Genomic characterisation of an *mcr-1* and *mcr-3*-producing *Escherichia coli* strain isolated from pigs in France

Afaf Hamame^{1,2}, Bernard Davoust², Jean-Marc Rolain^{1,2}, Seydina M. Diene^{1,2*}

Running title : ***mcr-1/mcr-3* in *Escherichia coli* from pigs, in France**

1. Aix Marseille Univ, IRD, APHM, MEPHI, Faculté de Pharmacie, 13005 Marseille, France.

2. IHU Méditerranée Infection, 13005 Marseille, France.

*** Corresponding author :**

Adresse : MEPHI, IHU-Méditerranée Infection, Faculté Pharmacie, 19-21 Boulevard Jean

Moulin, 13385 Marseille CEDEX 05, France.

Phone: (33) 4 13 73 24 01.

Email: seydina.diene@univ-amu.fr

KEYWORDS: *mcr* genes, *mcr-1*, *mcr-3*, colistin resistance, whole-genome sequencing.

ABSTRACT

The current study is about genomic characterisation of an atypical multidrug-resistant *Escherichia coli* harbouring two mobilised colistin resistance (*mcr*) genes isolated from pigs in France. Stool samples taken from a pig farm in Avignon in the department of the Vaucluse were subjected to a molecular screening for the detection of *mcr* gene variants. These samples were cultured on selective LBJMR medium. Growing bacteria were identified using MALDI-TOF, followed by antibiotic susceptibility testing (AST). Whole genome sequencing (WGS) and bioinformatic genome analysis was performed. The selective culture of stools revealed the presence of an *E. coli* strain named Q4552 which was simultaneously positive for the *mcr*-1.1 and *mcr*-3.5 genes. This strain exhibited resistance phenotype to fourteen antibiotics, including colistin. Genome sequencing revealed a circular chromosome and eight plasmids. Genomic analysis revealed a chromosomal integration of a mobile genetic element (MGE) harbouring the *mcr*-1.1 gene, while the *mcr*-3.5 gene was plasmidic (i.e., an IncFII plasmid). Its resistome exhibited twenty-two resistance genes, explaining its multidrug resistance phenotype. The Q4552 strain is an ST-843 clone belonging to the clonal complex Cplx-568 and is the only ST type of this cplx-568 which has been isolated from animals, humans, and the environment. Here, we report the first co-occurrence of the *mcr*-1 and *mcr*-3 genes in France from a pathogenic *E. coli* strain isolated from a pig farm. Since this clone (ST-843) has been reported in zoonotic transmissions, programs to monitor such colistin resistant bacterium are urgently required to avoid its spread and zoonotic transmission to humans.

INTRODUCTION

Colistin (polymyxin E) is a polycationic antibiotic that interacts with the lipid A of lipopolysaccharide (LPS) in the outer membrane of Gram-negative bacteria (GNB) leading to cell lysis (1). This antibiotic is considered as one of the last-line antimicrobial agents to treat infections caused by multidrug resistant (MDR) Gram-negative bacteria, especially *Enterobacteriaceae* such as *Escherichia coli* and *Klebsiella pneumoniae* carbapenemases-producers (2). Furthermore, the use of polymyxins in veterinary medicine has become very common within the European Union and European Economic Area (EU/EEA). (3) The widespread use of this antibiotic has caused a significant increase in colistin resistance, especially in *E. coli* from pigs. Consequently, as reported, the emergence of colistin resistant bacteria in animals and humans is higher than before (4). Previously, it has been shown that colistin resistance is mediated by chromosomal gene mutations leading to amino acid substitutions in protein sequences associated with this resistance (5–7). Gram-negative bacteria, especially *Enterobacteriaceae*, have developed several mechanisms against colistin including amino acid substitutions in the two-component system *PhoP/PhoQ* and *PmrA/PmrB*, leading to the addition of phosphoethanolamine and 4-amino-4-deoxy-L-arabinose to the lipid A (8). Other mechanisms, such as the inactivation of the *phoP/phoQ* negative feedback regulator (*mgrB*) or overexpression efflux pumps have also been reported (9). Liu *et al.* reported the first *E. coli* harbouring the plasmid bearing mobile colistin resistance *mcr-1* gene, which they isolated from pigs in China (10). After this, ten other *mcr* gene variants were discovered (11–18). Almost all of these variants are abundant in pigs and poultry farming due to the use of polymyxin to prevent infections caused by bacterial pathogens (19).

In this study, we report the first co-occurrence of two mobile colistin resistance genes, *mcr-1*, and *mcr-3*, in an *E. coli* Q4552 strain isolated from pigs in France.

