upon request.

Ethics

This study was approved by the Ethics Committee of Nagasaki University Hospital (16072509). Data regarding clinical ESBL-producing Enterobacteriaceae and CRE isolates were anonymized and individually numbered when they were collected from the hospitals.

Results

Colistin Resistance

167 The rate of colistin resistance amongst all strains was 7.7% (Table 1). The rate of
168 colistin resistance in the CRE strains was higher than that in the ESBL-producing
169 strains (20.4% versus 1.1%, Table 1). The MIC of colistin in both ESBL-producing
170 strains and CRE formed a bimodal distribution (Fig. 1A). The *Enterobacter cloacae*
171 complex had the highest rate of colistin resistance (50.0%) (Table 1). The rate of
172 colistin resistance was not significantly different based on the site of infection or region
173 where the strain was isolated (Table 2). In the CRE strains, carbapenemase-producing
174 Enterobacteriaceae (CPE) had a higher rate of colistin resistance (40.0%) than non-CPE
175 strains (16.6%) (Fig. 1B).
176
177 *Detection of mcr genes*
178 The 273 strains (ESBL and CRE) were screened using PCR for the presence of the
179 nine *mcr* genes, *mcr-1* to *mcr-9*. The *mcr-5* gene was detected in only one ESBL-
180 producing *E. coli* strain (1/273, 0.37%), and the *mcr-9* gene was detected in three CRE
181 *Enterobacter cloacae* complex strains (3/273, 1.1%). The two of three *mcr-9* positive
182 strains were isolated in the same hospital, but the ward and the department were
183 different. All the *mcr* positive strains had very low MIC for colistin (Table S1).
184

185 *Whole-genome sequencing of mcr-5 gene-positive strain*

186 Since there were no reports of *mcr-5* positive ESBL-producing *E. coli* isolated from
187 humans in Japan, we performed a detailed analysis for one strain by whole-genome
188 sequencing. The draft genome of *the mcr-5* gene-positive *E. coli* strain (DDBJ
189 accession no. DRA010253) comprised 5,027,748 bp with an overall GC content of
190 50.76%. The genome consisted of 22 rRNA operons, 80 tRNA genes, and 4686
191 protein-coding genes (CDSs). The *mcr-5* gene-positive *E. coli* was classified as
192 ST1642 and *fimH* subtype 31. The isolate harbored two distinct plasmids, pN-ES-6-1
193 (DDBJ accession no. LC553463, 94901 bp), and pN-ES-6-2 (DDBJ accession no.
194 LC553464, 79974 bp) (Figure 2). pN-ES6-1 had various resistance genes, including
195 *mph*(A), *dfrA17*, $bla_{\text{TEM-1B}}$, *sul2*, *tet*(B), *aac(3)-lld*, *aac(3'')-lb*, and *aph(6)-ld*, whereas
196 pN-ES-6-2 had only one resistance gene (*mcr-5.1*) (Table 3). The Plasmid Inc. groups
197 and transposons of pN-ES-6-2 were IncFII and TnShfr1 (Tn3-family), respectively
198 (Table 3). Phylogenetic analysis of *mcr-5* carrying plasmid revealed that pN-ES-6-2
199 showed similarities to *Escherichia coli* (Gene accession no. BENI01000099.1) isolated
200 in Japan, but less to the first reported *mcr-5* carrying plasmid (Gene accession no.
201 KY807921.1, *Salmonella enterica*, Germany) (Figure 3A). pN-ES-6-2 lacked some

major facilitator superfamily (MFS) gene in comparison with other *mcr-5* carrying plasmids (Figure 3B).

Discussion

In this study, we studied the prevalence of colistin resistance in ESBL-producing Enterobacteriaceae and CRE. The rates of colistin resistance were 0.7% for *E. coli*, 2.6% for *K. pneumoniae*, and 50.0% for *Enterobacter cloacae* complex. The Surveillance of Multicenter Antimicrobial Resistance in Taiwan reported that the resistance rate of colistin was 0.3% in *E. coli* and 2.4% in *K. pneumoniae* [20] Global surveillance in 2015 reported that the resistance rate of colistin was 0.3% in *E. coli*, 2.4% in *K. pneumoniae*, and 39.1% in *Enterobacter asburiae* [21]. These results are consistent with our study. In this study, *Enterobacter cloacae* complex showed a higher colistin resistance rate than *E. coli* and *K. pneumoniae.* In the previous report, *Enterobacter cloacae* complex also showed a higher colistin resistance rate than *E. coli* and *K. pneumoniae* including *K. aerogenes* [22]. Although there is a study that reported the mechanism of colistin-resistance in *Enterobacter* spp. [23], the reason for the high rates of colistin-resistant *Enterobacter cloacae* complex remains unclear.

