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

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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.[1–3] 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.[4–7] Colistin resistance is caused by decreases in the net negative charge of the outer membrane, loss of lipid A, or efflux pumps.[8–10] The most common resistance mechanism in *Enterobacteriaceae* is the covalent modification of the lipid A moiety of lipopolysaccharide (LPS) via cationic substitution.[11–13] These modifications neutralize the negative charge of LPS and subsequently reduce the binding affinity of colistin for its target.[13,14]

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A plasmid-mediated colistin resistance gene, *mcr-1*, was first reported in *Escherichia coli* from China in 2015,[15] and several variants of the *mcr* gene were identified.[16,17] The *mcr* genes encode phosphoethanolamine (PEtN) transferase enzyme that adds PEtN to lipid A, and consequently results in a more cationic LPS.[13,18] 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.[19,20] 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).[13,20–23] The PEtN phosphotransferase PmrC adds a PEtN group to the LPS.[22] The *arnBCADTEF* operon (also called the *pmrHFIJKLM* or *pbgPE* operon) and the *pmrE* gene are responsible for the biosynthesis of L-Ara4N and its transfer to lipid A.[24] Addition of these cationic groups to the LPS decreases the net negative charge of the LPS and induces colistin resistance.[25–27] Specific mutations in the *pmrAB* and *phoPQ* genes have been found in colistin-resistant gram-negative bacteria.[25–28] The MgrB and PmrD are also associated with colistin resistance in the *Enterobacteriaceae*.[13,29,30] 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.[29,30] 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*,[13,22] 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.[31] 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 KRNV4F 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).[32] 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, 10^9 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	TET	SXT
CL-1	Cattle	Feces	2006	297	16	≤1	≤2	4	4	8/4	8	2	≤0.5	4	≤0.12	4	4	≤2	≤0.12/2.28
CL-2	Cattle	Feces	2006	New	8	≤1	≤2	8	≤2	4/2	16	2	≤0.5	≤2	≤0.12	8	4	≤2	≤0.12/2.28
CL-3	Pig	Feces	2007	6488	8	≤1	>32	128	64	4/2	4	2	≤0.5	≤2	≤0.12	4	4	64	0.25/4.75
CL-4	Pig	Feces	2007	515	>32	≤1	>32	64	>64	4/2	16	≤1	>8	8	≤0.12	4	4	64	>4/76
CL-5	Cattle	Feces	2008	2035	8	1	32	128	64	4/2	16	16	0.5	128	8	16	64	32	0.5/9.5
CL-6	Cattle	Feces	2008	278	32	8	32	128	64	8/4	32	2	0.5	2	0.12	8	4	128	0.25/4.75
CL-7	Cattle	Feces	2008	448	8	1	2	128	64	8/4	16	4	0.5	128	16	8	4	64	4/76
CL-8	Cattle	Feces	2008	448	16	64	32	128	64	8/4	8	2	0.5	128	16	4	2	128	4/76
CL-9	Cattle	Feces	2008	906	32	4	8	32	32	8/4	32	2	0.5	2	0.12	8	4	2	0.12/2.28
CL-10	Pig	Feces	2008	10	8	64	32	64	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	128	64	8/4	16	2	1	128	0.5	64	64	128	4/76
CL-12	Cattle	Feces	2009	2111	8	1	2	8	8	8/4	16	2	1	2	0.12	8	4	2	0.12/2.28
CL-13	Cattle	Feces	2009	388	32	8	32	128	64	64/32	64	64	8	128	16	64	64	128	4/76
CL-14	Chicken	Feces	2010	224	8	32	4	32	64	8/4	8	4	0.5	128	16	64	8	64	4/76
CL-15	Chicken	Feces	2010	3054	8	1	2	16	64	8/4	16	4	0.5	128	16	32	8	128	4/76
CL-16	Pig	Feces	2010	3054	8	1	2	128	64	8/4	16	4	0.5	128	0.12	64	64	128	4/76
CL-17	Chicken	Diarrheal stool	2010	226	16	1	2	16	64	8/4	16	4	0.5	128	16	16	4	128	0.12/2.28
CL-18	Pig	Diarrheal stool	2010	100	8	4	32	32	64	8/4	8	32	8	128	16	64	8	32	4/76
CL-19	Cattle	Feces	2011	5564	8	1	2	8	2	2/1	4	2	0.5	2	0.12	8	4	2	0.12/2.28