RESULTS

Gene screening, culture, and bacterial identification from stool samples. the RT-PCR screening on extracted DNA from the collected stool samples revealed one positive result. This sample was positive for the *mcr-1* and *mcr-3* genes, with CT values of 24 and 21, respectively. According to this result, the selective culture of this sample on the LBJMR medium gave pure bacterial colonies after 24 hours of incubation, then was identified using MALDI-TOF as *E. coli*, with an identification score of 2.4, and was named the Q4552 strain. As shown in **Figures 1A** and **1B**, the *E. coli* Q4552 strain was resistant to colistin with MIC_{COL} = 4 µg/ml. Moreover, the standard PCR performed on this Q4552 strain and sequencing confirmed the co-presence of the *mcr-1.1* and *mcr-3.5* gene variants (**Fig. 1C**). The AST results revealed that the *E. coli* Q4552 strain was resistant to different β-lactams, including amoxicillin, amoxicillin/clavulanic acid, and penicillin G, but remained susceptible to cephalosporins and carbapenems (**Table 1**). Overall, this Q4552 strain was resistant to fourteen different antibiotics tested (**Table 1**).

Genomic analysis of the colistin resistant Q4552 strain. genome assembly using combined raw reads from the Illumina and Nanopore technologies resulted in the circular chromosome with 4,778,049 bps with 80.87% GC content and eight circular plasmids with sizes which ranged from 84,331 bps to 3,607 bps and a %GC content ranging from 59.94% to 43.39% (**Table 2**). As presented in **Table 1**, resistome analysis reveals the presence in the genome of several genes encoding for resistance to β-lactams (namely *bla*_{AMPC2}, *bla*_{AMPC1}, *bla*_{TEM}, and *bla*_{ampH}), to aminoglycosides, especially streptomycin (*aadA1* and *aadA2*), to macrolides, especially clindamycin and erythromycin (*ermB* and *mphA*), and to quinolones (*mdtH*).

Resistance to colistin was mediated by the presence of the *mcr-1.1* and *mcr-3.5* genes. Interestingly, sequence analysis shown that the *mcr-1.1* gene was located a mobile genomic

element (MGE) with 51,089 bps from the Q4552 chromosome. As shown in **Figure 2**, the *E. coli* Q4552 chromosome was compared with published sequences, including the chromosome sequence of *E. coli* K-12 MG1655, *E. coli* CP66_6, and *E. coli* plasmid pPN43. Our analysis reveals that the MGE: *mcr*-1.1 was integrated within a specific integration site upstream of a tRNA gene and mediated by a phage integrase *intA*, as compared to the *E. coli* K-12 MG1655 chromosome (**Fig. 2B**). Moreover, within this MGE, the *mcr*-1 gene is flanked on both sides by the IS30, which is suggestive of a transposition event within this MGE. While this MGE is also identified within the chromosome of the *E. coli* CP66_6 strain isolated from a pig in Sichuan (China), this MGE is, furthermore, identified within the published plasmid *E. coli* pPN43 strain from a human sample in Thailand (**Fig. 2B**). Interestingly, in addition to the *mcr*-1 gene, a restriction-modification system type 1 (RMS type-1) and toxin/antitoxin system were identified within this MGE (**Fig. 2B**) suggestive of a stabilization of MGE within the genome. Regarding *mcr*-3.5, our genomic analysis revealed that this was located in one of the eight identified plasmids (pQ4552_*mcr*-3, an IncFII type) (**Fig. 3**). As shown in **Figure 3**, this plasmid pQ4552_*mcr*-3 (84,331 bps; 96.56%CG) was compared with the six most similar plasmids from the NCBI database, with an alignment percentage between 78% and 98% and an identity percentage between 98.60 and 99.96%. This analysis shows that *mcr*-3 is only present in two out of the six plasmids, including the *E. coli* plasmid pVE796 (AP018353.1) and the unnamed *K. pneumoniae* plasmid (CP041102.1). A toxin/antitoxin (*pemI/pemK*) system allows this plasmid to be maintained within this *E. coli* Q4552 strain (**Fig. 3**).

Multilocus sequence typing analysis. to investigate the clonality of the Q4552 strain, an MLST analysis was performed from the genome sequence, which classified the colistin-resistant Q4552 strain into ST-843, belonging to the clonal complex Cplx-568. As presented in **Figure 4**, the Cplx-568 complex is thus far composed of 14 ST types from the Institut Pasteur database. The investigation of the isolation sources of these ST types revealed

diverse sources, including humans, animals, and the environment. Interestingly, three ST types, namely ST-80, ST-1946, and ST-843, were isolated from humans and animals, suggesting the ability of this clone to be transferred from animals to humans and vice versa. However, only the ST-843 (our Q4552) was isolated from the three different sources (animal, human, and the environment) (**Fig. 4**).

DISCUSSION

According to the literature, the main source of the strains with a co-occurrence of *mcr*-1 and *mcr*-3 are animals. Here, the ecosystem of *E. coli* Q4552 is also farm animals, since this strain was isolated from a pig sample. Pigs are in close contact with humans. Depending on culture and traditions, pig meat is consumed around the entire world in various forms, and humans are consequently exposed to contamination by strains with the *mcr* genes, as already reported in farm workers and the surrounding environment being largely contaminated by the *mcr*-1 gene (20). So far, few studies have reported the co-occurrence of two *mcr* genes within a bacterium. This co-occurrence has been reported in an *E. coli* strain isolated from cattle (21) in Spain, pigs in Thailand (22), and in dog faeces from China (23). Moreover, the co-occurrence of *mcr*-1 and *mcr*-3 has also been reported in humans, such as the *E. coli* strain isolated from a human urine sample in New Zealand (24), and a *K. pneumoniae* strain isolated from a stool sample from a healthy volunteer in Thailand (25). The identified MGE harbouring the *mcr*-1.1 gene from the Q4552 chromosome appears to be extremely rare in available genomes from the NCBI database, and has only been detected in an *E. coli* CP66-6 strain with *mcr*-1 which was isolated, as was ours, from a pig in China (26), but also from a recombinant plasmid of an *E. coli* strain isolated from a human in Thailand (27). However, both *E. coli* strains are not ST-843 like our strain, but share this MGE. This MGE seems to be stabilised on the *E. coli* Q4552 chromosome given the presence of RMS type 1 and the