219 In the current study, the colistin-resistance rate in ESBL-producing and CRE strains was
220 7.7%. Accordingly, in Japan, it is considered that colistin-resistant bacteria have not
221 become widespread. However, in the CRE strains, the rate of colistin resistance in CPE
222 strains was higher than that in non-CPE strains. A previous study reported a strong
223 association between the presence of carbapenemase and increased resistance to colistin
224 in Enterobacteriaceae strains [21]. In addition, other investigators have reported clonal
225 spread of colistin resistance due to multiple mutational mechanisms in CPE [24]. Since
226 the number of CPE strains has been increasing in Japan, it will be necessary to
227 continually monitor the MIC of colistin in carbapenemase-producing
228 Enterobacteriaceae strains[25].
229 Nine *mcr* genes in ESBL-producing Enterobacteriaceae and CRE strains were
230 investigated in this study. The positive rate of *mcr* genes in all strains was 1.5%, which
231 was similar to that in other previous reports from clinical samples (0.2-3.2%) [9, 26-29].
232 Therefore, in Japan, it seems that *mcr* genes have not become widespread in bacteria.
233 One of the reasons for this is that colistin is currently used only in limited situations in
234 Japan [30]. On the other hand, large amounts of colistin has been used in animals [31,
235 32]. The first plasmid-mediated colistin resistance gene in Enterobacteriaceae, *mcr-1*,
236 was detected in food-producing animals and humans in China, and it is likely that *mcr-*

237 *1*-mediated colistin resistance originated in animals and subsequently spread to humans
238 [9]. In addition, the presence of plasmids containing *mcr* genes in *E. coli* from livestock
239 animals has previously been reported in Japan. High prevalence of *mcr-1* and *mcr-5*
240 have been observed among strains isolated from diseased pigs [12]. Thus, we need to be
241 wary of the future spreading of *mcr* gene-positive strains in humans.
242 The *mcr-1* and *mcr-9* are distributed worldwide, *mcr-4*, *mcr-2,* and *mcr-8* has a limited
243 distribution, and other *mcr* are rarely reported[33]. In this study, we identified three
244 *mcr-9* positive *Enterobacter cloacae* complex. Although the *Enterobacter cloacae*
245 complex harboring carbapenemase gene and *mcr-9* has previously been reported in
246 Japan[34], carbapenemase genes were not detected in the three *mcr-9* positive stains.
247 We also identified one *mcr-5* positive *E. coli*, but there were no reports of *mcr-5*
248 positive *E. coli* isolated from humans in Japan. The patient infected with the *mcr-5*
249 gene-positive strain had recurrent urinary tract infection, but the patient had never been
250 treated with colistin. The acquisition of *mcr* gene-positive strains in humans has been
251 reported to be transmitted from livestock[9], but the patient had no history of contact
252 with animals. There was a possibility that the *mcr-5*-positive strain resulted from meat
253 consumption in this case because the number of *mcr-5* positive strains isolated in
254 livestock was higher in Japan than in other countries [12]. In this case, there was no

255 history of treatment with colistin, whereas the patient was previously treated with
256 levofloxacin and piperacillin-tazobactam. Since a previous study reported that the risk
257 factors for *mcr*-positive Enterobacteriaceae were immunosuppression and history of
258 antibiotic use, particularly carbapenems and fluoroquinolones [35], past use of
259 levofloxacin may have been a risk factor for acquiring the *mcr-5*-positive strain in this
260 patient.

261 Whole-genome sequencing analysis revealed that the *mcr-5*-positive strain was
262 ST1642 and carried two distinct plasmids, the ESBL gene-carrying pN-ES-6-1 and *mcr-*
263 *5*-carrying pN-ES-6-2. Although one study reported three *mcr-1*-harboring ESBL-
264 producing *E. coli* ST1642 strains from bovine fecal samples[36], the *mcr-5.1*-harboring
265 ESBL-producing *E. coli* ST1642 has not been reported previously. We found
266 transposons of TnShfr1 (Tn3-family) in the *mcr-5* carrying *E. coli*. The *mcr-5.1* gene is
267 reportedly located on the Tn3-family transposon of the *Salmonella enterica* Paratyphi B
268 and *Cupriavidus gilardii* [37]. Therefore, there is a possibility that the *E. coli* strain
269 received plasmid from these bacteria. In this study, the *mcr* genes-positive strain
270 exhibited low MIC for colistin, which was not interpreted as resistant according to
271 EUCAST. The results are the same as the previous reports [12, 38]. These results

indicate that *mcr-5* and *mcr-9* may silently spread among Enterobacteriaceae, such as the stealth phenotype CPE [39].

This study has some limitations. We investigated the prevalence of colistin-resistant and plasmid-mediated colistin resistance genes in ESBL-producing and CRE strains. Since *mcr*-positive strains have also been reported detected in non-ESBL-producing and non-CRE strains [40], we need to perform surveillance in all Enterobacteriaceae including drug-sensitive strains. In addition, we did not investigate intrinsic resistance to colistin. In this study, we performed a WGS analysis for the strain harbored *mcr*-gene that had not been reported from patients in Japan. Thus, we didn't perform a WGS analysis for *mcr-9* positive strains. However, further investigation for *mcr-9* positive strains is necessary to verify whether the same *mcr-9* positive strains detected in this study are similar to the previous report.

In conclusion, we revealed that the rates of colistin-resistance and *mcr*-positive strains are not high in Japan. Since the MIC for colistin was low in the *mcr*-5 or *mcr-9* gene-positive strain, continuous monitoring of plasmid-mediated *mcr* genes in Enterobacteriaceae is necessary.

Notes

290 ***Author contributions.***

291 NK, NA, KS, KK, YM, and KY contributed to study design and data interpretation. K
292 I, YY, HM, MK, and KO contributed to the study design and collection of isolates and
293 data. N. K. provided expert advice, critically reviewed the manuscript, including for
294 aspects related to the English language, and contributed to its content. All authors
295 reviewed and approved the final version of the manuscript.

