



# Characterization Of Chromosome-Mediated Colistin Resistance In *Escherichia coli* Isolates From Livestock In Korea

This article was published in the following Dove Press journal:  
*Infection and Drug Resistance*

Shukho Kim<sup>1</sup>  
Jung Hwa Woo<sup>1</sup>  
Nayeong Kim<sup>1</sup>  
Mi Hyun Kim<sup>1</sup>  
Se Yeon Kim<sup>1</sup>  
Joo Hee Son<sup>1</sup>  
Dong Chan Moon<sup>2</sup>  
Suk-Kyung Lim<sup>2</sup>  
Minsang Shin <sup>1</sup>  
Je Chul Lee <sup>1</sup>

<sup>1</sup>Department of Microbiology, School of Medicine, Kyungpook National University, Daegu, Republic of Korea;

<sup>2</sup>Bacterial Disease Division, Animal and Plant Quarantine Agency, Gimcheon-si, Gyeongsangbuk-do, Republic of Korea

**Purpose:** Colistin resistance in gram-negative bacteria from humans and livestock has been increasingly reported worldwide. The aim of this study was to investigate the underlying mechanisms of chromosome-mediated colistin resistance in *Escherichia coli* isolates from livestock in Korea.

**Materials and methods:** Thirty *mcr-1*-negative isolates were selected from a collection of colistin-resistant *E. coli* isolates collected from livestock in 2005 and 2015 in Korea. Amino acid alterations in PmrAB, PhoPQ, MgrB, and PmrD were investigated. Colistin-resistant derivatives were produced by serial passage of colistin-susceptible *E. coli* isolates in colistin-containing media.

**Results:** Thirty colistin-resistant *mcr*-negative *E. coli* isolates were classified into 26 sequence types. Twenty-two isolates carried diverse amino acid alterations in PmrB, PhoP, PhoQ, MgrB, and/or PmrD, whereas no mutation in any of these genes was found in the remaining eight isolates. Sixteen out of the 22 isolates shared a total of nine polymorphic positions that were found in colistin-susceptible *E. coli* strains. Colistin-resistant derivatives from two colistin-susceptible isolates showed the same genetic alterations that were observed in colistin-resistant clinical isolates.

**Conclusion:** Our results suggest that the mechanism underlying chromosome-mediated colistin resistance remain to be discovered in *E. coli*. Selective pressure of colistin in vitro induced the same genetic mutations associated with colistin resistance in vivo. Efforts to reduce colistin consumption in livestock should be redoubled, to prevent the occurrence of colistin-resistant *E. coli* strains.

**Keywords:** colistin resistance, two-component system, livestock, genetic mutation, *mcr* gene

## Introduction

Colistin (polymyxin E) is a cationic amphipathic lipopeptide antimicrobial agent that is an important drug of last resort against multidrug-resistant (MDR) gram-negative bacteria, including *Enterobacteriaceae*, *Pseudomonas aeruginosa*, and *Acinetobacter baumannii*, in human medicine.<sup>1–3</sup> However, colistin has also been used extensively for veterinary medicine in many countries, and colistin resistance in livestock-derived *Enterobacteriaceae* has been increasingly reported worldwide.<sup>4–7</sup> Colistin resistance is caused by decreases in the net negative charge of the outer membrane, loss of lipid A, or efflux pumps.<sup>8–10</sup> The most common resistance mechanism in *Enterobacteriaceae* is the covalent modification of the lipid A moiety of lipopolysaccharide (LPS) via cationic substitution.<sup>11–13</sup> These modifications neutralize the negative charge of LPS and subsequently reduce the binding affinity of colistin for its target.<sup>13,14</sup>

Correspondence: Je Chul Lee  
Department of Microbiology, School of Medicine, Kyungpook National University, 680 Gukchaebosang-ro, Jung-gu, Daegu 41944, Republic of Korea  
Tel +82-53-420-4844  
Fax +82-53-427-5664  
Email leejc@knu.ac.kr

A plasmid-mediated colistin resistance gene, *mcr-1*, was first reported in *Escherichia coli* from China in 2015,<sup>15</sup> and several variants of the *mcr* gene were identified.<sup>16,17</sup> The *mcr* genes encode phosphoethanolamine (PEtN) transferase enzyme that adds PEtN to lipid A, and consequently results in a more cationic LPS.<sup>13,18</sup> Chromosome-mediated colistin resistance involving the modification of lipid A with PEtN and/or 4-amino-4-deoxy-L-arabinose (L-Ara4N) has also been identified in gram-negative bacteria.<sup>19,20</sup> The LPS modification is associated with the overexpression of the *pmrCAB*, *pmrE*, and *arnBCADTEF* genes through the activation of the PmrAB and PhoPQ two-component systems (TCS).<sup>13,20–23</sup> The PEtN phosphotransferase PmrC adds a PEtN group to the LPS.<sup>22</sup> The *arnBCADTEF* operon (also called the *pmrHFLJKLM* or *pbgPE* operon) and the *pmrE* gene are responsible for the biosynthesis of L-Ara4N and its transfer to lipid A.<sup>24</sup> Addition of these cationic groups to the LPS decreases the net negative charge of the LPS and induces colistin resistance.<sup>25–27</sup> Specific mutations in the *pmrAB* and *phoPQ* genes have been found in colistin-resistant gram-negative bacteria.<sup>25–28</sup> The MgrB and PmrD are also associated with colistin resistance in the *Enterobacteriaceae*.<sup>13,29,30</sup> MgrB is a negative regulator of the PhoPQ system, and inactivation of *mgrB* leads to overexpression of the *phoPQ* operon, whereas the PmrD activated by PhoP upregulates PmrAB.<sup>29,30</sup> Mutations in the TCS and their regulators lead to the synthesis of PEtN or L-Ara4N and their transfer to lipid A through the upregulation of the *pmrCAB* operon, the *arnBCADTEF* operon, or the *pmrE* gene. Mutations in the *pmrAB*, *phoQ*, and *mgrB* genes have been described as being responsible for colistin resistance in *E. coli*,<sup>13,22</sup> but the association of genetic polymorphisms in these genes with colistin resistance has not been fully understood. The aim of this study was to investigate the chromosome-mediated colistin resistance underlying the genetic polymorphism of TCS and their regulators, including PmrAB, PhoPQ, MgrB, and PmrD, among the *mcr*-negative *E. coli* isolates from livestock in Korea.