toxin/antitoxin system within this acquired MGE. While the *mcr-1* gene was identified from the Q4552 chromosome, the *mcr-3.5* gene variant was identified on a conjugative and recombinant IncFII type plasmid. Highly similar plasmids harbouring the *mcr-3.5* gene have been reported in the literature from *E. coli* strains isolated in Vietnam (AP018353.1), Thailand (Cp041102.1) and China (26). *E. coli* Q4552 is an atypical bacterium which belongs to the clonal complex Ccpx-568 and has an ST-843. ST-843 appears closely related to ST-80 and ST-1946 which are two *E. coli* clones well reported in zoonotic transmissions from animals and humans. Indeed, ST-80 has well been reported as a uropathogenic *E. coli* clone from retail food such as chicken and is able to cause community acquired infections (28).

In conclusion, *E. coli* Q4552 exhibited a multidrug resistance phenotype mediated by various resistance genes. The most unusual co-occurrence is the fact that *mcr-1.1* is chromosomal, with more than 4 IS transposases and a plasmidic *mcr-3.5*. As reported in the literature, *mcr* genes encode for phosphoethanolamine transferase proteins and could serve as defensive enzymes against phage attacks (29). These results give rise to questions about the real functions of these *mcr* genes within this Q4552 strain, which was not more resistant to colistin, despite this co-occurrence.

MATERIALS AND METHODS:

Decree and law authorising the use of animal products for research and diagnosis. Prefectorial authorisation (Bouches-du-Rhône) No.13 205 107 of 4 September 2014 authorising the IHU Méditerranée Infection to use unprocessed animal by-products in categories 1, 2 and 3 for research and diagnostic purposes. Order of 8 December 2011 laying down health rules concerning animal by-products and derived products in application of Regulation (EC) No. 1069/2009 and Regulation (EU) No. 142/2011. In 2020, pig faecal samples were collected from a pig fattening farm near Avignon (43° 50' 32.5''N - 4° 57' 33.9''E), in the department of the Vaucluse, France. The pigs are crossbreeds (mother: Large White X Landrace cross - father: Pietrain X Duroc cross). These animals (2 to 8 months old) were sampled in several batches. After fattening, they are intended for human consumption in Corsica, France.

DNA extraction. DNA was extracted from the pig faecal samples according to a protocol used in previous studies (30). 1g of faeces, Buffer G2 and proteinase K were incubated at 56°C overnight and extracted using the EZ1 DNeasy Blood Tissue Kit (Qiagen GmbH, Hilden, Germany) in line with the manufacturer's protocol.

Molecular screening of colistin resistance genes. the presence of genes encoding for colistin resistance (*mcr-1*, *mcr-2*, *mcr-3*, *mcr-4*, *mcr-5*, *mcr-8*) was investigated by Real-Time PCR assay(31)(32) using the CFX96 TM Real time System / C1000 TM Touch thermal Cycler (Bio-Rad, Singapore). Targeted genes were detected using specific primers and probes (**Suppl. Table S1**) (33). Results were considered positive when the cycle threshold value of real-time PCR was ≤ 30 . Standard PCR multiplex was then performed to confirm positive qPCR results, followed by sequencing.

Screening of colistin resistant Gram-negative bacteria. the selected faecal sample was enriched in TSB for 72 hours then cultured on a selective medium known as LBJMR

(Lucie Bardet Jean Marc Rolain), containing colistin sulfate salt at a concentration of 4µg/ml, and vancomycin (50µg/ml), with glucose as the fermentative substrate, on a Purple Agar Base. Using this LBJMR medium, *Enterobacteriaceae* isolates are yellow in colour, contrasting with the purple agar and are of a different size (2–3 mm), whereas *Enterococci* isolates are small and round (0.1–1 mm) (34).

MALDI-TOF MS identification. colonies with different morphologies were picked up from the LBJMR agar plate. Those colonies were spotted and identified using a Microflex LS spectrometer (Bruker Daltonics, Bremen, Germany) (35). The strain was properly identified at the species level when the score is ≤ 2 .