296

297 ***Disclaimer.***

298 The funders had no role in the design of the study, the collection or analysis of data, or
299 the decision to publish.

300

301 ***Financial support.***

302 This work was supported by the Japanese Society of Laboratory Medicine Fund for the
303 Promotion of Scientific and the Health and Labour Sciences Research Grants from the
304 Ministry of Health, Labour and Welfare, Japan (H28-Shinkou-Ippan-003).

305

306 ***Potential conflicts of interest.***

307 All authors: No reported conflicts of interest.

Acknowledgment

We would like to thank Editage (www.editage.com) for English language editing.

312 **References**

313 [1] Takesue Y, Kusachi S, Mikamo H, Sato J, Watanabe A, Kiyota H, et al.
314 Antimicrobial susceptibility of pathogens isolated from surgical site infections in
315 Japan: Comparison of data from nationwide surveillance studies conducted in
316 2010 and 2014-2015. Journal of infection and chemotherapy : official journal of
317 the Japan Society of Chemotherapy. 2017;23:339-48.
318 [2] Salah FD, Soubeiga ST, Ouattara AK, Sadji AY, Metuor-Dabire A, Obiri-
319 Yeboah D, et al. Distribution of quinolone resistance gene (qnr) in ESBL-
320 producing Escherichia coli and Klebsiella spp. in Lomé, Togo. Antimicrob Resist
321 Infect Control. 2019;8:104.
322 [3] Pana ZD, Zaoutis T. Treatment of extended-spectrum β-lactamase-
323 producing Enterobacteriaceae (ESBLs) infections: what have we learned until
324 now? F1000Res. 2018;7.
325 [4] Ku YH, Lee MF, Chuang YC, Chen CC, Yu WL. In vitro activity of colistin
326 sulfate against Enterobacteriaceae producing extended-spectrum β-lactamases.
327 J Microbiol Immunol Infect. 2015;48:699-702.
328 [5] Suay-García B, Pérez-Gracia MT. Present and Future of Carbapenem-
329 resistant Enterobacteriaceae (CRE) Infections. Antibiotics (Basel). 2019;8.

330 [6] Parchuri S, Mohan S, Cunha BA. Extended spectrum beta-lactamase-
331 producing Klebsiella pneumoniae chronic ambulatory peritoneal dialysis
332 peritonitis treated successfully with polymyxin B. Heart Lung. 2005;34:360-3.
333 [7] Mlynarcik P, Kolar M. Molecular mechanisms of polymyxin resistance and
334 detection of mcr genes. Biomed Pap Med Fac Univ Palacky Olomouc Czech
335 Repub. 2019;163:28-38.
336 [8] Olaitan AO, Morand S, Rolain JM. Mechanisms of polymyxin resistance:
337 acquired and intrinsic resistance in bacteria. Frontiers in microbiology.
338 2014;5:643.
339 [9] Liu Y-Y, Wang Y, Walsh TR, Yi L-X, Zhang R, Spencer J, et al. Emergence
340 of plasmid-mediated colistin resistance mechanism MCR-1 in animals and
341 human beings in China: a microbiological and molecular biological study. The
342 Lancet Infectious Diseases. 2016;16:161-8.
343 [10] Sun J, Zhang H, Liu YH, Feng Y. Towards Understanding MCR-like
344 Colistin Resistance. Trends in microbiology. 2018;26:794-808.
345 [11] Kawanishi M, Abo H, Ozawa M, Uchiyama M, Shirakawa T, Suzuki S, et al.
346 Prevalence of Colistin Resistance Gene mcr-1 and Absence of mcr-2 in

Escherichia coli Isolated from Healthy Food-Producing Animals in Japan. Antimicrobial Agents and Chemotherapy. 2017;61.

[12] Fukuda A, Sato T, Shinagawa M, Takahashi S, Asai T, Yokota SI, et al. High prevalence of mcr-1, mcr-3 and mcr-5 in Escherichia coli derived from diseased pigs in Japan. International journal of antimicrobial agents. 2018;51:163-4.

[13] Ohsaki Y, Hayashi W, Saito S, Osaka S, Taniguchi Y, Koide S, et al. First Detection of an Escherichia coli Strain Harboring the mcr-1 Gene in Retail Domestic Chicken Meat in Japan. Japanese journal of infectious diseases. 2017;70:590-2.

[14] Tada T, Uechi K, Nakasone I, Shimada K, Nakamatsu M, Kirikae T, et al. Emergence of a colistin-resistant Escherichia coli clinical isolate harboring mcr-1 in Japan. Int J Infect Dis. 2017;63:21-2.

[15] CLSI. Performance standards for antimicrobial susceptibility testing. 25th ed. CLSI guideline M100. Wayne, PA: Clinical and Laboratory Standards Institute.