## Materials And Methods

### Bacterial Isolates

A total of 30 *mcr-1*-negative isolates were selected from a collection of 154 colistin-resistant *E. coli* isolates in the Korean Veterinary Antimicrobial Resistance Monitoring System during 2005 and 2015.<sup>31</sup> The representative

isolates were selected based on animal species, healthy or diseased condition of animals, isolation year, isolation area, and minimum inhibitory concentrations (MICs) of colistin (Table 1). Fourteen isolates were from pigs (eight from fecal samples of healthy animals and six from clinical samples of diseased animals), 11 were from cattle (11 from fecal samples of healthy animals), and five were from chicken (three from fecal samples of healthy animals and two from clinical samples of diseased animals). Two colistin-susceptible *E. coli* isolates were obtained: EC6 from the fecal sample of a healthy pig, and EC7 from the liver of a diseased chicken. All *E. coli* isolates were obtained from Korea Veterinary Culture Collection (KVCC).

### Antimicrobial Susceptibility Test

The MICs of 15 antimicrobials were determined by the broth microdilution method using the KRN4F Sensititre panel (Trek Diagnostic Systems) according to the manufacturer's instructions. *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 were used as quality control strains. Interpretation of antimicrobial susceptibility was based on the guidelines of the Clinical Laboratory Standards Institute (CLSI).<sup>32</sup> Susceptibility to colistin was interpreted according to the European Committee on Antimicrobial Susceptibility Testing breakpoint of > 2 µg/mL.

### Multi-Locus Sequence Typing (MLST) Analysis

Sequence types (STs) were determined using the Achtman scheme available at <https://pubmlst.org/escherichia/>.

### Induction Of Colistin Resistance In Colistin-Susceptible Isolates

Colistin-susceptible *E. coli* isolates were repeatedly cultured in Luria-Bertani (LB) broth with increasing concentrations of colistin. Briefly, 10<sup>9</sup> colony forming units/mL from overnight cultures of isolates were inoculated in LB broth and cultured overnight at 37°C. Cultures were diluted 1:100 in LB broth containing 1/4 sub-MIC of colistin (0.25 µg/mL) and incubated overnight. Thereafter, in vitro-selected mutants were passaged in LB broth containing increasing concentrations of colistin (from 0.5 to 8 µg/mL). Five colistin-resistant derivatives were picked randomly from LB plates containing 8 µg/mL of colistin, and stored at –80°C until use.

**Table 1** Characteristics Of The *Mcr*-Negative Colistin-Resistant *E. coli* Isolates In This Study

Isolate No.	Animals	Samples	Isolated Year	ST	MIC (µg/mL)												
					COL	GEN	NEO	STR	AMP	AMC	CEF	FOX	XNL	NAL	CIP	CHL	FFL
CL-1	Cattle	Feces	2006	297	≤1	16	4	8/4	8	2	≤0.5	4	≤0.12	4	4	≤2	≤0.12/2.28
CL-2	Cattle	Feces	2006	New	≤1	8	≤2	4/2	16	2	≤0.5	≤2	≤0.12	8	4	≤2	≤0.12/2.28
CL-3	Pig	Feces	2007	6488	≤1	8	>32	4/2	4	2	≤0.5	≤2	≤0.12	4	4	64	0.25/4.75
CL-4	Pig	Feces	2007	515	≤1	>32	64	4/2	16	≤1	>8	8	≤0.12	4	4	64	>4/76
CL-5	Cattle	Feces	2008	2035	1	8	32	4/2	16	16	0.5	128	8	16	32	0.5/9.5	
CL-6	Cattle	Feces	2008	278	8	32	32	8/4	32	2	0.5	2	0.12	8	4	128	0.25/4.75
CL-7	Cattle	Feces	2008	448	1	2	128	8/4	16	4	0.5	128	16	8	64	4/76	
CL-8	Cattle	Feces	2008	448	64	32	128	8/4	8	2	0.5	128	16	4	2	128	4/76
CL-9	Cattle	Feces	2008	906	4	8	32	8/4	32	2	0.5	2	0.12	8	4	2	0.12/2.28
CL-10	Pig	Feces	2008	10	64	8	64	8/4	8	2	0.5	128	0.25	64	64	128	0.5/9.5
CL-11	Pig	Feces	2008	4038	8	8	32	8/4	16	2	1	128	0.5	64	64	128	4/76
CL-12	Cattle	Feces	2009	2111	1	8	8	8/4	16	2	1	2	0.12	8	4	2	0.12/2.28
CL-13	Cattle	Feces	2009	388	8	32	128	64/32	64	64	8	128	16	64	64	128	4/76
CL-14	Chicken	Feces	2010	224	32	8	32	8/4	8	4	0.5	128	16	64	8	64	4/76
CL-15	Chicken	Feces	2010	3054	1	2	16	8/4	16	4	0.5	128	16	32	8	128	4/76
CL-16	Pig	Feces	2010	3054	1	2	128	8/4	16	4	0.5	128	0.12	64	64	128	4/76
CL-17	Chicken	Diarrheal stool	2010	226	1	2	16	8/4	16	4	0.5	128	16	16	4	128	0.12/2.28
CL-18	Pig	Diarrheal stool	2010	100	4	32	32	8/4	8	32	8	128	16	64	8	32	4/76
CL-19	Cattle	Feces	2011	5564	1	2	8	2/1	4	2	0.5	2	0.12	8	4	2	0.12/2.28
CL-20	Cattle	Feces	2011	2035	1	2	4	2/1	4	2	0.5	2	0.12	8	4	2	0.12/2.28
CL-21	Pig	Intestinal lesion	2011	450	64	2	128	4/2	4	1	0.5	128	16	4	2	32	4/76
CL-22	Chicken	Diarrheal stool	2011	156	1	2	4	8/4	16	4	0.5	128	16	8	4	128	0.12/2.28
CL-24	Pig	Feces	2012	641	1	4	32	8/4	16	4	0.5	2	0.12	64	64	128	0.25/4.75
CL-25	Pig	Feces	2013	5903	1	2	8	4/2	8	8	0.5	4	0.12	4	4	2	0.12/2.28
CL-26	Pig	Intestinal lesion	2013	752	≤1	16	32	8/4	16	8	≤0.5	≤2	≤0.12	>64	>64	128	0.25/4.75
CL-27	Pig	Feces	2013	1257	>64	>32	>128	4/2	≤2	64	>8	128	>16	>64	>64	>128	>4/76
CL-28	Pig	Intestinal lesion	2014	93	64	32	128	8/4	16	8	8	128	16	64	64	128	4/76
CL-31	Chicken	Feces	2014	548	4	4	1	4/2	64	2	2	8	0.12	4	4	2	0.5/9.5
CL-29	Pig	Urine	2015	1	8	8	64	8/4	8	4	4	32	≤0.12	>64	>64	64	≤0.12/2.28
CL-30	Pig	Abscess	2015	1	≤1	32	128	8/4	8	4	4	16	≤0.12	>64	64	64	≤0.12/2.28