Antibiotic susceptibility testing (AST). the Disk diffusion (DD) test (Kirby-Bauer procedure) is a routine susceptibility testing method which was implemented according to the CLSI and EUCAST guidelines (https://www.eucast.org/clinical_breakpoints/). Sixteen antibiotics were tested, including ampicillin, amoxicillin-clavulanic acid, ceftazidime, cefotaxime, cefepime, cefoxitin, piperacillin-tazobactam, aztreonam, meropenem, ertapenem, imipenem, tigecycline, ciprofloxacin, gentamicin, trimethoprim-sulfamethoxazole, and colistin (Bio-Rad, Marne-la-Coquette, France). Additional AST methods such as the E-test (BioMérieux) and microdilution using UMIC microdilution (Biocentric, Bandol, France) were performed to confirm some AST results, as previously reported.

Genome sequencing and bioinformatic analysis. whole genome sequencing was performed using MiSeq Illumina (36) (Illumina Inc., San Diego, CA, United states) and Nanopore MINION technologies (37). Genome assembly was performed using a Canu assembler, which combines Illumina and Nanopore sequence reads, and genome annotation was performed using Prokka.(38) Resistome analysis was investigated using different databases including Resfinder (39), ARG-ANNOT (40), Card (41), and Plasmid Finder (42).

201 CGview software was then used for circular representation of chromosome and plasmid

202 sequences (43).

203 **Multilocus Sequence Typing (MLST).** sequence type analysis was carried out from

204 genomic sequences and the set of close typing sequences was determined by PubMLST then

205 compared with the 11,808 ST of *Escherichia* spp (<https://pubmlst.org/>) using Cytoscape and

206 layout software (44).

Sequence accession number. Complete genome sequence including the chromosome and the eight plasmids of the *E. coli* Q4552 strain have been deposited in the NCBI GenBank database under accession numbers: CP077063, CP077064, CP077065, CP077066, CP077067, CP077068, CP077069, CP077070, and CP077071.

Funding information. This work was supported by the French Government under the “Investissements d’avenir” (Investments for the Future) programme managed by the Agence Nationale de la Recherche (ANR, fr: National Agency for Research), (reference: Méditerranée Infection 10-IAHU-03).

Conflicts of interest. The authors declare that they have no competing interests.

Authors’ contributions. SMD, BD, and JMR designed the study; AF performed microbiology analyses; AF and SMD drafted the manuscript; AF, BD, JMR, and SMD made corrections and critical revisions. All the authors have read and approved the final version of the manuscript.

220 **Figure legends**

221 **Figure 1:** Phenotypic characterisation of the *E. coli* Q4552 strain carrying the two *mcr*
 222 variants. **A:** colistin susceptibility test according to EUCAST Microdilution UMIC. **B:**
 223 Colistin E-test on *E. coli* Q4552 strain. **C:** Multiplex standard PCR targeting both *mcr*-1 and
 224 *mcr*-3.

225 **Figure 2:** **A.** Circular maps of the chromosomal genome with *mcr*-1. **B.** Zoom on the genetic
 226 environment of the transposon carrying the *mcr*-1.1 gene compared with the top best hit blast
 227 *E. coli* CP66-6 and plasmid *E. coli* PN43.

228 **Figure 3:** Circular map of the plasmid carrying *mcr*-3.5 compared to six homologous
 229 plasmids with considerable query coverage.

230 **Figure 4:** MLST and cytoscape analysis of *E. coli* Q4552 (ST843) clone compared with the
 231 other clones associated with Ccpx568.