363 [16] The European Committee on Antimicrobial Susceptibility Testing.
364 Breakpoint tables for interpretation of MICs and zone diameters.version 9.0,
365 2019.
366 [17] Rebelo AR, Bortolaia V, Kjeldgaard JS, Pedersen SK, Leekitcharoenphon P,
367 Hansen IM, et al. Multiplex PCR for detection of plasmid-mediated colistin
368 resistance determinants, mcr-1, mcr-2, mcr-3, mcr-4 and mcr-5 for surveillance
369 purposes. Euro surveillance : bulletin Europeen sur les maladies transmissibles =
370 European communicable disease bulletin. 2018;23.
371 [18] Borowiak M, Baumann B, Fischer J, Thomas K, Deneke C, Hammerl JA, et
372 al. Development of a Novel mcr-6 to mcr-9 Multiplex PCR and Assessment of
373 mcr-1 to mcr-9 Occurrence in Colistin-Resistant Salmonella enterica Isolates
374 From Environment, Feed, Animals and Food (2011-2018) in Germany.
375 Frontiers in microbiology. 2020;11:80.
376 [19] Motoshima M, Yanagihara K, Morinaga Y, Matsuda J, Sugahara K, Yamada
377 Y, et al. Genetic diagnosis of community-acquired MRSA: a multiplex real-time
378 PCR method for Staphylococcal cassette chromosome mec typing and detecting
379 toxin genes. Tohoku J Exp Med. 2010;220:165-70.

[20] Lee YL, Lu MC, Shao PL, Lu PL, Chen YH, Cheng SH, et al. Nationwide surveillance of antimicrobial resistance among clinically important Gram-negative bacteria, with an emphasis on carbapenems and colistin: Results from the Surveillance of Multicenter Antimicrobial Resistance in Taiwan (SMART) in 2018. International journal of antimicrobial agents. 2019;54:318-28.

[21] Bradford PA, Kazmierczak KM, Biedenbach DJ, Wise MG, Hackel M, Sahm DF. Correlation of beta-Lactamase Production and Colistin Resistance among Enterobacteriaceae Isolates from a Global Surveillance Program. Antimicrob Agents Chemother. 2015;60:1385-92.

[22] Mushtaq S, Reynolds R, Gilmore MC, Esho O, Adkin R, García-Romero I, et al. Inherent colistin resistance in genogroups of the Enterobacter cloacae complex: epidemiological, genetic and biochemical analysis from the BSAC Resistance Surveillance Programme. J Antimicrob Chemother. 2020;75:2452-61.

[23] Telke AA, Olaitan AO, Morand S, Rolain JM. soxRS induces colistin hetero-resistance in Enterobacter asburiae and Enterobacter cloacae by regulating the acrAB-tolC efflux pump. J Antimicrob Chemother. 2017;72:2715-21.

397 [24] Otter JA, Doumith M, Davies F, Mookerjee S, Dyakova E, Gilchrist M, et
398 al. Emergence and clonal spread of colistin resistance due to multiple mutational
399 mechanisms in carbapenemase-producing Klebsiella pneumoniae in London. Sci
400 Rep. 2017;7:12711.
401 [25] Ohno Y, Nakamura A, Hashimoto E, Matsutani H, Abe N, Fukuda S, et al.
402 Molecular epidemiology of carbapenemase-producing Enterobacteriaceae in a
403 primary care hospital in Japan, 2010-2013. Journal of infection and
404 chemotherapy : official journal of the Japan Society of Chemotherapy.
405 2017;23:224-9.
406 [26] Quan J, Li X, Chen Y, Jiang Y, Zhou Z, Zhang H, et al. Prevalence of mcr-1
407 in Escherichia coli and Klebsiella pneumoniae recovered from bloodstream
408 infections in China: a multicentre longitudinal study. Lancet Infect Dis.
409 2017;17:400-10.
410 [27] Zheng B, Xu H, Yu X, Jiang X, Zhang J, Chen Y, et al. Low prevalence of
411 MCR-1-producing Klebsiella pneumoniae in bloodstream infections in China.
412 Clin Microbiol Infect. 2018;24:205-6.

413 [28] Chan WS, Au CH, Ho DN, Chan TL, Ma ES, Tang BS. Prospective study
414 on human fecal carriage of Enterobacteriaceae possessing mcr-1 and mcr-2
415 genes in a regional hospital in Hong Kong. BMC Infect Dis. 2018;18:81.
416 [29] Wise MG, Estabrook MA, Sahm DF, Stone GG, Kazmierczak KM.
417 Prevalence of mcr-type genes among colistin-resistant Enterobacteriaceae
418 collected in 2014-2016 as part of the INFORM global surveillance program.
419 PLoS One. 2018;13:e0195281.
420 [30] Hamada Y, Hirai J, Suematsu H, Yamagishi Y, Nicolau DP, Mikamo H.
421 Clinical experience with colistin in 9 Japanese patients with infection due to
422 multi-drug resistance pathogens. The Japanese journal of antibiotics.
423 2016;69:319-26.
424 [31] Catry B, Cavaleri M, Baptiste K, Grave K, Grein K, Holm A, et al. Use of
425 colistin-containing products within the European Union and European
426 Economic Area (EU/EEA): development of resistance in animals and possible
427 impact on human and animal health. International journal of antimicrobial
428 agents. 2015;46:297-306.

[32] Antimicrobial-resistant Bacteria Arising from the Use of Colistin Sulfate in the Livestock (Antimicrobial-resistant Bacteria). Food Saf (Tokyo). 2017;5:24-8.

[33] Ling Z, Yin W, Shen Z, Wang Y, Shen J, Walsh TR. Epidemiology of mobile colistin resistance genes mcr-1 to mcr-9. J Antimicrob Chemother. 2020;75:3087-95.