**Abbreviations:** ST, sequence type; COL, colistin; GEN, gentamicin; NEO, neomycin; STR, streptomycin; AMP, ampicillin; AMC, amoxicillin/clavulanic acid; CEF, cephalothin; FOX, cefoxitin; XNL, nalidixic acid; CIP, ciprofloxacin; CHL, chloramphenicol; FFL, florfenicol; TET, tetracycline; SXT, trimethoprim/sulfamethoxazole.

## Polymerase Chain Reaction (PCR) Amplification And Sequencing

The genomic DNA isolated from bacteria was subjected to PCR using specific primers listed in [Supplementary Table S1](#). The carriage of the *mcr* genes, *mcr-1* to *mcr-4*, was verified by PCR as previously described.<sup>33</sup> The *pmrA*, *pmrB*, *phoP*, *phoQ*, *mgrB*, and *pmrD* genes were amplified and sequenced. The amino acid sequences of the colistin-resistant isolates were compared with those of the reference strains *E. coli* K-12 MG1655 and *E. coli* ATCC 25922, and other reported *E. coli* strains (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>).

## Results

### Detection Of *mcr* Genes, Antimicrobial Susceptibility, And STs Of Colistin-Resistant *E. coli* Isolates

To determine the carriage of the *mcr* genes (*mcr-2* to *mcr-4*) among the colistin-resistant *E. coli* isolates, PCR was performed. No *mcr* gene was identified in the *E. coli* isolates tested. The MIC range of colistin against the 30 *mcr*-negative colistin-resistant *E. coli* isolates was 4 - >32 µg/mL (MIC<sub>50</sub> = 8 µg/mL and MIC<sub>90</sub> = 32 µg/mL) ([Table 1](#)). Twenty-three colistin-resistant *E. coli* isolates showed an MDR phenotype, and one isolate, CL-31, was resistant to colistin, ampicillin, and cephalothin. The remaining six isolates from fecal samples of healthy animals, five from cattle and one from pig, were resistant to colistin only. High resistance rates to ampicillin ( $n = 24$ ) and tetracycline ( $n = 22$ ) were observed among the colistin-resistant isolates. All *E. coli* isolates, except CL-13, were susceptible to amoxicillin/clavulanic acid. MLST classified 29 colistin-resistant *E. coli* isolates into 26 STs, but one isolate, CL-2, was not typable (allelic profile, 2, 6, 4, 18, 9, 8, and 6 in *adk*, *fumC*, *gyrB*, *icd*, *mdh*, *purA*, and *recA*, respectively). ST1, ST448, ST2035, and ST3054 were each identified in two isolates, but the antimicrobial susceptibility of the two isolates belonging to the same ST was different.

### Amino Acid Alterations In TCS And Their Regulators

To evaluate whether the 30 *mcr*-negative colistin-resistant *E. coli* isolates carried the genetic mutations in TCS and their regulators, amino acid alterations in PmrAB, PhoPQ, MgrB, and PmrD were analyzed. *E. coli* K-12 MG1655 and ATCC 25922 were used as reference strains for the