REFERENCES

1. Hamel M, Rolain JM, Baron SA. 2021. The history of colistin resistance mechanisms in bacteria: Progress and challenges. *Microorganisms* 9:1–18.
2. Poirel L, Jayol A, Nordmann P. 2017. Polymyxins: Antibacterial activity, susceptibility testing, and resistance mechanisms encoded by plasmids or chromosomes. *Clin Microbiol Rev.* American Society for Microbiology.
3. Catry B, Cavaleri M, Baptiste K, Grave K, Grein K, Holm A, Jukes H, Liebana E, Navas AL, Mackay D, Magiorakos AP, Romo MAM, Moulin G, Madero CM, Pomba MCMF, Powell M, Pyörälä S, Rantala M, Ružauskas M, Sanders P, Teale C, Threlfall EJ, Törneke K, Van Duijkeren E, Edo JT. 2015. Use of colistin-containing products within the European Union and European Economic Area (EU/EEA): development of resistance in animals and possible impact on human and animal health. *Int J Antimicrob Agents.* Elsevier B.V.
4. Hadjadj L, Riziki T, Zhu Y, Li J, Diene SM, Rolain JM. 2017. Study of mcr-1 gene-mediated colistin resistance in enterobacteriaceae isolated from humans and animals in different countries. *Genes (Basel)* 8:1–16.
5. Johansen HK, Moskowitz SM, Ciofu O, Pressler T, Høiby N. 2008. Spread of colistin resistant non-mucoid *Pseudomonas aeruginosa* among chronically infected Danish cystic fibrosis patients. *J Cyst Fibros* 7:391–7.
6. Arduino SM, Quiroga MP, Ramírez MS, Merkier AK, Errecalde L, Di Martino A, Smayevsky J, Kaufman S, Centrón D. 2012. Transposons and integrons in colistin-resistant clones of *Klebsiella pneumoniae* and *Acinetobacter baumannii* with epidemic or sporadic behaviour. *J Med Microbiol* 61:1417–1420.
7. Tenover FC, McGowan JE. 1996. Reasons for the Emergence of Antibiotic Resistance. *Am J Med Sci* 311:9–16.
8. Baron S, Hadjadj L, Rolain JM, Olaitan AO. 2016. Molecular mechanisms of polymyxin resistance: knowns and unknowns. *Int J Antimicrob Agents* 48:583–591.
9. Olaitan AO, Morand S, Rolain JM. 2014. Mechanisms of polymyxin resistance: Acquired and intrinsic resistance in bacteria. *Front Microbiol* 5:1–18.
10. Liu Y-Y, Wang Y, Walsh TR, Yi L-X, Zhang R, Spencer J, Doi Y, Tian G, Dong B, Huang X, Yu L-F, Gu D, Ren H, Chen X, Lv L, He D, Zhou H, Liang Z, Liu J-H, Shen J. 2016. Emergence of plasmid-mediated colistin resistance mechanism MCR-1 in animals and human beings in China: a microbiological and molecular biological study. *Lancet Infect Dis* 16:161–8.
11. Xavier BB, Lammens C, Ruhel R, Malhotra-Kumar S, Butaye P, Goossens H, Malhotra-Kumar S. 2016. Identification of a novel plasmid-mediated colistin resistance gene, mcr-2, in *Escherichia coli*, Belgium, June 2016. *Eurosurveillance* 21:30280.
12. Yin W, Li H, Shen Y, Liu Z, Wang S, Shen Z, Zhang R, Walsh TR, Shen J, Wang Y. 2017. Novel Plasmid-Mediated Colistin Resistance Gene mcr-3 in *Escherichia coli*. *MBio* 8.
13. Carattoli A, Villa L, Feudi C, Curcio L, Orsini S, Luppi A, Pezzotti G, Magistrali CF. 2017. Novel plasmid-mediated colistin resistance mcr-4 gene in *Salmonella* and *Escherichia coli*, Italy 2013, Spain and Belgium, 2015 to 2016. *Euro Surveill Bull Eur sur les Mal Transm = Eur Commun Dis Bull* 22.
14. Borowiak M, Fischer J, Hammerl JA, Hendriksen RS, Szabo I, Malorny B. 2017. Identification of a novel transposon-associated phosphoethanolamine transferase gene, mcr-5, conferring colistin resistance in d-tartrate fermenting *Salmonella enterica* subsp. *enterica* serovar Paratyphi B. *J Antimicrob Chemother* 72:3317–3324.