CL-20	Cattle	Feces	2011	2035	8	1	2	4	2	2/1	4	2	0.5	2	0.12	8	4	2	0.12/2.28
CL-21	Pig	Intestinal lesion	2011	450	8	64	2	128	64	4/2	4	1	0.5	128	16	4	2	32	4/76
CL-22	Chicken	Diarrheal stool	2011	156	8	1	2	4	64	8/4	16	4	0.5	128	16	8	4	128	0.12/2.28
CL-24	Pig	Feces	2012	641	4	1	32	128	64	8/4	16	4	0.5	2	0.12	64	64	128	0.25/4.75
CL-25	Pig	Feces	2013	5903	4	1	2	8	4	4/2	8	8	0.5	4	0.12	4	4	2	0.12/2.28
CL-26	Pig	Intestinal lesion	2013	752	16	≤1	32	16	>64	8/4	16	8	≤0.5	≤2	≤0.12	>64	>64	128	0.25/4.75
CL-27	Pig	Feces	2013	1257	>32	>64	>128	>128	>64	4/2	≤2	64	>8	128	>16	>64	>64	>128	>4/76
CL-28	Pig	Intestinal lesion	2014	93	32	64	128	128	64	8/4	16	8	8	128	16	64	64	128	4/76
CL-31	Chicken	Feces	2014	548	4	4	1	0.12	32	4/2	64	2	2	8	0.12	4	4	2	0.5/9.5
CL-29	Pig	Urine	2015	1	8	≤1	64	64	>64	8/4	8	4	4	32	≤0.12	>64	>64	64	≤0.12/2.28
CL-30	Pig	Abscess	2015	1	32	≤1	128	128	>64	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, ceftifur; NAL, 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.[33] 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_{50} = 8 μg/mL and MIC_{90} = 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)	PmrB1		PhoP	PhoQ					MgrB	PmrD					
K-12 MG1655 (ATCC25922)		S138[a]	D(G)283	V108	D101	S138	I175	V386	E464	Q33	N11	M20	A27	K35	A52	K82
CL-1, CL-2, CL-10, CL-19, CL-21, CL-24, CL-26, CL27	4, 8, 16, >32															
CL-4	>32													N[d]		
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[b] (frameshift)													T
CL-17	16				E[b]			L								
CL-18	8			M[c]					D							
CL-25	4														V[b]	T
CL-28	32								D							
CL-29, CL-30	8, 32	N[c]				T				R[c]	D	K	T			

Notes: [a]Amino acid is described by a one letter code. [b]Mutations that have not been described previously. [c]Mutations that have been described in colistin-resistant *E. coli* strains. [d]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 (μg/mL)	PmrB	PhoQ			MgrB	PmrD		
K-12 MG1655		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.[6,34] In Korea, the colistin resistance rate was 1.46% among *E. coli* isolates from livestock during 2005 and 2015.[31] 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.[31] 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.[31] 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.[31] 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*.[13,22] However, colistin resistance mechanisms in *E. coli* remain to be characterized. Qesada et al[35] 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*.[36–39] 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.[35–39] 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*.[40–42] 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,[39,43,44] 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*.[13,45,46] 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,[37] 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.[39] 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,[36] 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.[37] 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.[30] 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.[36] Mutations in MgrB were more frequent in colistin-resistant *K. pneumoniae* than colistin-resistant *E. coli*.[13,47,48] PmrD is a connector protein that links the PhoPQ and PmrAB systems in *Salmonella* Enterica.[49] 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.[50] 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,[13] 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.

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Disclosure

The authors report no conflicts of interest in this work.

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