comparison of the nucleotide and amino acid sequences. Amino acid substitutions in the TCS and their regulators were found in 22 *E. coli* isolates, whereas eight isolates did not carry any amino acid alterations in these genes ([Table 2](#)). Amino acid alterations were found at two sites in PmrB (S138N and G200R); one site in PhoP (V108M); five sites in PhoQ (D101E, S138T, I175F, V386L, and E464D); one site in MgrB (Q33R); and six sites in PmrD (N11D, M20K, A27T, K35N, A52V, and K82T). Phylogenetic trees of PhoQ and PmrD were presented in [Figure S1](#). Amino acid substitution K82T in PmrD was the most commonly identified mutation in 13 isolates. Two isolates, CL-29 and CL-30, belonging to ST1, showed six mutations, S138N in PmrB, S138T in PhoQ, Q33R in MgrB, and N11D, M20K, and A27T in PmrD. The addition of cytosine in nucleotide 598 of the *pmrB* gene and subsequent frameshift mutation was observed in the CL-13 isolate. The CL-13 isolate was found to have a high level of resistance to colistin (MIC, 32 µg/mL). Next, we evaluated whether mutations in TCS and their regulators found in this study were unique to colistin-resistant *E. coli* isolates. Amino acid alterations S138T, I175F, V386L, and E464D in PhoQ and N11D, M20K, A27T, and K82T in PmrD were observed in colistin-susceptible *E. coli* strains. Mutations S138N in PmrB, V108M in PhoP, and Q33R in MgrB were identified only in colistin-resistant *E. coli* strains. Mutation K35N in PmrD was identified in *E. coli*, but its association with colistin resistance has not been determined. Mutations G200R and a frameshift in PmrB, D101E in PhoQ, and A52V in PmrD were found in colistin-resistant *E. coli* isolates in this study.

### Induction Of Colistin Resistance And Amino Acid Alterations In TCS And Regulators

To determine whether in vitro exposure of colistin-susceptible *E. coli* strains to colistin induced amino acid alterations in TCS and regulators that were observed in colistin-resistant *E. coli* isolates, colistin-resistant derivatives were produced by the serial passage of colistin-susceptible bacteria in colistin-containing media. Five colistin-resistant derivatives were randomly selected from each of two colistin-susceptible *E. coli* isolates. Isolates EC6 and EC7 were allocated to ST641 and ST118. Colistin MICs increased from 1 µg/mL in the wild-type strains to 8 µg/mL in the mutant derivatives ([Table 3](#)). Of the 10 colistin-resistant derivatives, nine showed the same amino acid alterations:

Table 2 Amino Acid Alterations In PmrAB, PhoPQ, MgrB, And PmrD In The Mcr-Negative Colistin-Resistant *E. coli* Isolates

Isolate	Colistin MICs (µg/mL)	PmrBI		PhoP	PhoQ				MgrB	PmrD																
		S138 <sup>a</sup>	D(G)283		V108	D101	S138	I175		V386	E464	Q33	N11	M20	A27	K35	A52	K82								
K-12 MGI655 (ATCC25922)																										
CL-1, CL-2, CL-10, CL-19, CL-21, CL-24, CL-26, CL27	4, 8, 16, >32																									
CL-4	>32																						N <sup>d</sup>			
CL-3, CL-5, CL-6, CL-7, CL-8, CL-9, CL-11, CL-14, CL-15, CL-16, CL-20, CL-22, CL-31	8-32																								T	
CL-12	8										F														T	
CL-13	32		G200R <sup>b</sup> (frameshift)																						T	
CL-17	16				E <sup>b</sup>						L															
CL-18	8			M <sup>c</sup>											D											
CL-25	4																								V <sup>b</sup>	T
CL-28	32													D												
CL-29, CL-30	8, 32	N <sup>c</sup>								T									R <sup>c</sup>	D	K	T				

Notes: <sup>a</sup>Amino acid is described by a one letter code. <sup>b</sup>Mutations that have not been described previously. <sup>c</sup>Mutations that have been described in colistin-resistant *E. coli* strains. <sup>d</sup>Mutations that have been described previously in *E. coli* strain, but the association with colistin resistance was not known.

**Table 3** Amino Acid Substitutions In The PmrAB, PhoPQ, MgrB, And PmrD In The Colistin-Resistant *E. coli* Mutants Derived From Colistin-Susceptible EC6 And EC7 Isolates

Strain	Colistin MIC ( $\mu\text{g/mL}$ )	PmrB	PhoQ			MgrB	PmrD		
		S138	S138	T348	E464	Q33	N11	M20	A27
EC6, EC7	1								
EC6-1, EC6-2, EC6-3, EC6-4, EC7-1, EC7-2, EC7-3, EC7-4, EC7-5	8	N	T			R	D	K	T
EC6-5	8			N	D				

S138N in PmrB, S138T in PhoQ, Q33R in MgrB, and N11D, M20K, and A27T in PmrD. This mutation profile was identical to that of two of the colistin-resistant isolates, CL-29 and CL-30. One colistin-resistant derivative, E6-5, from the E6 isolate showed amino acid alterations T348N and E464D in PhoQ. Mutation T348N in PhoQ was not identified in the clinical colistin-resistant isolates.

## Discussion

Colistin resistance in *E. coli* strains from healthy animals was found to have a prevalence of less than 1% in European countries, despite the extensive use of colistin in veterinary medicine.<sup>6,34</sup> In Korea, the colistin resistance rate was 1.46% among *E. coli* isolates from livestock during 2005 and 2015.<sup>31</sup> The annual consumption of colistin in veterinary medicine was gradually decreased from 16.3 tons in 2005 to 9.3 tons in 2015 in Korea, and the occurrence of colistin resistance among *E. coli* isolates from animals and animal carcasses also decreased, from 4.11% in 2008 to 0.94% in 2015.<sup>31</sup> Of the 10,576 *E. coli* isolates obtained from animals and animal carcasses during 2005 and 2015, 154 were resistant to colistin. The plasmid-mediated colistin resistance gene *mcr-1* was identified in 11 (7.1%) isolates, whereas the remaining 143 (92.9%) isolates were *mcr-1* negative.<sup>31</sup> In the present study, 30 representative *mcr-1*-negative colistin-resistant *E. coli* isolates were selected for the investigation of chromosome-mediated colistin resistance mechanisms. No *mcr-2*, *-3*, and *-4* genes were identified in the *E. coli* isolates tested. Thirty *mcr*-negative colistin-resistant *E. coli* isolates were originated from diverse clones based on the STs. In addition, 11 *mcr-1* gene-carrying *E. coli* isolates showed different STs and pulsotypes in a previous study.<sup>31</sup> These results suggest that colistin resistance in *E. coli* isolates from livestock during 2005 and 2015 in Korea is mainly due to chromosomal mutations associated with

LPS modification or unknown mechanisms occurring in sporadic clones, but not to the horizontal transfer of *mcr* genes or the spread of specific colistin-resistant clones. Extensive use of colistin in veterinary medicine may contribute to the sporadic occurrence of colistin resistance in *E. coli* in Korea.