- 277 15. Teale C, Anjum MF, Kirchner M, Stubberfield EJ, Nunez-Garcia J, Lemma F, Randall LP,
278 AbuOun M, Dormer L, Duggett NA, Crook DW, Smith RP. 2017. mcr-1 and mcr-2 (mcr-6.1)
279 variant genes identified in *Moraxella* species isolated from pigs in Great Britain from 2014 to
280 2015. *J Antimicrob Chemother* 72:2745–2749.
- 281 16. Yang Y-Q, Li Y-X, Lei C-W, Zhang A-Y, Wang H-N. 2018. Novel plasmid-mediated colistin
282 resistance gene mcr-7.1 in *Klebsiella pneumoniae*. *J Antimicrob Chemother* 73:1791–1795.
- 283 17. Carroll LM, Gaballa A, Guldimann C, Sullivan G, Henderson LO, Wiedmann M. 2019.
284 Identification of novel mobilized colistin resistance gene mcr-9 in a multidrug-resistant,
285 colistin-susceptible *salmonella enterica* serotype typhimurium isolate. *MBio* 10:1–6.
- 286 18. Wang C, Feng Y, Liu L, Wei L, Kang M, Zong Z. 2020. Identification of novel mobile colistin
287 resistance gene mcr-10. *Emerg Microbes Infect* 9:508–516.
- 288 19. Kempf I, Fleury MA, Drider D, Bruneau M, Sanders P, Chauvin C, Madec JY, Jouy E. 2013.
289 What do we know about resistance to colistin in Enterobacteriaceae in avian and pig
290 production in Europe? *Int J Antimicrob Agents*. Elsevier.
- 291 20. Dandachi I, Fayad E, Sleiman A, Daoud Z, Rolain JM. 2020. Dissemination of Multidrug-
292 Resistant and mcr-1 Gram-Negative Bacilli in Broilers, Farm Workers, and the Surrounding
293 Environment in Lebanon. *Microb Drug Resist* 26:368–377.
- 294 21. Hernández M, Iglesias MR, Rodríguez-Lázaro D, Gallardo A, Quijada NM, Miguela-Villoldo
295 P, Campos MJ, Píriz S, López-Orozco G, de Frutos C, Sáez JL, Ugarte-Ruiz M, Domínguez L,
296 Quesada A. 2017. Co-occurrence of colistin-resistance genes mcr-1 and mcr-3 among
297 multidrug-resistant *Escherichia coli* isolated from cattle, Spain, September 2015.
298 *Eurosurveillance* 22:30586.
- 299 22. Khine NO, Lugsomya K, Kaewgun B, Honhanrob L, Pairojrit P, Jermprasert S, Prapasarakul
300 N. 2020. Multidrug Resistance and Virulence Factors of *Escherichia coli* Harboring Plasmid-
301 Mediated Colistin Resistance: mcr-1 and mcr-3 Genes in Contracted Pig Farms in Thailand.
302 *Front Vet Sci* 7:582899.
- 303 23. Du C, Feng Y, Wang G, Zhang Z, Hu H, Yu Y, Liu J, Qiu L, Liu H, Guo Z, Huang J, Qiu J.
304 2020. Co-occurrence of the mcr-1.1 and mcr-3.7 genes in a multidrug-resistant *escherichia coli*
305 isolate from China. *Infect Drug Resist* 13:3649–3655.
- 306 24. Creighton J, Anderson T, Howard J, Dyet K, Ren X, Freeman J. 2019. Co-occurrence of mcr-1
307 and mcr-3 genes in a single *Escherichia coli* in New Zealand. *J Antimicrob Chemother*. Oxford
308 University Press.
- 309 25. Yu Y, Andrey DO, Yang RS, Sands K, Tansawai U, Li M, Portal E, Gales AC, Niumsup PR,
310 Sun J, Liao X, Liu YH, Walsh TR. 2020. A *Klebsiella pneumoniae* strain co-harboring mcr-1
311 and mcr-3 from a human in Thailand. *J Antimicrob Chemother* 75:2372–2374.
- 312 26. Li R, Du P, Zhang P, Li Y, Yang X, Wang Z, Wang J, Bai L. 2021. Comprehensive Genomic
313 Investigation of Coevolution of mcr genes in *Escherichia coli* Strains via Nanopore Sequencing
314 . *Glob Challenges* 5:2000014.
- 315 27. Yang Q, Li M, Spiller OB, Andrey DO, Hinchliffe P, Li H, MacLean C, Niumsup P, Powell L,
316 Pritchard M, Papkou A, Shen Y, Portal E, Sands K, Spencer J, Tansawai U, Thomas D, Wang
317 S, Wang Y, Shen J, Walsh T. 2017. Balancing mcr-1 expression and bacterial survival is a
318 delicate equilibrium between essential cellular defence mechanisms. *Nat Commun* 8:1–12.
- 319 28. Yamaji R, Friedman CR, Rubin J, Suh J, Thys E, McDermott P, Hung-Fan M, Riley LW.
320 2018. A Population-Based Surveillance Study of Shared Genotypes of *Escherichia coli* Isolates
321 from Retail Meat and Suspected Cases of Urinary Tract Infections. *mSphere* 3.
- 322 29. Khedher M Ben, Baron SA, Riziki T, Ruimy R, Raoult D, Diene SM, Rolain JM. 2020.