[34] Kananizadeh P, Oshiro S, Watanabe S, Iwata S, Kuwahara-Arai K, Shimojima M, et al. Emergence of carbapenem-resistant and colistin-susceptible Enterobacter cloacae complex co-harboring bla(IMP-1) and mcr-9 in Japan. BMC Infect Dis. 2020;20:282.

[35] Wang Y, Tian GB, Zhang R, Shen Y, Tyrrell JM, Huang X, et al. Prevalence, risk factors, outcomes, and molecular epidemiology of mcr-1-positive Enterobacteriaceae in patients and healthy adults from China: an epidemiological and clinical study. Lancet Infect Dis. 2017;17:390-9.

[36] Hassen B, Saloua B, Abbassi MS, Ruiz-Ripa L, Mama OM, Hassen A, et al. mcr-1 encoding colistin resistance in CTX-M-1/CTX-M-15- producing Escherichia coli isolates of bovine and caprine origins in Tunisia. First report of

446 CTX-M-15-ST394/D E. coli from goats. Comp Immunol Microbiol Infect Dis.
447 2019;67:101366.
448 [37] Borowiak M, Fischer J, Hammerl JA, Hendriksen RS, Szabo I, Malorny B.
449 Identification of a novel transposon-associated phosphoethanolamine transferase
450 gene, mcr-5, conferring colistin resistance in d-tartrate fermenting Salmonella
451 enterica subsp. enterica serovar Paratyphi B. J Antimicrob Chemother.
452 2017;72:3317-24.
453 [38] Tyson GH, Li C, Hsu CH, Ayers S, Borenstein S, Mukherjee S, et al. The
454 mcr-9 Gene of Salmonella and Escherichia coli Is Not Associated with Colistin
455 Resistance in the United States. Antimicrob Agents Chemother. 2020;64.
456 [39] Shigemoto N, Kuwahara R, Kayama S, Shimizu W, Onodera M, Yokozaki
457 M, et al. Emergence in Japan of an imipenem-susceptible, meropenem-resistant
458 Klebsiella pneumoniae carrying blaIMP-6. Diagn Microbiol Infect Dis.
459 2012;72:109-12.
460 [40] Sato T, Fukuda A, Usui M, Shinagawa M, Shiraishi T, Tamura Y, et al.
461 Isolation of a mcr-1-harbouring Escherichia coli isolate from a human clinical
462 setting in Sapporo, Japan. J Glob Antimicrob Resist. 2018;13:20-1.
463

Table 1. Colistin resistance in ESBL-producing and CRE strains

Organisms	Colistin -resistance		Carbapenemase genes (number)	*mcr* genes (number)
	n	(%)		
ESBL-producing strains (180)	2	(1.1%)		
Escherichia coli (147)	1	(0.68%)	N.D.	*mcr-5* (1)
Klebsiella pneumoniae (28)	1	(3.6%)	N.D.	N.D.
Klebsiella oxytoca (5)	0	(0.0%)	N.D.	N.D.
CRE strains (93)	19	(20.4%)		
Klebsiella aerogenes (42)	1	(2.4%)	IMP-1 group (1)	N.D.
Enterobacter cloacae complex (36)	18	(50.0%)	IMP-1 group (6)	*mcr-9* (3)
Klebsiella pneumoniae (11)	0	(0.0%)	IMP-1 group (7), NDM and OXA-48 (1)	N.D.
Escherichia coli (2)	0	(0.0%)	N.D.	N.D.
Citrobacter spp. (2)	0	(0.0%)	N.D.	N.D.
Total (273)	21	(7.7%)		

N.D., not detected

464

Table 2. Characteristics of colistin-resistant strains

Characteristics	Resistance of colistin
The region where the strain was isolated	
Western Japan (A hospital)	8/90 (8.9%)
Central Japan (B and C hospitals)	4/65 (6.2%)
Eastern Japan (D and E hospitals)	9/118 (7.6%)
Sites of infection	
Urinary tract	6/120 (5.0%)
Intra-abdominal	3/41 (7.3%)
Respiratory tract	8/55 (14.5%)
Blood	1/23 (4.3%)
Stool	2/20 (10.0%)
Others	1/14 (7.1%)

465

466

Table 3. Inc groups and antimicrobial resistance genes of *mcr-5* gene positive strain

Plasmid	Size(bp)	Inc groups	Resistance gene	Identity (%)	Query / Template length	Position in contig	Accession number
pN-ES-6-1	94,901	IncFIA	*mph*(A)	100	906/906	15559..16464	D16251
		IncFIB	*dfrA17*	100	474/474	21482..21955	FJ460238
		IncQ1	*bla* $_{\text{TEM-1B}}$	100	861/861	41557..42417	AY458016
			sul2	100	816/816	45621..46436	HQ840942
			tet(B)	100	1206/1206	24758..25963	AF326777
			aac(3)-lld	99.88	860/861	11179..12039	EU022314
			aac(3")-lb	100	804/804	46497..47300	AF321551
			aph(6)-ld	100	837/837	47300..48136	M28829
pN-ES-6-2	79,974	IncFII	*mcr-5.1*	100	1644/1644	6837..8480	KY807921

467

468

469 **Figure Legends**

470 **Figure 1.** The Distribution of colistin minimum inhibitory concentration (MIC) in
471 ESBL producing strains and CRE strains, Japan, August 2016- March 2017 (A). B) The
472 comparison of colistin resistance rates between non-CPE and CPE. The MICs of
473 colistin were measured using the microdilution method. White indicates non-colistin-
474 resistant; Black, colistin-resistant.