Specific mutations in the TCS PmrAB and PhoPQ and their regulators MgrB and PmrD are associated with colistin resistance in *Enterobacteriaceae*, including *Klebsiella pneumoniae*, *K. aerogenes*, and *Salmonella* Enterica, as well as *P. aeruginosa*, and *A. baumannii*.<sup>13,22</sup> However, colistin resistance mechanisms in *E. coli* remain to be characterized. Qesada et al<sup>35</sup> first described the association of mutations S39I and R81S in PmrA and V161G in PmrB with colistin resistance in *E. coli* isolates from pigs. Thereafter, several molecular mechanisms involved in mutations in TCS and their regulators have been identified in *E. coli*.<sup>36-39</sup> In the present study, missense mutations in TCS were frequently found in sensor kinases PhoQ or PmrB rather than their response regulators PhoP or PmrA. No amino acid substitution was identified in PmrA. Previous studies have also reported that mutations in the sensor kinases were more frequently found in colistin-resistant *E. coli* isolates than in those of response regulators.<sup>35-39</sup> These results suggest that the sensor kinase of TCS is more susceptible to the occurrence of mutations associated with colistin resistance than its response regulator. Missense mutations, deletions, or insertion of insertion sequences (IS) in MgrB were identified most frequently in colistin-resistant *K. pneumoniae*.<sup>40-42</sup> However, a mutation in MgrB was found in only two *E. coli* isolates in this study. These results suggest that mutation type or the genes associated with colistin resistance are different between *K. pneumoniae* and *E. coli*.

Five different mutations in PhoQ were observed in six isolates. Of them, four mutations, S138T, I175F, V386L, and E464D, were observed in colistin-susceptible *E. coli* isolates



in previous studies,<sup>39,43,44</sup> whereas mutation D101E in the phosphorelay signal transduction system domain (PhoQ\_sensor) of PhoQ was first detected in the CL-17 isolate in this study. Many different mutations in PhoQ\_sensor and histidine kinase-like ATPases (HATPase\_c) domains have been described in colistin-resistant gram-negative bacterial species, as well as *E. coli*.<sup>13,45,46</sup> With respect to PhoP, the missense mutation V108M was observed only in the CL-18 isolate. Although mutation V108M in PhoP was also observed in colistin-resistant *E. coli* isolates from pigs,<sup>37</sup> the association of this mutation with colistin resistance is unknown. Other mutations in PhoP sequences have been described, such as I44L in colistin-resistant and -susceptible *E. coli* isolates.<sup>39</sup> The 222 amino acid sequences of PmrA in *E. coli* isolates in this study were identical to the reference strain *E. coli* K-12 MG1655 and *E. coli* ATCC 25922. Although several mutations in PmrA (V89I, A111S, A115G, and A122G) have been described in two colistin-resistant *E. coli* isolates from pigs,<sup>36</sup> previous studies did not find specific mutations in PmrA in clinical *E. coli* isolates from humans and animals. Missense mutation S138N and frameshift mutation G200R in PmrB were observed in *E. coli* isolates in this study. An S138N mutation was previously observed in nine *mcr-1*-positive *E. coli* isolates from diseased pigs.<sup>37</sup> However, frameshift mutation G200R was described for the first time in this study. The *mgrB* gene encodes a short 47-amino acid transmembrane protein that negatively regulates the histidine kinase of PhoQ.<sup>30</sup> In the present study, a mutation Q33R in MgrB was observed in two isolates. This mutation was also observed in three colistin-resistant *E. coli* isolates from pigs.<sup>36</sup> Mutations in MgrB were more frequent in colistin-resistant *K. pneumoniae* than colistin-resistant *E. coli*.<sup>13,47,48</sup> PmrD is a connector protein that links the PhoPQ and PmrAB systems in *Salmonella* Enterica.<sup>49</sup> This protein stabilizes and protects the phosphorylated form of PmrA from dephosphorylation by PmrB, leading to lipid A modifications. In *E. coli*, PmrD positively regulates the expression of *pmrA* and its downstream target gene, including genes coding for the LPS modification enzymes.<sup>50</sup> The association of a mutation in PmrD with colistin resistance in *E. coli* has not yet been determined. In the present study, six different mutations were observed in PmrD. Four mutations, N11D, M20K, A27T, and K82T, were frequently observed in colistin-susceptible *E. coli* isolates according to the sequences available in GenBank (<https://www.ncbi.nlm.nih.gov/genbank/>). A K35N mutation was observed in *E. coli*, but its association with colistin susceptibility was not known. Mutation A52V in PmrD was not detected in the reported sequences of *E. coli*. The