- 323 Massive analysis of 64,628 bacterial genomes to decipher water reservoir and origin of mobile
324 colistin resistance genes: is there another role for these enzymes? *Sci Rep* 10:1–10.
- 325 30. Ngaiganam EP, Pagnier I, Chaalal W, Leangapichart T, Chabou S, Rolain JM, Diene SM.
326 2019. Investigation of urban birds as source of β -lactamase-producing Gram-negative bacteria
327 in Marseille city, France. *Acta Vet Scand* 61:1–7.
- 328 31. Edelstein M, Pimkin M, Palagin I, Edelstein I, Strachounski L. 2003. Prevalence and
329 Molecular Epidemiology of CTX-M Extended-Spectrum β -Lactamase-Producing *Escherichia*
330 *coli* and *Klebsiella pneumoniae* in Russian Hospitals. *Antimicrob Agents Chemother* 47:3724–
331 3732.
- 332 32. Chabou S, Leangapichart T, Okdah L, Le Page S, Hadjadj L, Rolain JM. 2016. Real-time
333 quantitative PCR assay with Taqman®probe for rapid detection of MCR-1 plasmid-mediated
334 colistin resistance. *New Microbes New Infect.* Elsevier Ltd.
- 335 33. Leangapichart T, Dia NM, Olaitan AO, Gautret P, Brouqui P, Rolain JM. 2016. Acquisition of
336 extended-spectrum β -lactamases by *Escherichia coli* and *Klebsiella pneumoniae* in gut
337 microbiota of pilgrims during the hajj pilgrimage of 2013. *Antimicrob Agents Chemother*
338 60:3222–3226.
- 339 34. Bardet L, Le Page S, Leangapichart T, Rolain JM. 2017. LBJMR medium: A new polyvalent
340 culture medium for isolating and selecting vancomycin and colistin-resistant bacteria. *BMC*
341 *Microbiol* 17:1–10.
- 342 35. Singhal N, Kumar M, Kanaujia PK, Viridi JS. 2015. MALDI-TOF mass spectrometry: An
343 emerging technology for microbial identification and diagnosis. *Front Microbiol.* Frontiers
344 Research Foundation.
- 345 36. Caporaso JG, Lauber CL, Walters W a, Berg-Lyons D, Huntley J, Fierer N, Owens SM, Betley
346 J, Fraser L, Bauer M, Gormley N, Gilbert J a, Smith G, Knight R. 2012. Ultra-high-throughput
347 microbial community analysis on the Illumina HiSeq and MiSeq platforms. *ISME J* 6:1621–
348 1624.
- 349 37. Mikheyev AS, Tin MMY. 2014. A first look at the Oxford Nanopore MinION sequencer. *Mol*
350 *Ecol Resour* 14:1097–1102.
- 351 38. Seemann T. 2014. Prokka: Rapid prokaryotic genome annotation. *Bioinformatics* 30:2068–
352 2069.
- 353 39. Bortolaia V, Kaas RS, Ruppe E, Roberts MC, Schwarz S, Cattoir V, Philippon A, Allesoe RL,
354 Rebelo AR, Florensa AF, Fagelhauer L, Chakraborty T, Neumann B, Werner G, Bender JK,
355 Stingl K, Nguyen M, Coppens J, Xavier BB, Malhotra-Kumar S, Westh H, Pinholt M, Anjum
356 MF, Duggett NA, Kempf I, Nykäsenoja S, Olkkola S, Wiczorek K, Amaro A, Clemente L,
357 Mossong J, Losch S, Ragimbeau C, Lund O, Aarestrup FM. 2020. ResFinder 4.0 for
358 predictions of phenotypes from genotypes. *J Antimicrob Chemother* 75:3491–3500.
- 359 40. Gupta SK, Padmanabhan BR, Diene SM, Lopez-Rojas R, Kempf M, Landraud L, Rolain J-M.
360 2014. ARG-ANNOT, a new bioinformatic tool to discover antibiotic resistance genes in
361 bacterial genomes. *Antimicrob Agents Chemother* 58:212–20.
- 362 41. Alcock BP, Raphenya AR, Lau TTY, Tsang KK, Bouchard M, Edalatmand A, Huynh W,
363 Nguyen AL V., Cheng AA, Liu S, Min SY, Miroshnichenko A, Tran HK, Werfalli RE, Nasir
364 JA, Oloni M, Speicher DJ, Florescu A, Singh B, Faltyn M, Hernandez-Koutoucheva A, Sharma
365 AN, Bordeleau E, Pawlowski AC, Zubyk HL, Dooley D, Griffiths E, Maguire F, Winsor GL,
366 Beiko RG, Brinkman FSL, Hsiao WWL, Domselaar G V., McArthur AG. 2020. CARD 2020:
367 Antibiotic resistome surveillance with the comprehensive antibiotic resistance database.
368 *Nucleic Acids Res* 48:D517–D525.
- 369 42. Carattoli A, Hasman H. 2020. PlasmidFinder and In Silico pMLST: Identification and Typing

- 370 of Plasmid Replicons in Whole-Genome Sequencing (WGS), p. 285–294. *In* Methods in
371 Molecular Biology. Humana Press Inc.
- 372 43. Stothard P, Grant JR, Van Domselaar G. 2018. Visualizing and comparing circular genomes
373 using the CGView family of tools. *Brief Bioinform* 20:1576–1582.
- 374 44. Shannon P, Markiel A, Ozier O, Baliga NS, Wang JT, Ramage D, Amin N, Schwikowski B,
375 Ideker T. 2003. Cytoscape: A software Environment for integrated models of biomolecular
376 interaction networks. *Genome Res* 13:2498–2504.
- 377 45. Rebelo AR, Bortolaia V, Kjeldgaard JS, Pedersen SK, Leekitcharoenphon P, Hansen IM,
378 Guerra B, Malorny B, Borowiak M, Hammerl JA. 2018. Multiplex PCR for detection of
379 plasmid-mediated colistin resistance determinants, *mcr-1*, *mcr-2*, *mcr-3*, *mcr-4* and *mcr-5* for
380 surveillance purposes. *Eurosurveillance* 23.
- 381 46. Nabti LZ, Sahli F, Ngaiganam EP, Radji N, Mezaghcha W, Lupande-Mwenebitu D, Baron SA,
382 Rolain JM, Diene SM. 2020. Development of real-time PCR assay allowed describing the first
383 clinical *Klebsiella pneumoniae* isolate harboring plasmid-mediated colistin resistance *mcr-8*
384 gene in Algeria. *J Glob Antimicrob Resist* 20:266–271.

Table 1: Phenotypic and genotypic comparison of *E. coli* Q4552 according to antibiotic family