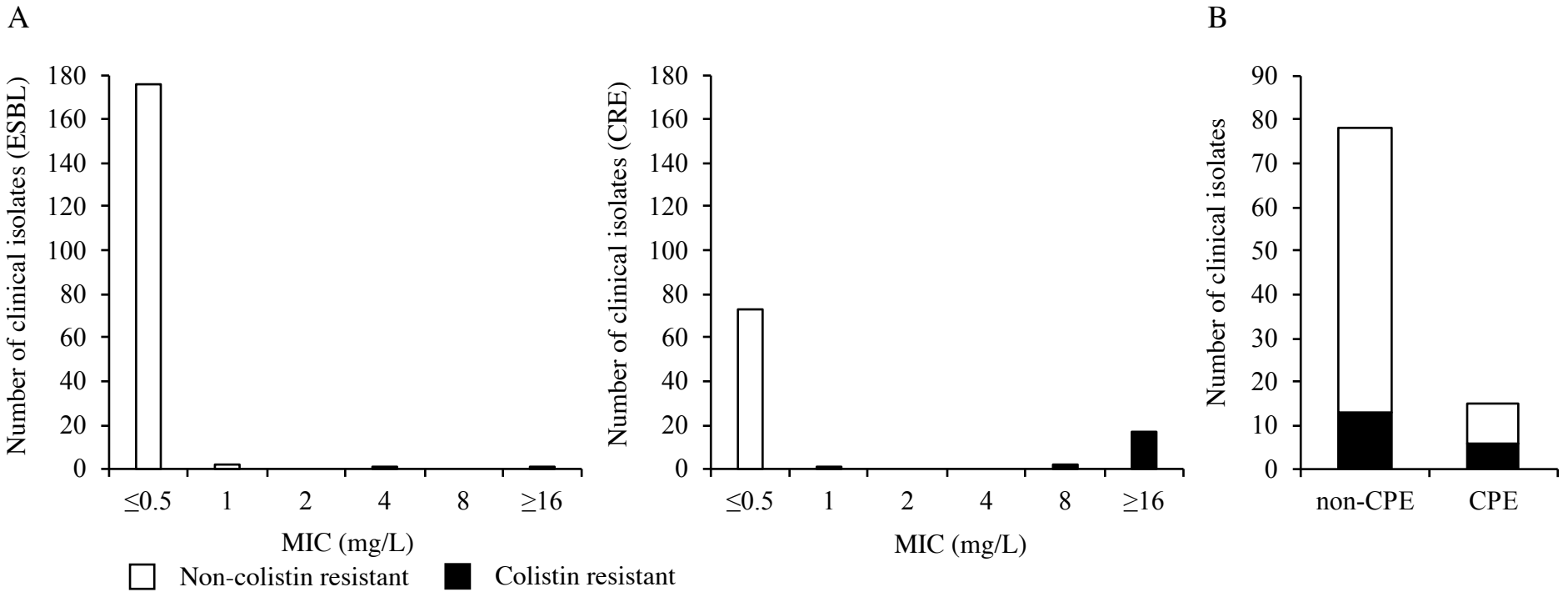
475

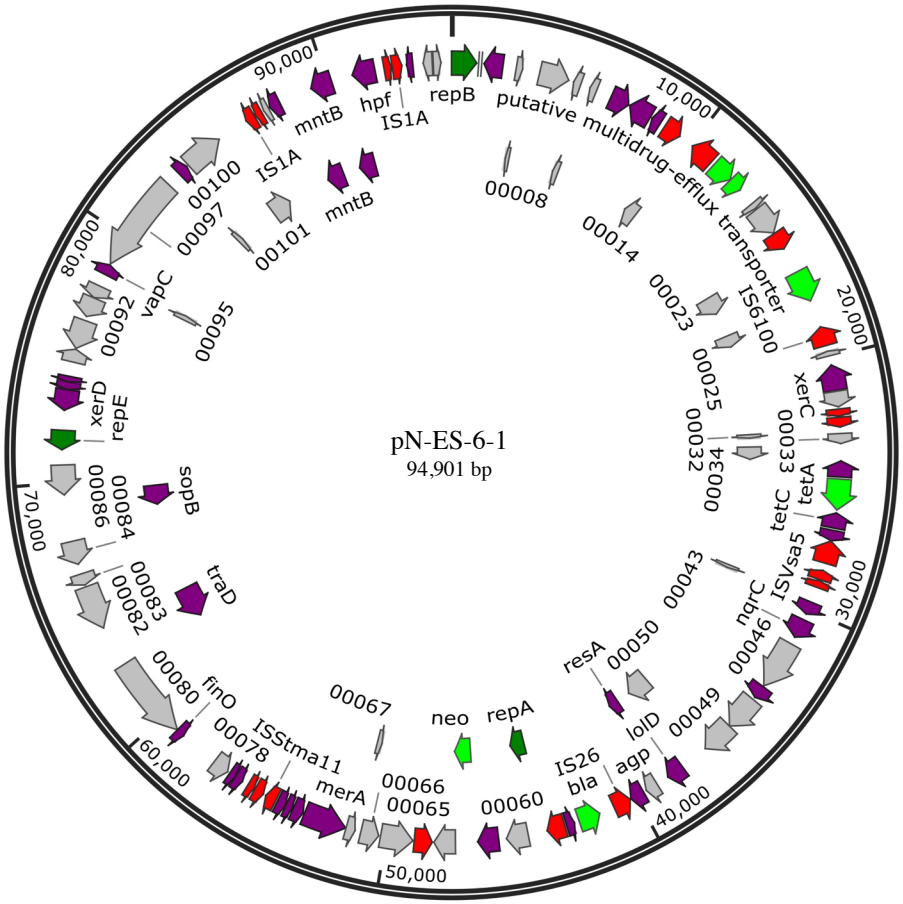
476 **Figure 2**. Structure of plasmid pN-ES-6-1 and pN-ES-6-2 from *Escherichia coli*.
477 Yellow-green indicates antimicrobial resistance; gray, hypothetical protein; red,
478 insertion sequence; green, plasmid replication; purple, other protein.