combinations of genetic mutations in TCS and their regulators led to nine different mutation profiles (Table 2). Of the 30 colistin-resistant *E. coli* isolates, seven (CL-4, CL-13, CL-17, CL-18, CL-25, CL-29 and CL-30) carried mutations that were first observed in this study, or previously observed only in colistin-resistant strains. However, the remaining 23 isolates carried no mutation or mutations found in colistin-susceptible *E. coli* strains. For these isolates, other genetic alterations in *mgrR*, *eptB*, *lpxM*, or *QseB/QseC* may explain colistin resistance. Further studies are now needed to identify the colistin resistance mechanisms in *E. coli*. Moreover, further complementation studies are needed to evaluate the impact of mutations in TCS and their regulators on colistin resistance. This study also found that nine out of 10 colistin-resistant derivatives induced by in vitro-selection showed the same genetic mutation profile in PmrB, PhoQ, MgrB, and PmrD, as observed in EC-29 and EC-30 isolates from the diseased pigs. These results suggest that selective pressure of colistin either in vitro or in vivo may induce the same genetic mutations in hot spots of TCS and their regulators in *E. coli*. In the present study, we could not analyze the impact of mutations in TCS and their regulators on the MICs of colistin because many isolates carried no mutation or mutations found in colistin-susceptible isolates. Moreover, CL-29 and CL-30 isolates carrying the same mutation showed the different MICs of colistin. Chromosome-mediated colistin resistance up-regulated the *pmrCAB* operon, the *arnBCADTEF* operon, or the *pmrE* gene,<sup>13</sup> but we did not analyze the expression of LPS-modifying genes in this study. This is limitation of this study.

In summary, the present study demonstrates diverse genetic mutations in TCS PmrB and PhoPQ and their regulators MgrB and PmrD in *mcr*-negative colistin-resistant *E. coli* isolates from livestock in Korea. Some mutations in these genes are unique to colistin-resistant isolates, but others are commonly identified in both colistin-resistant and -susceptible isolates. In addition, colistin-resistant isolates carried no mutations in PmrAB, PhoPQ, MgrB, and PmrD. These results suggest that the mechanisms underlying colistin resistance remain to be discovered in *E. coli*. The in vitro selection of colistin-resistant derivatives from colistin-susceptible strains produced the same genetic alterations that were observed in colistin-resistant isolates. Efforts to reduce colistin consumption in livestock should be reinforced to prevent the occurrence of colistin-resistant strains.

## Conclusions

Colistin resistance in livestock-derived *E. coli* strains causes serious public concern worldwide. Although the

plasmid-mediated *mcr* genes contribute to the transfer and occurrence of colistin resistance in *Enterobacteriaceae* from both humans and livestock, the majority of colistin resistance in *E. coli* from livestock was associated with mutations in TCS and their regulators by antibiotic selective pressure. However, the exact mechanisms underlying colistin resistance in *E. coli* remain unclear and will be investigated in future studies.

## Acknowledgments

This study was supported by a grant from the Animal and Plant Quarantine Agency, Ministry of Agriculture, Food, and Rural Affairs, Republic of Korea (Z-1543081-2017-18-01). The abstract of this paper was presented at the 27th Federation Meeting of Korean Basic Medical Scientists 2019 as a poster presentation with interim findings.

## Disclosure

The authors report no conflicts of interest in this work.