Antibiotic Family	Antibiotics	CMI (mm)	CMI (mg/ml)	ARGs	encoded gene for	% COVERAGE ARG ANNOT	% COVERAGE Resfinder
Penicillins	Penicillin A: Amoxicillin	R:0		(Bla)AmpC2	β -lactamase Class C	99%	100%
	Amoxicillin + Clavulanic acid	R:16	>256	(Bla)AmpC1	β -lactamase Class C	99.88%	100%
	Oxacillin	R	>256	(Bla)blaTEM	BLSE Class A	100%	100%
	Piperacillin	S	2			100%	100%
	Aztreonam	S	0.46				
	Penicillin G	R	>32		Carboxypeptidase endopeptidase		
	Tazobactam piperacillin	S: 24.2	0.38				
Cephalosporins	Cephalosporin G1: cefalotin	I:16,1	0.32	acrF	Inner membrane transporter	100%	
	Cephalosporin G2: cefotaxime	S	<0.25	AcrE	Membrane fusion protein	99.66 %	
	Cephalosporin G3: ceftriaxone	S:38,8		acrS	Efflux pump	99%	
	Cephalosporin G4: cefepime	S:30					
Carbapenems	Meropenem	S	<0.125				
	Ertapenem	S: 39.3	0.03				
	Imipenem	S:35.6	<0.32				
Macrolides	Clindamycin	R	>256	erm(B)_1		100%	100%
	Erythromycin	R	>256	mph(A)		100%	100%
	Levofloxacin	S	0.125	emrA, emrB, emrR	Multidrug efflux pump	99%	
Quinolone	Ciprofloxacin	R	1,024	mdtH	Multidrug resistance protein	97.87%	
Tetracycline	Doxycycline	R:10.4	32	(Tet)tetA,	Tetracycline efflux pump	94.12%	99,92
	Minocycline		12	(Tet)tetR	Tetracycline efflux pump	100%	
Sulfamids	Trimethoprim sulfamethoxazole	R:0		dfrA12_8			100%
	Sulfamide	R	64	(Sul)sul3		100%	100%

Aminoglycosides	Streptomycin	R		(AGly)aadA1-pm (AGly)aadA2	Aminoglycoside 6- Acetyltransferase	93.86% 100%	98.48% 95.24%
	Gentamicin	S:37.6	<0.64				
	Netilmicin		0.25				
	Kanamycin		1.5				
	TGC Tigecycline		0.5				
	Amikacin	S:25.6	1.5			100%	
Phenicol	chloramphenicol			(Phe)cmlA1	Mutation	100%	
Polymyxin E	Colistin	R: 13,6	4	(Col) mcr-1.1	Phosphatidylethanolamine Transferase	100%	100%
				(Col) mcr-3.5		100%	100%
OTHERS	Fosfomycin	R	0.125	mdtG	Multidrug efflux pump	99.27 %	
	Metronidazole	R	>256				
	Rifampicin	R	>32				

1 **Table 2:** Genomic features of the *E. coli* Q4552 strain

Type	Size (bp)	%GC content	Replication type	Genes	CDS	rRNAs	tRNAs
Chromosome	4,778,049	50.87		5317	4965	15	87
Plasmid_pECQ4552_mcr-3	84,331	52.61	incFII	87	87	0	0
Plasmid_pECQ4552_IHU02	57,888	49.02	incA/C2	69	69	0	0
Plasmid_pECQ4552_IHU03	17,382	54.86	Unknown	23	23	0	0
Plasmid_pECQ4552_IHU04	9,982	59.94	Unknown	12	12	0	0
Plasmid_pECQ4552_IHU05	8,913	50.12	Unknown	9	9	0	0
Plasmid_pECQ4552_IHU06	7,870	54.35	Unknown	11	11	0	0
Plasmid_pECQ4552_IHU07	4,229	51.97	Unknown	2	2	0	0
Plasmid_pECQ4552_IHU08	3,607	43.39	Unknown	6	6	0	0

A **B** **C**

A

B

Supplementary Table S1: Sequences of primers and probes used for real-time PCRs and conventional PCRs in this study (45, 46).

Gene	PCR Type	Primers / Probes	PCR product (bp)
<i>mcr-1</i>	RT-PCR	F: GCAGCATACTTCTGTGTGGTAC R: ACAAAGCCGAGATTGTCCGCG Probe : FAM-GACCGCGACCGCCAATCTTACC-TAMRA	180
	Standard PCR and sequencing	F: GCAGCATACTTCTGTGTGGTAC R: TATGCACGCGAAAGAAACTGGC	893
<i>mcr-2</i>	RT-PCR	F: CTGTGCCGTGTATGTTTCAGC R: TTATCCATCACGCCTTTTGAG Probe: VIC-TGACCGCTTGGGTGTGGGTA-TAMRA	151
<i>mcr-3</i>	RT-PCR	F: TGAATCACTGGGAGCATTAGGGC R: TGCTGCAAACACGCCATATCAAC Probe: FAM-TGCACCGGATGATCAGACCCGT-TAMRA	144
	Standard PCR and sequencing	F: AAATAAAAATTGTTCCGCTTATG R: ATGGAGATCCCCGTTTTT	556
<i>mcr-4</i>	RT-PCR	F: GCCAACCAATGCTCATACCCAAAA R: CCGCCCCATTCGTGAAAACATAC Probe : FAM-GCCACGGCGGTGTCTCTACCC-TAMRA	112
<i>mcr-5</i>	RT-PCR	F: TATCCCGCAAGCTACCGACGC R: ACGGGCAAGCACATGATCGGT Probe: FAM-TGCGACACCACCGATCTGGCCA-TAMRA	126
<i>mcr-8</i>	RT-PCR	F: TCCGGGATGCGTGACGTTGC R: TGCTGCGCGAAT-GAAGACG Probe: FAM-TCATGGAGAATCGCTGGGG-GAAAGC-TAMRA	158

3

4