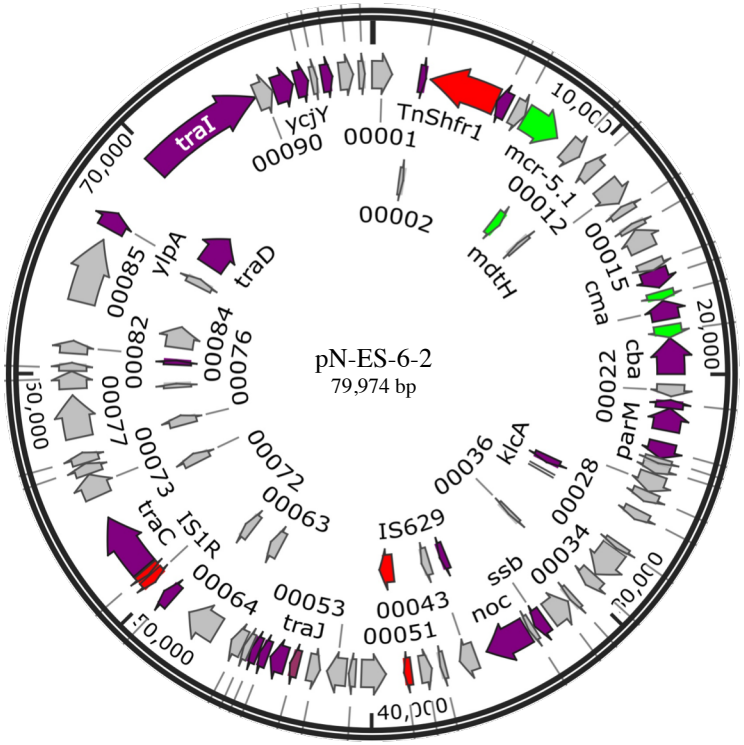
479

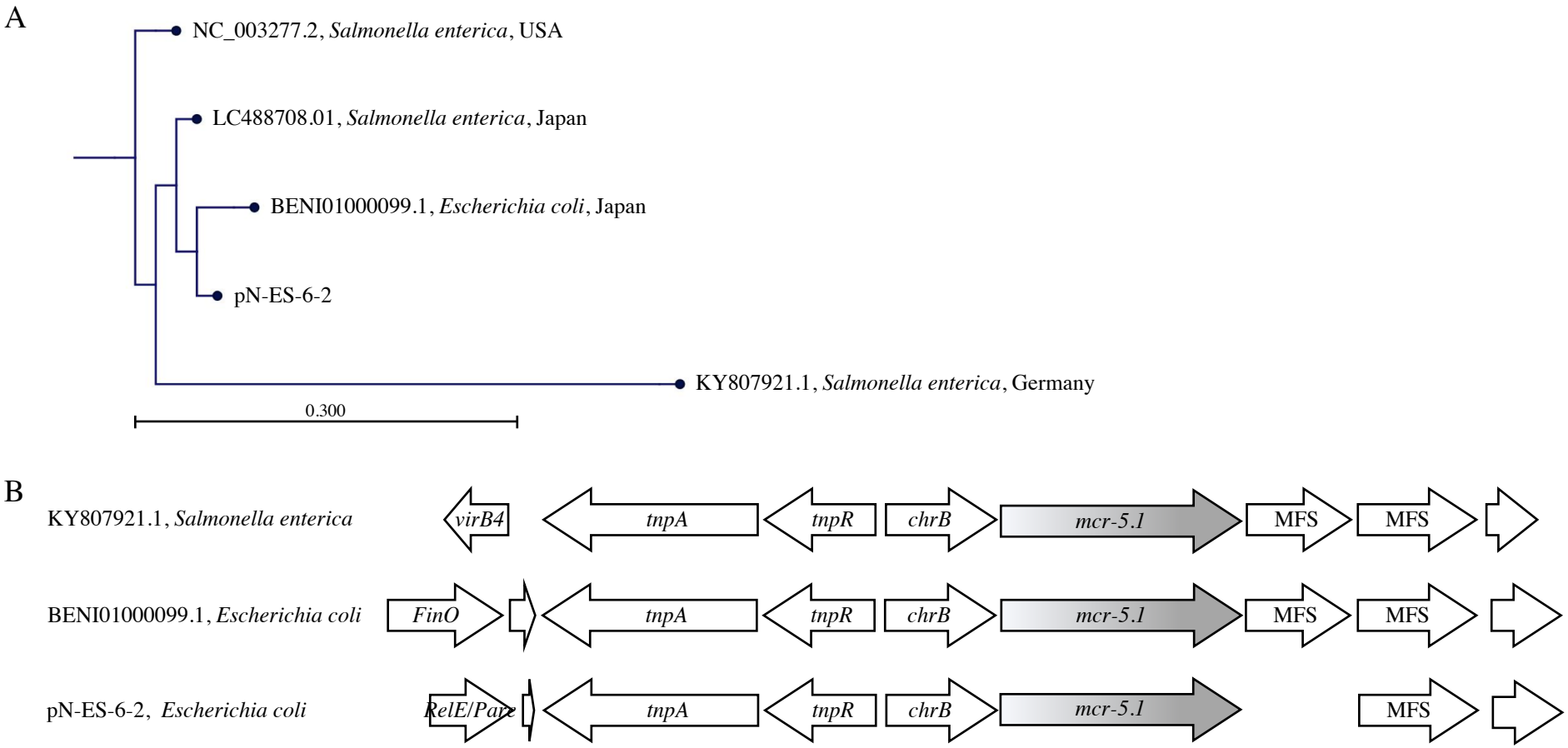
480 **Figure 3**. Comparison of the *mcr-5-carrying* plasmid. A) Phylogenetic tree of *mcr-5-*
481 *carrying* plasmid from *Salmonella enterica* (Gene accession no. NC_003277.2),
482 *Salmonella enterica* (Gene accession no. LC488708.01), *Escherichia coli* (Gene
483 accession no. BENI01000099.1), pN-ES-6-2 *Escherichia coli,* and *Salmonella enterica*
484 (Gene accession no. KY807921.1). Arrow represents coding sequences (gray arrows,
485 *mcr-5*) and indicated direction of transcription. B) The genetic environment of *mcr-5*