## References

- Nation RL, Velkov T, Li J. Colistin and polymyxin B: peas in a pod, or chalk and cheese? *Clin Infect Dis*. 2014;59:88–94. doi:10.1093/cid/ciu213
- Biswas S, Brunel JM, Dubus JC, Reynaud-Gaubert M, Rolain JM. Colistin: an update on the antibiotic of the 21st century. *Expert Rev Anti Infect Ther*. 2012;10:917–934. doi:10.1586/eri.12.78
- Li J, Nation RL, Turmidge JD, et al. Colistin: there-emerging antibiotic for multidrug-resistant Gram-negative bacterial infections. *Lancet Infect Dis*. 2006;6:589–601. doi:10.1016/S1473-3099(06)70580-1
- Kieffer N, Poirel L, Nordmann P, Mavec JY, Haenni M. Emergence of colistin resistance in *Klebsiella pneumoniae* from veterinary medicine. *J Antimicrob Chemother*. 2015;70:1265–1267. doi:10.1093/jac/dku485
- Catry B, Cavaleri M, Baptiste K, et al. 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*. 2015;46:297–306. doi:10.1016/j.ijantimicag.2015.06.005
- Kempf I, Fleury MA, Drider D, et al. What do we know about resistance to colistin in *Enterobacteriaceae* in avian and pig production in Europe? *Int J Antimicrob Agents*. 2013;42:379–383. doi:10.1016/j.ijantimicag.2013.06.012
- Shafiq M, Huang J, Shah JM, et al. High incidence of multidrug-resistant *Escherichia coli* coharboring *mcr-1* and *bla<sub>CTX-M-15</sub>* recovered from pigs. *Infect Drug Resist*. 2019;12:2135–2149. doi:10.2147/IDR.S209473
- Raetz CR, Reynolds CM, Trent MS, Bishop RE. Lipid A modification systems in gram-negative bacteria. *Annu Rev Biochem*. 2007;76:295–329. doi:10.1146/annurev.biochem.76.010307.145803
- Moffatt JH, Harper M, Harrison P, et al. Colistin resistance in *Acinetobacter baumannii* is mediated by complete loss of lipopolysaccharide production. *Antimicrob Agents Chemother*. 2010;54:4971–4977. doi:10.1128/AAC.00834-10
- Padilla E, Llobet E, Domenech-Sanchez A, et al. *Klebsiella pneumoniae* AcrAB efflux pump contributes to antimicrobial resistance and virulence. *Antimicrob Agents Chemother*. 2010;54:177–183. doi:10.1128/AAC.00715-09
- Nikaido H. Molecular basis of bacterial outer membrane permeability revisited. *Microbiol Mol Biol Rev*. 2003;67:593–656. doi:10.1128/mmbr.67.4.593-656.2003
- Wright MS, Suzuki Y, Jones MB, et al. Genomic and transcriptomic analyses of colistin-resistant clinical isolates of *Klebsiella pneumoniae* reveal multiple pathways of resistance. *Antimicrob Agents Chemother*. 2015;59:536–543. doi:10.1128/AAC.04037-14
- Poirel L, Jayol A, Nordmann P. Polymyxins: antibacterial activity, susceptibility testing, and resistance mechanisms encoded by plasmids or chromosomes. *Clin Microbiol Rev*. 2017;30:557–596. doi:10.1128/CMR.00064-16
- Tamayo R, Choudhury B, Septer A, Merighi M, Carlson R, Gunn JS. Identification of *cptA*, a PmrA-regulated locus required for phosphoethanolamine modification of the *Salmonella enterica* serovar Typhimurium lipopolysaccharide core. *J Bacteriol*. 2005;187:3391–3399. doi:10.1128/JB.187.10.3391-3399.2005
- Liu YY, Wang Y, Walsh TR, et al. 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*. 2016;16:161–168. doi:10.1016/S1473-3099(15)00424-7
- Partridge S, Di Pilato V, Doi Y, et al. Proposal for assignment of allele numbers for mobile colistin resistance (*mcr*) genes. *J Antimicrob Chemother*. 2018;73:2625–2630. doi:10.1093/jac/dky262
- Wang X, Wang Y, Zhou Y, et al. Emergence of colistin resistance gene *mcr-8* and its variant in *Raoultella ornithinolytica*. *Front Microbiol*. 2019;10:228. doi:10.3389/fmicb.2019.00228
- Liassine N, Assouvie L, Descombes MC, et al. Very low prevalence of MCR-1/MCR-2 plasmid-mediated colistin resistance in urinary tract *Enterobacteriaceae* in Switzerland. *Int J Infect Dis*. 2016;51:4–5. doi:10.1016/j.ijid.2016.08.008
- Aquilini E, Merino S, Knirel YA, Regue M, Tomas JM. Functional identification of *Proteus mirabilis* *epiC* gene encoding a core lipopolysaccharide phosphoethanolamine transferase. *Int J Mol Sci*. 2014;15:6689–6702. doi:10.3390/ijms15046689
- Lin QY, Tsai YL, Liu MC, Lin W-C, Hsueh P-R, Liaw S-J. *Serratia marcescens* *arn*, a PhoP-regulated locus necessary for polymyxin B resistance. *Antimicrob Agents Chemother*. 2014;58:5181–5190. doi:10.1128/AAC.00013-14
- Schurek KN, Sampaio JLM, Kiffer CRV, et al. Involvement of pmrAB and phoPQ in polymyxin B adaptation and inducible resistance in non-cystic fibrosis clinical isolates of *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother*. 2009;53:4345–4351. doi:10.1128/AAC.01267-08
- Olaitan AO, Morand S, Rolain JM. Mechanisms of polymyxin resistance: acquired and intrinsic resistance in bacteria. *Front Microbiol*. 2014;5:643. doi:10.3389/fmicb.2014.00547
- Gunn JS. The *Salmonella* PmrAB regulon: lipopolysaccharide modifications, antimicrobial peptide resistance and more. *Trends Microbiol*. 2008;16:284–290. doi:10.1016/j.tim.2008.03.007
- Yan A, Guan Z, Raetz CR. An undecaprenyl phosphateaminoarabinose flippase required for polymyxin resistance in *Escherichia coli*. *J Biol Chem*. 2007;282:36077–36089. doi:10.1074/jbc.M706172200
- Gunn JS, Lim KB, Krueger J, et al. PmrA-PmrB-regulated genes necessary for 4-amino arabinose lipid A modification and polymyxin resistance. *Mol Microbiol*. 1998;27:1171–1182. doi:10.1046/j.1365-2958.1998.00757.x
- Gunn JS, Ryan SS, Van Velkinburgh JC, Ernst RK, Miller SI. Genetic and functional analysis of a PmrA-PmrB-regulated locus necessary for lipopolysaccharide modification, antimicrobial peptide resistance, and oral virulence of *Salmonella enterica* serovar typhimurium. *Infect Immun*. 2000;68:6139–6146. doi:10.1128/iai.68.11.6139-6146.2000
- Lee H, Hsu FF, Turk J, Groisman EA. The PmrA-regulated pmrC gene mediates phosphoethanolamine modification of lipid A and polymyxin resistance in *Salmonella enterica*. *J Bacteriol*. 2004;186:4124–4133. doi:10.1128/JB.186.13.4124-4133.2004



28. Guo L, Lim KB, Gunn JS, et al. Regulation of lipid A modifications by *Salmonella* Typhimurium virulence genes phoP-phoQ. *Science*. 1997;276:250–253. doi:10.1126/science.276.5310.250
29. Cannatelli A, D'Andrea MM, Giani T, et al. *In vivo* emergence of colistin resistance in *Klebsiella pneumoniae* producing KPC-type carbapenemases mediated by insertional inactivation of the PhoQ/PhoP mgrB regulator. *Antimicrob Agents Chemother*. 2013;57:5521–5526. doi:10.1128/AAC.01480-13
30. Cheng HY, Chen YF, Peng HL. Molecular characterization of the PhoPQ-PmrD-PmrAB mediated pathway regulating polymyxin B resistance in *Klebsiella pneumoniae* CG43. *J Biomed Sci*. 2010;17:60. doi:10.1186/1423-0127-17-74
31. Lim SK, Kang HY, Lee K, Moon D-C, Lee H-S, Jung S-C. First detection of the *mcr-1* gene in *Escherichia coli* isolated from livestock between 2013 and 2015 in South Korea. *Antimicrob Agents Chemother*. 2016;60:6991–6993. doi:10.1128/AAC.01472-16
32. CLSI. Performance standards for antimicrobial susceptibility testing. 29th ed. M100. Wayne, Pennsylvania: Clinical and Laboratory Standards Institute; 2019.
33. Rebelo AR, Bortolaia V, Kjeldgaard JS, et al. Multiplex PCR for detection of plasmid-mediated colistin resistance determinants, *mcr-1*, *mcr-2*, *mcr-3*, *mcr-4* and *mcr-5* for surveillance purposes. *Euro Surveill*. 2018;23.
34. de Jong A, Thomas V, Simjee S, et al. Pan-European monitoring of susceptibility to human-use antimicrobial agents in enteric bacteria isolated from healthy food-producing animals. *J Antimicrob Chemother*. 2012;67:638–651. doi:10.1093/jac/dkr539
35. Quesada A, Porrero MC, Tellez S, Palomo G, García M, Domínguez L. Polymorphism of genes encoding PmrAB in colistin-resistant strains of *Escherichia coli* and *Salmonella enterica* isolated from poultry and swine. *J Antimicrob Chemother*. 2015;70:71–74. doi:10.1093/jac/dku320
36. Anjum MF, Duggett NA, AbuOun M, et al. Colistin resistance in *Salmonella* and *Escherichia coli* isolates from a pig farm in Great Britain. *J Antimicrob Chemother*. 2016;71:2306–2313. doi:10.1093/jac/dkw149
37. Delannoy S, Le Devendec L, Jouy E, Fach P, Drider D, Kempf I. Characterization of colistin-resistant *Escherichia coli* isolated from diseased pigs in France. *Front Microbiol*. 2017;8:2278. doi:10.3389/fmicb.2017.02278
38. Cannatelli A, Giani T, Aiezza N, et al. An allelic variant of the PmrB sensor kinase responsible for colistin resistance in an *Escherichia coli* strain of clinical origin. *Sci Rep*. 2017;7:5071. doi:10.1038/s41598-017-05167-6
39. Luo Q, Yu W, Zhou K, et al. Molecular epidemiology and colistin resistant mechanism of *mcr*-positive and *mcr*-negative clinical isolated *Escherichia coli*. *Front Microbiol*. 2017;8:2262. doi:10.3389/fmicb.2017.02262
40. Poirel L, Jayol A, Bontron S, et al. The mgrB gene as a key target for acquired resistance to colistin in *Klebsiella pneumoniae*. *J Antimicrob Chemother*. 2015;70:75–80. doi:10.1093/jac/dku323
41. Cannatelli A, Giani T, D'Andrea MM, et al. MgrB inactivation is a common mechanism of colistin resistance in KPC-producing *Klebsiella pneumoniae* of clinical origin. *Antimicrob Agents Chemother*. 2014;58:5696–5703. doi:10.1128/AAC.03110-14
42. Aires CAM, Pereira PS, Asensi MD, Carvalho-Assef APDA. MgrB mutations mediating polymyxin B resistance in *Klebsiella pneumoniae* isolates from rectal surveillance swabs in Brazil. *Antimicrob Agents Chemother*. 2016;60:6969–6972. doi:10.1128/AAC.01456-16
43. Ogura Y, Ooka T, Iguchi A, et al. Comparative genomics reveal the mechanism of the parallel evolution of O157 and non-O157 enterohemorrhagic *Escherichia coli*. *Proc Natl Acad Sci U S A*. 2009;106:17939–17944. doi:10.1073/pnas.0903585106
44. Kwon SK, Kim SK, Lee DH, Kim JF. Comparative genomics and experimental evolution of *Escherichia coli* BL21(DE3) strains reveal the landscape of toxicity escape from membrane protein overproduction. *Sci Rep*. 2015;5:16076. doi:10.1038/srep16076
45. Olaitan AO, Thongmalayvong B, Akkhavong K, et al. Clonal transmission of a colistin-resistant *Escherichia coli* from a domesticated pig to a human in Laos. *J Antimicrob Chemother*. 2015;70:3402–3404. doi:10.1093/jac/dkv252
46. Cheng YH, Lin TL, Pan YJ, et al. Colistin resistance mechanisms in *Klebsiella pneumoniae* strains from Taiwan. *Antimicrob Agents Chemother*. 2015;59:2909–2913. doi:10.1128/AAC.04763-14
47. Nordmann P, Jayol A, Poirel L. Rapid detection of polymyxin resistance in *Enterobacteriaceae*. *Emerg Infect Dis*. 2016;22:1038–1043. doi:10.3201/eid2206.151840
48. Olaitan AO, Diene SM, Kempf M, et al. Worldwide emergence of colistin resistance in *Klebsiella pneumoniae* from healthy humans and patients in Lao PDR, Thailand, Israel, Nigeria and France owing to inactivation of the PhoP/PhoQ regulator *mgrB*: an epidemiological and molecular study. *Int J Antimicrob Agents*. 2014;44:500–507. doi:10.1016/j.ijantimicag.2014.07.020
49. Kato A, Groisman EA. Connecting two-component regulatory systems by a protein that protects a response regulator from dephosphorylation by its cognate sensor. *Genes Dev*. 2004;18:2302–2313. doi:10.1101/gad.1230804
50. Rubin EJ, Herrera CM, Crofts AA, Trent MS. PmrD is required for modifications to *Escherichia coli* endotoxin that promote antimicrobial resistance. *Antimicrob Agents Chemother*. 2015;59:2051–2061. doi:10.1128/AAC.05052-14

## Infection and Drug Resistance

### Publish your work in this journal

Infection and Drug Resistance is an international, peer-reviewed open-access journal that focuses on the optimal treatment of infection (bacterial, fungal and viral) and the development and institution of preventive strategies to minimize the development and spread of resistance. The journal is specifically concerned with the epidemiology of

antibiotic resistance and the mechanisms of resistance development and diffusion in both hospitals and the community. The manuscript management system is completely online and includes a very quick and fair peer-review system, which is all easy to use. Visit <http://www.dovepress.com/testimonials.php> to read real quotes from published authors.

Submit your manuscript here: <https://www.dovepress.com/infection-and-drug-resistance-journal>

Dovepress