from *Salmonella enterica* (Gene accession no. KY807921.1), *Escherichia coli* (Gene accession no. BENI01000099.1), and pN-ES-6-2 *Escherichia coli*.


A
B
Number of clinical isolates (ESBL)
Number of clinical isolates (CRE)
Number of clinical isolates
180
160
140
120
100
80
60
40
20
0
90
70
50
30
10
≤0.5
1
2
4
8
≥16
MIC (mg/L)
non-CPE
CPE
Non-colistin resistant
Colistin resistant


pN-ES-6-1
94,901 bp
repB
putative multidrug-efflux transporter
IS6100
xerC
tetA
tetC
ISVsa5
ngrC
resA
lolD
agp
IS26
bla
neo
repA
merA
ISStma11
finO
traD
sopB
repE
xerD
vapC
IS1A
mntB
hpf
10,000
20,000
30,000
40,000
50,000
60,000
70,000
80,000
90,000


pN-ES-6-2
79,974 bp
traI
TnShfr1
mcr-5.1
mdtH
cma
parM
IS629
traJ
noc
ssb
klcA
traC
IS1R
traD
ylpA
ycjY
10,000
20,000
30,000
40,000
50,000
60,000
70,000


A
NC_003277.2, Salmonella enterica, USA
LC488708.01, Salmonella enterica, Japan
BENI01000099.1, Escherichia coli, Japan
pN-ES-6-2
KY807921.1, Salmonella enterica, Germany
0.300
B
KY807921.1, Salmonella enterica
virB4
tnpA
tnpR
chrB
mcr-5.1
MFS
MFS
BENI01000099.1, Escherichia coli
FinO
tnpA
tnpR
chrB
mcr-5.1
MFS
MFS
pN-ES-6-2, Escherichia coli
RelE/ParE
tnpA
tnpR
chrB
mcr-5.1
MFS

Supplementary data

Table S1. Antibiotic susceptibility testing of *mcr* gene positive strains

Antibiotics	*mcr-5* *E. coli* ESBL	*mcr-9* *Ent. cloacae* CRE	*mcr-9* *Ent. cloacae* CRE	*mcr-9* *Ent. cloacae* CRE
Piperacillin	>64	≤2	16	8
Ceftazidime	2	≤1	8	16
Ceftriaxone	≤0.5	≤0.5	2	2
Cefpodoxime	>4	≤4	>4	>4
Cefepime	≤0.5	≤1	≤1	≤1
Cefmetazole	32	>32	>32	>32
Aztreonam	4	2	8	>32
Piperacillin-tazobactam	16	≤2	16	16
Ampicillin-sulbactam	>16	≤2	16	16
Imipenem	≤0.5	2	2	2
Meropenem	≤0.5	≤0.5	≤0.5	≤0.5
Gentamicin	>8	≤0.5	≤0.5	≤0.5
Amikacin	≤4	≤2	≤2	≤2
Minocycline	>8	2	2	4
Ciprofloxacin	>4	≤0.5	2	≤0.5
Levofloxacin	>4	≤1	2	≤1
Sulfamethoxazole-trimethoprim	>4	≤1	≤1	≤1
Colistin	1	≤0.5	≤0.5	≤0.5